UNIVERSIDADE DE LISBOA
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PHYTOCHEMICAL STUDY AND BIOLOGICAL ACTIVITIES OF DITERPENES AND DERIVATIVES FROM *PLECTRANTHUS* SPECIES

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DOUTORAMENTO EM FARMÁCIA
(QUÍMICA FARMACÊUTICA E TERAPÊUTICA)

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ABSTRACT

This study focused on the research of new bioactive constituents from four species of the *Plectranthus* plants. Previous works on plants of the genus *Plectranthus* (Lamiaceæ) evidenced that some of their constituents possess interesting biological activities.

The antimicrobial activity of the plant extracts and of the isolated metabolites was thoroughly searched. Antioxidant, anticholinesterase and anti-inflammatory properties of some compounds were also screened.

The phytochemical study of the acetone extracts of *Plectranthus ornatus* Codd., *P. ecklonii* Benth., *P. porcatus* Winter & Van Jaarsv and *P. saccatus* Benth. rendered several terpenoid constituents mostly diterpenes.

From *P. ornatus* three new forskolin-like labdane diterpenes (6-*O*-acetylforskolin, 1,6-di-*O*-acetylforskolin and 1,6-di-*O*-acetyl-9-deoxyforskolin), a new diterpene with the rare halimane skeleton (11*R**-acetoxyhalima-5,13*E*-dien-15-oic acid), and two known labdane diterpenes were isolated; the rhinocerotinoic acid which was found in *Plectranthus* species for the first time, and plectrornatin C. Six known triterpenoids were also identified as mixtures. The study of *P. ecklonii* led to the isolation of two known abietanes, sugiol and parvifloron D. Sugiol was obtained from *Plectranthus* species for the first time. Four known triterpenoids were also identified as mixtures. *P. porcatus*, a plant not hitherto studied, yield a new spiro-abietane diterpene [(13*S*,15*S*)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione]. A new beyerane diterpene (*ent*-7α-acetoxy-15-beyeren-18-oic acid) was isolated from *P.* saccatus.

Attempting to find novel bioactive prototypes from the more potent antibacterial diterpenes, isolated in higher yields, some diterpene derivatives were prepared. Nine new derivatives were obtained from (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid (*P. ornatus*). A new 2β-(4-hydroxy)benzoyloxy derivative of microstegiol was prepared from parvifloron D (*P. ecklonii*). From the 7α-acetoxy-6β-hydroxyroyleanone (isolated in the past from *P. grandidentatus*) thirteen ester derivatives were synthesized, whereof ten were new compounds.

The unequivocal chemical structures of pure compounds (natural and derivatives) were deduced from their spectroscopic (IR, MS, 1D and 2D NMR experiments) and physico-chemical data, as well as from literature information.

The preliminary antimicrobial activity screenings of all the isolated metabolites showed that several diterpenes inhibited the growth of the Gram positive bacteria tested. In addition, the minimum inhibitory concentration against standard and clinical isolates of sensitive and resistant *Staphylococcus* and *Enterococcus* strains was determined for the antibacterial metabolites and their synthesized derivatives.

The (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid and its (11*R**,13*E*)-halima-5,13-diene-11,15-diol derivative were the more active halimanes. Parvifloron D was less active than its microstegiol 2β-(4-hydroxy)benzoate derivative, but both showed more potent antibacterial activities than the halimane diterpenoids.

The three 12-*O*-benzoyl esters derivatives of the 7α-acetoxy-6β-hydroxyroyleanone prototype revealed to be more potent growth inhibitors against *Staphylococcus* and *Enterococcus* strains than the prototype. The 6β-propionyloxy-12-*O*-propionyl derivative also showed to be more active against *Enterococcus* than the prototype. Generally, the 12-esters and the 6,12-diesters were more active against *Enterococcus* than *Staphylococcus* strains. The hydrophobic extra-interactions with the bacterial targets seem to play an important role on the activity of royleanones derivatives prepared.

Taking into account the IC_{50} values which expressed the scavenging DPPH radical ability, the isolated metabolite parvifloron D as well as 7α-acetoxy-6β-hydroxyroyleanone showed *in vitro* antioxidant activity.

The *in vitro* acetylcholinesterase assay did not detect any activity for all the newly isolated diterpenes and 7α-acetoxy-6β-hydroxyroyleanone.

The COX inhibitor screening assay was tested on 6-*O*-acetylforskolin, rhinocerotinoic acid, plectrornatin C, (11*R**,13*E*)-halima-5,13-diene-11,15- diol, 11*R**-acetoxyhalima-5,13*E*-dien-15-oic acid and on its methyl ester, for their ability to inhibit COX-2. The preliminary results encourage further studies aiming to confirm and to examine its potential anti-inflammatory activity in a more robust approach.

Keywords: Lamiaceae, *Plectranthus*, *Plectranthus ornatus*, *Plectranthus ecklonii, Plectranthus saccatus, Plectranthus porcatus*, diterpenes, abietane, halimane, forskolin-like diterpenoids, labdane, beyerane, antimicrobial activity, antioxidant activity, anticholinesterase activity and anti-inflammatory activity.

RESUMO

Este estudo teve como objectivo a pesquisa de novos constituintes bioactivos de quatro espécies de plantas do género *Plectranthus.*

A actividade antimicrobiana dos extractos obtidos e dos metabolitos isolados foi realizada e foram testadas as propriedades anti-oxidante, anti-colinesterase e anti-inflamatória de alguns compostos.

O estudo fitoquímico dos extractos de acetona de *Plectranthus ornatus* Codd., *P. ecklonii* Benth., *P. porcatus* Winter & Van Jaarsv. e *P. saccatus* Benth. originou diversos constituintes terpénicos, principalmente diterpenos.

Três novos diterpenos do tipo forskolina (6-*O*-acetilforskolina; 1,6-di-*O*-acetilforskolina e 1,6-di-*O*-acetil-9-deoxiforskolina) foram isolados de *P. ornatus.* Foram também identificados um novo diterpeno com o raro esqueleto de halimano (ácido 11*R**-acetoxihalima-5,13*E*-dien-15-óico), dois diterpenos labdânicos conhecidos; o ácido rinocerotinóico encontrado pela primeira vez em espécies do género *Plectranthus*, e a plectrornatina C. Seis triterpenos já conhecidos foram igualmente identificados na forma de misturas. O estudo de *P. ecklonii* originou o isolamento de dois abietanos conhecidos: o sugiol e a parviflorona D. O sugiol foi isolado pela primeira vez de espécies *Plectranthus*. Outros quatro triterpenos conhecidos foram identificados também como misturas. A planta *P. porcatus*, até à data não estudada, originou um novo diterpeno *spiro*-abietânico [(13*S*,15*S*)-6β,7α,12α,19-tetrahidroxi-13β,16-ciclo-8-abietene-11,14-diona]. Um novo diterpeno com esqueleto de beierano (ácido *ent*-7α-acetoxi-15-beieren-18-óico) foi isolado de *P. saccatus*.

Na tentativa de obter novos protótipos bioactivos, vários derivados foram preparados, a partir dos diterpenos antibacterianos mais potentes e isolados em maior quantidade. Nove novos derivados foram obtidos do ácido (11*R**,13*E*)-11-acetoxihalima-5,13-dien-15-óico (*P. ornatus*). Um novo derivado 2β-(4-hidroxi)benzoilado do microstegiol, foi preparado a partir da parviflorona D (*P. ecklonii*). Treze ésteres derivados da 7α-acetoxi-6β-hidroxiroyleanona (isolada anteriormente de *P. grandidentatus*) foram sintetizados, sendo de assinalar que dez dos derivados são compostos novos.

A determinação estrutural dos compostos puros (naturais e derivados) foi deduzida por espectroscopia (IV, EM, RMN 1D e 2D), propriedades físico-químicas e com base na informação obtida da literatura.

O estudo preliminar da actividade antimicrobiana de todos os metabolitos isolados, mostrou que diversos diterpenos inibem o crescimento de bactérias de Gram positivo. A concentração mínima inibitória (CMI) dos metabolitos e seus derivados foi determinada em estirpes de *Staphylococcus* e *Enterococcus,* tanto em bactérias padrão como em isolados clínicos resistentes e sensíveis a antibióticos. O ácido (11*R**,13*E*)-11-acetoxihalima-5,13-dien-15-óico e o seu derivado (11*R**,13*E*)-halima-5,13-diene-11,15-diol foram os halimanos mais activos. A parviflorona D foi menos activa do que o seu correspondente derivado 2β-(4-hidroxi)benzoilado, mas ambos apresentaram uma actividade antibacteriana mais potente do que os diterpenos com esqueleto de halimano.

Os três 12-*O*-benzoil-ésteres derivados do protótipo 7α-acetoxi-6β-hidroxiroyleanona revelaram ser inibidores mais potentes do que a royleanona-protótipo, contra as estirpes testadas de *Staphylococcus* e *Enterococcus*. O derivado 6β-propioniloxi-12-*O*-propionilo mostrou ser o mais activo contra as estirpes testadas de *Enterococcus* do que o protótipo. De um modo geral, os derivados 12-ésteres e os 6,12-diésteres foram mais activos contra as estirpes de *Enterococcus* do que as estirpes de *Staphylococcus* testadas. As interacções hidrofóbicas com os alvos bacterianos parecem ter um papel importante na actividade antibacteriana dos derivados de royleanona preparados.

Os metabolitos parviflorona D e a 7α-acetoxi-6β-hidroxiroyleanona demostraram possuir actividade antioxidante *in vitro,* tendo em conta os valores de IC_{50} que expressam a actividade anti-oxidante com base na captura do radical DPPH.

Todos os novos diterpenos isolados e derivados obtidos neste trabalho foram testados e não revelaram possuir actividade inibitória da acetilcolinesterase *in vitro*.

A actividade anti-inflamatória foi testada nos compostos 6-*O*-acetilforskolina, ácido rinocerotinóico, plectrornatina C, (11*R**,13*E*)-halima-5,13-diene-11,15-diol, ácido 11*R**-acetoxihalima-5,13*E*-dien-15-óico e no seu éster metílico, através da sua capacidade de inibir a COX-2. Os resultados preliminares obtidos apoiam a necessidade de estudos futuros de forma a confirmar, explorar e discutir uma potencial actividade anti-inflamatória.

Palavras-chave: Lamiaceae, *Plectranthus*, Plectranthus ornatus, Plectranthus *ecklonii,* Plectranthus *saccatus, Plectranthus porcatus*, diterpenos, abietano, halimano, derivados da forskolina, labdano, beierano, actividade antimicrobiana, actividade anti-oxidante, actividade anti-colinesterase, actividade anti-inflamatória.

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List of publications

The majority of the results described in this dissertation were presented in various scientific meetings, published in peer-reviewed journals or sent for publication.

A- Papers

- Rijo P., Duarte A., Francisco A. P., Simões M.F., Royleanone derivatives as potential anti-Gram positive agents, **2010**, in preparation.

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- Rijo P., Esteves M., Simões M.F., Silva A., Duarte A., Rodriguez B., Antimicrobial activity of 7α-acetoxy-6β-hydroxyroyleanone 12-*O*-benzoyl esters, 7th Joint Meeting of AFERP, ASP, GA, PSE & SIF, Athens, Greece, 3-8 August 2008.

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ABBREVIATIONS AND SYMBOLS

Ac	Acetyl
AcOH	Acetic Acid
ATCC	American Type Culture Collection
ax	Axial
BHT	2,6-di-*tert*-butyl-4-methylphenol
Bu	Butanoyl
Bz	Benzyl
c	Concentration
^{13}C NMR	^{13}C Nuclear Magnetic Resonance
calcd	Calculated
CC	Column chromatography
$CDCl_3$	Deuterated Chloroform
CH_2Cl_2	Dichloromethane
COSY	Correlation Spectroscopy
COX	Cycloxygenase
°C	Degree Celsius
d	Doublet
dd	Double doublet
ddd	Double doublet of doublets
dddd	Double doublet of doublet of doublets
ddddd	Double doublet of doublet of doublet of doublets
dq	Double quartet
dddt	Double doublet of doublet of triplets
ddt	Double doublet of triplets
dt	Double triplet
DMAPP	Dimehylallyl Diphosphate
DMSO	Dimethylsulphoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl radical
DXP	Deoxyxylulose Phosphate Pathway
EIMS	Electronic Impact Mass Spectrometry
eq	Equatorial
et al.	and others
EtOAc	Ethyl acetate
EtOH	Ethanol
eV	Electron volt
FPP	Farnesyl Diphosphate
GGPP	Geranyl Geranyl Diphosphate
GPP	Geranyl Diphosphate
h	Hour
^{1}H NMR	^{1}H Nuclear Magnetic Resonance
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Coherence
HR-ESIMS	High Resolution Electrospray Mass Spectrometry
HR-LSIMS	High Resolution Liquid Secondary Ion Mass Spectrometry

Hz	Hertz
IC_{50}	Sample concentration causing 50% inhibition
IPP	Isopentenyl Diphosphate
IR	Infrared
J	Coupling Constant
L	Litre
M	Molar concentration
m	Multiplet
m.p.	Melting Point
m/z	Ratio of mass to charge
max	Maximum
Me	Methyl
Me_2CO	Acetone
MeOH	Methanol
MEP	Methylerythritol Phosphate pathway
mg	Milligram
MHz	Megahertz
MIC	Minimum inhibitory concentrations
min	Minute
mL	Millilitre
MVA	Mevalonoic Acid
n.d.	Not Described or Not Determined
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Enhancement Spectroscopy
ppm	Parts per million
QSAR	Quantitative Structure Activity Relationship
Rel. int.	Relative intensity
R_f	Retention fraction
sept	Septuplet
SAR	Structure Activity Relationship
s	Singlet
T	Temperature
t	Triplet
td	Triple doublet
tt	Triple triplet
tdd	Triple doublet of doublets
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
UV	Ultraviolet
VRE	Vancomycin resistant *Enterococcus faecalis*
v/v	Volume/volume
$W_{1/2}$	Width at half height (^{1}H NMR)
$[\alpha]^T_D$	Specific rotation for a sample at a temperature T (given in degrees Celsius) and wavelength of the light used is the sodium D line
δ	Chemical shift

δ_c	Carbon chemical shif
λ_{max}	Maximum Wave Lenght
µg	Microgram
µL	Microlitre
µM	Micromolar
µmol	Micromole
ν_{max}	Maximum Wave Number
$[M]^+$	Molecular ion

CHAPTER I

Introduction

I.1. Drug discovery from medicinal plants

Current research in drug discovery from medicinal plants involves a multifaceted approach combining botanical, phytochemical, biological, and molecular techniques. Medicinal plant drug discovery continues to provide new and important lead-compounds against various pharmacological targets. Plant secondary metabolites and their semi-synthetic derivatives continue to play an important role in drug discovery. These compounds are representative of a wide structural diversity. Despite the recent interest in molecular modeling, combinatorial chemistry, and other synthetic chemistry techniques by the pharmaceutical companies and funding organizations, natural products, and particularly medicinal plants, remains an important source of new drugs, new drug leads, and new chemical entities. Considering the example of anticancer drug market, a recent analysis in North America, Europe, and Japan during the period 1981–2006 revealed that 47.1% of a total of 155 clinically approved anticancer drugs were either unmodified natural products or their semi-synthetic derivatives, or synthesized molecules based on natural product compound pharmacophores (Pan L. et al., 2010). Approximately one quarter of the bestselling drugs worldwide (2001 and 2002) were natural products or derived from natural products. Drugs derived from medicinal plants can serve not only as new drugs themselves but also as drug leads suitable for optimization by medicinal and synthetic chemists. Even when new chemical structures are not found during drug discovery from medicinal plants, known compounds with new biological activity can provide important drug leads. Several known compounds isolated from traditionally used medicinal plants have already been shown to act on newly validated molecular targets.

In the last few decades the medicinal plants which form the backbone of traditional medicine have been the subject of very intense pharmacological studies. In developing countries, it is estimated that about 80% of the population rely on traditional medicine for their primary health care. Therefore, arises a need to screen medicinal plants for bioactive compounds as a basis for further pharmacological studies (Matu E.N. and Van Staden J., 2003).

The natural products are typically isolated in small quantities that are insufficient for lead optimization and development. Thus, considering the drug discovery from medicinal plants, the collaboration with synthetic and medicinal chemists is necessary, since the natural products isolated remains an essential component in the search for new medicines (Balunas M.J. et al., 2005).

Recently, there has been a renewed interest in natural product research (due to the failure of alternative drug discovery methods) to deliver lead compounds in key therapeutic areas such as immunosuppression, anti-infectives, and metabolic diseases (Butler M.S., 2004).

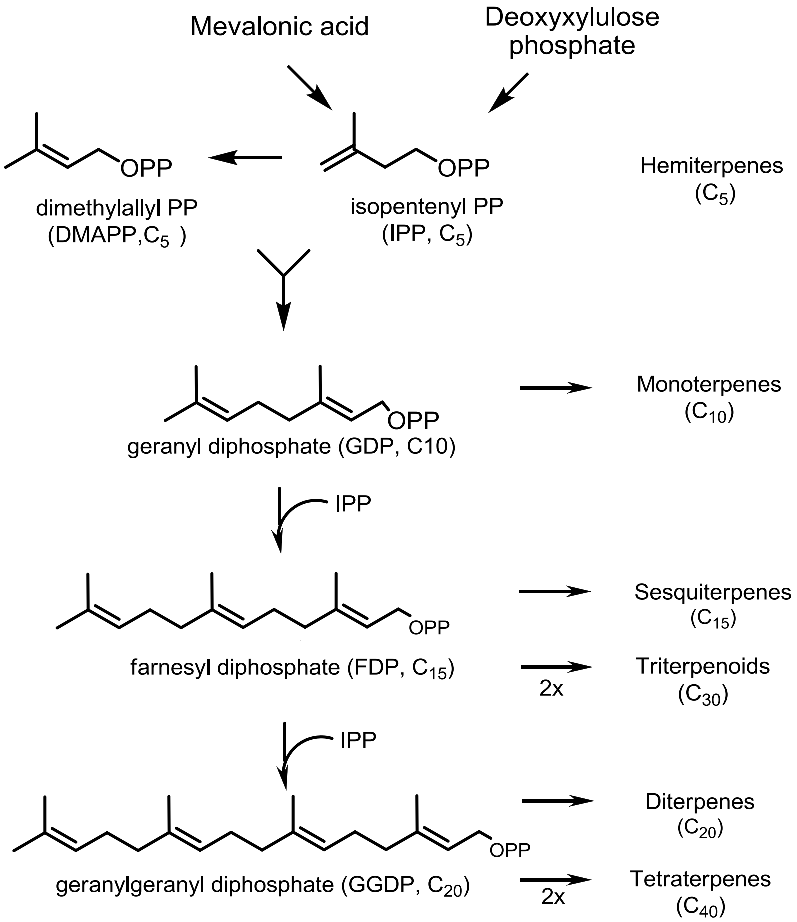
In the last decades, developments in spectroscopic techniques at molecular levels, improvements in immunology and enzymologie as well as in searching for structure–activity relationships (SAR studies) and more sensitive bioassays led to determinate important drugs from natural sources. Plants are the most important source of new drugs. Several recent drugs are derived from their secondary metabolites or prepared from them as semi-synthetic and synthetic derivatives for clinical uses (Topcu G. et al., 2007).

I.2. Terpenoids: biogenetic considerations

Terpenoids are an enormous class of plant metabolites with many diverse roles in growth, development, and resistance to environmental stresses. Terpenoids have several applications as industrial biomaterials, including pharmaceuticals, fragrances and flavors, and insecticides. Some terpenoids may serve in the production of novel biofuels (Bohlmann J. and Gershenzon J., 2009).

Isoprene had been characterized as a decomposition product from various natural cyclic hydrocarbons, and was suggested as the fundamental building block for terpenoids (isoprenoids). Isoprene is produced naturally but is not involved in the formation of these compounds, and the biochemically active isoprene units were identified as the diphosphate (pyrophosphate) esters dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) (**Scheme I.1**). The term ‘prenyl’ is in general use to indicate the dimethylallyl substituent. In macromolecules like proteins can be modified by attaching terpenoids chains, like cysteine residues alkylated with farnesyl or geranylgeranyl groups increasing the lipophilicity of the protein and its ability to associate with membranes (Dewick P.M., 2002).

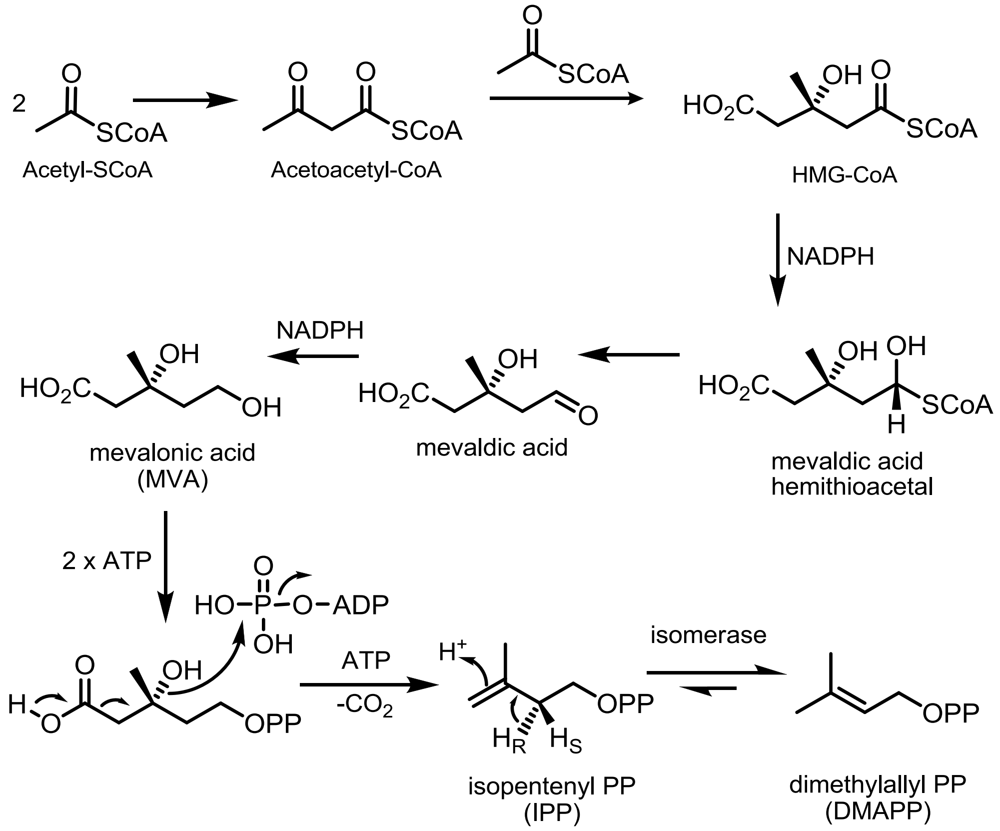
Terpenoids derived from C_5 isoprene units in a head-to-tail arrangement. Typical structures containing carbon skeletons represented by $(C_5)_n$ are classified as hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}) and tetraterpenes (C_{40}) (see **Scheme I.1**). The monoterpene, sesquiterpene, and diterpene synthases of plant origin use the corresponding C_{10}, C_{15}, and C_{20} prenyl diphosphates as substrates to generate the enormous diversity of carbon skeletons characteristic of the terpenoid family of natural products. The synthesis of isopentenyl diphosphate, isomerization to dimethylallyl diphosphate, prenyltransferase-catalyzed condensation of these two C_5-units to geranyl diphosphate (GDP), and the subsequent 1´-4 additions of isopentenyl diphosphate generate farnesyl (FDP) and geranylgeranyl (GGDP) diphosphate (**Scheme I.1**). The prenyl diphosphates undergo a range of cyclizations based on variations on the same mechanistic theme to produce the parent skeletons of each class. Thus, GDP (C_{10}) gives rise to monoterpenes, FDP (C_{15}) to sesquiterpenes, and GGDP (C_{20}) to diterpenes. These transformations catalyzed by the terpenoid synthases (cyclases) may be followed by a variety of redox modifications of the parent skeletal types to produce the many thousands of different terpenoid metabolites. More than 3000 different diterpenoid structures have been defined, all of which appear to be derived from GGDP. Most diterpenoids are cyclic, and there appear to be two major, and fundamentally different, modes of cyclization in this class. The macrocyclic diterpenes, are formed by cyclizations analogous to those of the monoterpene and sesquiterpene series. The second mode of cyclization involves generation of copalyl diphosphate (CDP) as the initial intermediate (Mann J., 1987; Bohlmann J. et al, 1998; Dewick P.M., 2002).



Scheme I.1. Isoprenoids obtention from isoprene units identified as the diphosphate (pyrophosphate) esters dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP; Dewick P.M., 2002)

I.2.1 The mevalonate and deoxyxylulose phosphate pathways

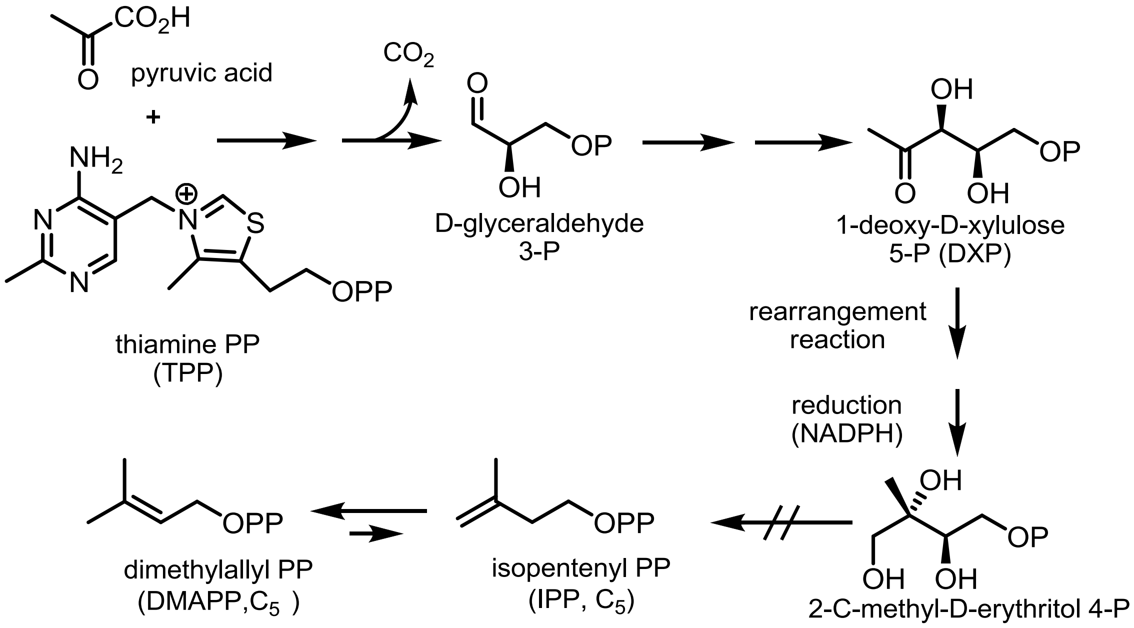
Since the initial discovery of the mevalonate pathway in the 1950s, it was widely accepted that isopentenyl diphosphate (IPP), the fundamental unit in terpenoid biosynthesis, was only formed by condensation of acetyl CoA through the ubiquitous mevalonate pathway (**Scheme I.2**).



Scheme I.2. Pathway of mevalonic acid (MVA; Dewick P.M., 2002).

However, it has been disclosed that many organisms including higher plants, that use an alternative mevalonate-independent pathway (non-mevalonate pathway) for the formation of IPP. According to Rohmer and coworkers, the initial step of this pathway is the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) by condensation of pyruvate and glyceraldehyde 3-phosphate (**Scheme I.3**). In the second step the intramolecular rearrangement of DXP is assumed to give a hypothetical rearranged intermediate, 2-*C*-methylerythrose 4-phosphate, which is then converted to 2-*C*-methyl-D-erythritol 4-phosphate (MEP) by an unspecified reduction process. Thus, precise details about the reductive mechanism for the formation of MEP remained unclear (Takahashi S. et al., 1998; Kuzuyama T., 2002).

The biochemical isoprene units (IPP and DMAPP) may be derived from two pathways either the intermediate is mevalonic acid (MVA; **Scheme I.2**) or 1-deoxy-D-xylulose 5-phosphate (DXP; **Scheme I.3**). This latter pathway is also referred to as the mevalonate-independent pathway or the methylerythritol phosphate pathway (**Scheme I.3**).



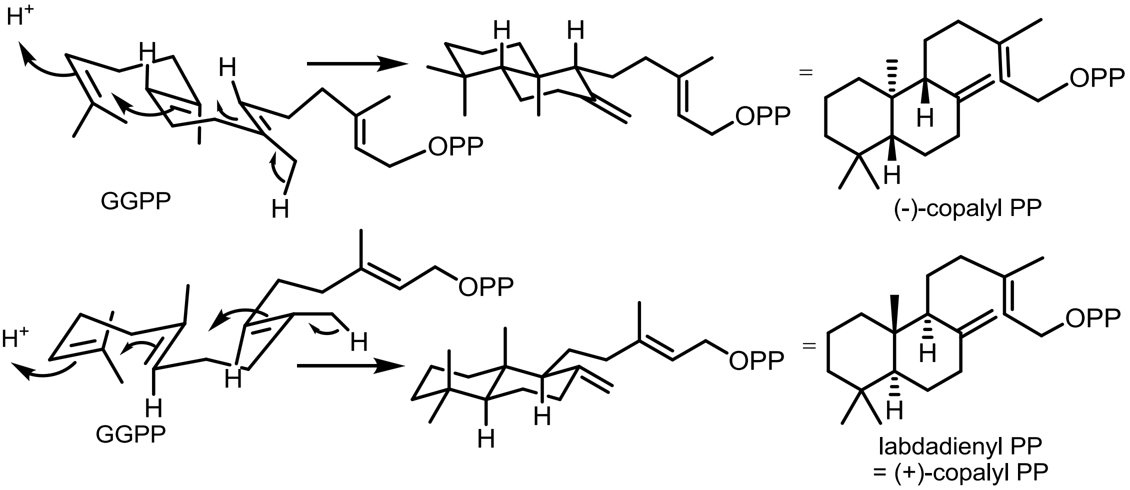
Scheme I.3. Pathway via 1-deoxy-D-xylulose 5-phosphate (DXP; Dewick P.M., 2002).

1-Deoxy-D-xylulose 5-phosphate (DXP) is formed from the glycolytic pathway intermediates pyruvic acid and glyceraldehyde 3-phosphate. The methylerythritol phosphate contains the branched-chain system equivalent to the isoprene unit, but the complete sequence of steps leading to the intermediate isopentenyl phosphate has yet to be elucidated (**Scheme I.3)**.

In plants, the two pathways appear to be compartmentalized, so the mevalonate pathway enzymes are located in the cytosol, whereas the deoxyxylulose phosphate pathway enzymes are found in chloroplasts. Accordingly, triterpenoids and steroids (cytosolic products) are formed by the mevalonate pathway, whilst most other terpenoids are formed in the choloplasts and are deoxyxylulose phosphate derived, with some exceptions. There are also examples where the two pathways can supply different portions of a molecule, or where there is exchange of late-stage common intermediates between the two pathways resulting in a contribution of isoprene units from each pathway (Dewick P.M., 2002). Several isoprenoids were found to be of mixed origin suggesting that some exchange and/or cooperation exists between these two pathways of different biosynthetic origin. Contradictory results presented by Wanke *et al.* could indicate that these two pathways are operating under different physiological conditions of the cell and are dependent on the developmental state of plastids (Wanke M. et al., 2001).

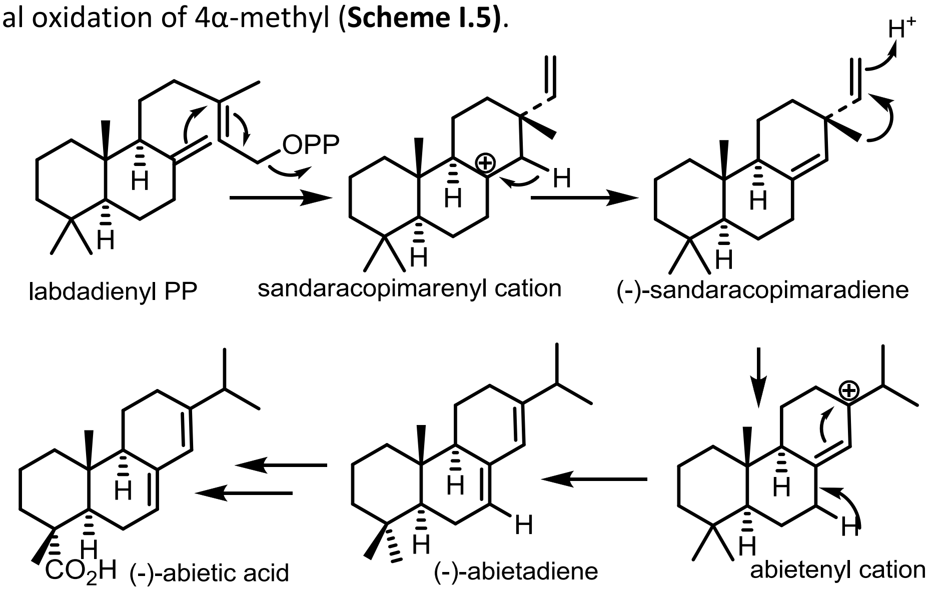
The diterpenes arise from geranylgeranyldiphosphate (GGPP), which is formed by addition of a further IPP molecule to farnesyl diphosphate in the same manner as described for the lower terpenoids. Cyclization reactions of GGPP mediated by carbocations formation, plus the potential Wagner-Meerwein rearrangements, will allow many structural variants of diterpenes to be produced. In addition, individual enzyme systems present in a particular organism will then control the folding of the substrate molecule and thus define the stereochemistry of the final product.

Protonation of GGPP can initiate a concerted cyclization sequence, terminated by loss of proton from a methyl, yielding copalyl PP. The stereochemistry in this product is controlled by the folding of the substrate on the enzyme surface, though an alternative folding can lead to labdadienyl PP, the enantiomeric product having opposite configurations at the newly generated chiral centres. The most commun stereochemistry is found in labdadienyl PP and derivatives and so the enanteriomeric series use the prefix *ent*. (**Scheme I.4**).



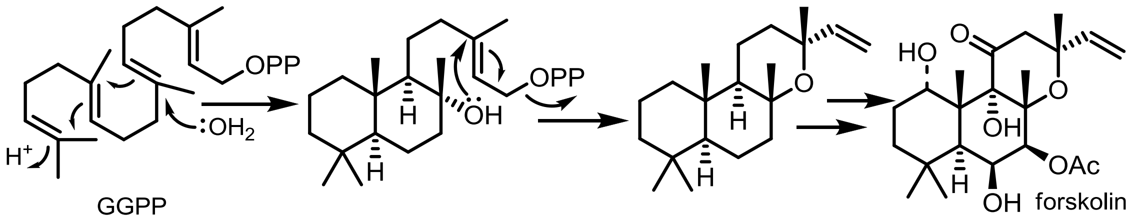
Scheme I.4. Protonation of GGPP yielding copalyl PP though an alternative folding leading to labdadienyl PP (Dewick P.M., 2002).

The alternative stereochemistry typified by labdadienyl PP can be seen in the structure of abietic acid. Initially, the tricyclic system is built up as in the pathway to *ent*-kaurene (with (-)-copalyl PP as precursor) via the same mechanism, but generating the enantiomeric series of compounds. The cation loses a proton to give sandaracopimaradiene, which undergoes a methyl migration to modify the side-chain, and further proton loss to form the diene abietadiene. Abietic acid results from sequential oxidation of 4α-methyl (**Scheme I.5)**.



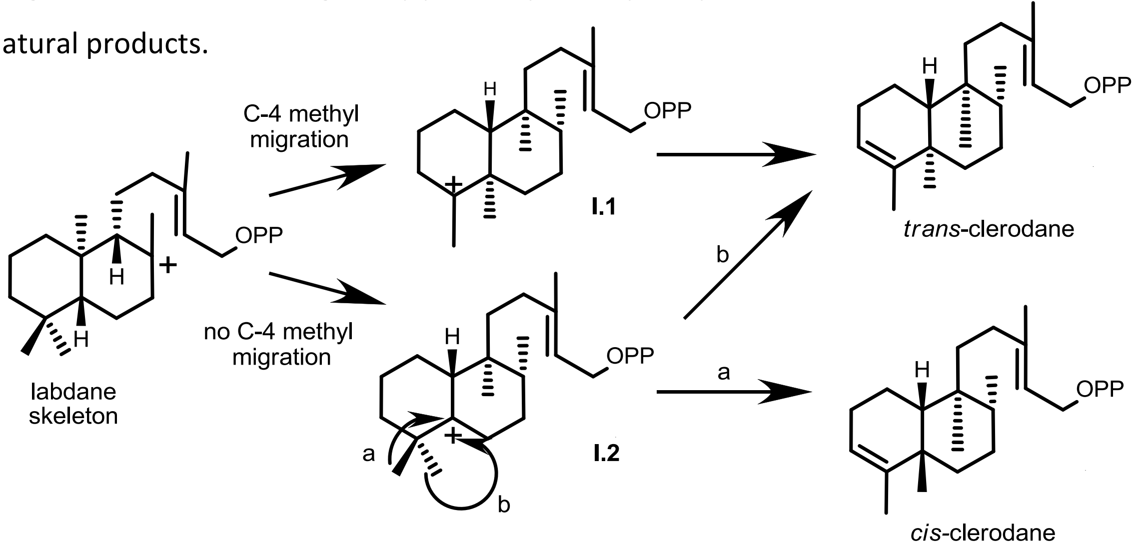
Scheme I.5. Biosynthetic pathway of abietic acid from labdadienyl PP (Dewick P.M., 2002).

In forskolin (**Scheme I.6**), the third ring is heterocyclic rather than carbocyclic. The basic skeleton of forskolin can be viewed as the result of quenching of the cation by water as opposed to proton loss, followed by S_N2 nucleophilic substitution on to the allylic diphosphate (or nucleophilic substitution on to the allylic cation generated by loss of diphosphate). A series of oxidative modifications will then lead to forskolin (a valuable pharmacological tool as a potent stimulator of adenyl cyclase activity, and it is being investigated for its cardiovascular and bronchospasmolytic effects; Dewick P.M., 2002).



Scheme I.6. Biosynthetic pathway of forskolin from GGPP (Dewick P.M., 2002).

The clerodanes appear to be related biosynthetically to the labdanes, via a series of methyl and hydride shifts. The labdane skeleton is itself derived from geranylgeranylpyrophosphate (GGPP), although this represents a simplification of the overall biogenetic route, involving many parallel pathways to yield the multitude of clerodane natural products.



Scheme I.7. Biosynthetic pathway of clerodanes from GGPP (Merritt A.T. and Ley *S*.V., 1992).

The *trans* clerodanes can arise via a concerted migration process to intermediate **I.1,** whilst the *cis* compounds require a stepwise process, with a ‘pause’ at intermediate **I.2.** Then this can lead to either *cis* or *trans* compounds, depending on which of the C-4 methyl groups that migrate.

It is possible that the various genera/families have developed independently the capacity to biosynthesize the clerodanes, or simply that there are a large number of families producing as yet unisolated clerodanes. Within individual families and genera, however, the species have been grouped to indicate chemical similarities of the isolated natural products, or trends in the productivity of species, rather than adhering to strict taxonomic ordering (Merritt A.T. and Ley *S* V., 1992).

Geranylfarnesyl PP (GFPP) arises by a continuation of the chain extension process, adding a further IPP unit to GGPP (**Scheme I.1)**. Triterpenes (C_{30}) are not formed by an extension of the now familiar process of adding IPP to the growing chain. Instead, two molecules of farnesyl PP are joined tail to tail to yield the hydrocarbon squalene, originally isolated from the liver oil shark (*Squalus* sp.) which was used to study its biosysthetic role as a precursor of triterpenes and steroids. The tetraterpenes has its skeleton formation involving tail-to-tail coupling of two molecules of geranylgeranyl diphosphate (GGPP) in a sequence essentially analogous to that seen for squalene and triterpenes.

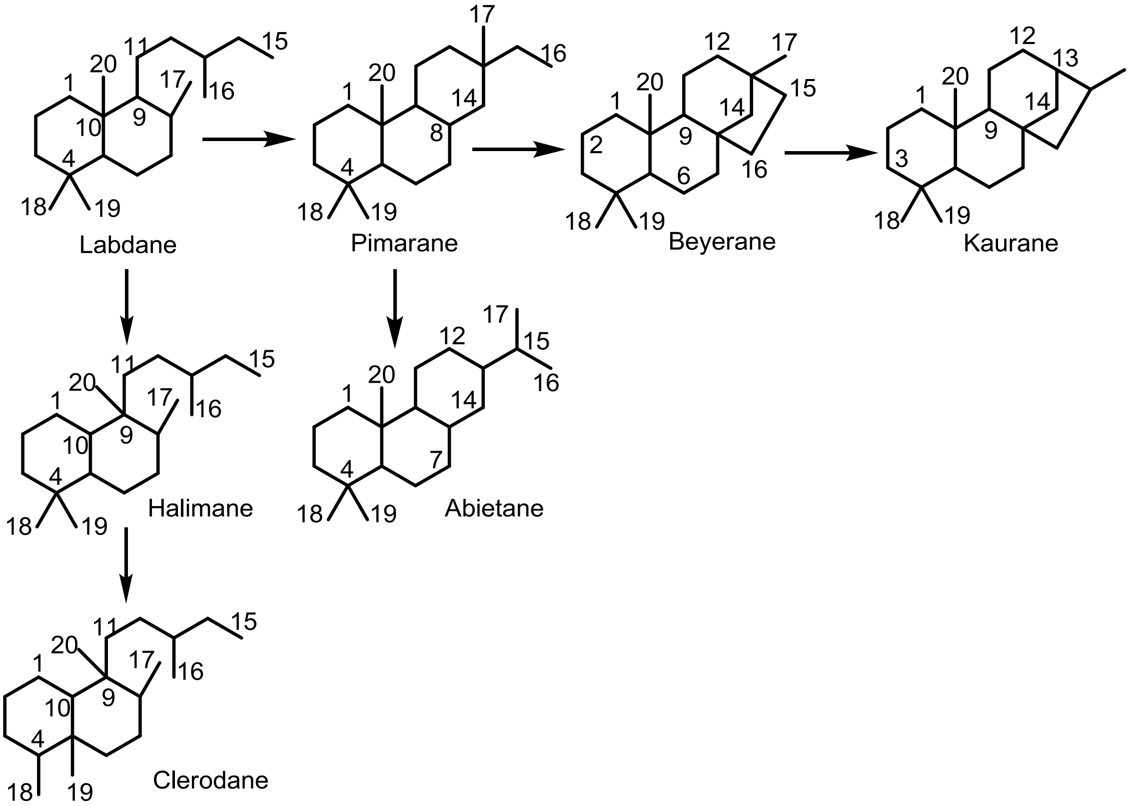
I.2.2. Diterpenoids

Diterpenes constitute the second largest class of terpenes, with over 2200 compounds belonging to 130 distinct skeletal types. The interest in the isolation of these compounds is growing due to their biological activity, ecological function, use as templates for synthesis and taxonomic function (Hanson J.R., 1995-2009; Alvarenga S.A.V. et al., 2001).

As Lukhoba *et al.* reported, the majority of phytochemical studies on *Plectranthus* species have focused on the isolation of a wide range of diterpenoids. One of the most studied *Plectranthus*-derived compounds is the labdane forskolin isolated from *P. barbatus*. It has a range of pharmacological properties and could explain many of the diverse medicinal uses of *P. barbatus.* Other biologically active diterpene is taxol isolated from *Taxus brevifolia* that displays anticancer properties. Terpentecin is a microbial clerodane diterpene isolated from *Streptomyces* spp. that has shown to be an antitumor antibiotic that targets DNA topoisomerase II. (Wang S.-Y. et al, 2002; Lukhoba C.W. et al., 2006; Lozama A. and Prisinzano T.E., 2009).

Lamiaceae is one of the families in which most of the diterpenes are found. The frequent occurrence of specific diterpene skeletons is observed and has been used, together with the presence of other secondary metabolite classes, in chemotaxonomic studies. In this way, diterpenes had been used as chemotaxonomic markers. Alvarenga *et al.* showed that the Lamiaceae diterpenes can be divided into 91 skeletons. *Plectranthus* (*Coleus*) species were characterized by the accumulation of abietanes and its derivatives, cyclopropylabietanes and its derivatives and kauranes skeletons. The main type of diterpene structures found was the abietane skeletons. The majority of diterpenes were found in *Salvia* and *Plectranthus* genera. *Salvia* presentes the highest number of species studied with the highest occurrence of diterpenes distributed among 50 diterpene skeletons (Alvarenga S.A.V. et al., 2001).

Some of the most common structural diterpene types in *Plectranthus* species are included in the biosynthetic grid presented in **Scheme I.8**.



Scheme I.8. Metabolic grid for some common diterpenoid skeletons (Figueiredo M.R. et al., 1995).

Diterpenes diversify mainly through labdane-derived pathways bifurcating at a pimarane stage into kaurane or abietane-centered biosynthetic groups. There is a strong predominance of abietanes over kauranes in gymnosperms (Figueiredo M.R. et al., 1995).

Abietane (**Scheme I.8**) is the skeleton with the highest occurrence and the most widespread in Lamiaceae. The *Plectranthus* abietanes found were not oxidized at carbons 1 and 20. In *Coleus*, the carbons preserved were 1,2,15, 18 (equatorial methyl) and 19 (axial methyl). The abietanes more oxidized were produced by *Salvia* in which any carbon can be oxidized. The derivatives with a 17(15→16)*abeo*abietane skeleton (**a**, see **Figure I.1**) were also found in *Plectranthus* genus and these diterpenes preserved positions 1, 2, 15, 18 (equatorial methyl group) and 20. The skeleton 17(15→16)*abeo*triptolidane (**b**, see **Figure I.1**) was also found in *Plectranthus* species. These substances isolated, maintained positions 1, 15, 19 and 20 as in the original skeleton and carbon 4 was always sp^2 hybridised. The skeleton 13(16)-cyclopropylabietane (**c**, see **Figure I.1**) is derived from abietane and was reported as being present in *Coleus* and *Plectranthus*. The substances isolated from *Coleus* and *Plectranthus* displayed some similarities. In both genera an oxygen atom bound at positions 6 and 12, the formation of a carbonyl at C-11 and C-14, and a double bond between C-8 and C-9 were detected. The substitution pattern from the original skeleton was retrieved at positions 1, 2, 5 and 20. The cyclopropylabietanes from *Plectranthus* were additionally preserved at 15, 16, 17 and 18 while those isolated from *Coleus* presented an oxygen attached to C-7. The skeleton 13(16)-cyclopropyltriptolidane (**d**, see **Figure I.1**) was also found in *Coleus* and *Plectranthus*. The compounds isolated from *Plectranthus* presented oxygen bound at positions 6 and 12, formation of a carbonyl at C-11 and C-14 and a double bond between C-8 and C-9. The original skeleton was maintained at positions 1, 5 and 20 and carbon 4 was a sp^2 hybridised in all substances. The structures reported in *Plectranthus* were also preserved at C-15, C-16 and C-17, but had an oxygen bound to C-7.

In Lamiaceae, the skeleton of beyeranes were found exclusively in *Sideritis*. The occurrence of kauranes was reported for eight Lamiaceae genera including *Plectranthus*.

Labdane diterpenes were found in 20 genera of Lamiaceae. All *Coleus* labdanes were derived from manoyl oxide, i.e. labdanes skeletons with an epoxide bridge at C-8 and C-13. They always possessed a carbonyl group at C-11 and a double bond between C-14 and C-15. The carbons at 2, 3, 5, 12, 16, 17, 18, 19 and 20 remained unchanged when compared with the original labdane skeleton. The presence of clerodanes was reported in 13 Lamiaceae genera (Alvarenga et al., 2001).

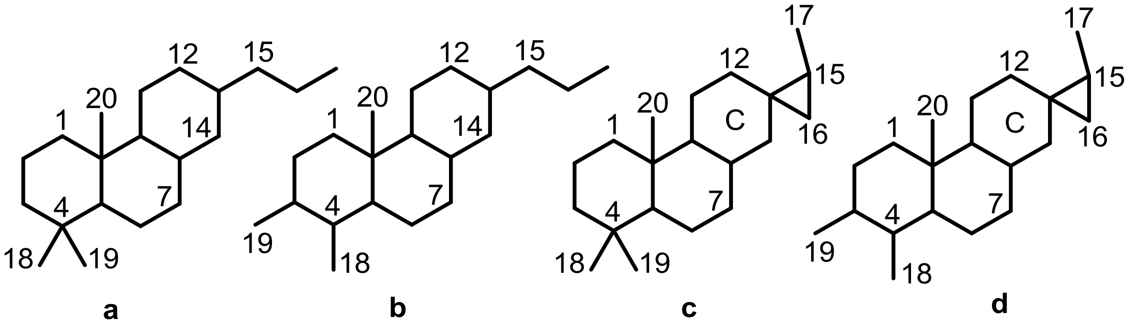


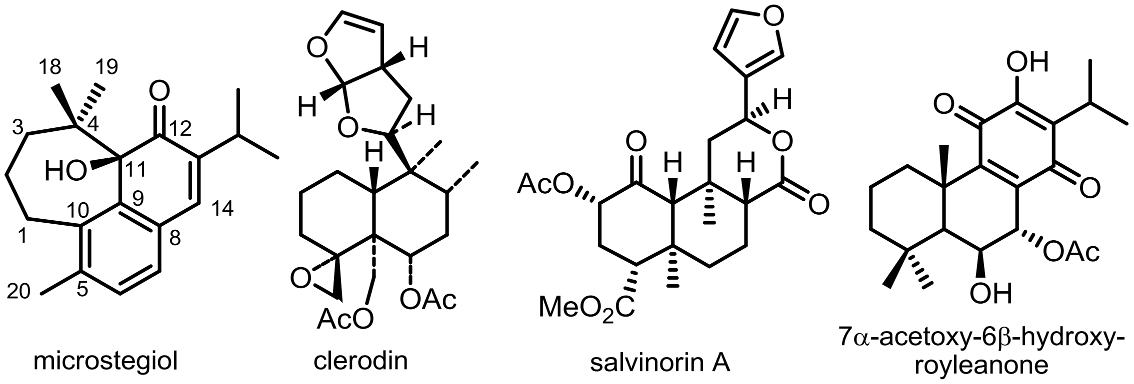
Figure I.1. 17(15→16)*Abeo*abietane (**a**), 17(15→16)*abeo*triptolidane (**b**), 13(16)-cyclopropylabietane (**c**) and 13(16)-cyclopropyltriptolidane (**d**) skeletons.

Salvia species are important medicinal plants due to their diverse secondary metabolites, especially abietane diterpenoids with a *p*-quinone C ring moiety. In fact, in *Salvia* genus, the diversity of abietane diterpenes is very rich. The most interesting abietane diterpenes were rearranged abietanes and norabietanes. A new serie of rearranged abietanes, which have 7 or 8 membered ring A was isolated by Ulubelen *et al.* The first member of this serie rearranged abietanes was microstegiol (see **Figure I.2**), isolated from the aerial parts of *S. microstegia*. Like *Plectranthus* genus, *Salvia* species also afforded many triterpenes, however, their structures were not as much interesting and diverse as abietane diterpenes. Most of them have oleanane or ursane type skeleton, and some lupanes (Topcu G. et al., 2007).

Clerodanes (**Scheme I.8**) are found in many different plant families and contain four contiguous stereocenters contained in a *cis* or *trans* decalin. (Lozama A., Prisinzano T. E., 2009). During the last thirty years, over six hundred and fifty diterpenoids and nor-diterpenoids with the clerodane carbon skeleton have been isolated. Confusion has arisen in the literature over the absolute stereochemistry of the various clerodanes isolated. The revision of the absolute stereochemistry of clerodin (see **Figure I.2**), a *neo*-clerodane that displays antifeedant properties and inhibits insect growth, (Lozama A. and Prisinzano T.E., 2009), result in the first member of the clerodane series. This *neo*-clerodane has led to those compounds with the same absolute stereochemistry as clerodin being termed *neo*-clerodanes and those compounds enantiomeric to clerodin being termed *ent-neo*-clerodanes. A further division of the clerodanes has been to *cis* and *trans* compounds, depending on the stereochemistry of the decalin ring junction (**Scheme I.7**).

Six genera of the Lamiaceae family have been shown to produce clerodanes. Only a small number of the clerodanes have been shown to exhibit any biological activity. However, only a handful of compounds have been reported as having no activity in the tests carried out, thus leaving the large majority as simply untested or unreported. The clerodanes are best known for their insect antifeedant properties, and related insecticidal properties, with an emphasis placed on the safety aspects of such natural insect antifeedants in relation to mammalian and piscial life (Merritt A.T. and Ley *S.*V., 1992).

One neoclerodane diterpene that has been investigated recently is salvinorin A (see **Figure I.2**), isolated from the leaves of *Salvia divinorum*, possessing hallucinogenic effects. Because it is active at opioid receptors, its chemical reactivity is a useful example for the development of methodology to modify other neoclerodane diterpenes. Further advances in the synthesis of neoclerodane diterpenes are likely to further develop this structural class of terpenes into useful biological probes (Lozama A., Prisinzano T.E., 2009).



microstegiol clerodin salvinorin A 7α-acetoxy-6β-hydroxy-royleanone

Figure I.2. Chemical structures of diterpenes microstegiol, clerodin, salvinorin A and 7α-acetoxy-6β-hydroxyroyleanone.

I.2.2.1. The abietane 7α-acetoxy-6β-hydroxyroyleanone

The constituents of *Plectranthus* plants are mainly diterpenoids belonging to abietane, kaurane and labdane classes, being royleanone abietanes a well represented subclass. In a few royleanone abietanes were found antimycobacterial activities, for example, in derivatives of carnosic acid, isolated from *Salvia africana-lutea* (McGaw L. J. et al., 2008), and in horminone, obtained from *S. reptans* (Martínez-Vázquez M. et al., 1998) and *S. multicaulis* (Ulubelen A. et al., 1997). These last abietanes were similar to royleanones isolated from *P. grandidentatus* and *P. hereroensis*, that revealed anti-methicillin resistant *Staphylococcus aureus* (MRSA) proprieties (Gaspar-Marques C.C. et al., 2006).

Royleanones are hydroquinonic abietanes with a 12-hydroxy-11,14-di-oxo-quinone moiety in C ring, vastly distributed in Lamiaceae family. Several natural quinones metabolites showed antimycobacterial activity (Copp B.R. et al., 2007; Mahapatra A. et al., 2007; Rijo P. et al, 2010) and other interesting pharmacological activities, namely, antimicrobial, antitumoral and antiviral (Batista O. et al., 1995; Batista O. et al., 1996; Teixeira A.P., 1997; Gaspar-Marques C.C., 2006; Gaspar-Marques C. et al., 2008). 7α-Acetoxy-6β-hydroxyroyleanone (see **Figure I.2**) is often isolated from *Plectranthus* species (Gaspar-Marques C. et al., 2006; Lukhoba C.W. et al., 2006). The antioxidant activity of this royleanone was studied by Chang *et al.* (Chang S.-T. *et al.,* 2002) and recently this diterpene was studied for its bioactivity (selectively modify C-type inactivation of Kv1.2 channels; Leung Y.-M. et al., 2010).

Its possible biosynthesis pathway was proposed from the diterpene (-)-abietic acid, that originates by cyclization of the corresponding C_{20} isoprenoid precursor, followed by sequential oxidation of A-ring α-methyl of the olefin to a carboxyl function (Wang S.-Y. et al., 2002).

The quinones are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive. These compounds, being colored, are responsible for the browning reaction in cut or injured fruits and vegetables and are an intermediate in the melanin synthesis pathway in human skin. Their presence gives the material its dyeing properties. The switch between diphenol (or hydroquinone) and diketone (or quinone) occurs easily through oxidation and reduction reactions. The individual redox potential of the particular quinone-hydroquinone pair is very important in many biological systems; witness the role of ubiquinone (coenzyme Q) in mammalian electron transport systems. Vitamin K is a complex naphthoquinone and its antihemorrhagic activity may be related to its ease of oxidation in body tissues. Hydroxylated amino acids may be made into quinones in the presence of suitable enzymes, such as a polyphenoloxidase.

In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins, often leading to inactivation of the protein and loss of function. For that reason, the potential range of quinone antimicrobial effects is great. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism. As with all plant-derived antimicrobials, the possible toxic effects of quinones must be thoroughly examined (Cowan M.M., 1999).

The diterpenoid 6β-acetoxy-7α-hydroxyroyleanone (see **Figure I.2**), isolated from *Plectranthus grandidentatus*, is associated with various biological activities (Cerqueira F. et al., 2004; Marques C.G. et al., 2002). Previous studies on *Plectranthus* spp. revealed that their royleanones metabolites were active against the Gram-positive *Enterococcus* and *Staphylococcus aureus* strains. Preliminary SAR reasoning led to the conclusion that the presence of a C ring with a 12-hydroxy-*p*-benzoquinone feature and an oxidized B ring at C-6/C-7 positions were significant for the activity (Gaspar-Marques C. et al., 2006). In addition, Young *et al.* (Yang Z. et al., 2001) reported that the presence of additional benzyl groups influenced the anti-MRSA activities of phenolic abietanoids. Furthermore, a number of diterpene metabolites were found to be active against Gram positive bacteria, as totarol and abietic acid (**Scheme I.5**). These diterpenes have the ability to cross or damage the bacterial cytoplasmatic membrane due to their amphipathic character. Besides that, it was described that antibacterial activity may be modulated through an increase in lipophilicity and/or in hydrogen-bond donor/acceptor groups of the hydrophilic moiety (Yang Z. et al., 2001; Urzúa A. et al., 2008; Bernabeu A. et al., 2002). However, nor the complete mechanism of action neither their biological targets are known.

I.3. *Plectranthus* L'Hérit genus

Plectranthus is an Old World genus belonging to the Mint family (Lamiaceae), and there are about 350 known species which are widely distributed in Africa, Madagascar, India, Australia and a few Pacific islands. The family Lamiaceae contains several genera, such as sage (*Salvia*) and mint (*Mentha*), with a rich diversity of ethnobotanical uses. The popularity of *Plectranthus* is explained by their ornamental leaves, make free-flowering, easy and fast-growing and resist most pests and diseases. Most of the *Plectranthus* species of the world are soft trailing semi-succulent to sucullent herbs or shrubs widely distributed in the summer-rainfall savannahs and forested regions.

The name *Plectranthus* means spurflower (*plectron* = spur and *anthos* = flower) and refers to the characteristic spur at the base of the corolla tube of *Plectranthus fruticosus*, the first plectranthus to be placed in the genus. The name is confusing because very few plectranthus actually have this spur, but the French botanist L'Heritier, who described the genus in 1788, was unaware of this fact (Van Jaarsveld E., 2006; Lukhoba C.W. et al., 2006).

Some species of *Plectranthus* are difficult to identify because of a lack of clear-cut morphological criteria to discriminate not only among species within the genus but also among the closely related genera. This has resulted in numerous taxonomic problems in the naming of species with the result that species have often been placed in several closely related genera like *Coleus*. Phylogenetically they are divided into two major clades: clade 1 (corresponding to the formally recognized genus *Coleus*) were richer in number and diversity of uses than members of clade 2; and clade 2 (compromising the remaining species of *Plectranthus*). The high incidence of synonymy can lead to problems in uncovering a species ethnobotanical profile. Members of the '*Coleus*' clade are the most studied group both taxonomically and economically.

Plectranthus species in clade 1 are frequently used as medicines and are used to treat a range of ailments, particularly digestive, skin, infective and respiratory problems. *Plectranthus* used as foods, flavours, fodder and materials are also mostly found in Clade 1. Many species of *Plectranthus* grown as ornaments are resistant to diseases including *P. ecklonii* and *P. saccatus* (Lukhoba C.W. et al., 2006). A subsequent work to Lukhoba C.W. *et al.* shows that there are three main clades in the genus *Plectranthus*, (a sigmoid '*Coleus*' clade; a sigmoid *Plectranthus* clade; and a straight *Plectranthus* clade (Potgieter C.J. et al., 2009).

I.3.1. *Plectranthus ornatus* Codd.

The name '*ornatus*' means ornamental spurflower (tuin spoorsalie or skutblaarsalie), synonym *Coleus comosus*. Is a decumbent aromatic succulent herb, to 130-300 mm, freely branched, neat and cushion-shaped. *Corolla* 20-25 mm long, light blue to blue-purple, tube slightly deflexed, expanding to the mouth. *P. ornatus* is widespread in Central Africa from Ethiopia in the north to Tanzania in the south. It is semi-naturalized in South Africa. It occurs in glassland above the forest zone (Van Jaarsveld E., 2006).

Figure I.3. *Plectranthus ornatus* Codd. (Van Jaarsveld E., 2006).

Previous studies on the secondary metabolites of *Plectranthus ornatus* Codd. (see **Figure I.3**; synonym *P. comosus* Hochst. ex Gürke) reported the isolation of labdane, clerodane and halimane diterpenoids (Oliveira P. et al., 2005; Rijo P. et al., 2002; Rijo P. et al., 2005; Rijo P. et al., 2007), some of which have shown to possess a moderate antimicrobial activity against *Candida* species and selected Gram positive and Gram negative bacteria strains.

The first chemical study of this specie was on the acetone extract and yielded a novel neoclerodane derivative, plectrornatin A, and two labdane derivatives, plectrornatins B and C (see **Figure I.4**). Plectrornatins A and C showed moderate antimicrobial activity against five *Candida* species and selected Gram negative and Gram positive bacteria strains (Rijo P. et al., 2002).

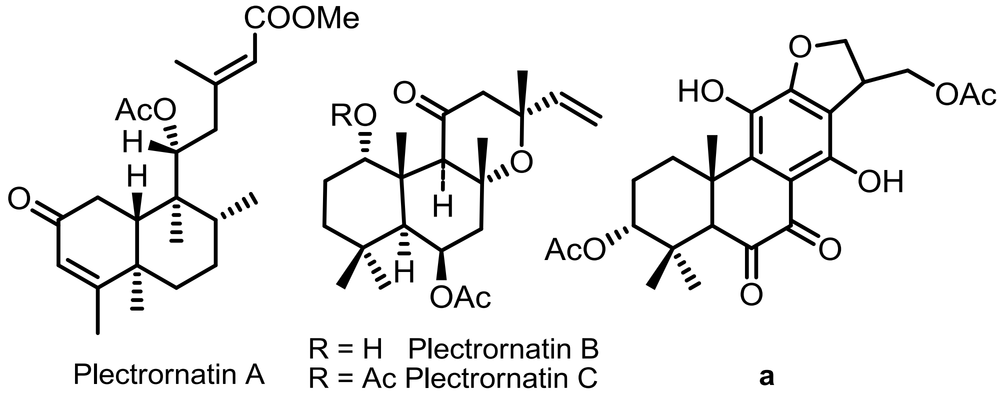


Figure I.4 Chemical structures of diterpenoids **a** and plectrornatins A, B and C.

Further phytochemical studies on the leaves of *P. ornatus* resulted on the isolation of a new diterpene (**a**; see **Figure I.4**; Albuquerque R.L. et al., 2003).

In Brasil, this specie is very used and is popular known as ‘boldinho’, ‘boldo-rasteiro’, ‘boldo gambá’ and ‘boldo-de-folha-miúda’. According to an ethnopharmacological survey, the leaves of *P. ornatus* are often used for stomach and liver diseases as substitute to *P. barbatus* (common name ‘falso-boldo’) also used for the same reason. Both are used for the same kind of biological activities: diuretic, antipyretic, analgesic, antibiotic and anti-inflammatory (Oliveira P. et al., 2005). A study of *P. ornatus* hexane and ethanol extracts on the anti-inflammatory activity mediated by glycocorticoide receptor revealed that only the hexane extract showed agonist activity on the glycocorticoide receptor (Franzotti E. M. et al., 2006). Mauro C. *et al.* described an anatomical study of the vegetative organs of *P. ornatus* (Mauro C. et al, 2008).

A phytochemical investigation of a hexane extract of the aerial parts of *P. ornatus* yielded three new neoclerodane diterpenoids, two labdane diterpenes (see **Figure I.5**) obtained for the first time as natural products, and several previously known substances, namely plectrornatin C (**Figure I.4**) and triterpenoids (Oliveira P. et al., 2005).

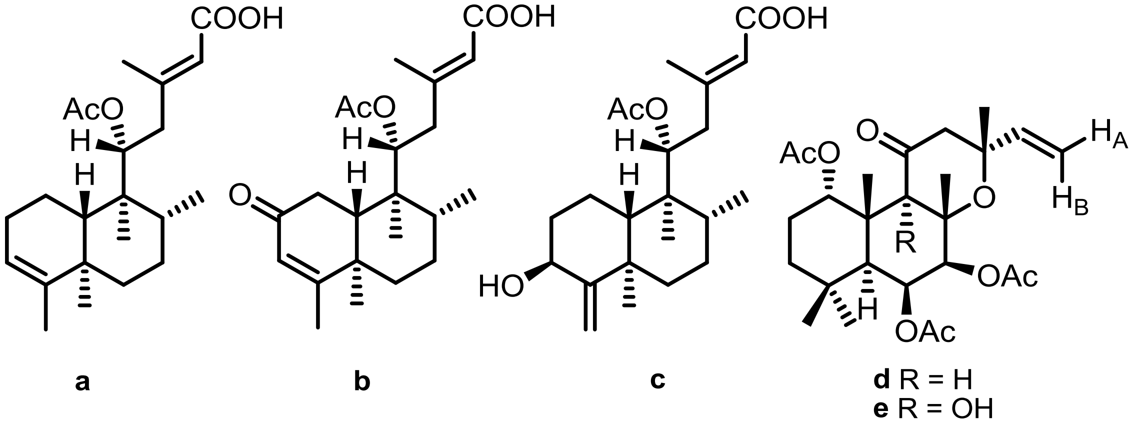


Figure I.5. Chemical structures of neoclerodane diterpenoids (**a**, **b** and **c**) and two labdane derivatives isolated from *P. ornatus* (**d** and **e;** Oliveira P. et al., 2005).

An extension of a previous study (Rijo P. et al., 2002) from the same acetone extract of *P. ornatus,* yielded the isolation of three labdane diterpenoids (see **Figure I.6**) that were found, together with known triterpenoids. The isolated compounds have structures closely related to that of forskolin, a very interesting substance isolated from *Coleus forskohlii* Briq. (Lamiaceae). This was the first report on those labdane diterpenoids as naturally occurring substances, although they were already known as semisynthetic derivatives (Rijo P. et al., 2005).

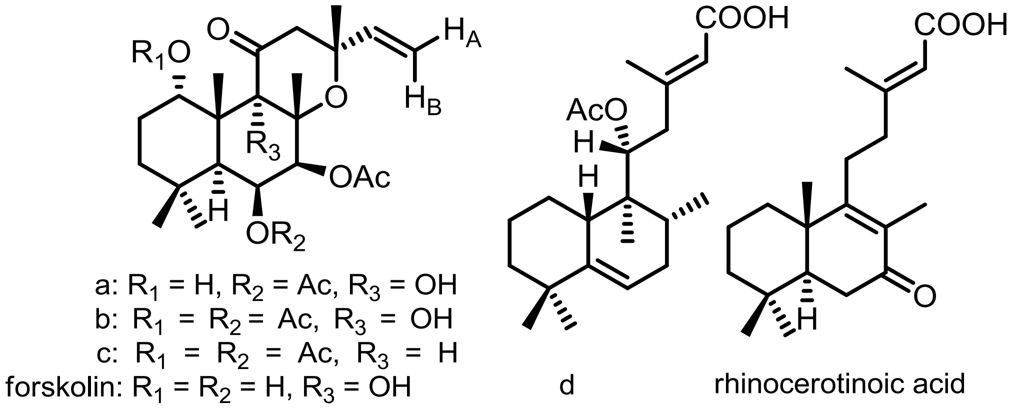


Figure I.6. Chemical structures of labdane diterpenoids **a**, **b** and **c**, forskolin, halimane **d** and rhinocerotinoic acid (Rijo P. et al., 2005; Rijo P. et al., 2007).

The persistence of the phytochemical study of the same acetone extract of *P. ornatus* (Rijo P. et al., 2002) yielded the isolation of a new halimane and a known labdane (see **Figure I.6**). This was the first report on the isolation of rhinocerotinoic acid from a Lamiaceae species and also on the presence of a halimane-type diterpene in a plant belonging to the genus Plectranthus (see **Figure I.6**). Moreover, only few 5-halimene derivatives have been isolated so far, and they were found in Euphorbiaceae and Asteraceae plants. It is of chemotaxonomic interest that *P. ornatus* contains labdane, halimane and clerodane diterpenoids (Oliveira et al., 2005; Rijo et al., 2002; Rijo P. et al., 2005).

The essencial oils from the leaves of *P. ornatus* were analysed and the antioxidant activity and chemical composition was also studied (Albuquerque R.L. et al., 2007).

I.3.2. *Plectranthus ecklonii* Benth.

Plectranthus ecklonii Benth. (see **Figure I.7**) was first collected in 1813 by the naturalist William Burchell in the Eastern Cape, according to Dr. Codd (1983). When Bentham (British taxonomist 1800-1884) described the plant in 1848, he named it after Ecklon (plant collector and traveler in South Africa, 1795-1868) who had collected it on the Katberg (South Africa). Bentham was apparently unaware of William Burchell's original collection. It thrives in partial shade where frost is not severe, and in cultivation gets to 1-3 m. The common names are Ecklon spurflower and Ecklon spoorsalie and is an erect aromatic freely branched shrub. Leaves are ovale to elliptical, the lower surface of the glands reddish-brown and with the corolla bluish-purple, rarely pink or white (Van Jaarsveld E., 2006).

Figure I.7. *Plectranthus ecklonii* Benth. (Van Jaarsveld E., 2006).

P. ecklonii is used in Zimbabwe to treat skin infections for its antibacterial and antifungal activities and in South Africa is used as ornamentals (Lukhoba C.W. et al., 2006) and traditionally used for treating stomach aches, nausea, vomiting and meningitis (Nyila M.A. et al., 2009). Studies on pollination of Plectranthus spp. (Lamiaceae) in southern Africa also refers to *P. ecklonii* (Potgieter, C.J. et al., 1999; Potgieter C.J. et al., 2009).

The phytochemical studies that have been reported to *P. ecklonii* includes the isolation of two novel isomeric *o*-quinones, ecklonoquinone A and B, two flavones (see **Figure I.8**) and the diterpenoid *p*-quinomethane parvifloron F (see **Figure I.9**; Uchida M. et al., 1980).

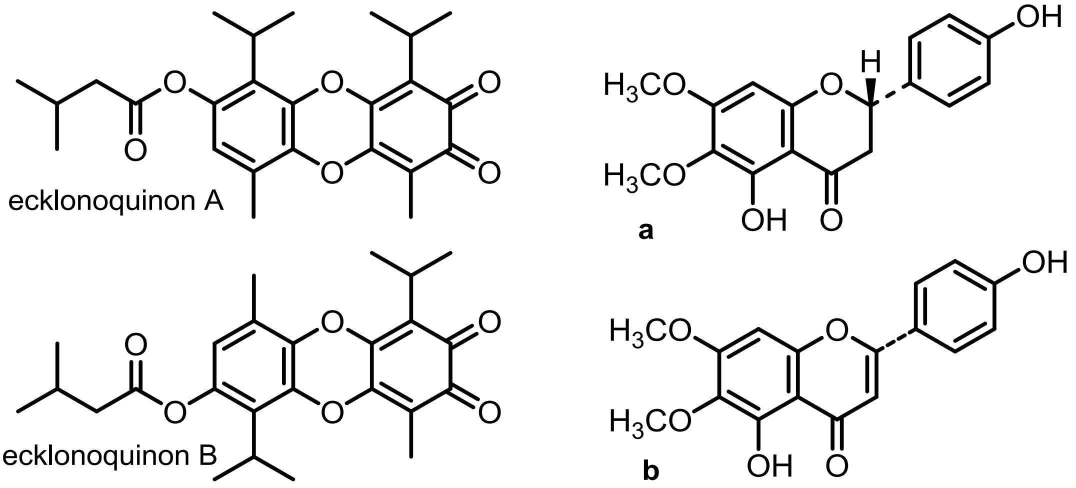


Figure I.8. Chemical structures of ecklonoquinone A and B and two flavones **a** and **b** (Uchida M. et al., 1980).

The *Plectranthus ecklonii* aqueous extract was studied and the major compound found was rosmarinic acid (see **Figure I.9**). The presence of this compound could explain the inhibition of acetylcholinesterase and antioxidant activities studied, but the extract was stronger inhibitor than rosmarinic acid (Figueiredo N.L., et al. 2010).

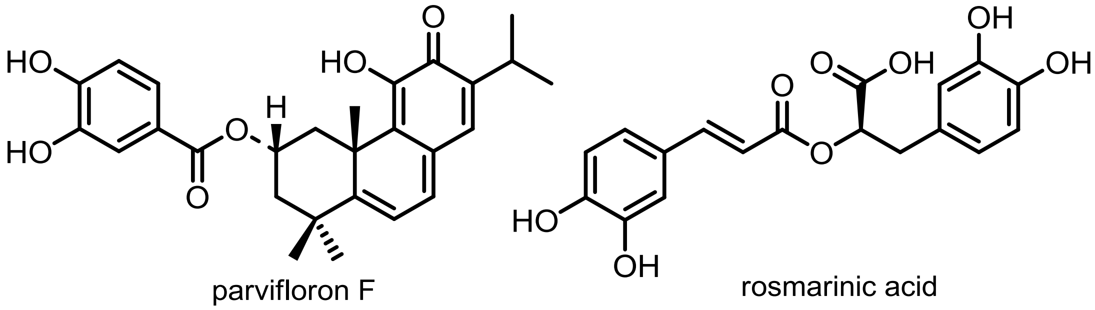


Figure I.9. Chemical structures of parvifloron F and rosmarinic acid (Uchida M. et al., 1980; Figueiredo N.L. et al., 2010).

A first report on the bioactivity of the ethyl acetate *P. ecklonii* extract and its constituents was done. The study tested the *P. ecklonii* extract and its isolated compounds for their activity on tyrosinase inhibition. Bioassay-guided fractionation of the ethyl acetate extract of the plant led to the isolation of two known compounds, parvifloron D (**Figure I.10**) and parvifloron F (**Figure I.9**; Rüedi P., Eugster C.H., 1978). The antibacterial activity of the extract and its isolated compounds correlated with the traditional use of the plant (Nyila M.A. et al., 2009). Van Zyl *et al.* also isolated those two known abietane diterpenes from *P. ecklonii* dichloromethane extract and the compounds showed antiplasmodial activity (van Zyl R.L. et al., 2008). Parvifloron D was also isolated on the acetone extract of *P. ecklonii* together with sugiol and showed antibacterial activity (see **Figure I.10**; Simões M.F. et al., 2010b).

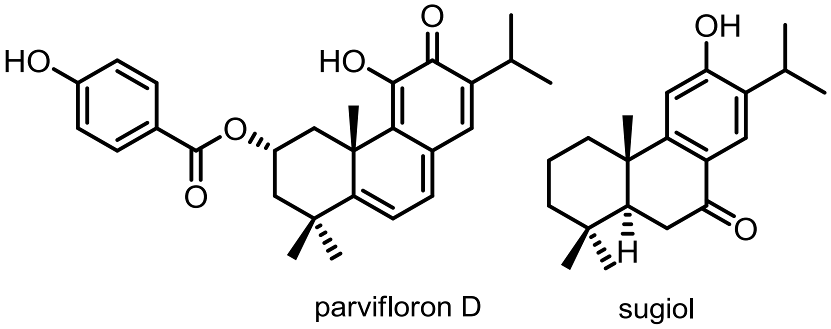


Figure I.10. Chemical structures of parvifloron D and sugiol (Simões M.F. et al., 2010b).

I.3.3. *Plectranthus porcatus* Winter & Van Jaarsv.

P. porcatus (see **Figure I.11**) was discovered by Pieter Winter of the Natural Herbarium in Pretoria whilst searching for more populations of new species of *Raphionacme* in the Leolo Mountains (South Africa). Its distribution is from northern Leolo Moutains to Sekukuniland and Limpopo Province. As habitat the climate is subtropical, with hot summers and dry, sunny winters with light frost. Common names are ribbed spurflower and Sekukuni spoorsalie. The epithet '*porcatus*' alludes to the ridged nature of most stem internodes. *P. porcatus* are perennial soboliferous multi-stemmed aromatic shrub to ± 1.2 m and 1.5 m in diameter, aerial stems sparingly branched. Corolla 2-lipped, somewhat sigmoid, white to slightly mauve-tinged, glandular hairy and sparsely dotted with sessile orange glandular trichomes.

Figure I.11. *Plectranthus porcatus* Winter & Van Jaarsv. (Van Jaarsveld E., 2006).

The only phytochemical study from *P. porcatus* yielded a new diterpenoid, (13*S*,15*S*)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione (see **Figure I.12**). This was the first study either chemically or pharmacologically, on this specie and the compound showed no antibacterial activity against Gram-negative bacteria and *Candida albicans* (yeast strain) tested (Simões M. F. et al., 2010a).

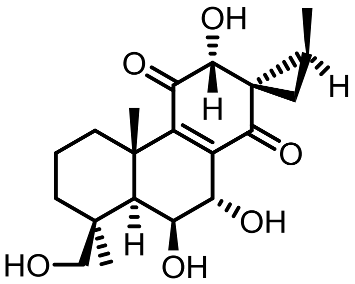


Figure I.12. Chemical structure of (13*S*,15*S*)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione (Simões M.F. et al., 2010a).

I.3.4. *Plectranthus saccatus* Benth.

The name from the latin ‘sacattus’ means bag-shaped or pouched, referring to the base of the flower. *P. saccatus* (see **Figure I.13)** was first collected in 1832 by Drège, a German botanical collector and traveler. Medley Wood, who collected it in Ngoye Forest, rated it hightly. The common name is stoep jacaranda, stoep jacaranda and sak-spoorsalie. *P. saccatus* is procumbent, decumbent or erect aromatic shrub up to 2m, stems puberulous. *Corolla* 11-30 mm long, mauve to blue (rarely white). Two subspecies are recognized. The recognition of two varieties within this subspecies is contentious, and a careful consideration of corolla lengths shows that they are highly variable, representing a continuum rather than forming clearcut groups within the species. Subsp. *saccatus* is a decumbent to erect shrub with a widespread distribution and a large saccate corolla and is very variable with several local forms or ecotypes. *P. pondoensis* (Pondo stoep jacaranda) is distinguished its trailing habit, and its flexible stems up to 4 m long bearing distinctly succulent glandular pubescent leaves. It usually has a short corolla tube 6-10 mm long, compared to the much larger corolla of subsp. *saccatus*. As habitat it prefers forest or forest margins, in hilly terrain or deep river gorges (Van Jaarsveld E., 2006).

There are few studies involving *P. saccatus,* Lukhoba *et al.* reported *P. saccatus* as having horticultural use (Lukhoba C.W. et al., 2006) and Potgieter *et al.* presented studies of pollination of *Plectranthus* spp. (Lamiaceae) where *P. saccatus* was described as having a sigmoid corolla shape that limits the type and size of insects that can access nectar and act as pollinators (Potgieter C.J. et al., 1999; Potgieter C.J. et al., 2009).

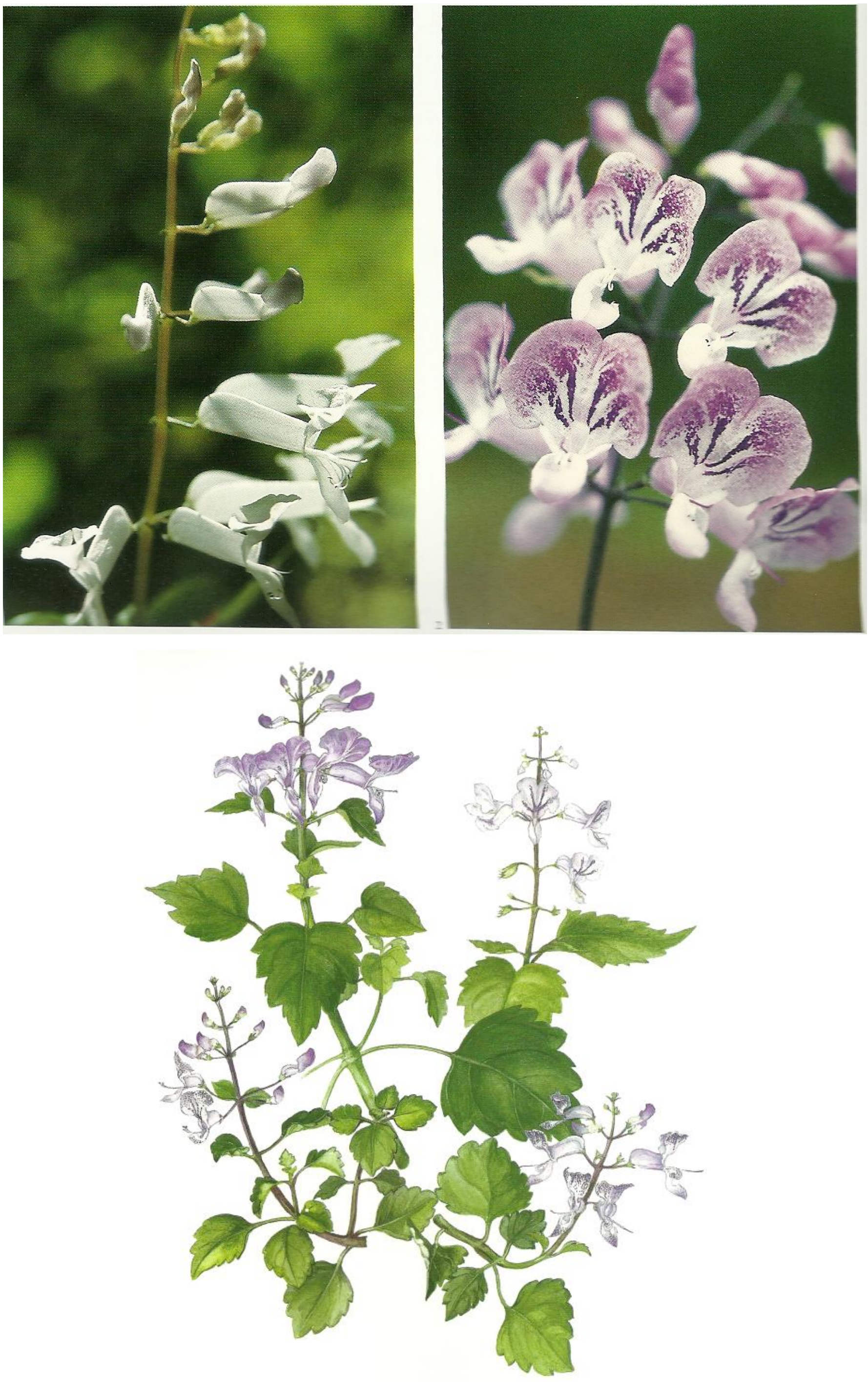


Figure I.13. *Plectranthus saccatus* Benth. (Van Jaarsveld E., 2006).

The first chemical study of *Plectranthus saccatus* Benth. described a bio-assay guided fractionation of an acetone extract of leaf material, that resulted in the isolation of a novel beyerane diterpenoid and other minor beyerane (**a** and **b** respectively; see **Figure I.14**). The new compound showed insect antifeedant activity against *Spodoptera littoralis* (Wellsow J. et al., 2006). A new diterpenoid, ent-7α-acetoxy-15-beyeren-18-oic acid (**c**; see **Figure I.14**) have been isolated recently from an acetone extract and showed no antimicrobial activity against Gram-negative bacteria and *Candida albicans* (yeast strain) tested (Simões M.F. et al., 2010).

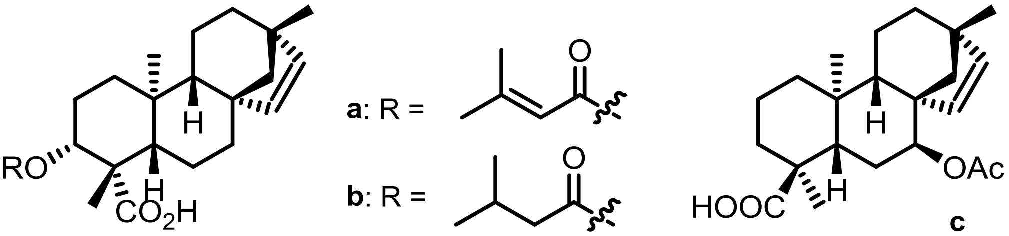


Figure I.14. Chemical structures of beyeranes **a, b** (Wellsow J. et al., 2006) and **c** (Simões M.F. et al., 2010).

I.4. Biological activity of *Plectranthus*

Many *Plectranthus* species are plants of ornamental, economic and medicinal interest, and with a rich diversity of ethnobotanical uses. Several species are used all around the world as vermicides, antiseptics and purgatives, for the treatment of ear infections, toothache and stomach ache, as a remedy for vomiting and nausea, and against a vast array of other diseases (Rijo P. et al., 2007). Some examples: *P. barbatus* and *P. bojeri* are indicated for the treatment of pneumonia and *P. amboinicus* revealed anti-MTB activity. Moreover *P. aegypticus*, *P. ambiguus*, *P. caninus*, *P. edulis*, *P. elegans*, *P. glandulosus*, *P. hadiensis*, *P. lanceolatus*, *P. laxiflorus*, *P. madagascarensis*, *P. mollis*, *P. montanus*, and *P. stolzii* are used to relieve several respiratory diseases (Lukhoba C.W., 2006).

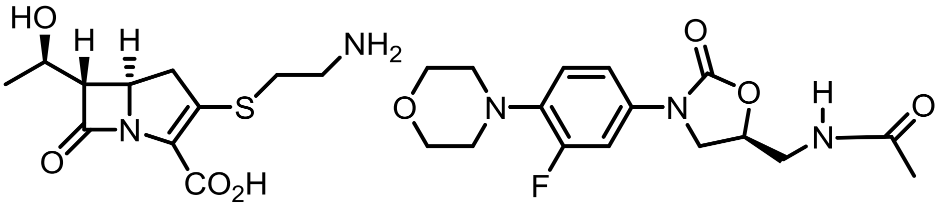
Several studies on species from the genus Plectranthus describe various biological activities, providing scientific evidence for their use in traditional herbal preparations (Maistry K., 2003). The isolation of compounds from *Plectranthus* is important to validate the popular use of the plant. The active components indicate that these compounds contribute for the activity of *Plectranthus* species (Rodrigues P.A. et al., 2010).

Many biological activities have been attributed to this genus and antimicrobial activity is very cited (Maistry K., 2003). Other examples are extensive like acaricidal activity (Rasikari H., 2007), herpetic inhibitory and antioxidant properties (Gaspar-Marques C. et al., 2008) or gastroprotective effect (Rodrigues P.A. et al., 2010).

Some biological activities described for Plectranthus species (and tested in chapter **IV**) will be presented, namely the main antimicrobial activity, antioxidant, acetylcholinesterase inhibitory activity and anti-inflammatory activity.

I.4.1. Antimicrobial activity

Natural products have been a rich source in providing leads for the development of drugs for the treatment of bacterial infections. However, beyond the discovery of the natural product, thienamycin and the synthetic lead, oxazolidinone in the 1970s, there has been a dearth of new compounds (see **Figure I.15**).



thienamycin

oxazolidinone

Figure I.15. Chemical structures of thienamycin and the synthetic lead, oxazolidinone.

A number of natural products, e.g. aspirin and morphine (see **Figure I.16**), are still in use today.

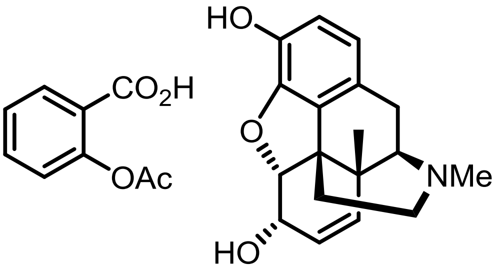


Figure I.16. Chemical structures of aspirin and morphine.

Drugs derived from microbial fermentations have played an equally seminal role in modern discovery and have revolutionized medicine, saving both human and animal lives. Like other areas of drug discovery, there are two sources for antibiotic leads—natural products and synthetic compounds. Natural products have been the mainstay in providing novel chemical scaffolds for many drugs, as well as leads that were chemically modified and developed as antibacterial agents. They generally possess complex architectural scaffolds and densely deployed functional groups, affording the maximal number of interactions with molecular targets, often leading to exquisite selectivity for pathogens versus the host. The successes of the Golden Age of antibiotics led to considerable excitement. Antibiotic resistance began to emerge, and vancomycin becoming the antibiotic of last resort for the treatment of Gram-positive bacterial infections in hospitals. After a number of years of use, emergence of nosocomial vancomycin-resistant *Staphylococcus aureus* (VRSA) and multidrug-resistant (MDR) strains of this organism and *Enterococcus faecalis* strains have become a common occurrence. It is now accepted that resistance is inevitable, and that resistance management will be part of the process for all new antibiotics (Singh S.B. and Barrett J.F., 2006; Gibbons S., 2008). There is therefore a continuing need to discover and characterize new classes of antibiotics to reduce the pressures of bacterial resistance (Cordell G.A., 2000; Xiao Z.Y. et al., 2007).

Considering the multidrug resistance (MDR), Gibbons S. *et al.* revealed the molecular similarity of MDR in an attempt to find some common features of structurally unrelated inhibitors. The shape of the molecule, aromatic rings and the presence of some polar atoms determine the potency of MDR inhibitors (Zloh M. and Gibbons S., 2004).

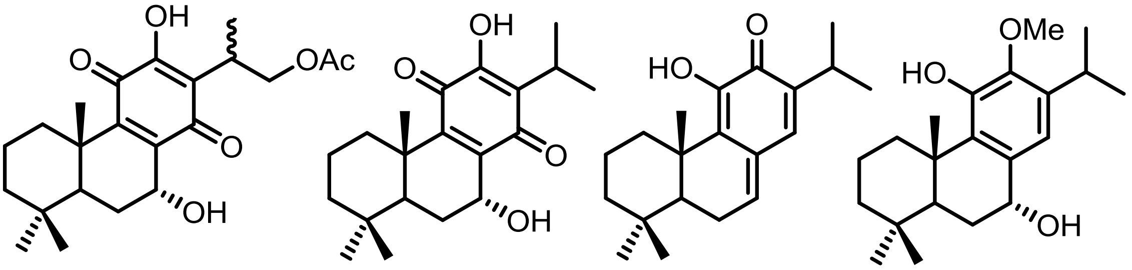
Staphylococcus aureus is a commensal organism that is commonly cited as being a major hospital-acquired pathogen. Strains of this species that are resistant to β-lactams, notably the methicillin-resistant *Staphylococcus aureus* (MRSA) strains, have been described from clinical sources for over forty years. It is the ability of this Gram-positive organism to acquire resistance to practically all useful antibiotics that is the cause for considerable concern. For example, in the UK there has been a significant increase in the number of death certificates which mention MRSA with 47 citations in 1993 rising to 398 in 1998. The occurrence of a fully vancomycin resistant strain of MRSA in the US in 2002 indicated that the successful treatment of MRSA strains by the use of the glycopeptides antibiotic is not guaranteed (Gibbons S., 2004).

Pressure to find novel antibacterials with new modes of action will drive exploitation of plant sources as antimicrobials. The choice is logical given the ecological rationale that plants produce natural products as a chemical defence against microbes in their environment. Pharmaceutical companies have neglected natural products preferring to utilise combinatorial chemistry libraries as a source of chemical diversity. Unfortunately such libraries lack the true chemical diversity that natural products display (extensive functional group chemistry and chirality) and these libraries are poor for discovery purposes but have potential in lead optimization (Gibbons S., 2004).

A review study found a number of articles published on the antimicrobial activity of medicinal plants in PubMed during the period between 1966 and 1994, and found 115; however, in the following decade between 1995 and 2004, this number more than doubled to 307. In these studies is possible to find a wide range of criteria. Many focus on determining the antimicrobial activity of plant extracts found in folk medicine (*Plectranthus hadiensis extracts,* Mothana R.A. et al., 2008; *Plectranthus amboinicus,* Gurgel A.P.A.D. et al., 2009b), essential oils or isolated compounds. Some of these compounds were isolated or obtained by bio-guided isolation after previously detecting antimicrobial activity on the part of the plant. A second block of studies focuses on the natural flora of a specific region or country; the third relevant group of papers is made up of specific studies of the activity of a plant or principle against a concrete pathological microorganism (Ríos J.L., Recio M.C., 2005).

In this way, it is of interest, to explore the potential medicinal and economic uses of *Plectranthus*, for the reasons that some are African medicinal plants with potential use in primary healthcare, and that this genus is related to Salvia, Coleus and Rabdosia genera used in ethnomedicine all over the world (Gaspar-Marques C. et al., 2006).

Diterpenes is one of the largest groups of plant derived natural products with anti-staphylococcal activity and certain plant taxa and diterpene classes are well represented, in particular, the genus from the Lamiaceae or mint plant family. (Ulubelen A. et al., 2002). Other taxa in the mint family include *Plectranthus hereroensis*, which produces an acetylated abietane quinone (**a**; **Figure I.17**; Baptista O. et al., 1995) related to horminone, and *P. elegans* (**b** and **c**; **Figure I.17**; Dellar J.E et al., 1996) with similar activities against Gram-positive bacteria. The authors suggested that these compounds may have a role in the chemical defence of *Plectranthus.* Interesting sources of antibacterial leads, such as abietane type diterpenes are potential to exploit this taxonomic group and the abietane diterpene class (Gibbons S., 2004).



a horminone b c

Figure I.17. Chemical structures of abietanes **a**, **b**, **c** and horminone (Gibbons S., 2004).

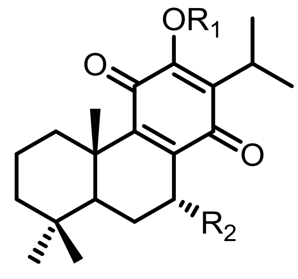
A Structure-Activity Study of Antibacterial diterpenoids (several kaurane, abietane, labdane, clerodane and totarane skeletons), led to the identification of some structural requirements for their action and suggested two structural requirements for activity of these and related compounds. These structural features included a substituted decalinic system, capable of insertion into a lipophilic region, and a hydrophilic fragment possessing one hydrogen-bond-donor group, capable of interactions with hydrogen-bond-acceptor groups in the membrane. These structural requirements are responsible for an optimal insertion of these and related compounds into cell membranes, as suggested by the results of docking of some of these compounds into a model phospholipid bilayer. The analysis of the antibacterial activities on related diterpenoids thus offers an insight into the structural requirements for the antibacterial activity of these and related compounds and contributes to the future design of other antibacterial molecules (Urzúa A. et al., 2008).

In previous Plectranthus genus isolation works were reported several antimicrobial abietane diterpenoids from *Plectranthus hereroensis* (Batista O. et al., 1994; 1995), *P. grandidentatus* (Teixeira A.P. et al., 1997) and *P. elegans* (Dellar J. et al., 1996). Salvia is another genus that have been study for the isolation of abietane diterpenoids (Martínez-Vázquez M. et al., 1998; Chen X. et al., 2002; Ulubelen A. et al., 2000; 1996; 2001) but antimicrobial diterpenoids were also isolated from other family plants (Dellar et al, 1996; Zgoda-Pols J.R. et al., 2002; Politi M. et al, 2003; Smith E. et al., 2005; Jeong S.-I. et al., 2006; Yoshikawa et al., 2008). Considering the antibacterial activity, abietane diterpenoids (Woldemichael G.M. et al., 2003) namely, oxidized abietane diterpenes (Gaspar-Marques C. et al., 2006; Mitchell M.O., 2007; Mulvey M.R. and Simor A.E., 2009) are frequently reported on reviews (San Feliciano A., 1993), or structural effects and structure-activity studies (Gigante B. et al., 2002; Feio S.S., 1999; Moujir L.M., 1993). Due to the multiple pharmacological activities described for these oxidized abietane metabolites, it is thinkable to use them as models for the search of further bioactive drugs (Gaspar-Marques C. et al., 2006). This prompted Michalet *et al*. and other researchers groups to synthesize derivatives in order to provide structure–activity relationships and to access more potent inhibitors. Synthesis of derivatives is a strategy to potentiate antimicrobials, considering the natural product molecule a good starting point for structure–activity relationships (SARs). Searching for inhibitors from natural sources is therefore an attractive strategy to access a greater range of active compounds (Michalet S. et al., 2007).

Natural products have inspired many developments in organic chemistry, leading to advances in synthetic methodologies and to the possibility of making analogues of the original lead compound with improved pharmacological or pharmaceutical properties. Natural product scaffolds have also been well recognized as being ‘privileged’ structures in terms of their ability to be the basis for successful drugs (Harvey A.L., 2008; Saleem M. et al., 2010).

There are several examples in the literature of chemical modifications of abietane (San Feliciano A. et al., 1993; Moujir L. et al., 1993; Feio S.S. et al., 1999; Yang Z. et al., 2001; Gigante B. et al., 2002; Tada M. and Ishimaru K., 2006; Rijo P. et al, 2010), totarane (Evans G.B. et al., 1999; Evans G.B., Furneaux R.H., 2000a; Evans G.B., 2000b), pimarane, podocarpane and kaurane diterpenes to evaluate their antibacterial activity, in an attempt to define some of the structural parameters needed for improving antimicrobial activity (Tada M. et al., 2010).

Another study described some derivatives prepared in an attempt to define some of the structural parameters needed for antimicrobial activity. 12-Benzoylhorminone was found to be more active against the tested organisms than the starting materials. The lack of antimicrobial activity of the 6,7-dehydroroyleanone clearly demonstrated that the presence of a hydroxyl group at C-7 in these molecules is essencial for such activity (see **Figure I.18**; Martínez-Vázquez M. et al., 1998).



12-benzoyl horminone: R_1 = COPh, R_2 = OH
6,7-dehydroroyleanone: R_1 = H, R_2 = H, $\Delta^{6,7}$

Figure I.18. Chemical structures of 12-Benzoylhorminone and 6,7-dehydroroyleanone.

The ability of many *Plectranthus* species to produce antibacterial metabolites, particularly of the diterpene class, prompted Gibbons S. *et al.* to investigate the chemistry and antibacterial activity of extracts from *Plectranthus ernstii*. Three new diterpenoids including two pimaranes (**a** and **b**; **Figure I.19**) and a labdane (**c**; see **Figure I.19**) were isolated from the whole herb of *P. ernstii*. Pimarane **a** exhibited moderate antistaphylococcal activity against a range of multidrug-resistant (MDR) and methicillin-resistant (MRSA) strains of *Staphylococcus aureus* and all three diterpenes exhibited antimycobacterial activity. Certain diterpenes, such as totarol, have been shown to possess multifaceted activities as potent antibacterials and at the same time behaving as efflux pump inhibitors. The simple change (from 7-hydroxy of compound **a** to 7-oxo of compound **b**) resulted in a loss of antistaphylococcal activity, presumably as a result of increased lipophilicity and poorer uptake (Stavri M. et al., 2009).

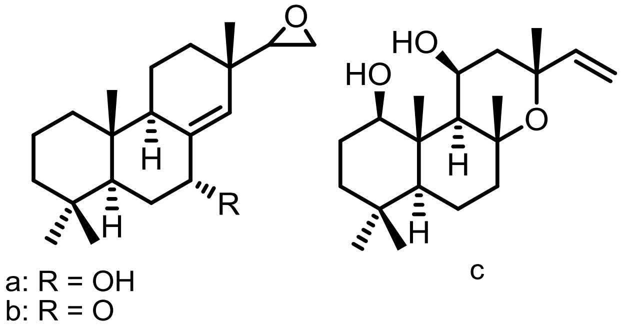


Figure I.19. Chemical structures of pimaranes (**a** and **b**) and labdane (**c**) isolated from *P. ernstii.*

The mechanism of action of terpenes as antimicrobial agents is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds. A study revealed that increasing the hydrophilicity of kaurene diterpenoids (Mendonza L. et al., 1997) by addition of a methyl group drastically reduced their antimicrobial activity (Cowan M.M., 1999).

Considering the mode of action of antibacterial diterpenes there is a theoretical study on the structural and electronic parameters of the horminone molecule (see **Figure I.18)**, an abietane diterpene quinone, by means of all-electron calculations. The results allow the identification of the negative site of horminone (HM) most favorable for its binding to the Mg^{2+} ion. The HM–Mg^{2+} complex is assumed to play a significant role in the antibacterial activity. First, it penetrates the membrane cell. Then, through its interaction with rRNA, it inhibits the protein synthesis in several types of bacteria and it was also found to be cytotoxic against mammalian tumor cells. It is worth mentioning that horminone and derivatives are structurally related to the tetracycline moieties, specifically with oxitetracycline, a well-known commercial antibiotic. These results suggest that the carbonyl and hydroxyl groups, attached at the “7” position of ring B and at the “11,” “12,” and “14” positions of the quinoide ring, may play a significant role in the reactivity properties of these compounds (Nicolás I. et al., 2003). Further studies of Castro *et al.* continued on another theoretical study of the complexes of horminone with Mg^{2+} and Ca^{2+} ions and their relation with the bacteriostatic activity (Nicolás I. and Castro M., 2006).

I.4.2. Antioxidant activity

Antioxidants are vital in combating the free radicals, which damage human cells under oxidative stress conditions and an imbalance of free radicals may cause grave disturbances in cell metabolism. Free radicals are instable species because they have unpaired electrons and seek stability through electron pairing with biological macromolecules (Ozyurt D. et al., 2007). Active oxygen (hydroxyl, peroxyl radicals and singlet oxygen) is highly toxic and an important causative agent of many diseases including cancer, heart disease, cataract and congestive disorders. Antioxidant compounds block the oxidation processes that produce free radicals which contribute towards these chronic diseases and aging (Muraina I.A. et al., 2009). In addition, it is also speculated to be pathologically important in various neurodegenerative processes including cognitive deficits and oxidative stress, that is associated with the pathogenesis of Alzheimer’s disease (AD) and cellular characteristics of this disease are either causes or effects of oxidative stress. Interestingly, intake of polyphenols through diets rich in fruits, vegetables and beverages such as red wine was stated to reduce incidence of certain age related neurological disorders including macular degeneration and dementia. Therefore, the data suggests that high dietary or supplemental consumption of antioxidants in people may reduce the risk of AD (Orhan I. et al., 2007).

Recently there has been an increased concern about synthetic antioxidants. This is partly due to their possible toxicity against animal DNA. This may explain the interest in examining plant extracts as a source of cheaper and effective antioxidants and the growing interest in nutraceuticals (Ozyurt D. et al., 2007).

Vitamin C plays an important role in animal health as an antioxidant supplement by inactivating free radicals produced through normal cellular activity and diverse stressors. Plant-derived antioxidants exert their effects by enhancing the levels of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase or by lowering the levels of lipid peroxides in the blood or liver. It is recognized that antioxidant (mainly polyphenolic) compounds from plant extracts can act by either free radical scavenging, singlet oxygen quenching, chelating of transitional metal such as iron, as well as a reducing agents and activator of antioxidative defense enzyme systems to suppress radical damage in biological system (Muraina I.A. et al., 2009).

Interest has increased in naturally occurring antioxidants since they may be used to protect humans from oxidative stress damage. For example the use of antioxidants may slow the progression of AD and minimize neuronal degeneration. The chemical investigations on plants of the *Lamiaceae* and their antioxidant activitiy was investigated, as well as, the isolated diterpenoids with the *ent*-kaurane skeleton, oxidized on carbons C-7, or C-15 and C-18 (Ertas A. et al., 2009). Other study resulted in two new antioxidative diterpenoids (**a** and **b**, see **Figure I.20**), having the abietane nucleus, along with two known diterpenoids, parvifloron E and F (see **Figure I.20** and **Figure I.9)**. The compounds were isolated from the leaves of *Plectranthus nummularius* Briq. (Narukawa Y. et al., 2001).

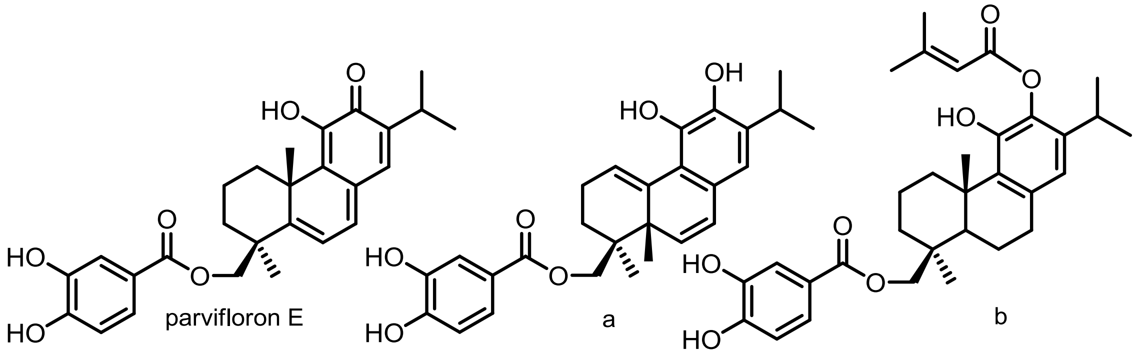


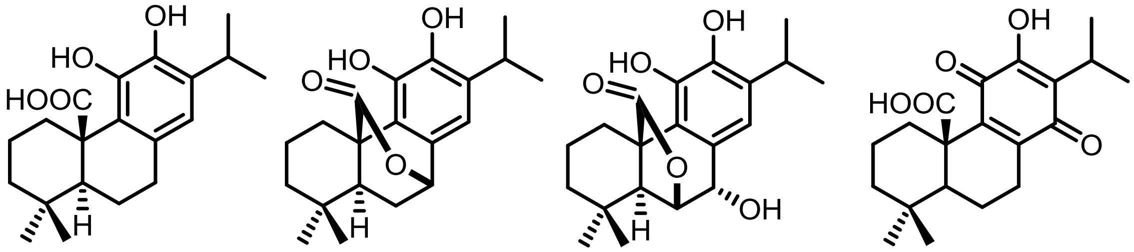
Figure I.20. Chemical structures of diterpenoids **a**, **b** and parvifloron E.

A study on Plectranthus plants reported the antioxidant activity and chemical composition of the essential oils of *P. grandis* and *P. ornatus* fresh leaves. The oil of *P. ornatus* showed a higher antioxidant activity than that of *P. grandis*, probably due to its higher yield of the phenolic compounds (Albuquerque R.L. et al., 2007). Another study of methanolic extracts of plants including *Plectranthus hadiensis* were analyzed for their antioxidant activity, and showed high free radical scavenging activity. The phytochemical screening demonstrated the presence of different types of compounds like flavonoids, terpenoids and others, which could be responsible for the obtained activities (Mothana A. et al., 2008). Recent works, revealed studies concerning the antioxidant activity of *Plectranthus* sp., the aqueous extract of *P. barbatus* leaves (Maioli M.A. et al., 2010) and the ethanol extract of *P. amboinicus* (Palani S. et al., 2010).

Essential oils and various extracts of plants (Lamiaceae) have been screened for their potential capacity to scavenge free radicals. It is known that many plants with medicinal value are used as condiment or aromatic and the inclusion of this kind of plants in those studies may determine their ability as functional foods or even pharmafoods. In addition, considering questions about the quality and safety of foods, only selected food additives are allowed. Because of the possible toxicities of the synthetic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), increasing attention has been directed toward natural antioxidants. Antioxidant activities of plants from *Salvia* genus, whose isolated compounds are closely related to

Plectranthus, were analyzed and the free radical scavenger activity was higher for the polar extracts (Tepe B. et al., 2004; Ferreira A. et al., 2006).

In a recent study, sage (*Salvia officinalis*) and some other spices were used as antioxidant, and it was reported that the addition of sage and the other spices retarded the process of oxidation which proved sage to be more effective than the mixture of the other spices. *Salvia* is the largest genus in mint family (Lamiaceae) and those species can be one of natural sources with good culinary qualities, and their extracts are commonly used to increase the shelf life of foods. Besides their antioxidant, antiseptic, and antibacterial properties they possess antifungal, antiviral, cytotoxic, carminative, diuretic, hypoglycemic, hemostatic, wound healing, spasmolytic, tranquilizer and sedative activities. Abietane diterpenoids carnosic acid, royleanonic acid, carnosol and rosmanol have good antioxidant abilities isolated from sage plants (see **Figure I.21)**. The oxidation cascade of carnosic acid, which causes formation of other phenolic and quinone abietanes in Lamiaceae plants, was proposed and antioxidant activity mechanism studies on abietanes have continued.



carnosic acid carnosol rosmanol royleanonic acid

Figure I.21. Chemical structures of carnosic acid, royleanonic acid, carnosol and rosmanol.

For this purpose, *Salvia barrelieri* extract and its diterpenoids were investigated for potential antioxidant activity. The results indicate that the extract and the isolated abietane diterpenes, related to royleanone and horminone structures, particularly in uroyleanol (**Figure I.22**) are promising antioxidants (Kabouche A. et al., 2007).

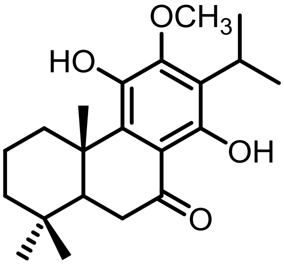


Figure I.22. Chemical structure of uroyleanol (Kabouche A. et al., 2007).

The antioxidant activity of some extracts from *Salvia* sp., particularly, the ethyl acetate and methanol extracts were observed to be highly active. The data indicates that the polar extracts for antioxidant activity are worth further phytochemical evaluation for identifying their active components (Orhan I. et al., 2007). Concerning the antioxidant properties of Lamiaceae family, and future phytochemical evaluation of active compounds, there are several reports of *Salvia* sp. extracts in literature (Erdemoglu N. et al., 2006; Tosun M. et al., 2009; Nickavar B. et al., 2007; Orhan I. et al., 2007).

I.4.3. Acetylcholinesterase inhibitory activity

The major biological role of acetylcholinesterase (AChE) is the termination of nerve impulse transmission at the cholinergic synapses, by rapid hydrolysis of the cationic neurotransmitter acetylcholine (ACh). According to the cholinergic hypothesis, memory impairments in patients with this senile dementia disease are due to a selective and irreversible deficiency in the cholinergic functions in brain. This serves as a rationale for the use of AChE inhibitors for the symptomatic treatment of Alzheimer's disease (AD) in its early stages (Ahmad V.U. et al., 2005).

AD is a degenerative neurological disorder characterized by senile plaques containing amyloid β protein and loss of cholinergic neuromediators in the brain. The most remarkable biochemical change in AD patients is a reduction of acetylcholine (ACh) levels in the hippocampus and cortex of the brain. Therefore, inhibition of acetylcholinesterase (AChE), the enzyme responsible for hydrolysis of ACh at the cholinergic synapse, is currently the most established approach to treating AD. While

AChE is found in all excitable tissue, whether nerve or muscle, in most erythrocytes and in placental tissue, BChE (butyrylcholinesterase) is present more commonly in the body including within the central and peripheral nervous system, liver and plasma (Orhan I. et al., 2007).

Inhibition of AChE serves as a strategy for the treatment of Alzheimer's disease (AD), senile dementia, ataxia, myasthenia gravis and Parkinson's disease. There are a few synthetic medicines, e.g. tacrine, and the natural product-based rivastigmine for treatment of cognitive dysfunction and memory loss associated with AD (see **Figure I.23**). These compounds have been reported to have their adverse effects including gastrointestinal disturbances and problems associated with bioavailability which necessitates the interest in finding better AChE inhibitors from natural resources. Acetylcholinesterase (AChE) inhibitors are the only registered drugs used to treat Alzheimer's disease.

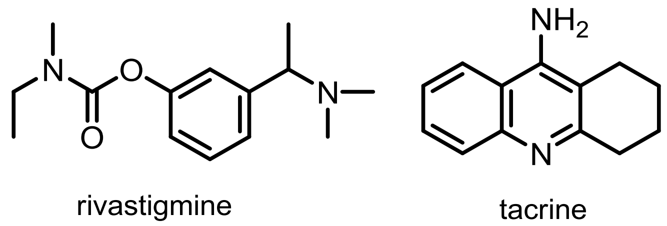


Figure I.23. Chemical structure of tacrine, and rivastigmine.

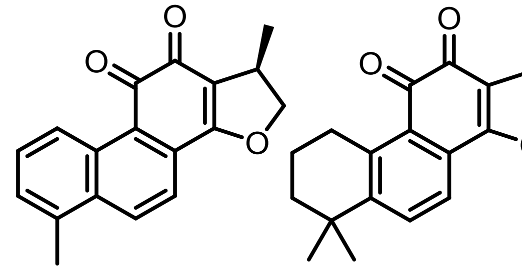
The majority of studies have focused on enhancement of cholinergic function, with particular attention being paid to the anti-ChE alkaloids, such as galantamine. This is perhaps a reflection of the relative success of the use of AChE inhibitors in AD patients, and a lack of understanding of the pathological mechanisms that occur in AD and the subsequent targets for treatment. However, numerous studies have shown that a diverse array of compounds, and not just the anti-ChE alkaloids, may have potential for efficacy in cognitive disorders (Howes M.-J.R. and Houghton P.J., 2003). Literature indicates that plants may yield novel AChE inhibitors, other than alkaloids and monoterpenes, which may have advantages in relation to efficacy and adverse-effect profile (Howes M.-J.R. et al., 2003). Triterpene ursolic acid has also been reported to possess AChE inhibitory activity (Mukherjee P.K. et al., 2007).

New AChE inhibitors may contribute to the design of new pharmaceuticals and supply information which will facilitate the understanding of the interaction between inhibitors and the enzyme (Ren Y. et al., 2004).

Amongst plants that have been investigated for dementia therapy, *Salvia* is one of the most numerous genera within the family Lamiaceae and grows in many parts of the world (Mukherjee P.K. et al., 2007). *Salvia* species (Lamiaceae) have been recorded to be used against memory loss in European folk medicine (Orhan I. et al., 2007). *Salvia officinalis* (sage) has previously been shown to possess *in vitro* cholinesterase inhibiting properties, enhancing mnemonic performance and improving mood in healthy young participants. The history of the use of sage (several plants of the Salvia genus) as a medicinal treatment spans several millennia, and both ancient and modern cultures. A series of studies have shown that both *Salvia lavandulaefolia* (Spanish Sage) and *Salvia officinalis* (Garden Sage) inhibit the cholinesterase group of enzymes. These Salvia species are both edible, not toxic (at the doses that are liable to be utilized in humans) and have long histories of safe usage (Kennedy D.O. et al., 2006; Adams M. et al., 2007; Kamatou G.P.P. et al., 2008; Savelev S. et al., 2003; Perry N.S.L. et al., 2000). Several extracts from *Salvia* species (Lamiaceae) were examined for the anticholinesterase activity and the results indicated that nonpolar extracts are worth further phytochemical evaluation for identifying their active components (Orhan I. et al., 2007).

Considering the anti-cholinesterase activity of terpenoids reported to date relatively weak, the analogues of active terpenoid compounds may be developed to enhance efficacy (Mukherjee P.K. et al., 2007).

The first example of diterpenoids as acetylcholinesterase (AChE) inhibitors were two *o*-quinone diterpenes (dihydrotanshinone and cryptotanshinone, see **Figure I.24**) isolated from *Salvia miltiorhiza* (Ren Y. et al., 2004). Dihydrotanshinone had a seven-fold higher activity than cryptotanshinone suggesting that an aromatic A ring may contribute more to inhibitory activity than hexane A ring. The abietane *o*-quinones still remain in study and could serve as interesting templates for the development of new drugs against AD (Wong K.K.-K. et al., 2010a; Wong K.K.-K. et al., 2010b).

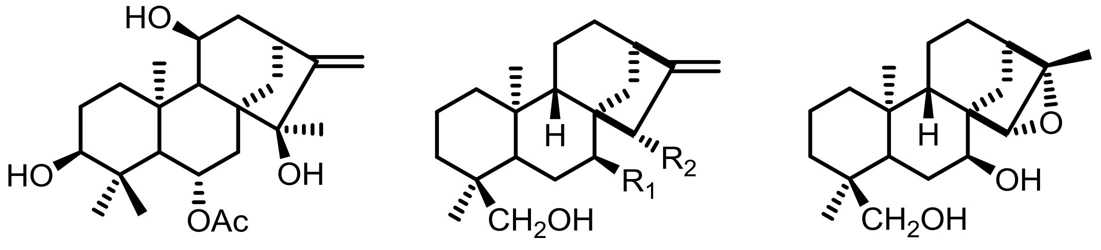


dihydrotanshinone cryptotanshinone

Figure I.24. Chemical structures of dihydrotanshinone and cryptotanshinone.

So far, there are few terpene-type AChE inhibitors ever reported, which are of relatively low potency, with IC_{50} in milli-molar level (Thirugnanasampandan R. et al., 2008). However, two diterpenoid (dihydrotanshinone and cryptotanshinone, see **Figure I.24**) showed potent anti-AChE activity. Wong K.K.-K. *et al.* reported, in order to have a better understanding on the mode of action of those compounds on AChE inhibition, through detailed analyses of AChE enzyme kinetics and molecular docking (Wong K.K.-K. et al., 2010b).

Concerning the anticholinesterase properties of diterpenoids, there are reports from Lamiaceae family, of oxidized *ent*-kauranes. The melissoidesin (**Figure I.25**), isolated from *Isodon*, a species formally placed in Plectranthus, and now recognised as a more distantly related genus (Thirugnanasampandan R. et al., 2008) that exhibited moderate antiacetylcholinesterase activity; and others bioactive *ent*-kaurane diterpenoids: eubol, 7-epi-candicandiol and sideroxol (see **Figure I.25**; Ertas A. et al., 2009).



melissoidesin

eubol: R_1 = $OCOCH_3$, R_2 = OH

7-*epi*-candicandiol: R_1 = OH, R_2 = H_2

sideroxol

Figure I.25. Chemical structures of melissoidesin, eubol, 7-epi-candicandiol and sideroxol (Ertas A. et al., 2009).

Ahmad V.U. *et al.* also reported three new tricyclic *cis*-clerodane type diterpenoids (**a**, **b** and **c**, see **Figure I.26**) which have shown inhibitory activity against AChE and BChE (Ahmad V. U. et al., 2005).

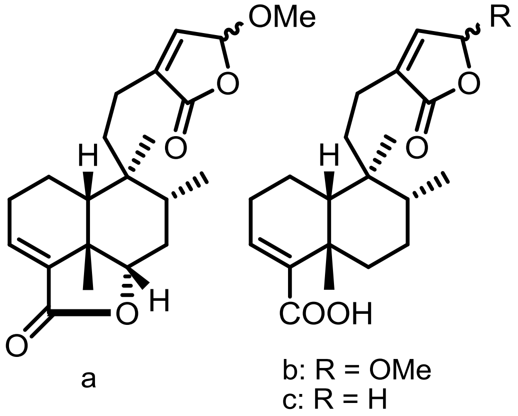


Figure I.26. Chemical structures of *cis*-clerodane type diterpenoids **a**, **b** and **c** (Ahmad V.U. et al., 2005).

I.4.4. Anti-inflammatory activity

Inflammation is a complex process and many different mediators are involved. The inflammatory process is necessary for survival against pathogens and injury, but sometimes the inflammatory response is aggravated and sustained without benefit. (de las Heras B. et al., 2003) No definite model covering all aspects of inflammation exists. Arachidonic acid is released from the cell membrane by chemical and mechanical stimuli and converted by the cyclooxygenase enzymes (COX-1/COX-2) to the unstable prostaglandin intermediates PGG_2 and PGH_2. The fate of the cyclooxygenase products, PGG_2 and PGH_2, differs from tissue to tissue depending on the metabolizing enzymes present. COX-1 is constitutively expressed and is mainly responsible for the synthesis of cytoprotective prostaglandins in the gastrointestinal tract and of the proaggregatory thromboxane in blood platelets while COX-2 is induced in the inflamed tissue. COX-2 plays a major role in prostaglandin biosynthesis in inflammatory cells (monocytes/macrophages) and in the central nervous system. Modulation of the activity of the enzyme implies that the inflammation process can be modified (Matu E.N. and Van Staden. J., 2003). The screening of compounds isolated from medicinal plants is motivated by the possibility of discovering new biologically active chemotypes for later use in clinical medicine. The anti-inflammatory activities of isolated compounds also made it possible to rationalize the ethnomedicinal use of species of the plants used. This rationalization of the ethnomedicinal use of plants is important in developing countries like South Africa because the services of traditional healers play an important role in primary health care systems (Kalgutkar A.S. et al., 2000; Toit K. et al., 2005).

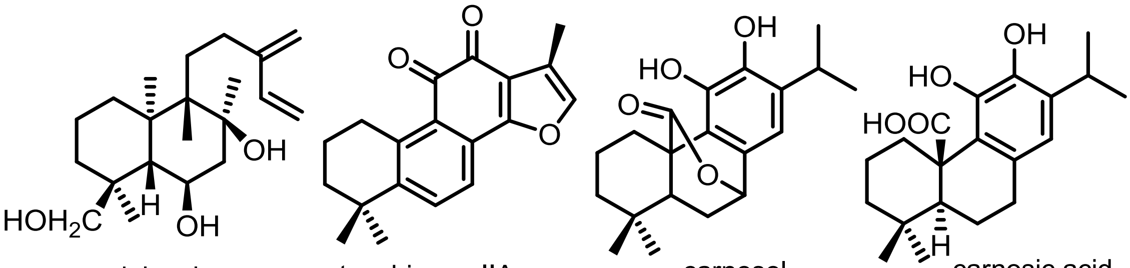
The extracts of plant species of Plectranthus, as *Plectranthus barbatus* (Matu E.N. and Van Staden. J., 2003), or *Plectranthus amboinicus (*Gurgel A.P.A.D. et al., 2009) traditionally used for treatment of ailments of infectious and/or inflammatory nature have been screened. The evaluation of the anti-inflammatory activity of the extracts was done to support the folk use of this medicinal species (Gurgel A.P.A.D. et al., 2009).

A vast number of terpenoids have been evaluated as potential anti-inflammatory molecules. The use of plant extracts rich in these terpenoids and administered in traditional medicine, point to the existence of likely candidates to act as potent anti-inflammatory drugs. It has been difficult to define precise molecular motifs, broadly distributed among these terpenes and involved in their anti-inflammatory activity. Some terpenoids act as plant hormones regulating different physiological functions but other are secondary metabolites involved in host defence and in the protection of plant/animal from potential pathogens.

The isolation of triterpenoids and their biological activity against COX-1 and COX-2 have been investigated (Hasmeda M. et al., 1999; Rajic A. et al., 2001; Mitaine-Offer A.-C. et al., 2002; Yoshikawa K. et al., 2005; Angeh J.E. et al., 2007). The search for selective inhibitors of COX-2 is considered important, on the basis of the theory that side effects, such as gastric lesions, that occurred from inhibition of COX-1 activity were observed with aspirin and others non-steroidal anti-inflammatory drugs (NSAIDs). Until now, very few compounds of natural origin have been reported to possess COX-2 inhibitory effects.

Concerning the anti-inflammatory properties of diterpenes (Liu Q. et al., 2006), there are reports of labdane (Abe M. et al., 2006), kaurane (de las Heras B. et al., 2007), clerodane, abietane (as abietic acid, see **Scheme I.5**; Fernandez M.A. et al., 2001) and pimarane (Kim T. D. et al., 2010) diterpenes (de las Heras B. et al., 2003).

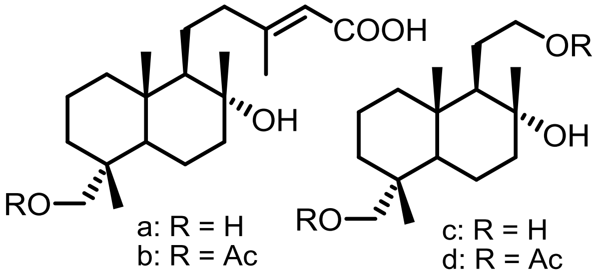
S. Hortelano *et al.* reported the potential of labdane diterpenoids from Lamiaceae, like andalusol, as anti-inflammatory agents. The future development of this class of compounds, as anti-inflammatory drugs requires the introduction of novel molecular targets of therapeutic relevance and thus are very promising candidates as leads for developing useful therapeutics. The diterpene tanshinone IIA, isolated from *Salvia,* and the abietanes carnosol and carnosic acid, were also described as anti-inflammatory agents (see **Figure I.27**; de las Heras B. and Hortelano S., 2009).



andalusol tanshinone IIA carnosol carnosic acid

Figure I.27. Chemical structures of andalusol, tanshinone IIA, carnosol and carnosic acid (de las Heras B. and Hortelano S., 2009).

In fact, a variety of biological activities have been determined for labdane diterpenes including antibacterial, antifungal, antiprotozoal, and anti-inflammatory activities, and additionally, recent studies reported the anti-inflammatory activity of labdane diterpenes through their inhibitory activity against cyclooxygenase. Selective inhibitory activity for two labdane diterpenes **a** and **c** and their acetate derivatives, **b** and **d**, against cyclooxygenases (COX-1 and COX-2) was reported (**a**, **b**, **c** and **d**; see **Figure I.28**; Hegazy M.-E.F. et al., 2008).



a: R = H
b: R = Ac

c: R = H
d: R = Ac

Figure I.28. Chemical structures of labdane diterpenes **a** and **c** and their acetate derivatives, **b** and **d** (Hegazy M.-E.F. et al., 2008).

Several reports indicate that nonselective anti-inflammatory drugs which inhibit COX-1 and COX-2 and microglial COX-1 may lead to a protective effect reducing the incidence of neurodegenerative disorders. Thus the use of anti-inflammatory drugs has been proposed as a therapeutic target implicated in the pathology of many diseases including neurodegenerative disorders such as Alzheimer's disease. A new bioactive diterpene, was tested for antibacterial activity, anti-inflammatory activity using the COX-1 and COX-2 assays and investigated for inhibitory effect against acetylcholinesterase. The results obtained support the traditional uses of this plant in African traditional medicine for the treatment of some ailments that relate to microbial diseases, inflammation and central nervous system disorders. The isolated compound in this study showed activities against both cyclooxygenases (involved with inflammation) and acetylcholinesterase (associated with central nervous disorders) (Eldeen I.M.S. et al., 2010). Plants and their constituents with pharmacological activities may be relevant to the treatment of cognitive disorders, including, anti-cholinesterase (anti-ChE), anti-inflammatory and antioxidant (Howes M.-J.R. et al., 2003; Tabet N., 2006; Pavlov V.A. et al., 2009; Kamal M.A. et al., 2009).

From all the above mentioned, it was decided to undertake a study of some *Plectranthus* species for the isolation and complete structural characterization of their diterpenoids components. In addition, it was decided to prepare suitable derivatives from the more abundant bioactive compounds. Finally, studies to evaluate mostly the antimicrobial activity, but also the antioxidant, antiacetylcholinesterase and anti-inflammatory activities, for all the compounds obtained, including the plant extracts, should be performed. This has been the focus of the work compiled in this thesis report.

CHAPTER II

Phytochemical study of *Plectranthus ornatus, Plectranthus ecklonii, Plectranthus porcatus and Plectranthus saccatus*

Results and Discussion

II.1. Introduction

This phytochemical study is focused on the acetone extracts of *Plectranthus ornatus* Codd. and *Plectranthus ecklonii* Benth. (whole plants) and *P. porcatus* Winter & Van Jaarsv. and *P. saccatus* Benth. (aerial parts).

Several diterpenoids were isolated from *P. ornatus*: three new forskolin-like diterpenoids, a known labdane found for the first time in *Plectranthus* species and other two isolated in major quantities (a novel diterpenoid with the rare halimane skeleton and the previously isolated plectrornatin C). Mixtures of six known triterpenoids have also been isolated and characterized.

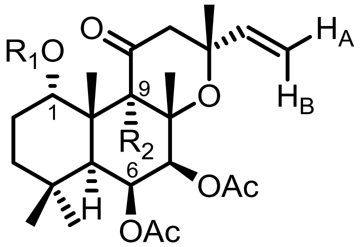
The study of *P. ecklonii* has afforded two known abietane diterpenoids: one of which was found in *Plectranthus* species for the first time, and the other one was isolated in a very large amount. Mixtures of four known triterpenoids were also isolated and characterized.

A spiro-abietane and a beyerane diterpenoids were isolated, from *P. porcatus* and *P.* saccatus respectively.

In this chapter the physical and spectroscopic data of the isolated substances which allow the structural elucidation of all the compounds are described and discussed.

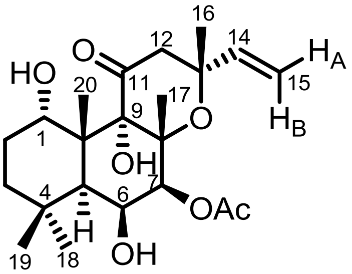
II.2. Structure elucidation of terpenic compounds from *Plectranthus ornatus* Codd.

II.2.1. Forskolin-like diterpenoids

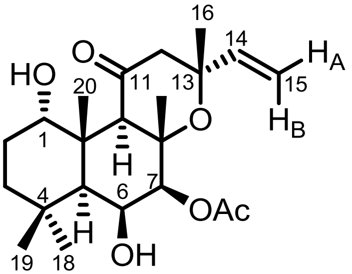


II.1 R_1 = H, R_2 = OH
II.2 R_1 = Ac, R_2 = OH
II.3 R_1 = Ac, R_2 = H

Compounds **II.1**-**II.3** possess structures closely related to that of forskolin **II.4**, a very interesting substance isolated from *Coleus forskohlii* Briq. (Lamiaceae) whose synthesis and chemical reactivity have been extensively studied (Bhat S.V., 1993). The present study is the first on compounds **II.1**-**II.3** as naturally occurring substances, although they were already known as semisynthetic derivatives. 6-*O*-Acetylforskolin **II.1** and 1,6-di-*O*-acetylforskolin **II.2** have previously been obtained by acetylation of forskolin **II.4**, (Bhat S.V. et al., 1982) whereas **II.3** (1,6-di-*O*-acetyl-9-deoxyforskolin) has been reported as an intermediate in a semisynthesis of **II.4** starting from 9-deoxyforskolin **II.5** (Hrib N.J., 1987a; Hrib N.J., 1987b).



II.4 Forskolin



II.5

The forskolin **II.4**, possesses a labdane skeleton with: five methyl groups (Me-16 to Me-20) and a double bond between C-14 and C-15; the characteristic olefinic proton H-14 that correlates in the HMBC experiment, with the totally substituted carbon C-13 which in turn, correlates with the C-16 methyl group; and a system of AB protons on C-12 that have no protons in vicinal positions and are only mutually coupled (Kogler H. and Fehlhaber H.-W., 1991). Structures **II.1-II.3** were established by NMR spectroscopic studies. For the assignment of the ^{1}H and ^{13}C NMR spectra of **II.1-II.3** (**Tables II.1.** and **II.2.**, respectively), two-dimensional COSY, HSQC and HMBC experiments were carried out, as well as 1D NOESY spectra for the determination of the relative stereochemistry and conformations of these substances.

Table II.1. ^{1}H NMR data of compounds **II.1**[a], **II.2**[b] and **II.3**[b] ([a]pyridine-d_5, [b]$CDCl_3$, 400 MHz; δ in ppm, *J* (H,H) in Hz).

Proton	**II.1**[a]	**II.1**[b]	**II.2**[b]	**II.3**[b]	*J*(H,H)	**II.1**[a]	**II.1**[b]	**II.2**[b]	**II.3**[b]
1β	5.01 ddd[§]	4.60 ddd[§]	5.56 t	5.50 t	1β,2α	3.2	3.3	2.4	2.7
2α	1.54 dq	1.43dq	1.63 dq	1.72 dddd	1β,2β	2.4	2.5	2.4	2.7
2β	2.16 dddd	2.11dddd	2.12 dddd	1.97 dddd	2α,2β	14.4	14.0	15.4	14.4
3α	1.98 td	1.78 td	1.58 td	1.45 ddd	2α,3α	3.2	3.3	3.4	2.7
3β	1.04 dt	1.09 dt	1.15 dt	1.12 dt	2α,3β	3.2	3.3	3.4	3.4
5α	2.72 d	2.38 d	2.41 d	1.60 d	2β,3α	13.1	13.7	13.0	13.0
6α	6.20 dd	5.79 dd	5.80 dd	5.74 dd	2β,3β	3.2	3.3	3.4	3.4
7α	6.15 d	5.50 d	5.54 d	5.10 d	3α,3β	13.1	13.7	13.0	13.5
9α	---	---	---	3.32 s	5α,6α	2.8	2.9	2.7	2.4
12α	3.48 d	3.21 d	3.14 d	2.64 d	6α,7α	4.6	4.4	4.6	3.9
12β	2.60 d	2.48 d	2.42 d	2.59 d	12α,12β	16.4	17.1	16.5	18.8
14	6.28 dd	5.94 dd	5.90 dd	5.94 dd	14,15A	10.7	17.3	10.6	10.8
15A	4.91 dd	5.26 dd	4.93 dd	5.05 dd	14,15B	17.4	10.7	17.2	17.4
15B	5.35 dd	4.97 dd	5.20 dd	5.24 dd	15A,15B	1.5	1.0	1.1	1.0
Me-16	1.42 s	1.34 s	1.33 s	1.21 s	1β,1α-OH[#]	3.7	2.0	---	---
Me-17	1.84 s	1.64 s	1.64 s	1.50 s					
Me-18	0.97 s	1.02 s	1.03 s	0.98 s					
Me-19	1.00 s	0.97 s	0.98 s	0.94 s					
Me-20	1.64 s	1.42 s	1.52 s	1.44 s					
1α-OH	8.49 d[#]	2.9 d[#]	---	---					
9α-OH	8.63 s[#]	6.04 s[#]	4.74 s[#]	---					
1α-OAc	---	---	2.09 s	1.96 s					
6β-OAc	2.13 s	2.08 s	2.02 s[*]	2.07 s[*]					
7β-OAc	2.00 s	2.02 s	2.01 s[*]	2.08 s[*]					

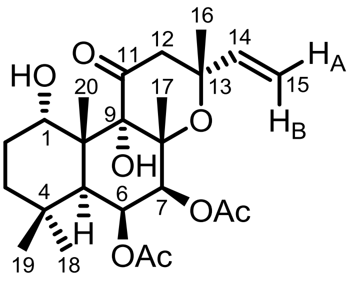
[§] Collapsed into a dd ($J_{1β,2α}$ and $J_{1β,2β}$) after addition of D_2O; [#] Disappeared after addition of D_2O; [*] Interchangeable assignments.

Table II.2. ^{13}C NMR data of compounds **II.1**^{a}, **II.2**^{b} and **II.3**^{b} (^{a}pyridine-d_5, ^{b}CDCl$_3$, 100 MHz; δ in ppm, J (H,H) in Hz).

Carbon	**II.1**^{a}	**II.1**^{b}	**II.2**^{b}	**II.3**^{b}
1	73.54 d	74.18 d	73.94 d	74.78 d
2	27.06 t	26.48 t	23.11 t	21.61 t
3	36.82 t	36.23 t	37.00 t	36.74 t
4	34.23 s	34.09 s	33.84 s	33.77 s
5	42.61 d	41.84 d	43.00 d	47.23 d
6	70.08 d	69.73 d	69.36 d	69.24 d
7	75.54 d	74.05 d	76.57 d	78.51 d
8	81.29 s	81.00 s	81.65 s	77.21 s
9	82.74 s	82.36 s	81.44 s	57.32 d
10	43.28 s	43.17 s	43.44 s	40.62 s
11	206.82 s	205.28 s	205.26 s	205.21 s
12	49.66 t	48.77 t	48.74 t	49.01 t
13	75.83 s	75.17 s	75.70 s	74.61 s
14	147.54 d	146.12 d	145.83 d	146.30 d
15	110.13 t	110.81 t	110.38 t	112.89 t
16	30.80 q	31.35 q	31.00 q	31.80 q
17	23.69 q	23.01 q	23.22 q	24.03 q
18	32.74 q	32.78 q	32.69 q	32.63 q
19	23.54 q	23.49 q	23.39 q	22.91 q
20	20.07 q	19.58 q	19.64 q	17.25 q
1α-$\underline{\text{C}}\text{OCH}_3$	---	---	168.40 s	169.88 s
1α-$\text{CO}\underline{\text{C}}\text{H}_3$	---	---	21.74 q	21.33 q
6β-$\underline{\text{C}}\text{OCH}_3$	170.49 s	169.89 s	169.87 s	169.45 s
6β-$\text{CO}\underline{\text{C}}\text{H}_3$	21.28 q	21.45 q	21.44 q*	20.93 q
7β-$\underline{\text{C}}\text{OCH}_3$	170.31 s	169.85 s	169.87 s	170.35 s
7β-$\text{CO}\underline{\text{C}}\text{H}_3$	20.95 q	20.83 q	20.82 q*	21.33 q

*Interchangeable assignments.

II.2.1.1. 6-*O*-Acetylforskolin II.1



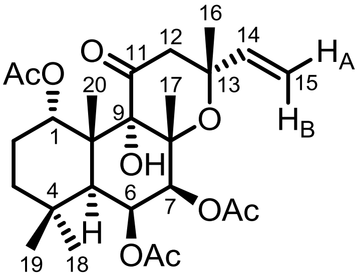
II.1

6-*O*-Acetylforskolin **II.1** (9 mg; 0.00026 %) is new as natural compound that was obtained as colorless prisms from EtOAc. Low-resolution mass spectrometry and combustion analysis indicated a molecular formula of $C_{24}H_{36}O_8$ for **II.1** and its ^{1}H and ^{13}C NMR spectra (**Tables II.1** and **II.2**, respectively) were almost identical to those reported for forskolin (**II.4**, $C_{22}H_{34}O_7$; Kogler H. and Fehlhaber H.-W., 1991). In fact, the observed differences between the ^{1}H and ^{13}C NMR spectra of **II.1** and **II.4** were compatible with the presence in the former of a 6β-acetoxyl group instead of the 6β-hydroxyl group in the latter. This conclusion is supported by the following facts: compound **II.1** possesses two acetoxyl groups, as was evidenced by its molecular formula and confirmed by the presence of two three-proton singlets (δ 2.13 and 2.00) and two pairs of acetoxyl carbons (δ 170.49 s, 170.31 s, 21.28 q and 20.95 q) in the ^{1}H and ^{13}C NMR spectra, respectively. The H-6α proton of **II.1** appeared down-field shifted (δ 6.20) with respect to that of **II.4** (δ 4.44) (Kogler H. and Fehlhaber H.-W., 1991). The C-1 – C-4 and C-8 – C-20 carbon atom resonances of **II.1** (**Table II.2**) were almost identical to those of **II.4**, whereas the observed differences in the chemical shifts of the C-5, C-6 and C-7 carbons [Δδ = δ (**II.1**) – δ (**II.4**): –0.2, +1.2 and –1.6 ppm, respectively] are compatible only with the presence in **II.1** of an acetoxyl substituent at the 6β-position. Finally, the HMBC spectrum of **II.1** (**Table II.3**) showed connectivity between the carbonyl carbon of the 6-*O*-acetyl group (δ 170.49 s) and the H-6α proton (δ 6.20 dd) which, in turn, was connected with the C-5, C-7, C-8 and C-10 carbons, whereas the H-7α proton showed HMBC cross-peaks with the carbonyl carbon of the other acetate group (δ 170.31 s) and the C-6, C-8, C-9 and C-17 carbons. Thus, **II.1** is 6-*O*-acetylforskolin.

Table II.3. Significant assignments observed on HMBC experiment for compound **II.1**

Position	HMBC correlations
OH-9α	C-8, C-9 and C-10
H-14	C-13
H-6α	C-5, C-7, C-8, C-10 and OAc:CO
H-7α	C-6, C-8, C-9, C-17 and OAc:CO
H-15A + H-15B	C-13 and C-14
H-1β	C-3 and C-10
H-12α	C-11, C-13, C-14 and C-16
OH-1α	C-10
H-12β	C-11, C-13, C-14 and C-16
H-5α	C-3, C-4, C-7, C-9, C-10, C-20, C-18 and C-19
Me-16	C-12, C-13 and C-14
Me-17	C-7, C-8 and C-9
Me-18	C-3, C-4, C-5 and C-19
Me-19	C-3, C-4, C-5 and C-18
Me-20	C-1, C-5, C-9 and C-10

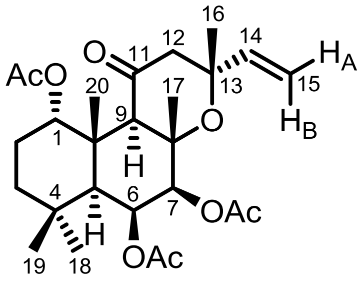
II.2.1.2. 1,6-Di-*O*-acetylforskolin II.2



II.2

1,6-Di-*O*-acetylforskolin **II.2** (21.1 mg; 0.000622 %) was obtained as colorless fine needles from EtOAc-pentane. Compound **II.2** is the 1-*O*-acetyl derivative of **II.1**, as was evidenced by its molecular formula ($C_{26}H_{38}O_9$) and the presence of three acetoxyl groups in its ^{1}H and ^{13}C NMR spectra [δ_H 2.09 s, 2.02 s and 2.01 s, 3H each; δ_C 169.87 s (2C), 168.40 s, 21.74 q, 21.44 q and 20.82 q]. Comparison of the chemical shift of the H-1β proton of **II.2** (δ 5.56 t) with respect to those of **II.1** (δ 5.01 ddd, see **Table II.1**) and **II.4** (δ 4.54 ddd; Kogler H. and Fehlhaber H.-W., 1991) together with the diamagnetic shift of the C-2 carbon of **II.2** (δ 23.11) with respect to those of **II.1** (δ 27.06) and **II.4** (δ 26.46; Kogler H. and Fehlhaber H.-W., 1991) strongly support that **II.2** is 1,6-di-*O*-acetylforskolin.

II.2.1.3. 1,6-Di-*O*-acetyl-9-deoxyforskolin II.3



II.3

1,6-Di-*O*-acetyl-9-deoxyforskolin **II.3** (11.6 mg; 0.000342 %) was obtained as colorless fine needles from EtOAc-pentane. Diterpenoid **II.3** is the 9-deoxy derivative of **II.2**. The molecular formula established for **II.3** was $C_{26}H_{38}O_8$ and $C_{26}H_{38}O_9$ for **II.2**. The IR spectrum of **II.3** was devoid of hydroxyl absorptions, whereas **II.2** showed a hydroxyl band at 3468 cm^{-1}. The C-9 methine carbon of **II.3** appeared at δ 57.32 in its ^{13}C NMR spectrum and it was connected (HSQC) with one proton singlet signal at δ 3.32. In the HMBC spectrum of **II.3,** the H-9 proton showed connectivities with the C-1, C-5, C-7, C-8, C-11, C-17 and C-20 carbons. Comparison of the ^{13}C NMR spectra of **II.2** and **II.3** (**Table II.2**) clearly revealed that the 9α-hydroxyl substituent of the former is a 9α-hydrogen in the latter, because upfield shifts for the α-carbon (C-9) and the C-8 and C-10 β-carbons [Δδ = δ (**II.3**) – δ (**II.2**): -24.1, -3.4 and –2.8 ppm, respectively], and paramagnetic shifts in the C-1, C-5, C-7, C-12 and C-17 γ-carbons (Δδ: +0.8, +4.2, +1.9, +0.3 and +0.8 ppm, respectively) were observed for **II.3** with respect to those of **II.2**. Moreover, the shielding effect (Δδ = -2.4 ppm) observed on the γ-*trans* C-20 carbon of **II.3**, caused by the change of the 9α-OH substituent of **II.2** by a 9α-hydrogen **II.3**, has also been reported in 9-deoxyforskolin **II.5** with respect to forskolin **II.4** ($\Delta\delta_{C\text{-}20}$ = -1.5

ppm; Hrib N.J., 1987b). Consequently, compound **II.3** is 1,6-di-*O*-acetyl-9-deoxyforskolin.

NOE (nuclear Overhauser effect) experiments on **II.1**-**II.3** (**Table II.4**) were in agreement with the relative stereochemistry of these compounds as shown in the previously presented structures and allowed the unequivocal assignment of both 12-methylene protons. Irradiation at δ 2.72 (H-5α proton of **II.1**) produced strong NOE enhancements in the signals of the H-3α, H-6α, H-7α and 9α-OH protons and a weak NOE in the signal of the Me-18 group. On irradiating at δ 3.32 (H-9α proton of **II.3**) the signals of the H-5α, H-7α, H-14 and H_B-15 protons were enhanced. In addition, when the Me-17 protons of **II.3** (δ 1.50) were irradiated, NOEs in the signals of Me-16 and Me-20 were observed. Moreover, irradiation at δ 1.44 (Me-20 of **II.3**) caused NOE enhancements in the H-1β, H-2β, Me-17 and Me-19 signals. These and other NOE results (**Table II.4**) established that the H-1 proton and the Me-16, Me-17, Me-19 and Me-20 groups of **II.1**-**II.3** are on the same side of the plane of the molecule and possess a β-configuration. By the same reasoning, the H-5, H-6 and H-7 protons, the 9-substituent (a hydroxyl group in **II.1** and **II.2**, and a hydrogen in **II.3**) and the vinyl group at C-13 are α-substituents. On the other hand, the data in **Table II.4** clearly established that the 12-methylene proton of **II.1**-**II.3** appearing at lower field (H_B-12, δ 3.48, 3.14 and 2.64, respectively) is the α-hydrogen (observed NOEs between the H-14 olefinic and the H_B-12 protons in **II.1**-**II.3**, and between the H-9α and H_B-12 protons in **II.3**), whereas the H_A-12 proton (δ 2.60, 2.42 and 2.59, respectively) is the 12-methylene β-hydrogen (observed NOE between the H_A-12 and Me-17 protons in **II.3**).

Table II.4. Significant NOEs for compounds **II.1**-**II.3**

Compound	Irradiated proton (δ)	Observed NOE with protons (% NOE enhancement)
II.1	H-1β (5.01)	H-2α (+3.7), H-2β (+3.5), 9α-OH (+1.9), Me-20 (+7.7)
	H-5α (2.72)	H-3α (+7.9), H-6α (+11.7), H-7α (+11.7), 9α-OH (+15.6), Me-18 (+4.6)
	H-6α (6.20)	H-5α (+10.5), H-7α (+3.5), Me-18 (+9.3), Me-19 (+1.8)
	H-14 (6.28)	9α-OH (+0.1), H-12α (+1.9), H_A-15 (+1.7), Me-16 (+1.9)
II.2	H-14 (5.90)	9α-OH (+0.2), H-12α (+1.4), H_A-15 (+1.1), Me-16 (+1.1)
II.3	H-1β (5.50)	H-2α (+3.7), H-2β (+4.3), H-9α (+1.9), Me-20 (+6.1)
	H-6α (5.74)	H-5α (+5.9), H-7α (+7.0), Me-18 (+5.9), Me-19 (+0.7)
	H-9α (3.32)	H-1β (+1.7), H-5α (+8.4), H-7α (+6.4), H-12α (+1.4), H-14 (+1.3), H_B-15 (+0.9)
	H-14 (5.94)	H-9α (+0.9), H-12α (+2.3), H_A-15 (+3.9), Me-16 (+2.4)
	Me-17 (1.50)	H-12β (+1.7), Me-16 (+1.5), Me-20 (+1.4)
	Me-20 (1.44)	H-1β (+2.2), H-2β (+3.0), Me-17 (+3.6), Me-19 (+3.8)

A careful study of the ^{1}H NMR spectra of **II.1**-**II.3** provided the ^3J (H,H) values (**Table II.1**) which allowed to establish the conformation for rings A and B of the *trans*-decalin part of these diterpenoids. Both these six-membered rings possess a chair ($^4C_1$ and $^6C_9$, respectively) conformation in which the 1α-substituent and the H-2β and H-3α protons are *trans* diaxially oriented ($J_{1\beta,2\beta}$ = 2.7 – 2.4 Hz, $J_{2\beta,3\alpha}$ = 13.1 – 13.0 Hz, $J_{1\beta,2\alpha}$ = 3.2 – 2.4 Hz), and the H-5α and H-7α protons and the 6β-acetoxyl group are also axial ($J_{5\alpha,6\alpha}$ = 2.8 – 2.4 Hz, $J_{6\alpha,7\alpha}$ = 4.6 – 3.9 Hz; see **Figure II.1**). The behaviour of **II.1**-**II.3** under NOE experiments further supported these deductions (see **Table II.4**).

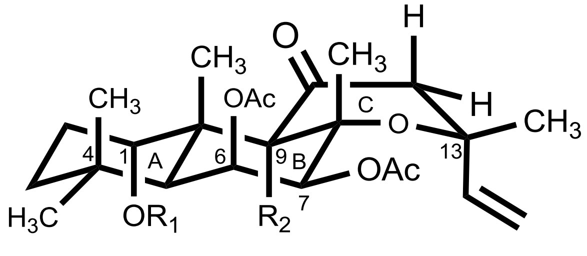
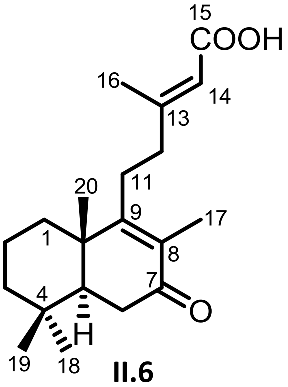


Figure II.1. Three-dimensional structure of compounds **II.1** (R_1=H, R_2=OH), **II.2** (R_1=Ac, R_2=OH) and **II.3** (R_1= Ac, R_2=H) derived from NOE experiments and interpretation of vicinal H-H coupling constants (Kogler H. and Fehlhaber H.-W., 1991).

The analysis of the conformation of the substituted tetrahydropyran fragment (ring C) of **II.1**-**II.3** was carried out on the basis of NOE results. If the tetrahydropyran was in a half-chair ($^{13}C_9$) conformation, the H-12β proton assumed an equatorial orientation and the Me-16 and Me-17 groups were axial. On the contrary, if this heterocycle was in a boat-like ($B_{9,13}$) conformation, the H-12β proton and the Me-17 group were *cis* diaxial, and the Me-16 group will adopt a pseudoequatorial orientation. The NOEs observed in the H-12β proton and the Me-16 and Me-20 groups of **II.3** (+1.7, +1.5 and +1.4 % NOE enhancement, respectively) when the Me-17 protons (δ 1.50) were irradiated, suggest (Kogler H. and Fehlhaber H.-W., 1991) that the second conformational possibility is more likely. In fact, a boat-like ($B_{9,13}$) conformation of ring C in forskolin **II.4** has previously been established by NOE measurements, although, in the crystalline state, ring C of **II.4** shows a chair conformation (Kogler H. and Fehlhaber H.-W., 1991). Since the chemical shifts of the C-11 – C-13, C-16 and C-17 carbons of **II.3** were almost identical to those of **II.1** and **II.2** (**Table II.2**), it´s plausible that, in solution, all these diterpenoids possess a boat ($B_{9,13}$) conformation of the tetrahydropyran structural part, as in forskolin **II.4** (see **Figure II.1**; Kogler H. and Fehlhaber H.-W., 1991; Rijo P. et al., 2005).

II.2.2. A previously known labdane and a new halimane diterpenoids

II.2.2.1. Rhinocerotinoic acid (7-oxo-labda-8,13E-dien-15-oic acid) II.6



II.6

This was the first report on the isolation of rhinocerotinoic acid **II.6** from a Lamiaceae species. The labdane diterpenoid isolated from *P. ornatus* **II.6** showed physical (mp., $[\alpha]_D$) and spectroscopic (IR, ^{1}H and ^{13}C NMR and mass spectra) data identical to those reported for rhinocerotinoic acid, an labdane diterpene previously isolated (Dekker et al., 1988) from *Elytropappus rhinocerotis* (L.f.) Less. (Asteraceae) which structure has recently been confirmed by partial synthesis from (–)- sclareol (Gray et al., 2003).

Compound **II.6.** was isolated as white fine needles with m.p. 187-189 ˚C and $[\alpha]_D^{18}$ +38.9˚ (*c* 0.236, $CHCl_3$). Its mass spectrum showed the molecular ion at m/z 318 and exhibited an ion at m/z 205 $[M-C_8H_{11}O_4]^+$ originated by the loss of the side chain.

The ^{13}C NMR spectrum showed the presence of a carboxylic carbon on C-15 at δ_C 171.13, and a ketone on C-7 at δ_C 200.13, in agreement with IR absorptions at 3306-2894 br, 1720, 1630 cm^{-1} implying the presence of a hydroxyl and two carbonyl groups. Those α-β unsaturated oxygen functions were identified by four olefinic carbons, two belonging to the double bond between C-8 and C-9, adjacent to the C-7 ketone (δ_C 130.57 and δ_C 166.23; C-8 and C-9 respectively), and the other two of the C-13 and C-14 double bond, adjacent to the carboxylic acid (δ_C 161.70 and δ_C 115.18; C-13 and C-14 respectively).

Observation of the ^{1}H NMR and ^{13}C NMR spectra of **II.6** corresponding to carbons from C-1 to C-5; C-10 and from C-18 to C-20 hydrocarbon structural part were very similar to the data already reported for rhinocerotinoic acid **II.6** (Dekker et al., 1988; see **Table II.5**). In addition, a long-range coupling of four bonds was observed between H-1β (δ 1.91, dddd, $J_{1\beta,3\beta}$ = 1.3 Hz) and H-3β (1.48, dt, $J_{3\beta,1\beta}$ = 1.3 Hz) with w-shaped arrangement of the bonds.

Table II.5. Comparison of the ^{1}H and ^{13}C NMR data of rhinocerotinoic acid **II.6**^{a} with that reported by Dekker *et al.*^{b} (^{1}H NMR, $CDCl_3$, ^{a}400 MHz, ^{b}500 MHz and ^{13}C NMR,$CDCl_3$, ^{a}100 MHz and ^{b}127.5MHz; δ in ppm, *J* (H,H) in Hz).

Position	$\delta_H{}^a$	$\delta_H{}^b$	$J_{H,H}$		$\delta_C{}^a$	$\delta_C{}^b$	**HSQC**
1α	1.36 td	1.35	1α,1β	12.7	35.89	35.9	t
1β	1.91 dddd	1.90	1α,2α	3.7	---	---	---
2α	1.70 m*	1.68	1α,2β	12.7	18.56	18.6	t
2β	1.60 ddddd	1.58	1β,2α	4.0	---	---	---
3α	1.21 td	1.20	1β,2β	3.7	41.23	41.2	t
3β	1.48 dt	1.47	2α,2β	13.5	---	---	---
4			2α,3α	4.1	33.12	33.1	s
5α	1.70 dd	1.68	2α,3β	3.2	50.26	50.3	d
6α	2.50 dd	2.49	2β,3α	13.5	35.19	35.2	t
6β	2.35 dd	2.35	2β,3β	3.2	---	---	---
7			3α,3β	13.5	200.13	200.1	s
8			5α,6α	3.7	130.57	130.6	s
9			5α,6β	14.3	166.23	166.3	s
10			6α,6β	17.5	41.01	41.0	s
11A+11B	2.35 m*	2.35	1β,3β	1.3	27.64	27.6	t
12A+12B	2.28 m*	2.28	14,16	1.3	39.79	39.8	t
13	**---**	---	---	---	161.70	161.5	s
14	5.74 q	5.73			115.18	115.4	d
15					171.13	171.6	s
Me-16	2.23 d	2.20			19.12	19.1	q
Me-17	1.77 s	1.75			11.42	11.4	q
Me-18	0.88 s	0.86			32.49	32.5	q
Me-19	0.91 s	0.90			21.29	21.3	q
Me-20	1.08 s	1.07			18.15	18.2	q

* overlapped signals. δ values were measured from the HSQC spectrum.

The significant correlations observed on the HMBC spectrum of compound **II.6** confirmed its structure and are compiled in **Table II.6**.

Table II.6. Significant correlations observed in HMBC for compound **II.6**.

Position	HMBC
H-14	C-12, C-13, C-15 and C-16
H-6α	C-4, C-5, C-7, C-8 and C-10
Me-16	C-12, C-13 and C-14
Me-17	C-7, C-8 and C-9
Me-20	C-1, C-5, C-9 and C-10,
Me-19	C-3, C-4, C-5 and C-18
Me-18	C-3, C-4, C-5 and C-19

Irradiation of the Me-20 at δ 1.08 in an NOE experiment showed an effect on H-6β (+3.4%), H-1β (+0.8%), H-2β (+1.1%) and Me-19 (+2.1%) establishing that those protons were all on the same face of compound **II.6**. The irradiation of the downfield proton H-14 at δ 5.74, showed a bigger effect on the methylene H-12 (+7.5%) than Me-16 (+1.1%) defining the *E* stereochemistry of the double bond Δ^{13} where proton H-14 and both protons H-12 were on the same side of the double bond.

The three-dimensional structure of compound **II.6** was derived from NOE effects and the interpretation of the angular dependencies of the vicinal proton-proton coupling constants described above. The A and B rings conformation of the decalin part of the molecule is $^{4}C_{1}$, with a chair conformation of the A ring with C-4 on the upper side of the molecule and C-1 on the lower part of the A ring. The B ring conformation is $^{6}HC_{5}$, a half-chair with C-6 on the upper side of the B ring and C-5 on the lower part of the molecule (see **Figure II.2**) (Rijo P. et al., 2007; Kogler H. and Fehlhaber H.-W., 1991).

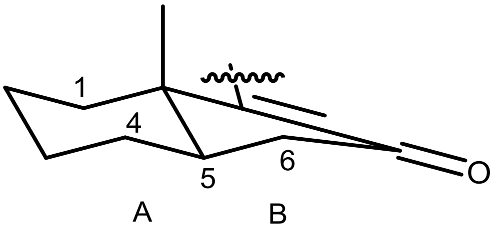
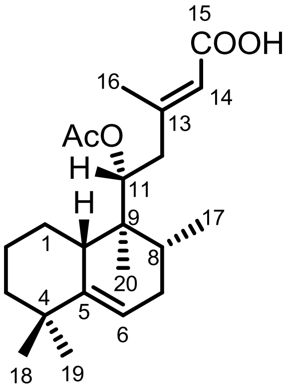


Figure II.2. A and B rings conformation (A: $^{4}C_{1}$ and B: $^{6}HC_{5}$) of compound **II.6**.

II.2.2.2. 11*R**-Acetoxyhalima-5,13*E*-dien-15-oic acid II.7



II.7

11*R**-Acetoxyhalima-5,13*E*-dien-15-oic acid **II.7** (0.70 g, 0.0286 %) is a new halimane diterpenoid isolated as colourless hexagonal plates (from EtOAc – *n*-pentane). I.R. absorptions at 3450-2576, 1736 and 1674 cm^{-1} revealed the presence of an ester and an α,β-unsaturated carboxylic acid. Combustion analysis and low-resolution mass spectrometry indicated a molecular formula of $C_{22}H_{34}O_4$ for diterpenoid **II.7**. Mass spectrum didn´t show the molecular ion but exhibited an ion at m/z 302 $[M\text{-}HOAc]^+$. The base peak at m/z 191 $[M\text{-}C_8H_{11}O_4]^+$ corresponds to the fragment obtained after the loss of the side chain.

The ^{13}C-NMR spectrum displayed twenty two carbon resonances corresponding to six methyl groups (one of which belongs to the acetyl group δ 20.8), five methylenes, five methines (two of them corresponding to olefinic carbons) and six quaternary carbons (one of the acetyl group δ 170.7 and the other of a carboxyl group at δ 171.1; see **Table II.7**).

Table II.7. ^{1}H and ^{13}C NMR data of 11*R**-acetoxyhalima-5,13*E*-dien-15-oic acid **II.7** (^{1}H NMR, $CDCl_3$, 400 MHz and ^{13}C NMR,$CDCl_3$, 100 MHz; δ in ppm, *J* (H,H) in Hz).

Position	**δ_H**	**$J_{H,H}$**	**δ_C**	**HSQC**
1α	1.10 qd	(1α,1β)12.8	28.9	t
1β	1.92 dtd	(1α,2α)4.2	---	---
2α	1.64 ddddd	(1α,2β)12.8	22.5	t
2β	1.52 qt	(1α,10β)12.8	---	---
3α	1.19 td	(1β,2α)4.0	40.9	t
3β	1.42 ddd	(1β,2β)4.1	---	---
4	---	(1β,10β)2.0	36.3	s
5	---	(2α,2β)13.2	145.2	s
6	5.45 dt	(2α,3α)3.7	116.0	d
7α	1.78 ddd	(2α,3β)4.0	31.3	t
7β	1.84 ddd	(2β,3α)13.1	---	---
8β	1.56 ddq	(2β,3β)3.2	32.9	d
9	---	(3α,3β)13.1	41.6	s
10β	2.07 ddd	(6,7α)2.2	40.3	d
11	5.31 dd	(6,7β)5.2	75.0	d
12A	2.44 ddd	(6,10β)2.2	41.7	t
12B	2.36 dd	(7α,7β)17.1	---	---
13	---	(7α,8β)12.4	159.8	s
14	5.68 qd	(7β,8β)2.8	117.7	d
15	---	(8β,17) 6.8	171.1	s
Me-16	2.19 d	(11,12A) 2.6	19.0	q
Me-17	0.99 d	(11,12B) 10.2	17.2	q
Me-18	0.98 s	(12A,12B) 13.4	28.1	q
Me-19	1.05 s	(12A,14) 0.4	29.7	q
Me-20	0.69 s	(14,16) 1.3	11.8	q
11-OAc	2.00 s		170.7	s
11-OAc	---		20.8	q

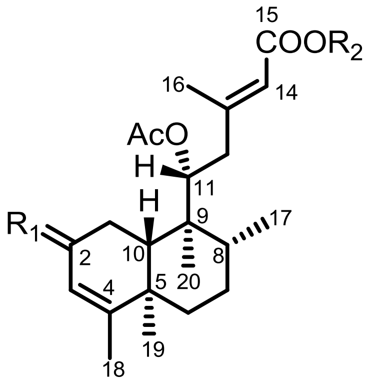
The ^{1}H-NMR spectrum showed signals corresponding to six methyl groups, five bonded to tertiary carbons (a doublet δ 2.19 (CH_3-16) and four singlets δ 2.00, 1.05, 0.98, 0.69 – $OCCH_3$, CH_3-19, CH_3-18 and CH_3-20 respectively) and one to a secondary carbon (doublet δ 0.99 – CH_3-17). Two olefinic protons [a double triplet at δ 5.45 (H-6) and a quadruple doublet at δ 5.68 (H-14)] and a highfield proton signal of a methine group [double doublet at δ 5.31 (H-11)] could also be seen. The highfield signals of protons H-12A (δ 2.44) and H-12B (δ 2.46) were consistent with the proximity of the acetyl group and the double bond. The proton H-12A (ddd multiplicity) showed a geminal coupling constant (*J*=13.4 Hz) with proton H-12B, a vicinal coupling constant (*J*=2.6 Hz) with proton H-11 and an allylic coupling constant (*J*=0.4 Hz) with proton H-14 (**Table II.7**). The significant correlations observed in the HMBC spectrum of compound **II.7** are shown in **Table II.8**.

Table II.8. Significant correlations observed in the HMBC spectrum of compound **II.7**.

Position	HMBC
H-6	C-8, C-7 and C-10
H-10β	C-1, C-5 and C-8
11-COC$\underline{H}_3$	C-8, C-9, C-10, C-11, C-12, C-13 and C-20
H-12A + H-12B	C-9, C-11, C-13, C-14 and C-16
H-14	C-12, C-13, C-15 and C-16
Me-16	C-12, C-13, and C-14
Me-17	C-7, C-8 and C-9
Me-20	C-9 and C-11
Me-19	C-3, C-4, C-5 and C-18
Me-18	C-3, C-4, C-5 and C-19

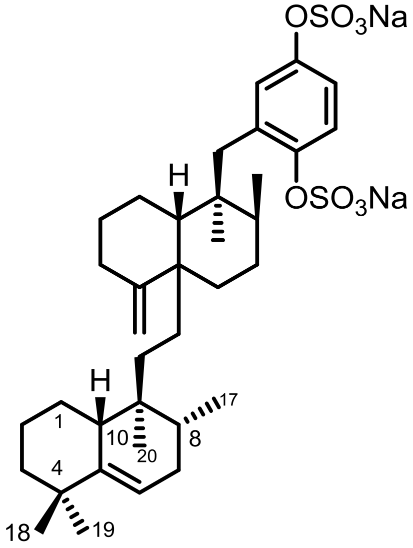
The ^{1}H and ^{13}C NMR spectra signals of the C-9 side chain (C-11– C-16) of **II.7** (**Table II.7**) are identical with those of 11*R**-acetoxykolavenic acid **II.8** (Oliveira P. et al., 2005) and plectrornatin A **II.9** (Rijo P. et al., 2002), two clerodane diterpenoids previously isolated from this species (**Table II.9**). Moreover, the ^{1}H and ^{13}C NMR data of **II.7** corresponding to the C-1 – C-10 and C-17 – C-20 hydrocarbon structural part (**Table II.7**) were very similar to those reported for the same structural moiety of akaterpin **II.10**, a triterpenoid found in a marine sponge (Fukami A. et al., 1997; see **Table II.10**). These spectral analogies between compounds **II.8**-**II.10** and the new diterpene of *P. ornatus* suggested a 5-halimene hydrocarbon skeleton for this compound, and its structure, as depicted in **II.7,** was confirmed as follows. The HMBC spectrum of **II.7** showed correlations between the olefinic H-6 proton (δ 5.45) and the C-4, C-7, C-8 and C-10 carbons (δ_C 36.3, 31.3, 32.9 and 40.3, respectively), the protons of both C-4 methyl groups (δ_H 0.98 and 1.05, Me-18 and Me-20, respectively) were connected with the C-3 – C-5 carbons (δ_C 40.9, 36.3 and 145.2, respectively), and the

Me-20 protons (δ_H 0.69) displayed correlations with the C-8 – C-11 carbons (δ_C 32.9, 41.6, 40.3 and 75.0, respectively). Moreover, the C-5 carbon (δ_C 145.2) showed HMBC connectivities with the H-1α, H-3β, H-7α, H-7β, H-10β, Me-18 and Me-19 protons (**Table II.7**), and the C-8 carbon (δ 32.9) was correlated with the H-6, H-7α, H-7β, H-10β, H-11 and Me-17 protons (**Table II.8**). In addition, the protons and carbons of the side chain of compound **II.7**, corresponding to its C-11 – C-16 at C-9 (**Table II.9**), showed HMBC connectivities identical to those observed for the same structural part of **II.8** and **II.9** (Rijo P. et al., 2002; see **Table II.8**). All the HMBC data acquired for this diterpenoid are only compatible with the structure of **II.7** herein presented.



Compound **II.8** R$_1$ = H$_2$, R$_2$ = H

Compound **II.9** R$_1$ = O, R$_2$ = Me



Compound **II.10**

Table II.9. Comparison between the ^{1}H and ^{13}C NMR data of the C-9 side chain (C-11 – C-16) of compounds 11*R**-acetoxyhalima-5,13*E*-dien-15-oic acid **II.7**, **II.8** (Oliveira P. et al., 2005) and **II.9** (Rijo P. et al., 2002; ^{1}H NMR, CDCl$_3$, 400 MHz and ^{13}C NMR,CDCl$_3$, 100 MHz; δ in ppm, *J* (H,H) in Hz).

^{1}H Position	**δ_H II.7**	**δ_H II.9**	**δ_H II.8**	**^{13}C Position**	**δ_C II.7**	**δ_C II.9**	**δ_C II.8**
H-11	5.31 dd	5.28 dd	5.29 dd	C-11	75.0	74.5	74.4
H-12A	2.44 ddd	2.28 dd	2.30 dd	C-12	41.7	41.7	42.0
H-12B	2.36 dd	2.34 ddd	2.37 dd	C-13	159.8	155.9	158.6
H-14	5.68 qd	5.62 qd	5.66 br s	C-14	117.7	118.4	118.3
H-15	---	---	---	C-15	171.1	166.4	170.4
Me-16	2.19 d	2.12 d	2.14 d	C-16	19.0	18.8	19.0
11-OCC$\underline{H}_3$	2.00 s	1.98 s	2.01 s	11-O$\underline{C}$CH$_3$	170.7	170.3	170.7
---	---	---	---	11-OC$\underline{C}$H$_3$	20.8	20.7	20.7

Table II.10. Comparison of the ^{1}H and ^{13}C NMR data of the C-1 – C-10 and C-17 – C-20 hydrocarbon structural part obtained for 11*R**-acetoxyhalima-5,13*E*-dien-15-oic acid **II.7**[a] with that of akaterpin **II.10**[b] (Fukami A. et al., 1997) (^{1}H NMR, CDCl$_3$[a] and CD$_3$OD[b], 400 MHz and ^{13}C NMR,CDCl$_3$[a] and CD$_3$OD[b], 100 MHz; δ in ppm, *J* (H,H) in Hz).

Position	**δ_H II.7**	**δ_H II.10**	**δ_C II.7**	**δ_C II.10**
1α	1.10 qd	1.68 m	28.9	28.9
1β	1.92 dtd	0.98 m	---	---
2α	1.64 ddddd	1.57 m	22.5	23.3
2β	1.52 qt	1.57 m	---	---
3α	1.19 td	1.42 m	40.9	42.2
3β	1.42 ddd	1.20 m	---	---
4	---	---	36.3	37.1
5	---	---	145.2	147.4
6	5.45 dt	---	116.0	d
7α	1.78 ddd	1.84 ddd	31.3	32.7
7β	1.84 ddd	1.77 ddd	---	---
8β	1.56 ddq	1.47 m	32.9	34.6
9	---	---	41.6	37.6
10β	2.07 ddd	2.23 m	40.3	41.0
Me-17	0.99 d	0.86 d	17.2	15.6
Me-18	0.98 s	1.04 s	28.1	29.5
Me-19	1.05 s	1.06 s	29.7	30.3
Me-20	0.69 s	0.59 s	11.8	16.8

The relative configuration of **II.7** was established by NOE experiments (**Table II.11**). Irradiation at δ 5.68 (H-14 proton) produced NOE enhancement in the signals of the H-11 and both H-12 protons, but not in the Me-16 signal, thus establishing a 13*E* configuration for the Δ^{13} – trisubstituted olefin. Furthermore, irradiation at δ 2.07 (H-10β proton) caused NOE enhancements in the signals of the H-1β, H-8β and Me-18 protons, whereas irradiation of the Me-20 protons (δ 0.69), caused an enhancement in the signals of the H-1α, H-7α, H-11 and Me-17 protons. These results established that the H-1β, H-8β, H-10β and Me-18 hydrogens are on the same side of the plane of the decalin part of **II.7**, and that the H-1α, H-7α, Me-17 and Me-20 hydrogens are on the opposite side. This conclusion was also supported by the coupling constant values observed for the axial H-8β and H-10β protons ($J_{8\beta,7\alpha}$ = 12.4 Hz, $J_{8\beta,7\beta}$ = 2.8 Hz, $J_{10\beta,1\alpha}$ = 12.8 Hz and $J_{10\beta,1\beta}$ = 2.0 Hz, **Table II.7**). Therefore, it was ascertained that compound **II.7** is the 11-acetoxyhalima-5,13*E*-dien-15-oic acid.

The configuration of the C-11 stereogenic centre of **II.7** was not ascertained. However, it´s likely that it should have the *R** stereochemistry, because the ^{1}H and ^{13}C NMR data of the C-9 side chain of **II.7** were almost identical to those reported for compounds **II.8** (Oliveira P. et al., 2005) and **II.9** (**Table II.9**; Rijo P. et al., 2002).

The A ring has a $^{4}C_{1}$ chair conformation with protons H-1α, H-2β, H-3α, Me-18(β) and H-10β with an axial orientation, and protons H-1β, H-2α, H-3β and Me-19(α) in the equatorial positions. However the C-5—C-6 double bond indicates that the A ring adopts a distorted $^{4}C_{1}$ chair conformation. The B ring presents a half chair $^{8}HC_{9}$ conformation, in which the axial substituents are protons H-10β, H-8β, H-7α and Me-20 and the equatorial positions are occupied by protons H-6, H-7β and Me-17. These conformations are in agreement with the values observed for the coupling constants (see **Table II.7**).

Table II.11. Significant NOEs for compounds **II.7**

Irradiated proton (δ)	Observed NOE with protons (% NOE enhancement)
H-14 (5.68)	δ 5.31, H-11 (+0.8), δ 2.44 + 2.36, 2H-12 (+5.3), δ 2.19,Me-16 (0.6), δ 2.00, 11-OAc (0.8), δ 1.10, H-1α (+0.7);
H-6 (5.45)	δ 1.78 + 1.84, 2H-7 (+6.6); δ 1.05, Me-19 (α- equatorial, +8.8).
H-11 (5.31)	δ 5.68, H-14 (+1.6); δ 2.44 + 2.36, 2H-12 (+3.4); δ 2.19, Me-16 (+7.9); δ 2.07, H-10β (+4.7); δ 1.92, H-1β (+10.2); δ 0.69, Me-20 (+2.9).
Me-16 (2.19)	δ 2.19, H-14 (+0.4); δ 5.31, H-11 (2.1); δ 2.44 + 2.36, 2H-12 (+1.6).
H-10β (2.07)	δ 5.31, H-11 (+4.4); δ 2.44 + 2.36, 2H-12 (+6.7); δ 1.92, H-1β (+3.2); δ 1.56, H-8β (+5.43); δ 0.98, Me-18 (β- axial, 5.7).
Me-19 (1.05)	δ 5.45, H-6 (+16.7); δ 0.98, Me-18 (+6.0); δ 1.42, H-3β (+~4.0).
Me-20 (0.69)	δ 5.31, H-11 (+4.8); δ1.78 , H-7α (+2.0); δ 1.10 + 0.99, H-1α and Me-17 (+ 8.9).

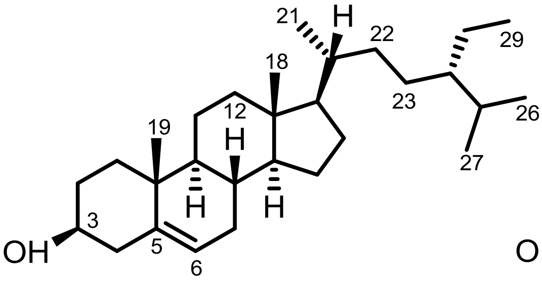
II.2.2.3. Chemotaxonomic and biogenetic significance

This is the first report on the isolation of rhinocerotinoic acid **II.6** from a Lamiaceae species and also on the presence of a halimane-type diterpene **II.7** in a plant belonging to the genus *Plectranthus*. Moreover, only few 5-halimene derivatives like **II.7** have been isolated so far, and they were all found in Euphorbiaceae and Asteraceae plants.

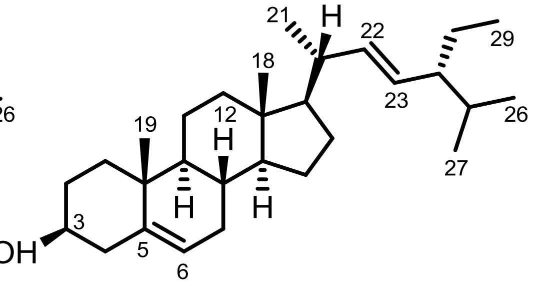
The isolation of the 5-halimene derivative **II.7** from *P. ornatus* is of biogenetic significance, because 5- and 5(10)-halimenes arise from an intermediate in the biosynthetic transformation of labdanes into clerodanes (Merritt A. T. and Ley S. V., 1992). Diterpenoids belonging to both labdane- and clerodane-type had previously been found in *P. ornatus* (Oliveira P. et al., 2005; Rijo P. et al., 2002, 2005), therefore compound **II.7** could be considered as the missing link of that biosynthetic pathway in this species (Rijo P. et al., 2007).

II.2.3. Mixtures of six known triterpenoids

II.2.3.1. β-Sitosterol II.11 and stigmasta-5,22*E*-dien-3β-ol II.12



II.11



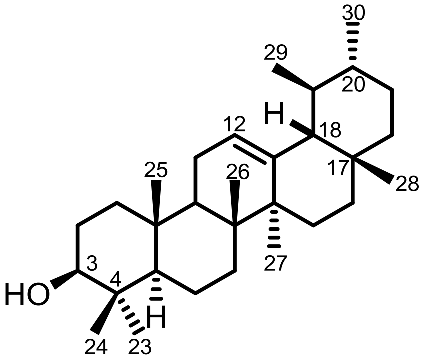
II.12

A mixture of β-sitosterol **II.11** and stigmasta-5,22*E*-dien-3β-ol **II.12** in a (1:1) ratio was isolated from many chromatographic fractions of the extract of this plant. The mixture crystallized spontaneously, as white needles with m.p. 139 - 146 ˚C. The identification of **II.11** and **II.12** was done by tlc by comparison with an authentic sample. The tlc, prepared with co-aplication, revealed the characteristic pink colour of compounds β-sitosterol **II.11** and stigmasta-5,22*E*-dien-3β-ol **II.12** confirming their presence.

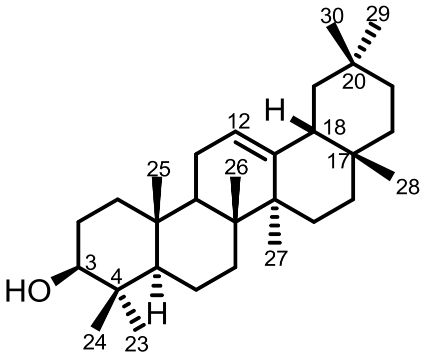
The ^{1}H NMR spectrum of the mixture, showed the olefinic H-6 proton at δ 5.35 and the geminal proton to hydroxyl group H-3α at δ 3.52, for both phytosterols. The (1:1) ratio was established through the comparison of the integral of the olefinic protons H-22 and H-23 of compound stigmasta-5,22*E*-dien-3β-ol **II.12** at δ 5.15 (1H, dd, $J_{22,23}$=15.2 Hz, $J_{22,20}$ =8.4 Hz, H-22) and δ 5.01 (1H, dd, $J_{23,22}$ =15.2 Hz, $J_{23,24}$ = 8.8 Hz, H-

23) with the integral of the H-6 and/or H-3α protons. The methyl signals seen from δ 1.02 to 0.67 were complex but it was possible to assign the singlet at δ 1.00 to methyl Me-19 of both compounds **II.11** and **II.12**, the doublet signal at δ 1.01 (3H, d, $J_{21,20}$ =6.8 Hz, Me-21) to Me-21 group of compound **II.12** and the more shielded signal at δ 0.91 (3H, d, $J_{21,20}$ =6.4 Hz, Me-21) of compound **II.11**. Two singlet signals at δ 0.69 and δ 0.67 were assigned to C-18 methyl groups of compounds **II.12** and **II.11**, respectively (Kojima H. et al., 1990).

II.2.3.2. α-Amyrin II.13 and β-amyrin II.14



II.13

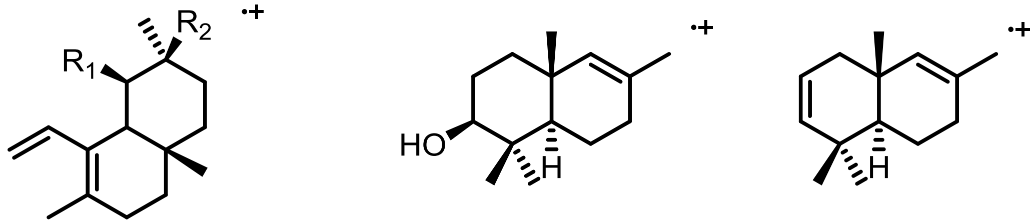


II.14

A mixture of α-amyrin **II.13** and β-amyrin **II.14** in a (3:1) ratio was isolated as white crystals needles with m.p. 175-180 ˚C. The identification of **II.3** and **II.4** was done by tlc, comparing with an authentic sample (co-aplication). The characteristic purple colour presence of α-amyrin **II.13** and β-amyrin **II.14** was confirmed by tlc.

The ^{1}H NMR spectrum of the mixture showed the olefinic protons H-12 at δ 5.18 (1H, t, $J_{12,11}$ = 3.8 Hz, H-12) of compound **II.14** and the more shielded signal at δ 5.12 (1H, t, $J_{12,11}$ = 3.6 Hz, H-12) of compound **II.13**. The (3:1) ratio was established by comparison of the integral of the olefinic H-12 proton present on both compounds. The geminal proton to hydroxyl group, H-3α at δ 3.22 (1H, dd, $J_{3\alpha,2\beta}$ = 10.8 Hz, $J_{3\alpha,2\alpha}$ = 5.0 Hz, H-3α) was identified for both compounds **II.13** and **II.14**. The ^{1}H NMR spectrum also showed the allylic H-18 proton at δ 2.00 (1H, dd, $J_{18,19}$ = 13.4 Hz, $J_{18,19\beta}$ = 4.4 Hz, H-18, compound **II.14**) and the more shielded signal at δ 1.19 (1H, d, $J_{18,19\beta}$ = 3.6 Hz, H-18, compound **II.13**). The mixture of compounds showed a complex signal at δ from 1.13 to 0.78, corresponding to methyl signals of Me-23 to Me-30. The identification of the mixture was definitely established by comparing its ^{1}H NMR spectrum with that of an authentic sample (Shingu T. et al., 1973).

The mass spectrum confirmed the identification of α-amyrin **II.13** and β-amyrin **II.14** showing the presence of fragments m/z 218 (100%), m/z 208 (4%) and m/z 190 ($[a\text{-}H_2O]^{+\cdot}$, 10%) but with no discrimination between compounds **II.13** and **II.14** (**Figure II.3;** Djerassi C. et al., 1962; Budzikiewicz H. et al., 1963).



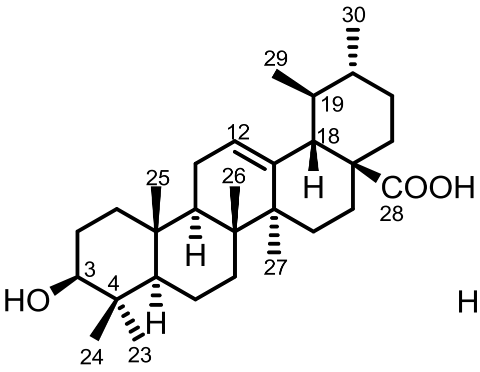
m/z = 218 m/z = 208 a: m/z = 190

α-amyrin **II.13.** R_1 = Me; R_2 = H

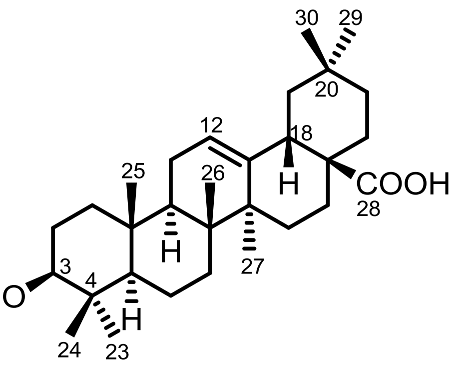
β-amyrin **II.14.** R_1 = H; R_2 = Me

Figure II.3. Fragments m/z 218, m/z 208 and m/z 190 of the mixture of α-amyrin **II.13** and β-amyrin **II.14** (Djerassi C. et al., 1962; Budzikiewicz H. et al., 1963).

II.2.3.3. Ursolic and oleanolic acids II.15 and II.16



II.15

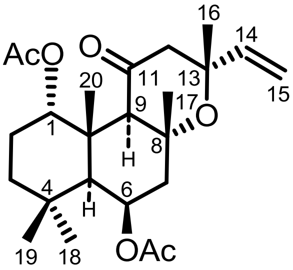


II.16

The mixture of ursolic and oleanolic acids **II.15** and **II.16** in (1:4) ratio was isolated as white crystals needles with m.p. 243-249 ˚C. The tlc comparison with a standard sample (co-aplication), confirmed the characteristic pink colour presence of ursolic and oleanolic acids **II.15** and **II.16**.

The ^{1}H NMR spectrum of the mixture showed the olefinic H-12 proton at δ 5.12 (1H, br s, H-12) of compound **II.16** and at δ 5.09 (1H, br s, H-12) of compound **II.15**. The (1:4) ratio was determined using the integral area of the H-12 proton of the corresponding compounds. The E ring methyl substitution is the only structural difference between compounds **II.15** and **II.16**. The methyl group on carbon C-29 presence, of compound **II.15,** displays the shielding effect of proton H-18. The geminal proton to hydroxyl group, H-3α at δ 3.04 (1H, dd, $J_{3\alpha,2\beta}$ = 8.0 Hz, $J_{3\alpha,2\alpha}$ = 6.0 Hz, H-3α) was identified for both compounds **II.15** and **II.16**. The ^{1}H NMR spectrum showed the allylic proton H-18 at δ 2.04 (1H, dd, $J_{18,19}$ = 11.6 Hz, $J_{18,12}$ = 2.0 Hz, H-18, compound **II.15**) and the more shielded doublet proton at δ 1.84 (1H, ddd, $J_{18,19\alpha\ (a,a)}$= 13.2 Hz, $J_{18,19\beta\ (a,e)}$ = 8.6 Hz, $J_{18,12\ (allylic)}$ = 2.0 Hz, H-18 compound **II.16**). The methyl signals of both compounds **II.15** and **II.16** from δ 1.00 to 0.62 (Me-23 to Me-30) were complex. The highfield part of the spectrum showed seven intense signals that could correspond to the seven methyl groups of oleanane skeleton, of compound **II.16.** The other seven methyl groups, including two doublet signals for C-29 and C-30 methyl groups, could correspond to the ursane skeleton, of compound **II.15** (Janicsak G. et al., 2006).

II.2.4. Plectrornatin C II.17



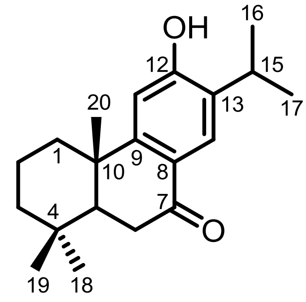
II.17

Labdane **II.17** was isolated before from a fraction studied earlier on the same plant extract (Rijo P. et al., 2002). The ^{1}H and ^{13}C NMR spectra showed characteristic signals identical to those reported before (Rijo P. et al., 2002). The five methyl groups attached to fully substituted sp^3 carbon atoms, a vinyl group also on a quaternary carbon, an acetoxyl group at carbon C-1 (δ_H 1.95, 3H, s; δ_C 169.5 s and 21.4 q) in an axial configuration (geminal H-1β proton at δ 5.53 t, J = 2.8 Hz; δ_C 75.1 d) and placed between a fully substituted sp^3 carbon and a methylene grouping. A (C)- CH-CHOAc-CH_2-(C) structural moiety in which the acetoxyl group must also be in an axial configuration (geminal H-6α proton of the acetate at δ 5.57 (ddd, J = 3.8, 2.9, and 2.6 Hz, δ_C 69.5 d); acetoxyl group at carbon C-6 (δ_H 2.05, 3H, s, and δ_C 169.9 s and 21.8 q); methine group at carbon C-5 (δ_H 1.47 d, J = 2.6 Hz, δ_C 49.1 d); methylene grouping at carbon C-7 (δ_H 1.90 dd, J = 14.6 and 3.8 Hz, H-7α and δ 2.26 dd, J = 14.6 and 2.9 Hz, H-7β, δ_C 46.2 t), and finally, another (C)-CH-CO-CH_2-(C) partial structure (ketone at C-11, δ_C 206.2 s; methine at C-9, δ_H 3.23 s and δ_C 58.2 d; methylene protons as an AB system at carbon C-12, δ 2.60 and 2.67, both d, $J_{geminal}$ = 18.6 Hz).

Comparing all these spectral data with those previously obtained (Rijo P. et al., 2002), established the structure of this compound as **II.17**.

II.3. Structure elucidation of terpenic compounds from *Plectranthus ecklonii* Benth.

II.3.1. The diterpenoid sugiol II.18



II.18

Sugiol **II.18**, is an abietane type diterpene, former isolated from several plants including *Salvia* (Ulubelen A. et al., 2000), and was isolated from *Plectranthus ecklonii* Benth. for the first time.

Sugiol **II.18** was isolated as white crystals with m.p. 288 – 290 ˚C with $[\alpha]_D^{27}$ + 30.8 (c 0.133, $CHCl_3$). Its EIMS showed a molecular ion at *m/z* 300, consistent with the molecular formula of $C_{20}H_{28}O_2$. The I.R. spectrum confirmed the presence of a hydroxyl group (3140 cm^{-1}), and in addition absorption band for an α,β-unsaturated carbonyl group at 1650 cm^{-1}. The spectral assignments of ^1H and ^{13}C NMR spectra of sugiol **II.18** (**Table II.12**) are in agreement with the previously data reported for this compound (Rodríguez B., 2003).

Table II.12. NMR data of sugiol **II.18**, (acetone-d$_6$, ^1H 400 MHz, ^{13}C 100 MHz; δ in ppm, *J* in Hz).

Position	δ_H	$J_{H,H}$	δ_C	HSQC
1α	1.47 ddd	(1α, 1β) 12.8	37.34	t
1β	2.20 ddd	(1α, 2α) 3.6		
2α	1.63 ddddd	(1α, 2β) 13.2	18.81	t
2β	1.79 ddddd	(1β, 2α) 1.6		
3α	1.27 ddd	(1β, 2β) 3.2	40.82	t
3β	1.50 ddd	(2α, 2β) 14.0		
4		(2α, 3α) 3.6	32.58	s
5α	1.80 dd	(2α, 3β) 1.8	49.14	d
6α	2.50 dd	(2β, 3α) 13.6	35.21	t
6β	2.55 dd	(2β, 3β) 3.5		
7	---	(3α, 3β) 13.6	196.11	s
8	---	(5α, 6α) 8.8	123.18	s
9	---	(5α, 6β) 8.8	155.59	s
10	---	(6α, 6β) 17.6	37.32	s
11	6.82 s	(15,16(17)) 6.9	109.00	d
12	---	(14,15) 0.6	159.25	s
13	---	(1α, 20) 0.8	132.41	s
14	7.79 d		125.23	d
15	3.23 sept of d		26.07	d
Me-16*	1.21 d		21.58	q
Me-17*	1.19 d		21.46	q
Me-18	0.92 s		31.71	q
Me-19	0.99 s		20.46	q
Me-20	1.20 d		22.32	q
12-OH	8.90 br		---	---

*Interchangeable assignments.

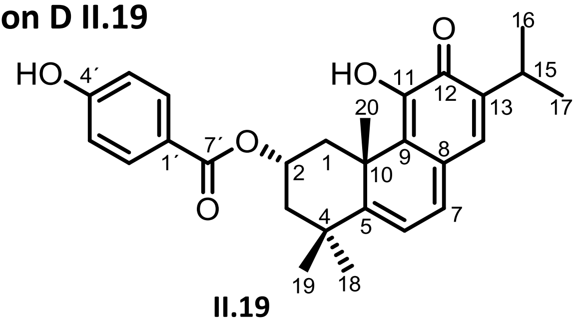
The HSQC and HMBC experiments were also in agreement with the literature (Rodríguez B., 2003) and the latter significant assignments are shown in **Table II.13**.

Table II.13. Significant assignments observed on HMBC experiment for sugiol **II.18.**

Position	HMBC
12-OH	C-11, C-12 and C-13
H-14	C-7, C-9, C-12, C-15
H-11	C-8, C-9, C-10, C-12 and C-13
H-15	C-12, C-13, C-14, C-16 and C-17
H-6α and H-6β	C-4, C-5, C-7, C-8 and C-10
H-1β	C-2, C-5, C-10, C-20
Me-19	C-3, C-4, C-5cand C-18
Me-18	C-3, C-4, C-5 and C-19

The vicinal coupling constant values for C-1 - C-3 methylene protons (**Table II.12**) established that ring A of this diterpene is in chair conformation ($^4C_1$) from the vicinal $J_{5\alpha,6\alpha}$ and $J_{5\alpha,6\beta}$ values (**Table II.12**). Sugiol **II.18** possesses a half-boat conformation for its ring B ($^{7,10}B$, $J_{5\alpha,6\alpha} = J_{5\alpha,6\beta}$ = 8.8 Hz; Rodríguez B., 2003).

II.3.2. Parvifloron D II.19



II.19

Parvifloron D **II.19** (19.2 g, 0.27%), is an abietane type diterpene, previously isolated from several Plectranthus species including *P. parviflorus* (Ruedi P., Eugster C.H., 1978) and *P. strigosus* (Alder A.C. et al., 1984; Gaspar-Marques C., 2008) and reported as an antibacterial metabolite from *P. ecklonii* (Nyila M.A. et al., 2009).

Parvifloron D **II.19** was isolated from *P. ecklonii* as orange crystals of m.p. 157-159 ˚C. The I.R. spectrum confirmed the presence of hydroxyl and phenol groups (3329 cm^{-1}) and the absorption bands for an aromatic ring (3100-300, 1600 and 773 cm^{-1})

and carbonyl (1669 cm^{-1}) groups. The ^{1}H and ^{13}C NMR spectral data obtained of compound **II.19** (**Table II.14**) were in agreement with the data described in the literature (Alder A.C. et al., 1984; Gaspar-Marques C. et al., 2008) for parvifloron D **II.19**. The structure of parvifloron D **II.19** was confirmed by two-dimensional NMR experiments (COSY, HMQC and HMBC) and the significant correlations of long-range H-C coupling were determinate by HMBC experiment spectrum and shown on **Table II.15**.

Table II.14. NMR data of parvifloron D **II.19,** ($CDCl_3$,^{1}H 400 MHz, ^{13}C 100 MHz; δ in ppm, *J* in Hz).

Position	δ_H	$J_{H,H}$	δ_C	HSQC
1α	1.74 dd	(1α, 1β) 13.0	38.37	t
1β	3.76 ddd	(1α, 2β) 11.4		
2β	5.59 tt	(1β, 2β) 4.4	67.87	d
3α	1.56 dd	(2β, 3α) 11.4	45.06	t
3β	2.15 ddd	(2β, 3β) 4.4		
4	---	(3α, 3β) 12.5	38.58	s
5	---	(6, 7) 6.9	164.84	s
6	6.41 d	(15,16(17)) 6.8	118.69	d
	---	(14,15) 0.8		
7	6.79 d	(1β,3β) 2.4	139.13	d
8	---	(2′,3′;5′,6′) 8.9	127.45	s
9	---		127.17	s
10	---		43.91	s
11	---		146.40	s
12	---		178.24	s
13	---		141.61	s
14	6.96 d		133.57	d
15	3.15 sept of d		26.52	d
Me-16*	1.18 d		21.84	q
Me-17*	1.16 d		21.63	q
Me-18	1.29 s		33.03	q
Me-19	1.42 s		30.58	q
Me-20	1.64 s		25.52	q
1′	---		122.43	s
2′, 6′	7.93 d		131.89	d
3′,5′	6.88 d		115.23	d
4′	---		160.58	s
7′	---		166.18	s
4′-OH	~7.70 br			
11-OH	~7.20 br			

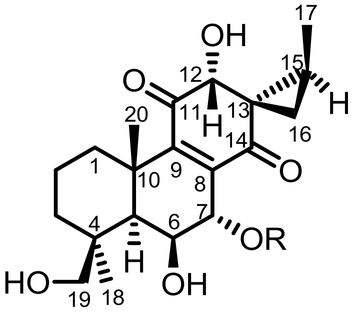
*Interchangeable assignments, ~ not assigned

Table II.15. Significant assignments observed on HMBC experiment for parvifloron D **II.19**.

Position	HMBC
H-2´,6´	C-7´ and C-4´
H-14	C-7, C-8, C-9, C-12, C-15
H-3´,5´	C-1´ and C-4´
H-7	C-5, C-6, C-8, C-9 and C-14
H-6	C-4, C-5, C-7, C-8 and C-10
H-2β	CO-(Ph)
H-1β	C-2, C-3, C-5 and C-20
H-15	C-12, C-13, C-14 and C-16(17)
H-3β	C-1, C-2, C-4, C-5, C-18 and C-19
H-1α	C-2, C-3, C-5, C-9 and C-20
H-3α	C-1, C-2, C-4, C-5, C-18 and C-19
Me-20	C-1, C-5, C-9 and C-10
Me-19	C-3, C-4, C-5 and C-18
Me-18	C-3, C-4, C-5 and C-19
Me-16(17)	C-13 and C-15

II.4. Structure elucidation of (13*S*,15*S*)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione II.20 from *P. porcatus* Winter & Van Jaarsv.

Repeated chromatographic processes of the acetone extract of the aerial parts of *P. porcatus* (see Chapter **V**) allowed the isolation of the new (13*S*,15*S*)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione **II.20** as yellowish fine needles (0.009 g; 0.009 %), which possessed a molecular formula of $C_{20}H_{28}O_6$ (HRESIMS). The ^{1}H NMR spectrum of **II.20** (**Table II.16**) was almost identical to that of spirocoleon 13 (*7*α-acetyl-12-O-desacetyl-19-hydroxycoleon Q); (13S,15S)-*7*α-acetyl-6β,12α,19-trihydroxy-13β,16-cyclo-8-abietene-11,14-dione **II.21**, a compound previously isolated (Matloubi-Moghadam F. et al.,1984) from *Coleus somaliensis* S. Moore. In fact, the only observed differences were consistent with the absence in the former of the 7-*O*-acetyl group of the latter [**II.20**: $\delta_{H\text{-}7\beta}$ 4.63 dd, $J_{7\beta,6\alpha}$ = 1.9 Hz, $J_{7\beta,7\alpha\text{-}OH}$ = 4.9 Hz; a *doublet* (*J* = 1.9 Hz) after addition of D_2O. **II.21:** $\delta_{H\text{-}7\beta}$ = 5.75 d, $J_{7\beta,6\alpha}$ = 2.00 Hz; δ 1.97, 3H, s, 7α-OAc (Matloubi-Moghadam F. et al., 1984)].



II.20, R=H;
II.21 R = Ac

Moreover, the ^{13}C NMR spectrum of **II.20** (**Table II.16**) was in complete agreement with those reported (Ruedi P. et al., 1983) for other spirocoleons possessing the same oxidation pattern and an identical stereochemistry. The stereochemistry at the C-12, C-13 and C-15 chiral centers of **II.20** was established by comparing the ^{1}H and ^{13}C NMR chemical shifts and $J_{H,H}$ values corresponding to the C-12–C-17 structural fragment (**Table II.16**) with those reported for the spirocoleon 13 **II.21** (Matloubi-Moghadam F. et al., 1984) and other related spirocoleons (Künzle J.M. et al., 1987; Rüedi P. et al., 1983; Schmid J.M. et al., 1982).

Table II.16. NMR data of compound **II.20** (acetone-d$_6$, ^{1}H 500 MHz, ^{13}C 125 MHz; δ in ppm, *J* in Hz).

Position	δ$_H$	$J_{H,H}$	δ$_C$	HSQC
1α	1.29 td	(1α, 1β) 13.5	38.76	t
1β	2.05 m**	(1α, 2α) 3.6		
2α	1.5 ddddd	(1α, 2β) 13.5	20.11	t
2β	1.79 ddddd	(1β, 2α) 3.1		
3α	1.26 ddd	(1β, 2β) 3.4	40.27	t
3β	1.55 br d	(2α, 2β) 14.1		
4	---	(2α, 3α) 3.6	39.81	s
5α	1.72 d	(2α, 3β) 3.1	50.33	d
6α	4.23 m*	(2β, 3α) 13.5	70.30	d
7β	4.63 dd	(2β, 3β) 3.4	67.16	d
8	---	(3α, 3β) 14.0	142.49	s
9	---	(5α,6α) 1.6	156.17	s
10	---	(6α,7β) 1.9	40.08	s
11	---	(15,16A) 8.5	200.28	s
12β	3.82 d	(15,16B) 6.7	78.57	d
13	---	(15,17) 6.1	37.47	s
14	---	(16A,16B) 3.7	198.00	s
15	1.44 m**	(19A, 19B) 11.1	21.63	d
16A	1.08 dd	(6α, 6β-OH) 1.8	27.28	t
16B	0.92 dd	(7β, 7α-OH) 4.9		
Me-17	1.26 d	(12β, 12α-OH) 5.5	14.15	q
Me-18	1.09 s	(19A, 19OH) 4.4	29.37	q
19A	4.24 dd	(19B, 19OH) 5.5	68.72	t
19B	3.33 dd			
Me-20	1.74 s		22.73	q
6β-OH	4.99 d			
7α-OH	4.26 d			
12α-OH	5.12 br d			
19-OH	4.98 br dd			

* overlapped or partially overlapped signals. ** Signal overlapped with the solvent signals.

In the ^{13}C NMR spectrum (**Table II.16**), were observed two singlet signals corresponding to two α,β-unsaturated carbonyl groups (δ$_C$ 200.28 and 198.00) in agreement with the I.R. spectrum (1699 and 1670 cm^{-1}). Only the C-11 carbonyl carbon is correlated with the broad doublet hydroxyl proton 12α-OH at δ 5.12 and the H-12β proton at δ 3.82. The methine H-12β correlates with both carbonyl carbons C-11 at δ 200.28 and C-14 at δ 198.00, as observed in the HMBC spectrum (**Table II.17**).

Table II.17. Significant assignments observed on HMBC experiment for compound **II.20**.

Position	HMBC
OH-12α	C-11, C-12 and C-13
OH-6β	C-5, C-6 and C-7
OH-19	C-19
H-7β	C-5, C-6, C-8, C-9 and C-14
H-6α	C-4, C-5, C-7, C-8 and C-10,
H-19A	C-13, C-4, C-5 and C-18
OH-7α	C-6, C-7 and C-8
H-12β	C-9, C-11, C-13, C-14, C-15 and C-16
H-19B	C-3, C-4, C-5 and C-18
H-15	C-13, C-14 and C-16
Me-17	C-13, C-15 and C-16
Me-18	C-3, C-4, C-5 and C-.19
H-16A	C-12, C-13, C-14, C-15 and C-17
H-16B	C-12, C-13, C-14, C-15 and C-17
Me-20	C-1, C-5, C-9 and C-10
H-5α	C-1, C-3, C-4, C-6, C-7, C-9, C-18, C-19 and C-20

The methylcyclopropane group was identified by two double doublet proton signals (1.08, dd, $J_{16A,15}$ = 8.5 Hz, H-16A and 0.92, dd, $J_{16B,15}$ = 6.7 Hz, H-16B) that were correlated with a signal at δ 2.02 (H-15) that on its turn was correlated with a doublet methyl group at δ 1.26 (1.26, d, $J_{17,15}$= 6.1 Hz, Me-17).

The downfield protons H-6α and H-7β were assigned as δ 4.23 and the double doublet at δ 4.63 respectively. They were correlated with each other and with 6β-OH and 7α-OH hydroxyl protons (4.99, d, $J_{6\beta\text{-OH},6\alpha}$ = 1.8 Hz, 6β-OH and 4.26, d, $J_{7\alpha\text{-OH},7\beta}$ = 1.8 Hz, 7α-OH) in the COSY spectrum. The COSY spectrum also showed that proton H-6α correlated with the methine H-5α at δ1.72. The observation of HMBC spectrum showed the correlation of the double bond carbons C-8 (δc 142.49, s) and C-9 (δc 156.17, s) with the proton H-7β at δ 4.63. The methine H-5α at δ 1.72 only correlated with the methine H-6α. The significant assignments observed on HMBC spectrum for compound **II.20** are shown in **Table II.17**.

The ^{1}H -^{1}H COSY spectrum was in complete agreement with the assigned structure. Proton at δ 5.12 (br d, 12α-OH) correlates with proton at δ 3.82 (d, H-12β). Hydroxyl proton signals at ~ δ 5.00 (d, 6β-OH and dd, 19-OH) correlates with the correspondents protons at δ 4.23 (m, H- 6α) and 4.24 (dd, H_A-19). Proton at δ 4.63 (dd, H-7β) correlates with hydroxyl proton at δ 4.26 (d, 7α-OH) and with proton at δ 4.23 (m, 6α-H).

The remaining hydroxyl group to assign was OH-19 at δ 4.98 ($J_{19OH,19A}$ = 4.4 Hz, $J_{19\text{-}OH,19B}$= 5.5 Hz) as a broad double doublet. The double doublet protons at δ 4.24 and δ 3.33, H-19A and H-19B respectively, showed connectivities with each other ($J_{19A,19B}$=11.1 Hz) and with the hydroxyl group (19-OH). The difference in δ values of the diastereotopics H-19A at δ 4.24 and H-19B at δ 3.33 is due to the intramolecular hydrogen bridge between the hydroxyl group 19-OH and the hydroxyl group at 6β-OH.

Some signals were overlapped (H-1β, H-6α and H-15) with the solvent (acetone-d_6) signal, so it was decided to repeat the ^{1}H-NMR spectrum in CD_3OD as presented in **Table II.18.** The proton H-1β appeared at δ 2.07 as a ddd, showing all three coupling constants ($J_{1\beta,\ 1\alpha}$=13.0 Hz, $J_{1\beta,\ 2\alpha}$=3.0 Hz, $J_{1\beta,\ 2\beta}$=3.7 Hz), proton H-6α as a broad singlet at δ 4.21 ($J_{6\alpha,\ 5\alpha}$=~1.5 Hz, $J_{6\alpha,\ 7\beta}$=2.5 Hz) and proton H-15 as a ddq at δ 2.11 ($J_{15,16A}$= 8.6 Hz, $J_{15,16B}$ = 6.8 Hz, $J_{15,17}$= 6.1 Hz).

Table II.18. NMR data of compound **II.20** (CD$_3$OD, ^{1}H 500 MHz; δ in ppm, *J* in Hz).

Position	**δ_H**	**$J_{H,H}$**
1α	1.29 ddd	(1α, 1β) 13.0
1β	2.07 ddd	(1α, 2α) 3.7
2α	1.53 ddddd	(1α, 2β) 14.2
2β	1.81 qt	(1β, 2α) 3.0
3α	1.26 m*	(1β, 2β) 3.7
3β	1.56 ddd	(2α, 2β) 14.2
4	---	(2α, 3α) 3.7
5α	1.68 br s§	(2α, 3β) 3.0
6α	4.21 br s$^{\#}$	(2β, 3α) 14.2
7β	4.63 d	(2β, 3β) 3.7
8	---	(3α, 3β) 14.2
9	---	(5α,6α) ~1.5
10	---	(6α,7β) 2.5
11	---	(15,16A) 8.6
12β	3.75 s	(15,16B) 6.8
13	---	(15,17) 6.1
14	---	(16A,16B) 3.7
15	2.11 ddq	(19A, 19B) 11.1
16A	1.15 dd	
16B	0.93 dd	
Me-17	1.26 d	
Me-18	1.09 s	
19A	4.19 d	
19B	3.29 d	
Me-20	1.70 s	
6β-OH	---	
7α-OH	---	
12α-OH	---	
19-OH	---	

$^{\#}w_{1/2}$ = 6 Hz, § $W_{1/2}$ = 2.2 Hz, * overlapped signal.

The results of the NOE experiment are summarized in **Table II.19** and show the information of more significant NOEs irradiations.

Table II.19. Significant NOEs for compounds **II.20**.

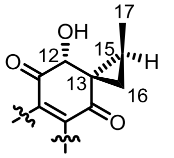
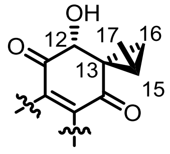
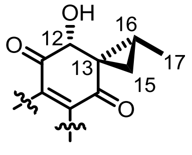
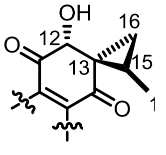
Irradiated proton[a] (δ)	Observed NOE with protons (% NOE enhancement)
H-12β (3.75)	δ 0.93, H-7α (+2.4);
	δ 1.26, Me-17 (+2.6);
H-16A (1.15)	δ 2.11, H-15 (+5.8);
	δ 0.93, H-16B (+25.4).
Me-18 (1.09)	δ 4.21, H-6α (+9.5);
	δ 4.19, H-19A (~+2.0);
	δ 3.29, H-19B (+3.6);
	δ 1.68, H-5α (+5.7);
	δ 1.56, H-3β (+1.3);
	δ 1.26, H-3α (+2.5).
H-16B (0.93)	δ 3.75, H-12β (+3.6);
	δ 2.11, H-15 (<0.5);
	δ 1.26, Me-17 (+2.1);
	δ 1.15, Me-16A (+15.8).

[a] CD_3OD spectrum.

Considering the protons H-16A and H-16B, the irradiation of proton H-16A at δ 1.15 on NOE experiment showed a big effect (+25.4%) on the geminal proton H-16B at δ 0.93 and a moderate effect on H-15 (+5.8%) thus H-16A is a *pro-S* proton. The irradiation of proton H-16B at δ 0.93 showed NOE effects on H-16A (+15.8%), H-12β (+3.6%), Me-17 (+2.1%), and H-15 (<0.5%), showing that H-16B is an enantiotopic proton, *pro-R*.

Compound **II.20** showed a positive sign for its specific rotation ($[\alpha]_D^{20}$ + 218.8) like **II.21** (Matloubi-Moghadametal F., 1984) and other spirocoleons previously found in plants belonging to the *Plectranthus* and *Coleus* genera (see **Table II.20;** Künzle J.M. et al., 1987; Matloubi-Moghadametal F., 1984; Rüedi P. et al., 1983; Schmid J.M. et al., 1982) and whose absolute stereochemistry has been rigorously established. For this reason, and also on biogenetic grounds, was assumed that **II.20** possesses a *normal*-abietane absolute configuration. Thus, this new diterpenoid must be (13S,15S)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14- dione **II.20**.

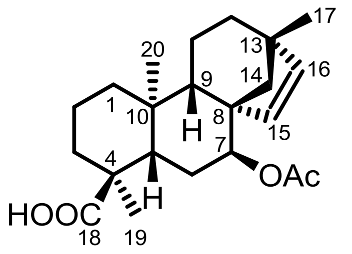
Table II.20. Comparation of spectral and chiroptical data of the diastereomers and compound **II.20** (Rüedi P. et al., 1983).

spectral and chiroptical data	**12R, 13S, 15S** 	**12R, 13R, 15R** 	**12R,13S,15R** 	**12R,13R15S** 	**II.20**
$[\alpha]_D^{23}$ (°)	+181.0	+100.0	+99.5	+147.5	+218.8
^{1}H NMR ($CDCl_3$) δ(H-12β)	4.11 s	4.41 s	4.69 s	3.93 s	3.82 d
δ(Me-17)	1.27 d	1.26 d	0.89 s	1.27 d	1.26 d
^{13}C NMR (Acetone)					
δ(C-12)	77.6	75.3	75.6	81.7	78.57
δ(C-15)	20.8	27.3	20.4	25.3	21.63
δ(C-16)	26.5	21.5	16.9	18.8	27.28
δ(C-17)	13.1	13.4	11.5	11.1	14.15

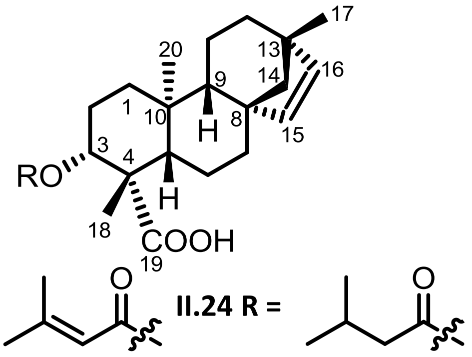
These values for **II.20** were identical with those of compounds with a (12R,13S,15S)-configuration and very different from those of the other diastereoisomers [(Künzle J.M. et al., 1987; Matloubi-Moghadam F., et al. , 1984; Rüedi P. et al., 1983; Schmid J.M., et al., 1982); see **Table II.20**].

II.5. Structure elucidation of *ent*-7α-acetoxy-15-beyeren-18-oic acid II.22 from *Plectranthus saccatus* Benth.

The new diterpenoid **II.22**, isolated from an acetone extract of the aerial parts of *P. saccatus* (see Chapter **V**) was a colourless crystalline solid (0.0024 g; 0.0024 %), for which a molecular formula of $C_{22}H_{32}O_4$ was established by HRESIMS. The ^{1}H and ^{13}C NMR spectra of *ent*-7α-acetoxy-15-beyeren-18-oic acid **II.22** (**Table II.21**) were very similar to those of 3α,15-beyerene **II.23** diterpenoid previously isolated from *P. saccatus* (Wellsow J. et al., 2006).



II.22



II.23 R = **II.24 R =**

Table II.21. NMR data of compound **II.22** (CDCl$_3$, ^{1}H 500 MHz, ^{13}C 125 MHz; δ in ppm, *J* in Hz).

Position	δ_H	$J_{H,H}$	δ_C	HSQC
1α	1.64 m*	(2α, 3β) 14.7	38.0	t
1β	1.02 m*	(2β, 3β) 2.5		
2β and 2α	1.53 (2H) m*	(3α, 3β) 14.7	17.6	t
3α	1.64 m*	(5β,6α) 12.8	36.8	t
3β	1.72 td	(5β,6β) 1.5		
4	---	(6α,7α) 2.7	46.7	s
5β	2.24 dd	(6β,7α) 2.7	42.4	d
6α	1.72 m*	(14α, 14β) 9.1	27.4	t
6β	1.49 m*	(15, 16) 5.8		
7α	4.76 t	(14α, 12α) 2.1	76.0	d
8	---	(16, 12[#]) 1.0	52.9	s
9β	1.45 m*		48.1	d
10	---		36.6	s
11α	1.28 m*		19.6	t
11β	1.53 m*			
12α and 12β	1.26 (2H) m*		32.7	t
13	---		43.9	s
14α	1.44 dd		56.6	t
14β	1.26 d			
15	5.53 d		132.1	d
16	5.55 dd		138.1	d
Me-17	1.01 s		24.7	q
18	---		184.1	s
Me-19	1.15 s		16.4	q
Me-20	0.79 s		14.9	q
OAc (CO)	---		170.8	s
OAc (CH$_3$)	2.04 s		21.1	q

[#] long range, * overlapped or partially overlapped signals. δ were measured from the HSQC spectrum.

The ^{1}H and ^{13}C NMR spectra of **II.22** showed signals, among others, for a carboxylic acid function (δ_C 184.1, q, C-18), a (*Z*)-1,2-disubstituted olefin (δ_H 5.53, d, H-15 and δ_H 5.55, dd, H-16, J_{vic} = 5.8 Hz) linked directly to quaternary carbon atoms C-8 and C-13 and an acetoxyl group at C-7β (δ_H 2.04, 3H, s; δ_C 170.8, q, and δ_C 21.1, CH_3). This acetoxyl substituent must be axially attached to a secondary carbon atom (δ_C 76.0, C-7; δ_H 4.76, 1H, t, $J_{e,a}$ = $J_{e,e}$ = 2.7 Hz, H-7α) which was placed between a fully substituted carbon C-8 and a methylene group (H-6α and H-6β). The observed HMBC connectivities (**Table II.22**) between the carbonyl carbon of the acetate (δ 170.8) and the proton at δ 4.76 (H-7α), and between this proton and carbons C-5, C-6, C-8, C-9, C-14 and C-15 (δ 42.4, 27.4, 52.9, 48.1, 56.6 and 132.1, respectively), established that the acetoxyl substituent of **II.22** was at the 7β-position (*ent*-7α). The α- or β- configuration for a substituent indicates that it is placed, respectively, below or above the plane of the formula depicted for **II.22**. However, since is assumed that this compound belong to the *enantio* series, those configurations should be described more rigorously as *ent*-β or *ent*-α, indicating that the substituent is placed, respectively, below or above the plane of the depicted structure.

The HMBC spectrum of **II.22** (**Table II.22**) showed long-range connectivities between the carboxylic carbon (δ_C 184.1, C-18) and the protons at δ 2.24 (H-5), 1.72 and 1.64 (H-3α and H-3β), and 1.15 (quaternary methyl group, Me-19), whereas the methyl protons at δ 1.15 were HMBC correlated with C-3, C-4, C-5 and with the carboxylic carbon (δ 36.8, 46.7, 42.4 and 184.1, respectively). These results established that both a carboxylic function and a quaternary methyl group at δ_H 1.15 (δ_C 16.4) were attached to the C-4 position (δ_C 46.7, s) of the beyerane skeleton. From the ^{13}C NMR chemical shifts of the carboxylic carbon (δ 184.1, C-18) and its geminal methyl group (δ 16.4, Me-19) it was evident (Bruno et al., 1986; Hussein A.A. et al., 1999; Hussein A.A. and Rodríguez B., 2000; Wellsow J. et al.,2006) that in **II.22** the carboxyl group was an equatorial substituent (18-position) and, consequently, the methyl group was axially oriented (19-position), with opposite stereochemistry to that in **II.23** (Wellsow J. et al. ,2006).

Table II.22. Significant assignments observed on HMBC experiment for compound **II.22**.

Position	**HMBC**
Me-20	C-1, C-5, C-9 and C-10
Me-17	C-12, C-13, C-14 and C-16
Me-19	C-3, C-4, C-5 and C-18
H-7α	C-5, C-6, C-8, C-9, C-14, C-15 and -CO-CH_3
H-5β	C-1, C-63, C-4, C-6, C-7, C-9, C-10, C-18, C-19 and C20
H-15	C-7, C-8, C-9, C-13, C-14 and C-16
H-16	C-8, C-12, C-13, C-14, C-15 and C-17

1D NOESY experiments confirmed the relative stereochemistry depicted in **II.22** for this new diterpenoid and allowed the complete assignment of the overlapped proton signals shown in **Table II.21**. Irradiation at δ 5.54 (both H-15 and H-16 protons) caused NOEs in H-6α, H-7α, H-11α, H-14α, Me-17 and Me-20, whereas the signal of the H-15 proton, among others, was strongly enhanced when the H-7α proton signal (δ 4.76) was irradiated. Moreover, on irradiation at δ 0.79 (Me-20) NOEs were observed in protons H-1α, H-2α, H-6α, H-11α, H-15 and, more important, in Me-19 (**Table II.23)**.

Table II.23. Significant NOEs for compound **II.22**.

Irradiated proton (δ)	Observed NOE with protons (% NOE enhancement)	Assigment
H-15 and H-16 (5.54)	δ 4.76, H-7α (0.8);	H-15 → H-7α
	δ 1.72, H-6α (0.8);	H-15 → H-6α
	δ 1.49, H-6β (0.3);	H-15 → H-6β
	δ 1.01, Me-17 (0.8);	H-16 → Me-17
	δ 0.79, Me-20 (1.5);	H-15 + H-16 → Me-20
	δ ~ 1.30, H-14α + H-11α (0.7).	H-15 + H-16 → H-14α + H-11α Discriminates H-11α from H-11β and H-14α from H-14β
H-7α (4.76)	δ 5.53, H-15 (1.4);	H-7α → H-15
	δ 2.24, H-5β (0.3);	H-7α → H-5β
	δ 1.72, H-6α (2.0);	H-7α → H-6α
	δ 1.49, H-6β (2.0);	H-7α → H-6β
	δ 1.44, H-14α (2.0);	H-7α → H-14α + H-14β and
	δ 1.26, H-14β (0.5).	Discriminates H-14α from H-14β
Me-20 (0.79)	δ 5.54, H-15>>H-16 (1.6);	
	δ 1.72, H-6α (1.8);	Me-20 → Me-19
	δ1.64, H-1α (0.8);	Defines configuration on C-4
	δ1.53, H-2α (0.7);	(Me-19 and COOH-18).
	δ1.28, H-11α (1.8);	
	δ1.15, Me-19 (2.1).	

→ corresponds to the irradiated proton that discriminate the assignment signals.

The absolute configuration of **II.22** was not ascertained, although on biogenetic grounds (Wellsow J. et al., 2006) can be assumed that this compound belongs to the *enantio* series, like the majority of the beyerane-type diterpenes isolated from natural sources and whose absolute stereochemistry has been rigorously determined (Connolly J.D. and Hill R.A., 1991; Jefferies et al., 1963; McMillan and Beale, 1999). From all the above data **II.22** was characterized as *ent*-7α-acetoxy-15-beyeren-18-oic acid.

It is of interest to comment that the co-occurrence in the same species of diterpenes oxidized at the C-18 **II.22** or C-19 (**II.23** and **II.24**) (Wellsow J. et al., 2006) positions is quite rare (Connolly J.D. and Hill R.A., 1991), and only a few cases have been reported hitherto (Connolly J.D. and Hill R.A., 1991; Matloubi-Moghadam et al., 1984; Simões M.F. et al., 2010a).

CHAPTER III

Synthesis of diterpenoid derivatives

Results and Discussion

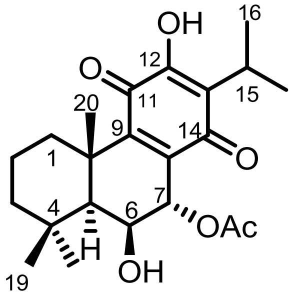
III.1. Introduction

In this chapter is described the synthesis of diterpenoid derivatives with the purpose of improving the biological activity showed by the analogous natural diterpenes. The antimicrobial activity was the main biological activity studied on the starting materials and on their derivatives. The diterpenoids 7α-acetoxy-6β-hydroxyroyleanone **III.1**, (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid **II.7** and parvifloron D **II.19** were used as starting materials to prepare the derivatives.

The previously isolated abietane 7*α*-acetoxy-6β-hydroxyroyleanone **III.1**, have shown to be active against various microorganisms namely, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VRE) (Gaspar-Marques C. et al., 2006). The diterpenoids (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid **II.7**, and parvifloron D **II.19** were isolated in significant yields (as described in Chapter **V**) and showed interesting antimicrobial activities (Chapter **VI**).

Thirteen 7*α*-acetoxy-6β-hydroxyroyleanone **III.1** derivatives were prepared using known methodologies (hydrolysis, esterification and glycosidation). The diterpenoid (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid **II.7** afforded nine derivatives using similar methodologies. From the parvifloron D **II.19** a benzoyloxy derivative of microstegiol was prepared via an easy and stereoselective rearrangement.

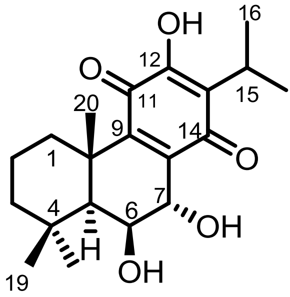
III.2. 7α-Acetoxy-6β-hydroxyroyleanone derivatives



7α-acetoxy-6β-hydroxyroyleanone **III.1**

7α-acetoxy-6β-hydroxyroyleanone **III.1** is a natural diterpene previously isolated from *Plectranthus grandidentatus* (Teixeira A.P. et al., 1997; Gaspar-Marques C et al., 2006). Due to the multiple pharmacological activities described for these oxidized abietane metabolites like royleanone **III.1**, it is plausible to think them as models for the search of further bioactive molecules (Gaspar-Marques C. et al., 2006; Cerqueira F. et al., 2004; Teixeira A.P. et al., 1997). Previous results have shown that royleanone abietanes were active against Gram-positive bacteria (Rijo P. et al., 2010; Gaspar-Marques C et al., 2006). As 7α-acetoxy-6β-hydroxyroyleanone **III.1**, showed to be a potent antimicrobial diterpenoid, **III.1** may be a lead for antimicrobial drug development (Gaspar-Marques C et al., 2006).

III.2.1. Basic hydrolysis of 7α-acetoxy-6β-hydroxyroyleanone III.1

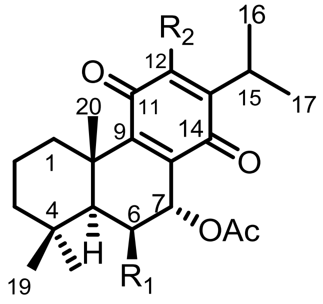


6β,7α-dihydroxyroyleanone **III.2**

6β,7α-dihydroxyroyleanone **III.2** is a known compound (Hersch M. et al., 1975) that was obtained by basic hydrolysis of 7α-acetoxy-6β-hydroxyroyleanone **III.1** using a potassium hydroxide solution. The ^{1}H NMR and ^{13}C NMR spectra confirmed the structure of compound **III.2** showing similar signals to compound **III.1**. Royleanone **III.2** showed an additional hydroxyl proton (s, OH-6β) on C-6 carbon at δ 7.25 comparing to royleanone **III.1**.

III.2.2. 7α-Acetoxy-6β-hydroxyroyleanone III.1 ester derivatives

In order to study the effect of the ester substituents on the antimicrobial activity of **III.1**, aromatic and acyl ester derivatives of **III.1** were synthesized using a known methodology (Martínez-Vázquez M. et al., 2004). Royleanone derivatives **III.3–III.13**, were prepared (**Figure III.1**) by esterification of the hydroxyl group at C-6 and/or C-12. The reaction with the correspondent aromatic acyl chloride afforded two highly lipophilic diesters, **III.3** and **III.4**, and the aromatic monoesters **III.5–III.8**. A set of five alkyl derivatives **III.9–III.13** was also prepared (**Figure III.1**; Martínez-Vázquez M. et al, 2004).



	R_1	R_2
III.3	BzO	BzO
III.4	4-Cl-C_6H_4COO	4-Cl-C_6H_4COO
III.5	OH	4-Me-C_6H_4COO
III.6	OH	4-Cl-C_6H_4COO
III.7	OH	4-NO_2-C_6H_4COO
III.8	4-NO_2-C_6H_4COO	OH
III.9	AcO	AcO
III.10	$MeCH_2COO$	$MeCH_2COO$
III.11	AcO	OH
III.12	$MeCH_2COO$	OH
III.13	$Me(CH_2)_2COO$	OH

Figure III.1. 7α-Acetoxy-6β-hydroxyroyleanone **III.1** ester derivatives **III.3–III.13.**

Despite the fact that compounds **III.9** (Hensch M. et al., 1975) and **III.11** (Chang C.-I. et al., 2005) were already known, the ^{1}H NMR data of **III.9** are now completed and its ^{13}C NMR spectrum is reported for the first time. The ^{1}H and ^{13}C NMR data of **III.11** were in good agreement with those reported in literature (Chang C.-I. et al., 2005), except for the assignments of the C–20 and the methyl carbon of the 6β-acetate, which must be reversed. Moreover, several *J*(H,H) values for **III.11**, not measured previously, are now reported (see Chapter **VI**).

Aromatic and acyl ester derivatives of 7α-acetoxy-6β-hydroxyroyleanone **III.1** are abietane diterpenoids with five methyl groups (Me-15 to Me-20), an 7α-acetate group and an isopropyl group at C-13 with a typical H-15 septuplet proton (δ from 3.09 to 3.18 of compounds **III.3-III.13**). The ^{1}H NMR data for the decalin part of these molecules showed closely related signals (see **Tables III.1** and **III.3**). The significant changes will be discussed below for each compound, comparing the signals of 7α-acetoxy-6β-hydroxyroyleanone **III.1** with the aromatic ester derivatives **III.3-III.8** and with the acyl ester derivatives **III.9-III.13**.

Structures **III.3-III.8** were established by NMR spectroscopic studies and the complete assignment of the ^{1}H and ^{13}C NMR spectra of **III.3-III.8** is reported in **Tables III.1** and **III.2**, respectively. A combination of two-dimensional COSY, HSQC and HMBC experiments was carried out, together with 1D NOESY spectra, in some cases, for establishing the relative stereochemistry and conformations of these substances (Pretsch E. et al., 2000).

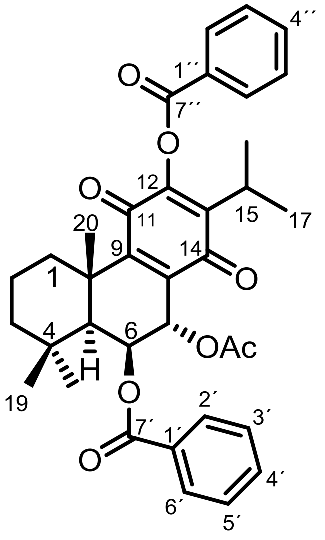
The ^{1}H NMR spectrum of the aromatic ester derivatives **III.3**, **III.4** and **III.8** showed, for the methine H-6α proton, downfield signals between δ 5.76 and 5.78, when comparing with the double doublet at δ 4.31 of royleanone **III.1.** This effect was caused by the introduction of the aromatic ester groups and was also observed for the correlated doublet protons H-5α and H-7β showing signals from δ 1.69 to 1.70 (H-5α) and from δ 5.87 to 5.90 (H-7β), respectively, when comparing with compound **III.1** signals at δ 1.32 (H-5α) and 5.65 (H-7β). The remaining derivatives **III.5**, **III.6** and **III.7** (with no modifications on the hydroxyl group of carbon C-6) showed similar signals, at δ 4.32, 4.33 and 4.34 respectively, for the methine H-6α proton comparing with the double doublet at δ 4.31 for the royleanone **III.1** (see **Table III.1**).

The ^{13}C NMR spectra of the derivatives **III.3**, **III.4** and **III.8** showed that the introduction of the aromatic ester groups on C-6 produced a paramagnetic shift (δ 68.4 to 69.44) with respect to the signal at δ 67.0 of carbon C-6 of compound **III.1.** The carbons C-5 and C-7, on β position with respect to the ester group of C-6, showed diamagnetically shifted signals (δ 49.24 to 49.30 and δ 64.94 to 65.20 for C-5 and C-7, respectively) with respect to signals at δ 49.8 (C-5) and 68.7 (C-7) of compound **III.1**. The introduction of the withdrawing electron groups on carbon C-12 of compounds **III.3**-**III.7** (δ from 179.2 to 180.0 for C-11 and δ from 149.5 to 150.0 for C-12) produced diamagnetic shifts comparing with compound **III.1** (at δ 183.3 and 150.9 for carbons C-11 and C-12, respectively); comparing with royleanone **III.1** at δ 124.7, was also observed the deshielding (from δ 139.5 to 140.0) of carbon C-13 signal; see **Table III.2**).

Table III.1. ^{1}H NMR data for 7α-Acetoxy-6β-hydroxyroyleanone **III.1** and for compounds **III.3**-**III.8** ($CDCl_3$, ^{a}400 MHz; ^{b}500 MHz; δ in ppm, *J* in Hz).

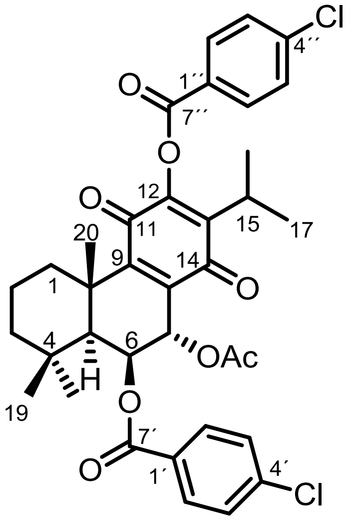
Proton	**III.1**^{b}	**III.3**^{a}	**III.4**^{a}	**III.5**^{b}	**III.6**^{a}	**III.7**^{a}	**III.8**^{a}
1α	~1.17*	1.29	~1.24*	~1.23*	~1.24	~1.23*	~1.25*
1β	2.62	2.58	2.58	2.49	2.49	2.49	2.75
2α	1.55	~1.58*	1.60	1.53	~1.54	~1.55*	~1.64*
2β	1.83	~1.78*	1.79	1.79	1.80	1.81	1.84
3α	~1.20*	~1.38	~1.28*	~1.19*	~1.20*	~1.20*	~1.28*
3β	1.46	1.49	1.48	1.45	1.45	1.46	1.52
5α	1.32	1.52	1.70	1.36	1.36	~1.37*	~1.70*
6α	4.31	1.69	5.76	4.32	4.33	4.34	5.78
7β	5.65	5.90	5.88	5.67	5.68	5.68	5.87
15	3.14	3.17	3.17	3.17	3.17	3.18	3.15
Me-16$^{\#}$	1.17	1.19	1.21	~1.19	~1.21*	~1.22*	1.21
Me-17$^{\#}$	1.20	1.21	1.19	~1.22	~1.21*	~1.22*	1.17
Me-18	0.93	1.06	1.06	0.94	0.94	0.95	0.98
Me-19	1.21	0.99	0.97	1.21	1.24	1.22	1.05
Me-20	1.59	1.77	1.74	1.62	1.62	1.62	1.73
6-OH	~2.03*	---	---	---	1.92	---	---
12-OH	7.19	---	---	---	---	---	7.19
7α-OAc	2.02	2.10	2.10	2.06	2.06	2.07	2.09
6β-OAc	---	2.04 s	---	---	---	---	---
12-OAc	---	2.34 s	---	---	---	---	---
6β-OBz: 2´ and 6´	---	7.99	7.92	---	---	---	8.14
6β-OBz: 3´ and 5´	---	~7.41	7.39	---	---	---	8.27
6β-OBz: 4´	---	~7.54	---	---	---	---	---
12-OBz: 2´´ and 6´´	---	8.15	8.08	8.09	8.07	8.32	---
12-OBz: 3´´ and 5´´	---	~7.52	7.51	6.98	7.49	8.37	---
12-OBz: 4´´	---	~7.66	---	---	---	---	---
Bz-OMe	---	---	---	3.89	---	---	---

*Overlapped signals, δ values measured from the HSQC and/or COSY spectra; $^{\#}$Interchangeable signals.



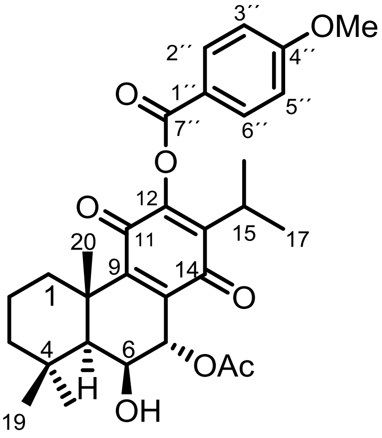
III.3

The compound **III.3** has two benzoyloxy groups attached on carbons C-6 and C-12. The H-6α methine at δ 5.77 (dd, $J_{6\alpha,7\beta}$ = 2.0 Hz, $J_{6\alpha,5\alpha}$ = 1.6 Hz, Hα–6), correlates in the HMBC spectrum with the carboxyl carbon at δ 165.3. This singlet carbon on its turn correlates with the *orto* protons of the benzoyl group at δ 8.15 (dt, $J_{2',3'}$ = 7.6 Hz, $J_{2',4'}$ = 2.0 Hz, H–2' and H–6', 6–OBz). As the carbonyl carbon of the acetyl group (δ 168.10) correlates in the HMBC with the methine H-7β (δ 5.90) the remaining carboxyl carbon at δ 164.00 must be linked to the C-12 position (**Table III.1** and **III.2**).



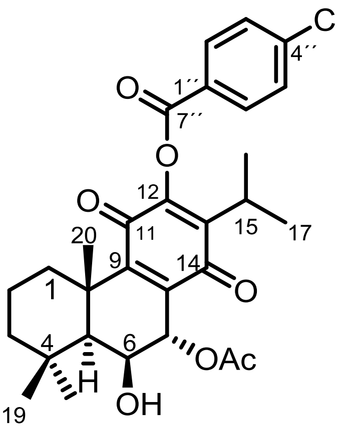
III.4

The diterpenoid **III.4** has two *p*-chlorobenzoyl groups that esterified the hydroxyl groups on carbons C-6 and C-12. The H-6α methine (δ 5.76, t, $J_{6\alpha,7\beta} = J_{6\alpha,5\alpha}$ = 1.6 Hz) correlates, in the HMBC spectrum, with the carbonyl carbon at δ 164.52 (C-7′). This singlet carbon correlates with the *orto* protons of the benzoyl group (δ 7.92, d, J_o = 8.6 Hz, H–2′ and H–6′). The carbonyl carbon of the acetyl group at C-7 (δ 168.13) correlates (HMBC) with the methine H-7β (δ 5.88) so the other carboxyl carbon (δ 163.19) belongs to the substituent group at the carbon C-12. The complete assignment of the two *p*-chlorobenzoyl groups (^{1}H and ^{13}C NMR spectra and HSQC and HMBC experiments) are shown in **Tables III.1** and **III.2**.



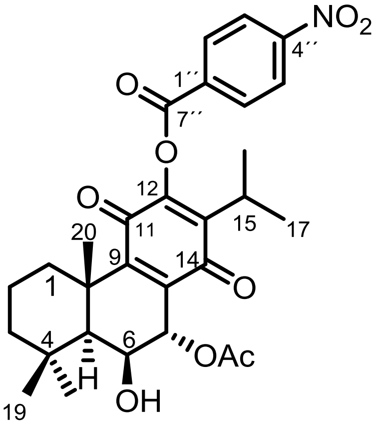
III.5

The derivative **III.5** has a *p*-methoxybenzoyloxy group on carbon C-12. The HMBC experiment showed connectivities between the H-7β methine at δ 5.67 (*d*, $J_{7\beta,6\alpha}$ = 1.7 Hz) and the carbonyl carbon at δ 169.7 (7α-OAc). This singlet carbon correlates with the methyl protons of the acethyl group at δ 2.06 (*s*, 7α-OAc). Though the hydroxyl proton of C-6 was not found in the ^{1}H NMR spectrum, in the ^{13}C NMR spectrum a signal at δ 67.3, similar to the C-6 signal of compound **III.1** (δ 67.0) was present. The remaining carboxyl carbon at δ 164.4 must be assigned to the *p*-methoxybenzoyl group at the C-12 position (see **Table III.1** and **III.2**). The *orto* and *meta* protons of the ring of the benzoyl group at δ 8.09 (*d*, $J_{2',3'(6',5')}$ = 8.9 Hz, H–2′ and H–6′) and at δ 6.98 (2H, *d*, $J_{3',2'(5',6')}$ = 8.9 Hz, H–3′ and H–5′) as well as the methoxyl protons at δ 3.89 (*s*, OMe-8'; see **Table III.1**) and the corresponding carbons were also assigned (see **Table III.2**).



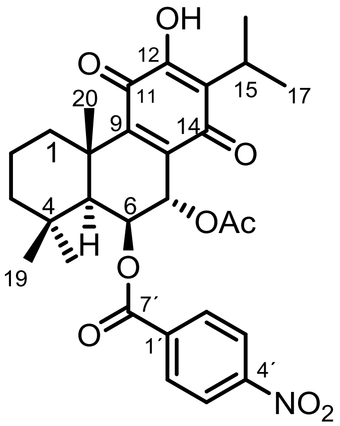
III.6

Compound **III.6** has a *p*-chlorobenzoyloxy group attached to carbon C-12. The HMBC experiment showed connectivities between the H-7β methine at δ 5.68 (dd, $J_{7\beta,6\alpha}$ = 2.0 Hz, $J_{7\beta,5\alpha}$ = 0.6 Hz) and the carbonyl carbon at δ 169.67, which correlates with the methyl protons of the acetyl group at δ 2.06 (*s*, 7α-OAc). Moreover, the ^{1}H NMR signals of H-5α, H-6α and H-7β and δ_C of C_5, C_6 and C_7 have almost the same values for **III.6** and **III.1**. this indicate that OH-6 is free (δ 1.92, *br*, 6β-OH) and OH-12 is esterified. The complete assignment of the *p*-chlorobenzoyl group was made by ^{1}H and ^{13}C NMR spectra and COSY, HSQC and HMBC experiments (see **Tables III.1** and **III.2**).



III.7

The royleanone derivative **III.7** bears a *p*-nitrobenzoyloxy group on carbon C-12. Once more, ^{1}H and ^{13}C NMR data of $–C_5H-C_6H(OH)-C_7H(OAc)-$ and HMBC experiments indicated that this moiety, is present in both **III.1** and **III.7.** So the signal at δ 164.6 must correspond to the ester group linked to C-12 and the complete assignment of the ester group were established by ^{1}H and ^{13}C NMR spectra and HSQC experiment (see **Tables III.1** and **III.2**).



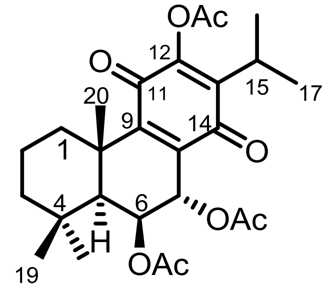
III.8

The compound **III.8** is a *p*-nitrobenzoyl derivative of **III.1**. Its H-6α methine (δ 5.78, t, $J_{6\alpha,7\beta} = J_{6\alpha,5\alpha}$ = 1.6 Hz) is correlated with the carbonyl carbon at δ 163.58 (C-7′) which correlates with the *orto* proton of the benzoyl group (δ 8.27, d, $J_{2',3'(6',5')}$ = 9.0 Hz, H–2’ and H–6’). The ^{1}H NMR spectrum showed a hydroxyl group attached to an aromatic carbon (δ 7.19, *s*; δ_C 150.63) similary to C_{12}-OH of **III.1** (see **Table III.1** and **III.2**).

Table III.2. ^{13}C NMR data for 7α-Acetoxy-6β-hydroxyroyleanone **III.1**[a] and for compounds **III.3**[b]-**III.8**[b] (CDCl$_3$, [a] 125.7 MHz; [b]100 MHz; δ in ppm, *J* in Hz).

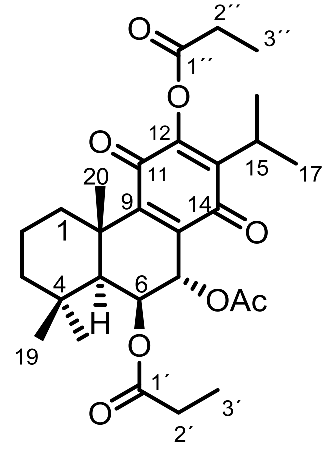
Carbon	**III.1**	**III.3**	**III.4**	**III.5**	**III.6**	**III.7**	**III.8**
1	38.37	38.37	38.37	38.30	38.33	38.35	38.46
2	19.00	18.83	18.81	18.86	18.87	18.86	18.86
3	42.28	42.48	42.47	42.28	42.26	42.22	42.39
4	33.67	33.76	33.77	33.52	33.72	33.51	33.27
5	49.76	49.31	49.30	48.80	49.77	49.78	49.24
6	67.00	68.43	68.72	67.26	67.22	67.21	69.44
7	68.75	65.25	65.19	68.88	68.85	68.80	64.94
8	137.12	135.20	135.67	135.52	135.74	135.96	136.74
9	149.91	152.50	152.40	153.04	152.5	152.94	149.23
10	38.63	38.77	38.78	38.86	38.93	39.00	38.35
11	183.29	180.00	179.7	179.89	179.55	179.20	183.13
12	150.90	150.00	149.5	149.90	149.8	149.70	150.63
13	124.69	140.00	139.8	139.51	139.5	139.80	125.03
14	185.74	185.33	185.21	185.90	185.69	185.50	185.17
15	24.17	25.16	25.23	25.07	25.18	25.30	24.20
16	19.84	20.36	20.37	20.44	20.44	20.40	19.81
17	19.70	20.00	20.21	20.18	20.23	20.43	19.64
18	33.52	33.33	33.29	33.71	33.51	33.73	33.74
19	23.81	23.21	22.20	23.83	23.83	23.83	23.27
20	21.51	22.20	21.23	21.75	21.73	21.72	22.03
7α-$\underline{C}OCH_3$	169.60	168.13	168.13	169.71	169.67	169.60	168.13
7α-$CO\underline{C}H_3$	20.93	20.84	20.81	20.91	20.91	20.89	20.81
6β-OBz 7´	---	165.33	164.52	---	---	---	163.58
6β-OBz 1´	---	127.91	128.14	---	---	---	135.04
6β-OBz 2´ and 6´	---	129.87	131.22	---	---	---	130.91
6β-OBz 3´ and 5´	---	128.47	128.88	---	---	---	123.68
6β-OBz 4´	---	133.22	139.79	---	---	---	150.92
12-OBz 7´´	---	~164.00*	163.19	164.38	163.18	164.60	---
12-OBz 1´´	---	129.71	126.36	120.21	126.45	133.38	---
12-OBz 2´´ and 6´´	---	130.51	131.84	132.72	131.84	131.63	---
12-OBz 3´´ and 5´´		128.81	129.24	114.05	129.18	123.90	---
12-OBz 4´´		134.30	141.02	163.66	140.89	151.19	---
12-OBz OMe		---	---	55.57	---	---	---

The five alkyl derivatives **III.9–III.13** will be described (**Figure III.1**; Martínez-Vázquez M. et al, 2004). The ^{1}H NMR spectrum of these acyl ester derivatives **III.9-III.13** showed, for the methine H-6α proton, downfield signals from δ 5.48 to 5.51, comparing with the double doublet at δ 4.31 for H-6α of the royleanone **III.1.** This effect was caused by the introduction of the electron withdrawing groups (see **Table III.3**).



III.9

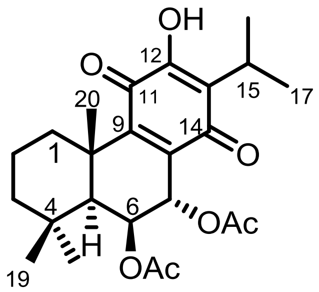
Compound **III.9** has two new acetyl groups linked to C-6 and C-12 carbons, when compared to **III.1** (see **Tables III.3** and **III.4**). The HMBC experiment showed connectivities of the H-6α (δ 5.48) with the carbonyl carbon at δ 169.04 (6β-O$\underline{C}$OCH$_3$), confirming the acetyl group at 6-β position.



III.10

Comparing to compound **III.1**, the derivative **III.10** presents two more propyloxy groups (O-CO-CH$_2$-CH$_3$) on both C-6 and C-12 carbons. The HMBC spectrum confirmed the correlation between proton H-6α (δ 5.49) and the carbonyl carbon C-1´

(δ 172.50). Thus the remaining not assigned carbonyl carbon must be the C-1´´ (δ 171.73). The two methylene protons (H-2´) at δ 2.32 (dq, $J_{2'A,2'B}$ = 16.8 Hz, $J_{2'A,3'}$ = 7.6 Hz, HA–2’) and 2.25 (q, $J_{2'B,2'A}$ = 16.8 Hz, $J_{2'B,3'}$ = 7.6 Hz, HB–2’) are attached to the secondary C-2´ carbon (confirmed by HSQC experiment) at δ 27.81. The C-2´ correlates with the carbonyl and methyl carbons at δ 172.50 (C-1´) and 8.80 (C-3´), respectively. The methyl protons of C-3´ (δ 1.11, t, $J_{3',2'A} = J_{3',2'B}$ = 7.6 Hz, H-3’) correlates, in HMBC experiment, to C-2´ carbon (δ 27.81) and carbonyl C-1´ (δ 172.50). The presence of other C-12 propanoate moiety was evidenced by HMBC and HSQC spectra which showed connectivities that assigned the protons and carbons of $OC_{1''}$-$C_{2''}H_2$-$C_{3''}H_3$ (see Chapter **VI**, **Tables III.3** and **III.4**).



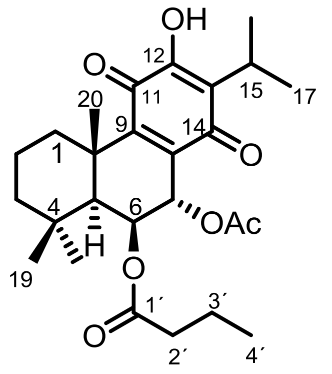
III.11

The 6,7-diacetyl **III.11** showed a hydroquinonic hydroxyl group (δ_H 7.17) also present in the protype **III.1.** The extra acetoxyl group (δ_H 2.04, s, 3H; δ_C 20.81, $CO\underline{C}H_3$; δ_C 168.99, $\underline{C}OCH_3$) was attributed to the 6β position. The introduction of the 6β-acetoxyl group was confirmed by correlations observed in the HMBC spectrum between the carbon δ_C 168.99 (6β-$\underline{C}OCH_3$) and the proton H-6α at δ 5.50 (**Tables III.3** and **III.4**).

Table III.3. ^{1}H NMR data for 7α-Acetoxy-6β-hydroxyroyleanone **III.1** and for compounds **III.9-III.13** ($CDCl_3$, ^{a}500 MHz; ^{b}400 MHz; δ in ppm, *J* in Hz).

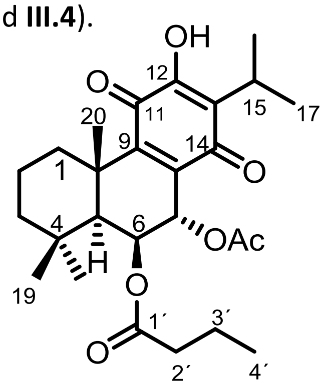
Proton	**III.1**^{a}	**III.9**^{b}	**III.10**^{b}	**III.11**^{a}	**III.12**^{b}	**III.13**^{b}
1α	~1.17*	~1.24*	~1.24*	~1.24*	~1.22*	~1.23*
1β	2.62	2.52	2.51	2.67	2.66	2.66
2α	1.55	1.56*	1.55	~1.60*	~1.58*	~1.58*
2β	1.83	1.78	1.77	1.82	1.82	1.82
3α	~1.20*	~1.23	~1.23*	1.24*	~1.22	~1.23*
3β	1.46	1.45	1.44	1.49	1.48	1.47
5α	1.32	1.52	1.53	1.56	1.54	~1.60*
6α	4.31	5.48	5.49	5.50	5.51	5.50
7β	5.65	5.68	5.68	5.70	5.70	5.69
15	3.14	3.09	3.09	3.16	3.16	3.16
Me-16$^\#$	1.17	1.18	1.17	1.22	1.22	1.22
Me-17$^\#$	1.20	1.17	1.16	1.19	1.19	1.19
Me-18	0.93	0.98	0.98	0.99	0.99	0.99
Me-19	1.21	0.97	0.96	0.98	0.97	0.98
Me-20	1.59	1.59	1.59	1.59	1.59	1.59
6-OH	~2.03*	---	---	---	---	---
12-OH	7.19	---	---	7.17	7.17	7.18
7α-OAc	2.02	2.03	2.04	2.03	2.04	2.04
6β-OAc	---	2.04	---	2.04	---	---
12-OAc	---	2.34	---	---	---	---
6β-Pr-2´A	---	---	2.32	---	2.29	---
6β-Pr-2´B	---	---	2.25	---	2.29	---
6β-Pr-Me3´	---	---	1.11	---	1.12	---
12-Pr-2´´A	---	---	2.66	---	---	---
12-Pr-2´´B	---	---	2.61	---	---	---
12-Pr-Me3´´	---	---	1.27	---	---	---
6β-Bu-2´A	---	---	---	---	---	2.28
6β-Bu-2´B	---	---	---	---	---	2.20
6β-Bu-3´A and 3´B	---	---	---	---	---	~1.63*
6β-Bu-4´	---	---	---	---	---	0.91

*Overlapped signals, δ values measured from HSQC and/or COSY spectra; $^\#$Interchangeable signals.



III.12

Compound **III.12** has only a propyloxy chain at the 6β position when compared with derivative **III.10.** The similar signals at δ 2.29 (m, H-2'), of the methylene and methyl at δ 1.12 (t, $J_{3',2'A} = J_{3',2'B}$ = 7.6 Hz, Me-3') protons, were correlated with each other in the COSY spectrum. The hydroxyl proton of C-12 appeared at δ 7.17 as a broad singlet signal (see **Tables III.3** and **III.4**).



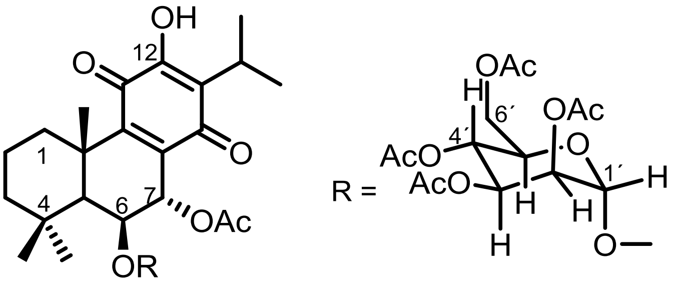
III.13

Compound **III.13** is a butanoate ester of HO-C-6. The C-12 hydroxyl proton was observed at δ 7.18 as a broad singlet signal. The attachment of the butanoxy moiety at C-6 position was evidenced by HMBC spectrum, which showed correlations between the H-6α methine (δ 5.50, t, $J_{6\alpha,7\beta} = J_{6\alpha,5\alpha}$ = 2.1 Hz), with carbonyl C-1´ (δ 171.7). This singlet carbon (C-1´) correlated with the methylene protons at δ 2.28 (dt, $J_{2'A,2'B}$ = 15.9 Hz, $J_{2'A,3'}$ = 7.1 Hz, HA–2'), and 2.20 (dt, $J_{2'B,2'A}$ = 15.9 Hz, $J_{2'B,3'}$ = 8.0 Hz, HB–2'). The remaining protons and carbons of the ester group were assigned on the basis of their coupling constants and multiplicities, being also in agreement with the COSY, HSQC and HMBC spectra (see **Tables III.3** and **III.4**; Chapter **VI**).

Table III.4. ^{13}C NMR data for 7α-Acetoxy-6β-hydroxyroyleanone **III.1** and for compounds **III.9-III.13** ($CDCl_3$, [a] 125.7 MHz; [b]100 MHz; δ in ppm, *J* in Hz).

Carbon	III.1[a]	III.9[b]	III.10[b]	III.11[b]	III.12[b]	III.13[b]
1	38.37	38.31	38.31	38.39	38.36	38.36
2	19.00	18.78	18.78	18.88	18.88	18.88
3	42.28	42.39	42.39	42.42	42.43	42.41
4	33.67	33.58	33.58	33.57	33.58	33.57
5	49.76	48.95	48.95	48.98	49.03	49.00
6	67.00	67.24	67.24	67.15	67.08	67.00
7	68.75	65.25	65.25	65.21	65.24	65.24
8	137.12	135.67	135.67	137.08	137.11	137.09
9	149.91	152.22	152.22	149.29	149.32	149.32
10	38.63	38.93	38.93	38.70	38.66	38.65
11	183.29	179.67	179.67	183.22	183.23	183.23
12	150.90	149.28	149.28	150.84	150.84	150.84
13	124.69	139.42	139.42	124.82	124.81	124.79
14	185.74	185.37	185.37	185.33	185.35	185.34
15	24.17	25.17	25.17	24.17	24.15	24.13
16	19.84	20.17	20.17	19.82	19.82	19.81
17	19.70	20.19	20.19	19.68	19.67	19.66
18	33.52	33.18	33.18	33.22	33.2	33.24
19	23.81	22.93	22.93	22.95	23.02	23.00
20	21.51	21.44	21.44	21.27	21.35	21.33
7α-$\underline{C}OCH_3$	169.60	168.15	168.15	168.09	168.04	168.06
7α-$CO\underline{C}H_3$	20.93	21.27	21.27	21.27	20.82	20.82
6β-$\underline{C}OCH_3$		169.04	169.04	168.99	---	---
6β-$CO\underline{C}H_3$		20.75	20.75	20.81	---	---
6β-$\underline{C}OCH_2CH_3$			172.50		172.46	---
6β-$CO\underline{C}H_2CH_3$			27.81		27.84	---
6β-$COCH_2\underline{C}H_3$			8.80		8.85	---
12-$\underline{C}OCH_3$		168.26				
12-$CO\underline{C}H_3$		20.39				
12-$\underline{C}OCH_2CH_3$			171.73			
12-$CO\underline{C}H_2CH_3$			27.21			
12-$COCH_2\underline{C}H_3$			8.86			
6β-$\underline{C}OCH_2CH_2CH_3$						171.78
6β-$CO\underline{C}H_2CH_2CH_3$						36.42
6β-$COCH_2\underline{C}H_2CH_3$						18.08
6β-$COCH_2CH_2\underline{C}H_3$						13.70

III.2.3. Synthesis of the α-mannopyranoside III.14 from 7α-acetoxy-6β-hydroxyroyleanone III.1



III.14

Compound **III.14** has the α-mannopyranosyl group at the C-6 carbon when compared with royleanone **III.1**. Both the ^{1}H NMR and ^{13}C NMR spectra are consistent with six additional carbons (C-1´ to C-6´), the five methine protons, of C-1´ to C-5´, and methylene protons of the C-6´ carbon, corresponding to the sugar group introduced. Besides the acetyl group at C-7α carbon, four more acetyl groups were assigned to positions C-2´, C-3´, C-4´ and C-6´ (singlet signals at δ 2.16, 1.98, 2.00 and 2.13, respectively) of the sugar moiety. The H-6α methine at δ 4.19 (br s, $J_{6\alpha,7\beta}$ = 1.4 Hz, $J_{6\alpha,5\alpha}$ = ~1.4 Hz, Hα–6) correlates, in the HMBC spectrum, with the C-1´carbon of the α-mannopyranosyl moiety (δ 99.98). The HMBC and HSQC correlations and the ^{1}H ^{1}H COSY spectrum were in complete agreement with the structure of **III.14** (see **Table III.5**).

Table III.5. ^{1}H[a] and ^{13}C[b] NMR spectroscopic data for compound **III.14**[a] ($CDCl_3$, [a]500 MHz; [b]125 MHz; δ in ppm, *J* in Hz).

Position	δ$_C$	δ$_H$	HMBC (H→C)
1	38.56 CH$_2$	1.22 m* (H-1α)	
		2.66 brd (H-1β)	
2	18.90 CH$_2$	1.56 m*(H-2α)	
3	42.37 CH$_2$	1.20 m* (H-3α)	
		1.52 m* (H-3β)	
4	33.60 qC	-	
5	49.63 CH	1.45 br s (H-5α)	C-1, C-3, C-4, C-6, C-10, C-18 and C-19
6	77.41 CH	4.19 br s (H-6α)	C-4, C-5, C-7, C-8, C-10 and C-´1
7	65.20 CH	6.05 d (H-7β)	C-5, C-6, C-8, C-9, C-14 and CO at δ 168.68
8	136.55 qC	-	
9	149.96 qC	-	
10	38.80 qC	-	
11	183.17 qC	-	
12	150.97 qC	-	
13	124.78 qC	-	
14	185.24 qC	-	
15	24.15 CH	3.18 sept (H-15)	C-12, C-13, C-14, C-16, C-17
16[#]	19.87 CH$_3$	1.23 d (Me-16)	C-13, C-15, C-17
17[#]	19.66 CH$_3$	1.22 d (Me-17)	C-13, C-15, C-16
18	33.51 CH$_3$	0.94 s (Me-18)	C-3, C-4, C-5, C-19
19	23.59 CH$_3$	1.20 s (Me-19)	C-3, C-4, C-5, C-18
20	21.93 CH$_3$	1.64 s (Me-20)	C-5, C-6, C-10
1´	99.98 CH	5.11 br s	
2´	69.71 CH	5.18 br s	
3´	68.85 CH	5.17 dd	
4´	65.73 CH	5.29 t	
5´	69.74 CH	3.75 ddd	
6´	62.13 CH$_2$	4.30 dd (HA-6)	
		4.04 dd (HB-6)	
7α-OAc	168.68 qC; 20.63[$] CH$_3$	2.02 s	
2´-OAc	168.68 qC; 20.63 CH$_3$	2.16 s	
3´-OAc	170.05 qC; 20.68[$] CH$_3$	1.98 s	
4´-OAc	169.53 qC; 20.70[$] CH$_3$	2.00 s	
6´-OAc	170.89 qC; 20.77[$] CH$_3$	2.13 s	

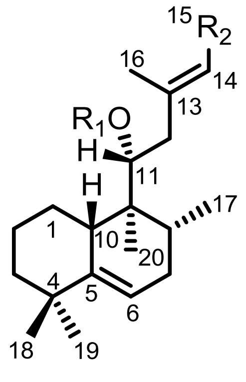
*Overlapped or partially overlapped signals. Approximate δ values were obtained from the HSQC spectrum. [#]Interchangeable signals. [$]Interchangeable assignments.

III.3. Halimane derivatives

III.3.1. Synthesis and identification of the derivatives III.15-III.23

To prepare the derivatives **III.15-III.23** a large quantity of pure (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid **II.7** was isolated from the acetone extract of *Plectranthus ornatus*, as described in Chapter **VI**.

Nine derivatives were synthesized taking advantage of the presence of both carboxylic and ester groups of **II.7**. Compound **II.7** gave the (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid methyl ester **III.15**, under methylation conditions. Reduction of **II.7** with lithium aluminium hydride yielded two derivatives: (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-ol **III.16** and (11*R**,13*E*)-halima-5,13-diene-11,15-diol **III.17**. Transacetylation of **III.16** yielded (11*R**,13*E*)-15-acetoxyhalima-5,13-dien-11-ol **III.18**. Esterification of **III.17**, using appropriate acyl anhydrides, allowed the preparation of three ester derivatives: (11*R**,13*E*)-15-propionyloxyhalima-5,13-dien-11-ol **III.19**, (11*R**,13*E*)-11,15-dipropionyloxyhalima-5,13-diene **III.20** and (11*R**,13*E*)-15-butyryloxyhalima-5,13-dien-11-ol **III.21**. Finally, (11*R**,13*E*)-15-benzoyloxyhalima-5,13-dien-11-ol **III.22** and (11*R**,13*E*)-15-(4-methoxy)benzoyloxyhalima-5,13-dien-11-ol **III.23** were also prepared from **III.17** by reaction with the adequate benzoyl chlorides (see **Figure III.2**).



	R_1	R_2
II.7	Ac	COOH
III.15	Ac	COOMe
III.16	Ac	CH_2OH
III.17	OH	CH_2OH
III.18	OH	CH_2OAc
III.19	OH	$CH_2OCOCH_2CH_3$
III.20	$COCH_2CH_3$	$CH_2OCOCH_2CH_3$
III.21	OH	$CH_2OCOCH_2CH_2CH_3$
III.22	OH	CH_2OCOPh
III.23	OH	CH_2OCOPh-*p*-OCH_3

Figure III.2. (11*R**,13*E*)-11-Acetoxyhalima-5,13-dien-15-oic acid **II.7** derivatives **III.15-III.23.**

The structures of the compounds **III.15-III.23** were unequivocally established from spectroscopic information – I.R., MS, ^{1}H and ^{13}C NMR, COSY, HSQC and HMBC – and by comparison with **II.7** data (Chapter **VI**).

Halimane **II.7** and its derivatives have common structural features, namely the presence of five methyl groups (Me-16 to Me-20), two double bonds (C5-C6 and C13-C14) with their typical downfield protons at δ from 5.41 to 5.44 (dt, $J_{6,7\beta}$ = 5.6 Hz, $J_{6,7\alpha}$ = $J_{6,10\beta}$ = 2.1 Hz, H-6 for compound **II.7**) and at δ from 5.34 to 5.67 (qd, $J_{14,16}$ = 1.2 Hz, $J_{14,12A}$ = 0.4 Hz, H-14 for halimane **II.7**). The significant characteristics of each halimane derivatives **III.15-III.23**, will be discussed below.

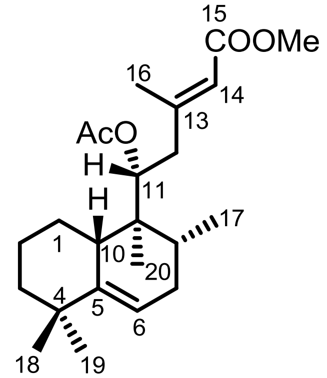
The ^{1}H NMR of C-11 acyloxy halimanes (**II.7, III.15**, **III.16** and **III.20**) presents the geminal methine H-11 protons more deshielded (δ_{H-11} 5.21 to 5.31) than the C-11 hydroxy derivatives **III.17-III.19** and **III.21-III.23** (δ_{H-11} from 3.66 to 3.69). Consistently, the C-11 chemical shift of the 11-hydroxyhalimanes appear at a more shielded values (δ_{C-11} from 72.9 to 73.08) than the analogous 11-acyloxy diterpenes (δ_{C-11} from 74.84 to 75.82; see **Tables III.6** and **III.7**).

Another ^{13}C NMR signal affected by the chemical transformations is the corresponding C-15 carbon. The higher δ_{C-15} values correspond to the carboxylic function (δ 171.05 of compound **II.7**) and the methyl ester (δ 166.61 of derivative **III.15**). A shielding effect observed for **III.16** and **III.17** (δ 59.21 and 59.27, respectively) is coherent with a hydroxyl attached to C-15. The C-15 of all esters from the 15-hydroxyl at halimane **III.17** showed deshielding values (δ from 61.7 to 61.04; **III.18-III.23**) when compared to **III.17** (δ 59.27; see **Tables III.8** and **III.9**).

Table III.6. ^{1}H NMR data for (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid **II.7** and for compounds **III.15-III.18** ($CDCl_3$, 400 MHz; δ in ppm, *J* in Hz).

Proton	**II.7**	**III.15**	**III.16**	**III.17**	**III.18**
1α	1.10	1.09	1.08	1.10	1.10
1β	1.92	1.92	1.91*	~1.81	~1.80*
2α	1.64	1.63	1.64*	~1.60	~1.60*
2β	1.52*	1.52	1.51	~1.55	~1.55*
3α	1.19	1.19	1.19	1.20	1.19
3β	1.42	1.41	1.41	1.42	1.42
6	5.45	5.44	5.43*	5.41	5.41
7α	1.78*	1.75	1.76	~1.78	~1.78*
7β	1.84*	1.84	1.84	1.87	1.87
8β	1.56*	1.56*	1.58*	~1.62	~1.62*
10β	2.07	2.06	2.09*	2.29	2.28
11	5.31	5.31	5.21	3.66	3.66
12A	2.44	2.42	2.30	2.33	2.34
12B	2.36	2.33	2.21	2.09	2.12
14	5.68	5.67	5.43*	5.53	5.46
Me-16[#]	2.19	2.18	1.71	1.72	1.75
Me-17[#]	0.99	0.98	0.98	0.97	0.97
Me-18	0.98	0.97	0.97	1.00	1.00
Me-19	1.05	1.05	1.05	1.05	1.05
Me-20	0.69	0.68	0.68	0.80	0.80
11-OAc	2.00	1.98	1.99	---	---
15-COOCH$_3$	---	3.66	---	---	2.04
15A	---	---	4.10	4.18	4.62
15B	---	---	4.06	4.18	4.58
2´A, 2´B	---	---	---	---	---
Me-3´	---	---	---	---	---
2´´A, 2´´B	---	---	---	---	---
Me-3´´	---	---	---	---	---
Me-4´´	---	---	---	---	---
11-OH	---	---	---	---	---

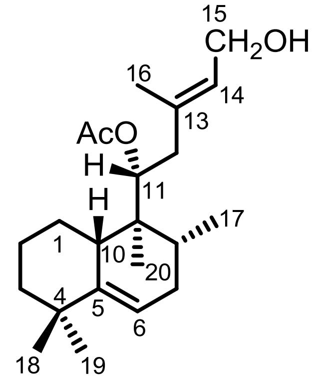
*Overlapped signals, δ values measured from the HSQC and/or COSY spectra.



III.15

Derivative **III.15** is the methyl ester of halimane **II.7** on carbon C-15. The HMBC experiment of the methoxyl group at δ 3.66 (correlated in HSQC with carbon at δ 50.89) showed connectivities to C-15 carbon (δ 166.61). The remaining ^{1}H NMR and ^{13}C NMR spectra signals were completely assigned (see **Tables III.6** and **III.8**) and were very similar to the halimane **II.7** signals.

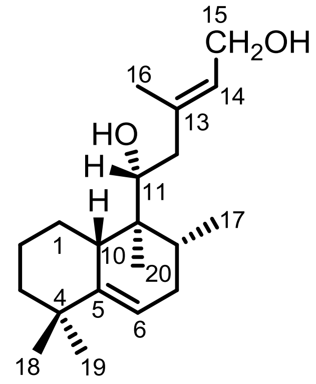
The reduction of compound **III.15** yielded a hydroxyl group on C-15, resulting the compound **III.16.** When an excess of the reducing reagent was used, the compound **III.17** was obtained, with two hydroxyl groups on carbons C-11 and C-15. The compound **III.18,** with a hydroxyl group on carbon C-11, was obtained at the purification step of compound **III.16,** by transesterification.



III.16

Halimane **III.16** has two additional double doublet proton signals at δ 4.10 (dd, $J_{15A,15B}$ = 12.3 Hz, $J_{15A,14}$ = 7.0 Hz, H-15A) and at δ 4.06 (1H, dd, $J_{15B,15A}$ = 12.3 Hz, $J_{15B,14}$ = 6.8 Hz, H-15B) caused by the presence of a hydroxyl on carbon C-15. Those geminal protons H-15A and H-15B show connectivity on COSY experiment with the vinylic proton H-14 at δ 5.43. The allylic protons H-15A and H-15B at δ 4.10 and at δ 4.06 (which correlated in HSQC with the triplet carbon at $\delta_{C\text{-}15}$ 59.21) showed connectivities, in the HMBC experiment, on C-13 and C-14 carbons at δ 136.86 and at δ 126.32, respectively. The complete assignment of NMR signals of **III.16** were established by ^{1}H and ^{13}C NMR spectra and HMBC, HSQC and COSY experiments. The hydroxyl group on carbon C-15 deshielded the signals of proton H-14 and methyl group at carbon C-16 from δ 5.67 and 2.18 (compound **III.15**), to δ 5.43 and 1.71 (compound **III.16**) respectively.

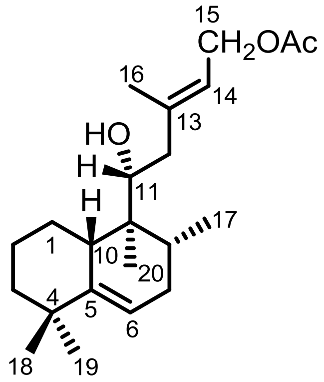
The ^{13}C NMR spectrum of compound **III.16** comparing to compound **III.15** were very similar, except for carbons from C-13 to C15. In this way, the hydroxyl group shielded the signal of carbon C-15 (from δ 166.61 to 59.21) and the same effect was observed for carbon C-13 (from δ 156.80 to 136.86). The double bond carbon C-14 deshielded the signals from δ 117.86 to 126.32, when the ester carbon C-15 of compound **III.15** was converted to the hydroxyl group of compound **III.16** (see **Tables III.6** and **III.8**).



III.17

^{1}H and ^{13}C NMR spectra and HMBC, HSQC and COSY experiments of compounds **III.17** and **III.16** were very similar. The signal assigned to the methyl (δ 1.99) of the acetoxyl group on C-11 of compound **III.16** disappeared. The ^{1}H NMR spectrum showed the methine proton H-11 shielded from δ 5.21 (with an acetyl group on C-11 of compound **III.16**) to δ 3.66 (with a hydroxyl group of compound **III.17**).

The differences on the ^{13}C NMR spectra signals, of compound **III.17** compared to compound **III.16** were**:** shielding carbons C-11 (δ from 75.82 to 73.08) and C-10 (δ from 40.16 to 38.91) and deshielding for the carbons C-13 (δ from 136.86 to 137.78) and C-12 (δ from 40.06 to 42.01), respectively (see **Tables III.6** and **III.8**).



III.18

Compound **III.18** was obtained from compound **III.16** by transesterification. The transesterification could resulte from the proximity of the hydroxyl group on carbon C-15, of the side chain, and the acetyl group on carbon C-11. Comparing the ^{1}H NMR spectra of compounds **III.17** and **III.18,** the latter has a methine proton H-11 at δ 3.66, an additional methoxyl group at δ 2.04, and the deshielded geminal protons H-15A and H-15B at δ 4.62 (dd, $J_{15A,15B}$ = 12.7 Hz, $J_{15A,14}$ = 7.0 Hz, H-15A), and at δ 4.58 (dd, $J_{15B,15A}$ = 12.7 Hz, $J_{15B,14}$ = 7.0 Hz, H-15B) by the ester group on carbon C-15.

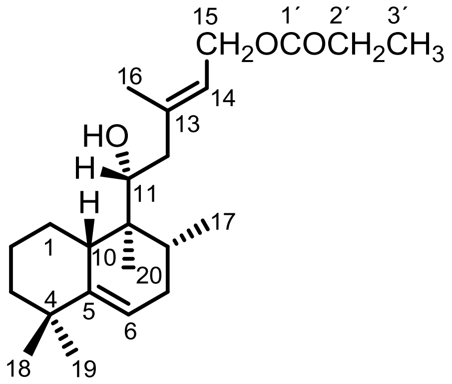
The ^{13}C NMR spectra ofcompounds **III.17** and **III.18** were also very similar**.** The significant differences were carbons C-13-C-15 and the acetoxyl group on C-15. The withdrawing group on C-15 deshielded the carbon C-15 (δ from 59.27 to 61.17), shielded the carbon C-14 (δ from 126.68 to 121.72) and deshielded the carbon C-13 (δ from 137.78 to 140.25).

The carbonyl at δ 171.12 (15-O$\underline{C}$OCH$_3$) showed connectivities, in the HMBC experiment, with the geminal protons H-15A and H-15B, at δ 4.62 and at δ 4.58, respectively (see **Tables III.6** and **III.8**).

Table III.7. ^{1}H NMR data for compounds (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid **II.7** and for **III.19-III.23** ($CDCl_3$, 400 MHz; δ in ppm, *J* in Hz).

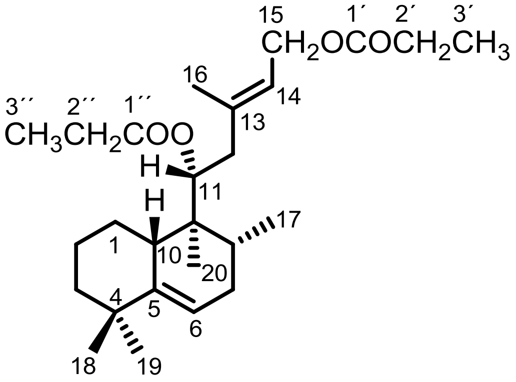
Proton	II.7	III.19	III.20	III.21	III.22	III.23
1α	1.10	1.09	~1.08*	1.10	1.10	1.10
1β	1.92	~1.80*	*	~1.80*	~1.80*	~1.80*
2α	1.64	~1.58*	*	~1.58*	~1.57*	~1.57*
2β	1.52*	1.53	*	~1.53*	~1.52*	~1.53*
3α	1.19	1.19	1.18	1.19	1.19	1.19
3β	1.42	1.42	*	~1.42*	1.42	~1.42*
6	5.45	5.41	5.44	5.41	5.41	5.41
7α	1.78	~1.78*	*	~1.78*	~1.78*	1.78*
7β	1.84	1.86	1.84	1.86	1.87	1.87
8β	1.56	~1.62*	*	~1.62*	1.62	1.61*
10β	2.07	2.28	*	2.28	2.30	2.30
11	5.31	3.66	5.26	3.66	3.69	3.68
12A	2.44	2.34	*	2.34	2.38	2.37
12B	2.36	2.12	2.24	2.12	2.16	2.15
14	5.68	5.46	5.34	5.46	5.60	5.59
Me-16$^{\#}$	2.19	1.75	1.73	1.75	1.81	1.81
Me-17$^{\#}$	0.99	0.97	0.98	0.96	0.97	0.97
Me-18	0.98	1.00	0.97	1.00	1.01	1.01
Me-19	1.05	1.05	1.05	1.05	1.05	1.05
Me-20	0.69	0.80	0.67	0.80	0.81	0.81
11-OAc	2.00	---	---	---	---	---
15-$COOCH_3$	---	---	---	---	---	---
15A	---	4.63	4.56	4.62	4.88	4.84
15B	---	4.59	4.49	4.59	4.84	4.81
2´A, 2´B	---	2.31	2.30	2.27	---	---
Me-3´	---	1.13	1.12	1.64	---	---
2´´A, 2´´B	---	---	2.27	---	---	---
Me-3´´	---	---	1.10	---	---	---
Me-4´´	---	---	---	0.93	---	---
11-OH	---	---	---	1.43	---	---
Ph-(2´, 6´)					8.03	7.98
Ph-(3´, 5´)					7.42	6.90
Ph-(4´)					7.54	---
PhOMe						3.85

*Overlapped signals, δ values measured from the HSQC and/or COSY spectra.



III.19

Compound **III.19** has a propyloxy group on carbon C-15. The ^{1}H and ^{13}C NMR spectra showed similar signals to the compound **III.18** spectra and the additional signals ($\delta_{H\text{-}2'}$ 2.31; $\delta_{C\text{-}2'}$ 27.57 and $\delta_{Me\text{-}3'}$ 1.13; $\delta_{C\text{-}3'}$ 9.12) were confirmed with the HMBC and HSQC experiments. The HMBC experiment revealed the correlation of the carbonyl carbon at δ 174.49 (C-1´) with the geminal protons H-15A and H-15B at δ 4.63 and δ 4.59, respectively (see **Tables III.6**-**III.9**).



III.20

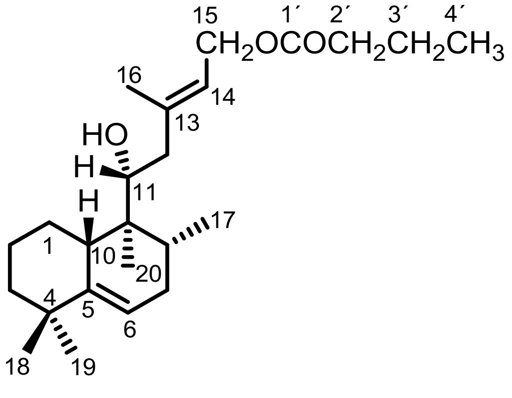
Compound **III.20** has two propyloxy groups on carbons C-15 and C-11. The ^{1}H NMR spectrum showed similarities with the signals of compound **III.19** for all assignments except for the proton H-11 deshielded at δ 5.26. Compound **III.20** like compounds **II.7, III.15** and **III.16** have an ester group on carbon C-11 with proton H-11 signals at δ from 5.21 to 5.31 in agreement with δ (5.26) of **III.20**. Additional H-

2´methylene signals at δ 2.27 (q, $J_{2',3'}$ = 7.6 Hz, H_2-2’ at C-11) and the methyl Me-3´ signal at δ 1.10 (t, $J_{3',2'}$ = 7.6 Hz, Me-3’at C-11) were also observed. The position of propyloxy group of C-11 was confirmed with HMBC experiments showing correlations of the carbonyl carbon at δ 173.97 with the methylene H-2´ protons at δ 2.27 (see **Tables III.7** and **III.9**).

The ^{13}C NMR spectrum also showed similarities with the signals of compound **III.19** except for carbons C-10 to C-13. The carbon C-11 showed a downfield signal at δ 74.84, similar to the derivatives with ester groups on carbon C-11, δ from 75.82 to 74.97 (halimanes **II.7, III.15** and **III.16**) with respect to hydroxyl group on carbon C-11, δ from 73.08 to 72.9 (derivatives **III.17-III.19** and **III.21-III.23**). The same effect was observed for carbon C-10 signal at δ 40.14, like halimane esters on carbon C-11 (δ from 40.27 to 40.16) comparing with hydroxyl group on carbon C-11 (at δ 38.9 to 38.93). The opposite effect was observed on carbon C-12 (signal at δ 40.24) like the ester derivatives (δ from 41.67 to 40.06) showing a higher field signal compared to the hydroxyl derivatives on carbon C-11 (δ from 42.24 to 42.06; see **Tables III.7** and **III.9**).

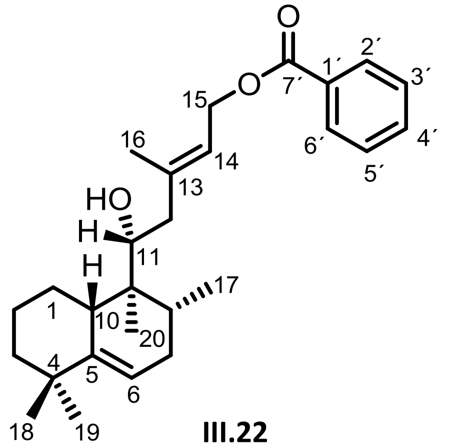
Table III.8. ^{13}C NMR data for (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid **II.7** and for compounds **III-15-III.18** ($CDCl_3$, 100 MHz; δ in ppm, *J* in Hz).

Carbon	**II.7**	**III.15**	**III.16**	**III.17**	**III.18**
1	28.89	28.87	28.96	29.87	29.69
2	22.51	22.50	22.58	22.90	22.88
3	40.88	40.89	41.31	41.37	41.33
4	36.30	36.29	36.31	36.52	36.51
5	145.17	145.20	145.33	146.12	146.10
6	116.02	116.04	115.94	115.15	115.19
7	31.34	31.36	31.36	31.03	31.04
8	32.85	32.83	32.78	32.79	32.79
9	41.61	41.64	40.98	41.20	41.18
10	40.25	40.27	40.16	38.91	38.90
11	74.97	75.07	75.82	73.08	72.93
12	41.69	41.37	40.06	42.01	42.06
13	159.78	156.80	136.86	137.78	140.25
14	117.72	117.86	126.32	126.68	121.72
15	171.05	166.61	59.21	59.27	61.17
16	18.99	18.75	16.20	16.34	16.44
17	17.15	17.15	17.18	17.27	17.25
18	28.14	28.14	28.12	27.68	27.69
19	29.72	29.72	29.73	29.87	29.87
20	11.84	11.77	11.99	12.64	12.61
$11\text{-O}\underline{\text{C}}\text{OCH}_3$	170.67	170.59	171.12	---	---
$11\text{-OCO}\underline{\text{C}}\text{H}_3$	20.82	20.85	21.04	---	---
$15\text{-COO}\underline{\text{C}}\text{H}_3$	---	50.89	---	---	---
$15\text{-O}\underline{\text{C}}\text{OCH}_3$	---	---	---	---	171.12
$15\text{-OCO}\underline{\text{C}}\text{H}_3$	---	---	---	---	21.03

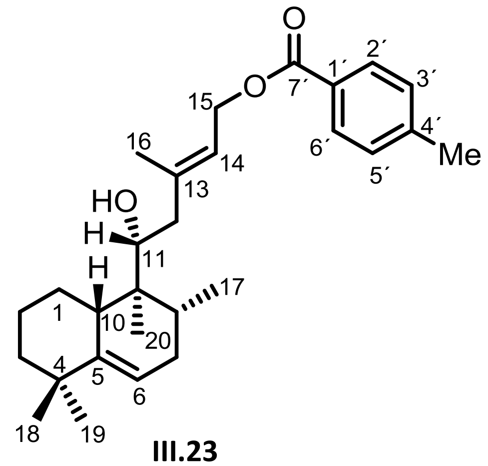


III.21

Compound **III.21** has a butanoate group on C-15 and the ^{1}H NMR spectrum showed similarities of signals with compound **III.17-III.19**. The ^{1}H and ^{13}C NMR spectra of derivative **III.21**, compared with **III.17,** showed the presence of a butanoxy on the C-15 position (H-2´ at δ 2.27 and C-2´ at δ 36.19, H-3´ at δ 1.64 and C-3´ carbon at δ 18.45, Me-4´ at δ 0.93 and C-4´ at δ 13.66; see **Tables II.7** and **III.9**). The presence of the butanoxy moiety at C-15 was evidenced by the HMBC spectrum, which showed correlations between the carbonyl carbon of the ester moiety at δ 173.70 and the C-15 methylene protons H-15A and H-15B at δ 4.62 and δ 4.59, respectively. The remaining protons and carbons of the ester group were assigned on the basis of their coupling constants, multiplicities and other data obtained from the HSQC and HMBC spectra (see **Tables III.7** and **III.9**).



III.22



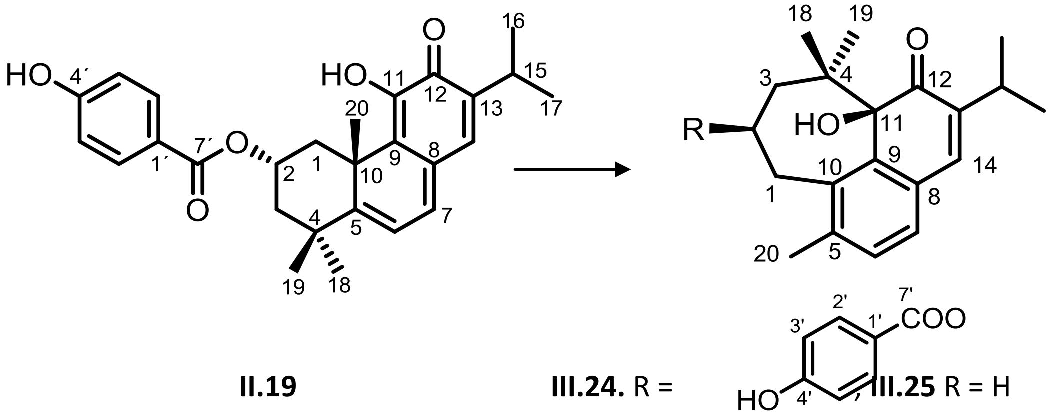
III.23

The aromatic ester derivatives **III.22** and **III.23** at carbon C-15 showed similarities of signals in the ^{1}H NMR spectrum with compounds **III.17-III.19** and **III.21** that also have a hydroxyl group on C-11 carbon ($\delta_{H\text{-}11}$ 3.69 and 3.68, for compounds **III.22** and **III.23** respectively, and $\delta_{H\text{-}11}$ 3.66 for compounds **III.17-III.19** and **III.21**). The H-15A and H-15B protons at δ 4.88 and 4.84, for compound **III.22,** and at δ 4.84 and 4.81 for compound **III.23**, are deshielded by the aromatic ester group to respect to acyl derivatives ($\delta_{H\text{-}15A}$ 4.63-4.56 and $\delta_{H\text{-}15B}$ 4.49-4.59). This effect was also observed for the methyl protons Me-16 at δ 1.81, comparing to the same signal from δ 1.71 to 1.75 (derivatives **III.16-III.21**). The ^{13}C NMR spectrum also showed similar signals with carbon C-15 ester derivatives (compounds **III.17-III.19** and **III.21**; see **Table III.9**) and all carbon assignments were confirmed with the HSQC experiment as expected (Pretsch E. et al., 2000).

Table III.9. ^{13}C NMR data for (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid **II.7** and for compounds **III.19-III.23** (CDCl$_3$, ^{a}100 MHz; δ in ppm, *J* in Hz).

Carbon	**II.7**	**III.19**	**III.20**	**III.21**	**III.22**	**III.23**
1	28.89	28.82	28.95	29.81	29.80	29.80
2	22.51	22.89	22.57	22.89	22.90	22.90
3	40.88	41.36	41.40	41.35	41.30	41.30
4	36.30	36.52	36.30	36.51	36.50	36.50
5	145.17	146.13	145.37	146.11	146.10	146.10
6	116.02	115.20	115.99	115.19	115.20	115.20
7	31.34	31.06	31.39	31.05	31.00	31.00
8	32.85	32.82	32.77	32.81	32.80	32.80
9	41.61	41.22	40.96	41.20	41.20	41.20
10	40.25	38.93	40.14	38.91	38.90	38.90
11	74.97	72.96	74.84	72.95	72.90	72.90
12	41.69	42.12	40.24	42.10	42.20	42.10
13	159.78	140.08	139.05	140.09	140.40	140.00
14	117.72	121.91	121.60	121.91	121.90	122.10
15	171.05	61.04	61.06	60.93	61.70	61.40
16	18.99	16.45	16.28	16.46	16.60	16.50
17	17.15	17.25	17.23	17.25	17.30	17.20
18	28.14	27.71	28.17	27.69	27.70	27.70
19	29.72	29.88	29.74	29.88	29.90	29.90
20	11.84	12.60	11.88	12.61	12.60	12.60
11-O$\underline{C}$OCH$_3$	170.67	---	---	---	---	---
11-OCO$\underline{C}$H$_3$	20.82	---	---	---	---	---
1´	---	174.49	174.43	173.70	---	---
2´	---	27.57	27.54	36.19	---	---
3´	---	9.12	9.10	18.45	---	---
4´	---	---	---	13.66	---	---
1´´	---	---	173.97	---	---	---
2´´	---	---	27.82	---	---	---
3´´	---	---	9.39	---	---	---
OBz-1´	---	---	---	---	130.30	122.70
OBz-2´ and 6´	---	---	---	---	129.60	131.60
OBz-3´ and 5´	---	---	---	---	128.30	113.50
OBz-4´	---	---	---	---	132.90	163.30
OBz-7´	---	---	---	---	166.60	166.40
PhOMe	---	---	---	---	---	55.40

III.4. 2β-(4-hydroxy)benzoyloxy-11β-hydroxy-4(5→11),20(10→5) di*abeo*-5(10),6,8,13-abietatetraen-12-one III.24



II.19 **III.24.** R = ; **III.25** R = H

The 2β-(4-hydroxy) benzoyloxy derivative of microstegiol **III.24** was obtained from parvifloron D **II.19** (Rüedi P.and Eugster C.H., 1978; Van Zyl R.L. et al., 2008) which was isolated from an acetone extract of *Plectranthus ecklonii* Benth. (see Chapter **II** and **V**).

Treatment of a dichloromethane solution of parvifloron D **II.19** with acid-washed molecular sieves, for 24 hours at room temperature, gave a complex mixture of compounds. Column chromatography allowed the isolation of **III.24**, the main and the more polar product of the reactional mixture (see Chapter **V**). 2β-(4-hydroxy)benzoyloxy-11β-hydroxy-4(5→11),20(10→5)di*abeo*-5(10),6,8,13-abietatetraen-12-one **III.24** had a molecular formula $C_{27}H_{30}O_5$ (the same of the starting material **II.19)**. The ^{1}H and ^{13}C NMR spectra of **III.24** (**Table III.10**) were almost identical to the microstegiol **III.25** data (Ulubelen A. et al., 1992). Moreover the observed differences were consistent with the presence, in the **III.24** of a 4-hydoxybenzoyloxy group attached to the C-2 position instead of the C-2 methylene group of **III.25**.

Table III.10. ^{1}H and ^{13}C NMR spectroscopic data for compound **III.24** (CDCl$_3$, 500 MHz(^{1}H) and 125 MHz (^{13}C); δ in ppm, *J* in Hz).

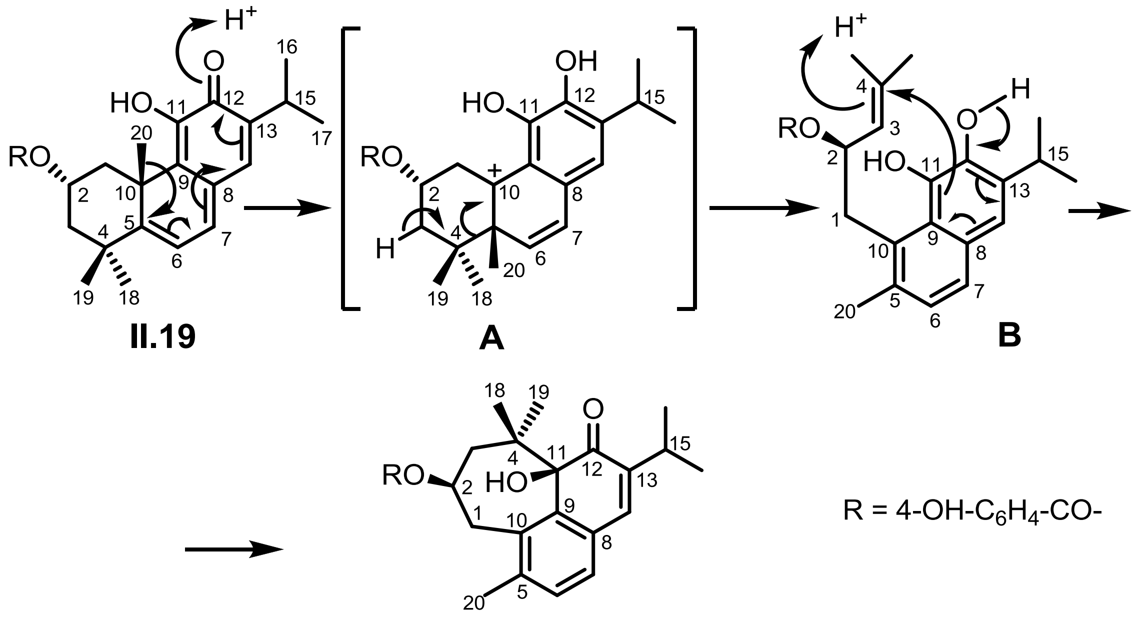
Position	δ_C	δ_H	HMBC (H→C)
1	33.60 CH$_2$	3.11 dt [*J* = 12.9,2.1 (*W*-type coupling)], eq H-1α	C-2, C-3, C-5, C-9, C-10
		4.04 dd (*J* = 12.9, 11.8), ax[b] H-1β	C-2, C-3, C-5, C-9, C-10
2	70.22 CH	4.85 dddd (*J* = 12.3, 11.8, 3.7, 2.1), ax H-2α	C-7´
3	47.61 CH$_2$	1.65 ddd [*J* = 12.8, 3.7, 2.1 (*W*-type coupling)], eq H-3α	C-1, C-2, C-4, C-11, C-18, C-19
		2.73 dd (*J* = 12.8, 12.3), ax H-3β	C-1, C-2, C-4, C-11, C-18, C-19
4	41.12 qC	-	
5	136.12 qC	-	
6	130.67 CH	7.13 br d (*J* = 7.9)	C-5, C-7, C-8, C-10
7	127.81 CH	6.97 d (*J* = 7.9)	C-5, C-6, C-8, C-9, C-14
8	128.90 qC	-	
9	139.54 qC	-	
10	139.43 qC	-	
11	83.51 qC	4.63 s, (OH-11)	C-9, C-11 and C-12
12	205.74 qC	-	
13	141.25 qC	-	
14	141.05 CH	7.00 t [*J* = 1.2(long-range coupled with both H-6 and H-15 protons)]	C-7, C-8, C-9, C-12, C-13, C-15
15	27.14 CH	3.03 septuplet of d (*J* = 6.8, 1.2)	C-12, C-13, C-14, C-16, C-17
16[a]	20.93 CH$_3$	1.22 d (*J* = 6.8)	C-13, C-15, C-17
17[a]	22.08 CH$_3$	1.16 d (*J* = 6.8)	C-13, C-15, C-16
18	28.96 CH$_3$	0.85 s, eq Me-18	C-3, C-4, C-11, C-19
19	21.87 CH$_3$	0.88 s, ax Me-19	C-3, C-4, C-11, C-18
20	21.38 CH$_3$	2.42 s	C-5, C-6, C-10
1´	123.20 qC	-	
2´,6´	131.92 CH	7.98 d (*J* = 8.8)	C-1´, C-3´, C-4´, C-7´
3´,5´	115.15 CH	6.86 d (*J* = 8.8)	C-1´, C-2´, C-4´
4´	159.85 qC	5.74 br, (*p* OH-Ph)	
7´	165.75 qC	-	

[a] Interchangeable signals; [b] ax and eq designate axial and equatorial hydrogen, respectively, and for positions 18 and 19 axial or equatorial methyl substituents.

The HMBC correlations observed between the protons Me-20 and the carbons C-5, C-6 and C-10, and between the protons Me-18 and Me-19 and the carbons C-3, C-4 and C-11, as well as those between the OH-11 and the carbons C-9, C-11 and C-12 (**Table III.10**), supported that **III.24** arises from the abietane skeleton of **II.19** by a 4(5→11),20(10→5)di*abeo*-rearrangement.

The absolute configuration of **III.24** and the stereochemistry of its seven-membered ring substituents were established as follows. The C-2 asymmetric centre must have the same absolute stereochemistry in **II.19** (Rüedi P. and Eugster C.H., 1978) and **III.24**, and the H-2α proton of **III.24** is axially oriented because it showed two *trans* diaxial ($J_{2\alpha,1\beta}$ = 11.8 Hz and $J_{2\alpha,3\beta}$ = 12.3 Hz) and two *cis* axial / equatorial ($J_{2\alpha,1\alpha}$ = 2.1 Hz and $J_{2\alpha,3\alpha}$ = 3.7 Hz) vicinal couplings (**Table III.10**). Moreover, NOESY-1D experiments were in agreement with a *cis* spatial relationship between the 2β-4-hydroxybenzoate and the hydroxyl group at C-11 because irradiation at δ 2.73 (H-3β axial proton) caused NOE enhancement in the signals of the H-1β, H-3α, OH-11 and Me-18 protons (δ 4.04, 1.65, 4.63 and 0.85, respectively), whereas the signals of the H-1α, H-3α and Me-19 (δ 3.11, 1.65 and 0.88, respectively) were enhanced when the proton H-2α (δ 4.85) was irradiated. In addition, irradiation of the C-11 hydroxyl proton (δ 4.63) produced NOE enhancement only in the signals of the H-1β and H-3β protons.

The formation of a compound such as **III.24** starting from parvifloron D **II.19** under acid catalysis is not surprising, and should be rationalized as it is outlined in **Figure III.3**. Compound **II.19** rearranges, via a C-10 carbonium ion intermediate **A** (Karanatsios D. et al., 1966; Matsumoto T. et al., 1995), to the 4,5-*seco*-20(10→5)*abeo*-abietane **B**, a class of rearranged abietanes that have been found in several Labiatae plants (Sexmero-Cuadrado M.J. et al., 1992; Li M. et al., 2000; Ulubelen A. et al.,1992, Ulubelen A. et al., 1997b). 12-*O*-Deprotonation of **B** followed by a stereoselective attack of the 9,11-aromatic bond from the *si*-face on the 3,4-olefin produces compound **III.24**. The total stereoselectivity for the conversion of **II.19** into **III.24** could be attributed to the presence in the former of a chiral centre at C-2 (Acuña A.U. et al., 2009).



III.24

Figure III.3. Formation of compound **III.24** from parvifloron D **II.19**.

The use of acid-washed molecular sieves to obtain 4(5→11),20(10→5) di*abeo*-rearranged derivatives from suitable abietane diterpenoids looks particularly attractive due to the noteworthy simplification of the experimental procedure, the avoidance of more acidic promoters (Karanatsios D. et al., 1966; Matsumoto T. et al., 1995; Sexmero-Cuadrado M.J. et al., 1992; Simões M.F. et al., 1986) and the mildness of the reaction conditions. This is the first report on the transformation of an abietane diterpene into a microstegiol derivative by an acid-catalyzed rearrangement.

CHAPTER IV

Biological activity

Results and Discussion

IV.1. Introduction

This chapter covers the biological activity namely antimicrobial, antioxidant and anti-inflammatory activities of the extracts and isolated compounds studied on Chapter **II** and of diterpenoid derivatives prepared on Chapter **III**.

The *P. ornatus* and *P. ecklonii* extracts were prepared by means of an increasing polarity set of solvents, *n*-hexane, ethyl acetate, dichloromethane, acetone, methanol and methanol-water (7:3) and its antimicrobial activities were evaluated. *P. ornatus* acetonic extract showed the more potent growth inhibitory effect against *E. faecalis* with a MIC value of 31.25 µg/mL. In addition, it demonstrated a MIC value of 125.00 µg/mL towards *S. aureus*, the same value obtained with the methanol-water extract. The *P. ecklonii* extracts and the remaining extracts were devoid of activity (MIC >125.00 or >250.00 µg/mL) or showing weak MIC values of 125.00 µg/mL against the standard Gram-positive, Gram-negative bacteria and *C. albicans* tested.

Antimicrobial assays of *P. ecklonii* and *P. ornatus* acetone extract column chromatographic fractions of the whole plants were determinated using the agar diffusion method. All the fractions of *P. ornatus* exhibited negative results against all the tested microorganisms and *P. ecklonni* fractions from hexane:EtOAc 25% to EtOAc:MeOH 10% to 40% (PE_2 and PE_5-PE_8) exhibited positive results against *S. aureus* and *M. smegmatis*, with the remaining fractions showing no antimicrobial activity. The fractions that showed antimicrobial activity were elected for exhausted gradient column chromatography which resulted in the isolation and elucidation of compounds studied in Chapter **V**. Parvifloron D **II.19**, previously reported as an antibacterial metabolite from *P. ecklonii* (Nyila M.A. et al., 2009), inhibited *E. faecalis* ATCC 51299 (low-VRE: vancomycin resistant Enterococcus) and *E. faecalis* FFHB with MIC values of 7.81 and 3.90 µg/mL, respectively, and showed lower MIC values (15.62 µg/mL) against both *S. aureus* ATCC 43866 and CIP 106760.

7α-Acetoxy-6β-hydroxyroyleanone **III.1**, isolated from *Plectranthus grandidentatus* revealed growth inhibitory activity against Gram-positive bacteria. Searching for additional royleanone abietane templates, the antibacterial activity of eleven derivatives of **III.1** (**III.3-III.13**) was studied. The esters **III.5**, **III.6** and **III.10** are more active than **III.1** against *Enterococcus* and *Staphylococcus* strains (MIC values ranging from 0.98 to 15.62 µg/mL). Moreover, 7α-acetoxy-6β-hydroxy-12-*O*-(4-chloro)benzoylroyleanone **III.6** gave rise to a new antibacterial-prototype (MIC values of 3.91-15.62 µg/mL against *Staphylococcus* and of 0.98-3.91 µg/mL against *Enterococcus spp.*). The hydrophobic extra-interactions with bacterial targets seem to play an important role on the activity of royleanones derivatives **III.3-III.13**.

11*R**-Acetoxyhalima-5,13*E*-dien-15-oic acid **II.7**, isolated from the acetone extract of *P. ornatus*, exhibited growth inhibitory activity against five *Staphylococcus* and five *Enterococcus* strains [MIC values 15.62 µg/mL (43.15 µM) – 62.50 µg/mL (172.65 µM)]. Diterpenes with a halimane skeleton are rarely isolated and their pharmacologic properties remain unknown. To study the potential of **II.7** as antibacterial prototype some derivatives **III.15-III.17**, **III.19-III.23** were obtained from **II.7** and the majority were tested against the same ten Gram positive bacteria. The more active was the (11*R**,13*E*)-halima-5,13*E*-diene-11,15-diol **III.17** with MIC values of 15.62 µg/mL (51.05 µM) to 31.25 µg/mL (102.12 µM).

Parvifloron D **II.19**, previously reported as an antibacterial metabolite from *P. ecklonii* (Nyila M.A. et al., 2009), inhibited *E. faecalis* ATCC 51299 (low-VRE: vancomycin resistant Enterococcus) and *E. faecalis* FFHB with MIC values of 7.81 and 3.90 µg/mL, respectively, and showed lower MIC values (15.62 µg/mL) against both *S. aureus* ATCC 43866 and CIP 106760.

The new microstegiol derivative **III.24** inhibited the growth of some *Staphylococcus* and *Enterococcus* strains with significant MIC values ranging from 3.91 to 7.81 µg/mL.

IV.2.Antimicrobial activity

IV.2.1. Antimicrobial activity of *P. ornatus* extracts

Plectranthus ornatus Codd. is reported to be used in Brazil folk medicine, due to the antibiotic properties of its leaves, besides the more referred use for digestive ailments. The antimicrobial activity of six extracts of *P. ornatus* were screened (see Chapter **V**; Rijo P. et al., 2007) and their MIC values were determined against a collection of Gram-positive and Gram-negative bacteria and a yeast using the microdilution method.

The initial screening of the antimicrobial activities was carried out on *P. ornatus* extracts (PO) prepared by maceration of dried plant, by means of an increasing polarity set of solvents from *n*-hexane (PO-H), ethyl acetate (PO-EA), dichloromethane (PO-D), acetone (PO-A), methanol (PO-M) to the solvent binary mixture MeOH-H_2O (7:3; PO-MW; see Chapter **V**).

The acetonic extract from *P. ornatus* showed the more potent growth inhibitory effect against *E. faecalis* with a MIC value of 31.25 µg/mL (see **Table IV.1**). Additionally, this extract revealed the same MIC value (125.00 µg/mL) towards *S. aureus* observed for the methanol-water extract. The *n*-hexane, ethyl acetate, dichloromethane, acetone, methanol and extracts were devoid of activity (MIC >125.00 µg/mL) against the standard Gram-positive, Gram-negative bacteria and *C. albicans* (see **Table IV.1**).

Table IV.1. MIC[a] values against a collection of Gram-positive and Gram-negative bacteria and a yeast, using the microdilution method, for six extracts[c] prepared from *P. ornatus* (PO).

Extracts and Controls[b]	*M. smegmatis* ATCC 607	*E. coli* ATCC 25922	*P.aeruginosa* ATCC 27853	*S.aureus* ATCC 25923	*C.albicans* ATCC 10231	*E. hirae* CIP 5855
PO-H	>250	>125	>125	>250	>125	>125
PO-EA	>250	>125	>125	>250	>125	>125
PO-D	>250	>125	>125	>250	>125	>125
PO-M	>250	>125	>125	>250	>125	>125
PO-MW	>250	>125	>125	125	>125	>125
PO-A	ND	ND	ND	125	ND	ND
DMSO	250	125	125	250	125	125
Positive control	RIF <0.48	NOR <0.48	VAN >125 Nor 0.97	VAN 1.95	KET 31.25	VAN 0.97

ND - not determined; [a]Minimal inhibitory concentrations of the extracts given in µg/mL; [b]concentrations of extracts and controls given in 1 mg/mL; [c]*P. ornatus* extracts (PO) from: *n*-hexane (PO-H), ethyl acetate (PO-EA), dichloromethane (PO-D), acetone (PO-A), methanol (PO-M) and MeOH-H_2O (7:3; PO-MW); RIF = Rifampicine, NOR = Norfloxacin, VAN = Vancomycin, KET = Ketoconazole.

Table IV.2. MIC[a] values against a four Gram-positive *Staphylococcus* strains using the microdilution method for acetone extract from *P. ornatus* (PO-A).

Extract and Controls	*S. aureus* ATCC 43866	*S. aureus* ATCC 700699	*S. aureus* CIP 106760	*S. aureus* FFHB 29593[b]
PO-A	125.00	ND	ND	125.00
Vancomycine	3.90	7.81	3.90	1.95
Meticillin	1.95	15.62	>250	0.97
DMSO	250.00	250.00	250.00	250.00

ND - not determined; [a]Minimal inhibitory concentrations of the extracts given in µg/mL; [b]FFHB species are clinical isolates from *Hospital do Barreiro*, deposited on the Microbiology Laboratory, Pharmacy Faculty of Lisbon University;

Table IV.3. MIC[a] values against a four Gram-positive *Enterococcus* strains using the microdilution method for acetone extract from *P. ornatus* (PO-A).

Extract and Controls	*E. faecium* FFHB 435628[b]	*E. faecalis* FFHB 427483[b]	*E. flavescens* ATCC 49996	*E. faecalis* ATCC 51299[c]
PO-A	62.50	>125.00	31.25	31.25
Vancomycine	0.97	1.95	3.90	62.50
DMSO	125.00	125.00	125.00	125.00

[a] Minimal inhibitory concentrations of the extracts given in µg/mL; [b]FFHB species are clinical isolates from *Hospital do Barreiro*, deposited on the Microbiology Laboratory, Pharmacy Faculty of Lisbon University; [c]VRE.

IV.2.2. Antimicrobial activity of *P. ecklonii* extracts

The antimicrobial activity of six extracts of *P. ecklonii* (PE; see Chapter **V**) were screened and their MIC values determined against a collection of Gram-positive and Gram-negative bacteria and a yeast using the microdilution method.

The initial screening of the antimicrobial activities was carried on extracts prepared from *P. ecklonii* by means of an increasing polarity set of solvents, *n*-hexane (PE-H), ethyl acetate (PE-EA), dichloromethane (PE-D), acetone (PE-A), methanol (PE-M), and methanol-water (7:3; PE-MW; see Chapter **V**).

Table IV.4. MIC[a] values against a collection of Gram-positive and Gram-negative bacteria and a yeast using the microdilution method for five extracts prepared from *P.ecklonii.*

Extracts and Controls[b]	*M.smegmatis* ATCC 607	*E. coli* ATCC 25922	*P.aeruginosa* ATCC 27853	*S.aureus* ATCC 25923	*C.albicans* ATCC 10231	*E. hirae* CIP 5855
PE-H	>250	>125	>125	125	>125	>125
PE-EA	125	>125	>125	125	>125	>125
PE-D	125	>125	>125	125	>125	>125
PE-M	>250	>125	>125	>250	>125	>125
PE-MW	>250	>125	>125	125	>125	>125
PE-A	ND	ND	ND	ND	ND	ND
DMSO	250	125	125	250	125	125
Positive control	RIF <0.48	NOR <0.48	NOR 0.97	VAN 1.95	KET 31.25	VAN 0.97

ND - not determined, [a]Minimal inhibitory concentrations of the extracts given in µg/mL; [b]concentrations of extracts and controls given in 1 mg/mL; [c]*P. ecklonii* extracts (PE) from: *n*-hexane (PE-H), ethyl acetate (PE-EA), dichloromethane (PE-D), acetone (PE-A), methanol (PE-M) and MeOH-H_2O (7:3; PE-MW); RIF = Rifampicine, NOR = Norfloxacin, VAN = Vancomycin, KET = Ketoconazole.

The *n*-hexane, ethyl acetate, dichloromethane, methanol, methanol:water (7:3) and acetone extracts were devoid of activity (MIC >125.00 or >250.00 µg/mL) or showing weak MIC values of 125.00 µg/mL against the standard Gram-positive, Gram-negative bacteria and *C. albicans* tested (see **Table IV.4**).

IV.2.3. Qualitative antimicrobial activity of *P. ornatus* and *P. ecklonii* acetone extract fractions

The antimicrobial assays of *Plectranthus ecklonii* Benth. and *P. ornatus* Codd. acetone extract fractions of the whole plants were determinated. Fractions with a similar composition (see Chapter **V**) were pooled to give eight major fractions for *P. ornatus* (PO_1-PO_8; **Table V.1**) and eight major fractions for *P. ecklonii* (PE_1-PE_8; **Table V.11**).

Antimicrobial activities were tested against *Psedomonas aeruginosa*, *Escherischia coli*, *Staphylococcus aureus*, *Candida albicans* and *Mycobacterium smegmatis* (see section **V.8.1**). The disc-diffusion method was used to determine the growth inhibition caused by plant extracts and fractions. The extracts and fractions in study were dissolved in DMSO with a concentration of 1 mg/mL and 15 µL or 30 µL were tested, using the agar diffusion method. The ratio of the inhibition zone (mm) produced by plant extract or fraction was used to express antibacterial activity. All the fractions and extracts of *P. ornatus* exhibited negative results for all biological assays, no inhibition zone (mm) was produced by plant extract or fraction. *P. ecklonni* fractions PE_2 and PE_5-PE_8 exhibited positive results against *S. aureus* and *M. Smegmatis* as presented in **Table IV.5**, with the remaining extract and fractions presenting no antimicrobial activity. The fractions that showed antimicrobial activity were elected for exhausted gradient column chromatography which resulted in the isolation and elucidation of compounds studied in Chapter **II**.

Table IV.5. Positive results of *P. ecklonni* fractions PE_2 and PE_5-PE_8 against *S. aureus* and *M. smegmatis*.

P. ecklonni fractions / g	Polarity	*S. aureus* inhibition zone (mm) 15 µL / 30 µL applied	*M. Smegmatis* inhibition zone (mm) 15 µL / 30 µL applied
PE_2 / 13.7	From hexane:EtOAc 25% to 50%	8.5 / 9.0	8.0 / 8.5
PE_5 / 37.7	From hexane:EtOAc 60% to 75%	9.0 / 8.0	8.0 / 8.0
PE_6 / 27.5	From hexane:EtOAc 75% to 90%	- / 7.5	- / 8.5
PE_7 / 5.5	From EtOAc 100% to 10% MeOH	8.0/ 7.0	- / -
PE_8 / 49.8	From EtOAc:MeOH 10% to 40%	9.0 / 8.0	- / -

IV.2.4. Antimicrobial activity of diterpenoids isolated from *P. ornatus*

The diterpenoids isolated from *P. ornatus* (see Chapter **V**) 6-*O*-acetylforskolin **II.1,** 1,6-di-*O*-acetylforskolin **II.2,** 1,6-di-*O*-acetyl-9-deoxyforskolin **II.3,** rhinocerotinoic acid **II.6,** 11*R**-acetoxyhalima-5,13*E*-dien-15-oic acid **II.7,** and plectrornatin C **II.17** were tested against a collection of Gram-positive and Gram-negative bacteria and a yeast using preliminary disk diffusion tests. The antibacterial activities against *M. smegmatis*, *S. aureus, E. coli* and *C. albicans,* showed the values of 9.0, 16.0, 7.0 and 7.0 mm, respectively, as diameters of inhibitory zones for compound 11*R**-acetoxyhalima-5,13*E*-dien-15-oic acid **II.7** and 6.5 mm against *E. coli*, for compound rhinocerotinoic acid **II.6**. The microdilution method was used against the same collection of Gram-positive and Gram-negative bacteria and a yeast for compounds **II.6** and **II.7** as shown in **Table IV.6.**

Table IV.6. MIC[a] values against a collection of Gram-positive and Gram-negative bacteria and a yeast using the microdilution method for diterpenoids **II.1-II.3**, **II.6**, **II.7** and **II.17** isolated from *P. ornatus.*

Compound	*M. smegmatis* ATCC 607	*E. coli* ATCC 25922	*P.aeruginosa* ATCC 27853	*S.aureus* ATCC 25923	*C.albicans* ATCC 10231	*B. subtillis ATCC 6633*
II.1	> 250	> 125	> 125	> 250	> 125	125
II.2	>250	> 125	> 125	> 250	> 125	ND
II.3	> 250	> 125	> 125	> 250	> 125	ND
II.6	>250	ND	ND	ND	ND	62.50
II.7	125	ND	ND	ND	ND	31.25
II.17	> 250	> 125	> 125	> 250	62.5	ND
DMSO	250	125	125	250	125	125
Positive control	RIF <0.48	NOR <0.48	NOR 0.97	VAN 1.95	KET 31.25	RIF 0.48

ND - not determined, [a]Minimal inhibitory concentrations of the extracts given in µg/mL; RIF = Rifampicine, NOR = Norfloxacin, VAN = Vancomycin, KET = Ketoconazole.

Compounds **II.6** and **II.7** were also tested against methicillin-resistant *Staphylococcus aureus* (MRSA) and low vancomycin-resistant *Enterococcus faecalis* (VRE) as presented in **Tables IV.7** and **IV.8.**

Table IV.7. MIC[a] values against four Gram-positive *Enterococcus* strains using the microdilution method for diterpenoids **II.6** and **II.7** isolated from *P. ornatus.*

Compounds	*E. faecalis* ATCC 51299[b]	*E. flavescens* *ATCC* 49996	*E. faecium* FFHB 435628[c]	*E. faecalis* FFHB 427483[c]
II.6	62.50	>125.00	>125.00	>125.00
II.7	0.48	62.50	1.95	15.60
DMSO	125.00	125.00	125.00	125.00
Vancomycin	>500.00	3.90	0.48	0.97

[a] Minimal inhibitory concentrations of the compounds is given in µg/mL; [b]Low level vancomycin-resistant *Enterococcus* (VRE); [c]FFHB species are clinical isolates from *Hospital do Barreiro*, deposited on the Microbiology Laboratory, Pharmacy Faculty of Lisbon University;

Table IV.8. MIC[a] values against four Gram-positive *Staphylococcus* strains using the microdilution method for diterpenoids **II.6** and **II.7** isolated from *P. ornatus.*

Compounds	*S. aureus* ATCC 700699	*S. aureus* CIP 106760[b]	*S. aureus* ATCC 43866	*S. aureus* FFHB 29593[c]
II.6	250.00	250.00	250.00	250.00
II.7	250.00	250.00	3.90	7.81
DMSO	250.00	250.00	250.00	250.00
Vancomycin	3.90	3.90	0.97	0.97

[a] Minimal inhibitory concentrations of the compounds is given in µg/mL; [b]MRSA; [c]FFHB species are clinical isolates from *Hospital do Barreiro*, deposited on the Microbiology Laboratory, Pharmacy Faculty of Lisbon University;

IV.2.5. Antimicrobial activity of diterpenoids isolated from *P. ecklonii*

IV.2.5.1. Antimicrobial activity of sugiol II.18

The growth inhibition properties of sugiol **II.18** were tested against *P. aeruginosa*, *E. coli*, *S. aureus*, *C. albicans, E. hirae* and *M. smegmatis* using the disc-diffusion method. Fraction PE_2 showed antibacterial activity against *S. aureus* and *M. smegmatis* (see **Table IV.5**). Bioassay-guided chromatographic fractionation of the antibacterial active PE_2 fraction led to the isolation of sugiol **II.18**. The Minimum Inhibitory Concentration value (MIC) of sugiol **II.18** was evaluated against four *Staphylococcus aureus* strains and five strains of *Enterococcus* strains. Sugiol **II.18** showed MIC value of 62.5 µg/mL against *E. faecalis* FFHB 427483 (the remaining MIC values were 125-250 µg/mL). The low activity exhibited by sugiol **II.18** was in agreement with a very low antibacterial activity previously reported for this compound (Politi et al., 2003; Ulubelen A. et al., 2000).

IV.2.5.2. Antimicrobial activity of parvifloron D II.19

Purification of the fractions PE_5-PE_8 (**Table IV.5**) led to the isolation of parvifloron D **II.19** and the antibacterial activity of this diterpenoid was carried out being outlined in **Table IV.9**.

Table IV.9 MIC[a] values against a collection of Gram-positive and Gram-negative bacteria and a yeast for parvifloron D **II.19** isolated from *P.ecklonii.*

Compounds	*M. smegmatis* ATCC 607	*E. coli* ATCC 25922	*P.aeruginosa* ATCC 27853	*S.aureus* ATCC 25923	*C.albicans* ATCC 10231	*E. hirae* CIP 5855
II.19	125	>125	>125	125	>125	62.5
DMSO	250	125	125	250	125	125
Positive	RIF	NOR	NOR	VAN	KET	VAN
control	<0.48	<0.48	0.97	1.95	31.25	0.97

[a]Minimal inhibitory concentrations given in µg/mL; RIF = Rifampicine, NOR = Norfloxacin, , VAN = Vancomycin, KET = Ketoconazole.

Table IV.10. MIC[a] values against a four Gram-positive *Staphylococcus* strains for parvifloron D **II.19** isolated from *P. ecklonii.*

Compound	*S. aureus* FFHB 29593[b,c]	*S. aureus* ATCC 43866	*S.aureus* CIP 106760[b]	*S.aureus* ATCC 700699
Parvifloron D **II.19**	125	15.62	15.62	62.5
DMSO	250	250	250	250
VAN	3.90	3.90	3.90	7.81
TET	<0.48	125	31.25	62.50
OXY	<0.48	31.25	31.25	62.50
AMP	125	>250	>250	<0.48
MET	>250	1.95	>250	15.62

[a]Minimal inhibitory concentrations given in µg/mL; VAN = Vancomycin; TET = Tetracycline; OXY = Oxytetracycline; AMP = Ampicillin; MET = Meticillin; [b]MRSA; [c] FFHB species are clinical isolates from *Hospital do Barreiro*, deposited on the Microbiology Laboratory, Pharmacy Faculty of Lisbon University.

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Table IV.11. MIC[a] values against Gram-positive *Enterococcus* strains for parvifloron D **II.19**.

Compound	*E. faecalis* ATCC 51299[b]	*E. flavescens* ATCC 49996	*E. faecalis* CIP 104476	*E. faecium* FFHB 435628[c]	*E. faecalis* FFHB 427483[c]
II.19	7.81	15.62	7.81	31.25	3.90
DMSO	125	125	125	125	125
Vancomycin	62.5	3.90	31.25	0.97	1.95
Tetracycline	<0.48	<0.48	<0.48	<0.48	31.25
Ampicillin	<0.48	<0.48	<0.48	>125	<0.48

[a]Minimal inhibitory concentrations given in μg/mL; [b]Low level vancomycin-resistant *Enterococcus* (VRE); [c] FFHB species are clinical isolates from *Hospital do Barreiro*, deposited on the Microbiology Laboratory, Pharmacy Faculty of Lisbon University.

IV.2.6. Antimicrobial activity of the spiro-abietane diterpenoid II.20 isolated from *P. porcatus* and of the beyerane diterpenoid II.22 isolated from *P. saccatus*

The antimicrobial activity against reference bacteria (*M. smegmatis*, *E. coli*, *P. aeruginosa* and *E. faecalis*) and yeast (*C. albicans)*, were evaluated for diterpenoids (13*S*,15*S*)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione **II.20** and *ent*-7α-acetoxy-15-beyeren-18-oic acid **II.22,** using the microdilution method. Among Gram-positive bacteria, two *Staphylococcus* spp. and two *Enterococcus* spp. were tested as shown in **Tables IV.12** and **IV.13**.

Table IV.12. MIC[a] values against two Gram-positive *Staphylococcus* spp. using the microdilution method for the spiro-abietane diterpenoid **II.20** isolated from *P. porcatus* and beyerane diterpenoid **II.22** from *P. saccatus*.

Compound	*S. aureus* ATCC 700699	*S. aureus* CIP 106760[b]	*S. aureus* ATCC 43866	*S. aureus* FFHB 29593[b]	*S. aureus* ATCC 6538	*S. epidermis* ATCC 12228
II.20	125	>250	125	125	62.50	>250
II.22	125	125	125	125	125	125
DMSO	250	250	250	250	250	250
Rifampicin	<0.49	500	<0.49	<0.49	<0.49	<0.49

[a]Minimal inhibitory concentrations given in μg/mL; [b]MRSA.

Table IV.13. MIC[a] values against a two Gram-positive *Enterococcus* spp. for the spiro-abietane diterpenoid **II.20** isolated from *P. porcatus* and of the beyerane diterpenoid **II.22** from *P. saccatus*.

Compound	*E. faecalis* ATCC 51299[b]	*E. faecalis* H164	*E. hirae* ATCC 10541
II.20	>125	>125	>125
II.22	>125	>125	>125
Rifampicin	<0.49	<0.49	<0.49
DMSO	125	125	125

[a]Minimal inhibitory concentrations given in µg/mL; [b]Low level vancomycin-resistant *Enterococcus* (VRE).

The new diterpenes showed no activity against Gram-negative bacteria and *Candida albicans* (yeast strain). Among Gram-positive bacteria, the lower MIC value evaluated was 62.50 µg/mL for the abietane **II.20** against *Staphylococcus aureus* ATCC 6538.

IV.2.7. Antimicrobial activity of royleanone derivatives III.3-III.13

A previous antimicrobial studies on royleanones revealed that they were active against *Staphylococcus* and *Enterococcus* species. Moreover, a preliminary SAR (structure activity relationships) analysis of 10 natural abietanes led to the conclusion that the presence of a C ring with a 12-hydroxy-*p*-benzoquinone moiety and an oxidized B ring at C-6/C-7 positions were significant for the activity (Gaspar-Marques C. et al., 2006). In addition, (Yang Z. et al., 2001) reported that the presence of additional benzyl groups influenced the anti-MRSA activities of phenolic abietanoids. Along with others similar diterpene metabolites active against Gram-positive bacteria are totarol and abietic acid. These diterpenes have the ability to transverse or damage the bacterial cytoplasmatic membrane due to their amphipathic character. Besides that, it was described that antibacterial activity may be modulated through an increase in lipophilicity and/or in hydrogen-bond donor/acceptor groups of the hydrophilic moiety. However, nor the complete mechanism of action neither their biological targets are entirely known (Urzúa A. et al. 2008; Bernabeu A. et al. 2002).

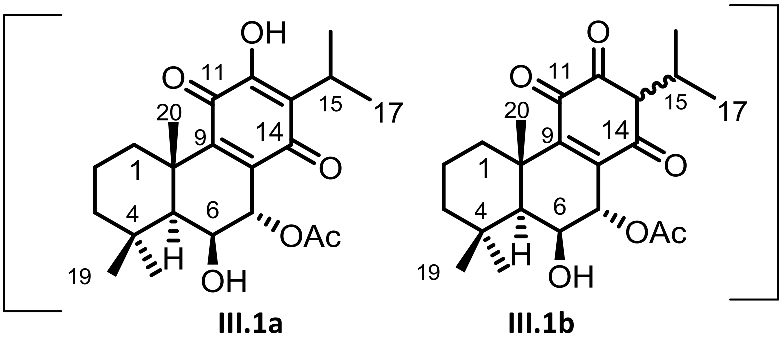
Trying to further identify the structural requirements involved in the activity against Gram-positive bacteria, 7α-acetoxy-6β-hydroxyroyleanone **III.1** have been selected as a template because it has revealed a potent *in vitro* anti-Gram positive activity (Gaspar-Marques C. et al., 2006). Thus, a set of eleven derivatives **III.3-III.13** (see Chapter **VI**), all being esters of HO-C_6 and/or HO-C_{12}, were hemisynthesised following the procedures as previously described (see Chapter **III**; Rijo P. et al., 2010). These analogues were prepared having in mind the plausible importance of the H-binding groups (hydroxyl, quinone and ester), van der Waals interactions and hydrophobic binding moieties (hydrocarbon skeleton, acyl and aromatic features) in the interaction with the bacterial targets.

The aim of the hemisynthesis of these derivatives was to evaluate the potential utility of the substituents groups, carried by ester diterpenoids **III.3-III.13**, on the antibacterial activity of **III.1** against methicillin-sensible and -resistant *S. aureus*, as well, vancomycin-sensible and -resistant *Enterococcus* spp.

A previous assay showed that all derivatives **III.3-III.13** were inactive against *Escherichia coli* and *Pseudomonas aeruginosa* (MIC values >125 μg/ml), in agreement with the former observations that royleanones were inactive towards most of Gram-negative bacteria (Gaspar-Marques C. et al., 2006; Yang Z. et al. 2001).

Despite template **III.1** to be almost equipotent towards both *Staphylococcus* and *Enterococcus* bacteria, all derivatives **III.3-III.13** showed higher inhibitory growth properties against *Enterococcus* spp. Than against *Staphylococcus* spp. Compounds **III.3-III.13** inhibited vancomycin-resistant *E. faecalis* strains (**Table IV.14**) showing MIC values ranging from 0.97 to 62.5 μg/mL (vancomycin MIC value of 62.50 μg/mL). These compounds **III.3-III.13** also inhibited the MRSA strains (MIC range 3.90–7.81 μg/mL) (**Table IV.15**). Nevertheless, the best MIC values resulting from the growth inhibition of MRSA and low-VRE strains were 1.95 to 7.81 μg/mL by **III.5-III.6** and **III.10**. This may be a key-step in the selection of the mentioned derivatives as new antibacterial leads against resistant bacteria.

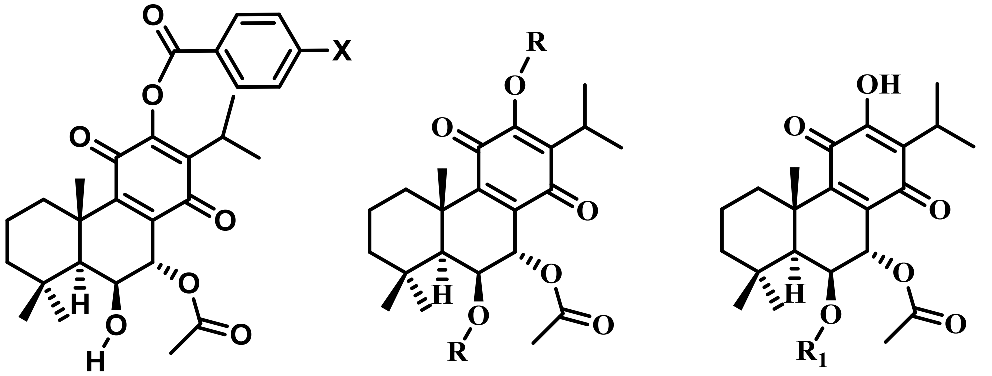
Prototype royleanone **III.1** has a lipophilic ring system, two hydroxyl groups both with potential hydrogen-bond donor/acceptor characteristics (at C_6 and C_{12}), but with different electronic and chemical behaviour, as represented in **Figure IV.1**, and three more oxygen atoms with hydrogen-bond acceptor abilities (carbonyl groups). Transformation of **III.1** resulted in more lipophilic esters with extra hydrogen-bond acceptors atoms (mostly oxygen from carbonyl) and with lower number of hydrogen-bond donor atoms (Rijo P. et al. 2010).



III.1a **III.1b**

Figure IV.1 Tautomers **a** and **b** (5:95) of 7α-acetoxy-6β-hydroxyroyleanone **III.1**. Horminone is the 7α-hydroxy analogue of **III.1.**

The derivatives may be chemically classified into three groups: **III.5-III.7** that preserved the alcoholic substituent (at C_6); **III.3-III.4** and **III.9-III.10** without any free hydroxyl groups; and the remaining **III.8** and **III.11-III.13** that kept the more acidic HO group (at C_{12}) (**Figure IV.2**). This division corresponds, nearly, to three levels of antibacterial activity (based on simple arithmetic mean calculations). The first group of compounds (**III.5-III.7**), together with **III.10**, are more active against *Enterococcus* spp. (MIC values ranging from 0.98 to 15.62 µg/mL) being also more active than **III.1** (MIC 7.81–15.62 µg/mL) (**Table IV.14**). Moreover, 12-*O*-*p*-chlorobenzoyl analogue **III.6** and 6, 12-*O*-propyonyl diester **III.10** are the most active compounds against *Staphylococcus* spp. (MIC ranging from 3.90 to 15.62 µg/mL) being also more active than **III.1** (MIC 7.81–31.25 µg/mL) (**Table IV.15**). The compounds **III.3-III.4** and **III.9** of the second group are less active, against all strains (MIC ranging from 7.81 to 125 µg/mL), than **III.5-III.7** and **III.10**. Even so, the abietanes **III.3** and **III.9,** with MIC values of 7.81 µg/mL, are significantly active against low VRE *E. faecalis* ATCC 51299 (vancomycin MIC of 62.5 µg/mL). Compounds of the third group **III.8** and **III.11-III.13** showed MIC values that ranges from 15.62 to 62.50 µg/mL against tested bacteria (**Tables IV.14** and **IV.15**) revealing, generally, to be less active than **III.1**.



R

R₁

III.5 X = -OMe
III.6 X = -Cl
III.7 X = NO_2

III.3 R = Bz
III.4 R = 4-Cl-C_6H_4CO
III.9 R = $COCH_3$
III.10 R = $COCH_2CH_3$

III.8 R_1 = 4-NO_2- C_6H_4CO
III.11 R_1 = $COCH_3$
III.12 R_1 = $COCH_2CH_3$
III.13 R_1 = $CO(CH_2)_2CH_3$

Figure IV.2. Ester derivatives **III.3-III.13** of 7α-acetoxy-6β-hydroxyroyleanone **III.1**.

The first partial conclusion is that oxygen atom attached to C_{12}, most certainly, does not act as hydrogen-bond donor and thus, this kind of interaction is not essential for activity. In fact, the more active compounds **III.5-III.7** and **III.10** have an ester functional group on C_{12}. On the contrary, those with a free hydroxyl on C_{12} (as **III.8** and **III.11-III.13**), which could act as hydrogen-bond donor, are less active (Radulović N. et al., 2010). The 1,2,4-quinone structure on the ring C of the major tautomeric form, (similarly to the tautomer **III.1b** represented on the **Figure IV.1**), may contribute to this behaviour. Moreover, the importance for activity of the OH–C_6 **III.5-III.7** and **III.10**, as hydrogen-bond donor, is questionable due to its low dissociation capacity and to its steric hindrance. However, the role of the hydroxyl groups *versus* the carbonyl groups of the ester functions as hydrogen-bond acceptors was not clarified, based on the results obtained in this work.

Most importantly, the significant increase of the activity revealed by compounds **III.5-III.7** and **III.10,** seems to result from the higher lipophilicity brought by the aromatic/alkylic motif linked to the ester groups. The aromatic rings may improve the activity of antibacterial prototypes through additional interactions both with the hydrophobic regions of the bacterial cell wall and cell membrane – through van der Walls interactions – and interacting with the ammonium ions – through induced dipole interactions or hydrogen bonding – with peptidoglycan and lipoteichoic acids constituents of the membrane system of Gram-positive bacteria (Patrick G.L. 2009 Chopra I. and Roberts M., 2001). Compound **III.6** showed the highest MIC values (0.98-15.62 µg/mL) due, must certainly, to the hydrophobic nature of chlorine, favouring the activity through extra hydrophobic interactions. Furthermore, analogues **III.5** and **III.7** also reveal interesting antibacterial activities, hypothetically, due to the peculiar electronic properties of both methoxyl and nitro groups attached to the benzoyl moiety. They would able to interact with an extra binding site of the cell wall and/or membrane acting as hydrogen-bond acceptors. The derivative **III.10** shows an unexpected good activity (MIC range 3.91-15.62 µg/mL) when compared with **III.9** (MIC ranges 7.81-62.50 µg/mL). The extra hydrophobic binding sites carried by the alkylic structures around C_6 and C_{12} of **III.10** (propyl) seems to fit better to different hydrophobic regions of the binding site.

Positional isomers 12-*O*-(4-nitro)benzoyl derivative **III.7** and 6*β*-(4-nitro)benzoyl analogue **III.8** showed a considerable difference in activity (MIC range 1.95-15.62 and 7.81-62.50 µg/mL, respectively) (**Tables IV.14** and **IV.15**), despite the presence of close structural features (**Figure IV.2**). The nitro-benzoyl group may confer a bulkier structure to **III.8** (around C_6) and a quite planar hydrophobic surface to **III.7** (around C_{12}). The extra hydrophobic moiety linked, to C_6, may not correspond to a hydrophobic binding site of the bacterial membrane systems, contrary when linked to C_{12}. The same reasoning can be applied when **III.5** (12-O-(4-methoxy)benzoyl ester) and **III.4** (6β,12-O-(4-chloro)benzoyl ester) are compared as potential antibacterial agents (**Tables IV.14** and **IV.15**). Derivative **III.4** may have either an unfavourable shape and a steric hindrance or a wrong spatial distribution of the hydrophobic moieties that prevent the efficient binding to bacterial wall, besides a too higher lipophilicity.

Finally, analogues **III.8** and **III.11-III.13** carrying dissimilar hydrophobic esters, attached to the C_6 position, are not good antibacterial agents (**Tables IV.14** and **IV.15**). These findings could be attributed either to a bulked steric structure or to an incorrect position of the hydrophobic substituents once oxygen atoms do not seem to play an important role on the activity (as hydrogen-bond donors or acceptors).

The studies carried out by Spiridonov *et al* (Spiridonov N. et al., 2003) suggested that the primary target of royleanones as antimicrobial (and antitumoral) agent would be the biological membranes. Their mode of action would be a consequence of the protonophoric activity due to the dissociation of the C_{12} hydroxyl group. Theoretical studies carried out by Nicolás *et al* (Nicolás I. et al., 2003; Nicolás I. et al., 2006) postulated that horminone, structurally close to **III.1** (**Figure IV.1**), could be correlated with oxytetracycline having a similar bacteriostatic mode of action. Both have chelating abilities to form complexes with divalent cations, with preference by Mg^{2+}. Oxygen atoms attached to C_7 and C_{14} would behave as Lewis bases and coordinate with Mg^{2+}. Thus, horminone should cross the membrane system of bacteria (a pH potential driven process) and, within the cytoplasm, should inhibit protein synthesis by interaction of the hydrated horminone-Mg^{2+} complex with ribosomal RNA (Nicolás I. et al. 2006; Chopra I. and Roberts M. 2001). Contrary to oxytetracycline, horminone and several royleanones (Tada M. and Ishimaru K., 2006; Gaspar-Marques C. et al, 2006; Yang Z. et al. 2001) as well as derivatives **III.3-III.13** are active mostly against Gram-positive bacteria. This may be a consequence of the differences on bacterial cell walls. These small molecules could cross the porous cell wall of Gram-positive bacteria, whilst they only could pass the membrane systems of Gram-negative bacteria through the protein porins channels which could reduce their diffusion probability.

Further information from literature, concerning the antimicrobial mode of action of other royleanones is unavailable. Therefore, as all compounds (**III.1** and **III.3-III.13**; **Figures IV.1** and **IV.2**) show a decreased basic character of the oxygen atoms of the acetyl group (C_7), when compared with the Lewis basic character of the hydroxyl (C_7) of horminone (**Figures IV.1**), it is difficult to believe that the mode of action might involve the coordination with Mg^{2+} cation as postulated for horminone, unless esters hydrolysis occur.

This first approach to find an antimicrobial prototype derivative provided compounds with better MIC values. Unfortunately, selectivity against bacterial cells is not satisfactory. Actually, calculations of the selective index (SI = GI50 (VERO cells) / MIC values), anchored in cytotoxicity data on Vero cells previously reported (Rijo P. et al., 2010) and on MIC values (**Tables IV.14 and IV.15**), show that just template **III.1**, derivatives **III.5** and **III.6** have SI values > 1 (Rijo P. et al., 2010) towards some strains.

The more relevant derivative showing the more interesting SI values, is 12-*O*-*p*-chlorobenzoyl analogue **III.6** which is less cytotoxic than **III.1** (Rijo, P. et al. 2010) and show the lowest MIC values, mainly, against the Enterococcus species. However, to improve potency and selectivity in order to reach compounds with attractive ADME/Tox (Absorption, Disposition, Metabolism, Excretion and Toxicity) properties, further efforts are required (Simpson, 2009).

Table IV.14. MIC[a] values against a five Gram-positive *Enterococcus* strains for compounds **III.1**, **III.3-III.13.**

Compound	*E. faecalis* ATCC 51299[b]	*E. faecalis CIP 104476*	*E. faecalis* FFHB[c] 427483	*E. flavescens* ATCC 49996	*E. faecium* FFHB 435628[c]
III.1	15.62	15.62	7.81	7.81	15.62
III.3	7.81	1.95	7.81	7.81	15.62
III.4	62.50	3.91	31.25	31.25	31.25
III.5	1.95	15.62	0.97	3.91	0.97
III.6	3.91	7.81	1.95	0.97	1.95
III.7	1.95	7.81	3.91	3.91	3.91
III.8	15.62	31.25	7.81	15.62	31.25
III.9	7.81	ND	7.81	15.62	15.62
III.10	3.90	ND	3.90	7.81	3.90
III.11	31.25	15.62	15.62	62.50	15.62
III.12	15.62	31.25	15.62	15.62	15.62
III.13	15.62	15.62	15.62	62.50	15.62
DMSO	125.00	125.00	125.00	125.00	125.00
VAN	62.50	31.25	1.95	3.91	0.98
AMP	<0.49	<0.49	>125.00	<0.49	<0.49
TET	<0.49	<0.49	<0.49	31.25	<0.49

[a]Minimal inhibitory concentrations given in µg/mL; [b]Low level vancomycin-resistant *Enterococcus* (VRE); [c] FFHB species are clinical isolates from *Hospital do Barreiro*, deposited on the Microbiology Laboratory, Pharmacy Faculty of Lisbon University; Control antibiotics: AMP (ampicillin), TET (tetracycline), VAN (vancomycin) ; ND = Not Determined.

Table IV.15. MIC[a] values against methicillin-sensitive *Staphylococcus aureus* (MSSA)[b] and methicillin-resistant *S. aureus* (MRSA)[c] strains for compounds **III.1, III.3-III.13.**

Compound	*S aureus* ATCC 700699[b]	*S aureus* CIP 106760[c]	*S aureus* ATCC 43866[b]	*S aureus* FFHB 29593[c]	*S aureus* ATCC 25923[b]
III.1	31.25	7.81	15.62	7.81	31.25
III.3	31.25	15.62	15.62	125	62.5
III.4	62.5	125	125	125	31.25
III.5	31.25	3.90	3.90	3.90	62.5
III.6	15.62	3.90	3.90	7.81	3.90
III.7	7.81	15.62	7.81	15.62	62.5
III.8	62.5	15.62	15.62	31.25	62.5
III.9	31.25	15.62	15.62	62.5	62.5
III.10	7.81	3.90	7.81	7.81	15.62
III.11	31.25	62.5	125	125	62.5
III.12	31.25	15.62	31.25	31.25	31.25
III.13	31.25	31.25	31.25	31.25	62.5
DMSO	250	250	250	250	250
VAN	7.81	3.90	3.90	1.95	7.81
AMP	< 0.49	>250	>250	125	<0.49
MET	15.62	>250	1.95	>250	098
OXY	62.50	31.25	31.25	<0.49	0.98
TET	62.50	31.25	125	<0.49	<0.49

[a] Minimal inhibitory concentrations given in µg/mL; Control antibiotics: AMP (ampicillin), MET (methicillin), OXY (oxytetracycline), TET (tetracycline), Van (vancomycin); [c]FFHB species are clinical isolates from *Hospital do Barreiro*, deposited on the Microbiology Laboratory, Pharmacy Faculty of Lisbon University.

IV.2.8. Antimicrobial activity of halimane derivatives III.15-III.23

The synthesis of derivatives (**III.15-III.23**; see Chapter **III**) of 11*R**-acetoxyhalima-5,13*E*-dien-15-oic acid **II.7** (see Chapter **V**) was carried out to screen the antimicrobial activity and determine their MIC values against a collection of Gram-negative and positive bacteria and *C. albicans* (see **Table IV.16**).

Further antibacterial studies on **II.7**, **III.15-III.17** and **III.19-III.23** were carried out against a collection of both antibiotic-sensible and antibiotic-resistant strains of *Staphylococcus* and *Enterococcus* (see **Tables IV.17** and **IV.18**) aiming to understand some plausible structure activity relationships and search for a valuable antibacterial halimane-prototype. Diterpene **II.7** revealed to be active against *Mycobacterium smegmatis*, *E. faecalis* and *S. aureus* with MIC values of 62.50, 15.62 and 31.25 µg/mL, respectively (see **Tables IV.16-IV.18**). From the data shown in **Tables IV.17** and **IV.18** it may be observed that compounds **II.7, III.15-III.17** showed the highest inhibitory growth activity, particularly against *Enterococci* than against *Staphylococci* spp (MIC values of 15.62 and 125.00 µg/mL). The same observations occurred with the acetone extract and the fraction containing **II.7** (PO-A3, see Chapter **V**).

Table IV.16. MIC[a] values against a collection of Gram-positive and Gram-negative bacteria and a yeast for halimanes **II.7** and hemisynthesised **III.15-III.17** and **III.19-III.23**.

Compound	*C. albicans* ATCC 10231	*M. smegmatis* ATCC 607	*E. coli* ATCC 25922	*P.aeruginosa* ATCC 27853
II.7	>125	62,5	>125	>125
III.15	>125	>250	>125	>125
III.16	>125	ND	>125	>125
III.17	62,5	>250	>125	>125
III.18	ND	ND	ND	ND
III.19	>125	>250	>125	>125
III.20	ND	ND	ND	ND
III.21	>125	>250	>125	>125
III.22	ND	ND	ND	ND
III.23	>125	>250	>125	>125
DMSO	125	250	125	125
Positive control	KET 31,25	RIF <0.48	NOR <0,48	NOR 0.97

[a] Minimal inhibitory concentrations given in µg/mL; Control antibiotics: KET (Ketoconazole), NOR (Norfloxacin), OXY (oxytetracycline), RIF (Rifampicine), VAN (vancomycin), ND = Not Determined.

The metabolite **II.7** revealed interesting MIC values, ranging from 15.62 to 125.50 µg/mL, against all five *Enterococcus* strains and against five of the *S. aureus* strains. It may be noticed that **II.7** is 4-fold more active (MIC of 15.62 µg/mL) than vancomycin (MIC 62.50 µg/mL) against *E. faecalis* ATCC 51299. Furthermore, **II.7** showed activity against the two MRSA strains (*S. aureus* CIP 106760 and FFHB 29593) exhibiting MIC values of 15.62 and 31.25 µg/mL (**Tables IV.17** and **IV.18**).

Generally, the halimenediol **III.17** shows higher inhibitory ability than the acidic metabolite **II.7** against *Staphylococcus* and *Enterococcus* spp., with MIC values of 15.62 or 31.25 µg/mL (**Tables IV.17** and **IV.18**). This suggests that **III.17** (5,13-diene-11,15-diol halimane) may be a valuable anti-Gram-positive prototype for further activity modulation.

Compound **III.16** (11-acetoxy-5,13-dien-15-ol derivative) exhibits lower MIC values (31.25 – 62.50 µg/mL) only against four strains, and the halimane diester **III.15** (methyl 11-acetoxy-5,13-dien-15-oate) revealed even lower MIC values (62.50 µg/mL) and only against two *Enterococcus* strain (**Tables IV.17** and **IV.18**).

Finally, results showed that the low-level VRE (*E. faecalis* ATCC 51299) strain is more susceptible to the halimanes **II.7**, **III.16** and **III.17** (MIC of 15.62 – 31.25 µg/mL) than to the vancomycin antibiotic (MIC of 62.50 µg/mL). Likewise, growth of both MRSA strains (*S. aureus* CIP 106760 and FFHB 29593; methicillin MIC >250 µg/mL) is inhibited, mostly, by **II.7** and **III.17** halimanes also with MIC values of 15.62 to 31.25 µg/mL.

Compound **III.17** was found to be the more potent derivative in inhibiting both *Staphylococcus* and *Enterococcus* at a micromolar concentration, with MIC values from 51.20 to 102.12 µM (15.62 to 31.25 µg/mL; **Tables IV.17** and **IV.18**).

These outcomes further support the need of assaying other antibiotic-resistant Gram-positive bacteria, and search for other halimane **II.7** derivatives with better antibacterial profiles.

Table IV.17. MIC[a] values against five Gram-positive *Staphylococcus* spp. using the microdilution method for halimanes **II.7** and hemisynthesised **III.15-III.17** and **III.19-III23**.

Compound[b]	*S. aureus* ATCC 700699	*S. aureus* CIP 106760[c]	*S. aureus* ATCC 43866	*S. aureus* FFHB 29593[c]	*S. aureus* ATCC 25923
II.7	125.00	15.62	62.50	31.25	31.25
II.7[d]	345.30	43.15	172.65	86.33	86.33
III.15	>250.00	>250.00	>250.00	125.00	125.00
III.16	>250.00	62.50	125.00	125.00	125.00
III.17	31.25	15.62	15.62	31.25	31.25
III.17[d]	102.12	51.05	51.05	102.12	102.12
III.18	ND	ND	ND	ND	ND
III.19	>250.00	>250.00	>250.00	>250.00	125.00
III.20	125.00	>250.00	>250.00	>250.00	125.00
III.21	>250.00	>250.00	>250.00	>250.00	125.00
III.22	>250.00	>250.00	>250.00	>250.00	125.00
III.23	>250.00	>250.00	>250.00	>250.00	125.00
PO-A	ND	ND	125.00	125.00	125.00
PO-A$_3$	ND	ND	62.50	62.50	125.00
VAN	7.81	3.90	3.90	1.95	7.81
VAN[d]	5.39	2.69	2.69	1.35	1.35
MET(8-16)	15.62	>250	1.95	>250	0.97
DMSO	250.00	250.00	250.00	250.00	250.00

ND = Not Determined; [a]Minimal inhibitory concentrations given in μg/mL; [b]Estimated log P: **II.7**=5.20(±0.48); **III.15**=5.58(±0.87); **III.16**=5.22(±0.81); **III.17**=4.59(±0.72); **III.18**=5.18(±0.77); **III.19**=5.62(±0.84); **III.20**=6.68(±1.07); **III.21**=6.05(±0.87); **III.22**=6.69(±-0.81); **III.23**=6.66(±0.93) (VCCLAB, **2005**); [c]MRSA; [d]Minimal inhibitory concentrations given in μM; PO-A = *P. ornatus* acetone extract; PO-A$_3$ = fraction containing **II.7** (see Chapter **V**); VAN = Vancomycin; MET = Meticillin.

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Table IV.18. MIC[a] values against a five Gram-positive *Enterococcus* spp. using the microdilution method for halimanes **II.7** and hemisynthesised **III.15-III.17** and **III.19-III23**.

Compound[b]	*E. faecalis* ATCC 51299[c]	*E. faecalis* CIP 104476	*E. faecalis* FFHB 427483	*E. flavescens* ATCC 49996	*E. faecium* FFHB 435628
II.7	15.62	62.50	31.25	15.62	31.25
II.7[d]	43.15	172.65	86.33	43.15	86.33
III.15	62.50	>125.00	>125.00	>125.00	62.50
III.16	31.25	ND	>125.00	31.25	62.50
III.17	31.25	15.62	15.62	15.62	15.62
III.17[d]	102.12	51.05	51.05	51.05	51.05
III.18	ND	ND	ND	ND	ND
III.19	62.50	ND	>125.00	>125.00	>125.00
III.20	>125.00	ND	>125.00	>125.00	>125.00
III.21	>125.00	ND	>125.00	>125.00	>125.00
III.22	>125.00	ND	>125.00	>125.00	>125.00
III.23	>125.00	ND	>125.00	>125.00	>125.00
PO-A	31.25	ND	>125.00	31.25	62.50
PO-A$_3$	15.62	ND	62.50	15.62	31.25
VAN	62.50	31.25	1.95	3.90	0.97
VAN[d]	43.12	21.56	1.35	2.69	0.67
DMSO	125.00	125.00	125.00	125.00	125.00

ND = Not Determined; [a]Minimal inhibitory concentrations given in μg/mL; [b]Estimated log P: **II.7**=5.20(±0.48); **III.15**=5.58(±0.87); **III.16**=5.22(±0.81); **III.17**=4.59(±0.72); **III.18**=5.18(±0.77); **III.19**=5.62(±0.84); **III.20**=6.68(±1.07); **III.21**=6.05(±0.87); **III.22**=6.69(±-0.81); **III.23**=6.66(±0.93) (VCCLAB, **2005**); [c]Low-level VRE; [d]Minimal inhibitory concentrations given in μM; VAN (Vancomycin); PO-A = *P. ornatus* acetone extract; PO-A$_3$ = fraction containing **II.7** (see Chapter **V**); VAN = Vancomycin.

IV.2.9. Antimicrobial activity of parvifloron D II.19 derivative: benzoyloxy derivative of microstegiol III.24

Compound **III.24** was tested against Gram-negative and Gram-positive bacteria, and *Candida albicans*. The results obtained revealed moderate activity against *C. albicans* and *M. smegmatis* (62.50 μg/mL; **Tables IV.19**). The Gram-positive *Staphylococcus* strains showed very interesting MIC values against four of the strains tested including a MRSA strain (methicillin resistant *S. aureus*; 3.91-7.81 μg/mL) for **III.24**. The *Enterococcus* strains evaluated together with *E. faecalis* ATCC 51299 (low-

MRSA) also showed antibacterial activity with MIC values from 7.81 to 15.62 µg/mL for derivative **III.24** (**Tables IV.20** and **IV.21**).

Table IV.19. MIC[a] values against a collection of Gram-positive and Gram-negative bacteria and a yeast for benzoyloxy derivative of microstegiol **III.24**.

Compound	*C. albicans* ATCC 10231	*M. smegmatis* ATCC 607	*E. coli* ATCC 25922	*P.aeruginosa* ATCC 27853
III.24	62.50	62.50	>125.00	>125.00
Positive Control	KET <0.49	RIF <0.49	AMP <0.49	AMP 31.25
DMSO	125.00	250.00	125.00	125.00

[a]Minimal inhibitory concentrations given in µg/mL; KET = Ketoconazole, RIF = Rifampicin, AMP = Ampicillin.

Derivative **III.24** revealed to be a strong antimicrobial diterpenoid (MIC values from 3.91 to 125.00 µg/mL). In literature, the microstegiol **III.25** was reported (Ulubelen A. et al., 2000) as a weak microbial growth inhibitory compound so these kind of diterpenoids are interesting molecules for further antimicriobial studies.

Table IV.20. MIC[a] values against a seven Gram-positive *Staphylococcus* strains using the microdilution method for benzoyloxy derivative of microstegiol **III.24**.

Compound	*S. aureus* ATCC 700699	*S. aureus*[b] CIP 106760	*S. aureus* ATCC 43866	*S. aureus*[b] FFHB 29593	*S. aureus* ATCC 6538	S. aureus ATCC 25923	*S. epidermis* ATCC 12228
III.24	31.25	3.91	3.91	31.25	3.91	62.50	7.81
Rifampicin	<0.49	500	<0.49	<0.49	<0.49	0.98	<0.49
DMSO	250	250	250	250	250	250	250

[a]Minimal inhibitory concentrations given in µg/mL; [b]MRSA.

Table IV.21. MIC[a] values against a three Gram-positive *Enterococcus* strains using the microdilution method for benzoyloxy derivative of microstegiol **III.24**.

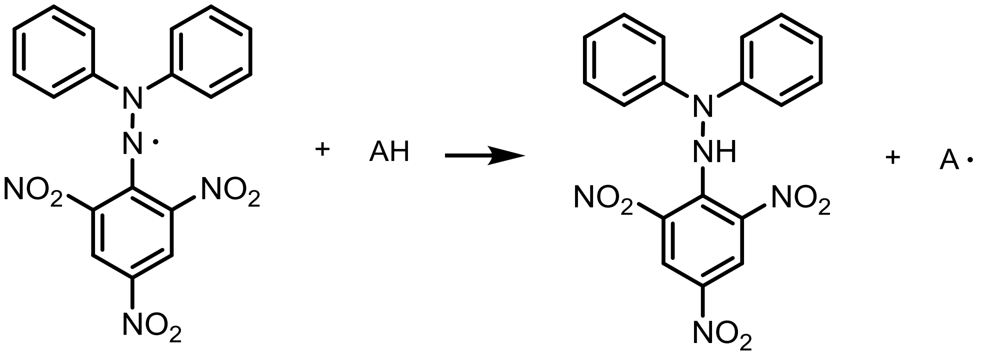
Compound	*E. faecalis* ATCC 51299[b]	*E. faecalis* H164	*E. hirae* ATCC 10541
III.24	7.81	15.62	7.81
Rifampicin	<0.49	<0.49	<0.49
DMSO	125	125	125

[a]Minimal inhibitory concentrations given in µg/mL; [b]VRE.

IV.3. Other biological activities

IV.3.1. Antioxidant activity - DPPH free radical-scavenging assay

There is an increasing interest in antioxidants, noting a preference for antioxidants from natural origin rather than from synthetic sources. A method that is currently popular is based upon the use of the stable free radical diphenylpicrylhydrazyl (DPPH; Molyneux P., 2003). The spectrophotometric method for assessing the total antioxidant activity is based on the absorbance decrease monitoring of the DPPH· radical (2,2-diphenyl-1-picrylhydrazyl) in the presence of antioxidants. DPPH· is characterized as a stable free radical due to the delocalization of the spare electron over the molecule. Thus, the molecule cannot dimerise, as would happen with other free radicals. The delocalization gives rise to a deep violet colour characterized by an absorption band at about 520 nm. When a DPPH· solution is mixed with a substance which can donate a hydrogen atom, the reduced form is generated accompanied by the loss of the violet colour (see **Scheme IV.1**). To obtain a preliminary evaluation of the antioxidant capacity of organic compounds, a DPPH bleaching assay can be used as a rapid tlc screening method (Pisoschi A.M. et al., 2009).



Scheme IV.1. Reaction of DPPH· free radical with an antioxidant (Pisoschi A.M. et al, 2009).

IV.3.2. TLC-DPPH bleaching assay

All the isolated compounds in Chapter **II** and 7α-acetoxy-6β-hydroxyroyleanone **III.1** were submitted to TLC-DPPH bleaching assay. Compounds with capacity to reduce DPPH appeared as yellow spots against a purple background. Only parvifloron D **II.19** and the royleanone **III.1** appeared as yellow spots, the remaining spots maintained the purple background. Considering the results of this preliminary study, the interaction of active compounds with DPPH was quantitatively measured by a UV-visible spectroscopy method (Bernardi A.P.M. et al, 2007; Pisoschi A.M. et al., 2009; Sofidiya M.O. et al, 2009).

IV.3.3. Measurement of DPPH Radical Scavenging Activity

The diterpenoids, parvifloron D **II.19** and 7α-acetoxy-6β-hydroxyroyleanone **III.1** were investigated for quantitative antioxidant activity through their ability to quench the synthetic DPPH radical. The percentage of DPPH radical reduction by abietanes **II.19** and **III.1** was evaluated at different concentrations in a spectrophotometric assay (Narukawa Y., 2001; Molyneux P., 2003; Sofidiya M.O. et al, 2009). Experiments were performed in triplicate and results were expressed as means ± standard deviation. The results obtained for the reference compounds, BHT and quercetin, are in good agreement with the ones previously published in literature (Gaspar-Marques C. et al., 2008; Sofidiya M.O. et al, 2009).

Both diterpenoids **II.19** and **III.1** showed a dose dependent scavenging activity as expected (Chang S.-Y. et al., 2002). Parvifloron D **II.19** showed antioxidant properties (IC_{50} 0.1125 ± 0.0177 mM) equivalent to BHT but lower than quercetin. The IC_{50} of royleanone **III.1** (1.8500 ± 0.0707 mM) revealed antioxidant ability inferior to butylated hydroxytoluene (BHT; see **Table IV.22**).

Table IV.22. The scavenging activity expressed in terms of IC_{50} for parvifloron D **II.19** 7α-acetoxy-6β-hydroxyroyleanone **III.1** and standard positive controls[a].

Compounds	IC_{50} (mM)
BHT[a]	0.1050 ± 0.0071
Quercetin[a]	0.0075 ± 0.0007
Parvifloron D **II.19**	0.1125 ± 0.0177
7α-Acetoxy-6β-hydroxyroyleanone **III.1**	1.8500 ± 0.0707

The results of DPPH radical scavenging assays suggest an interesting efficacy of the screened compounds (when compared with the standards) concerning the inhibition of reactive oxygen species, tested compounds react with free radicals, converting them to more stable products and terminating the radical chain reaction), which may be associated with the medicinal use of the *Plectranthus* plants.

IV.4. Anticholinesterase activity

The literature indicates that the terpenoids (Savelev S. et al., 2003; Mukherjee P. K. et al., 2007; Kamatou G.P.P. et al., 2008) and, particularly, some diterpenoids (Ren Y. et al., 2004; Ahmad V.U. et al., 2005; Ertas A. et al., 2009) may have anticholinesterase activity. Thus, the isolated compounds in Chapter **II** and **III.1** were evaluated for their anticholinesterase activity.

An extremely rapid method to screen a large numbers of compounds to uncovering new inhibitors of acetylcholinesterase (AChE) was performed. A qualitative method on a tlc plate for AchE activity measurement was described by Kiely *et al.*, which is based on the reliable and most widely used method according to Ellman *et al*. (Ellman G.L et al., 1961). Solutions of the compounds tested were spotted on silica gel tlc plates in a matrix pattern. The silica gel plate was sprayed with a solution of acetylthiocholine iodide and 5,5-dithiobis(2-nitrobenzoic acid) -DTNB- followed by a solution of acetylcholinesterase. The enzyme reaction produced a yellow background color with inhibitor compounds exposed as white zones where color has failed to develop. The enzyme hydrolyses the substrate acetylthiocholine and the product, thiocholine, reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoic-5-mercaptothiocholine (thiocholine-thionitrobenzoate disulphide) and 5-thio-2-nitrobenzoic acid (thionitrobenzoate) which may be detected at 405 nm (see **Figure IV.3**; Kiely J. S. et al., 1991; Ree I.K. et al., 2001 and 2003).

$$\text{Acetylthiocholine} + H_2O \xrightarrow{\text{AChE}} \text{acetate} + \text{thiocholine} + 2H^+$$

$$\text{Thiocholine} + \text{DTNB} \longrightarrow \underset{\text{(yellow color)}}{\text{5-thio-2-nitrobenzoate}} + \underset{\text{mercaptothiocholine}}{\text{2-nitrobenzoate-5-}}$$

Figure IV.3. The detection of acetylcholinesterase activity by Ellman´s method.

IV.4.1. Determination of AChE inhibitory activity - tlc assay

Enzyme inhibitory activities of the samples, on silica gel layer, were detected by spraying the substrate, dye and enzyme according to Ellman's method (**Figure IV.3**). A yellow background appeared with white spots caused by the inhibiting compounds (Rhee I. K. et al., 2003).

All the compounds isolated in Chapter **II** and 7α-acetoxy-6β,12-dihydroxy-8,12-abietadiene-11,14-dione **III.1** were tested and the tlc plate only appeared with white spots on a yellow background for the positive controls (physostigmine and tacrine hydrochloride). If some spot had revealed the presence of inhibiting compounds, their anticholinesterase activities could be evaluated by the quantitative spectrophotometric method using the same Ellman´s method.

Another method was used and the same negative results were obtained for all compounds tested. The method for the screening of acetylcholinesterase inhibition by the compounds consisted in a bioautographic enzyme assay on tlc plates. The enzyme activity is detected by the conversion of naphthyl acetate into naphthol and the formation of the corresponding purple-coloured diazonium dye with Fast Blue B salt. Inhibitors of cholinesterases should produce white spots on the dye-coloured background of the tlc plates and all the compounds revealed purple colour (Marston A. et al., 2002).

IV.5. Anti-inflammatory activity

There is a need for the design of COX inhibitors based on new structural templates (Chen Q.-H. et al., 2005) since the inhibition of the enzymes COX-1 and COX-2 are considered one of the mechanisms of anti-inflammatory actions. In this way, the isolated compounds in Chapter **II** and 7α-Acetoxy-6β-hydroxyroyleanone **III.1** were evaluated for their anti-inflammatory activity. The colorimetric COX (ovine) Inhibitor Screening Assay Kit was used in order to screen isozyme-specific inhibitors. Cayman's Colorimetric COX (ovine) Inhibitor Screening Assay measures the peroxidase component of COXs. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm (Cayman Chemical Company, 2010).

Cyclooxygenase (COX, also called Prostaglandin H Synthase or PGHS) is a bifunctional enzyme exhibiting both COX and peroxidase activities. The COX component converts arachidonic acid to a hydroperoxy endoperoxide (Prostaglandin G_2; PGG_2), and the peroxidase component reduces the endoperoxide to the corresponding alcohol (Prostaglandin H_2; PGH_2), the precursor of Prostaglandins (PGs), thromboxanes, and prostacyclins. It is now well established that there are two distinct isoforms of cyclooxygenase (COX). Cyclooxygenase-1 (COX-1) is constitutively expressed in a variety of cell types and is involved in normal cellular homeostasis. A variety of mitogenic stimuli such as phorbol esters, lipopolysaccharides, and cytokines lead to the induced expression of a second isoform of COX, cyclooxygenase-2 (COX-2). COX-2 is responsible for the biosynthesis of PGs under acute inflammatory conditions. This inducible COX-2 is believed to be the target enzyme for the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs (Nugteren D.H. and Hazelhof E., 1973; Hamberg M. and Samuelsson B., 1973; Xie W. et al., 1991; Kulmacz R.J. and Lands W.E.M., 1983; Jang M., et al., 1997).

IV.5.1. In Vitro Cyclooxygenase (COX) Inhibitory Assay

Cyclooxygenase inhibitor screening assay kits (Cayman Chemicals-Catalog No. 760114) were used in order to investigate the anti-inflammatory activity of all the isolated compounds in Chapter **II** against the inflammation pathway enzymes cyclooxygenase 2 (COX-2). The COX inhibitory activity of compounds **II.1**, **II.2**, **II.3**, **II.6**, **II.7** and **II.17**, isolated on Chapter **II**, and compound **II.7** derivatives **III.15** and **III.17**, prepared on Chapter **III,** was measured using ovine COX-2 enzyme by the COX inhibitor screening assay. Naproxen and SC-560 (Cayman chemicals, catalog nº 70340, IC_{50} = 6.3 µM) were used as controls in the COX assay. Any antioxidant could interfere with the assay and could appear to be a COX inhibitor. Thus, all the compounds tested were previously proved to be non-antioxidant as shown earlier (IV.2 Antioxidant activity). As the appropriate concentration of each eventual inhibitor was completely unknown, several dilutions of the inhibitor were tested, with compounds being examined around a final concentration of 1000 µM. The percent COX-2 activity values (Percent Initial Activity) were determinated as described in Chapter **V** and are shown on **Table IV.23**.

Table IV.23. Ovine COX-2 inhibition by SC-560[a], Naproxen[a], Plectrornatin C **II.17**, forskolin-like diterpenoids **II.1**, **II.2:II.3** (1:1), halimane **II.7** and its derivatives (**II.15** and **III.17)** and rinocerotinoic acid **II.6**.

Compounds	Percent activity (% ± SD)	CV (%)	Concentration / µM
SC-560[a]	60.30 ± 1.36	2.24	6.3
naproxen[a]	67.36 ± 5.51	8.04	1087
6-*O*-acetylforskolin **II.1**	89.93 ± 9.47	11.2	553
1,6-di-*O*-acetylforskolin **II.2 :** 1,6-di-*O*-acetyl-9-deoxyforskolin **II.3** (1:1)	76.05 ± 9.33	10.8	506
rinocerotinoic acid **II.6**	54.56 ± 10.13	15.3	785
halimane **II.7**	79.20 ± 6.93	7.96	1379
plectrornatin C **II.17**	59.42 ± 14.97	18.0	595
halimane ester **III.15**	76.20 ± 2.78	3.53	664
halimane dialcohol **III.17**	59.84 ± 12.35	16.9	1631

[a]Positive control.

In the COX inhibitor screening assay for COX-2, two positive controls were used: SC-560 (COX-1 IC50 = 0.009 µM; COX-2 IC50 = 6.3 µM; Smith C.J. et al., 1998), a highly selective COX-1 inhibitor that showed 60.30% percent of COX-2 activity, and naproxen (1087 μM) as a nonselective inhibitor that exhibited 67.36% for COX-2 activity (Hegazy M.-E.F. et al., 2008).

The compounds under evaluation seem to show weak to moderate COX-2 activity from 6-*O*-acetylforskolin **II.1** at 553 µM with 89.93%, to rinocerotinoic acid **II.6** at 785 µM with 54.56%, respectively. Because antioxidants could not be screened with this assay some derivatives were experienced. The derivatives halimane ester **III.15** (76.20 % at 664 μM) and halimane dialcohol **III.17** (59.84 % at 1631 μM) appear to inhibit COX-2 strongly than the starting material halimane **II.7** (79.20%) at 1379 μM. Plectrornatin C **II.17** showed 59.42 % of COX-2 activity at 595 μM.

Though randomly performed, without a range of test concentrations and the possibility of calculate the IC_{50} values for comparison purposes, these preliminary experiences led to results that encourages the pursue of a further and more systematic investigation.

CHAPTER V

Experimental Section

V.1. General experimental procedures

Melting-points were determined on a Kofler block and are uncorrected. Optical rotations were measured on a Perkin-Elmer 241 MC polarimeter (in $CHCl_3$ solution).

UV spectra were recorded on a Perkin-Elmer Lambda 2 UV/vis spectrophotometer using quartz or plastic cuvettes with an internal width of 1 cm (in MeOH solution).

Infrared spectra were obtained on a Perkin-Elmer Spectrum One spectrophotometer (in KBr or neat).

Mass spectra: positive EI mode, 70 eV, CH_2Cl_2 (Hewlett-Packard 5973 spectrometer), or electro spray ionization in the positive (ESI^+) mode on a Hewlett-Packard 1100 spectrometer. For derivative **III.24**: HRESIMS: Agilent 6520 Accurate-Mass QTOF LC/MS apparatus. Elemental analysis: LECO CHNS-932 apparatus.

Elemental analyses were performed with a Carlo Erba EA 1108 apparatus for compounds **II.1-II.3** and with a LECO CHNS-932 apparatus for compounds **II.6** and **II.7**.

NMR spectra were recorded on a Varian INOVA-400 Spectrometer equipped with a 5 mm inverse detection *z*-gradient probe. The ^1H and ^{13}C NMR spectra (at 400 and 100 MHz, respectively) were measured at room temperature (22–23 °C) using $CDCl_3$ as solvent, except for **II.1** (pyridine-*d*5). ^1H and ^{13}C NMR chemical shifts are reported with respect either to the residual $CHCl_3$ signal (δ 7.25) or to the solvent signals (δ_{CDCl3} 77.00), respectively. NMR spectra were also recorded at room temperature on a Varian SYSTEM 500 MHz spectrometer in the case of compounds **II.20, II.22 and III.24.** The ^1H and ^{13}C NMR spectra (at 500 MHz and 125 MHz, respectively) were recorded in solutions of $CDCl_3$ (**II.20** and **III.24)**, acetone-d_6 (**II.22**) and CD_3OD (**II.22**).

One-dimensional ^1H and ^{13}C-NMR spectra were acquired under standard conditions. The pulse programs of the COSY, gHSQC and gHMBC experiments were taken from the Varian software library. Homonuclear two-dimensional spectra (COSY) and inverse proton-detected heteronuclear two-dimensional spectra (gHSQC) were acquired in the phase-sensitive mode and gHMBC spectra were acquired in the absolute value mode.

Column chromatography was carried out on silica-gel Merck 9385 [40-63 µm (230-400 mesh)] and Merck 7734 [63-200 µm (70–230 mesh)].

Hydrated silica gel was prepared using silica-gel (Merck 7734, 230-400 mesh) with 10% (w/v) of water.

Analytical and preparative thin layer chromatography (tlc and ptlc) were performed on precoated silica gel F_{254} plates [Merck 5554 (0.2 mm) and 5744 (0.5 mm), respectively] or on prepared plates with silica gel 60 F_{254} Merck 7747 (5-50 µm). Visualisation was performed under the visible light and UV light (λ 254 and 366 nm) followed by spraying with a mixture of H_2SO_4:AcOH:H_2O (4:80:16) and heating. Light petroleum (b.p. 50-70 ˚C) was used for column chromatography.

The reagents used in the determination of AChE inhibitory activity (V.9.2.1) were commercial and used without further purification. The acetylcholinesterase used was electric eel (type V-s; lyophilized powder; 1430 U/mg solid; 2380 U/mg protein).

The anti-inflammatory activity (V.9.3.1) was performed using the *in vitro* Cyclooxygenase (COX) Inhibitory Assay – 760111 Colorimetric COX (ovine) Inhibitor Screening Assay Kit (Cayman Chemicals).

V.2. Study of *Plectranthus ornatus* Codd.

Plectranthus ornatus Codd. was cultivated in the Faculty of Pharmacy *Hortum*, Lisbon University, from seeds provided by the Herbarium of the Botanical Garden of Lisbon, Portugal. *P. ornatus* whole plants were collected in 1997 and 1998 and voucher specimens were deposited in the Herbarium of the “Instituto de Investigação Científica Tropical”, Lisbon (ref. C. Marques S/N° LISC).

V.2.1. Plant material: study of *Plectranthus ornatus* whole plants

Extraction and isolation

The air dried and powdered whole plants of *P. ornatus* Codd. (3392.32 g) were extracted with Me_2CO (6 x 34 L) at room temperature for 9 days. Filtration and evaporation of the solvent (under *vacuum*, 40 ˚C) yielded a residue of 183.91 g (5.42%

of dry material), which was subjected to column chromatography over silica gel (Merck 9385, 1088 g), using mixtures of *n*-hexane:EtOAc (1:0 to 0:1) and EtOAc:MeOH (1:0 to 0:1) as eluents collecting 10 fractions. The precipitate of the first fraction (24.95 g), consisting mainly of waxes, was eliminated by filtration. Fractions PO-A5 and PO-A6 were studied in a previous work (Rijo P., 2003).

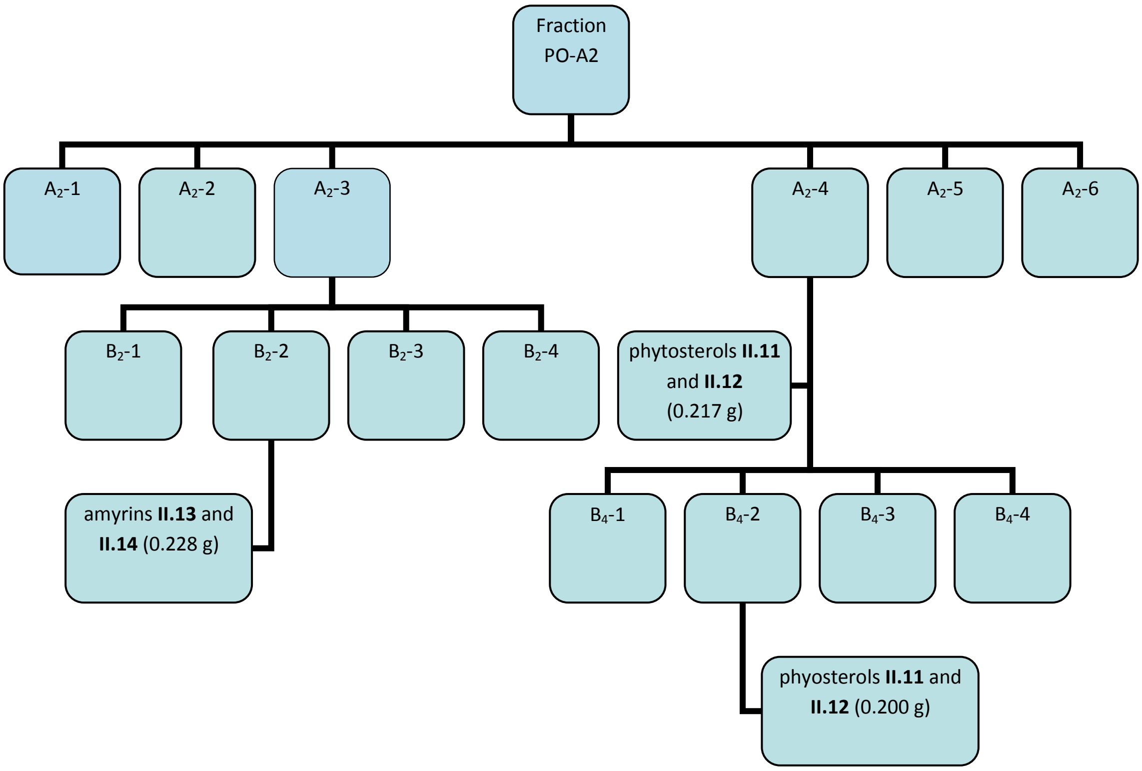
According to differences in composition, as indicated by tlc, ten crude fractions were obtained (PO-A1 to PO-A10, **Table V.1**).

Table V.1. Column chromatography A of the acetone extract (*P. ornatus*).

Fraction	Quantity (g)	Eluent (%) *n*-hexane : EtOAc	Eluent (%) EtOAc: MeOH
PO-A1	29.02	90:10 to 75:25	---
PO-A2	5.37	75:25	---
PO-A3	17.00	50:50	---
PO-A4	20.57	50:50	---
PO-A5	9.13	25:75	---
PO-A6	3.28	25:75	---
PO-A7	8.19	25:75	---
PO-A8	9.19	0:100	---
PO-A9	14.48	0:100	---
PO-A10	41.17	---	75:25 to 0:100

V.2.1.1. Study of fraction PO-A2 (Column chromatography A_2)

The fraction PO-A2 was fractionated by column chromatography A_2. Fraction A_2-3 was studied by subsequent column chromatography B_2 originating mixtures of amyrins **II.13** and **II.14** (0.228 g), and fraction A_2-4 was studied by column chromatography B_4, giving phytosterols **II.11** and **II.12** (0.417 g), as shown in the **Scheme V.1.**



Scheme V.1. Study of *P. ornatus:* isolated compounds from fraction PO-A2.

The fraction PO-A2 eluted with *n*-hexane:EtOAc (75:25) was fractionated by column chromatography A_2 (hydrated silica gel, 250 g) eluted with mixtures of *n*-hexane:EtOAc and EtOAc:MeOH. After tlc monitoring, the chromatographic fractions, were combined into six fractions (A_2-1 to A_2-6, **Table V.2**).

Table V.2. Column chromatography A_2 of fraction PO-A2 (*P. ornatus*).

Fraction	**Quantity / g**	**Eluents (%) *n*-hexane: EtOAc**	**Eluents (%) EtOAc : MeOH**
A_2-1	0.0304	1:0	---
A_2-2	0.0695	1:0 to 95:5	---
A_2-3	0.4562	95:5	---
A_2-4	2.2883	95:5 to 90:10	---
A_2-5	1.0498	90:10	---
A_2-6	1.6466	90:10	to 0:1

The fraction A_2-4 eluted with mixtures of *n*-hexane:EtOAc (95:5 to 90:10) was recrystallized from *n*-hexane:AcOEt to give 0.217 g of a mixture of β-sitosterol (stigmast-5-en-3β-ol) **II.11** and stigmasta-5,22*E*-dien-3β-ol **II.12** in the proportion (1:2). The mother liquors of the fraction A_2-4 were submitted to column chromatography B_4 (silica gel, 150 g) using mixtures of CH_2Cl_2:EtOAc (0:1 to 97:3) as eluents; after tlc monitoring, chromatographic fractions were combined into four fractions (B_4-1 to B_4-4, **Table V.3**).

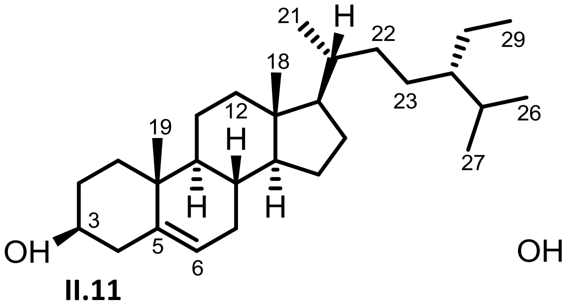
Table V.3. Column chromatography B_4 of mother liquors of the fraction A_2-4 (*P. ornatus*).

Fraction	Quantity / g	Eluents / % CH_2Cl_2: EtOAc
B_4-1	0.2350	1:0
B_4-2	0.5980	1:0
B_4-3	0.3522	1:0
B_4-4	0. 6466	0:1 to 97:3

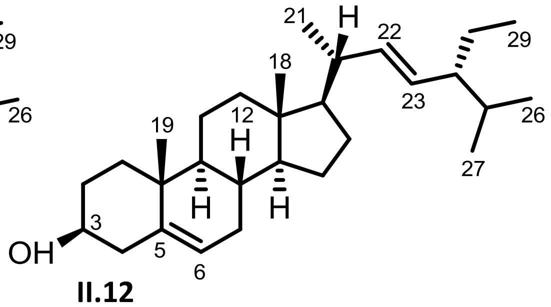
The fraction B_4-2 was recrystallized from *n*-hexane:EtOAc to give 0.200 g of a mixture of β-sitosterol (stigmast-5-en-3β-ol) **II.11** and stigmasta-5,22*E*-dien-3β-ol **II.12** in the proportion (1:2).

The residue of the fractions eluted (A_2-3) with *n*-hexane:EtOAc (95:5) was submitted to column chromatography B_2 (silica gel, 50 g) using mixtures of CH_2Cl_2:*n*-hexane (7:3 to 100:0) as eluents; after tlc monitoring, chromatographic fractions were combined into four fractions. The fractions eluted (B_2-2) with a mixture of CH_2Cl_2: *n*-hexane (7:3) were also recrystallized from the same solvent; through this procedure 0.228 g of a mixture of α-amyrin **II.13** and β-amyrin **II.14** (1:2) was obtained.

V.2.1.1.1. β-Sitosterol (stigmast-5-en-3β-ol) II.11 and stigmasta-5,22*E*-dien-3β-ol II.12 in a (1:1) ratio



II.11



II.12

White crystals needles (0.217 g; 0.00639 % dry material);

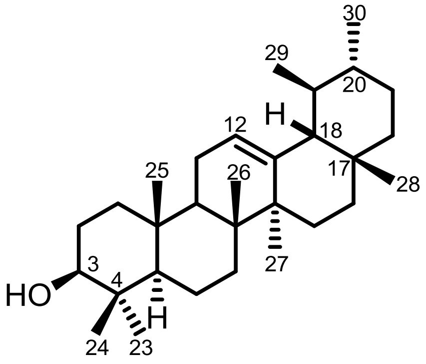
m.p. 139 - 146 ˚C;

R_f (SiO_2; n-hexane:ethyl acetate, 7:3): 0.33;

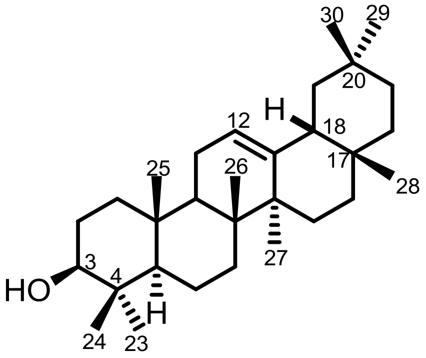
The tlc chromatogram upon revelation by a mixture of H_2O : CH_3COOH : H_2SO_4 (16: 80: 4) and posterior heat with a standard sample (co-aplication) confirmed the presence of β-sitosterol **II.11** and stigma-5,22*E*-dien-3β-ol **II.12** giving a characteristic pink colour;

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.35 (1H, m, H-6 of both compounds), 3.52 (1H, m, H-3α of both compounds), 1.00 (3H, s, Me-19 of both compounds), 1.01 (3H, d, $J_{21,20}$ = 6.4 Hz, Me-21 compound **II.12**), 0.91 (3H, d, $J_{21,20}$ = 6.8 Hz, Me-21 compound **II.11**), 0.82 (2 x 9H, m, Me-26, Me-27, Me-29 for both compounds), 0.69 (3H, s, Me-18 compound **II.12**), 0.67 (3H, s, Me-18 compound **II.11**).

V.2.1.1.2. α-Amyrin II.13 and β-amyrin II.14 in a (3:1) ratio



α-amyrin **II.13**



β-amyrin **II.14**

White crystals needles (228.3 mg; 0.00673 % dry material);

m.p. 175-180 ˚C;

***R*$_f$** (SiO_2, *n*-hexane: ethyl acetate; 1:1): 0.63;

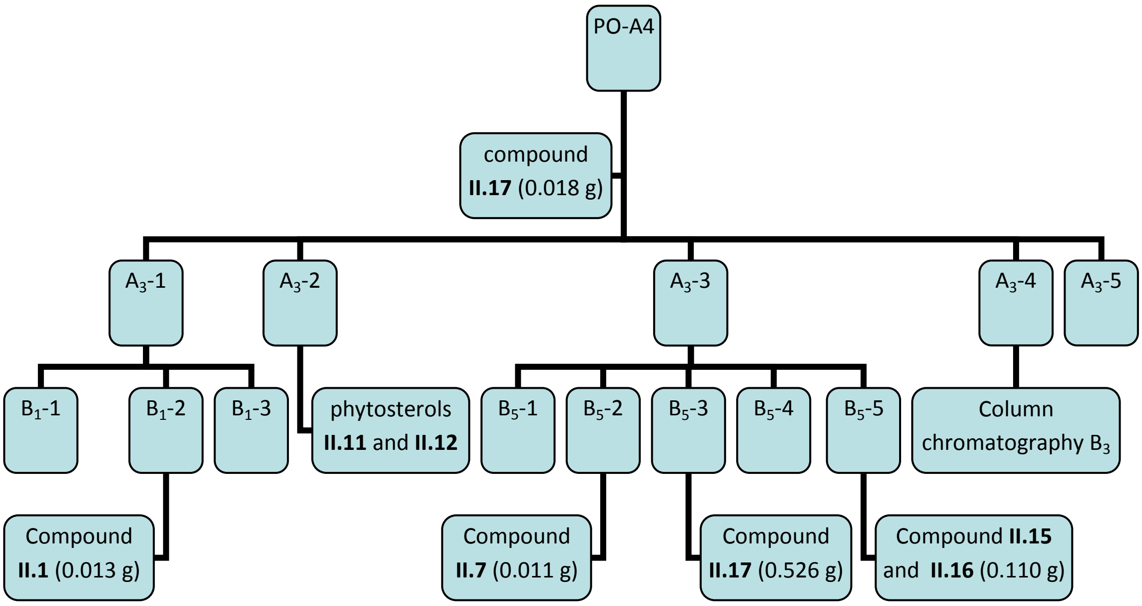
The tlc chromatogram upon revelation by a mixture of H_2O : CH_3COOH : H_2SO_4 (16: 80: 4) and posterior heat with a standard sample (co-aplication) confirmed the presence of α-amyrin **II.13** and β-amyrin **II.14** giving a characteristic purple colour;

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.18 (1H, t, $J_{12,11}$ = 3.8 Hz, H-12 compound **II.14**), 5.12 (1H, t, $J_{12,11}$ = 3.6 Hz, H-12 compound **II.13**), 3.22 (1H, dd, $J_{3\alpha,2\beta}$ = 10.8 Hz, $J_{3\alpha,2\alpha}$ = 5.0 Hz, H-3α for both compounds), 2.00 (1H, dd, $J_{18,19}$ = 13.4 Hz, $J_{18,19\beta}$ = 4.4 Hz, H-18 compound **II.14**), 1.19 (1H, d, $J_{18,19\beta}$ = 3.6 Hz, H-18 compound **II.13**), 1.13-0.78 (16*3H, m, Me-23 to Me-30 of compound **II.14** and **II.13)**;

EIMS *m/z* (rel. Int.): 426 $[M]^+$ (10.0); 411 $[M\text{-}CH_3]^+$ (4.0); 218 (100), 208 (4), 203 (50), 190 (10), 189 (5).

V.2.1.2. Column chromatography A_3 **(Study of fraction PO-A4)**

The fraction PO-A4 was pooled and recrystallized to give 0.018 g of compound **II.17** (plectrornatin C). The remaining residue was fractionated by column chromatography A_3 originating five fractions. Fraction A_3-2 was pooled and recrystallized to give phytosterols **II.11** and **II.12** (0.300 g), and fractions A_3-1 and A_3-3 were studied by subsequent column chromatographies, B_1 and B_5, respectively, giving 0.013 g of 6-*O*-acetylforskolin **II.1** (B_1) and 0.0011g of 11*R**-acetoxyhalima-5,13E-dien-15-oic acid **II.7**, 0.526 g of compound **II.17** and 0.110 g of ursolic and oleanolic acids **II.15** and **II.16** (B_5) as shown in the **Scheme V.2**. Fraction A_3-4 was studied by column chromatography B_3 and will be described later in **Scheme V.3.**



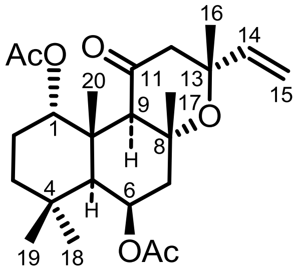
Scheme V.2. Study of *P. ornatus:* isolated compounds from fraction PO-A4.

The fraction PO-A4 eluted with *n*-hexane:EtOAc (50:50) was pooled and recrystallized from the same mixture of solvents to give 0.018 g of compound **II.17** (plectrornatin C). The remaining fraction was submitted to column chromatography A_3 in silica gel (9385; 200 g) to give five fractions (A_3-1 to A_3-5; **Table V.4**).

Table V.4. Column chromatography A_3 of fraction PO-A4 (*P. ornatus*).

Fraction	**Quantity / g**	**Eluents (%)** ***n*-hexane: EtOAc**	**Eluents (%)** **EtOAc : MeOH**
A_3-1	0.1139	1:0 to 85:15	---
A_3-2	5.4876	85:15	---
A_3-3	5.3710	85:15 to 80:20	---
A_3-4	6.9930	80:20 to 75:25	---
A_3-5	5.8438	75:25	to 75:25

V.2.1.2.1. Plectrornatin C (1α,6β-diacetoxy-8α,13R^*-epoxy-14-labden-11-one) II.17



II.17

Colorless needles (EtOAc-*n*-pentane);

m.p. 234-235 °C (EtOAc-*n*-pentane);

$[\alpha]_D^{20}$ − 81.5° (*c* 0,114; $CHCl_3$);

IR (KBr) ν_{max}: 3090, 1642, 948 (vinyl group), 1732, 1238 (OAc), 2953, 2861, 1453, 1394, 1364, 1210, 1144, 1036, 993, 913 cm^{-1};

^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.92 (1H, dd, $J_{14,15B}$ = 17.4 Hz, $J_{14,15A}$ = 10.7 Hz, H-14), 5.57 (1H, ddd, $J_{6\alpha,7\alpha}$ = 3.8 Hz, $J_{6\alpha,7\beta}$ = 2.9 Hz, $J_{6\alpha,5\alpha}$ = 2.6 Hz, H-6α), 5.53 (1H, t, $J_{1\beta,2\beta}$ =$J_{1\beta,2\alpha}$ = 2.8 Hz, H-1β), 5.19 (1H, dd, $J_{15B,14}$ = 17.4 Hz, $J_{15B,15A}$ = 1.1Hz, H_B-15), 5.04 (1H, dd, $J_{15A,14}$ = 10.7 Hz, $J_{15A,15B}$ = 1.1 Hz, H_A-15), 3.23 (1H, s, H-9α), 2.67 (1H, d, $J_{12\beta,12\alpha}$ = 18.6 Hz, H-12β), 2.60 (1H, d, $J_{12\alpha,12\beta}$ = 18.6 Hz, H-12α), 2.26 (1H, dd, $J_{7\alpha,7\beta}$ = 14.6 Hz, $J_{7\beta,6\alpha}$ = 2.9 Hz, H-7β), 2.05 (3H, s, 6β-OAc), 2.00 (1H, dddd, $J_{2\beta,2\alpha}$ = 15.5 Hz, $J_{2\beta,3\alpha}$ = 13.6 Hz, $J_{2\beta,3\beta}$ = 3.6 Hz, $J_{2\beta,1\beta}$ = 2.8 Hz, H-2β), 1.95 (3H, s, 1α-OAc), 1.90 (1H, dd, $J_{7\alpha,7\beta}$ = 14.6 Hz, $J_{7\alpha,6\alpha}$ = 3.8 Hz, H-7α), 1.71 (1H, dddd, $J_{2\alpha,2\beta}$ = 15.5 Hz, $J_{2\alpha,3\alpha}$ = 3.4 Hz, $J_{2\alpha,1\beta}$ = 2.8 Hz,

$J_{2\alpha,3\beta}$ = 2.7 Hz, H-2α), 1.47 (1H, d, $J_{5\alpha,6\alpha}$ = 2.6 Hz, H-5α), 1.46 (3H, s, Me-17), 1.45 (1H, ddd, $J_{3\alpha,3\beta}$ = 13.8 Hz, $J_{3\alpha,2\beta}$ = 13.6 Hz, $J_{3\alpha,2\alpha}$ = 3.4 Hz, H-3α), 1.42 (3H, s, Me-20), 1.26 (3H, s, Me-16), 1.11 (1H, ddd, $J_{3\beta,3\alpha}$ = 13.8 Hz, $J_{3\beta,2\beta}$ = 3.6 Hz, $J_{3\beta,2\alpha}$ = 2.7 Hz, H-3β), 0.99 (3H, s, Me-19), 0.97 (3H, s, Me-18);

^{13}C NMR ($CDCl_3$, 100 MHz): δ 75.1 (C-1, d); 21.7 (C-2, t); 36.9 (C-3, t); 33.7 (C-4, s); 49.1 (C-5, d); 69.5 (C-6, d); 46.2 (C-7, t); 75.7 (C-8, s); 58.2 (C-9, d); 40.5 (C-10, s); 206.2 (C-11, s); 49.1 (C-12, t); 74.6 (C-13, s); 146.7 (C-14, d); 112.4 (C-15, t); 31.7 (C-16, q); 29.5 (C-17, q); 32.9 (C-18, q); 22.9 (C-19, q); 17.4 (C-20, q); 169.5 (1α-O$\underline{C}$OOCH$_3$, s); 21.4 (1α-OCOO$\underline{C}$H$_3$, q); 169.9 (6β-O$\underline{C}$OOCH$_3$, s); 21.8 (6β-OCOO$\underline{C}$H$_3$, q);

EIMS *m/z* (rel. Int.): 420 $[M]^+$ (0.5); 405 (0.3), 377 (1), 360 (2), 300 (2), 285 (5), 247 (8), 215 (11), 190 (8), 173 (11), 163 (7), 147 (7), 119 (10), 109 (11), 107 (11), 95 (13), 91 (11), 81 (13), 69 (14), 67 (11), 55 (20), 43 (100), 41 (13);

Anal. C 68.39%, H 8.58%, calcd for $C_{24}H_{36}O_6$, C 68.54% and H 8.63%.

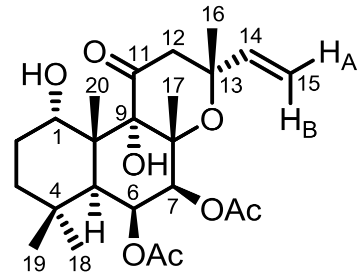
Fraction A_3-2 eluted with *n*-hexane:EtOAc (85:15), was filtered through charcoal and celite (1:1) to eliminate chlorophylls and was pooled and recrystallized from the same mixture of solvents to give phytosterols **II.1** and **II.2** (0.300 g). Fraction A_3-1 was studied by subsequent column chromatography B_1, in silica gel (9385; 30 g) to give three fractions (B_1-1 to B_1-3; **Table V.5**).

Table V.5. Column chromatography B_1 of fraction A_3-1 (*P. ornatus*).

Fraction	Quantity / g	Eluents (%) CH_2Cl_2: EtOAc
B_1-1	0.028	1:0 to 90:10
B_1-2	0.238	90:10
B_1-3	0.039	90:10 to 0:1

Fraction B_1-2 eluted by CH_2Cl_2: EtOAc (90:10) was pooled and recrystallized from the same mixture of solvents giving 0.013 g of compound **II.1**.

V.2.1.2.2. 6-*O*-acetylforskolin II.1



II.1

Colorless prisms from EtOAc;

m.p. 296-298 ˚C (m.p. 299-301 ˚C; Bhat S.V. et al., 1982);

$[\alpha]_D^{20}$ +11.4˚ (MeOH; *c* 0.236);

IR (KBr), υ_{max}: 3399, 3188, 3057, 2968, 1739, 1711, 1396, 1370, 1264, 1238, 1098, 1054, 995, 922 cm^{-1};

^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.63 (1H, s, 9α-OH), 8.49 (1H, d, $J_{5\alpha,6\alpha}$ = 2.8 Hz, 1α-OH), 6.28 (1H, dd, $J_{14,15A}$ = 10.7 Hz, $J_{14,15B}$ = 17.4 Hz, 14-H), 6.20 (1H, dd, $J_{6\alpha,5\alpha}$ = 2.8 Hz, $J_{6\alpha,7\alpha}$ = 4.6, 6α-H), 6.15 (1H, d, $J_{7\alpha,6\alpha}$ = 4.6, 7α-H), 5.35 (1H, dd, $J_{15B,14}$ = 17.4 Hz, $J_{15B,15A}$ = 1.5, 15B-H), 5.01 (1H, ddd, $J_{1\beta,2\alpha}$ = 3.2 Hz, $J_{1\beta,2\beta}$ = 2.4 Hz, 1β-H), 4.91 (1H, dd, $J_{15A,15B}$ = 1.5, $J_{15A,14}$ = 10.7 Hz, 15A-H), 3.48 (1H, d, $J_{12\alpha,12\beta}$ = 16.4 Hz, 12α-H), 2.72 (1H, d, $J_{5\alpha,6\alpha}$ = 2.8 Hz, 5α-H), 2.60 (1H, d, $J_{12\beta,12\alpha}$ = 16.4 Hz, 12β-H), 2.16 (1H, dddd, $J_{2\beta,1\beta}$ = 2.4 Hz, $J_{2\beta,2\alpha}$ = 14.4, $J_{2\beta,3\alpha}$ = 13.1 Hz, $J_{2\beta,3\beta}$ = 3.2 Hz, 2β-H), 2.13 (1H, s, 6β-OAc), 2.00 (1H, s, 7β-OAc), 1.98 (1H, td, $J_{3\alpha,2\alpha}$ = 3.2 Hz, $J_{3\alpha,2\beta}$ = 13.1 Hz, $J_{3\alpha,3\beta}$ = 13.1 Hz, 3α-H), 1.84 (3H, s, Me-17), 1.64 (3H, s, Me-20), 1.54 (1H, dq, $J_{2\alpha,1\beta}$ = 3.2 Hz, $J_{2\alpha,2\beta}$ = 14.4 Hz, $J_{2\alpha,3\alpha}$ = 3.2 Hz, $J_{2\alpha,3\beta}$ = 3.2 Hz, 2α-H), 1.42 (3H, s, Me-16), 1.04 (1H, dt, $J_{3\beta,2\alpha}$ = 3.2 Hz, $J_{3\beta,2\beta}$ = 3.2 Hz, $J_{3\beta,3\alpha}$ = 13.1 Hz, 3β-H), 1.00 (3H, s, Me-19), 0.97 (3H, s, Me-18);

^{13}C NMR ($CDCl_3$, 100 MHz): δ 73.54 (C-1, d); 27.06 (C-2, t); 36.82 (C-3, t); 34.23 (C-4, s); 42.61 (C-5, d); 70.08 (C-6, d); 75.54 (C-7, d); 81.29 (C-8, s); 82.74 (C-9, s); 43.28 (C-10, s); 206.82 (C-11, s); 49.66 (C-12, t); 75.83 (C-13, s); 147.54 (C-14, d); 110.13 (C-15, t); 30.80 (C-16, q); 23.69 (C-17, q); 32.74 (C-18, q); 23.54 (C-19, q); 20.07 (C-20, q); 170.49 (6β-$\underline{C}OCH_3$, s); 21.28 (6β-$CO\underline{C}H_3$, q); 170.31 (7β-$\underline{C}OCH_3$, s); 20.95 (7β-$CO\underline{C}H_3$, q);

EIMS *m/z* (relative intensity, %): 452 $[M]^+$ (1), 419 (1), 416 (1), 392 (2), 282 (2), 253 (2), 233 (3), 219 (3), 207 (13), 165 (21), 152 (12), 147 (12), 135 (10), 123 (16), 121

(11), 111 (11), 109 (15), 107 (10), 99 (16), 95 (18), 85 (11), 69 (13), 68 (13), 55 (14), 43 (100), 41 (12);

Anal. C, 63.49%, H, 8.11%, calcd for $C_{24}H_{36}O_8$ C, 63.70% and H 8.02 %.

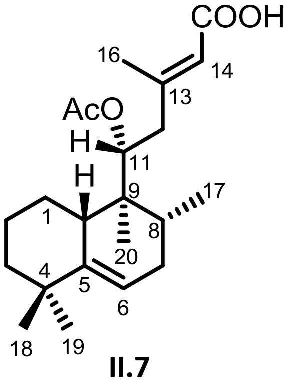
The fraction A_3-3 eluted with *n*-hexane:EtOAc (85:15 to 80:20) was filtered through a pad of charcoal and celite (1:1) to eliminate chlorophylls and was submitted to column chromatography B_5 of silica gel (9385; 30 g) using mixtures of CH_2Cl_2:EtOAc (1:0 to 65:35) as eluents; after tlc monitoring, chromatographic fractions were combined into five fractions (B_5-1 to B_5-5; **Table V.6**).

Table V.6. Column chromatography B_5 of fraction A_3-3 (*P. ornatus*).

Fraction	**Quantity / g**	**Eluents (%) *n*-hexane: EtOAc**
B_5-1	0.400	1:0 to 88:12
B_5-2	0.490	88:12
B_5-3	1.745	88:12
B_5-4	0.809	88:12 to 80:20
B_5-5	0.350	80:20 to 65:35

The fractions B_5-2, B_5-3 and B_5-5 were pooled and recrystallized from a mixture of solvents of EtOAc:CH_2Cl_2 (1:1), EtOAc:CH_2Cl_2 (5:95) and *n*-hexane:EtOAc (70:30) to give 11 mg of the compound **II.7**, 0.526 g of the compound **II.17** (Plectrornatin C) and 0.110 g of the compound **II.15** and **II.16**, respectively.

V.2.1.2.3. 11*R**-Acetoxyhalima-5,13*E*-dien-15-oic acid II.7



II.7

Colourless hexagonal plates from EtOAc – *n*-pentane;

m.p. 158-161 ˚C;

[α]$^{18}_{D}$ - 48,1˚ (*c* 0.162, $CHCl_3$);

IR (KBr) ν_{max}: 3450-2576 br, 2925, 1736, 1692, 1674, 1638, 1460, 1375, 1243, 1028, 718 cm^{-1};

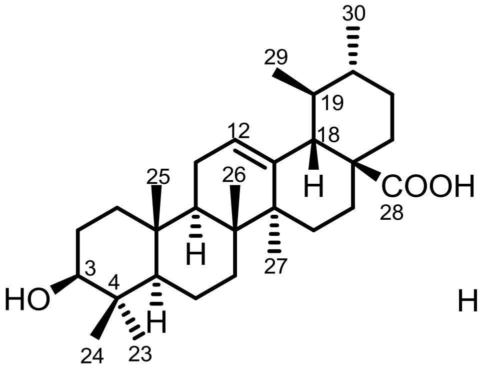
^{1}**H RMN** ($CDCl_3$, 400 MHz) : δ 5.68 (1H, qd, $J_{14,12A}$= 0.4 Hz, $J_{14,16}$= 1.3 Hz, H-14), 5.45 (1H, dt, $J_{6,7\alpha,}$= 2.2 Hz, $J_{6,7\beta}$= 5.2 Hz, $J_{6,10\beta}$= 2.2 Hz, H-6), 5.31 (1H, dd, $J_{11,12A}$= 2.6 Hz, $J_{11,12B}$ = 10.2, H-11), 2.44 (1H, ddd, $J_{12A,11}$= 2.6 Hz, $J_{12A,12B}$= 13.4 Hz, $J_{12A,14}$= 0.4 Hz, H-12A), 2.36 (1H, dd, $J_{12B,11}$= 10.2 Hz, $J_{12B,12A}$= 13.4 Hz, H-12B), 2.19 (3H, d, $J_{16,14}$= 1.3 Hz, Me-16), 2.07 (1H, ddd, $J_{10\beta,1\alpha}$= 12.8 Hz, $J_{10\beta,1\beta}$= 2.0 Hz, $J_{10\beta,6}$= 2.2 Hz, H-10β), 2.00 (3H, s, OAc-11), 1.92 (1H, dtd, $J_{1\beta,1\alpha}$= 12.8 Hz, $J_{1\beta,2\alpha}$= 4.0 Hz, $J_{1\beta,2\beta}$= 4.1 Hz, $J_{1\beta,10\beta}$= 2.0 Hz, H-1β), 1.84 (1H, ddd, $J_{7\beta,6}$= 5.2 Hz, $J_{7\beta,7\alpha}$= 17.1 Hz, $J_{7\beta,8\beta}$= 2.8 Hz, H-7β), 1.78 (1H, ddd, $J_{7\alpha,6}$= 2.2 Hz, $J_{7\alpha,7\beta}$= 17.1 Hz, $J_{7\alpha,8\beta}$= 12.4 Hz, H-7α), 1.64 (1H, ddddd, $J_{2\alpha,1\alpha}$ = 4.2 Hz, $J_{2\alpha,1\beta}$= 4.0 Hz, $J_{2\alpha,2\beta}$= 13.2 Hz, $J_{2\alpha,3\alpha}$= 3.7 Hz, $J_{2\alpha,3\beta}$= 4.0 Hz, H-2α), 1.56 (1H, dqd, $J_{8\beta,7\alpha}$= 12.4 Hz, $J_{8\beta,7\beta}$= 2.8 Hz, $J_{8\beta,17}$= 6.8 Hz, H-8β), 1.52 (1H, qt, $J_{2\beta,1\alpha}$= 12.8 Hz, $J_{2\beta,1\beta}$= 4.1 Hz, $J_{2\beta,2\alpha}$= 13.2 Hz, $J_{2\beta,3\alpha}$= 13.1 Hz, $J_{2\beta,3\beta}$= 3.2 Hz, H-2β), 1.42 (1H, ddd, $J_{3\beta,2\alpha}$= 4.0 Hz, $J_{3\beta,2\beta}$= 3.2 Hz, $J_{3\beta,3\alpha}$= 13.1 Hz, H-3β), 1.19 (1H, td, $J_{3\alpha,2\alpha}$= 3.7 Hz, $J_{3\alpha,2\beta}$= 13.1 Hz, $J_{3\alpha,3\beta}$= 13.1 Hz, H-3α), 1.10 (1H, qd, $J_{1\alpha,1\beta}$= 12.8 Hz, $J_{1\alpha,2\alpha}$= 4.2 Hz, $J_{1\alpha,2\beta}$ = 12.8 Hz, $J_{1\alpha,10\beta}$= 12.8 Hz, H-1α), 1.05 (3H, s, CH_3-19), 0.99 (3H, d, $J_{17,8\beta}$= 6.8 Hz, CH_3-17), 0.98 (3H, s, CH_3-18), 0.69 (3H, s, CH_3-20);

^{13}**C RMN** ($CDCl_3$, 100 MHz): δ 28.9 (C-1, t), 22.5 (C-2, t), 40.9 (C-3, t), 36.3 (C-4, s), 145.2 (C-5, s), 116.0 (C-6, d), 31.3 (C-7, t), 32.9 (C-8, d), 41.6 (C-9, s), 40.3 (C-10, d), 75.0 (C-11, d), 41.7 (C-12, t), 159.8 (C-13, s), 117.7 (C-14, d), 171.1 (C-1, s), 19.0 (C-16, q), 17.2 (C-17, q), 28.1 (C-18, q), 29.7 (C-19, q), 11.8 (C-20, q), 170.7 ($-0\underline{C}CH_3$, s), 20.8 ($-0C\underline{C}H_3$, q);

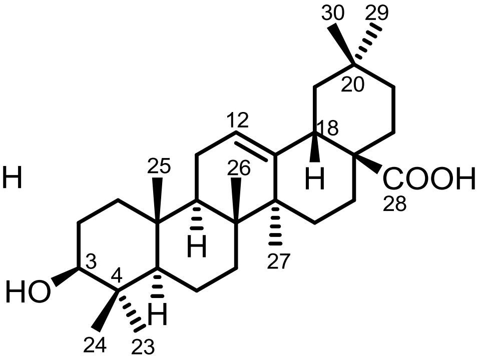
EIMS *m/z* (relative intensity, %): $[M]^+$ absent, 302 $[M-HOAc]^+$ (1), 287 (4), 260 (5), 245 (15), 203 (17), 191 $[M-C_8H_{11}O_4]^{+\cdot}$ (100), 175 (11), 147 (15), 133 (18), 119 (40), 105 (23), 95 (30), 80 (19), 69 (15), 55 (23);

Anal. calculated for $C_{22}H_{34}O_4$: C 72.89 %, H 9.45 %; found: C 72.71 %, H 9.33 %.

V.2.1.2.4. Ursolic and oleanolic acids II.15 and II.16 in a (4:1) ratio



II.15



II.16

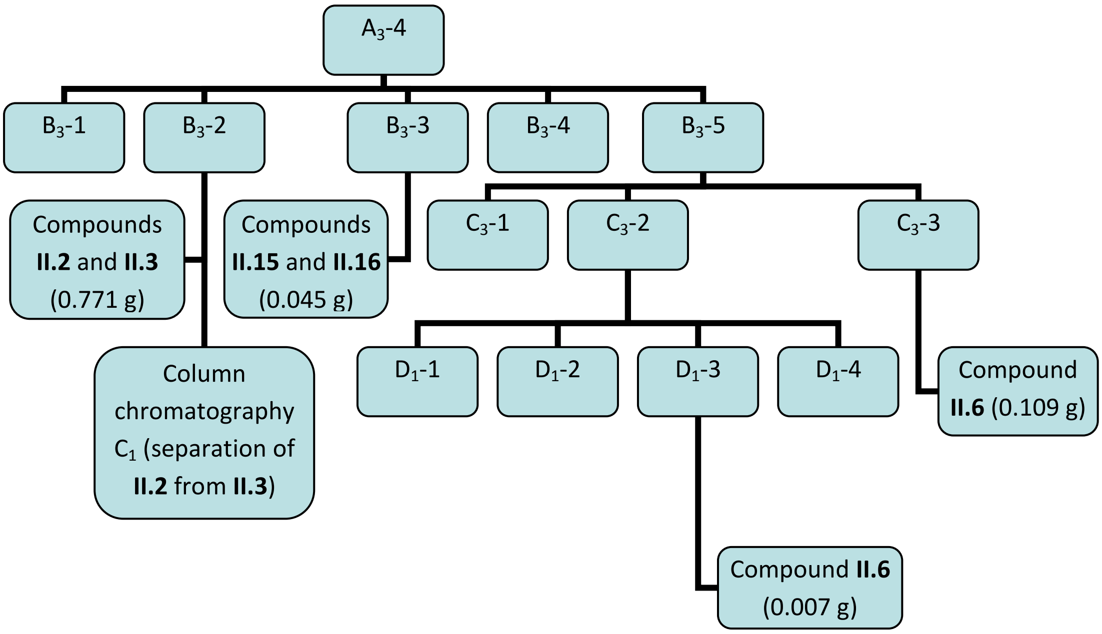
White crystals needles;

m.p. 243.249 ˚C;

***R*$_f$** (silica gel, dichloromethane: ethyl acetate; 8:2) 0.62;

^{1}H NMR (400 MHz, CDCl$_3$): δ 5.12 (1H, br s, H-12 of compound **II.16**), 5.09 (1H, s, H-12 of compound **II.15**), 3.04 (1H, dd, $J_{3\alpha,2\beta}$= 8.0 Hz, $J_{3\alpha,2\alpha}$= 6.0 Hz, H-3α), 2.04 (1H, d, $J_{18,19\alpha}$ = 11.6 Hz, H-18 of compound **II.15**), 1.84 (1H, dd, $J_{18,19\alpha}$ = 13.2 Hz, $J_{18,19\beta}$ = 4.4 Hz, H-18 of compound **II.16**), 1.00-0.62 (14*3H, s, Me-23 to Me-30 of compound **II.16** and **II.15**).

The study of fraction A$_3$-4 was fractionated by column chromatography B$_3$ in five fractions. Fraction B$_3$-2 was pooled and recrystallized to give 0.771 g of compound **II.2** and **II.3** (isolation of **II.2** from **II.3** was done by subsequent column chromatographies C$_1$). Fraction B$_3$-3 was pooled and recrystallized to give 0.045 g of compound **II.15** and **II.16**, and finally fraction B$_3$-5 was rechromatographed by column chromatographies C$_3$, which gave three fractions. The fraction C$_3$-2 was fractionated by column chromatographies D$_1$, and from fraction D$_1$-3 was obtain 0.007 g of compound **II.6.** The most polar fraction (C$_3$-3) was pooled and recrystallized to give 0.108 g of compound **II.6** (**Scheme V.3**).



Scheme V.3. Study of *P. ornatus:* isolated compounds from fraction A_3-4 (from fraction PO-A4).

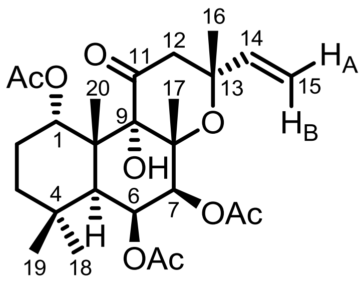
The fraction A_3-4 was filtered through a pad of charcoal and celite (1:1) to eliminate chlorophyls giving 5.993 g of the residue that was submitted to column chromatography B_3 of silica gel (9385; 200 g) using mixtures of CH_2Cl_2:EtOAc (1:0 to 0:1) as eluent; after tlc monitoring, chromatographic fractions were combined into five fractions (B_3-1 to B_3-5; **Table V.7**).

Table V.7. Column chromatography B_3 of fraction A_3-4 (*P. ornatus*).

Fraction	Quantity / g	Eluents (%) CH_2Cl_2: EtOAc
B_3-1	0.2155	1:0 to 95:5
B_3-2	1.2016	90:10 to 85:15
B_3-3	0.6193	85:15
B_3-4	0.3188	80:20
B_3-5	0.8089	80:20 to 0:1

The fraction B_3-2 was pooled and recrystallized from a mixture of solvents of CH_2Cl_2:EtOAc (1:1) to give 0.771 g of compound **II.2** and **II.3**. The isolation of **II.2** from **II.3** (0.150 g) was achieved by column chromatography C_1 using as eluent a mixture of CH_2Cl_2:EtOAc (95:5) to give 0.031 g of 1,6-di-*O*-acetylforskolin **II.2** and 0.015 g of 1,6-di-*O*-acetyl-9-deoxyforskolin **II.3.** The fraction B_3-3 recrystallized from CH_2Cl_2 to give 0.045 g of compounds **II.15** and **II.16** (ursolic and oleanolic acids) in the proportion (1:1).

V.2.1.2.5. 1,6-Di-*O*-acetylforskolin II.2



II.2

Colorless fine needles from EtOAc-pentane;

m.p. 213-215 ˚C (m.p. 216-218 ˚C; Bhat S.V. et al., 1982);

[α]$^{20}_{D}$ +10.3˚ ($CHCl_3$; *c* 0.136);

IR (KBr), υ_{max}: 3468, 2942, 1736, 1714, 1393, 1367, 1246, 1226, 1205, 1042 cm^1;

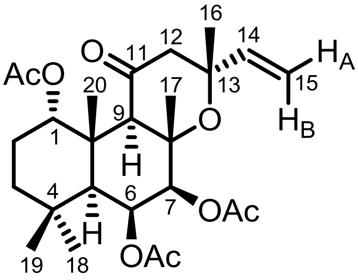
^{1}**H NMR** ($CDCl_3$, 400 MHz): δ 5.90 (1H, dd, $J_{14,15A}$ = 10.6 Hz, $J_{14,15B}$ = 17.2 Hz, 14-H), 5.80 (1H, dd, $J_{6\alpha,5\alpha}$ = 2.7 Hz, $J_{6\alpha,7\alpha}$ = 4.6 Hz, 6α-H), 5.56 (1H, t, $J_{1\beta,2\alpha}$ = 2.4 Hz, $J_{1\beta,2\beta}$ = 2.4 Hz, 1β-H), 5.54 (1H, d, $J_{7\alpha,6\alpha}$ = 4.6, 7α-H), 5.20 (1H, dd, $J_{15B,14}$ = 17.2 Hz, $J_{15B,15A}$ = 1.1, 15B-H), 4.93 (1H, dd, $J_{15A,15B}$ = 1.1, $J_{15A,14}$ = 10.6 Hz, 15A-H), 4.74 (1H, s, 9α-OH), 3.14 (1H, d, $J_{12\alpha,12\beta}$ = 16.5 Hz, 12α-H), 2.42 (1H, d, $J_{12\beta,12\alpha}$ = 16.5 Hz, 12β-H), 2.41 (1H, d, $J_{5\alpha,6\alpha}$ = 2.7 Hz, 5α-H), 2.12 (1H, dddd, $J_{2\beta,1\beta}$ = 2.4 Hz, $J_{2\beta,2\alpha}$ = 15.4, $J_{2\beta,3\alpha}$ = 13.0 Hz, $J_{2\beta,3\beta}$ = 3.4 Hz, 2β-H), 2.09 (1H, s, 1α-OAc), 2.02 (1H, s, 6β-OAc), 2.01 (1H, s, 7β-OAc), 1.64 (3H, s, Me-17), 1.63 (1H, dq, $J_{2\alpha,1\beta}$ = 2.4 Hz, $J_{2\alpha,2\beta}$ = 15.4 Hz, $J_{2\alpha,3\alpha}$ = 3.4 Hz, $J_{2\alpha,3\beta}$ = 3.4 Hz, 2α-H), 1.58 (1H, td, $J_{3\alpha,2\alpha}$ = 3.4 Hz, $J_{3\alpha,2\beta}$ = 13.0 Hz, $J_{3\alpha,3\beta}$ = 13.0 Hz, 3α-H), 1.52 (3H, s, Me-20), 1.33 (3H, s, Me-16), 1.15 (1H , dt, $J_{3\beta,2\alpha}$ = 3.4 Hz, $J_{3\beta,2\beta}$ = 3.4 Hz, $J_{3\beta,3\alpha}$ = 13.0, 3β-H), 1.03 (3H, s, Me-18), 0.98 (3H, s, Me-19);

^{13}C NMR (CDCl$_3$, 100 MHz): δ 73.94 (C-1, d); 23.11 (C-2, t); 37.00 (C-3, t); 33.84 (C-4, s); 43.00 (C-5, d); 69.36 (C-6, d); 76.57 (C-7, d); 81.65 (C-8, s); 81.44 (C-9, s); 43.44 (C-10, s); 205.26 (C-11, s); 48.74 (C-12, t); 75.70 (C-13, s); 145.83 (C-14, d); 110.38 (C-15, t); 31.00 (C-16, q); 23.22 (C-17, q); 32.69 (C-18, q); 23.39 (C-19, q); 19.64 (C-20, q); 168.40 (1α-$\underline{C}$OCH$_3$, s); 21.74 (1α-*CO*$\underline{C}$H$_3$, q); 169.87 (6β-$\underline{C}$OCH$_3$, s); 21.44 (6β-*CO*$\underline{C}$H$_3$, q); 169.87 (7β-$\underline{C}$OCH$_3$, s); 20.82 (7β-*CO*$\underline{C}$H$_3$, q);

EIMS *m/z* (relative intensity, %) 494 [M]$^+$ (2), 434 (8), 384 (8), 355 (25), 324 (5), 295 (5), 282 (13), 207 (28), 193 (14), 191 (20), 175 (15), 165 (30), 152 (16), 147 (17), 123 (19), 121 (12), 109 (13), 107 (12), 97 (10), 95 (19), 85 (13), 81 (15), 68 (19), 67 (13), 55 (12), 43 (100), 41 (8);

Anal. C, 63.28%, H 7.70%, calcd for $C_{26}H_{38}O_9$, C 63.14% and H 7.74 %.

V.2.1.2.6. 1,6-Di-*O*-acetyl-9-deoxyforskolin II.3



II.3

Colorless fine needles from EtOAc-pentane;

m.p. 199-200 ˚C;

[α]$^{20}_{D}$ –46.9˚ (CHCl$_3$; *c* 0.149);

IR (KBr), υ_{max}: 3090, 2971, 2942, 1736, 1715, 1635, 1393, 1366, 1252, 1234, 1039, 984, 948 cm^{-1};

^{1}H NMR (CDCl$_3$, 400 MHz): δ 5.94 (1H, dd, $J_{14,15A}$ = 10.8 Hz, $J_{14,15B}$ = 17.4 Hz, 14-H), 5.74 (1H, dd, $J_{6\alpha,5\alpha}$ = 2.4 Hz, $J_{6\alpha,7\alpha}$ = 3.9, 6α-H), 5.50 (1H, t, $J_{1\beta,2\alpha}$ = 2.7 Hz, $J_{1\beta,2\beta}$ = 2.7 Hz, 1β-H), 5.10 (1H, d, $J_{7\alpha,6\alpha}$ = 3.9, 7α-H), 5.24 (1H, dd, $J_{15B,14}$ = 17.4 Hz, $J_{15B,15A}$ = 1.0, 15B-H), 5.05 (1H, dd, $J_{15A,15B}$ = 1.0, $J_{15A,14}$ = 10.8 Hz, 15A-H), 3.32 (1H, s, 9α-H), 2.64 (1H, d, $J_{12\alpha,12\beta}$ = 18.8 Hz, 12α-H), 2.59 (1H, d, $J_{12\beta,12\alpha}$ = 18.8 Hz, 12β-H), 2.08 (1H, s, 7β-OAc), 2.07 (1H, s, 6β-OAc), 1.97 (1H, dddd, $J_{2\beta,1\beta}$ = 2.7 Hz, $J_{2\beta,2\alpha}$ = 14.4, $J_{2\beta,3\alpha}$ = 13.0 Hz, $J_{2\beta,3\beta}$ = 3.4 Hz, 2β-H), 1.96 (1H, s, 1α-OAc), 1.72 (1H, dddd, $J_{2\alpha,1\beta}$ = 2.7 Hz, $J_{2\alpha,2\beta}$ = 14.4 Hz, $J_{2\alpha,3\alpha}$ = 2.7 Hz, $J_{2\alpha,3\beta}$ = 3.4 Hz, 2α-H), 1.60 (1H, d, $J_{5\alpha,6\alpha}$ = 2.4 Hz, 5α-H), 1.50 (3H, s, CH_3-17), 1.45 (1H, ddd, $J_{3\alpha,2\alpha}$ = 2.7 Hz, $J_{3\alpha,2\beta}$ = 13.0 Hz, $J_{3\alpha,3\beta}$ = 13.5 Hz, 3α-H), 1.44 (3H, s, CH_3-20), 1.21 (3H, s, Me-16), 1.12 (1H , dt, $J_{3\beta,2\alpha}$ = 3.4 Hz, $J_{3\beta,2\beta}$ = 3.4 Hz, $J_{3\beta,3\alpha}$ = 13.5, 3β-H), 0.98 (3H, s, CH_3-18), 0.94 (3H, s, CH_3-19);

^{13}C NMR ($CDCl_3$, 100 MHz): δ 74.78 (C-1, d); 21.61 (C-2, t); 36.74 (C-3, t); 33.77 (C-4, s); 47.23 (C-5, d); 69.24 (C-6, d); 78.51 (C-7, d); 77.21 (C-8, s); 57.32 (C-9, d); 40.62 (C-10, s); 205.21 (C-11, s); 49.01 (C-12, t); 74.61 (C-13, s); 146.30 (C-14, d); 112.89 (C-15, t); 31.80 (C-16, q); 24.03 (C-17, q); 32.63 (C-18, q); 22.91 (C-19, q); 17.25 (C-20, q); 169.88 (1α-$\underline{C}OCH_3$, s); 21.33 (1α-$CO\underline{C}H_3$, q); 169.45 (6β-$\underline{C}OCH_3$, s); 20.33 (6β-$CO\underline{C}H_3$, q); 170.35 (7β-$\underline{C}OCH_3$, s); 21.33 (7β-$CO\underline{C}H_3$, q);

EIMS *m/z* (relative intensity, %): $[M]^+$ absent, 463 $[M-Me]^+$ (2), 435 (3), 418 $[M-HOAc]^+$ (7), 403 (2), 361 (3), 351 (7), 308 (8), 263 (10), 231 (10), 205 (10), 203 (24), 175 (10), 95 (14), 81 (11), 69 (14), 55 (12), 43 (100), 41 (9);

Anal. C, 65.31%, H, 7.89%, calcd for $C_{26}H_{38}O_8$, C 65.25% and H, 8.00 %.

The fraction B_3-3 eluted from a mixture of solvents of CH_2Cl_2:EtOAc (85:15) was recrystallized from the same mixture of solvents to give 0.045 g of compounds **II.15** and **II.16.**

The fraction B_3-5 was submitted to C_3 column chromatography of silica gel (9385; 70 g) using as eluent mixtures of *n*-hexane:EtOAc (1:0 to 0:100); after tlc monitoring, chromatographic fractions were combined into three fractions (C_3-1 to C_3-3 **Table V.8**).

Table V.8. Column chromatography C_3 of fraction B_3-5 (*P. ornatus*).

Fraction	**Quantity / g**	**Eluents (%) *n*-hexane: AcOEt**
C_3-1	0.0600	1:0 to 80:20
C_3-2	0.4387	80:20 to 75:25
C_3-3	0.0300	75:25 to 0:100

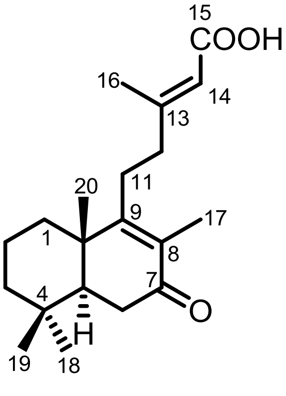
The fraction C_3-3 was recrystallized from a mixture of solvents of *n*-hexane:EtOAc (1:1) to give 6.6 mg of rhinocerotinoic acid **II.6** (1,7-oxo-labda-8-13E-dien-15-oic acid). The fraction C_3-2 was submitted to D_1 column chromatography using as eluents a mixture of CH_2Cl_2:EtOAc (1:0 to 0:1); after tlc monitoring, chromatographic fractions were combined into four fractions (D_1-1 to D_1-4 **Table V.9**).

Table V.9. Column chromatography D_1 of fraction eluted by *n*-hexane:EtOAc (75:25) of column chromatography C_1 (*P. ornatus*).

Fraction	Quantity / g	Eluents (%) CH_2Cl_2: EtOAc
D_1-1	0.0320	1:0 to 80:20
D_1-2	0.0500	78:22 to 74:26
D_1-3	0.3467	74:28 to 58:42
D_1-4	0.0100	58:42 to 0:1

The fraction D_1-3 was recrystallized from a mixture of solvents of *n*-hexane:EtOAc (1:1) to give 108 mg (0.0032 %, on dry plant material) of rhinocerotinoic acid **II.6** (1,7-oxo-labda-8-13*E*-dien-15-oic acid).

V.2.1.2.7. Rhinocerotinoic acid (1, 7-oxo-labda-8-13E-dien-15-oic acid) II.6



II.6

White fine needles;

m.p. 187-189 ˚C (EtOAc-light petroleum); m.p. 189-190 ˚C (Gray C.A. et al., 2003);

$[\alpha]^{18}_{D}$ +38.9° (c 0.236, $CHCl_3$); $[\alpha]_D$ +42° (Dekker T.G. et al., 1988); $[\alpha]^{27}_{D}$ +40° (c 2.52) (Gray C.A. et al., 2003);

IR (KBr), υ_{max}: 3306-2894 br, 1720, 1630, 1587, 1448, 1373, 1328, 1223, 1146, 860, 679 cm^{-1};

^{1}H RMN ($CDCl_3$, 400 MHz): δ 1.36 (1H, td, $J_{1\alpha,1\beta}$= $J_{1\alpha,2\beta}$ = 12.7 Hz, $J_{1\alpha,2\alpha}$ = 3.7 Hz, H-1α), 1.91 (1H, dddd, $J_{1\beta,1\alpha}$ = 12.7 Hz, $J_{1\beta,2\alpha}$= 4.0 Hz, $J_{1\beta,2\beta}$= 3.7 Hz, $J_{1\beta,3\beta}$= 1.3 Hz, H-1β), 1.70 (1H, m, $J_{2\alpha,1\alpha}$ = 3.7 Hz, $J_{2\alpha,1\beta}$= 4.0 Hz, $J_{2\alpha,2\beta}$= 13.5 Hz, $J_{2\alpha,3\alpha}$= 4.1 Hz, $J_{2\alpha,3\beta}$= 3.2 Hz, H-2α), 1.60 (1H, ddddd, $J_{2\beta,1\alpha}$ = 12.7 Hz, $J_{2\beta,1\beta}$= 3.7 Hz, $J_{2\beta,2\alpha}$= 13.5 Hz, $J_{2\beta,3\alpha}$= 13.5 Hz, $J_{2\beta,3\beta}$= 3.2 Hz, H-2β), 1.21 (1H, d largo, $J_{3\alpha,2\alpha}$= 4.1 Hz, $J_{3\alpha,2\beta}$= 13.5 Hz, $J_{3\alpha,3\beta}$= 13.5 Hz, H-3α), 1.48 (1H, dd, $J_{3\beta,1\beta}$= 1.3 Hz, $J_{3\beta,2\alpha}$= 3.2 Hz, $J_{3\beta,2\beta}$= 3.2 Hz, $J_{3\beta,3\alpha}$= 13.5 Hz, H-3β), 1.70 (1H, dd, $J_{5\alpha,6\alpha}$ = 3.7 Hz, $J_{5\alpha,6\beta}$= 14.3 Hz, H-5), 2.50 (1H, dd, $J_{6\alpha,5\alpha}$ = 3.7 Hz, $J_{6\alpha,6\beta}$ = 17.5 Hz, H-6α), 2.35 (1H, dd, $J_{6\beta,5\alpha}$ = 14.3 Hz, $J_{6\beta,6\alpha}$= 17.5 Hz, H-6β), 2.35 (2H, m, CH_2-11), 2.28 (2H, m, CH_2-12), 5.74 (1H, q, $J_{14,16}$ = 1.3 Hz, H-14), 2.23 (3H,d, $J_{16,14}$ = 1.3 Hz, CH_3-16), 1.77 (3H, s, CH_3-17), 0.88 (3H, s, CH_3-18), 0.91 (3H, s, CH_3-19), 1.08 (3H, s, CH_3-20);

^{13}C RMN ($CDCl_3$, 100 MHz): δ 35.8 (C-1, t), 18.56 (C-2, t), 41.23 (C-3, t), 33.12 (C-4, s), 50.26 (C-5, d), 35.19 (C-6, t), 200.13 (C-7, s), 130.57 (C-8, s), 166.23 (C-9, s), 41.01 (C-10, s), 27.64 (C-11, t), 39.79 (C-12, t), 161.70 (C-13, s), 115.18 (C-14, d), 171.13 (C-15, s), 19.12 (C-16, q), 11.42 (C-17, q), 32.49 (C-18, q), 21.29 (C-19, q), 18.15 (C-20, q);

EIMS m/z (relative intensity, %): 318 $[M]^+$ (3), 205 $[M-C_6H_9O_2]^+$ (53), 177 (18), 149 (18), 135 (100), 123 (47), 121 (27), 109 (20), 107 (20), 95 (17), 91 (23), 69 (25), 55 (31), 53 (18).

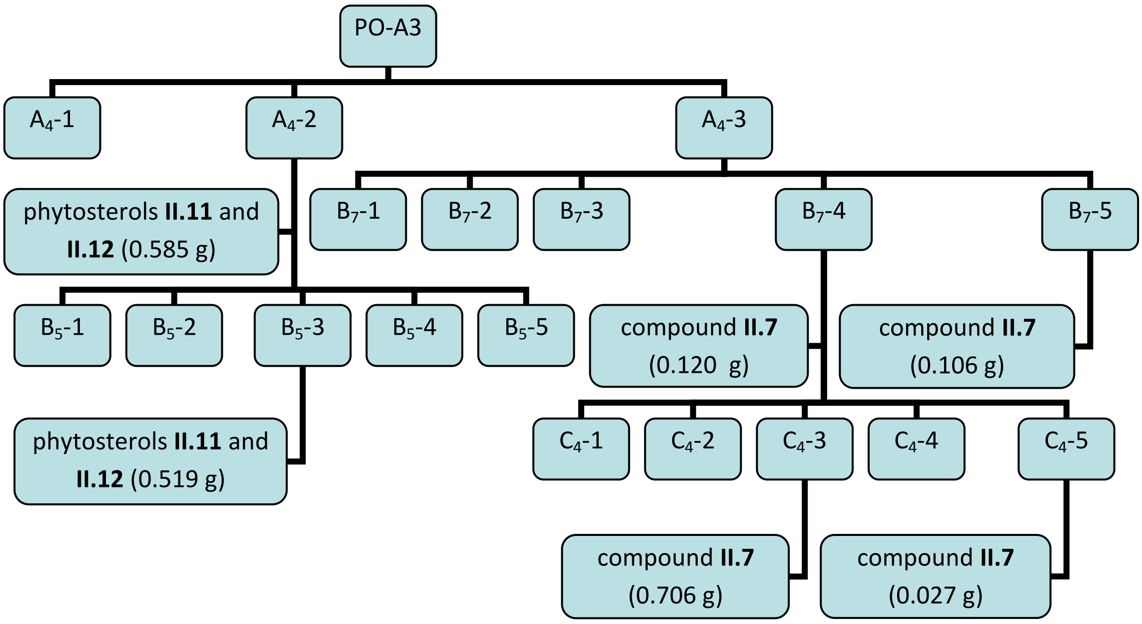
V.2.1.3. Column chromatography A_4 **(Study of fraction PO-A3)**

The fraction PO-A3 eluted with *n*-hexane:EtOAc (50:50) was filtered through a pad of charcoal and celite (1:1) to eliminate chlorophylls and the residue was submitted to column chromatography A_4 of silica gel(9385; 150 g) using mixtures of CH_2Cl_2:EtOAc (1:0 to 0:1) as eluent; after tlc monitoring, the chromatographic fractions were combined into three fractions (A_4-1 to A_4-3, **Table V.10**).

Table V.10. Column chromatography A_4 of fraction PO-A3 (*P. ornatus*).

Fraction	Quantity / g	Eluents (%) DCM: EtOAc
A_4-1	2.8600	1:0
A_4-2	4.2380	1:0 to 50:50
A_4-3	4.0000	50:50 to 0:1

The fraction PO-A3 was fractionated by column chromatography A_4 giving three fractions. Fractions A_4-2 were recrystallized to give 0.585 g a of a mixture of phytosterols **II.11** and **II.12** in the proportion (1:2) and a subsequent column chromatography B_5 gave 0.519 g of the same mixture of phytosterols **II.11** and **II.12.** Fraction A_4-3 was submitted to other two column chromatographies, B_7 and C_4, for purifying compound **II.7** (0.959 g) as shown in the **Scheme V.4.**



Scheme V.4. Study of *P. ornatus:* isolated compounds from fraction PO-A3.

The fraction A_4-2 (4.238g) was filtered through a pad of charcoal and celite (1:1) to eliminate chlorophylls and recrystallized from a mixture of solvents of *n*-hexane:EtOAc (1:1) to give 0.585 g of a mixture of β-sitosterol (stigmast-5-en-3β-ol) **II.11** and stigmasta-5,22*E*-dien-3β-ol **II.12** in the proportion (1:2) and the remain residue was submitted to column chromatography B_5 of silica gel (9385, 100 g) eluted with CH_2Cl_2 to obtain more 0.519 g of the mixture of β-sitosterol (stigmast-5-en-3β-ol) **II.11** and stigmasta-5,22*E*-dien-3β-ol **II.12** in the same proportion (1:2). The fraction A_4-3 (4.00 g) eluted with CH_2Cl_2:EtOAc (50:50 to 0:1) was submitted to column chromatography B_7 of silica gel (9385, 100 g) eluted with CH_2Cl_2:EtOAc (1:0 to 0:1) to give five fractions. The fraction B_7-4 (2.220 g) eluted with EtOAc, was submitted to another column chromatography C_4 of silica gel (9385, 200 g) eluted with CH_2Cl_2:acetone (1:0 to 0:1) for purifying compound **II.7** in order to obtain more five fractions. Fractions (0.800 g and 0.700 g) eluted with CH_2Cl_2:acetone (80:20 to 75:25) and CH_2Cl_2:acetone (65:45 to 0:1) gave 0.733 g and 0.027 g of the compound 11*R**-acetoxyhalima-5,13*E*-dien-15-oic acid **II.7**, respectively**.** The fraction B_7-5 (0.423 g) eluted with EtOAc was recrystallized giving 0.106 g of compound **II.7.**

V.2.2. *P. ornatus* extracts

The *P. ornatus* extracts (PO) were prepared by maceration of dried plant, by means of an increasing polarity set of solvents from *n*-hexane (PO-H), ethyl acetate (PO-EA), dichloromethane (PO-D), acetone (PO-A), methanol (PO-M) and solvent binary mixture MeOH-H_2O (7:3; PO-MW).

Five of the extracts were obtained by maceration of 25 g of dried and powdered plant material with 250 ml of each solvent, under agitation at room temperature, during a week. After filtration and solvents evaporation the following extracts were obtained: 0.351 g (1.404 % of dry plant) of a hexane extract, 0.772 g (3.088%) of an ethyl acetate extract, 0.696 g (2.784%) of a dichloromethane extract, 2.248 g (8.993%) of a methanol extract and 1.777 g (7.108 %) of a MeOH-H_2O (7:3) extract and 183.91 g (5.41%) of the acetonic extract (Rijo P. et al., 2010 submitted).

V.3. Study of *Plectranthus ecklonii* Benth.

Plectranthus ecklonii Benth. was cultivated in the Faculty of Pharmacy Hortum, Lisbon University, from seeds provided by the Herbarium of the National Botanical Garden of Kirstenbosch, South Africa. *P. ecklonii* whole plants were collected in July 1998 and voucher specimens were deposited in the Herbarium of the Instituto de Investigação Científica Tropical, Lisbon (ref. C. Marques S/N° LISC).

V.3.1. Plant material: study of *Plectranthus ecklonii* whole plants *Extraction and isolation*

The air dried and powdered whole plants of *P. ecklonii* Benth. (7035.5 g) were extracted with Me_2CO (6 x 7 L) at room temperature for 15 days. Filtration and evaporation of the solvent (under *vacuum*, 40˚C) yielded a residue of 284.4 g (4.04 % of dry material), which was subjected to column chromatography over silica gel (7734, 3000 g), using mixtures of *n*-hexane:EtOAc (1:0 to 0:1) and EtOAc:MeOH (1:0 to 0:1) as eluents yielding eight fractions. The precipitate of the first fraction, consisting mainly of waxes (21.34 g), was eliminated by filtration.

According to differences in composition, as indicated by tlc, the eight crude fractions were obtained (PE-A1 to PE-A8, **Table V.11**).

Table V.11. Column chromatography A of the acetone extract (*P. ecklonii*).

Fraction	Quantity (g)	Eluent (%) *n*-hexane : EtOAc	Eluent (%) EtOAc: MeOH
PE-A1	2.68	70:30	---
PE-A2	13.72	70:30 to 50:50	---
PE-A3	7.47	50:50	---
PE-A4	20.57	40:60	---
PE-A5	37.70	40:60 to 25:75	---
PE-A6	27.50	25:75 to 10:90	---
PE-A7	5.55	0:100	---
PE-A8	49.83	0:100	to 40:60

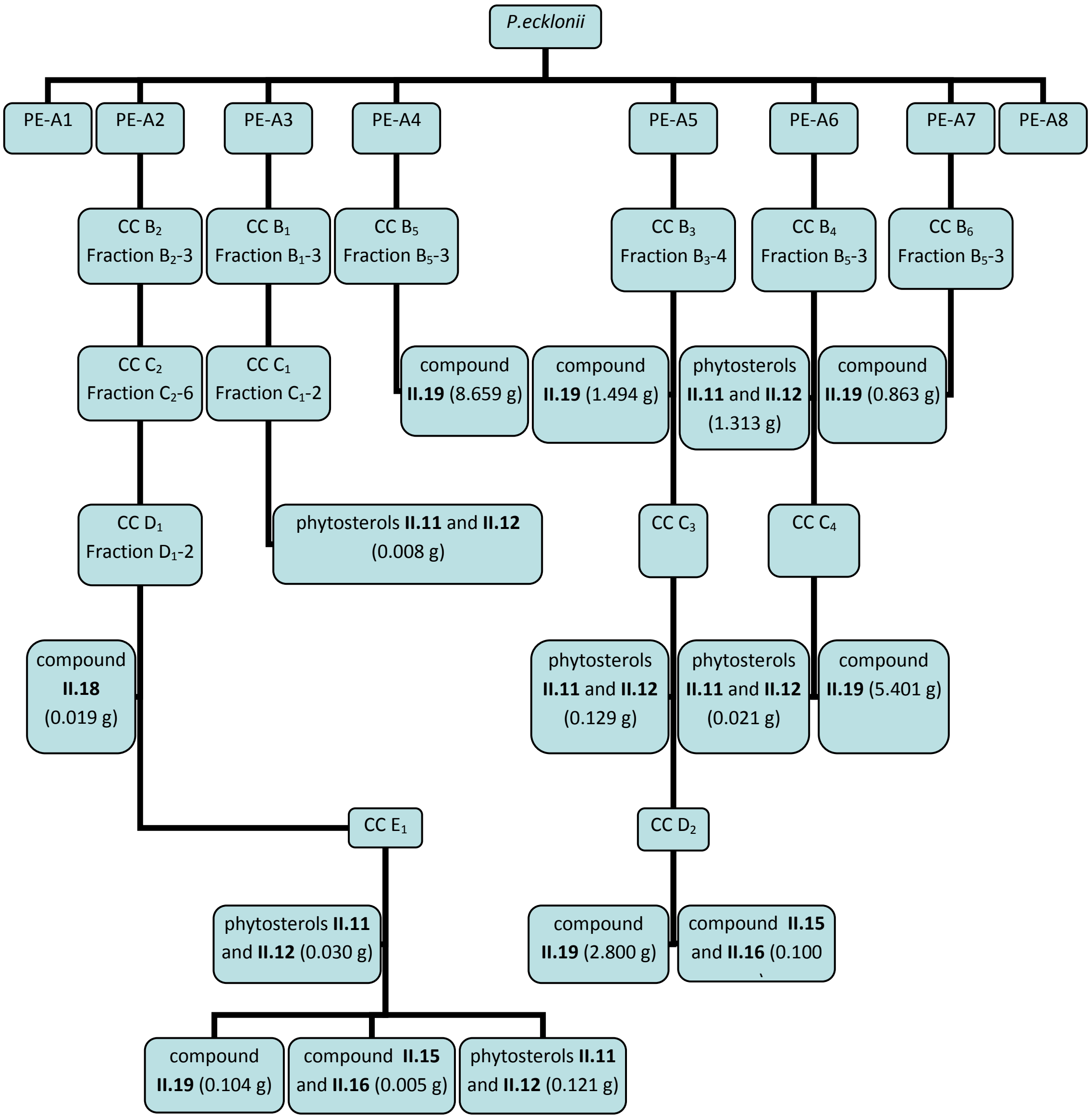
The fraction PE-A2 was studied by three column chromatographies (CC) B_2, C_2 and D_1 and from a fraction of the latter compound **II.18** (0.019 g) was obtained. The residue obtained was pooled and recrystallized to afford 0.030 g of phytosterols **II.11** and **II.12.** The remain residue was submitted to column chromatography E_1 and from three different fractions compound **II.19** (0.104 g), compound **II.15** and **II.16** (0.005 g) and phytosterols **II.11** and **II.12** (0.121 g) were obtained.

The fraction PE-A3 was fractionated by two column chromatographies (CC) B_1 and C_1 and from a fraction of the latter, phytosterols **II.11** and **II.12** (0.008 g) were obtained.

The fractions PE-A4 and PE-A7 were studied by column chromatographies (CC) B_5 and B_6, respectively, and compound **II.19** (8.659 g and 0.863 g, respectively) was obtained.

The fraction PE-A5 was studied by column chromatography B_3 and one of the fraction was pooled and recrystallized to afford compound **II.19** (1.494 g). The remaining residue was submitted to column chromatography C_3 and the crude obtained was pooled and recrystallized to afford phytosterols **II.11** and **II.12** (0.129 g). The subsequent column chromatography D_2 gave two different fractions that afforded the compound **II.19** (2.800 g) and the compounds **II.15** and **II.16** (0.100 g).

The fraction PE-A6 was studied by column chromatography B_4 and from one of the fractions the phytosterols **II.11** and **II.12** (1.313 g) were isolated. The remaining residue was submitted to column chromatography C_4 and the crude obtained from different fractions was pooled and recrystallized to afford phytosterols **II.11** and **II.12** (0.021 g) and compound **II.19** (5.401 g).



Scheme V.5. Study of *P. ecklonii:* isolated compounds from PE-A2 to PE-A7.

V.3.1.1. Study of fraction PE-A3 (Column chromatography B_1)

The fraction PE-A3 eluted with *n*-hexane:EtOAc (50:50) was fractionated by column chromatography B_1 (hydrated silica gel, 80 g) eluted with mixtures of *n*-hexane:EtOAc. The chromatographic fractions, after tlc monitoring were combined into five fractions (B_1-1 to B_1-5, **Table V.12**).

Table V.12. Column chromatography B_1 of the fraction PE-A3 (*P. ecklonii*).

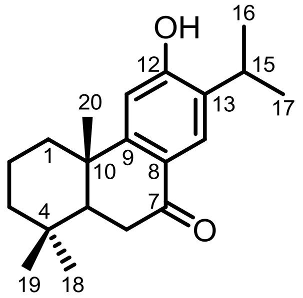
Fraction	**Quantity / g**	**Eluents (%) *n*-hexane: EtOAc**
B_1-1	5.689	100:0
B_1-2	0.024	100:0 to 85:15
B_1-3	3.689	80:20 to 85:25
B_1-4	0.500	70:30 to 45:55
B_1-5	0.833	40:60 to 0:100

The fraction B_1-3 was filtered through a pad of charcoal and celite (1:1) to eliminate chlorophylls; a quantity of 0.183 g of the resulting residue was submitted to column chromatography C_1 of silica gel (9385; 14 g) using mixtures of *n*-hexane:EtOAc (1:0 to 0:1) as eluent; after tlc monitoring, chromatographic fractions were combined into four fractions. The fractions eluted with *n*-hexane:EtOAc (91:9 to 90:10) were pooled and recrystallized from a mixture of solvents of *n*-hexane:EtOAc (1:1) to give 8.1 mg of β-sitosterol **II.11** and stigmasta-5,22*E*-dien-3β-ol **II.12** in the proportion (1:2).

V.3.1.2. Column chromatography B_2 **(Study of fraction PE-A2)**

The fraction PE-A2 eluted with *n*-hexane:EtOAc (70:30 to 50:50) was fractionated by column chromatography B_2 (silica gel, 130 g) eluted with mixtures of *n*-hexane:EtOAc. The chromatographic fractions, after tlc monitoring were combined into seven fractions (B_2-1 to B_2-7). The fraction B_2-3 was filtered through charcoal and celite (1:1) to eliminate chlorophylls and 1.948 g of the resulting residue was submitted to column chromatography C_2 of silica gel (9385; 100 g) using as eluent mixtures of *n*-hexane:EtOAc (1:0 to 0:1); after tlc monitoring, chromatographic fractions were combined into seven fractions. The fraction C_2-6 was fractionated by column chromatography D_1 (silica gel, 100 g) eluted with mixtures of *n*-hexane:EtOAc. The fraction eluted with *n*-hexane:EtOAc (92:8) was pooled and recrystallized from a mixture of solvents of *n*-hexane:EtOAc (1:1) to give 19.3 mg (0.0003%, on dry plant material) of sugiol **II.18.** The remaining residue was chromatographed and the fraction eluted with *n*-hexane:EtOAc (94:6 to 93:7) crystallized giving 30 mg of β-sitosterol **II.11** and stigmasta-5,22*E*-dien-3β-ol **II.12**. The remaining residue was re-chromatographed and further column chromatography for the purification gave 103.6 mg of parvifloron D **II.19**, 5.2 mg of compounds **II.15** and **II.16** (ursolic and oleanolic acids) and 121.2 mg of β-sitosterol **II.11** and stigmasta-5,22*E*-dien-3β-ol **II.12.**

V.3.1.2.1. Sugiol II.18



II.18

m.p. 288-290˚ C, 295-297 ˚C (Glasby J. S. 1982);

$[\boldsymbol{\alpha}]_D^{27}$ + 30.8 ˚ (*c* 0.133, $CHCl_3$); $[\boldsymbol{\alpha}]_D^{25}$ + 28.3 ˚ (*c* 1.000, $CHCl_3$; Chao K.P., 2005);

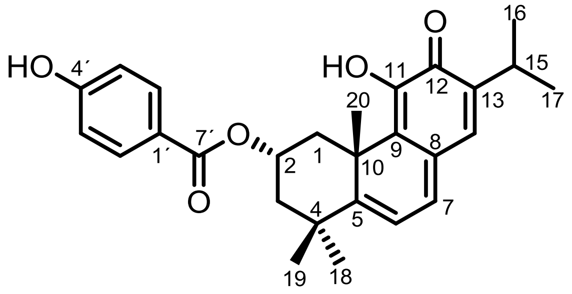
I.R. (KBr) ν_{max}: 3140, 2940, 2880, 1650, 1610, 1580, 1570, 1510, 1460, 1380, 1345, 1315, 1270, 1185, 1100, 920, 880, 780, 670 cm^{-1};

^{1}H RMN ($(CD_3)_2CO$, 400 MHz): δ 8.90 (1H, br, 12-OH), 7.79 (1H, s, H-14), 6.82 (1H, s, H-11), 3.23 (1H, sept of d, $J_{15,16(17)}$ = 6.9 Hz, H-15), 2.55 (1H, dd, $J_{6\beta,5\alpha}$= 8.8 Hz, $J_{6\beta,6\alpha}$= 17.6 Hz, H-6β), 2.50 (1H, dd, $J_{6\alpha,5\alpha}$= 8.8 Hz, $J_{6\alpha,6\beta}$= 17.6 Hz, H-6α), 2.20 (1H, ddd, $J_{1\beta,1\alpha}$= 12.8 Hz, $J_{1\beta,2\alpha}$= 1.6 Hz, $J_{1\beta,2\beta}$= 3.2 Hz, H-1β), 1.80 (1H, dd, $J_{5\alpha,6\alpha}$ = 8.8 Hz, $J_{5\alpha,6\beta}$ = 8.8 Hz, H-5α), 1.79 (1H, ddddd, $J_{2\beta,1\beta}$ = 3.2 Hz, $J_{2\beta,2\alpha}$ = 14.0 Hz, $J_{2\beta,3\alpha}$= 13.6 Hz, $J_{2\beta,3\beta}$ = 3.5 Hz, H-2β), 1.63 (1H, ddddd, $J_{2\alpha,1\alpha}$= 3.6 Hz, $J_{2\alpha,1\beta}$= 1.6 Hz, $J_{2\alpha,2\beta}$= 14.0 Hz, $J_{2\alpha,3\alpha}$= 3.6 Hz, $J_{2\alpha,3\beta}$ = 1.8 Hz, H-2α), 1.50 (1H, ddd, $J_{3\beta,2\alpha}$= 1.8 Hz, $J_{3\beta,2\beta}$= 3.5 Hz, $J_{3\beta,3\alpha}$= 13.6 Hz, H-3β), 1.47 (1H, ddd, $J_{1\alpha,1\beta}$ = 12.8 Hz, $J_{1\alpha,2\alpha}$ = 3.6 Hz, $J_{1\alpha,2\beta}$ = 13.2 Hz, H-1α), 1.27 (1H, ddd, $J_{3\alpha,2\alpha}$ = 3.6 Hz, $J_{3\alpha,2\beta}$ = 13.6 Hz, $J_{3\alpha,3\beta}$ = 13.6 Hz, H-3α), 1.21 (3H, d, CH_3-16), 1.20 (3H, d, CH_3-20), 1.19 (3H, d, CH_3-17), 0.99 (3H, s, CH_3-19), 0.92 (3H, s, CH_3-18);

^{13}C RMN ($(CD_3)_2CO$, 100 MHz): δ 37.34 (C-1, t), 18.31 (C-2, t), 40.82 (C-3, t), 32.58 (C-4,s), 49.14 (C-5, d), 35.21 (C-6, t), 196.11 (C-7, s), 123.18 (C-8, s), 155.59 (C-9, s), 37.32 (C-10, s), 109.00 (C-11, d), 159.25 (C-12, s), 132.41 (C-13, s), 125.23 (C-14, d), 26.07 (C-15, d), 21.58 (C-16, q), 21.46 (C-17, q), 31.71 (C-18, q), 20.46 (C-19, q), 22.32 (C-20, q);

EIMS *m/z* (relative intensity, %): 300 $[M]^+$ (81), 285 [M-15] (100), 257 (6), 243 (23), 229 (9), 217 (43), 215 (30), 203 (36), 201 (21), 189 (15), 187 (11), 163 (15), 145 (9), 141 (8), 129 (9), 128 (13), 115 (13), 91 (11), 69 (28), 55 (21).

V.3.1.2.2. Parvifloron D II.19



II.19

m.p. 157-158 ˚C;

[α]$^{20}_{D}$ + 128.1 ˚ (c̲ 0.367, $CHCl_3$);

IR (KBr) ν_{max}: 3568-3092, 3329, 3059, 2959, 1669, 1594, 1520, 1444, 1315, 1259, 1232, 1165, 1110, 1049, 1008, 985, 849, 773, 654 cm^{-1};

^{1}H RMN ($CDCl_3$, 400 MHz) : δ 7.93 (2H, *d*, $J_{2',3'}$ = 8.9 Hz, H-2´ and H-6´), ~7.70 and ~ 7.20 (br s, HO–4´ and HO–11), 6.96 (1H, *d*, $J_{14,15}$ = 0.8 Hz, H-14), 6.88 (2H, d, $J_{3',2'}$ = 8.9 Hz, H-3´ and H-5´), 6.79 (1H, d, $J_{7,6}$ = 6.9 Hz, H-7), 6.41 (1H, d, $J_{6,7}$ = 6.9 Hz, H-6), 5.59 (1H, tt, $J_{2\beta,1\alpha}$ = 11.4 Hz, $J_{2\beta,1\beta}$ = 4.4 Hz, $J_{2\beta,3\alpha}$ = 11.4 Hz, $J_{2\beta,3\beta}$ = 4.4 Hz, H-2β), 3.76 (1H, ddd, $J_{1\beta,1\alpha}$ = 13.0 Hz, $J_{1\beta,2\beta}$ = 4.4 Hz, $J_{1\beta,3\beta\ (W)}$ = 2.4 Hz, H-1β), 3.15 (1H, sept of d, $J_{15,16(17)}$ = 6.8 Hz, $J_{15,14}$ = 0.8 Hz, H-15), 2.15 (1H, ddd, $J_{3\beta,2\beta}$ = 4.4 Hz, $J_{3\beta,3\alpha}$ = 12.5 Hz, $J_{3\beta,1\beta(W)}$ = 2.4 Hz, H-3β), 1.74 (1H, dd, $J_{1\alpha,1\beta}$ = 13.0 Hz, $J_{1\alpha,2\beta}$ = 11.4 Hz, H-1α), 1.64 (3H, s, CH_3-20), 1.56 (1H, dd, $J_{3\alpha,2\beta}$ = 11.4 Hz, $J_{3\alpha,3\beta}$ = 12.5 Hz, H-3α), 1.42 (3H, s, CH_3-19), 1.29 (3H, s, CH_3-18), 1.16 (3H, d, $J_{16(17),15}$ = 6.8 Hz, CH_3-16 or CH_3-17), 1.18 (3H, d, $J_{17(16),15}$ = 6.8 Hz, Me-17 or Me-16);

^{13}C RMN ($CDCl_3$, 100 MHz): δ 38.37 (C-1, t), 67.87 (C-2, d), 45.06 (C-3, t), 38.58 (C-4, s), 164.84 (C-5, s), 118.69 (C-6, d), 139.13 (C-7, d), 127.45 (C-8, s), 127.17 (C-9, s), 43.91 (C-10, s), 146.40 (C-11, s), 178.24 (C-12, s), 141.61 (C-13, s), 133.57 (C-14, d), 26.52 (C-15, d), 21.84 (C-16 or C-17, q), 21.63 (C-17 or C-16, q), 33.03 (C-18, q), 30.58 (C-19, q), 25.52 (C-20, q), 122.43 (C-1´, s), 131.89 (C-2´ and C-6´, d), 115.23 (C-3´ and C-5´, d), 160.58 (C-4´, s), 166.18 (C-7´, s);

EIMS *m/z* (relative intensity, %): 434 $[M]^+$ (1), 297 (17), 296 (75), 240 (13), 228 (22), 227 (100), 121 (27).

V.3.1.3. Column chromatography B_3 **(Study of fraction PE-A5)**

The fraction PE-A5 eluted with *n*-hexane:EtOAc (40:60 to 25:75) was fractionated by column chromatography B_3 (silica gel, 300 g) eluted with mixtures of *n*-hexane:EtOAc and EtOAc:MeOH (1:0 to 0:1). The chromatographic fractions, after tlc monitoring, were combined into eight fractions (B_3-1 to B_3-8, **Table V.13**).

Table V.13. Column chromatography B_3 of the fraction PE-A5 (*P. ecklonii*).

Fraction	Quantity / g	Eluents (%) *n*-hexane : EtOAc	Eluent (%) EtOAc: MeOH
B_3-1	0.186	1:0 to 85:15	---
B_3-2	0.956	80:20 to 65:35	---
B_3-3	5.412	60:40	---
B_3-4	19.763	60:40 to 50:50	---
B_3-5	2.650	50:50 to 45:55	---
B_3-6	1.976	45:55	---
B_3-7	3.025	45:55 to 40:60	---
B_3-8	2.318	40:60 to	80:20

Fraction B_3-4 (1.739 g) was filtered through a pad of charcoal and celite (1:1) to eliminate chlorophylls and was pooled and recrystallized from a mixture of solvents of *n*-hexane:EtOAc (1:1) to give 1.494 g of parvifloron D **II.19.** The resulting residue was submitted to column chromatography (silica gel, 1000 g) using a mixture of solvents of *n*-hexane:EtOAc (0:1 to 1:0); after tlc monitoring, chromatographic fractions were combined into eight fractions. Fractions eluted with of *n*-hexane:EtOAc (85:15 to

75:25) crystallized from a mixture of solvents of *n*-hexane:EtOAc (1:1) to give 0.129 mg of β-sitosterol **II.11** and stigmasta-5,22*E*-dien-3β-ol **II.12** in the proportion (1:2).

Fraction eluted with *n*-hexane:EtOAc (80:20) was submitted to column chromatography (silica gel, 1200 g) using a mixture of solvents of *n*-hexane:EtOAc (0:1 to 1:0), after tlc monitoring, chromatographic fractions were combined into five fractions giving 2.800 g of parvifloron D **II.19** and 0.100 g of compounds **II.15** and **II.16** (ursolic and oleanolic acids).

V.3.1.4. Column chromatography B_4 **(Study of fraction PE-A6)**

The fraction PE-A6 eluted with *n*-hexane:EtOAc (25:75 to 10:90) was fractionated by column chromatography B_4 (silica gel, 2000 g) eluted with mixtures of *n*-hexane:EtOAc. The chromatographic fractions, after tlc monitoring were combined into seven fractions giving 1.3130 g of β-sitosterol **II.11** and stigmasta-5,22*E*-dien-3β-ol **II.12.** The fraction eluted with *n*-hexane:EtOAc (65:35) was submitted to column chromatography of silica gel (7734; 400 g) using as eluents mixtures of *n*-hexane:EtOAc (1:0 to 0:1) for the purification of compound **II.19** giving 5.4008 g and 0.0207 g β-sitosterol **II.11** and stigmasta-5,22*E*-dien-3β-ol **II.12** in the proportion of (1:2).

V.3.1.5. Column chromatography B_5 **(Study of fraction PE-A4)**

The fraction PE-A4 eluted with *n*-hexane:EtOAc (40:60) was fractionated in five by column chromatography B_5 (silica gel, 400 g) eluted with mixtures of *n*-hexane:EtOAc (1:0 to 0:1), giving 8.6594 g of compound **II.19.**

V.3.1.6. Column chromatography B_6 **(Study of fraction PE-A7)**

The fraction PE-A7 eluted with EtOAc was fractionated in five by column chromatography B_6 (silica gel, 150 g) eluted with mixtures of *n*-hexane:EtOAc (1:0 to 0:1), giving 0.8634 g of compound **II.19.**

V.3.2. *P. ecklonii* extracts

The six *P. ecklonii* extracts were prepared by maceration of dried plant, by means of an increasing polarity set of solvents from *n*-hexane (PE-H), ethyl acetate (PE-EA), dichloromethane (PE-D), acetone (PE-A), methanol (PE-M), and methanol-water (7:3; PE-MW) solvents extract. The first five extracts were obtained by maceration of 25 g of dried and powdered plant material with 250 ml of each solvent, under agitation at room temperature, during a week. So, after filtration and solvents evaporation 1.176 g (4.704 % of dry plant) of a hexane extract, 1.506 g (6.024 %) of an ethyl acetate extract, 1.870 g (7.480 %) of a dichloromethane extract, 4.336 g (1.320%) of a methanol extract and 1.777 g (7.108 %) of a MeOH-H_2O (7:3) extract were obtained. The acetone extract was described in section **V.3.1.**

V.4. Study of *Plectranthus porcatus* Winter & Van Jaarsv.

Plectranthus porcatus Winter & Van Jaarsv was cultivated in the *Departamento de Protecção das Plantas e de Fitoecologia* (DPPF) of *Instituto Superior de Agronomia (ISA)* of *Universidade Técnica de Lisboa* (UTL), from cuttings provided by the National Botanical Garden of Kirstenbosch (South Africa). Aerial parts of *P. porcatus* were collected in June 2007 and June and September 2008 and voucher specimens were deposited in the Herbarium João de Carvalho e Vasconcellos of the Instituto Superior de Agronomia, Lisbon (ref. 109/2008).

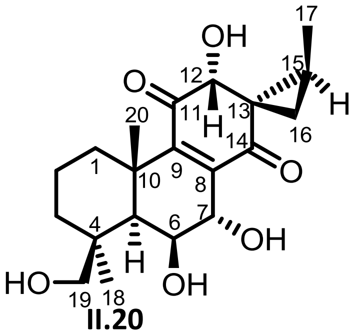
V.4.1. Plant material: study of *Plectranthus porcatus* aerial parts

Extraction and isolation

The air dried and powdered aerial parts of *P. porcatus* Winter & Van Jaarsv (99 g) were extracted with Me_2CO (5 x 0.2 L) at room temperature for 5 days. Filtration and evaporation of the solvent (under *vacuum*, 40 ˚C) from the crude extract yielded a residue of 8.46 g (8.54 % of dry material), which was subjected to column chromatography over silica gel (Merck 9385, 180 g), using mixtures of *n*-hexane:EtOAc

(1:0 to 0:1) and MeOH as eluents; after tlc monitoring, chromatographic fractions were combined into fourteen fractions (from 0.011 g to 1.403 g). The fraction (1.403 g) eluted with *n*-hexane:EtOAc (3:7) was fractionated in nine by column chromatography (silica gel, 20 g) eluted with the same mixtures of solvents, giving 0.286 g of impure **II.20** that was washed with cold *n*-hexane, and after recrystallization with MeOH-pentane, yielded 0.009 g (0.009 %, on dry plant material) of pure compound **II.20**.

V.4.1.1. (13*S*,15*S*)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione **II.20**



II.20

m.p. 200-203 ˚C, (yellowish fine needles, from MeOH-pentane);

[α]$_D^{20}$ + 218.8 ˚ (*c* 0.637, MeOH);

IR (KBr) ν_{max}: 3407, 3346, 3030, 2927, 2868.1, 1699, 1670, 1458, 1372, 1317, 1282, 1215, 1091, 1020, 909 cm^{-1};

^{1}**H RMN** (Acetone-d$_6$, 500 MHz) : δ 5.12 (br d, $J_{12\alpha\text{-OH},\text{H-12}\beta}$ = 5.5 Hz, 12α-OH), 4.99 (1H, d, $J_{6\beta\text{-OH, H-6}\alpha}$ = 1.8 Hz, HO–6β), 4.98 (br dd, $J_{19\text{-OH, 19B}}$ = 5.5 Hz, $J_{19\text{-OH, 19A}}$ = 4.4 Hz, HO–19), 4.63 (1H, *dd*, $J_{7\beta,7\alpha\text{-OH}}$ = 4.9 Hz, H-7β), 4.26 (1H, d, $J_{7\alpha\text{-OH, H-7}\beta}$ = 4.9 Hz, HO–7α), 4.24 (dd, $J_{19\text{A, 19-OH,}}$ = 4.4 Hz, H-19A), 4.23 (1H, m, $J_{\text{H-6}\alpha,\ 6\beta\text{ -OH}}$ = 1.8 Hz, $J_{\text{H-6}\alpha,\text{H-7}\beta}$ = 1.9 Hz, HO–6α), 3.82 (1H, d, $J_{\text{H-12}\beta,\ 12\alpha\text{-OH}}$ = 5.5 Hz, H-12β), 3.33 (dd, $J_{19\text{A, 19-OH,}}$ = 5.5 Hz, H-19B), 2.05 (1H, *m*, $J_{1\beta,1\alpha}$ = 13.5 Hz, $J_{1\beta,2\alpha}$ = 3.1 Hz, $J_{1\beta,2\beta}$ = 3.4 Hz, H-1β), 2.02 (1H, m, $J_{15,16\text{A}}$ = 8.5 Hz, $J_{15,16\text{B}}$ = 6.7 Hz, $J_{15,17}$ = 6.1 Hz, H-15), 1.79 (1H, ddddd, $J_{2\beta,1\alpha}$ = 13.5 Hz, $J_{2\beta,1\beta}$ = 3.4 Hz, $J_{2\beta,2\alpha}$ = 14.1 Hz, $J_{2\beta,3\alpha}$ = 13.5 Hz, H-2β), 1.74 (3H, s, CH$_3$-20), 1.72 (1H, d, $J_{5\alpha,6\alpha}$ = 1.6 Hz, H-5α), 1.55 (1H, br d, $J_{3\beta,2\alpha}$ = 3.1 Hz, $J_{3\beta,2\beta}$ = 3.4 Hz, $J_{3\beta,3\alpha}$ = 14.0 Hz, H-3β), 1.50 (1H, ddddd, $J_{2\alpha,1\alpha}$ = 3.6 Hz, $J_{2\alpha,1\beta}$ = 3.1 Hz, $J_{2\alpha,2\beta}$ = 14.1 Hz, $J_{2\alpha,3\alpha}$ = 3.6 Hz, $J_{2\alpha,3\beta}$ = 3.1

Hz,H-2α), 1.29 (1H, td, $J_{1\alpha,1\beta}$ = 13.5 Hz, $J_{1\alpha,2\alpha}$ = 3.6 Hz, $J_{1\alpha,2\beta}$ = 13.5 Hz, H-1α), 1.26 (1H, ddd, $J_{3\alpha,2\alpha}$ = 3.6 Hz, $J_{3\alpha,2\beta}$ = 13.5 Hz, $J_{3\alpha,3\beta}$ = 14.0, H-3α), 1.26 (3H, d, $J_{17,15}$ = 6.1 Hz, CH_3-17), 1.09 (3H, s, CH_3-18), 1.08 (3H, dd, $J_{16A,16B}$ = 3.7 Hz, $J_{16A,15}$ = 8.5 Hz, CH_3-16A), 0.92 (1H, dd, $J_{16B,15}$ = 6.7 Hz, $J_{16B,16A}$ = 3.7 Hz, H-16B);

^{13}C RMN (Acetone-d_6, 125 MHz): δ 38.76 (C-1, t), 20.11 (C-2, t), 40.27 (C-3, t), 39.81 (C-4, s), 50.33 (C-5, d), 70.30 (C-6, d), 67.16 (C-7, d), 142.49 (C-8, s), 156.17 (C-9, s), 40.08 (C-10, s), 200.28 (C-11, s), 78.57 (C-12, d), 37.47 (C-13, s), 198.00 (C-14, s), 21.63 (C-15, d), 27.28 (C-16 , t), 14.15 (C-17, q), 29.37 (C-18, q), 68.72 (C-19, t), 22.73 (C-20, q);

EIMS *m/z* (relative intensity, %): 364 $[M]^+$ (12), 346 $[M-H_2O]^+$ (36), 328 $[M-2H_2O]^+$ (100), 313 (31), 300 (73), 285 (72), 271 (72), 257 (38), 243 (48), 229 (55), 217 (72), 201 (50), 189 (42), 177 (40), 151 (36), 123 (36), 109 (39), 95 (50), 91 (58), 77 (41), 67 (33), 55 (66).

V.5. Study of *Plectranthus saccatus* Benth.

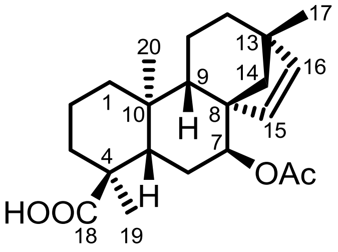
Plectranthus saccatus Benth. was cultivated in the Hortum of the *Departamento de Protecção das Plantas e de Fitoecologia* (DPPF) of *Instituto Superior de Agronomia* (ISA) of *Universidade Técnica de Lisboa* (UTL), from cuttings provided by the Herbarium of the National Botanical Garden of Kirstenbosch (South Africa). Aerial parts of *P. saccatus* were collected in 2007 and voucher specimens were deposited in the *Herbarium* João de Carvalho e Vasconcellos of *Instituto Superior de Agronomia*, Lisbon (ref. 109/2008).

V.5.1. Plant material: study of *Plectranthus saccatus* aerial parts

Extraction and isolation

The air dried and powdered aerial parts of *P. saccatus* Benth (100 g) were stored at 5 ˚C and then extracted with Me_2CO (8 x 0.6 L) at room temperature for 10 days. Filtration and evaporation of the solvent (under *vacuum*, 40 ˚C) from the crude extract yielded a residue of 6.89 g (6.89 % of dry material), which was subjected to column chromatography over silica gel (Merck 9385, 75 g), using mixtures of *n*-hexane:EtOAc (1:0 to 0:1) and MeOH as eluents, after tlc monitoring, chromatographic eluates were combined into six fractions (weights from 0.302 g to 2.419 g). The fraction (2.419 g) eluted with light petroleum:EtOAc (1:1) was filtered through a pad of charcoal and celite (1:1) to eliminate chlorophylls and the resulting residue was fractionated in four by column chromatography (silica gel Merck 7734, 8 g) eluted with mixtures of light petroleum:EtOAc (7:3), giving 47 mg of impure **II.22** that, after recrystallization from light petroleum, yielded 0.024 g (0.024 % of the plant material) of pure compound **II.22.**

V.5.1.1. *Ent*-7α-acetoxy-15-beyeren-18-oic acid II.22



II.22

m.p. 240-242 ˚C, (colourless rectangular prisms, from petroleum ether, *n*-hexane);

[α] $^{20}_{D}$ + 43.5 ˚ (c̲ 0.299, $CHCl_3$);

IR (KBr) ν_{max}: 3413 br (COOH), 3038 (C=C), 2949, 2866, 1728 (C=O, OAc), 1695 (C=O, COOH), 1452, 1387, 1267 (OAc), 1246 (OA cor COOH), 1184, 1022, 950, 858, 756, 745 cm^{-1};

^{1}H RMN ($CDCl_3$, 500 MHz) : δ 5.55 (1H, dd, $J_{16,15}$ = 5.8 Hz, $J_{16,\text{long range}}$ = 1.0 Hz, H-16), 5.53 (1H, d, $J_{15,16}$ =5.8 Hz, H-15), 4.76 (1H, t, $J_{7\alpha,6\alpha}$ = $J_{7\alpha,6\beta}$ = 2.7 Hz, H-7α), 2.24 (1H, dd, $J_{5\beta,6\alpha}$ = 12.8 Hz, $J_{5\beta,6\beta}$ = 1.5 Hz, H-5β), 2.04 (3H, s, 7β-OAc), 1.72 (1H, m, H-6α), 1.72 (1H, td, $J_{3\beta,2\alpha}$ = $J_{3\beta,3\alpha}$ = 14.7 Hz, $J_{3\beta,2\beta}$ = 2.5 Hz, H-3β), 1.64 (1H, m, $J_{1\alpha,2\alpha}$ = $J_{1\alpha,1\beta}$ = $J_{1\alpha,2\beta}$ = *, H-1α), 1.64 (1H, m, $J_{3\alpha,2\alpha}$ = $J_{3\alpha,2\beta}$ = $J_{3\alpha,3\beta}$ = *, H-3α), 1.53 (2H, m, $J_{2\alpha,1\alpha}$ = $J_{2\alpha,2\beta}$ = $J_{2\alpha,3\alpha}$ =

$J_{2\alpha,2\beta}$ = *, $J_{2\alpha,3\beta}$ = 14.7 Hz, $J_{2\beta,1\alpha} = J_{2\beta,1\beta} = J_{2\beta,2\alpha} = J_{2\beta,3\alpha}$ =* , $J_{2\beta,3\beta}$ = 2.5 Hz, H-2α and H-2β), 1.53 (1H, m, $J_{11\beta,11\alpha} = J_{11\beta,9\beta} = J_{11\beta,12\alpha} = J_{11\beta,12\beta}$ =*, H-11β), 1.49 (1H, m, $J_{6\beta,5\beta}$ = 1.5 Hz, $J_{6\beta,7\alpha}$ = 2.7 Hz, $J_{2\beta,6\alpha}$ =*, H-6β), 1.45 (1H, m, $J_{9\beta,11\beta} = J_{9\beta,11\alpha}$ = *, H-9β), 1.44 (1H, dd, $J_{14\alpha,14\beta}$ = 14.0 Hz, $J_{14\alpha,12\alpha}$ = 2.1 Hz, H-14α), 1.28 (1H, m, $J_{11\alpha,12\alpha} = J_{11\alpha,9\beta} = J_{11\alpha,11\beta} = J_{11\alpha,12\beta}$ = *, H-11α), 1.26 (2H, m, $J_{12\alpha,11\alpha} = J_{12\alpha,11\beta} = J_{12\alpha,12\beta}$ = *, $J_{12\alpha,14\alpha}$ = 2.1 Hz, $J_{12\beta,11\alpha} = J_{12\beta,11\beta} = J_{12\beta,12\alpha}$ = *, H-12α and H-12β), 1.26 (1H, d, $J_{14\beta,14\alpha}$ = 9.1 Hz, H-14β), 1.15 (3H, s, Me-19), 1.02 (1H, m, $J_{1\beta,1\alpha} = J_{1\beta,2\alpha} = J_{1\beta,2\beta}$ = *, H-1β), 1.01 (3H, s, Me-17), 0.79 (3H, s, Me-20);

*overlapped signal;

^{13}C RMN ($CDCl_3$, 125 MHz): δ 38.0 (C-1, t), 17.6 (C-2, t), 36.8 (C-3, t), 46.7 (C-4, s), 42.4 (C-5, d), 27.4 (C-6, t), 76.0 (C-7, d), 52.9 (C-8, s), 48.1 (C-9, d), 36.6 (C-10, s), 19.6 (C-11, t), 32.7 (C-12, t), 43.9 (C-13, s), 56.6 (C-14, t), 132.1 (C-15, d), 138.1 (C-16 , d), 24.7 (C-17, q), 184.1 (C-18, s), 16.4 (C-19, q), 14.9 (C-20, q), 170.8 (CO, s), 21.1 (CH_3, q);

EIMS *m/z* (relative intensity, %): 360 $[M]^+$ (0.5), 318 (2), 300 (42), 285 (17), 272 (100), 255 (12), 239 (13), 185 (14), 157 (19), 146 (65), 131 (29), 118 (48), 105 (34), 91 (30), 79 (22), 67 (12), 55 (17);

HRMS (ESI-HRMS negative): m/z 359.2229 $[M-H]^-$; Calculated for $C_{22}H_{31}O_4$: 359.2228.

V.6. Synthesis of diterpenoid derivatives

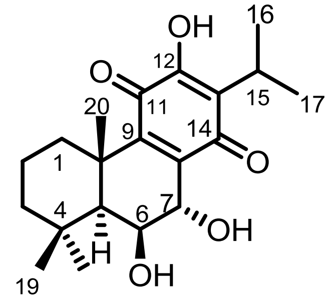
V.6.1. 7α-Acetoxy-6β-hydroxyroyleanone III.1 derivatives

V.6.1.1. Basic hydrolysis of 7α-acetoxy-6β-hydroxyroyleanone III.1

To a solution of 7α-acetoxy-6β-hydroxyroyleanone **III.1** (70 mg; 0.1792 mmol) in ethanol (1 mL), 2.5 mL of a potassium hydroxide solution (8%; w/v; 3.59 mmol) were added at room temperature, under agitation for 30 minutes. The reaction was followed by tlc (AcOEt : n-hexane; 3:7).

After this time the reaction mixture was poured into water (10 mL) and extracted with AcOEt (10 mL x 5). The combined organic layers were dried over anhydrous sodium sulphate filtered and evaporated in the rotavapor. The yellow residue obtained was purified by thin layer chromatography (silica gel plates Merck nº 7747 eluted with 7:3 n-hexane:AcOEt) to give 6β,7α-dihydroxyroyleanone **III.2** quantitatively (60.0 mg; 0.1722 mmol) as yellow crystals. This reaction was repeated under similar conditions and the same yield was obtained.

6β,7α-dihydroxyroyleanone III.2



III.2

Yellow needles;

m.p. 200-201 °C (*n*-hexane:AcOEt), 203-205°C (Hensch M. et al., 1975);

IR (KBr) ν_{max}: 3568, 3529, 3448, 3348, 2958, 2854, 2366, 1737, 1720, 1706, 1653, 1629, 1606, 1461, 1390, 1378, 1323, 1255, 1165, 1103, 1030, 998, 618, 493 cm^{-1};

^{1}**H NMR** (CDCl$_3$, 400 MHz): δ 7.27 (1H, s, OH-12), 7.25 (1H, s, OH-6β), 4.51 (1H, dd, $J_{7\beta,OH}$ = 3.3 Hz, $J_{7\beta,6\alpha}$ = 2.0 Hz, H-7β), 4.45 (1H, dd, $J_{6\alpha,5\alpha}$ = 4.0 Hz, $J_{6\alpha,7\beta}$ = 2.0 Hz, H-6α), 3.16 (1H, sept, $J_{15,16(17)}$ = 7.1 Hz, H-15), 2.93 (1H, d, $J_{OH,7\beta}$ = 3.3 Hz, OH-7α), 2.59 (1H, dddd, $J_{1\beta,1\alpha}$ = 12.8 Hz, $J_{1\beta,2\alpha}$ = 3.5 Hz, $J_{1\beta,2\beta}$ = 3.5 Hz, $J_{1\beta,3\beta(W)}$ = 1.3 Hz, H-1β), 1.83 (1H, ddddd, $J_{2\beta,1\alpha}$ = 13.4 Hz, $J_{2\beta,1\beta}$ = 3.5 Hz, $J_{2\beta,2\alpha}$ = 13.9 Hz, $J_{2\beta,3\alpha}$ = 13.4 Hz, $J_{2\beta,3\beta}$ = 3.4 Hz, H-2β), 1.60 (3H, s, Me-20), ~1.56 (1H, *, $J_{2\alpha,1\alpha}$ = 3.8 Hz, $J_{2\alpha,1\beta}$ = 3.5 Hz, $J_{2\alpha,2\beta}$ = 13.9 Hz, $J_{2\alpha,3\alpha}$ = 4.1 Hz, $J_{2\alpha,3\beta}$ = 3.4 Hz, H-2α), 1.47 (1H, dddd, $J_{3\beta,2\alpha}$ = 3.4 Hz, $J_{3\beta,2\beta}$ = 3.4 Hz, $J_{3\beta,3\alpha}$ = 13.4 Hz, $J_{3\beta(W),1\beta}$ = 1.3 Hz, H-3β), 1.40 (1H, d, $J_{5\alpha,6\alpha}$ = 4.0 Hz, H-5α), 1.25 (3H, s, Me-19), 1.22 (1H, ddd, $J_{3\alpha,2\alpha}$ = 4.1 Hz, $J_{3\alpha,2\beta}$ = 13.4 Hz, $J_{3\alpha,3\beta}$ = 13.4 Hz, H-3α), 1.22$^{\#}$ (3H, d,

$J_{16(17),15}$ = 7.1 Hz, Me-16), 1.21$^{\#}$ (3H, d, $J_{17(16),15}$ = 7.1 Hz, Me-17), 1.18 (1H, ddd, $J_{1\alpha,1\beta}$ = 12.8 Hz, $J_{1\alpha,2\alpha}$ = 3.8 Hz, $J_{1\alpha,2\beta}$ = 13.4 Hz, H-1α), 1.04 (3H, s, Me-18);

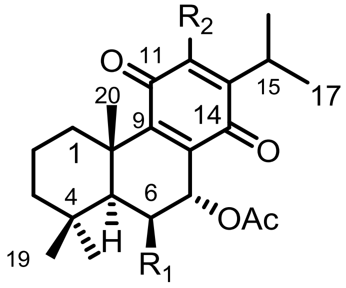
*overlapped signal, $^{\#}$ Interchangeable signals;

^{13}**C NMR** ($CDCl_3$, 100 MHz): δ 38.5 (C-1, t); 19.0 (C-2, t); 42.3 (C-3, t); 33.8 (C-4, s); 49.5 (C-5, d); 69.4 (C-6, d); 69.2 (C-7, d); 141.0 (C-8, s); 147.5 (C-9, s); 38.6 (C-10, s); 183.5 (C-11, s); 151.1 (C-12, s); 124.3 (C-13, s); 189.1 (C-14, s); 24.3 (C-15, d); 19.9$^{\#}$ (C-16, q); 19.8$^{\#}$ (C-17, q); 33.5 (C-18, q); 24.0 (C-19, q); 21.6 (C-20, q);

$^{\#}$ Interchangeable signals.

V.6.1.2. Synthesis of 7α-acetoxy-6β,12-dihydroxy-8,12-abietadiene-11,14-dione III.1 esters

The natural diterpene 7α-acetoxy-6β-hydroxyroyleanone **III.1** was re-isolated from an acetone extract of *P. grandidentatus.* The 7α-acetoxy-6β-hydroxyroyleanone **III.1** was used as starting material for the esterification reactions. Eleven royleanone derivatives **III.3-III.13** were prepared by esterification at C-6 and/or C-12 positions (**Figure V.1**).



	R_1	R_2
III.3	BzO	BzO
III.4	4-Cl-C_6H_4COO	4-Cl-C_6H_4COO
III.5	OH	4-Me-C_6H_4COO
III.6	OH	4-Cl-C_6H_4COO
III.7	OH	4-NO_2-C_6H_4COO
III.8	4-NO_2-C_6H_4COO	OH
III.9	AcO	AcO
III.10	$MeCH_2$COO	$MeCH_2$COO
III.11	AcO	OH
III.12	$MeCH_2$COO	OH
III.13	$Me(CH_2)_2$COO	OH

Figure V.1. Ester derivatives **III.3-III.13** of 7α-acetoxy-6β-hydroxyroyleanone **III.1.**

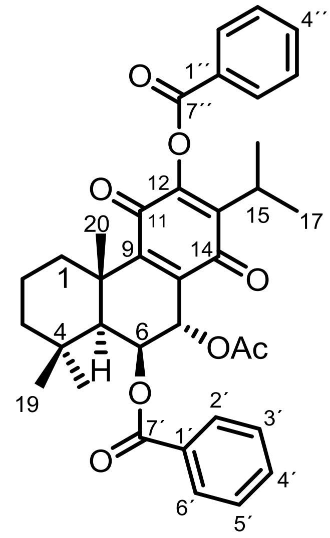
Hemisynthesis of royleanone derivatives III.3-III.13

A solution of 7α-acetoxy-6β-hydroxyroyleanone **III.1** in pyridine (Py) CH_2Cl_2 if necessary and the suitable benzoyl chloride or appropriate alkyl anhydride, was allowed to stand until total disappearance of **III.1** (tlc analysis). The reaction mixture was then diluted with CH_2Cl_2 and successively washed with water and NaOH solution (5%). The organic layer was dried with anhydrous Na_2SO_4. The residue was purified by preparative thin layer chromatography over silica gel plates eluted with CH_2Cl_2:CH_3COCH_3 (98:2) to afford **III.3-III.13** (Martínez-Vázquez M. et al, 2004; Rijo P. et al., 2010). The conditions for obtaining each one of the derivatives were as follows and shown in **Table V.14**.

Table V.14. Preparation of the 6β-hydroxy-7α-acetoxyroyleanone **III.1** derivatives **III.3-III.13**.

Starting material **III.1** mg; mmol	Benzoyl chloride/ alkyl anhydride	Reactional conditions	Product (yield)
30.0; 0.077	benzoyl chloride (2 mL; 17 mmol)	Py (0.5 ml), r.t., 3 days,	**III.3** (22.4 %)
20.0; 0.051	4-chlorobenzoyl chloride (0.032 mL; 0.25 mmol)	Py (2 ml), CH_2Cl_2 (2 ml), r.t., 7 days	**III.4** (66.4 %) and **III.6** (27.9 %)
32.2; 0.082	4-methoxybenzoyl chloride (0.027 mL; 0.24 mmol)	Py (2 ml), CH_2Cl_2 (2 ml), r.t, 24 h	96.6 % of **III.5**
30.3; 0.078	4-nitrobenzoyl chloride (141 mg; 0.76 mmol)	Py (2 ml), CH_2Cl_2 (2 ml), r.t., 40 min.	**III.7** (16.8 %) and **III.8** (11.9 %)
30.6; 0.078	acetic anhydride (2 mL; 21 mmol)	Py (2 ml), CH_2Cl_2 (1 ml), r.t., 24 h	**III.9 (**65.6%) and **III.11** (8.5 %)
40.5; 0.104	propionic anhydride (1 mL; 7.8 mmol)	Py (1 ml), 0˚ C, 7 days	73.5 % of **III.10** and **III.12** (8.6 %)
15.0; 0.038	butyric anhydride (1 mL; 6.1 mmol)	Py (1 ml), 0˚ C, 24 h	**III.13** (35.2%)

V.6.1.2.1. 7α-Acetoxy-6β-benzoyloxy-12-*O*-benzoylroyleanone III.3



III.3

Yellow quadrangular plates (EtOAc–*n*-pentane);

m.p. 238-241 ˚C;

$[\alpha]_D^{18}$ = –29.2 ˚ (*c* = 0.24, CHCl$_3$);

IR (KBr) ν_{max}: 3062, 2962, 2933, 2867, 1755, 1738, 1726, 1666, 1612, 1601, 1452, 1371, 1315, 1269, 1220, 1177, 1138, 1093, 1061, 1016, 932, 887, 808, 754, 712 cm^{-1};

^{1}H-NMR (400 MHz, CDCl$_3$): 8.15 (2H, dt, $J_{2',3'}$ = 7.6 Hz, $J_{2',4'}$ = 2.0 Hz, H–2′ and H–6′, 6–OBz), 7.99 (2H, dt, $J_{2',3'}$ = 7.2 Hz, $J_{2',4'}$ = 1.2 Hz, H-2′ and H–6′, 12–OBz), 7.69–7.40 (6H, complex signal, H–3′, H–4′, H–5′, 6– and 12–OBz), 5.90 (1H, dd, $J_{7\beta,6\alpha}$ = 2.0 Hz, $J_{7\beta,5\alpha}$ = 0.9 Hz, Hβ–7), 5.77 (1H, dd, $J_{6\alpha,7\beta}$ = 2.0 Hz, $J_{6\alpha,5\alpha}$ = 1.6 Hz, Hα–6), 3.17 (1H, sept, $J_{15,16(17)}$ = 7.1 Hz, H–15), ~2.58 (1H, *, Hβ–1), 2.10 (3H, *s*, OAc-7*α*), ~1.78 (1H, *, Hβ–2), 1.77 (3H, s, Me-20), 1.69 (1H, br d, $J_{5\alpha,6\alpha}$ = 1.6 Hz, Hα–5), 1.58 (1H, *, H*α*–2), 1.49 (1H, ddd, $J_{3\beta,3\alpha}$ = 13.2 Hz, $J_{3\beta,2\alpha}$ = 3.6 Hz, $J_{3\beta,2\beta}$ = 2.8 Hz, Hβ–3), 1.29 (1H, td, $J_{1\alpha,1\beta}$ = $J_{1\alpha,2\beta}$ = 13.2 Hz, $J_{1\alpha,2\alpha}$ = 3.8 Hz, H*α*–1), ~1.38 (1H, *, H*α*–3), 1.21 (3H, d, $J_{17,15}$ = 7.1 Hz, Me-17), 1.19 (3H, d, $J_{16,15}$ = 7.1 Hz, Me-16), 1.06 (3H, s, Me-18), 0.99 (3H, *s*, Me-19);

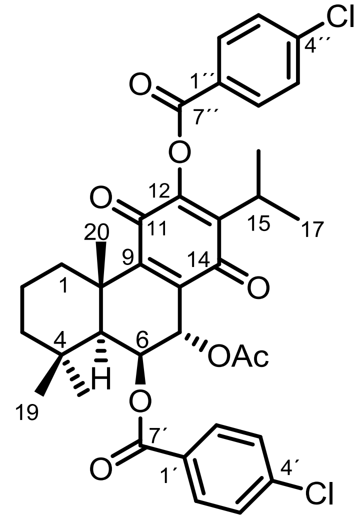
*Overlapped signals;

^{13}C NMR (100 MHz, CDCl$_3$): 185.33 (C-14, s); 180.00 (C-11, s); 168.13 (7*α*–OAc, s); 165.33 (C-7′, 6–OBz, s); 164.00 (C-7′, 12–OBz, s); 152.50 (C-9, s); 150.00 (C-12, s);

140.00 (C-13, s); 136.20 (C-8, s); 134.30 (C-4’, 12–OBz, d); 133.22 (C-4’, 6–OBz, s); 130.51 (C-3’ and C-5’, 12–OBz, d); 129.87 (C-3’ and C-5’, 6–OBz, d); 129.71 (C-1’, 6–OBz, s); 128.81 (C-2’ and C-6’, 12–OBz, d); 128.47 (C-2’ and C-6’, 6–OBz, d); 127.91 (C-1’, 12–OBz, s); 68.43 (C-6, d); 65.25 (C-7, d); 49.31 (C-5, d); 42.48 (C-3, t); 38.77 (C-10, s); 38.37 (C-1, t); 33.76 (C-4, s); 33.33 (C-18, q); 25.16 (C-15, d); 23.21 (C-19, q); 22.20 (C-20, q); 20.84 (7α- OAc, q); 20.36 (C-16, q); 20.00 (C-17, q); 18.83 (C-2, t);

EI-MS: 539 ($[M–AcO]^+$, 1), 510 (1), 451 (2), 434 ($[M–AcO–C_6H_5CO]^+$, 3), 406 (20), 391 (5), 313 (7), 284 (6), 269 (12), 105 ($[C_6H_5CO]^+$, 100), 77 (15); ($C_{36}H_{38}O_8$, M*r* 598).

V.6.1.2.2. 7α-Acetoxy-6β-(4-chloro)benzoyloxy-12-*O*-(4-chloro)-benzoyl-royleanone III.4



III.4

Yellow amorphous powder;

[α]$_D^{22}$ = −40.8 ˚ (*c* = 0.262, $CHCl_3$);

IR (KBr) ν_{max}: 3074, 3097, 2961, 2932, 2867, 1751, 1726, 1669, 1594, 1488, 1402, 1371, 1268, 1216, 1173, 1137, 1092, 1064, 1012, 931, 887, 847, 757, 732 cm^{-1};

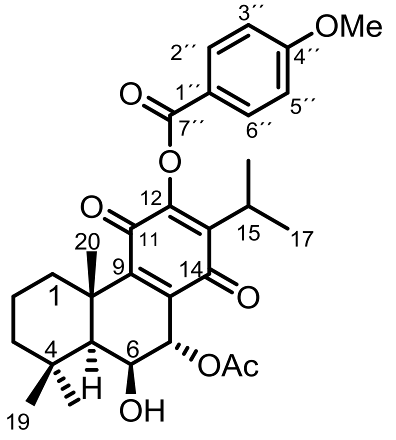
^{1}H-NMR (400 MHz, $CDCl_3$): 8.08 (2H, d, J_o = 8.6 Hz, H–2’ and H–6’, 12–OBzCl), 7.92 (2H, d, J_o = 8.6 Hz, H–2’ and H–6’, 6–OBzCl), 7.51 (2H, d, J_o = 8.6 Hz, H–3’ and H–5’, 12–OBzCl), 7.39 (2H, d, J_o = 8.6 Hz, H–3’ and H–5’, 6–OBzCl), 5.88 (1H, d, $J_{7\beta,6\alpha}$ = 1.6 Hz, Hβ–7), 5.76 (1H, t, $J_{6\alpha,7\beta} = J_{6\alpha,5\alpha}$ = 1.6 Hz, Hα–6), 3.17 (1H, sept, $J_{15,16(17)}$ = 7.0 Hz, H–15), 2.58 (1H, *, Hβ–1), 2.10 (3H, s, 7α–OAc), 1.79 (1H, qt, $J_{2\beta,1\alpha} = J_{2\beta,2\alpha} = J_{2\beta,3\alpha}$ = 14.0 Hz, $J_{2\beta,1\beta} = J_{2\beta,3\beta}$ = 3.7 Hz, Hβ–2), 1.74 (3H, s, Me-20), 1.70 (1H, d, $J_{5\alpha,6\alpha}$ = 1.6 Hz, Hα–5), 1.60 (1H, *dquint*, $J_{2\alpha,2\beta}$ = 14.0 Hz, $J_{2\alpha,1\alpha} = J_{2\alpha,1\beta} = J_{2\alpha,3\alpha} = J_{2\alpha,3\beta}$ = 3.6 Hz, H*α*–2); 1.48 (1H, dtd, $J_{3\beta,3\alpha}$ = 13.4 Hz, $J_{3\beta,2\beta}$ = 3.7 Hz, $J_{3\beta,2\alpha}$ = 3.6 Hz, $J_{3\beta,1\beta}$ = 1.0 Hz, Hβ–3), 1.30 (1H, *, Hα–1), 1.28 (1H, *, H*α*–3), 1.21 (3H, d, $J_{16,15}$ = 7.0 Hz, Me-16)), 1.19 (3H, d, $J_{17,15}$ = 7.0 Hz, Me-17), 1.06 (3H, s, Me-18), 0.97 (3H, s, Me-19);

*overlapped signals;

^{13}C NMR (100 MHz, $CDCl_3$): 185.21 (C-14, s); 179.70 (C-11, s); 168.13 (OAc-7α, s); 164.52 (C-7′, 6–OBzCl, s), 163.19 (C-7′, 12–OBzCl, s); 152.40 (C-9, s); 149.50 (C-12, s); 141.02 (C-4′, 12–OBzCl, s); 139.80 (C-13, s); 139.79 (C-4′, 6–OBzCl, s); 135.67 (C-8, s); 131.84 (C-2′ and C-6′, 12–OBzCl, d); 131.22 (C-2′ and C-6′, 6–OBzCl, d); 129.24 (C-3′ and C-5′, 12–OBzCl, d); 128.88 (C-3′ and C-5′, 6–OBzCl, d); 128.14 (C-1′, 6–OBzCl, s); 126.36 (C-1′, 12–OBzCl, s); 68.72 (C-6, d); 65.19 (C-7, d); 49.30 (C-5, d); 42.47 (C-3, t); 38.78 (C-10, s); 38.37 (C-1, t); 33.77 (C-4, s); 33.29 (C-18, q); 25.23 (C-15, d); 23.20 (C-19, q); 21.23 (C-20, q); 20.37 (C-16, q); 20.21 (C-17, q); 18.81 (C-2, t);

EI-MS: 607 ([M–AcO]$^+$, 3), 468 ([M–AcO–OC–C_6H_5Cl]$^+$, 3), 440 (9), 313 (6), 269 (9), 139 (OC–C_6H_5Cl , 100), 111 (16); ($C_{36}H_{36}O_8Cl_2$, M*r* 666, 668, 670).

V.6.1.2.3. 7α-Acetoxy-6β-hydroxy-12-*O*-(4-methoxy)benzoylroyleanone III.5



III.5

Yellow fine needles (EtOAc–*n*-pentane);

m.p. 215–218 ˚C;

$[\alpha]_{D}^{18}$ = +43.9˚ (*c* = 0.353, $CHCl_3$);

IR (KBr) ν_{max}: 3474, 2962, 2931, 2867, 1745, 1724, 1668, 1607, 1581, 1512, 1463, 1371, 1256, 1221, 1168, 1141, 1168, 1141, 1100, 1063, 1019, 1002, 963, 936, 898, 843, 816, 759, 746 cm^{-1};

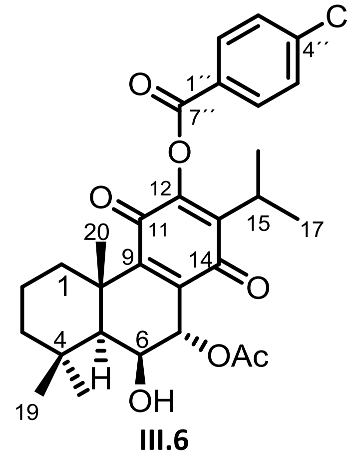
^{1}H-NMR (400 MHz, $CDCl_3$): 8.09 (2H, *d*, $J_{2',3'(6',5')}$ = 8.9 Hz, H–2’ and H–6’); 6.98 (2H, *d*, $J_{3',2'(5',6')}$ = 8.9 Hz, H–3’ and H–5’); 5.67 (1H, *d*, $J_{7\beta,6\alpha}$ = 1.7 Hz, H–7β); 4.32 (1H, *dd*, $J_{6\alpha,5\alpha}$ = 1.9 Hz, $J_{6\alpha,7\beta}$ = 1.7 Hz, Hα–6); 3.89 (3H, *s*, OMe-8’); 3.17 (1H, *sept*, $J_{15,16(17)}$ = 7.0 Hz, H–15); 2.49 (1H, br *m*, Hβ–1); 2.06 (3H, *s*, OAc-7α); 1.79 (1H, *qt*, $J_{2\beta,1\alpha} = J_{2\beta,2\alpha} = J_{2\beta,3\alpha}$ = 13.7 Hz, $J_{2\beta,1\beta} = J_{2\beta,3\beta}$ = 3.4 Hz, Hβ–2); 1.62 (3H, *s*, Me-20); 1.53 (1H, *dquint*, $J_{2\alpha,2\beta}$ = 13.7 Hz, $J_{2\alpha,1\alpha} = J_{2\alpha,1\beta} = J_{2\alpha,3\alpha} = J_{2\alpha,3\beta}$ = 3.7 Hz, Hα–2); 1.45 (1H, *ddd*, $J_{3\beta,3\alpha}$ = 13.2 Hz, $J_{3\beta,2\alpha}$ = 3.7 Hz, $J_{3\beta,2\beta}$ = 3.4 Hz, Hβ–3); 1.36 (1H, *d*, $J_{5\alpha,6\alpha}$ = 1.9 Hz, Hα–5); 1.23 (1H, *, Hα–1); 1.22 (3H, *d*, $J_{17,15}$ = 7.1 Hz, Me-17); 1.21 (3H, *s*, Me-19); 1.19 (1H, *, Hα–3); 1.19 (3H, *d*, $J_{16,15}$ = 7.0 Hz, Me-16); 0.94 (3H, *s*, Me-18);

*overlapped signals;

^{13}C NMR (100 MHz, $CDCl_3$): 185.90 (C–14, s); 179.89 (C–11, s); 169.71 (7α–OAc, s); 164.38 (C-7´´, s); 163.66 (C-4´´, s); 153.04 (C-9, s); 149.90 (C-12, s); 139.51 (C-13, s); 135.52 (C-8, s); 132.72 (C-2´´ and C-6´´, d); 120.21 (C-1´´, s); 114.05 (C-3´´ and C-5´´, d); 68.88 (C-7, d); 67.26 (C-6, d); 55.57 (C-8’, OMe, q); 49.80 (C-5, d); 42.28 (C-3, t); 38.86 (C-10, s); 38.30 (C-1, t); 33.71 (C-18, q); 33.52 (C-4, s); 25.07 (C-15, d); 23.83 (C-19, q); 21.75 (C-20, q); 20.91 (OAc-7α, q); 20.44 (C-16, q); 20.18 (C-17, q); 18.86 (C-2, t);

EI-MS: 524 ($[M]^+$, 0.5), 496 ($[M–CO]^+$, 1), 464 ($[M–AcOH]^+$, 0.5), 436 ($[M–AcOH–CO]^+$, 3), 137 (2),136 (17), 135 ($[H_3COC_6H_4CO]^+$, 100), 93 (1), 92 (3), 91 (1), 79 (1), 78 (1), 77 (6), 55(1); ($C_{30}H_{36}O_8$, M*r* 524).

V.6.1.2.4. 7α-Acetoxy-6β-hydroxy-12-*O*-(4-chloro)benzoylroyleanone III.6



III.6

Yellow fine needles (EtOAc–*n*-pentane);

m.p. 221-223 ˚C;

[α]$^{18}_{\mathrm{D}}$ = +44.8˚ (*c* = 0.355, $CHCl_3$);

IR (KBr) ν_{max}: 3485, 2962, 2929, 2867, 1749, 1732, 1667, 1596, 1461, 1371, 1252, 1220, 1141, 1094, 1066, 1008, 896, 844, 749 cm^{-1};

^{1}H-NMR (400 MHz, $CDCl_3$): 8.07 (2H, d, J_o = 8.5 Hz, H–2’ and H–6’), 7.49 (2H, d, J_o = 8.5 Hz, H–3’ and H–5’), 5.68 (1H, dd, $J_{7\beta,6\alpha}$ = 2.0 Hz, $J_{7\beta,5\alpha}$ = 0.6 Hz, Hβ–7), 4.33 (1H, dd, $J_{6\alpha,7\beta}$ = 2.0 Hz, $J_{6\alpha,5\alpha}$ = 1.6 Hz, Hα–6), 3.17 (1H, sep*t*, $J_{15,16(17)}$ = 7.1 Hz, H–15), 2.49 (1H, m, H–1β); 2.06 (3H, s, OAc-7α), 1.92 (1H, br, OHβ–6), 1.80 (1H, qt, $J_{2\beta,1\alpha}$ = $J_{2\beta,2\alpha}$ = $J_{2\beta,3\alpha}$ = 13.6 Hz, $J_{2\beta,1\beta}$ = $J_{2\beta,3\beta}$ = 3.6 Hz, Hβ–2), 1.62 (3H, s, Me-20), 1.54 (1H, dquint, $J_{2\alpha,2\beta}$ = 13.6 Hz, $J_{2\alpha,1\alpha}$ = $J_{2\alpha,1\beta}$ = $J_{2\alpha,3\alpha}$ = $J_{2\alpha,3\beta}$ = 3.7 Hz, Hα–2), 1.45 (1H, dtd, $J_{3\beta,3\alpha}$ = 13.3 Hz, $J_{3\beta,2\alpha}$ = 3.7 Hz, $J_{3\beta,2\beta}$ = 3.6 Hz, $J_{3\beta,1\beta}$ = 0.9 Hz, Hβ–3), 1.36 (1H, dd, $J_{5\alpha,6\alpha}$ = 1.6 Hz, $J_{5\alpha,7\beta}$ = 0.6 Hz, Hα–5), 1.24 (3H, s, Me-19), 1.24 (1H, *, Hα–1), 1.21 (6H, d, $J_{16(17),15}$ = 7.1 Hz, Me-16 and Me-17), 1.20 (1H, *, Hα–3), 0.94 (3H, s, Me-18);

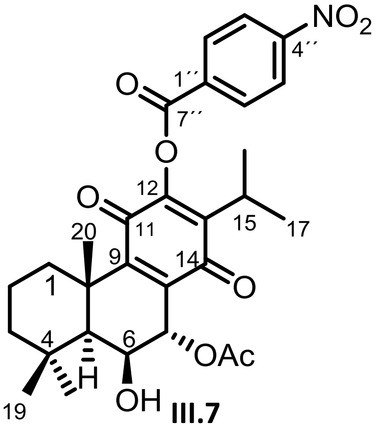
*overlapped signals;

^{13}C NMR (100 MHz, $CDCl_3$): 185.69 (C-14, s); 179.55 (C-11, s); 169.67 (OAc-7α, s); 163.18 (C-7´´, s); 152.50 (C-9, s); 149.80 (C–12, s); 140.89 (C-4´´, s); 139.50 (C-13, s); 135.74 (C-8, s), 131.84 (C-2´´ and C-6´´, d); 129.18 (C-3´´ and C-5´´, d); 126.45 (C-1´´, s); 68.85 (C-7, d); 67.22 (C-6, d); 49.77 (C-5)); 42.26 (C-3, t); 38.93 (C-10, s); 38.33 (C-1, t);

33.72 (C-4, s); 33.51 (C-18, q); 25.18 (C-15, d); 23.83 (C-19, q); 21.73 (C-20, q); 20.91 (OAc-7α, q); 20.44 (C-16, q); 20.23 (C-17, q), 18.87 (C-2, t);

EI-MS: 486 ([M−CH_2=C=O]$^+$, 1), 470 ([M−AcOH]$^+$, 3), 468 ([M−AcOH]$^+$, 7), 329 (9), 302 (8), 301 (8), 283 (6), 269 (7), 141 (43), 140 (10), 139 (100), 111 (14), 91 (2), 77 (1); ($C_{29}H_{33}O_7Cl$, M*r* 528 and 530).

V.6.1.2.5. 7α-Acetoxy-6β-hydroxy-12-*O*-(4-nitro)benzoylroyleanone III.7



III.7

Yellow rectangular plates (EtOAc–*n-pentane*);

m.p. 217–219 ˚C;

[α]$^{18}_{D}$ = +36.2˚ (*c* = 0.174, $CHCl_3$);

IR (KBr) ν_{max}: 3452, 3109, 3056, 2956, 2933, 2867, 1752, 1733, 1668, 1608, 1530, 1462, 1369, 1347, 1320, 1252, 1218, 1140, 1101, 1070, 1011, 966, 932, 897, 869, 854, 712 cm^{-1};

^{1}H-NMR (400 MHz, $CDCl_3$): 8.37 (2H, d, $J_{2',3'(6',5')}$ = 9.0 Hz, H–2’and H–6’), 8.32 (2H, d, $J_{3',2'(5',6')}$ = 9.0 Hz, H–3’ and H–5’), 5.68 (1H, d, $J_{7\beta,6\alpha}$ = 1.7 Hz, H*β*–7)), 4.34 (1H, dd, $J_{6\alpha,5\alpha}$ = 1.9 Hz, $J_{6\alpha,7\beta}$ = 1.7 Hz, H*α*–6), 3.18 (1H, sept, $J_{15,16(17)}$ = 7.1 Hz, H–15), 2.49 (1H, m, Hβ–1), 2.07 (3H, s, OAc-7*α*), 1.81 (1H, qt, $J_{2\beta,1\alpha}$ = $J_{2\beta,2\alpha}$ = $J_{2\beta,3\alpha}$ = 13.6 Hz, $J_{2\beta,3\beta}$ = 3.4 Hz, $J_{2\beta,1\beta}$ = 3.2 Hz, Hβ–2), 1.62 (3H, *s*, Me-20), 1.55 (1H, dquint, $J_{2\alpha,2\beta}$ = 13.6 Hz, $J_{2\alpha,3\beta}$ = 3.8 Hz, $J_{2\alpha,1\alpha}$ = $J_{2\alpha,1\beta}$ = $J_{2\alpha,3\alpha}$ = 3.7 Hz, Hα–2), 1.46 (1H, ddd, $J_{3\beta,3\alpha}$ = 13.4 Hz, $J_{3\beta,2\alpha}$ = 3.8 Hz, $J_{3\beta,2\beta}$ = 3.4 Hz, Hβ–3), 1.37 (1H, d, $J_{5\alpha,6\alpha}$ = 1.9 Hz, H*α*–5), ~1.23 (1H, *, Hα–1), ~1.22

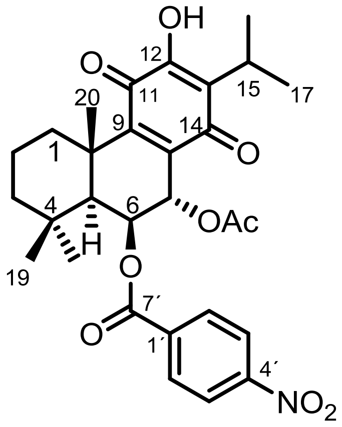
(3H, *, Me-16 and Me-17), 1.22 (3H, s, Me-19), ~1.20 (1H, *, H*α*–3), 0.95 (3H, s, Me-18);

*overlapped signals;

^{13}C NMR (100 MHz, CDCl$_3$): 185.50 (C-14, s); 179.20 (C-11, s); 169.60 (OAc-7*α*, s); 164.60 (C-7´´, s); 152.94 (C-9, s); 151.19 (C-4´´, s); 149.70 (C-12, s); 139.80 (C-13, s); 135.96 (C-8, s); 133.38 (C-1´´, s); 131.63 (C-2´´ and C–6´´, d); 123.90 (C-3´´ and C-5´´, d); 68.80 (C-7, d); 67.21 (C-6, d); 49.78 (C-5, d); 42.22 (C-3, t); 39.00 (C-10, s); 38.35 (C-1, t); 33.73 (C-18, q); 33.51 (C-4, s); 25.30 (C-15, d); 23.83 (C-19, q); 21.72 (C-20, q); 20.89 (OAc-7*α*, q); 20.40 (C-16, q); 20.43 (C-17, q); 18.96 (C-2, t);

EI-MS: 539 ([M]$^+$, 1), 521 ([M–H$_2$O]$^+$, 6), 506 ([M–H$_2$O–CH$_3$]$^+$, 2), 497 (14), 479 ([M–AcOH]$^+$, 15), 450 (10), 329 (18), 283 (15), 150 ([O$_2$N–C$_6$H$_4$CO]$^+$, 100), 120 (63), 92 (13), 76 (8), 55 (7); (C$_{29}$H$_{33}$O$_9$N, M*r* 539).

V.6.1.2.6. 7α-Acetoxy-6β-(4-nitro)benzoyloxyroyleanone III.8



III.8

Red amorphous solid;

$[\alpha]^{18}_{D}$ = −42.5° (*c* = 0.113, CHCl$_3$);

IR (KBr) ν_{max}: 3383, 3109, 3080, 3056, 2962, 2931, 2867, 1753, 1732, 1643, 1609, 1531, 1462, 1374, 1347, 1270, 1216, 1168, 1146, 1098, 1016, 981, 873, 756, 719 cm^{-1};

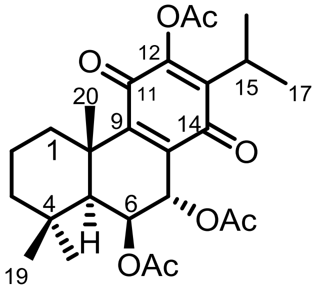
^{1}H-NMR (400 MHz, CDCl$_3$): 8.27 (2H, d, $J_{2',3'(6',5')}$ = 9.0 Hz, H–2’ and H–6’), 8.14 (2H, d, $J_{3',2'(5',6')}$ = 9.0 Hz, H–3’ and H–5’), 7.19 (1H, s, HO–12), 5.87 (1H, d, $J_{7\beta,6\alpha}$ = 1.6 Hz, Hβ–7), 5.78 (1H, t, $J_{6\alpha,5\alpha}$ = $J_{6\alpha,7\beta}$ = 1.6 Hz, Hα–6), 3.15 (1H, sept, $J_{15,16(17)}$ = 7.0 Hz, H–15), 2.75 (1H, br d, $J_{1\beta,1\alpha}$ = 12.9 Hz, Hβ–1), 2.09 (3H, s, OAc-7α), 1.84 (1H, qt, $J_{2\beta,1\alpha}$ = $J_{2\beta,2\alpha}$ = $J_{2\beta,3\alpha}$ = 14.0 Hz, $J_{2\beta,1\beta}$ = $J_{2\beta,3\beta}$ = 3.5 Hz, Hβ–2), 1.73 (3H, s, Me-20), 1.70 (1H, d, $J_{5\alpha,6\alpha}$ = 1.6 Hz, Hα–5), 1.64 (1H, dquint, $J_{2\alpha,2\beta}$ = 14.0 Hz, $J_{2\alpha,1\alpha}$ = $J_{2\alpha,1\beta}$ = $J_{2\alpha,3\alpha}$ = $J_{2\alpha,3\beta}$ = 3.4 Hz, Hα–2), 1.52 (1H, br d, $J_{3\beta,3\alpha}$ = 13.7 Hz, Hβ–3), 1.28 (1H, *, Hα–3), 1.25 (1H, *, Hα–1), 1.21 (3H, d, $J_{16,15}$ = 7.0 Hz, Me-16), 1.17 (3H, d, $J_{17,15}$ = 7.0 Hz, Me-17), 1.05 (3H, s, Me-19), 0.98 (3H, s, Me-18);

* overlapped signals;

^{13}C NMR (100 MHz, $CDCl_3$): 185.17 (C-14, s); 183.13 (C-11, s); 168.13 (OAc-7α, s); 163.58 (C-7', s); 150.92 (C-4', s); 150.63 (C-12, s); 149.23 (C-9, s); 136.74 (C-8, s); 135.04 (C-1', s); 130.91 (C-2' and C-6', d); 125.03 (C-13, s); 123.68 (C-3' and C-5', d); 69.44 (C-6, d); 64.94 (C-7, d); 49.24 (C-5, d); 42.39 (C-3, t); 38.46 (C-1, t); 38.35 (C-10, s); 33.74 (C-18, q); 33.27 (C-4, q); 24.20 (C-15, q); 23.27 (C-19, q); 22.03 (C-20, q); 20.81 (OAc-7α, q); 19.81 (C-16, q); 19.64 (C-17, q); 18.86 (C-2, t);

EI-MS: 539 ($[M]^+$, 0.5), 497 ($[M-CH_2CO]^+$, 1), 479 ($[M-AcOH]^+$, 7), 372 ($[M-HOOC-Ph-NO_2]^+$,1), 330 ($[M-CH_2CO-HOOC-Ph-NO_2]^+$, 43), 314 (100), 298 (46), 283 (20), 271 (23), 245 (68), 232 (84), 213 (25), 201 (18), 187 (24), 167 (54); ($C_{29}H_{33}NO_9$, M*r* 539).

V.6.1.2.7. 7α,6β-Diacetoxy-12-*O*-acetylroyleanone III.9



III.9

Yellow amorphous solid;

m.p., **[α]$_D$**, **IR** and **MS** data identical to those reported in literature (Hensch M. et al., 1975);

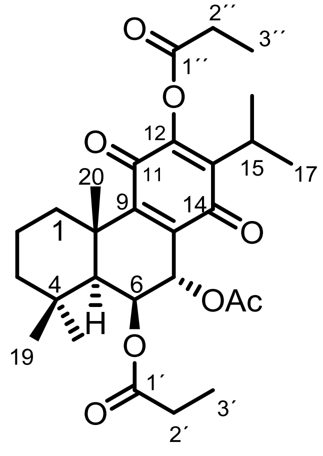
^{1}H-NMR (400 MHz, $CDCl_3$): 5.68 (1H, d, $J_{7\beta,6\alpha}$ = 2.0 Hz, Hβ–7), 5.48 (1H, dd, $J_{6\alpha,7\beta}$ = 2.0 Hz, $J_{6\alpha,5\alpha}$= 1.6 Hz, Hα–6), 3.09 (1H, sept, $J_{15,16(17)}$ = 7.0 Hz, H–15), 2.52 (1H, br d,

$J_{1\beta,1\alpha}$ = 12.5 Hz, Hβ–1), 2.34 (1H, s, OAc-12), 2.04 (3H, s, OAc-6β), 2.03 (3H, s, OAc-7*α*), 1.78 (1H, qt, $J_{2\beta,1\alpha} = J_{2\beta,2\alpha} = J_{2\beta,3\alpha}$ = 14.0 Hz, $J_{2\beta,1\beta} = J_{2\beta,3\beta}$ = 3.7 Hz, Hβ–2), 1.59 (3H, s, Me-20), 1.56 (1H, *, Hα–2), 1.52 (1H, d, $J_{5\alpha,6\alpha}$ = 1.6 Hz, Hα–5), 1.45 (1H, dddd, $J_{3\beta,3\alpha}$ = 13.4 Hz, $J_{3\beta,2\beta}$ = 3.7 Hz, $J_{3\beta,2\alpha}$ = 3.3 Hz, $J_{3\beta,1\beta}$ = 1.4 Hz, Hβ–3), 1.24 (1H, *, Hα–1), 1.23 (1H, *, Hα–3), 1.18 (3H, d, $J_{16,15}$ = 7.0 Hz, Me-16), 1.17 (3H, d, $J_{17,15}$ = 7.0 Hz, Me-17), 0.98 (3H, s, Me-18), 0.97 (3H, s, Me-19);

*Overlapped signals;

^{13}C NMR (100 MHz, $CDCl_3$): 185.37 (C-14, s); 179.67 (C-11, s); 169.04 (OAc-6β, s); 168.26 (OAc-12, s); 168.15 (OAc-7α, s); 152.22 (C-9, s); 149.28 (C-12, s); 139.42 (C-13, s); 135.67 (C-8, s); 67.24 (C-6, d); 65.25 (C-7, d); 48.95 (C-5, d); 42.39 (C-3, t); 38.93 (C-10, s); 38.31 (C-1, t); 33.58 (C-4, s); 33.18 (C-18, q); 25.17 (C-15, d); 22.93 (C-19, q); 21.44 (C-20, q); 21.27 (OAc-7α, q); 20.75 (OAc-6β, q); 20.39 (OAc-12α, q); 20.17 (C-16, q); 20.19 (C-17, q); 18.78 (C-2, t).

V.6.1.2.8. 7α-Acetoxy-6β-propionyloxy-12-*O*-propionylroyleanone III.10



III.10

Yellow rectangular plates, (EtOAc–*n*-pentane);

m.p. 137–139 ˚C;

[α]$^{18}_{D}$ = +29.9˚ (*c* = 0.257, $CHCl_3$);

IR (KBr) ν_{max}: 2962, 2940, 2873, 1776, 1764, 1744, 1668, 1612, 1462, 1375, 1275, 1210, 1172, 1136, 1113, 1079, 1027, 931, 893, 804, 750 cm^{-1};

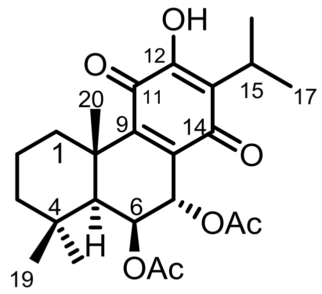
^{1}H-NMR (400 MHz, CDCl$_3$): 5.68 (1H, dd, $J_{7\beta,6\alpha}$ = 2.0 Hz, $J_{7\beta,5\alpha}$ = 0.5 Hz, Hβ–7), 5.49 (1H, br dd, $J_{6\alpha,7\beta}$ = 2.0 Hz, $J_{6\alpha,5\alpha}$ = 1.6 Hz, Hα–6), 3.09 (1H, sept, $J_{15,16(17)}$ = 7.1 Hz, H–15), 2.66 (1H, dq, $J_{2'A,2'B}$ = 17.2 Hz, $J_{2'A,3'}$ = 7.6 Hz, H2'A–12), 2.61 (1H, dq, $J_{2'B,2'A}$ = 17.2 Hz, $J_{2'B,3'}$ = 7.6 Hz, H2'B–12), 2.51 1H, (br d, $J_{1\alpha,1\beta}$ = 12.4 Hz, Hβ–1), 2.32 (1H, dq, $J_{2'A,2'B}$ = 16.8 Hz, $J_{2'A,3'}$ = 7.6 Hz, HA–2'), 2.25 (1H, q, $J_{2'B,2'A}$ = 16.8 Hz, $J_{2'B,3'}$ = 7.6 Hz, HB–2'), 2.04 (3H, s, OAc-7*α*), 1.77 (1H, dddt, $J_{2\beta,2\alpha}$ = 14.4 Hz, $J_{2\beta,1\alpha}$ = $J_{2\beta,3\alpha}$ = 13.8 Hz, $J_{2\beta,1\beta}$ = $J_{2\beta,3\beta}$ = 3.6 Hz, Hβ–2), 1.59 (3H, s, Me-20), 1.55 (1H, dquint, $J_{2\alpha,2\beta}$ = 14.4 Hz, $J_{2\alpha,1\alpha}$ = $J_{2\alpha,1\beta}$ = $J_{2\alpha,3\alpha}$ = $J_{2\alpha,3\beta}$ = 3.6 Hz, Hα–2), 1.53 (1H, dd, $J_{5\alpha,6\alpha}$ = 1.6 Hz, $J_{5\alpha,7\beta}$ = 0.5 Hz, Hα–5), 1.44 (1H, dtd, $J_{3\beta,3\alpha}$ = 13.8 Hz, $J_{3\beta,2\alpha}$ = $J_{3\beta,2\beta}$ = 3.6 Hz, $J_{1\beta,3\beta}$ = 1.6 Hz, Hβ–3), 1.27 (3H, t, $J_{3'',2''A}$ = $J_{3'',2''B}$ = 7.6 Hz, Me-3''), 1.24 (1H, *, Hα–1), 1.23 (1H, *, Hα–3), 1.17 (3H, d, $J_{16,15}$ = 7.1 Hz, Me-16), 1.16 (3H, d, $J_{17,15}$ = 7.1 Hz, Me-17), 1.11 (1H, t, $J_{3',2'A}$ = $J_{3',2'B}$ = 7.6 Hz, H-3'), 0.98 (3H, s, Me-18), 0.96 (3H, s, Me-19);

*Overlapped signals;

^{13}C NMR (100 MHz, CDCl$_3$): 185.37 (C-14, s); 179.67 (C-11, s); 172.50 (C-1', s); 171.73 (C-1'', s), 168.15 (OAc-7*α*, s); 152.22 (C-9, s); 149.28 (C-12, s); 139.42 (C-13, s); 135.67 (C-8, s); 67.24 (C-6, d); 65.25 (C-7, d); 48.95 (C-5, d); 42.39 (C-3, t); 38.93 (C-10, s); 38.31 (C-1, t); 33.58 (C-4, s); 33.18 (C-18, q); 27.81 (C-2', t), 27.21 (C-2'', t); 25.17 (C-15, d); 23.03 (C-19, q); 21.44 (C-20, q); 21.27 (OAc-7α, q); 20.17 (C-16, q); 20.19 (C-17, q); 18.78 (C-2, t), 8.86 (C-3'', q); 8.80 (C-3', q);

EI-MS: 502 ([M]$^+$, 0.3), 460 ([M$-$CH$_2$=C=O]$^+$, 1), 443 ([M$-$CH$_3$COO]$^+$, 2) 428, ([M$^+$$-CH_3CH_2$COOH], 0.5), 386 ([M$^+$$-CH_2CO-CH_3CH_2CO_2$H], 34), 330 (100), 312 (28), 302 (27), 269 (26), 57 (48); (C$_{28}$H$_{38}$O$_8$, M*r* 502).

V.6.1.2.9. 7α,6β-Diacetoxyroyleanone III.11



III.11

Yellow amorphous solid which softened at 60-70 ˚C;

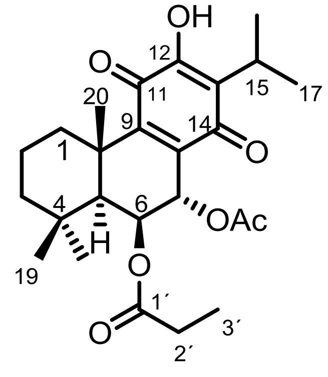
$[\alpha]^{18}_{\mathrm{D}}$ = −10.7 ˚ (c = 0.056, CHCl$_3$);

IR and **mass spectra** identical to those described previously (Chang C.-I. et al., 2005), whereas the **^{13}C NMR** spectrum is also identical to that described, but the signals at δ 21.3 (CH$_3$) and 20.8 (CH$_3$) must be assigned to C–20 and 6β–OCO$\underline{\mathrm{C}}$H$_3$ carbons, respectively;

^{1}H-NMR (500 MHz, CDCl$_3$): 7.17 (1H, br s, HO–12), 5.70 (1H, d, $J_{7\beta,6\alpha}$ = 1.7 Hz, Hβ–7), 5.50 (1H, dd, $J_{6\alpha,7\beta}$ = 1.7 Hz, $J_{6\alpha,5\alpha}$ =1.5 Hz, Hα–6), 3.16 (1H, sept, $J_{15,16(17)}$ = 7.1 Hz, H−15), 2.67 (1H, dddd, $J_{1\beta,1\alpha}$ = 12.9 Hz, $J_{1\beta,2\beta}$ = 3.1 Hz, $J_{1\beta,2\alpha}$ = 2.7 Hz, $J_{1\beta,3\beta}$ = 1.2 Hz, Hβ–1), 2.04 (3H, s, Me-2’), 2.03 (3H, s, OAc-7α), 1.82 (1H, qt, $J_{2\beta,1\alpha} = J_{2\beta,2\alpha} = J_{2\beta,3\alpha}$ = 14.0 Hz, $J_{2\beta,1\beta} = J_{2\beta,3\beta}$ = 3.1 Hz, Hβ–2), ~1.60 (1H, *, Hα–2), 1.59 (3H, s, Me-20), 1.56 (1H, d, $J_{5\alpha,6\alpha}$ = 1.5 Hz, Hα–5), 1.49 (1H, dt, $J_{3\beta,3\alpha}$ = 13.4 Hz, $J_{3\beta,2\beta} = J_{3\beta,2\alpha}$ = 3.1 Hz, Hβ–3), 1.24 (1H, *, Hα–1), 1.24 (1H, *, Hα–3), 1.22 (3H, d, $J_{16,15}$ = 7.1 Hz, Me-16), 1.19 (3H, d, $J_{17,15}$ = 7.1 Hz, Me-17), 0.99 (3H, s, Me–18), 0.98 (3H, s, H$_3$C(19));

* Overlapped signals.

V.6.1.2.10. 7α-Acetoxy-6β-propionyloxyroyleanone III.12



III.12

Yellow amorphous solid;

$[\alpha]^{18}_{546}$ = −25˚ (c = 0.044, CHCl$_3$);

^{1}H-NMR (400 MHz, CDCl$_3$): 7.17 (1H, br, HO−12), 5.70 (1H, br d, $J_{7\beta,6\alpha}$ = 2.0 Hz, $J_{7\beta,5\alpha}$ < 0.4 Hz, Hβ–7), 5.51 (1H, dd, $J_{6\alpha,7\beta}$ = 2.0 Hz, $J_{6\alpha,5\alpha}$ = 1.6 Hz, Hα-6), 3.16 (1H, sept, $J_{15,16(17)}$ = 7.1 Hz, H–15), 2.66 (1H, dtd, $J_{1\beta,1\alpha}$ = 12.9 Hz, $J_{1\beta,2\beta}$ = 3.6 Hz, $J_{1\beta,2\alpha}$ = 3.4 Hz, $J_{1\beta,3\beta}$ = 0.8 Hz, Hβ–1), 2.29 (2H, m, H-2’), 2.04 (3H, s, OAc-7α), 1.82 (1H, qt, $J_{2\beta,1\alpha} = J_{2\beta,2\alpha}$ =

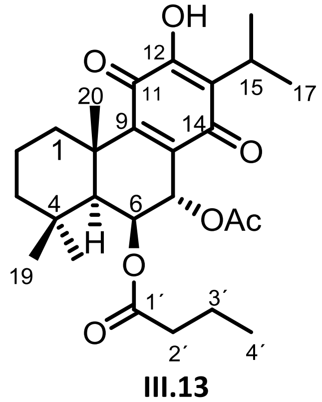
$J_{2\beta,3\alpha}$ = 14.0 Hz, $J_{2\beta,1\beta}$ = $J_{2\beta,3\beta}$ = 3.6 Hz, Hβ–2), 1.59 (3H, s, Me-20), 1.58 (1H, * m, Hα–2), 1.54 (1H, br d, $J_{5\alpha,6\alpha}$ = 1.6 Hz, $J_{5\alpha,7\beta}$ < 0.4 Hz, Hα–5), 1.48 (1H, dtd, $J_{3\beta,3\alpha}$ = 13.6 Hz, $J_{3\beta,2\alpha}$ = $J_{3\beta,2\beta}$ = 3.6 Hz, $J_{3\beta,1\beta}$ = 0.8 Hz, Hβ–3), 1.22 (1H, *, Hα–1) and Hα–3), 1.22 (3H, d, $J_{16,15}$ = 7.1 Hz, Me-16), 1.19 (3H, d, $J_{17,15}$ = 7.1 Hz, Me-17), 1.12 (3H, t, $J_{3',2'A}$ = $J_{3',2'B}$ = 7.6 Hz, Me-3'), 0.99 (3H, s, Me-18), 0.97 (3H, s, Me-19);

*Overlapped signals;

^{13}C NMR (100 MHz, CDCl$_3$): 185.35 (C-14, s), 183.23 (C-11, s); 172.46 (C-1′, s); 168.04 (OAc-7α, s); 150.84 (C-12, s); 149.32 (C-9, s); 137.11 (C-8, s); 124.81 (C-13, s); 67.08 (C-6, d); 65.24 (C-7, d); 49.03 (C-5, d); 42.43 (C-3, t); 38.66 (C-10, s); 38.36 (C-1, t); 33.58 (C-4, s); 33.20 (C-18, q); 27.84 (C-2', t), 24.15 (C-15, d); 23.02 (C-19, q); 21.35 (C-20, q); 20.82 (OAc-7α, q); 19.82 (C-16, q); 19.67 (C-17, q); 18.88 (C-2, t), 8.85 (C-3', q);

EI-MS: 446 ($[M]^+$, 0.4) 404 ($[M-CH_2CO]^+$, 1), 386 ($(M-AcOH]^+$, 4), 372 ($[M-CH_3CH_2CO_2H]^+$, 2), 348 (4), 330 ($[M-CH_3CH_2CO_2H-CH_2CO]^+$, 100), 315 (20), 298 (12), 287 (10), 261 (21), 260 (15), 248 (20),245 (13), 232 (12), 57 ($[C_2H_5C{=}O]^+$, 18); ($C_{25}H_{34}O_7$, M*r* 446).

V.6.1.2.11. 7α-Acetoxy-6β-butyryloxyroyleanone III.13



III.13

Amorphous solid;

$[\alpha]_D^{18}$ =+12.1° (c = 0.19, CHCl$_3$);

IR (KBr) ν_{max}: 3378, 2962, 2931, 2873, 1755, 1659, 1638, 1612, 1462, 1374, 1282, 1220, 1168, 1144, 1103, 1030, 959, 900, 756 cm^{-1};

^{1}H-NMR (400 MHz, CDCl$_3$): 7.18 (1H, br s, HO–12), 5.69 (1H, dd, $J_{7\beta,6\alpha}$ = 2.1 Hz, $J_{7\beta,5\alpha}$ = 0.6 Hz, Hβ–7), 5.50 (1H, t, $J_{6\alpha,7\beta}$ = $J_{6\alpha,5\alpha}$ = 2.1 Hz, Hα–6), 3.16 (1H, sept, $J_{15,16(17)}$ =

7.1 Hz, H–15), 2.66 (1H, ddd, $J_{1\beta,1\alpha}$ = 13.0 Hz, $J_{1\beta,2\alpha}$ = 3.3 Hz, $J_{1\beta,2\beta}$ = 2.9 Hz, Hβ–1), 2.28 (1H, dt, $J_{2'A,2'B}$ = 15.9 Hz, $J_{2'A,3'}$ = 7.1 Hz, HA–2'), 2.20 (1H, dt, $J_{2'B,2'A}$ = 15.9 Hz, $J_{2'B,3'}$ = 8.0 Hz, HB–2'), 2.04 (3H, s, OAc-7α), 1.82 (1H, qdd, $J_{2\beta,1\alpha}$ = $J_{2\beta,2\alpha}$ = $J_{2\beta,3\alpha}$ = 13.8 Hz, $J_{2\beta,1\beta}$ = $J_{2\beta,3\beta}$ = 2.9 Hz, Hβ–2), ~1.63 (2H, *, HA–3' and HB-3´), 1.60 (1H, *, Hα–5) 1.59 (3H, s, Me-20), 1.58 (1H, *, Hα–2), 1.47 (1H, ddd, $J_{3\beta,3\alpha}$ = 13.0 Hz, $J_{3\beta,2\alpha}$ = 3.6 Hz, $J_{3\beta,2\beta}$ =2.9 Hz, Hβ–3), 1.23 (2H, *, Hα–1 and Hα–3), 1.22 (3H, d, $J_{16,15}$ = 7.1 Hz, Me-16), 1.19 (3H, d, $J_{17,15}$ = 7.1 Hz, Me-17), 0.99 (3H, s, Me-18), 0.98 (3H, s, Me-19), 0.91 (1H, t, $J_{4',3'}$ = 7.4 Hz, H–4');

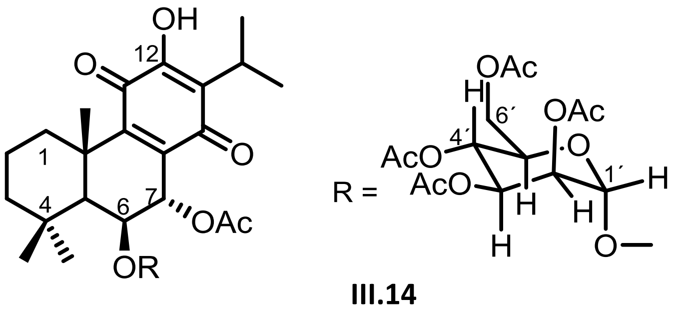
*Overlapped signals;

^{13}C NMR (100 MHz, $CDCl_3$): 185.34 (C-14, s); 183.23 (C-11, s); 171.78 (C-1', s); 168.16 (OAc-7α, s); 150.79 (C-12, s); 149.32 (C-9, s); 137.09 (C-8, s); 124.79 (C-13, s); 67.00 (C-6, d); 65.24 (C-7, d); 49.00 (C-5, d); 42.41 (C-3, t); 38.65 (C-10, s); 38.36 (C-1, t), 36.42 (C-2', t); 33.57 (C-4, s); 33.24 (C-18, q); 24.13 (C-15, d); 23.03 (C-19, q); 21.33 (C-20, q); 20.82 (OAc-7α, q); 19.81 (C-16, q); 19.66 (C-17, q); 18.88 (C-2, t); 18.08 (C-3', t); 13.70 (C-4', q);

EI-MS*:* 460 ($[M]^+$, 0.3) 418 ($[M-CH_2{=}C{=}O]^+$, 1), 401($[M-AcO]^+$, 5), 348 (5), 330 ($[M-AcO-CH_3CH_2CH_2CO]^+$, 100), 315 ($[M-AcO-CH_3CH_2CH_2CO-Me]^+$, 24), 297 (11), 287 (12), 261 (26), 248 (24), 232 (14), 217 (7), 201 (8), 187 (6), 83 (8), 71 (13), 55 (5); ($C_{26}H_{36}O_7$, M*r* 460).

V.6.1.3. Synthesis of α-mannopyranoside of 7α-acetoxy-6β-hydroxyroyleanone III.14

Acid-washed 4 Å molecular sieves AW-300 (2.5 g, 1.6 mm pellets) and three portions of (2*R*,3*R*,4*S*,5*S*,6*R*)-2-(acetoxymethyl)-6-(2,2,2-trichloro-1-iminoethoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (117 mg; 0.238 mmol) were added to a solution of **III.1** (40.2 mg; 0.103 mmol) in anhydrous dichloromethane (6 mL), under argon atmosphere. The reaction mixture was refluxed, for 48 h. The reaction was followed by tlc (AcOEt : *n*-hexane; 1:1). Then, after filtration and washing of the molecular sieves with CH_2Cl_2 (20 mL), the total organic solution was evaporated to dryness yielding 519 mg. The residue obtained was purified by column chromatography on silica gel 9385, with (AcOEt : *n*-hexane) from (3:1) to (1:1) solvents. The fraction containing the product (27.5 mg) was then further purified by preparative tlc (AcOEt : *n*-hexane; 1:1) to give the more polar derivative **III.14** (1.15 mg; 1.60 µmol; 1.55 %) as a yellowish crystals (from AcOEt : *n*-hexane; 1:1).



III.14

Yellow amorphous solid;

[α]$^{20}_{\mathrm{D}}$ =+56.8 ° (*c* = 0.018, CHCl$_3$);

^{1}**H-NMR** (500 MHz, CDCl$_3$): 6.05 (1H, d, $J_{7\beta,6\alpha}$ = 1.4 Hz, Hβ–7), 5.29 (1H, t, $J_{4',3'}$ = 9.8 Hz, $J_{4',5'}$ = 9.8 Hz, H–4’), 5.18 (1H, br s, $J_{2',1'}$ = ~1.8-2.4 Hz, $J_{2',3'}$ = 3.5 Hz, H–2’), 5.17 (1H, dd, $J_{3',2'}$ = 3.5 Hz, $J_{3',4'}$ = 9.8 Hz, H–3’), 5.11 (1H, br s, $J_{1',2'}$ = 2.8 Hz, H–1’), 4.30 (1H, dd, $J_{6'A,6'B}$ = 12.2 Hz, $J_{6'A,5'}$ = 4.9 Hz, H–6’A), 4.19 (1H, br s, $J_{6\alpha,7\beta}$ = 1.4 Hz, $J_{6\alpha,5\alpha}$ = ~1.4 Hz, Hα–6), 4.04 (1H, dd, $J_{6'B,6'A}$ = 12.2 Hz, $J_{6'B,5'}$ = 2.4 Hz, H–6’B), 3.75 (1H, ddd, $J_{4',3'}$ = 9.8 Hz, $J_{4',5'}$ = 9.8 Hz, H–4’), 3.18 (1H, sept, $J_{15,16(17)}$ = 6.8 Hz, H–15), 2.66 (1H, br d, $J_{1\beta,1\alpha}$ = 12.7 Hz, $J_{1\beta,2\alpha}$ = *, $J_{1\beta,2\beta}$ = 3.0 Hz, Hβ–1), 2.16 (3H, s, OAc-2´), 2.13 (3H, s, OAc-6´), 2.00 (3H, s, OAc-4´), 2.02 (3H, s, OAc-7α), 1.98 (3H, s, OAc-3´), 1.85 (1H, qdd, $J_{2\beta,1\alpha}$ = $J_{2\beta,2\alpha}$ = $J_{2\beta,3\alpha}$ = 13.2 Hz, $J_{2\beta,1\beta}$ = 3.0 Hz, $J_{2\beta,3\beta}$ = 3.0 Hz, Hβ–2), 1.64 (3H, s, Me-20), 1.56 (1H, m*, $J_{2\alpha,1\beta}$ = $J_{2\alpha,1\alpha}$ = $J_{2\alpha,3\alpha}$ = $J_{2\alpha,3\beta}$ = *, $J_{1\alpha,2\beta}$ = 13.2 Hz, $J_{2\alpha,2\beta}$ = 13.2 Hz, Hα–2), 1.52 (1H, m*, $J_{3\beta,3\alpha}$ = $J_{3\beta,2\alpha}$ = *, $J_{3\beta,2\beta}$ =3.0 Hz, Hβ–3), 1.45 (1H, br s, *, $J_{5\alpha,6\alpha}$ = 1.4 Hz, Hα–5), 1.23[#] (3H, d, $J_{16,15}$ = 6.8 Hz, Me-16), 1.22 (1H, m*, $J_{1\alpha,1\beta}$ = 12.7 Hz, $J_{1\alpha,2\alpha}$ = *, $J_{1\alpha,2\beta}$ = 13.2 Hz, Hα–1), 1.22[#] (3H, d, $J_{17,15}$ = 6.8 Hz, Me-17), 1.20 (3H, s, Me-19), 1.20 (1H, *, $J_{3\alpha,2\alpha}$ = $J_{3\alpha,3\beta}$ = *, $J_{3\alpha,2\beta}$ = 13.2 Hz, Hα–3), 0.94 (3H, s, Me-18);

*Overlapped or partially overlapped signals; approximate δ values obtained from the HSQC spectrum. [#]Interchangeable signals;

^{13}**C NMR** (125 MHz, CDCl$_3$): 185.24 (C-14, s); 183.17 (C-11, s); 170.89 (C-6’, s); 170.18 (C´-2, s); 170.05 (C-3´, s); 169.53 (C´4, s); 168.68 (OAc-7α, s); 150.97 (C-12, s);

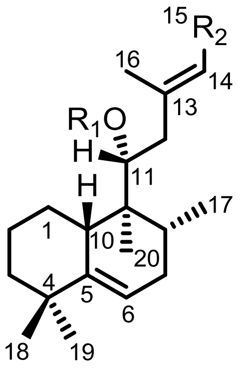
149.96 (C-9, s); 136.55 (C-8, s); 124.78 (C-13, s); 99.98 (C-1′, d); 77.41 (C-6, d); 69.74 (C-5′, d); 69.71 (C-2′, d); 68.85 (C-3′, d); 65.73 (C-4′, d); 65.20 (C-7, d); 62.13 (C-6′, t); 49.63 (C-5, d); 42.37 (C-3, t); 38.80 (C-10, s); 38.56 (C-1, t), 33.60 (C-4, s); 33.51 (C-18, q); 24.15 (C-15, d); 23.59 (C-19, q); 21.93 (C-20, q); 20.89 (OAc-2′, q); 20.77* (OAc-6′, q); 20.70* (OAc-4′, q); 20.68* (OAc-3′, q); 20.63* (OAc-7α, q); 19.87 (C-16, q); 19.66 (C-17, q); 18.90 (C-2, t);

*Interchangeable assignments;

HRESIMS *m/z:* 721.3051 $[M+H]^+$ (calcd 721.3066 for $C_{36}H_{49}O_{15}$).

V.6.2. (11*R**,13*E*)-11-Acetoxyhalima-5,13-dien-15-oic acid derivatives

The (11*R**,13*E*)-11-Acetoxyhalima-5,13-dien-15-oic acid **II.7** was used as starting material to prepare nine derivatives. Eight halimane derivatives **III.15-III.23** were obtained by esterification at C-11 and/or C-15 positions (**Figure V.2**). The natural diterpene (11*R**,13*E*)-11-Acetoxyhalima-5,13-dien-15-oic acid **II.7** used for obtaining the derivatives **III.15-III.23** was re-isolated from *P. ornatus* Codd. as described previously in Chapter **II** (Rijo P. et al., 2007; Rijo P. et al., accepted).



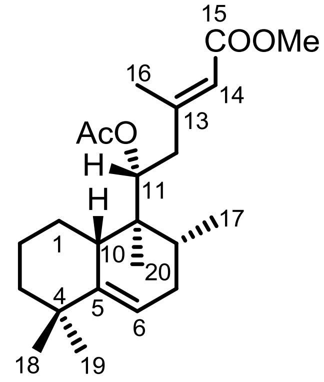
	R_1	R_2
II.7	Ac	COOH
III.15	Ac	COOMe
III.16	Ac	CH_2OH
III.17	H	CH_2OH
III.18	H	CH_2OAc
III.19	H	$CH_2OCOCH_2CH_3$
III.20	$COCH_2CH_3$	$CH_2OCOCH_2CH_3$
III.21	H	$CH_2OCOCH_2CH_2CH_3$
III.22	H	$CH_2OCOC_6H_5$
III.23	H	$CH_2OCO\text{-}C_6H_5\text{-}p\text{-}OCH_3$

Figure V.2. Derivatives **III.15-III.23** of (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid **II.7.**

VI.6.2.1. Methylation of (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid II.7

A solution of **II.7** (11.4 mg) in acetone (2 mL) was treated with Me_2SO_4 (16 mg) in the presence of K_2CO_3 (6.2 mg) at room temperature for 5 h. After this time the reaction mixture was poured into water (10 mL) and extracted with AcOEt (10 mL x 5). The combined organic layers were dried over anhydrous sodium sulphate filtered and evaporated in a rotavapor. The yellow residue obtained was purified by thin layer chromatography (precoated silica gel plates Merck nº 7747 eluted with 7:3 *n*-hexane:AcOEt) to give **III.15** (11.2 mg, 94.5 %).

(11*R**,13*E*)-11-Acetoxyhalima-5,13-dien-15-oic acid methyl ester III.15



III.15

Colourless fine needles (Et_2O – pentane);

m.p. 89-91 ˚C;

[α]$^{20}_{D}$ -33.7 ˚ (*c* 0.249, $CHCl_3$);

IR (KBr) ν_{max}: 3054, 2936, 1730, 1707, 1637, 1434, 1378, 1247, 1237, 1155, 1021, 975 cm^{-1};

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.67 (1H, qd, $J_{14,16}$ = 1.2 Hz, $J_{14,12A}$ = 0.4 Hz, H-14), 5.44 (1H, dt, $J_{6,7\beta}$ = 5.6 Hz, $J_{6,7\alpha}$ = $J_{6,10\beta}$ = 2.1 Hz, H-6), 5.31 (1H, dd, $J_{11,12A}$ = 2.5 Hz, $J_{11,12B}$ = 10.1 Hz, H-11), 3.66 (3H, s, COO$\underline{Me}$), 2.42 (1H, ddd, $J_{12A,12B}$ = 14.0, $J_{12A,11}$ = 2.5 Hz, $J_{12A,14}$ = 0.4 Hz, H-12A), 2.33 (1H, br dd, $J_{12B,12A}$ = 14.0 Hz, $J_{12B,11}$ = 10.1 Hz, $J_{12B,14}$ < 0.2 Hz, H-12B), 2.18 (3H, d, $J_{16,14}$ = 1.2 Hz, Me-16), 2.06 (1H, dm, $J_{10\beta,1\alpha}$ = 12.9 Hz, H-10β), 1.98 (3H, s, 11-OAc), 1.92 (1H, dm, $J_{1\beta,1\alpha}$ = 12.9 Hz, H-1β), 1.84 (1H, dtd, $J_{7\beta,7\alpha}$ = 17.7 Hz, $J_{7\beta,6}$ = $J_{7\beta,8\beta}$ = 5.6 Hz, $J_{7\beta,10\beta}$ =1.8 Hz, H-7β), 1.75 (1H, ddt, $J_{7\alpha,7\beta}$ = 17.7 Hz, $J_{7\alpha,8\beta}$ = 9.8 Hz, $J_{7\alpha,6}$ = $J_{7\alpha,10\beta}$ = 2.1 Hz, H-7α), 1.63 (1H, ddddd, $J_{2\alpha,2\beta}$ = 13.3 Hz, $J_{2\alpha,1\alpha}$ = 3.9 Hz, $J_{2\alpha,1\beta}$ = 3.6 Hz, $J_{2\alpha,3\alpha}$ = 4.3 Hz, $J_{2\alpha,3\beta}$ = 3.3 Hz, H-2α), 1.56 (1H, m, *, H-8β), 1.52 (1H, qt, $J_{2\beta,2\alpha}$ = $J_{2\beta,1\alpha}$ = $J_{2\beta,3\alpha}$ = 12.9 Hz, $J_{2\beta,1\beta}$ = $J_{2\beta,3\beta}$ = 3.3 Hz, H-2β), 1.41 (1H, dtd, $J_{3\beta,3\alpha}$ = 13.3 Hz, $J_{3\beta,2\alpha}$ = $J_{3\beta,2\beta}$ = 3.3 Hz, $J_{3\beta,1\beta}$ = 1.8 Hz, H-3β), 1.19 (1H, td, $J_{3\alpha,3\beta}$ = $J_{3\alpha,2\beta}$ = 13.3 Hz, $J_{3\alpha,2\alpha}$ = 4.3 Hz, H-3α), 1.09 (1H, qd, $J_{1\alpha,1\beta}$ = $J_{1\alpha,2\beta}$ = $J_{1\alpha,10\beta}$ = 12.9 Hz, $J_{1\alpha,2\alpha}$ = 3.9 Hz, H-1α), 1.05 (3H, s, Me-19), 0.98 (3H, d, $J_{17,8\beta}$ = 6.6 Hz, Me-17), 0.97 (3H, s, Me-18), 0.68 (3H, s, Me-20);

*overlapped signals;

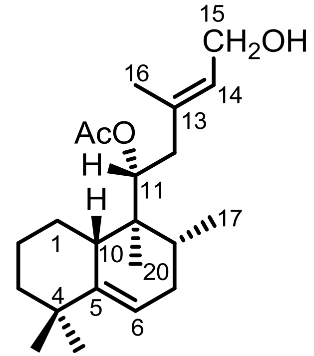
^{13}C NMR (100 MHz, $CDCl_3$) δ 170.6 (11-O$\underline{C}$OCH$_3$, s), 166.6 (C-15, s), 156.8 (C-13, s), 145.2 (C-5, s), 117.9 (C-14, d), 116.0 (C-6, d), 75.1 (C-11, d), 50.9 (COO$\underline{C}$H$_3$, q), 41.6 (C-9, s), 41.4 (C-12, t), 40.9 (C-3, t), 40.3 (C-10, d), 36.3 (C-4, s), 32.8 (C-8, d), 31.4 (C-7, t), 29.7 (C-19, q), 28.9 (C-1, t), 28.1 (C-18, q), 22.5 (C-2, t), 20.9 (11-OCO$\underline{C}$H$_3$, q), 18.8 (C-16, q), 17.2 (C-17, q), 11.8 (C-20, q);

EIMS: *m/z* (relative intensity) 376 (1), 316 (8), 301 (11), 274 (13), 259 (17), 203 (22), 191 (100), 175 (11), 147 (11), 119 (27) 105 (13); $C_{23}H_{36}O_4$, M_r 376.

V.6.2.2. Lithium aluminium hydride reduction of II.7: (11*R**, 13*E*)-11-acetoxyhalima-5,13-dien-15-ol III.16 and (11*R**, 13*E*)-halima-5,13-diene-11,15- diol III.17

To a solution of **II.7** (11.2 mg) in anhydrous ethyl ether (1.5 mL) H_4LiAl (1.7 mg) was added under N_2 atmosphere. The reaction mixture was left at room temperature for 2 h. The reaction mixture was evaporated in rotavapor, filtered and gave a mixture of **III.16** and **III.17**, which were isolated by tlc (*n*-hexane:EtOAc; 7:3) yielding **III.16** (2 mg, 18.6 %) and **III.17** (most polar compound, 6.5 mg, 68.7 %).

V.6.2.2.1. (11*R**, 13*E*)-11-Acetoxyhalima-5,13-dien-15-ol III.16



III.16

Colourless oil;

$[\alpha]_D^{20}$ -22.1 ° (*c* 0.70, CHCl$_3$);

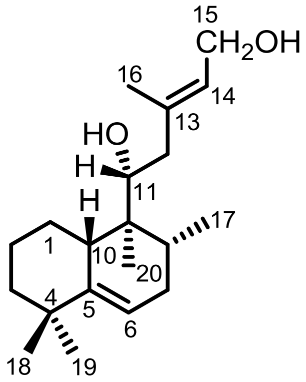
IR (neat) ν_{max}: 3424, 3051, 2930, 1735, 1458, 1375, 1244, 1216, 1026, 757 cm^{-1};

^{1}H NMR (400 MHz, CDCl$_3$): δ 5.43 (2H, m, H-6 and H-14), 5.21 (1H, dd, $J_{11,12A}$ = 2.3 Hz, $J_{11,12B}$ = 10.3 Hz, H-11), 4.10 (1H, dd, $J_{15A,15B}$ = 12.3 Hz, $J_{15A,14}$ = 7.0 Hz, H-15A), 4.06 (1H, dd, $J_{15B,15A}$ = 12.3 Hz, $J_{15B,14}$ = 6.8 Hz, H-15B), 2.30 (1H, br d, $J_{12A,12B}$ = 13.7, $J_{12A,11}$ + $J_{12A,14}$ < 3 Hz, H-12A), 2.21 (1H, dd, $J_{12B,12A}$ = 13.7 Hz, $J_{12B,11}$ = 10.3 Hz, H-12B), 2.09 (1H, dm, $J_{10\beta,1\alpha}$ = 12.7 Hz, H-10β), 1.99 (3H, s, 11-OAc), 1.91 (1H, m, *, H-1β), 1.84 (1H, dtd, $J_{7\beta,7\alpha}$ = 17.7 Hz, $J_{7\beta,6}$ = $J_{7\beta,8\beta}$ = 5.3 Hz, $J_{7\beta,10\beta}$ = 1.8 Hz, H-7β), 1.71 (3H, d, $J_{16,14}$ = 1.4 Hz, Me-16), 1.76 (1H, ddt, $J_{7\alpha,7\beta}$ = 17.7 Hz, $J_{7\alpha,8\beta}$ = 9.8 Hz, $J_{7\alpha,6}$ = $J_{7\alpha,10\beta}$ = 2.5 Hz, H-7α), 1.64 (1H, m, *, H-2α), 1.58 (1H, m, *, H-8β), 1.51 (1H, qt, $J_{2\beta,2\alpha}$ = $J_{2\beta,1\alpha}$ = $J_{2\beta,3\alpha}$ = 13.1 Hz, $J_{2\beta,1\beta}$ = $J_{2\beta,3\beta}$ = 3.4 Hz, H-2β), 1.41 (1H, dtd, $J_{3\beta,3\alpha}$ = 13.1 Hz, $J_{3\beta,2\alpha}$ = $J_{3\beta,2\beta}$ = 3.3 Hz, $J_{3\beta,1\beta}$ = 2.0 Hz, H-3β), 1.19 (1H, td, $J_{3\alpha,3\beta}$ = $J_{3\alpha,2\beta}$ = 13.1 Hz, $J_{3\alpha,2\alpha}$ = 4.5 Hz, H-3α), 1.08 (1H, qd, $J_{1\alpha,1\beta}$ = $J_{1\alpha,2\beta}$ = $J_{1\alpha,10\beta}$ = 12.7 Hz, $J_{1\alpha,2\alpha}$ = 3.9 Hz, H-1α), 1.05 (3H, s, Me-19), 0.98 (3H, d, $J_{17,8\beta}$ = 6.5 Hz, Me-17), 0.97 (3H, s, Me-18), 0.68 (3H, s, Me-20);

^{13}C NMR (100 MHz, CDCl$_3$): δ 171.1 (11-O$\underline{C}$OCH$_3$, s), 145.3 (C-5, s), 136.9 (C-13, s), 126.3 (C-14, d), 115.9 (C-6, d), 75.8 (C-11, d), 59.2 (C-15, t), 41.3 (C-3, t), 41.0 (C-9, s), 40.2 (C-10, d), 40.1 (C-12, t), 36.3 (C-4, s), 32.8 (C-8, d), 31.4 (C-7, t), 29.7 (C-19, q), 29.0 (C-1, t), 28.1 (C-18, q), 22.6 (C-2, t), 21.0 (11-OCO$\underline{C}$H$_3$, q), 17.2 (C-17, q), 16.2 (C-16, q), 12.0 (C-20, q);

EIMS *m/z* (relative intensity) $[M]^+$ absent, 330 $[M\text{-}H_2O]^+$ (1), 288 $[M\text{-HOAc}]^+$, (4), 273 $[M\text{-Me-HOAc}]^+$ (6), 270 $[M\text{-HOAc-}H_2O]^+$ (3), 231 (8), 203 (23), 191 (100), 189 (75), 175 (20), 173 (21), 147 (22), 119 (66) 105 (24); $C_{22}H_{36}O_3$, M_r 348.

V.6.2.2.2. (11*R**, 13*E*)-Halima-5,13-diene-11,15- diol III.17



III.17

Colourless thick oil;

[α] $^{20}_{D}$ -18.8 (*c* 0.33, $CHCl_3$);

IR (neat) ν_{max}: 3369, 3051, 2927, 1667, 1455, 1383, 1262, 1088, 1038, 1000, 802, 758 cm^{-1};

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.53 (1H, br tq, $J_{14,15}$ = 6.8 Hz, $J_{14,16}$ = 0.6 Hz, $J_{14,12A}$ < 0.6 Hz, H-14), 5.41 (1H, ddd, $J_{6,7\beta}$ = 5.9 Hz, $J_{6,7\alpha}$ = 2.1 Hz, $J_{6,10\beta}$ = 1.7 Hz, H-6), 4.18 (2H, d, $J_{15,14}$ = 6.8 Hz, H_2-15), 3.66 (1H, dd, $J_{11,12A}$ = 2.0 Hz, $J_{11,12B}$ = 10.8 Hz, H-11), 2.33 (1H, br dd, $J_{12A,12B}$ = 13.7, $J_{12A,11}$ = 2.0 Hz, $J_{12A,14}$ < 0.6 Hz, H-12A), 2.29 (1H, m, *, H-10β), 2.09 (1H, dd, $J_{12B,12A}$ = 13.7 Hz, $J_{12B,11}$ = 10.8 Hz, H-12B), 1.87 (1H, dtd, $J_{7\beta,7\alpha}$ = 17.5 Hz, $J_{7\beta,6}$ = $J_{7\beta,8\beta}$ = 5.1 Hz, $J_{7\beta,10\beta}$ = 2.1 Hz, H-7β), 1.81 (1H, m, *, H-1β), 1.78 (1H, m, *, H-7α), 1.72 (3H, d, $J_{16,14}$ = 0.6 Hz, Me-16), 1.62 (1H, m, *, H-8β), 1.60 (1H, m, *, H-2α), 1.55 (1H, m, *, H-2β), 1.42 (1H, dtd, $J_{3\beta,3\alpha}$ = 13.0 Hz, $J_{3\beta,2\alpha}$ = $J_{3\beta,2\beta}$ = 3.3 Hz, $J_{3\beta,1\beta}$ = 1.7 Hz, H-3β), 1.20 (1H, m, H-3α), 1.10 (1H, m, H-1α), 1.05 (3H, s, Me-19), 1.00 (3H, s, Me-18), 0.97 (3H, d, $J_{17,8\beta}$ = 6.7 Hz, Me-17), 0.80 (3H, s, Me-20);

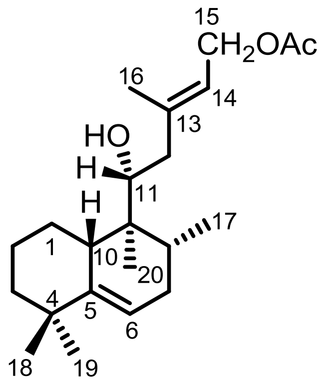
^{13}C NMR (100 MHz, $CDCl_3$) 146.1 (C-5,s), 137.8 (C-13, s), 126.7 (C-14, d), 115.5 (C-6, d), 73.1 (C-11, d), 59.3 (C-15, t), 42.0 (C-12, t), 41.4 (C-3, t), 41.2 (C-9, s), 38.9 (C-10, d), 36.5 (C-4, s), 32.8 (C-8, d), 31.0 (C-7, t), 29.9 (C-19, q and C-1, t), 27.7 (C-18, q), 22.9 (C-2, t), 17.3 (C-17, q), 16.3 (C-16, q), 12.6 (C-20, q);

EIMS: *m/z* (relative intensity) $[M]^+$ absent, 288 $[M-H_2O]^+$ (1), 231 (2), 203 (8), 191 (100), 175 (12), 147 (9), 135 (12), 119 (19) 105 (16), 95 (16); $C_{20}H_{34}O_2$, M_r 306.

V.6.2.3. Preparation of (11*R**,13*E*)-15-acetoxyhalima-5,13-dien-11-ol) III.18 from compound III.16

To a solution of **III.16** (15 mg) in 7:3 petroleum ether-EtOAc (10 mL) silica gel (Merck 7747, 20 mg) was added. The mixture was stirred for 48 h at room temperature. Filtration and evaporation of the solvents gave a mixture (14.8 mg) of the starting material **III.16** and another compound. After tlc of this mixture, compounds **III.16** (8 mg, 53.3 %) and **III.18** (6.2 mg, 41.3 %) were isolated.

(11*R**, 13*E*)-15-Acetoxyhalima-5,13-dien-11-ol III.18



III.18

Colourless oil;

$[\alpha]_D^{20}$ -21.6 ° (*c* 0.213, $CHCl_3$);

IR (neat) ν_{max}: 3445, 3049, 2928, 1739, 1731, 1668, 1455, 1383, 1235, 1039, 819 cm^{-1};

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.46 (1H, tqd, $J_{14,15}$ = 7.0 Hz, $J_{14,16}$ = 0.6 Hz, $J_{14,12A}$ = 0.4 Hz, H-14), 5.41 (1H, ddd, $J_{6,7\beta}$ = 4.7 Hz, $J_{6,7\alpha}$ = 3.0 Hz, $J_{6,10\beta}$ = 1.6 Hz, H-6), 4.62 (1H, dd, $J_{15A,15B}$ = 12.7 Hz, $J_{15A,14}$ = 7.0 Hz, H-15A), 4.58 (1H, dd, $J_{15B,15A}$ = 12.7 Hz, $J_{15B,14}$ = 7.0

Hz, H-15B), 3.66 (1H, dd, $J_{11,12A}$ = 1.6 Hz, $J_{11,12B}$ = 10.6 Hz, H-11), 2.34 (1H, ddd, $J_{12A,12B}$ = 13.7, $J_{12A,11}$ = 1.6 Hz, $J_{12A,14}$ = 0.4 Hz, H-12A), 2.28 (1H, dm, $J_{10\beta,1\alpha}$ = 12.8 Hz, $J_{10\beta,6}$ = 1.6 Hz, H-10β), 2.12 (1H, dd, $J_{12B,12A}$ = 13.7 Hz, $J_{12B,11}$ = 10.6 Hz, H-12B), 2.04 (3H, s, 15-OAc), 1.87 (1H, dtd, $J_{7\beta,7\alpha}$ = 17.6 Hz, $J_{7\beta,6}$ = $J_{7\beta,8\beta}$ = 5.0 Hz, $J_{7\beta,10\beta}$ = 2.0 Hz, H-7β), 1.80 (1H, m, *, H-1β), 1.78 (1H, m, *, H-7α), 1.75 (3H, d, $J_{16,14}$ = 0.6 Hz, Me-16), 1.62 (1H, m, *, H-8β), 1.60 (1H, m, *, H-2α), 1.55 (1H, m, *, H-2β), 1.42 (1H, dtd, $J_{3\beta,3\alpha}$ = 13.1 Hz, $J_{3\beta,2\alpha}$ = $J_{3\beta,2\beta}$ = 3.6 Hz, $J_{3\beta,1\beta}$ = 1.8 Hz, H-3β), 1.19 (1H, td, $J_{3\alpha,3\beta}$ = $J_{3\alpha,2\beta}$ = 13.1 Hz, $J_{3\alpha,2\alpha}$ = 3.8 Hz, H-3α), 1.10 (1H, qd, $J_{1\alpha,1\beta}$ = $J_{1\alpha,2\beta}$ = $J_{1\alpha,10\beta}$ = 12.8 Hz, $J_{1\alpha,2\alpha}$ = 4.0 Hz, H-1α), 1.05 (3H, s, Me-19), 1.00 (3H, s, Me-18), 0.97 (3H, d, $J_{17,8\beta}$ = 6.9 Hz, Me-17), 0.80 (3H, s, Me-20);

*Overlapped signals;

^{13}C NMR (100 MHz, CDCl$_3$): δ 171.1 (15-O$\underline{C}$OCH$_3$, s), 146.1 (C-5, s), 140.3 (C-13, s), 121.7 (C-14, d), 115.2 (C-6, d), 72.9 (C-11, d), 61.2 (C-15, t), 42.1 (C-12, t), 41.3 (C-3, t), 41.2 (C-9, s), 38.9 (C-10, d), 36.5 (C-4, s), 32.8 (C-8, d), 31.0 (C-7, t), 29.9 (C-19, q), 29.7 (C-1, t), 27.7 (C-18, q), 22.9 (C-2, t), 21.0 (15-OCO$\underline{C}$H$_3$, q), 17.3 (C-17, q), 16.4 (C-16, q), 12.6 (C-20, q);

EIMS *m/z* (relative intensity): $[M]^+$ absent, 330 $[M-H_2O]^+$ (1), 273 (1), 255 (1), 203 (6), 191 (100), 175 (12), 163 (10), 147 (8), 135 (13), 119 (23) 109 (16), 95 (18), 69 (19); $C_{22}H_{36}O_3$, M_r 348.

V.6.2.4. Preparation of the derivatives III.19-III.23

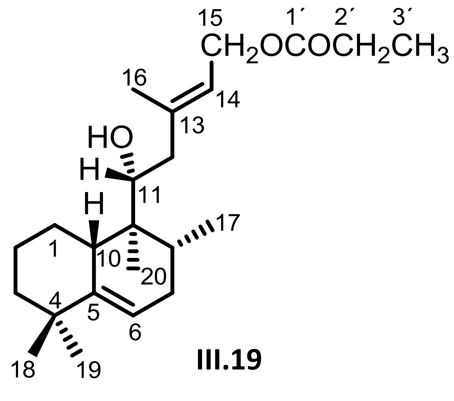
A solution of **III.17** in pyridine (Py) and eventually CH_2Cl_2 and the suitable alkyl anhydride (for obtaining **III.19-III.21**) or appropriate benzoyl chloride (for compounds **III.23** and **III.23**), was allowed to stand on adequate conditions. The reaction mixture was then diluted with EtOAc and successively washed with an aqueous saturated solution of $NaHCO_3$ and then with water. The organic layer was dried over anhydrous Na_2SO_4. The residue was then purified by tlc (7:3 petroleum ether-EtOAc as eluent) to afford **III.19-III.21**. The conditions for obtaining each one of the derivatives were as follows. Treatment of **III.17** (18.5 mg) with propionic anhydride (1 mL) in pyridine solution (1 mL) at 0 ˚C for 48 h gave a mixture of **III.19** and **III.20**. Chromatography yielded **III.19** (10.5 mg, 48.0 %) and **III.20** (9.5 mg, 37.5 %). A reaction mixture of **III.17** (37.5 mg), butyric anhydride (1 mL) and pyridine (1 mL) was allowed to stand at 0 ˚C for 30 min yielding **III.21** (19.1 mg, 41.4 %). Benzoylation of **III.17** (17 mg) with benzoyl chloride (30 µL) in pyridine (1 mL) at room temperature for 24 h gave **III.22** (9 mg, 39.5 %). A solution of **III.17** (19.8 mg) in pyridine (200 µL) and CH_2Cl_2 (2 mL) was treated with *p*-methoxybenzolyl chloride (40 µL) for 24 h at room temperature yielding **III.23** (14.7 mg, 51.6 %) (**Table V.15**).

Table V.15. Hemisynthesis conditions of derivatives **III.15-III.23**.

Starting material **III.17** mg;mmol	Benzoyl chloride/alkyl anhydride mL; mmol	Reactional conditions	Product mg (yield %)
18.5; 0.0603	propionic anhydride 1.00; 7.799	py (1 mL) 0 ˚C; 48 h	**III.19** 10.5 (48.0 %) **III.20** 9.5 (37.5 %)
37.5; 0.1223	butyric anhydride 1.00; 6.087	py (1 mL) 0 ˚C; 30 min	**III.21** 19.1 (41.4 %)
17.0; 0.0554	benzoyl chloride 0.030; 0.258	py (1 mL) r.t.; 24 h	**III.22** 9.0 (39.5 %)
19.8; 0.0646	*p*-methoxybenzolyl chloride 0.040; 0.295	py (0.2 mL) and CH_2Cl_2 (2 mL) 24 h; r.t.	**III.23** 14.7 (51.6 %)

Py = pyridine; r.t. = room temperature

V.6.2.4.1. (11*R**, 13*E*)-15-Propionyloxyhalima-5,13-dien-11-ol III.19



III.19

Colourless thick oil;

$[\alpha]^{20}_{\mathrm{D}}$ -21.3 ° (*c* 0.207, $CHCl_3$);

IR (neat) ν_{max}: 3543, 3050, 2929, 1738, 1462, 1382, 1363, 1277, 1184, 1081, 1009, 938 cm^{-1};

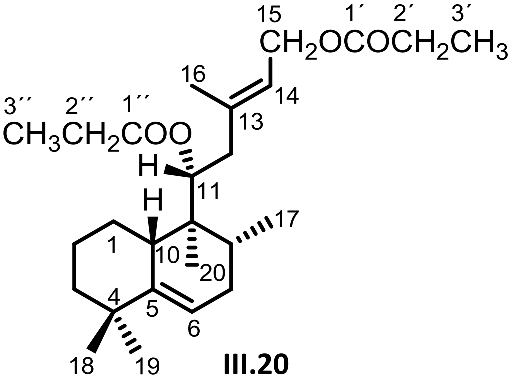
^{1}H NMR (400 MHz, $CDCl_3$): δ 5.46 (1H, br tq, $J_{14,15}$ = 7.0 Hz, $J_{14,16}$ = 0.9 Hz, $J_{14,12A}$ < 0.4 Hz, H-14), 5.41 (1H, ddd, $J_{6,7\beta}$ = 4.9 Hz, $J_{6,7\alpha}$ = 3.2 Hz, $J_{6,10\beta}$ = 1.6 Hz, H-6), 4.63 (1H, dd, $J_{15A,15B}$ = 12.9 Hz, $J_{15A,14}$ = 7.0 Hz, H-15A), 4.59 (1H, dd, $J_{15B,15A}$ = 12.9 Hz, $J_{15B,14}$ = 7.0 Hz, H-15B), 3.66 (1H, dd, $J_{11,12A}$ = 1.4 Hz, $J_{11,12B}$ = 10.7 Hz, H-11), 2.34 (1H, br dd, $J_{12A,12B}$ = 13.5, $J_{12A,11}$ = 1.4 Hz, $J_{12A,14}$ < 0.4 Hz, H-12A), 2.31 (2H, q, $J_{2',3'}$ = 7.7 Hz, H_2-2'), 2.28 (1H, dm, $J_{10\beta,1\alpha}$ = 12.7 Hz, H-10β), 2.12 (1H, dd, $J_{12B,12A}$ = 13.5 Hz, $J_{12B,11}$ = 10.7 Hz, H-12B), 1.86 (1H, dtd, $J_{7\beta,7\alpha}$ = 17.6 Hz, $J_{7\beta,6}$ = $J_{7\beta,8\beta}$ = 5.0 Hz, $J_{7\beta,10\beta}$ = 1.9 Hz, H-7β), 1.80 (1H, m, *, H-1β), 1.78 (1H, m, *, H-7α), 1.75 (3H, d, $J_{16,14}$ = 0.9 Hz, Me-16), 1.62 (1H, m, *, H-8β), 1.58 (1H, m, *, H-2α), 1.53 (1H, qt, $J_{2\beta,2\alpha}$ = $J_{2\beta,1\alpha}$ = $J_{2\beta,3\alpha}$ = 12.5 Hz, $J_{2\beta,1\beta}$ = $J_{2\beta,3\beta}$ = 3.5 Hz, H-2β), 1.42 (1H, dtd, $J_{3\beta,3\alpha}$ = 13.0 Hz, $J_{3\beta,2\alpha}$ = $J_{3\beta,2\beta}$ = 3.3 Hz, $J_{3\beta,1\beta}$ = 1.6 Hz, H-3β), 1.19 (1H, td, $J_{3\alpha,3\beta}$ = $J_{3\alpha,2\beta}$ = 13.0 Hz, $J_{3\alpha,2\alpha}$ = 4.3 Hz, H-3α), 1.13 (3H, t, $J_{3',2'}$ = 7.7 Hz, Me-3'), 1.09 (1H, qd, $J_{1\alpha,1\beta}$ = $J_{1\alpha,2\beta}$ = $J_{1\alpha,10\beta}$ = 12.7 Hz, $J_{1\alpha,2\alpha}$ = 4.6 Hz, H-1α), 1.05 (3H, s, Me-19), 1.00 (3H, s, Me-18), 0.97 (3H, d, $J_{17,8\beta}$ = 7.0 Hz, Me-17), 0.80 (3H, s, Me-20);

*Overlapped signals;

^{13}C NMR (100 MHz, $CDCl_3$): δ 174.5 (C-1', s), 146.1 (C-5, s), 140.1 (C-13, s), 121.9 (C-14, d), 115.2 (C-6, d), 73.0 (C-11, d), 61.0 (C-15, t), 42.1 (C-12, t), 41.4 (C-3, t), 41.2 (C-9, s), 38.9 (C-10, d), 36.5 (C-4, s), 32.8 (C-8, d), 31.1 (C-7, t), 29.9 (C-19, q), 29.8 (C-1, t), 27.7 (C-18, q), 27.6 (C-2', t), 22.9 (C-2, t), 17.3 (C-17, q), 16.5 (C-16, q), 12.6 (C-20, q), 9.1 (C-3', q);

EIMS *m/z* (relative intensity): $[M]^+$ absent, 288 $[M-CH_3CH_2COOH]^+$ (1), 287 (1), 255 (2), 203 (7), 191 (100), 175 (12), 163 (10), 147 (7), 135 (11), 119 (19), 109 (12), 97 (15), 69 (10), 57 (8); $C_{23}H_{38}O_3$, M_r 362.

V.6.2.4.2. (11*R**,13*E*)-11,15-Dipropionyloxyhalima-5,13-diene III.20



III.20

Colourless thick oil;

$[\alpha]^{20}_{D}$ -21.9 ° (*c* 0.187, $CHCl_3$);

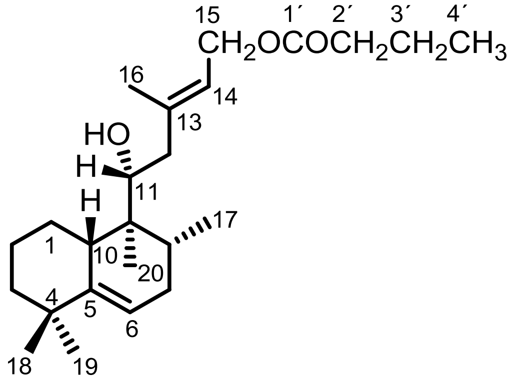
IR (neat) ν_{max}: 3049, 2930, 1737, 1733, 1463, 1385, 1276, 1183, 1082, 1015, 805 cm^{-1};

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.44 (1H, ddd, $J_{6,7\beta}$ = 5.0 Hz, $J_{6,7\alpha}$ = 3.2 Hz, $J_{6,10\beta}$ = 1.6 Hz, H-6), 5.34 (1H, br ddq, $J_{14,15A}$ = 7.2 Hz, $J_{14,15B}$ = 6.9 Hz $J_{14,16}$ = 1.2 Hz, $J_{14,12A}$ < 0.4 Hz, H-14), 5.26 (1H, dd, $J_{11,12A}$ = 2.5 Hz, $J_{11,12B}$ = 10.1 Hz, H-11), 4.56 (1H, dd, $J_{15A,15B}$ = 12.5 Hz, $J_{15A,14}$ = 7.2 Hz, H-15A), 4.49 (1H, dd, $J_{15B,15A}$ = 12.5 Hz, $J_{15B,14}$ = 6.9 Hz, H-15B), 2.30 (2H, q, $J_{2',3'}$ = 7.6 Hz, H_2-2' at C-15), 2.27 (2H, q, $J_{2',3'}$ = 7.6 Hz, H_2-2' at C-11), 2.24 (1H, dd, $J_{12B,12A}$ = 13.8 Hz, $J_{12B,11}$ = 10.1 Hz, H-12B), 1.84 (1H, dtd, $J_{7\beta,7\alpha}$ = 17.6 Hz, $J_{7\beta,6}$ = $J_{7\beta,8\beta}$ = 5.0 Hz, $J_{7\beta,10\beta}$ = 1.9 Hz, H-7β), 1.73 (3H, d, $J_{16,14}$ = 1.2 Hz, Me-16), 1.18 (1H, td, $J_{3\alpha,3\beta}$ = $J_{3\alpha,2\beta}$ = 13.1 Hz, $J_{3\alpha,2\alpha}$ = 4.5 Hz, H-3α), 1.12 (3H, t, $J_{3',2'}$ = 7.6 Hz, Me-3'at C-15), 1.10 (3H, t, $J_{3',2'}$ = 7.6 Hz, Me-3'at C-11), 1.05 (3H, s, Me-19), 0.98 (3H, d, $J_{17,8\beta}$ = 6.8 Hz, Me-17), 0.97 (3H, s, Me-18), 0.67 (3H, s, Me-20) (the remaining protons appeared as overlapped signals at almost identical field than in **III.19**, as was established from the HSQC spectrum);

^{13}C NMR (100 MHz, $CDCl_3$): δ 174.4 (C-1' at C-15, s), 174.0 (C-1' at C-11, s), 145.4 (C-5, s), 139.0 (C-13, s), 121.6 (C-14, d), 116.0 (C-6, d), 74.8 (C-11, d), 61.1 (C-15, t), 41.4 (C-3, t), 41.0 (C-9, s), 40.2 (C-12, t), 40.1 (C-10, d), 36.3 (C-4, s), 32.8 (C-8, d), 31.4 (C-7, t), 29.7 (C-19, q), 29.0 (C-1, t), 28.2 (C-18, q), 27.8 (C-2' at C-11, t), 27.5 (C-2' at C-15, t), 22.6 (C-2, t), 17.2 (C-17, q), 16.3 (C-16, q), 11.9 (C-20, q), 9.4 (C-3' at C-11, q), 9.1 (C-3' at C-15, q);

EIMS *m/z* (relative intensity): $[M]^+$ absent, 344 $[M\text{-}CH_3CH_2COOH]^+$ (1), 270 $[M\text{-}2CH_3CH_2COOH]^+$ (2), 203 (22), 191 (100), 173 (17), 147 (13), 135 (14), 133 (14), 119 (43), 109 (15), 105 (16), 95 (19), 69 (13), 57 (49); $C_{26}H_{42}O_4$, M_r 418.

V.6.2.4.3. (11*R**, 13*E*)-15-Butyryloxyhalima-5,13-dien-11-ol III.21



III.21

Colourless thick oil;

[α]$^{20}_{D}$ -20.9 ° (*c* 0.105, $CHCl_3$);

IR (neat) ν_{max}: 3546, 3051, 2962, 2929, 1736, 1667, 1458, 1383, 1364, 1275, 1178, 1090, 1040, 966 cm^{-1};

^{1}**H NMR** (400 MHz, $CDCl_3$): δ 5.46 (1H, br tq, $J_{14,15}$ = 7.0 Hz, $J_{14,16}$ = 1.0 Hz, $J_{14,12A}$ < 0.4 Hz, H-14), 5.41 (1H, ddd, $J_{6,7\beta}$ = 4.9 Hz, $J_{6,7\alpha}$ = 3.2 Hz, $J_{6,10\beta}$ = 1.6 Hz, H-6), 4.62 (1H, dd, $J_{15A,15B}$ = 12.8 Hz, $J_{15A,14}$ = 7.0 Hz, H-15A), 4.59 (1H, dd, $J_{15B,15A}$ = 12.8 Hz, $J_{15B,14}$ = 7.0 Hz, H-15B), 3.66 (1H, ddd, $J_{11,12A}$ = 1.9 Hz, $J_{11,12B}$ = 10.7 Hz, $J_{1,11OH}$ = 3.2 Hz, H-11), 2.34 (1H, br dd, $J_{12A,12B}$ = 13.6, $J_{12A,11}$ = 1.9 Hz, $J_{12A,14}$ < 0.4 Hz, H-12A), 2.28 (1H, dm, $J_{10\beta,1\alpha}$ = 12.8 Hz, H-10β), 2.27 (2H, t, $J_{2',3'}$ = 7.5 Hz, H_2-2′), 2.12 (1H, dd, $J_{12B,12A}$ = 13.6 Hz, $J_{12B,11}$ = 10.7 Hz, H-12B), 1.86 (1H, dtd, $J_{7\beta,7\alpha}$ = 17.6 Hz, $J_{7\beta,6}$ = $J_{7\beta,8\beta}$ = 5.0 Hz, $J_{7\beta,10\beta}$ = 1.9 Hz, H-7β), 1.80 (1H, m, *, H-1β), 1.78 (1H, m, *, H-7α), 1.75 (3H, d, $J_{16,14}$ = 1.0 Hz, Me-16), 1.64 (2H, sext, $J_{3',2'}$ = $J_{3',4'}$ = 7.5 Hz, H_2-3′), 1.62 (1H, m, *, H-8β), 1.58 (1H, m, *, H-2α), 1.53 (1H, m, *, H-2β), 1.43 (1H, d, $J_{11OH,11}$ = 3.2 Hz, 11-OH), 1.42 (1H, m, *, H-3β), 1.19 (1H, td, $J_{3\alpha,3\beta}$ = $J_{3\alpha,2\beta}$ = 13.0 Hz, $J_{3\alpha,2\alpha}$ = 4.4 Hz, H-3α), 1.10 (1H, qd, $J_{1\alpha,1\beta}$ = $J_{1\alpha,2\beta}$ = $J_{1\alpha,10\beta}$ =

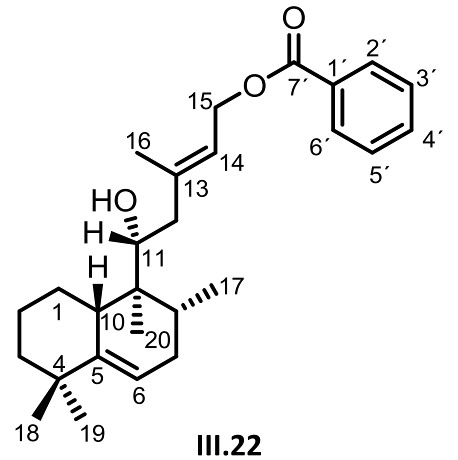
12.8 Hz, $J_{1\alpha,2\alpha}$ = 4.5 Hz, H-1α), 1.05 (3H, s, Me-19), 1.00 (3H, s, Me-18), 0.96 (3H, d, $J_{17,8\beta}$ = 6.8 Hz, Me-17), 0.93 (3H, t, $J_{4'3'}$ = 7.5 Hz, Me-4'), 0.80 (3H, s, Me-20);

*Overlapped signals;

^{13}C NMR (100 MHz, $CDCl_3$): δ 173.7 (C-1', s), 146.1 (C-5, s), 140.1 (C-13, s), 121.9 (C-14, d), 115.2 (C-6, d), 73.0 (C-11, d), 60.9 (C-15, t), 42.1 (C-12, t), 41.4 (C-3, t), 41.2 (C-9, s), 38.9 (C-10, d), 36.5 (C-4, s), 36.2 (C-2', t), 32.8 (C-8, d), 31.1 (C-7, t), 29.9 (C-19, q), 29.8 (C-1, t), 27.7 (C-18, q), 22.9 (C-2, t), 18.5 (C-3', t), 17.3 (C-17, q), 16.5 (C-16, q), 13.7 (C-4', q), 12.6 (C-20, q);

EIMS *m/z* (relative intensity): 376 $[M]^+$ (0.01), 358 $[M-H_2O]^+$ (0.1), 288 $[M-CH_3CH_2CH_2COOH]^+$ (1), 255 (2), 203 (8), 191 (100), 175 (12), 163 (10), 147 (7), 135 (11), 119 (19), 109 (12), 97 (18), 85 (6), 69 (11); $C_{24}H_{40}O_3$, M_r 376.

V.6.2.4.4. (11*R**, 13*E*)-15-Benzoyloxyhalima-5,13-dien-11-ol III.22



III.22

Colourless thick oil;

$[\alpha]^{20}_{D}$ -24.2 ° (*c* 0.062, $CHCl_3$);

IR (neat) ν_{max}: 3530, 2928, 1720, 1602, 1585, 1452, 1382, 1273, 1110, 1070, 1027, 758, 712 cm^{-1};

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (2H, dd, $J_{2',3'}$ = 8.4 Hz, $J_{2',4'}$ = 1.3 Hz, H-2' and H-6'), 7.54 (1H, tt, $J_{4',3'}$ = 7.4 Hz, $J_{4',2'}$ = 1.3 Hz, H-4'), 7.42 (2H, br dd, $J_{3',2'}$ = 8.4 Hz, $J_{3',4'}$ = 7.4 Hz, H-3' and H-5'), 5.60 (1H, br tq, $J_{14,15}$ = 6.9 Hz, $J_{14,16}$ = 0.7 Hz, $J_{14,12A}$ < 0.4 Hz, H-14), 5.41 (1H, ddd, $J_{6,7\beta}$ = 4.9 Hz, $J_{6,7\alpha}$ = 3.1 Hz, $J_{6,10\beta}$ = 1.6 Hz, H-6), 4.88 (1H, dd, $J_{15A,15B}$ =

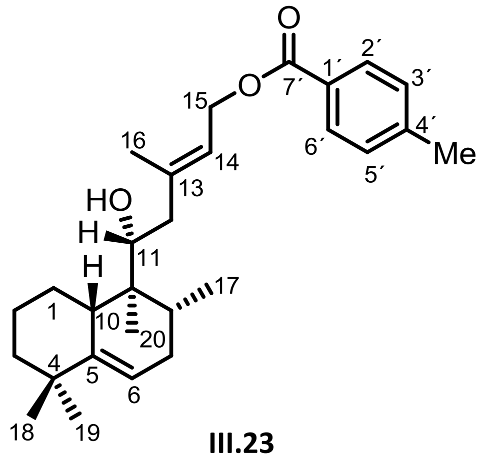
12.5 Hz, $J_{15A,14}$ = 6.9 Hz, H-15A), 4.84 (1H, dd, $J_{15B,15A}$ = 12.5 Hz, $J_{15B,14}$ = 6.9 Hz, H-15B), 3.69 (1H, br d, $J_{11,12A} < 0.4$ Hz, $J_{11,12B}$ = 10.6 Hz, H-11), 2.38 (1H, br d, $J_{12A,12B}$ = 13.6, $J_{12A,11} < 0.4$ Hz, $J_{12A,14} < 0.4$ Hz, H-12A), 2.30 (1H, dm, $J_{10\beta,1\alpha}$ = 12.8 Hz, H-10β), 2.16 (1H, dd, $J_{12B,12A}$ = 13.6 Hz, $J_{12B,11}$ = 10.6 Hz, H-12B), 1.87 (1H, dtd, $J_{7\beta,7\alpha}$ = 17.5 Hz, $J_{7\beta,6} = J_{7\beta,8\beta}$ = 5.0 Hz, $J_{7\beta,10\beta}$ = 1.9 Hz, H-7β), 1.81 (3H, d, $J_{16,14}$ = 0.7 Hz, Me-16), 1.80 (1H, m, *, H-1β), 1.78 (1H, m, *, H-7α), 1.62 (1H, m, *, H-8β), 1.57 (1H, m, *, H-2α), 1.52 (1H, m, *, H-2β), 1.42 (1H, m, *, H-3β), 1.19 (1H, td, $J_{3\alpha,3\beta} = J_{3\alpha,2\beta}$ = 13.0 Hz, $J_{3\alpha,2\alpha}$ = 4.8 Hz, H-3α), 1.10 (1H, qd, $J_{1\alpha,1\beta} = J_{1\alpha,2\beta} = J_{1\alpha,10\beta}$ = 12.8 Hz, $J_{1\alpha,2\alpha}$ = 4.7 Hz, H-1α), 1.05 (3H, s, Me-19), 1.01 (3H, s, Me-18), 0.97 (3H, d, $J_{17,8\beta}$ = 6.8 Hz, Me-17), 0.81 (3H, s, Me-20);

*Overlapped signals;

^{13}C NMR (100 MHz, $CDCl_3$): δ 166.6 (C-7′, s), 146.1 (C-5, s), 140.4 (C-13, s), 132.9 (C-4′, d), 130.3 (C-1′, s), 129.6 (C-3′ and C-5′, d), 128.3 (C-2′ and C-6′, d), 121.9 (C-14, d), 115.2 (C-6, d), 72.9 (C-11, d), 61.7 (C-15, t), 42.2 (C-12, t), 41.3 (C-3, t), 41.2 (C-9, s), 38.9 (C-10, d), 36.5 (C-4, s), 32.8 (C-8, d), 31.0 (C-7, t), 29.9 (C-19, q), 29.8 (C-1, t), 27.7 (C-18, q), 22.9 (C-2, t), 17.3 (C-17, q), 16.6 (C-16, q), 12.6 (C-20, q);

(**ESI$^+$**) MS: *m/z* 433 $[M+Na]^+$; $C_{27}H_{38}O_3$, M_r 410.

V.6.2.4.5. (11*R**, 13*E*)-15-(4-Methoxy)benzoyloxyhalima-5,13-dien-11-ol III.23



III.23

Colourless thick oil;

$[\alpha]^{20}_{D}$ -25.8 ° (*c* 0.159, $CHCl_3$);

IR (neat) ν_{max}: 3525, 2956, 2931, 1712, 1607, 1582, 1512, 1459, 1382, 1258, 1168, 1101, 1032, 847, 771, 758 cm^{-1};

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.98 (2H, d, $J_{2',3'}$ = 9.1 Hz, H-2' and H-6'), 6.90 (2H, d, $J_{3',2'}$ = 9.1 Hz, H-3' and H-5'), 5.59 (1H, br tq, $J_{14,15}$ = 6.9 Hz, $J_{14,16}$ = 0.8 Hz, $J_{14,12A}$ < 0.4 Hz, H-14), 5.41 (1H, ddd, $J_{6,7\beta}$ = 4.9 Hz, $J_{6,7\alpha}$ = 3.0 Hz, $J_{6,10\beta}$ = 1.6 Hz, H-6), 4.84 (1H, dd, $J_{15A,15B}$ = 12.5 Hz, $J_{15A,14}$ = 6.8 Hz, H-15A), 4.84 (1H, dd, $J_{15B,15A}$ = 12.5 Hz, $J_{15B,14}$ = 6.9 Hz, H-15B), 3.85 (3H, s, PhO*<u>Me</u>*), 3.68 (1H, dd, $J_{11,12A}$ = 1.9 Hz, $J_{11,12B}$ = 10.7 Hz, H-11), 2.37 (1H, br dd, $J_{12A,12B}$ = 13.7, $J_{12A,11}$ = 1.9 Hz, $J_{12A,14}$ < 0.4 Hz, H-12A), 2.30 (1H, dm, $J_{10\beta,1\alpha}$ = 12.8 Hz, H-10β), 2.15 (1H, dd, $J_{12B,12A}$ = 13.7 Hz, $J_{12B,11}$ = 10.7 Hz, H-12B), 1.87 (1H, dtd, $J_{7\beta,7\alpha}$ = 17.5 Hz, $J_{7\beta,6}$ = $J_{7\beta,8\beta}$ = 5.0 Hz, $J_{7\beta,10\beta}$ = 1.9 Hz, H-7β), 1.81 (3H, d, $J_{16,14}$ = 0.8 Hz, Me-16), 1.80 (1H, m, *, H-1β), 1.78 (1H, m, *, H-7α), 1.61 (1H, m, *, H-8β), 1.57 (1H, m, *, H-2α), 1.53 (1H, m, *, H-2β), 1.42 (1H, m, *, H-3β), 1.19 (1H, td, $J_{3\alpha,3\beta}$ = $J_{3\alpha,2\beta}$ = 13.0 Hz, $J_{3\alpha,2\alpha}$ = 4.3 Hz, H-3α), 1.10 (1H, qd, $J_{1\alpha,1\beta}$ = $J_{1\alpha,2\beta}$ = $J_{1\alpha,10\beta}$ = 12.8 Hz, $J_{1\alpha,2\alpha}$ = 4.6 Hz, H-1α), 1.05 (3H, s, Me-19), 1.01 (3H, s, Me-18), 0.97 (3H, d, $J_{17,8\beta}$ = 6.8 Hz, Me-17), 0.81 (3H, s, Me-20);

*Overlapped signals;

^{13}C NMR (100 MHz, $CDCl_3$): δ 166.4 (C-7', s), 163.3 (C-4', s), 146.1 (C-5, s), 140.0 (C-13, s), 131.6 (C-2' and C-6', d), 122.7 (C-1', s), 122.1 (C-14, d), 115.2 (C-6, d), 113.5 (C-3' and C-5', d), 72.9 (C-11, d), 61.4 (C-15, t), 55.4 (PhO<u>*C*</u>H$_3$, q), 42.1 (C-12, t), 41.3 (C-3, t), 41.2 (C-9, s), 38.9 (C-10, d), 36.5 (C-4, s), 32.8 (C-8, d), 31.0 (C-7, t), 29.9 (C-19, q), 29.8 (C-1, t), 27.7 (C-18, q), 22.9 (C-2, t), 17.2 (C-17, q), 16.5 (C-16, q), 12.6 (C-20, q);

(**ESI$^+$**) MS: *m/z* 463 $[M+Na]^+$; $C_{28}H_{40}O_4$, M_r 440.

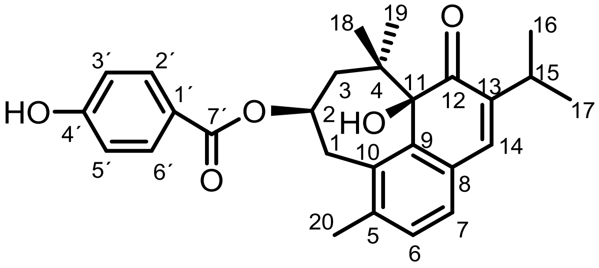
V.7. Parvifloron D II.19 derivatives

Parvifloron D **II.19** used to obtain the derivatives **III.24** was isolated from *Plectranthus ecklonii* as described previously in Chapter **II** (Simões M.F. et al., 2010b).

V.7.1. Skeletal rearrangement of parvifloron D II.19

To a solution of parvifloron D **II.19** (351 mg; 0.8077 mmol) in dichloromethane (30 mL), 12.5 g of molecular sieves AW-300 (acid-washed) were added at room temperature until disappearance of the initial red colour of the solution (24 hours). The reaction was followed by tlc (AcOEt : *n*-hexane; 3:1). Then, after filtration and washing of the molecular sieves with CH_2Cl_2 (50 mL), the organic solution was evaporated to dryness yielding 180 mg of a mixture of several compound (tlc). The residue obtained was purified by column chromatography on silica gel 9385 (eluted with AcOEt : *n*-hexane from 19:1 to 1:1). The more polar compound **III.24** (from AcOEt : *n*-hexane; 1:1) that did not decompose was isolated as a yellowish crystals (37 mg; 0.1239 mmol; 15.35 %).

[2β-(4-Hydroxy)benzoyloxy-11β-hydroxy-4(5→11),20(10→5)di*abeo*-5(10),6,8,13-abietatetraen-12-one] III.24



III.24

Colourless fine needles from EtOAc-light petroleum;

m.p. 192-195 ˚C;

$[\alpha]_D^{20}$ +271.2 ˚ ($CHCl_3$, *c* 0.229);

IR (KBr), ν_{max}: 3402, 2965, 2929, 2868, 1706, 1679, 1655, 1609, 1592, 1513, 1466, 1372, 1312, 1275, 1165, 1097, 1027, 950, 852, 773, 698 cm^{-1};

UV (MeOH), λ_{max} nm (log ε): 336 (3.89);

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.98 (2H, d, $J_{2',3'}$ = 8.8 Hz, H-2′ and H-6′), 7.13 (1H, br d, $J_{6,7}$ = 7.9 Hz, $J_{6,14}$ = 1.2 Hz,, H-6), 7.00 (1H, t, $J_{14,6}$ = $J_{14,15}$ = 1.2 Hz, H-14), 6.97 (1H, d $J_{7,6}$ = 7.9 Hz, H-7), 6.86 (2H, d, $J_{5',6'}$ = 8.8 Hz, H-3′ and H-5′), 5.74 (1H, br, 4′-OH), 4.85 (1H, dddd, $J_{2\alpha,3\beta}$ = 12.3 Hz, $J_{2\alpha,1\beta}$ = 11.8 Hz, $J_{2\alpha,3\alpha}$ = 3.7 Hz, $J_{2\alpha,1\alpha}$ = 2.1 Hz, H-2α), 4.63 (1H, s, 11β-OH), 4.04 (1H, dd, $J_{1\beta,1\alpha}$ = 12.9 Hz, $J_{1\beta,2\alpha}$ = 11.8 Hz, H-1β), 3.11 (1H, dt, $J_{1\alpha,1\beta}$ = 12.9 Hz, $J_{1\alpha,2\alpha}$ = 2.1 Hz, $J_{1\alpha,3\alpha(w)}$ = 2.1, H-1α), 3.03 (1H, sept of d, $J_{15,16(17)}$ = 6.8 Hz, $J_{15,14}$ = 1.2 Hz, H-15), 2.73 (1H, dd, $J_{3\beta,3\alpha}$ = 12.8 Hz, $J_{3\beta,2\alpha}$ = 12.3 Hz, H-3β), 2.42 (3H, s, Me-20), 1.65 (1H, ddd, $J_{3\alpha,3\beta\alpha}$ = 12.8, $J_{3\alpha,2\alpha}$ = 3.7 Hz, $J_{3\alpha,1\alpha(w)}$ = 2.1 Hz, H-3α), 1.22 (3H, d, $J_{16,15}$ = 6.8 Hz, Me-16*), 1.16 (3H, d, $J_{17,15}$ = 6.8 Hz, Me-17*), 0.88 (3H, s, Me-19), 0.85 (3H, s, Me-18);

*Interchangeable signals;

^{13}C NMR (100 MHz, $CDCl_3$): δ 205.74 (C-12, s); 165.75 (C-7′, s); 159.85 (C-4′, s); 141.25 (C-13, s); 139.54 (C-9, s); 136.12 (C-5, s); 131.92 (C-2′, and C-6′, d); 130.67 (C-6, d); 128.90 (C-8, s); 127.81 (C-7, d); 123.20 (C-1′, s); 115.15 (C-3′ and C-5′, d); 83.51 (C-11, s); 70.22 (C-2, d); 47.61 (C-3, t); 33.60 (C-1, t); 41.12 (C-4, s), 28.96 (C-18, q); 27.14 (C-15, d); 22.08 (C-17, q); 21.87 (C-19, q); 21.38 (C-20, q); 20.93 (C-16, q);

EIMS: *m/z* (relative intensity, %): 434 [M]$^+$ (1), 296 (76), 240 (11), 227 (100), 121 (27), 93 (5);

HRESIMS: *m/z* 435.2170 [M+H]$^+$ (calcd 435.2166 for $C_{27}H_{31}O_5$).

V.7.2. Acetylation of parvifloron D II.19

To a solution of parvifloron D **II.19** (321 mg; 0.739 mmol) in pyridine (30 mL) 5 mL of acetic anhydride were added at room temperature, under agitation for 72 hours. The reaction was followed by tlc (AcOEt : *n*-hexane; 1:1). After this period of time the reaction mixture was washed with water (50 mL) and extracted with dichloromethane (30 mL x 4). The combined organic layers were dried over anhydrous sodium sulfate filtered and evaporated in a rotavapor. The residue obtained was redissolved in toluene to eliminate acetic acid and pyridine, and re-evaporated. The mixture yielding 390 mg of a mixture of several compound (tlc) was purified by column chromatography (eluted with n-hexane :AcOEt; 2:1). A main product was isolated (105 mg), but it decomposed before the ^{1}H-NMR analysis).

V.7.3. Basic hydrolysis of parvifloron D II.19

To a solution of parvifloron D **II.19** (86 mg; 0.198 mmol) in methanol (5 mL) was added potassium hydroxide (5 mL, 10%) at room temperature, under agitation for 72 hours. After this time the reaction mixture was washed with water (30 mL) and HCl 5% (30 mL) and extracted with dichloromethane (20 mL x 4). The combined organic layers were dried over anhydrous sodium sulfate filtered and evaporated in rotavapor. The reaction mixture after analysed by tlc (n-hexane :AcOEt; 1:1) showed to be a mixture of several decomposition products.

V.8. Antimicrobial activity

V.8.1. Microbial strains

The *in vitro* screening for antimicrobial study was carried out using Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Enterococcus hirae* CIP 5855, *E. faecalis* ATCC 51299, *Mycobacterium smegmatis* ATCC 607), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) and the yeast *Candida albicans* ATCC 10231.

Comparative Gram-positive activities of the samples were performed against *E. faecalis* ATCC 51299 [low-level VRE (Vancomycin-Resistant *Enterococcus*)], *E. faecalis* FFHB 427483, *E. faecium* FFHB 435628, *E. flavescens* ATCC 49996, *S. aureus* ATCC 25923, *S. aureus* ATCC 43866, *S. aureus* ATCC 700699, *S. aureus* CIP 106760 [MRSA (Methicillin Resistant *S. aureus*)], and *S. aureus* FFHB 29593 (MRSA) strains. FFHB are clinical isolates from *Hospital do Barreiro* that were identified and deposited in the Laboratory of Microbiology of the Pharmacy Faculty, Lisbon University.

V.8.2. Antimicrobial activity assays

V.8.2.1. Agar diffusion method

Antimicrobial activity was evaluated using the agar diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI, 2005a) protocol with slight modifications. Briefly wells with 5 mm were made inside the Müller–Hinton agar plates. The plates were inoculated with 5 x 10^5 CFU/mL of the test microorganisms. All the compounds were dissolved in DMSO at a concentration of 1 mg/mL. Wells were filled with 30 µL of a solution of each tested compound, the positive control drug (vancomycin) and the negative control DMSO solvent, and were allowed to diffuse for 45 min. The plates were incubated at 37 ˚C for 24 h. The sensitivity was recorded by measuring the clear zone of growth inhibition around the wells (mm, diameter) with a micrometer. Each set was tested in triplicate.

V.8.2.2. Microdilution antimicrobial method

The minimum inhibitory concentration (MIC) values of compounds against the test strains, were performed by means of the two fold serial broth microdilution assay (CLSI, 2006). The compounds, dissolved in DMSO, were diluted at concentrations ranging from 500 to 0.49 µg/mL, with a Müeller-Hinton broth medium for bacteria, and a Sabouraud broth medium for the yeast strain. The antimicrobial activity of the solvent was evaluated. Ampicillin (AMP), ketoconazole (KTC), methicillin (MET), norfloxacin (NOR), rifampicin (RIF) and vancomycin (VAN) were used as control antibiotics. The MIC values were taken as the lowest concentration of the compound that inhibited the growth of the microorganisms, after 24h of incubation at 37 ˚C, and are presented in µg/mL unities. The bacterial growth was measured with an

Absorvance Microplate Reader set to 630nm (ELX808TM–BioteK). Assays were carried out in triplicate for each tested microorganism.

V.9. Other biological activities

V.9.1. Antioxidant activity - DPPH free radical-scavenging assay

To obtain a preliminary evaluation of the antioxidant capacity, a DPPH bleaching assay was used as a rapid tlc screening method (Pisoschi A.M. et al., 2009).

V.9.1.1. TLC-DPPH bleaching assay

A tlc [silica gel GF_{254} using as solvent *n*-hexane:AcOEt (7:3)] was performed for each of the isolated compounds in Chapter **II** and 7α-acetoxy-6β,12-dihydroxy-8,12-abietadiene-11,14-dione **III.1**. After development and drying, the tlc plates were sprayed with a 0.2% DPPH solution in MeOH and examined 10 min after spraying. Vitamin E was used as positive control. The compounds with capacity to reduce DPPH appeared as yellow spots against a purple background. In this way, all the isolated compounds in Chapter **II** and 7α-acetoxy-6β,12-dihydroxy-8,12-abietadiene-11,14-dione **III.1** were submitted to TLC-DPPH bleaching assay but only parvifloron D **II.19** and royleanone **III.1** appeared as yellow spots on purple background. After this preliminary study, the interaction of active compounds with DPPH were measured quantitatively by UV-visible spectroscopy (Bernardi A.P.M. et al, 2007; Pisoschi A.M. et al., 2009; Sofidiya M.O. et al, 2009).

V.9.1.2.Measurement of DPPH Radical Scavenging Activity

The percentage of DPPH radical reduction by abietanes **II.19** and **III.1** was evaluated at different concentrations in a spectrophotometric assay (Narukawa Y., 2001; Molyneux P., 2003; Sofidiya M.O. et al, 2009).

A solution of the tested compound in methanol (500 µL) was added to a methanol solution of the DPPH radical (0.100 mM, 500 µL). The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was spectrophotometrically measured at 516 nm. Quercetin and BHT (butylhydroxytoluene) were used as standards. The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(\mathrm{Abs}\,\mathit{control} - \mathrm{Abs}\,\mathit{sample})}{(\mathrm{Abs}\,\mathit{control})} \times 100$$

where $\mathrm{Abs}_{\mathrm{control}}$ was the absorbance of DPPH radical + methanol, $\mathrm{Abs}_{\mathrm{sample}}$ was the absorbance of DPPH radical + sample/standard. The scavenging activity was expressed in terms of IC_{50}, the concentration of the samples required to give a 50% of reduction in the intensity of the signal of the DPPH radical. The assays were done at least in triplicate.

V.9.2. Anticholinesterase activity

The isolated compounds in Chapter **II** and 7α-acetoxy-6β,12-dihydroxy-8,12-abietadiene-11,14-dione **III.1** were evaluated for their anticholinesterase activity. A qualitative method for AchE activity measurement on a tlc plate was described by Kiely *et al.*, which is based on the reliable and most widely used method according to Ellman *et al*. The solutions of the tested compounds were spotted on silica gel tlc plates in a matrix pattern. Each plate was sprayed with a solution of acetylthiocholine iodide and 5,5-dithiobis(2-nitrobenzoic acid) –DTNB- followed by a solution of acetylcholinesterase. The enzyme reaction produced a yellow background color where the inhibitor compounds were exposed as white zones (where the colour has failed to develop; Kiely J. S. et al., 1991; Ree I.K. et al., 2001 and 2003).

V.9.2.1. Determination of AChE inhibitory activity - tlc assay

Acetylthiocholine iodide chloride (ATCI) was employed as substrate of the reaction and 5,5´-Dithio-bis(2-nitrobenzoic)acid –DTNB- was used for the measurement of the cholinesterase activity. The experiments were done in triplicate. Physostigmine and tacrine hydrochloride were used as positive controls.

Buffers and enzyme preparation: the buffers employed were: A, 50 mM Phosphate buffer (pH 7.2); B, buffer A containing 0.1% bovine serum albumin (BSA). The lyophilised enzyme was dissolved in the buffer A to produce a stock solution containing 500 units/mL, and further diluted with buffer B to contain 8 units/mL for the tlc assay.

Substrate and Ellman’s reagent: acetylthiocholine iodide (ATCI) was used at a concentration of 60 mM in the buffer A for the tlc assay. DTNB was used at a concentration of 10 mM in the buffer A for the tlc assay.

Enzyme inhibitory activities of the samples on the silica gel layer were detected by spraying the substrate, dye and enzyme according to Ellman’s method (**Figure IV.3,** Chapter **IV**). Aliquots (10 µL each) of each compound sample at 0.1 % in methanol, were applied to silica gel layer. The plate was sprayed with DTNB/ATCI reagent (10 mM DTNB and 60 mM ATCI in buffer A) until the layer was just saturated. The plate was then allowed to dry for 3–5 min after which 8 units/mL of enzyme solution were applied by spraying. A yellow background appeared with white spots caused by inhibiting compounds being visible after about 5 min. These white spots had to be observed and recorded within 15 min because they disappeared in 20–30 min. (Rhee I. K. et al., 2003).

All the compounds isolated in Chapter **II** and **III.1** were tested and the tlc plate only appeared with white spots on a yellow background for the positive controls. If some spot had showed inhibiting compounds, their anticholinesterase activities could be evaluated by the spectrophotometric method for quantitative determination using the same Ellman´s method.

Bioautographic enzyme tlc assay: the method developed by Marston A. *et al* for the screening of acetylcholinesterase inhibition was also performed (Marston A. et al, 2002). Physostigmine and tacrine used as positive controls, were dissolved in methanol and applied to tlc plates in varying dilutions. Acetylcholinesterase (1000 U) was dissolved in 150 mL of 0.05 M Tris–hydrochloric acid buffer at pH 7.8; bovine serum albumin (150 mg) was added to the solution. The stock solution was kept at 4°C. Tlc plates were then sprayed with enzyme stock solution and thoroughly dried again. Incubation was performed at 37°C for 20 min. For detection of the enzyme, solutions of 1-naphthyl acetate (250 mg) in ethanol (100 mL) and of Fast Blue B salt (400 mg) in distilled water (160 mL) were prepared immediately before use. After incubation of the tlc plates, 10 mL of the naphthyl acetate solution and 40 ml of the Fast Blue B salt solution were mixed and sprayed onto the plates to give a purple coloration after 1–2 min. In the presence of an inhibitory activity, white spots should be observed against the dye-coloured background of the tlc plates. However, all the screened compounds revealed purple colour thus confirming the negative results verified in the previous method.

V.9.3. Anti-inflammatory activity

The isolated compounds in Chapter **II** and **III.1** were evaluated for their anti-inflammatory activity. The colorimetric COX (ovine) Inhibitor Screening Assay Kit was used in order to screen isozyme-specific inhibitors. Cayman’s Colorimetric COX (ovine) Inhibitor Screening Assay measures the peroxidase component of COXs. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N’,N’-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm.

V.9.3.1. *In vitro* Cyclooxygenase (COX) Inhibitory Assay

Cyclooxygenase inhibitor screening assay kits (Cayman Chemicals-Catalog No. 760114) were used in order to investigate the anti-inflammatory activity of all the isolated compounds in Chapter **II** against the inflammation pathway enzymes cyclooxygenase 2 (COX-2). The COX inhibitory activity of compounds **II.1**, **II.2**, **II.3**, **II.6**, **II.7** and **II.17**, isolated on Chapter **II**, and compound **II.7** derivatives **III.15** and **III.17**, prepared on Chapter **III**, was measured using ovine COX-2 enzyme by the COX inhibitor screening assay. Experimental procedures were performed according to the manufacturer's instructions. Naproxen and SC-560 (Cayman chemicals, catalog nº 70340, IC_{50} = 6.3 µM) were used as controls in the COX assay. Any antioxidant could interfere with the assay and could appear to be a COX inhibitor. Thus, all the compounds tested were previously proved to be non-antioxidant as shown earlier (IV.2 Antioxidant activity). The compounds under assessment and the controls were dissolved in dimethyl sulfoxide (DMSO). As the appropriate concentration of each eventual inhibitor was completely unknown, several dilutions of the inhibitor were tested, with compounds being examined around a final concentration of 1000 µM. For each inhibitor at least two independent experiments were performed. The experimental procedures were carried out as prescribed in the assay manual and percent COX-2 activity values (Percent Initial Activity) were determinated as each Inhibitor Sample multiplying by 100, then divided by the 100% Initial Activity Sample, to give the percent enzyme activity.

CHAPTER VI

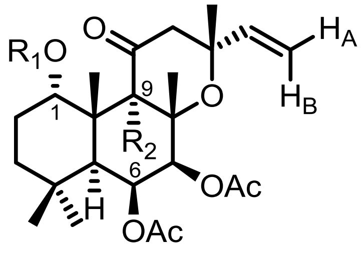
Conclusions

The main goal of this study was to search for new bioactive constituents from four species of the *Plectranthus* genus.

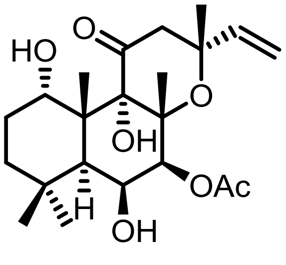
The antimicrobial activities of the plant extracts and of the isolated metabolites were thoroughly searched. Antioxidant, anticholinesterase and anti-inflammatory properties of some compounds were also screened.

The phytochemical study of the acetone extracts of *Plectranthus ornatus* Codd., *P. ecklonii* Benth., *P. porcatus* Winter & Van Jaarsv. and *P. saccatus* Benth. resulted in several terpenoid constituents mostly diterpenes.

From *P. ornatus* three new forskolin-like labdane diterpenes (6-*O*-acetylforskolin **II.1**, 1,6-di-*O*-acetylforskolin **II.2** and 1,6-di-*O*-acetyl-9-deoxyforskolin **II.3**) were isolated. Compounds **II.1**-**II.3** possess structures closely related to that of forskolin **II.4**, a very interesting bioactive substance which was also isolated from Lamiaceae plants. This was the first study on compounds **II.1**-**II.3** as naturally occurring substances, although they were already known as semisynthetic derivatives. 6-*O*-Acetylforskolin **II.1** and 1,6-di-*O*-acetylforskolin **II.2** have previously been obtained by acetylation of forskolin **II.4**, (Bhat S.V. et al., 1982) whereas **II.3** (1,6-di-*O*-acetyl-9-deoxyforskolin) has been reported as an intermediate in a semisynthesis of **II.4** (Hrib N.J., 1987a; Hrib N.J., 1987b).



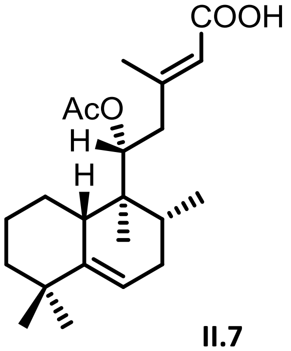
II.1 R_1 = H, R_2 = OH
II.2 R_1 = Ac, R_2 = OH
II.3 R_1 = Ac, R_2 = H



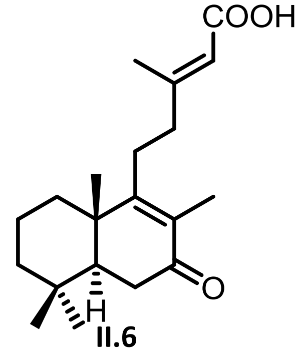
forskolin **II.4**

A new diterpene with the rare halimane skeleton [11*R**-acetoxyhalima-5,13*E*-dien-15-oic acid **II.7**] was also isolated from *P. ornatus*. This was the first report on the presence of a halimane-type diterpene in a plant belonging to the genus *Plectranthus*.

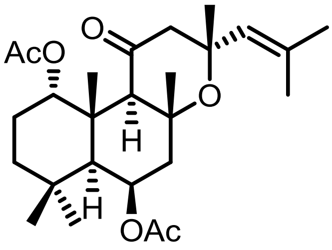
Moreover, only few 5-halimene derivatives like **II.7** have been isolated so far (found in Euphorbiaceae and Asteraceae). The isolation of the 5-halimene derivative **II.7** from *P. ornatus* is of biogenetic significance, because 5- and 5(10)-halimenes arise from an intermediate in the biosynthetic transformation of labdanes into clerodanes (Merritt A. T. and Ley S. V., 1992). Since diterpenoids belonging to both labdane- and clerodane-type have previously been found in *P. ornatus* (Oliveira P. et al., 2005; Rijo P. et al., 2002; Rijo P. et al., 2005), compound **II.7** may be considered as the missing link of that biosynthetic pathway in this species (Rijo P. et al., 2007).



II.7



II.6

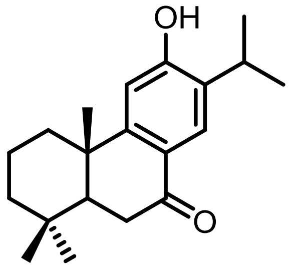


II.17

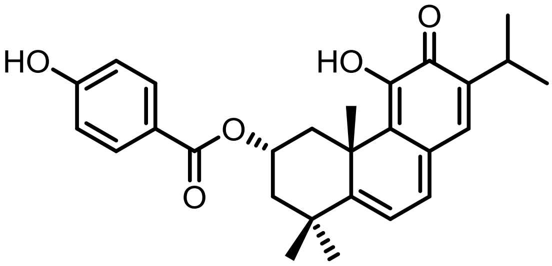
Two known labdane diterpenes were also isolated from *P. ornatus*. The rhinocerotinoic acid **II.6**, an anti-inflammatory labdane diterpene previously isolated (Dekker et al., 1988) from *Elytropappus rhinocerotis* (L.f.) Less. (Asteraceae) which was found in *Plectranthus* species and on any genus of Lamiaceae for the first time; and plectrornatin C **II.17** isolated from the same plant extract in an earlier study (Rijo P. et al., 2002).

Six known triterpenoids were also identified as mixtures. A mixture of β-sitosterol **II.11** and stigmasta-5,22*E*-dien-3β-ol **II.12**, a mixture of α-amyrin **II.13** and β-amyrin **II.14** and a mixture of ursolic and oleanolic acids **II.15** and **II.16**.

The study of *P. ecklonii* (acetone extract) led to the isolation of two known abietanes, sugiol **II.18** and parvifloron D **II.19**. Sugiol **II.18**, former isolated from several genera including *Salvia* (Ulubelen A. et al., 2000), was obtained from *Plectranthus* species for the first time.



II.18



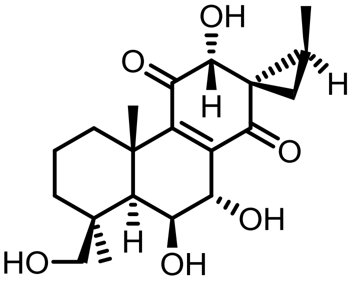
II.19

Parvifloron D **II.19** was previously isolated from several *Plectranthus* species including *P. parviflorus* (Ruedi P. Eugster C.H., 1978) and *P. strigosus* (Alder A.C. et al., 1984) and was also recently reported as an antibacterial metabolite from *P. ecklonii* (Nyila M.A. et al., 2009).

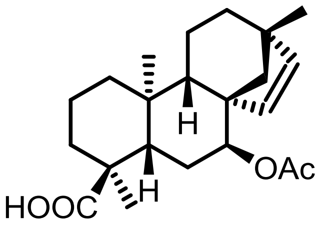
Four known triterpenoids were also isolated from *P. ecklonii* as mixtures. A mixture of β-sitosterol **II.11** and stigmasta-5,22*E*-dien-3β-ol **II.12** and the mixture of ursolic and oleanolic acids **II.15** and **II.16**.

Repeated chromatographic processes on the acetone extract of the aerial parts of *P. porcatus* Winter & Van Jaarsv. allowed the isolation of the new (13*S*,15*S*)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione **II.20**.

A new beyerane diterpene (*ent*-7α-acetoxy-15-beyeren-18-oic acid) **II.22** was isolated from an acetone extract of the aerial parts *P.* saccatus Benth..

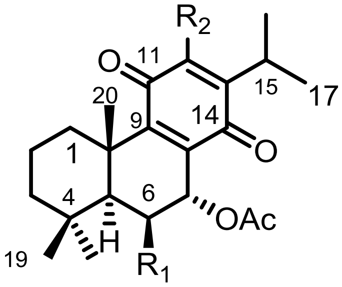


II.20



II.22

Attempting to find novel bioactive prototypes from the more potent antibacterial diterpenes isolated in higher yields, some diterpene derivatives were prepared. From the 7α-acetoxy-6β-hydroxyroyleanone **III.1** (isolated in the past from *P. grandidentatus*) thirteen derivatives (**III.2-III.13**) including a glycoside (**III.14**) were synthesized, whereof ten were new compounds. Nine new derivatives (**III.15-III.23**) were obtained from (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid **II.7** (*P. ornatus*). A new microstegiol derivative [2β-(4-hydroxy)benzoyloxy-11β-hydroxy-4(5→11),20(10→5)di*abeo*-5(10),6,8,13-abietatetraen-12-one] **III.24** was prepared from parvifloron D **II.19** (*P. ecklonii*).



	R_1	R_2
III.1	OH	OH
III.3	BzO	BzO
III.4	4-Cl-C_6H_4COO	4-Cl-C_6H_4COO
III.5	OH	4-Me-C_6H_4COO
III.6	OH	4-Cl-C_6H_4COO
III.7	OH	4-NO_2-C_6H_4COO
III.8	4-NO_2-C_6H_4COO	OH
III.9	AcO	AcO
III.10	$MeCH_2$COO	$MeCH_2$COO
III.11	AcO	OH
III.12	$MeCH_2$COO	OH
III.13	$Me(CH_2)_2$COO	OH

Figure VI.1. 7α-Acetoxy-6β-hydroxyroyleanone **III.1** ester derivatives **III.3**–**III.13.**

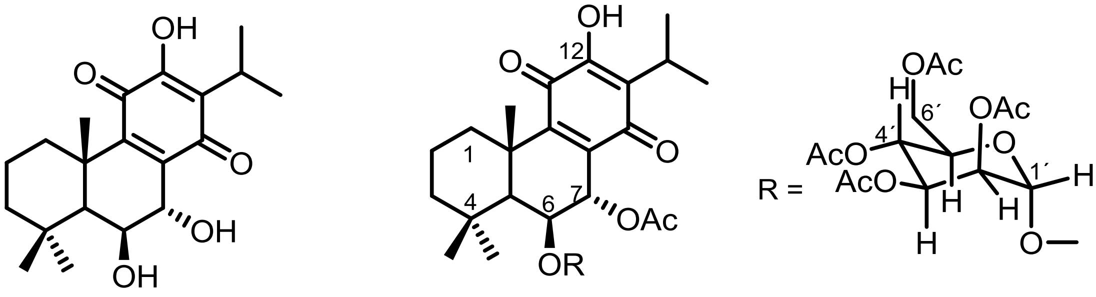
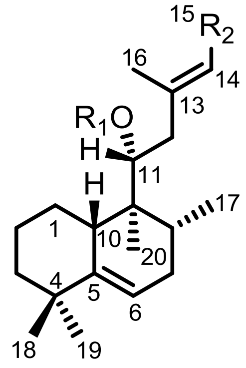
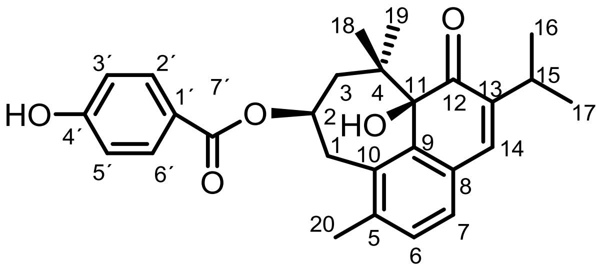


Figure VI.2. 6β,7α-dihydroxyroyleanone **III.2** and compound **III.14** (α-mannopyranoside of 7α-acetoxy-6β-hydroxyroyleanone **III.1**).



	R_1	R_2
II.7	Ac	COOH
III.15	Ac	COOMe
III.16	Ac	CH_2OH
III.17	OH	CH_2OH
III.18	OH	CH_2OAc
III.19	OH	$CH_2OCOCH_2CH_3$
III.20	$COCH_2CH_3$	$CH_2OCOCH_2CH_3$
III.21	OH	$CH_2OCOCH_2CH_2CH_3$
III.22	OH	CH_2OCOPh
III.23	OH	CH_2OCOPh-*p*-OCH_3

Figure VI.3. (11*R**,13*E*)-11-Acetoxyhalima-5,13-dien-15-oic acid **II.7** derivatives **III.15-III.23**.



III.24

The unequivocal chemical structures of pure compounds (natural and derivatives) were deduced from their spectroscopic (IR, MS, 1D and 2D NMR experiments) and physico-chemical data, as well as from literature information.

The antimicrobial activity screening of all the isolated diterpenes showed that the active compounds only inhibited the growth of the Gram positive bacteria tested. In addition, the minimum inhibitory concentration (MIC) against standard and clinical isolates of sensitive and resistant *Staphylococcus* and *Enterococcus* strains was determined for the antibacterial metabolites and their synthesized derivatives.

The three 12-*O*-benzoyl esters derivatives **III.5-III.7** of the 7*α*-acetoxy-6β-hydroxyroyleanone **III.1** prototype revealed to be more potent inhibitors against *Staphylococcus* and *Enterococcus* strains (MIC values ranging from 0.98 to 62.50 µg/mL) than the prototype (MIC values ranging from 7.81 to 31.25 µg/mL). The 6β-propionyloxy-12-*O*-propionyl derivative **III.10** also showed to be more active against

Enterococcus species (MIC values ranging from 3.90 to 7.81 µg/mL) than the prototype. Generally, the 12-esters and the 6,12-diesters were more active against *Enterococcus* than *Staphylococcus* strains. The hydrophobic extra-interactions with the bacterial targets seem to play an important role on the activity of royleanones derivatives prepared.

The (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid **II.7** and its (11*R**,13*E*)-halima-5,13-diene-11,15-diol derivative **III.17** were the more active halimanes (MIC values ranging from 15.62 to 125.00 µg/mL).

Parvifloron D **II.19** (MIC values ranging from 3.90 to 125.00 µg/mL) was less active than its microstegiol 2β-(4-hydroxy)benzoyl derivative **III.24** (MIC values ranging from 3.91 to 7.81 µg/mL), although showing both more potent antibacterial activities than the halimane diterpenoids (MIC values ranging from 15.62 to >250.00 µg/mL).

The compounds under study were also surveyed about their capacity to terminate radical chain processes which reflects the antioxidant activity. Taking into account the IC_{50} values which expressed the scavenging DPPH radical ability, both parvifloron D **II.19** and 7α-acetoxy-6β-hydroxyroyleanone **III.1** showed *in vitro* antioxidant activity. Parvifloron D **II.19** showed antioxidant properties (IC_{50} 0.1125 ± 0.0177 mM) equivalent to BHT (0.1050 ± 0.0070) but lower than quercetin (0.0075 ± 0.0010. The IC_{50} of royleanone **III.1** (1.8500 ± 0.0707 mM) revealed antioxidant ability inferior to butylated hydroxytoluene (BHT). In the literature similar oxidized abietanes bearing phenol groups, namely of the royleanone-type, are reported as promising antioxidants diterpenes (Kabouche A. et al., 2007), justifying thus the screening of this activity.

The preliminar *in vitro* acetylcholinesterase assays did not detect any activity for all the newly isolated diterpenes and 7α-acetoxy-6β-hydroxyroyleanone **III.1**.

The COX inhibitor screening assay were tested on 6-*O*-acetylforskolin **II.1**, rhinocerotinoic acid **II.6**, plectrornatin C **II.17**, (11*R**,13*E*)-halima-5,13-diene-11,15-diol **III.17**, 11*R**-acetoxyhalima-5,13*E*-dien-15-oic acid **II.7** and on its methyl ester **III.15**, for their ability to inhibit COX-2. The compounds tested seem to show weak to moderate COX-2 activity from 6-*O*-acetylforskolin **II.1** at 553 µM with 89.93%, to rinocerotinoic acid **II.6** at 785 μM with 54.56%, respectively. The results obtained for the derivatives halimane methyl ester **III.15** (76.20 % at 664 μM) and diol **III.17** (59.84 % at 1631 μM) suggest a strong inhibition of COX-2 than the starting material halimane **II.7** (79.20%) at 1379 μM. Plectrornatin C **II.7** showed 59.42 % of COX-2 activity at 595 μM. These preliminary anti-inflammatory activity results encourage further studies aiming to determinate the IC_{50} values (concentration at which there was 50% inhibition) of all the diterpenoids studied in this thesis, and to examine this potential bioactivity in a more robust approach.

The preliminar screening of various-types bioactivities of plants and their constituents (extracts, isolated compounds and even derivatives) namely anti-cholinesterase (anti-ChE), anti-inflammatory, antioxidant and antimicrobial activities can be relevant for the treatment of cognitive disorders (and other diseases), where anti-inflammatory process pathways are linked to the other two properties.

Previous research on plants of the genus *Plectranthus* (Lamiaceæ) evidenced that some of their constituents owned interesting biological activities. This research work revealed that the four *Plectranthus* species studied biosynthesize a wide range of bioactive diterpenes which can be transformed into more potent bioactive diterpenes analogous. In this way, *Plectranthus* species may be considered good raw material for bioassay-guided studies, phytochemical studies and biological studies. In order to obtain structure-activity relationships and elucidate the mode of action, these and future works may contribute for the development of new medicinal drugs.

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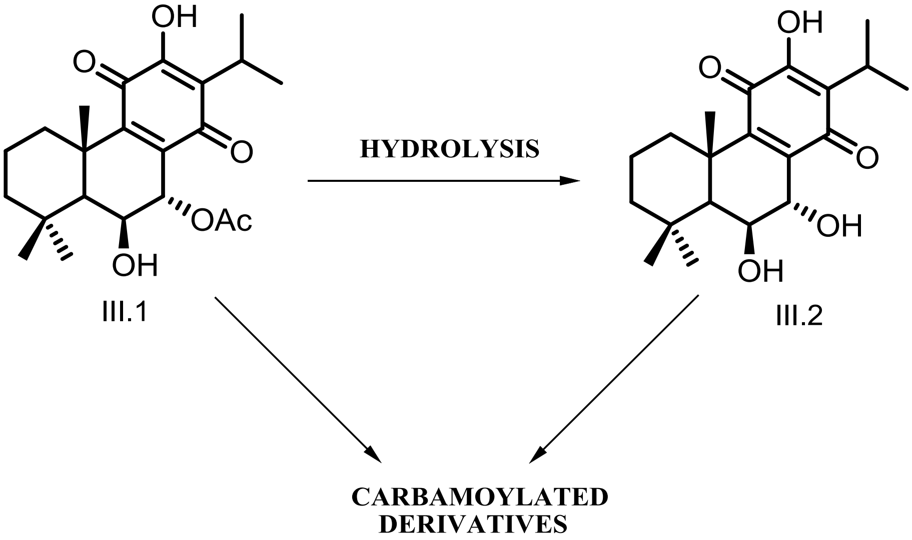
Annexes

A.1. Antimicrobial activity of carbamoyl derivatives of 7α-acetoxy-6β-hydroxyroyleanone III.1

The preparation of some derivatives is outlined in **Scheme A.1** 7α-acetoxy-6β-hydroxyroyleanone **III.1** and 6β,7α-dihydroxyroyleanone **III.2** were carbamoylated by two methods. Method A, with isocyanate (derivatives **A.1** and **A.2**) and method B, with carbonyldiimidazole (CDI) where the intermediate imidazolide (derivatives **A.3** and **A.4**), also isolated, was treated with an excess of the desired amine to form the carbamate derivative (derivatives **A.5**).

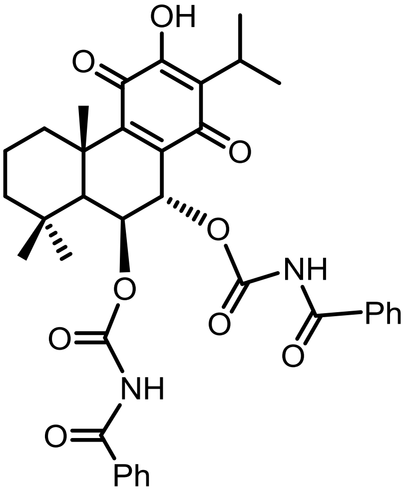


Scheme A.1. Carbamoylated derivatives from 7α-acetoxy-6β-hydroxyroyleanone **III.1** and its basic hydrolysis product 6β,7α-dihydroxyroyleanone **III.2.**

A.1.1. Royleanone carbamoylated derivatives using isocyanate (method A)

To a solution of 6β,7α-dihydroxyroyleanone **III.2** (12.4 mg; 0.0355 mmol) in dry toluene (2.5 mL) and triethylamine (0.005 mL; 0.0359 mmol) at 0 °C, benzyl isocyanate (0.010 mL; 0.0796 mmol) was added. The reaction mixture was kept at 0˚C under agitation for 1 hour (Leenders R.G.G. et al., 1996). The reaction was followed by tlc (diethyl ether).

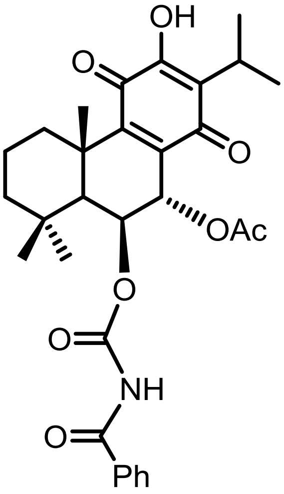
After this period of time the solvents were evaporated in rotavapor and the yellow residue obtained was purified by thin layer chromatography (precoated silica gel plates Merck nº 7747 eluted with 7:3 n-hexane:AcOEt) to give 7α,6β-di-(N-benzoyl)carbamoyloxyroyleanone **A.1** (3 mg; 0.0046 mmol) as yellow crystals (13.12 %). This reaction was repeated and similar yields were obtained.



A.1

^{1}H NMR (CDCl$_3$, 300 MHz): δ 7.98 and 7.96 (2H, br s, NH protons), 7.80-7.40 (10H, m, aromatic protons), 6.98 (1H, s, 12-OH), 5.94 (1H, d, $J_{7\beta,6\alpha}$ = 1.7 Hz, H-7β), 5.78 (1H, br s (W$_{1/2}$ = 7Hz), $J_{6\alpha,7\beta}$ = 1.7 Hz, H-6α), 3.19 (1H, sept, $J_{15,16(17)}$ = 7.1 Hz, H-15), 2.70 (1H, br d, $J_{1\beta,1\alpha}$ = 12.2 Hz, H-1β), 1.67 (3H, s, Me-20), 1.22 (3H, d, $J_{16(17),15}$ = 7.1 Hz, Me-16), 1.18 (3H, d, $J_{17(16),15}$ = 7.1 Hz, Me-17), 1.08 (3H, s, Me-19), 1.07 (3H, s, Me-18).

This reaction was repeated with 7α-acetoxy-6β-hidroxyroyleanone **III.1** and 7α-acetoxy-6β-(N-benzoyl)carbamoylroyleanone **A.2** (87,59%) was obtained.



A.2.

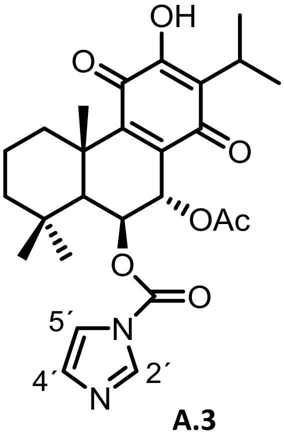
m.p. 213-215 °C (AcOEt: *n*-hexano);

$[\alpha]_D^{18}$ – 25.8° ($CHCl_3$; c 0.36).

The tlc chromatogram with a standard sample (co-aplication) upon revelation with a mixture of H_2O : CH_3COOH : H_2SO_4 (16: 80: 4) and posterior heat confirmed the presence of 7α-acetoxy-6β-(N-benzoyl)carbamoylroyleanone **A.2**.

A.1.2. Royleanone carbamoylated derivatives using carbonyldiimidazole (CDI) (method B)

To a solution of 7α-acetoxy-6β-hydroxyroyleanone **III.1** (10.0 mg; 0.0256 mmol) in dry dichloromethane (1 mL) carbonyldiimidazole (CDI) (0.0092 g; 0.0564 mmol) was added. The reaction mixture was kept at room temperature under agitation for 8 hours. An aliquot of the intermediate imidazolide **A.3** (6 mg) was isolated.



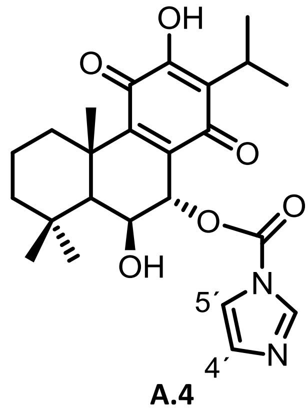
A.3

Yellow needles (*n*-hexane);

^{1}H NMR (CDCl$_3$, 400 MHz): δ 8.06 (1H, dd, $J_{2',4'}$ = 0.9 Hz, $J_{2',5'}$ = 1.5 Hz, H-2′), 7.37 (1H, t, $J_{5',2'}$ = $J_{5',4'}$ = 1.5 Hz, H-5′), 7.05 (1H, dd, $J_{4',2'}$ = 0.9 Hz, $J_{4',5'}$ = 1.5 Hz, H-4′), 6.98 (1H, br s, 12-OH), 5.92 (1H, dd, $J_{7\beta,6\alpha}$ = 1.8 Hz, $J_{7\beta,5\alpha}$ = 0.6 Hz, H-7β), 5.65 (1H, dd, $J_{6\alpha,5\alpha}$ = 1.5 Hz, $J_{6\alpha,7\beta}$ = 1.8 Hz, H-6α), 3.16 (1H, sept, $J_{15,16(17)}$ = 7.0 Hz, H-15), 2.74 (1H, ddd, $J_{1\beta,1\alpha}$ = 12.8 Hz, $J_{1\beta,2\alpha}$ = 3.8 Hz, $J_{1\beta,2\beta}$ = 2.7 Hz, H-1β), 2.06 (1H, s, 7α-OAc), 1.85 (1H, m, $J_{2\beta,1\alpha}$ = 13.1 Hz, $J_{2\beta,1\beta}$ = 2.7 Hz, $J_{2\beta,2\alpha}$ = $J_{2\beta,3\alpha}$ = *, $J_{2\beta,3\beta}$ = 13.2 Hz, H-2β), 1.69 (1H, *, $J_{5\alpha,6\alpha}$ = 1.5 Hz, $J_{5\alpha,7\beta}$ = 0.6 Hz, H-5α), 1.66 (3H, s, Me-20), ~1.60 (1H, *, $J_{2\alpha,1\alpha}$ = 3.8 Hz, $J_{2\alpha,1\beta}$ = 3.5 Hz, $J_{2\alpha,2\beta}$ = 13.9 Hz, $J_{2\alpha,3\alpha}$ = 4.1 Hz, $J_{2\alpha,3\beta}$ = 3.4 Hz, H-2α), 1.54 (1H, ddd, $J_{3\beta,2\alpha}$ = 3.8 Hz, $J_{3\beta,2\beta}$ = 13.2 Hz, $J_{3\beta,3\alpha}$ = 13.4 Hz, H-3β), ~1.30 (1H, *, $J_{3\alpha,2\alpha}$ = $J_{3\alpha,2\beta}$ = *, $J_{3\alpha,3\beta}$ = 13.4 Hz, H-3α), 1.22 (3H, d, $J_{17(16),15}$ = 7.0 Hz, Me-17), ~1.20 (1H, *, $J_{1\alpha,1\beta}$ = 12.8 Hz, $J_{1\alpha,2\alpha}$ = 3.7 Hz, $J_{1\alpha,2\beta}$ = 13.1 Hz, H-1α), 1.19 (3H, d, $J_{16(17),15}$ = 7.0 Hz, Me-16), 1.06 (3H, s, Me-19), 1.02 (3H, s, Me-18).

*overlapped signal

This carbamoylation reaction was repeated with the 6β,7α-dihydroxyroyleanone **III.2** (20.0 mg; 0.0574 mmol) and treated with carbonyldiimidazole (CDI; 0.036 g; 0.2263 mmol). The intermediate imidazolide was isolated, **A.4** (7.5 mg; 0.017mmol) as yellow needles (29.53%).



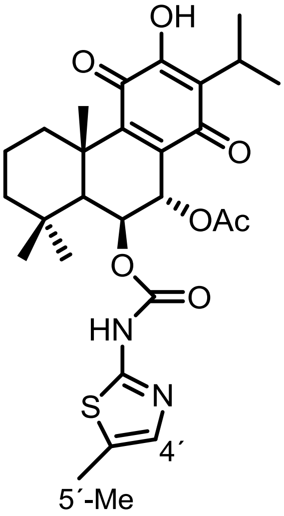
A.4

Yellow needles (*n*-hexane);

^{1}H NMR (CDCl$_3$, 400 MHz): δ 8.05 (1H, dd, $J_{2´,4´}$ = 0.9 Hz, $J_{2´,5´}$ = 1.5 Hz, H-2´), 7.37 (1H, t, $J_{5´,2´}$ = $J_{5´,4´}$ = 1.5 Hz, H-5´), 7.05 (1H, dd, $J_{4´,2´}$ = 0.9 Hz, $J_{4´,5´}$ = 1.5 Hz, H-4´), 6.98 (1H, s, 12-OH), 5.86 (1H, dd, $J_{7\beta,6\alpha}$ = 2.0 Hz, $J_{7\beta,5\alpha}$ = 0.9 Hz, H-7β), 4.50 (1H, t, $J_{6\alpha,5\alpha}$ = $J_{6\alpha,7\beta}$ = 2.0 Hz, H-6α), 3.14 (1H, sept, $J_{15,16(17)}$ = 7.1 Hz, H-15), 2.71 (1H, br d, $J_{1\beta,1\alpha}$ = 12.2 Hz, H-1β), 1.64 (3H, s, Me-20), ~1.20 (6H, *, Me-17 and Me-16), ~0.92 (6H, *, Me-19 and Me-18).

*overlapped signal

An excess of 5-methyl-2-aminothiazole (0.0199 g; 0.1745 mmol) was then added and the reaction mixture (containing the intermediate imidazolide), was kept at room temperature under agitation for 24 hours. The reaction was followed by tlc with n-hexane:AcOEt (7:3) (Robbins J.D. et al., 1996) The reaction mixture was poured into water (10 mL) and extracted with ethyl acetate (10 mL x 5). The combined organic layers were dried over anhydrous sodium sulphate filtered and evaporated in rotavapor. The yellow residue obtained was purified by thin layer chromatography (precoated silica gel plates Merck nº 7747 eluted with diethyl ether) to give 7α-acetoxy-6β-(*N*-2-(5-methylthiazolyl)carbamoyloxyroyleanone **A.5** (4.5 mg; 0.0084 mmol) as yellow crystals (33.11 %).



A.5

Yellow needles (*n*-hexane);

^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.90 (1H, *br s*, NH), 6.87 (1H, *q*, $J_{4',\text{Me-}5'}$ = 1.2 Hz, H-4´), 5.85 (1H, dd, $J_{7\beta,6\alpha}$ = 2.1 Hz, $J_{7\beta,5\alpha}$ = 0.6 Hz, H-7β), 5.52 (1H, dd, $J_{6\alpha,5\alpha}$ = 1.2 Hz, $J_{6\alpha,7\beta}$ = 2.1 Hz, H-6α), 3.17 (1H, sept, $J_{15,16(17)}$ = 7.0 Hz, H-15), 2.67 (1H, br d, $J_{1\beta,1\alpha}$ = 13.0 Hz, H-1β), 2.32 (3H, d, $J_{\text{Me-}5',4'}$ = 1.2 Hz, Me-5´), 2.06 (1H, s, 7α-OAc), 1.56 (3H, s, Me-20), 1.22 (3H, d, $J_{17(16),15}$ = 7.0 Hz, Me-17), 1.19 (3H, d, $J_{16(17),15}$ = 7.0 Hz, Me-16), 1.04 (3H, s, Me-19), 1.01 (3H, s, Me-18), 6.98 (1H, br s, 12-OH).

The antimicrobial activity screening on all the diterpenoids derivatives showed that those active only inhibited the growth of the Gram positive bacteria. In addition, the minimum inhibitory concentration against standard *Staphylococcus* and *Enterococcus* strains was determined for the antibacterial metabolites and their synthesized derivatives. None of the derivatives improved the antimicrobial activity of the starting material 7α-acetoxy-6β-hydroxyroyleanone **III.1** (MIC values ranging from 250 to 7.81 µg/mL).

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A.2. Antimycobacterial Metabolites from *Plectranthus.* Royleanone Derivatives against *Mycobacterium tuberculosis* Strains

The antimycobacterial activities of eight diterpenes, isolated previously from Plectranthus and eleven esters, of 7α-acetoxy-6β,12-dihydroxyabieta-8,12-diene-11,14-dione were evaluated against the MTB strains H37Rv and MDR. Only diterpenoids with a quinone framework revealed anti-MTB activity. 7α-Acetoxy-6β,12-dihydroxyabieta-8,12-diene-11,14-dione and its 6,12-dibenzoyl, 12-methoxybenzoyl, 12-chlorobenzoyl, and 12-nitrobenzoyl esters, showed potent activities against the MDR strain with MIC values between 0.39 and 3.12 μg/mL. Cytotoxic activities towards 3T3 and Vero cells were also evaluated. 12-Methoxybenzoyl derivative, with the best selectivity index, may be a suitable lead for further chemical modifications.

A.3. Screening of anti-*Helicobacter pylori* activities of five royleanone terpenoids

The diterpene 7α-acetoxy-6β-hydroxyroyleanone **III.1**, extracted from *P. grandidentatus*, is a suitable prototype with antibacterial activities. The bioactivities of four derivatives of **III.1**. 7α,6β-Diacetoxyroyleanone **III.11**, 7α,6β-diacetoxy-12-O-acetylroyleanone **III.9,** 7α-acetoxy-6β-hydroxy-12-O-(4-methoxy)benzoylroyleanone **III.5** and 7α-acetoxy-6β-hydroxy-12-O-(4-chloro)benzoylroyleanone **III.6** were evaluated and compared with the antimicrobial activity with **III.1** against four *H. pylori* clinical strains. The derivatives showed, on the whole, less antibacterial activity than 7α-acetoxy-6β-hydroxyroyleanone **III.1**. Natural diterpene **III.1** showed MIC values between 56.6 and 148.6 μg/mL against the *H. pylori* strains assayed, which is a good starting point range. Derivatives 7α,6β-Diacetoxyroyleanone **III.11** and **III.9** showed higher MIC values (92.8 to 222.7 μg/mL) towards the four assayed strains. Derivatives **III.5** and **III.6** showed no anti-*H. pylori* activity. In this set of derivatives the loss of anti-*H. pylori* activity may be related with the increase of the lipophilicity of 7α,6β-diacetoxyroyleanone **III.11**-**III.6**. Subsequently, more hydrophilic derivatives of **III.1** need to be prepared to carry out further SAR studies.

A.4. Isopimarane diterpenoids from *Aeollanthus rydingianus* and their antimicrobial activity

Four acyloxy-isopimarane derivatives along with two known isopimarane diterpenoids, the flavone cirsimaritin and the sterols β-sitosterol and stigmasterol were isolated from the aerial parts of *Aeollanthus rydingianus*. The structures of the compounds were established on the basis of spectroscopic analysis and chemical evidence. The isolated substances were screened for antimicrobial activity against Gram-positive and Gram-negative bacteria and a yeast strain. 19-Acetoxy-7,15-isopimaradien-3β-ol and 7,15-isopimaradien-19-ol showed minimum inhibitory concentration (MIC) values of 3.90–15.62 µg/mL for *Staphylococcus aureus* and of 7.81 µg/mL for *Enterococcus hirae*.

A.5. Prediction of diterpenes antibacterial activity against MRSA using Machine Learning Methods

Models for the qualitative prediction of antibacterial activity against MRSA strains were built using machine learning methods (MLM) like Random Forests (RFs), Associative Neural Networks (ASNNs) and Counter-Propagation Neural Networks (CPNNs) on the basis of MOLMAP and DRAGON descriptors (Latino D. A. R. S. et al., 2006; Pinheiro L. et al., 2010).

A dataset of 90 natural compounds from the class of diterpenes (abietanes, totaranes and pimaranes) was compiled (Gaspar-Marques C. et al., 2006; Yang Z. et al., 2001; Evans G.B. and Furneaux R.H., 2000; Rijo P. et al, 2009)

The Minimum Inhibitory Concentration (MIC) values reported for those diterpenes were used to divide them into two groups. Compounds with MIC values ≤ 8 µg/mL were classified as active and those with MIC values > 8 µg/mL as inactive antibacterial agents.

The relevant steps of the methodologies were:

- *(i)* generation of molecular descriptors (the input) through DRAGON software and the MOLMAP method;
- *(ii)* use of RFs were to check the most relevant descriptors for the prediction of anti-MRSA activity from an input vector that consists in DRAGON descriptors;
- *(iii)* performance of a selection of 74 descriptors (DRAGON descriptors) based on RFs;
- *(iv)* training of CPNNs and RFs to classify compounds as active or inactive against MRSA on the basis of their 15×15=225 MOLMAP descriptors;
- *(v)* employment of ASNNs and CPNNs to build models for the prediction of anti-MRSA activity using as input 93 descriptors – selection of 74 descriptors (DRAGON descriptors) based on a Random Forest plus 19 global molecular descriptors calculated with DRAGON software;
- *(vi)* validation of ASNNs and CPNNs by internal validation procedures (internal validation tests and leave-one-out measures) and a partition of the data in training and test sets.
- *(vii)* Utilization of the ASSNN program to perform the experiments.
- *(viii)* Corrections of the weights, during the training procedure, by means of the Levenberg-Marquardt algorithm.

The obtained models for the qualitative prediction of antibacterial activity against MRSA strains were able to classify correctly a compound as active or inactive in more than 70-80% of the cases both in training and tests sets, depending on the method used.

The trained CPNN showed a trend for clustering according to activity, and produced accurate predictions for a small random test set.

A regression model was also set up using Associative Neural Networks (ASNNs) and then applied to the qualitative prediction of bioactivity based on the threshold of 8 µg/mL. When the model was employed in qualitative prediction <90% of the compounds were correctly classified in the training, validation and Leave One Out (LOO) tests. Moreover, the results indicate that quantitative relationships with a good accuracy could not be found between the descriptors used and the activity against MRSA. However when these models were used for qualitative predictions, good results could be obtained.

In the future, the development of work conditions (in terms of descriptors, dataset and automatic learning techniques) are expected, aiming that the prediction of obtained models could assist in the design and synthesis of new antibacterial compounds namely against MRSA.

Machine Learning Methods: Drug discovery is a complex and costly process, with the main bottlenecks being the time and costs of finding, making and testing new chemical entities (NCE). The cost of developing a new drug today is estimated to be over $1 billion. A large part of this cost is the result of failed molecules: chemical compounds that appear to be promising drug candidates during initial stages of screening, but after several rounds of expensive preclinical and clinical testing, turn out to be unsuitable for further development. With chemical libraries today containing millions of structures for screening, there is an increasing need for computational methods that can help alleviate some of these challenges. In particular, computational tools that can rank chemical structures according to their chances of clinical success can be invaluable in prioritizing compounds for screening: such tools can be used to focus expensive biological testing on a small set of highly ranked, more promising candidates, leading to potentially huge savings in time and costs (Agarwal S. et al., 2010).

Machine learning methods (MLM) have already shown considerable promise for this task. In particular, since the early days of chemical informatics, regression methods from machine learning and pattern recognition have been used in quantitative structure-activity relationship (QSAR) analysis to predict biological activities of compounds. These include, for example, partial least-squares (PLS) regression, neural networks, genetic algorithms, regression trees and random forests, and more recently, support vector regression (SVR), all of which have been used extensively in QSAR as well as quantitative structure-property relationship (QSPR) applications. QSARs or QSPRs approaches represent probably the most robust well known tools to mathematically analyze the correlation between molecular properties and the corresponding property of interest (Michielan L. and Moro S., 2010).

The prediction of the biological activity of a compound can be obtained from inductive learning, where the knowledge of previous experiments is used to learn the problem, the information is incorporated in a model, and this is used to make predictions for new objects, establishing classification models.

A machine-learning method (inductive learning technique) takes as input a training-set of objects that have previously been classified into two or more classes (in the virtual screening context, this would be a set of molecules that had previously been tested and shown to be either active or inactive). These training-set molecules are then analysed to develop a decision rule that can be used to classify new molecules (the test-set) into one of the two classes. The concept of molecular similarity lies at the heart of such methods, since no machine-learning method can reasonably be expected to discriminate between active and inactive test-set molecules unless there are some structural commonalities (in terms of the descriptors available) between the training-set actives and/or structural dissimilarity between the training-set actives and inactives (Chen B. et al., 2007).

The foremost learning strategies in MLM are the supervised learning (Random Forests, Associative Neural Networks and Counter-Propagation Neural Networks) and unsupervised learning techniques (Kohonen Self-Organizing Map).

The main objective in any supervised learning technique is to build a system that, after the learning procedure, can associate the input data, Xs, with the output or target data, Ys. Supervised learning needs a set of pairs (Xs, Ys) as input. The input of the technique is the vector Xs and the target (correct answer) is the vector Ys. After the training, it is expected that the obtained model can give correct predictions for a new object X. During the learning procedure, the output of the system for a given object is compared with the corresponding target, and an error is calculated. The supervised learning methods use this error to make corrections that try to minimize it. In unsupervised learning there is no information about the classes or output of the training examples. Only the input data is given to the system to learn. It is said that the learning is performed without a "teacher". The main objective is to make a system learn the relations between objects, building a representation of the data, in the most part of the cases in a lower dimensionality thanthe input data. Generally, these techniques have an application in clustering, data compression and outlier detection. They are used to solve classification problems (Latino D.A.R.S., 2008).

The descriptors generation or features extraction is an important step in computational classification of molecular structures and other problems such as clustering and quantitative property/activity relationship modeling. A number of modelling tools are available that can be used to generate structural descriptors.

Dragon descriptors: The Dragon software can be used to generate around topological indices for the molecules. Topological indices are a set of features that characterize the arrangement and composition of the vertices, edges and their interconnections in a molecular bonding topology. These indices are calculated from the matrix information of the molecular structure using some mathematical formula. These are real numbers and possess high discriminative power and so are able to distinguish slight variations in molecular structure. This software can generate 99 topological indices which includes Zagreb index, quadratic index, Narumi simple topological index, total structure connectivity index, Wiener index, balaban index and etc. (Shah J.Z. and Salim N., 2006).

MOLMAP descriptors: The MOLMAP (MOLecular Maps of Atom-level Properties) descriptors encode local aspects of a chemical structure (exclusively on the basis of physicochemical properties) in a fixed-length code. The MOLMAP descriptor of a molecule represents the types of bonds available in that molecule. Their use by machine learning techniques for SAR applications can lead to the identification of structural features responsible for activity (Gupta, S. et al., 2006).

Generation of MOLMAP descriptors: The MOLMAP descriptor relies on a Kohonen SOM (Self-Organizing Map) that defines types of bonds on the basis of their physicochemical and topological properties (Latino D.A.R.S. and Aires-de-Sousa J., 2009).

Kohonen SOM: The Kohonen SOM is considered the most similar to biological neuron from all the neural networks types and architectures. SOMs learn by unsupervised training, distributing bonds through a grid of so-called neurons (each containing as many weights as there are input variables), on the basis of the chemical bonds properties. In order to use physicochemical properties of individual bonds for an entire molecule, and at the same time having a fixed-length molecular representation, all the bonds of a molecule are mapped into a fixed-size 2D self-organizing map. The algorithm is designed to reveal similarities in the bonds of a data set by mapping similar bonds into the same or closely adjacent neurons. (Latino D.A.R.S. and Aires-de-Sousa J., 2009).

Counter Propagation Neural Networks (CPNNs): A Counter Propagation Neural Network learn and map objects in a very similar manner to Kohonen SOMs, but have a second layer (output layer) that acts as a look-up table and stores output data. The first layer is a SOM and the second layer stores information about the output (ex: anti-MRSA activity) (Zupan J. and Gasteiger J., 1999).

Random Forests (RFs): A Random Forest is an ensemble of not pruned classification trees created by using bootstrap samples of the training data and random subsets of variables to define the best split at each node. It is a high-dimensional nonparametric method that works well on large numbers of descriptors. The final prediction is obtained by majority voting of the individual trees. Each tree is grown with a random subset of objects. A random subset of descriptors is made available for each node splitting. RFs associate a probability to each prediction, and report the importance of each descriptor in the global model (Breiman, L., 2001).

Associative Neural Networks (ASNNs): ASNNs is a combination of an ensemble of Feed-Forward Neural Networks (FFNNs), a memory-less method, with the K-Nearest Neighbor technique, a memory based method, that often allows more accurate predictions for non-linear problems (Tetko, I., 2002). The EnsFFNNs is combined with a memory into a so-called ASNN. The memory consists on a list of objects, represented by their input variables, and the corresponding targets.

Memory-less method: There is no explicit storage of the data in the system after the training. The information about the data is stored in the neural networks weights (Latino D.A.R.S., 2008).

Memory-based methods: The data used to train the models are stored in a "memory" and used to make predictions for new data based on some local approximations of the stored examples (Latino D.A.R.S., 2008).

Levenberg-Marquardt algorithm: The Levenberg-Marquardt (LM) algorithm is an iterative technique that can locate the minimum of a multivariate function. It can be thought of as a combination of steepest descent and the Gauss-Newton method. When the current solution is still far from the correct one, the algorithm behaves like a steepest descent method - with a slow progress but guaranteed to converge. When the current solution is close to the correct solution, it becomes a Gauss-Newton method. The LM algorithm has become a standard technique for non-linear least-squares problems that has been widely used in a broad spectrum of disciplines, namely to correct the weights of ASNNs in such way that the output of the network is as close as possible to the target (Latino D.A.R.S., 2008).

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A.6. Anti-influenza virus activity and cytotoxicity in MDCK* cell cultures of diterpenes from *Plectranthus*

Compounds parvifloron D **II.19**, (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid **II.7,** and their two derivatives (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid methyl ester **III.15** and (11*R**,13*E*)-halima-5,13-diene-11,15-diol **III.17**; 7α-Acetoxy-6β-hydroxyroyleanone **III.1** and their ester derivatives 7α-acetoxy-6β-benzoyloxy-12-*O*-benzoylroyleanone **III.3** and 7α-acetoxy-6β-propionyloxy-12-O-propionylroyleanone **III.10** were tested against Influenza A/H3N2 strain A/X-31, Influenza A/H3N2 strain A/HK/7/87 and Influenza B strain B/HK/5/72. None was able to inhibit the cytopathic effects of influenza A or B virus at subtoxic concentrations. In fact, all compounds had relatively high cytotoxicity. The reference compounds *oseltamivir carboxylate* (the active form of Tamiflu®) and ribavirin were active against influenza virus; their EC_{50} values were clearly lower than their MCC values (concentrations causing minimal toxicity). For amantadine and rimantadine, the best activity was seen with the H3N2 strain. These compounds are known to be inactive against influenza B. Also, the chimeric A/X-31 strain that was used in the tests is known to be less sensitive to amantadine and rimantadine.

(*MDCK cells: Madin Darby canine kidney cells)