Secondary Metabolites of Marine-Derived Fungi: Natural Product Chemistry and Biological Activity

D i s s e r t a t i on

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

Ahmed Abdel-Azim Mahdy Abdel-Lateff

aus Ägypten

Bonn 2004

Angefertig mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn

1. Referentin: Prof. Dr. G. M. König
2. Referent: PD. Dr. W. Knöss

Vorveröffentlichungen der Dissertation /In Advance Publications of the Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät, vertreten durch die Mentorin/Betreuerin der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Parts of the results of this study have been published in advance by permission of the Mathematisch-Naturwissenschaftlichen Fakultät, represented by the supervisor of this study:

Publikationen /Research Papers

Ahmed Abdel-Lateff, Gabriele M. König, Katja M. Fisch, Ulrich Höller, Peter G. Jones, and Anthony D. Wright; New Antioxidant Hydroquinone Derivatives from the Algicolous Marine Fungus *Acremonium* sp. *J. Nat. Prod*. 2002, *65*, 1605-1611.

Ahmed Abdel-Lateff, Christine Klemke, Gabriele M. König, and Anthony D. Wright; Two New Xanthone Derivatives from the Algicolous Marine Fungus *Wardomyces anomalus J. Nat. Prod*. 2003, *66*, 706-708.

Ahmed Abdel-Lateff, Katja M. Fisch, Gabriele M. König, and A. D. Wright; A New Antioxidant Isobenzofuranone Derivative from the Algicolous Marine Fungus *Epicoccum* sp. *Planta Med.* 2003, *69*, 831-834.

Ahmed Abdel-Lateff, Stefan Kehraus, Anja Krick, Anthony D. Wright, and Gabriele M. König, Novel Sorbicillinoid Derivatives from the Marine Fungus *Trichoderma viride* Associated with the Caribbean Sponge *Agelas dispar*, in preparation.

Tagungsbeiträge/Research Presentations

A. Abdel-Lateff, G. M. König, K. M. Fisch, U. Höller, P. G. Jones, A. D. Wright; New Antioxidant Hydroquinone Derivatives from the Algicolous Marine Fungus *Acremonium* sp. Poster presented at 50th G A Annual Congress, Barcelona, Spain, 8-12 September 2002.

A. Abdel-Lateff, G. M. König, A. D. Wright; A New Isobenzofuranone derivative from *Epicoccum* sp., a fungus associated with *Fucus vesiculosus* Poster presented at the third Euroconference on Marine Natural Products, Elmau Castle, Bavaria, 15-20 September 2002.

A. Abdel-Lateff, G. M. König, A. D. Wright; Three New Xanthone Derivatives from the Algicolous Marine Fungus *Wardomyces anomalus*. Poster presented at the third Euroconference on Marine Natural Products, Elmau Castle, Bavaria, 15-20 September 2002.

Acknowledgements

I wish to express my sincere gratitude to Prof. Dr. G. M. König for supervising this study, suggesting the research project, guiding, and sponsoring this work, and also for providing excellent both scientific and working facilities. She always had time for questions and discussions. I am very lucky being one of her students.

I would like to express my sincere gratitude to Prof. Dr. Mohamed M. Kalifa (Dean of faculty of Pharmacy, El-Menia University, Egypt) and Prof. Dr. Mohamed A. A. R. ElShanawany (Dean of faculty of Pharmacy, Assuit University, Egypt) for their help to gain my scholarship.

I would like to express my thanks to PD. Dr. W. Knöss for accepting the co-examination.

I would like to thank Dr. A. D. Wright for introducing me to the world of chemical structures, his scientific instructions in the spectral interpretation and sponsoring part of my work.

I would like to express my deep thanks to Dr. Ulrich Höller for guiding me in the first step of the spectral interpretation, helping in structural elucidation of *Acremonium* hydroquinones, fruitful and also effective cooperation.

I would like to express my deep gratitude to Dr. Stefan Kehraus for showing me NMR measurements, helping in structural elucidation of *Trichoderma* polyketides and solving most of the technical problems which occurred during this thesis.

This study is dealing with marine fungi which, were isolated by Dr. Ulrich Höller and Dr. Claudia Osterhage during their Ph.D. study. I am grateful for being allowed to work with this resource.

I would like to extend my thanks to Prof. Dr. P. G. Jones (Institut for Inorganic and Analytical Chemistry, TU Braunschweig) for performing the single crystal X-ray analysis of 2-(1-methylethylidene)-pentanedioic acid (**5**) and epicoccone (**17**).

Thanks also go to Prof. Dr. Heinz-Herbert Fiebig and his group (Institute for Experimental Onkology in Freiburg) for performing the antitumor activity of *Trichoderma* metabolites.

I would like to thank Dr. Anja Krick for measuring LC-ESIMS spectra of some *Trichoderma* polyketides, helping me in the ESIMS spectral interpretation, and for introducing me to HPLC-DAD.

Dr. G. Eckhardt, Ms. C. Sondag and Ms. K. Peters (Department of Chemistry, University Bonn) are grateful thanked for EI, FAB and ESI-MS measurements.

Part of this study was an excursion to Egypt financially supported from the Egyptian government. The aim was the collection of some algal samples useful for the isolation of fungi. Important arrangements were done by Prof. Ahmed Abdella (Department of Chemistry, Faculty of Science, El-Menia University, Egypt), Dr. Kamel Shaker (Department of Pharmaceutical Science, National Resaerch Centre, Egypt), and Dr. Mary Hana (National Resaerch Centre, Egypt). Finally, the algal samples were collected and identified by Dr. Mohamed Hegazi (Marine Science Department, Faculty of Science, Suiz Canal University). I am profoundly grateful to these scientists.

I would like to extend my thanks to Mr. Harald Gross for his assisting me in solving technical problems concerning computational ware.

Thanks go to Mr. C. Dreikorn ELISAs and agar diffusion assays; to Ms Edit Neu for performing a part of agar diffusion assays; to Ms. Katja M. Fisch for performing antioxidative activity assays, to Mr. Markus Nett for testing the *Trichoderma* metabolites for estrogenic activities. Finally, thanks go to Ms. Christine Klemke, Doris Akkermann, and Daniela Müller for performing ELISAs assays.

I would like express my deep thanks Ms. E. Gassen, and Ms. J. Dornbusch for their indispensable help concerning against administrative issues.

I would like express my deep thanks to Ms. Amal Abdoun, and Ms. Kamilia Sheha (Egyptian Culture Office, Berlin, Germany) for their valuable help regarding administrative issues of the scholarship.

Special thanks also go to all members of the Institut für Pharmazeutische Biologie, Universität Bonn, for their hospitality and friendliness.

I would like to express my sincere thanks to the Egyptian government for a PhD scholarship, and also for the financial support of the collection trip to Egypt in 2002.

Financial support from the Bundesministerium für Bildung und Forschung (BMBF) is also gratefully acknowledged.

Abbreviations

°C	Degrees Celsius
1D	One Dimensional
2D	Two Dimensional
[α]	Specific rotatory power
δ	NMR chemical shift [ppm]
λ	Wavelength [nm]
µL	10^{-6} litre
µm	10^{-6} metre
γ	Wave number [cm^{-1}]
ASW	Artificial Sea Water
B. m.	Bacillus megaterium
br	broad
c	concentration [g/100 mL]
C. f.	*Chlorella fusca*
c.f.	confer
CH_2Cl_2	Dichloromethane (see DCM)
cm	10^{-2} metre
COSY	COrrelated SpectroscopY
CT	Collection Trip
d	doublet
DAD	Diode Array Detector
DCM	Dichloromethane
DEPT	Distortionless Enhancement by Polarisation Transfer
dest.	distilled
E. c.	*Escherichia coli*
e.g.	example given
E. r.	*Eurotium repens*
EI	Electron Ionisation
ELISA	Enzyme Linked Immuno Sorbent Assay
EtOAc	Ethyl acetate
EtOH	Ethanol
FAB	Fast Atom Bombardment
GC	Gas Chromatography
HIV-1	Human Immunodeficiency Virus 1
HMBC	Hetero nuclear Multiple Bond Correlation

HMQC	Hetero nuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
HR	High Resolution
Hz	Hertz
IC	Inhibition Concentration
IR	Infrared
J	Spin-spin coupling constant [Hz]
m	multiplet (in connection with NMR data)
M. m.	*Mycotypha microspora*
M. v.	*Microbotryum violaceum*
MeOH	Methanol
MHz	Megahertz
min	minute
mL	10^{-3} litre
mM	10^{-3} Mol
m. p.	melting point
MS	Mass Spectrometry
nm	10^{-9} metre
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
ppm	parts per million
q	quartet
RI	Refractive Index
RP	Reversed Phase
RT	Room Temperature
RT	Reverse Transcriptase (in connection with HIV-1)
s	singlet
sp.	species
t	triplet
TK	Tyrosine Kinase
TLC	Thin Layer Chromatography
UV	Ultra Violet
VLC	Vacuum-Liquid Chromatography

Table of contents

Page

1 Introduction **1**

1.1 Marine microorganisms 1

1.2 Marine fungi 2
1.2.1 Secondary metabolites from algae-derived fungi 3
1.2.2 Secondary metabolites from sponge-derived fungi 9

2 Scope of the present study **15**

2.1 Biological and chemical screening of fungal extracts 15

2.2 Chemical investigations of selected fungal strains 15

2.3 Pharmacological potential of isolated pure compounds 15

3 Materials and methods **16**

3.1 Chromatography 16
3.1.1 Thin layer chromatography (TLC) 16
3.1.2 Vacuum-liquid chromatography (VLC) 16
3.1.3 High performance liquid chromatography (HPLC) and detectors 16
3.1.3.1 HPLC analysis of *Trichoderma viride* ethyl acetate extract 17

3.2 Structure elucidation 17
3.2.1 NMR spectroscopy 17
3.2.2 Mass spectrometry 18
3.2.3 UV measurements 18
3.2.4 IR spectroscopy 18
3.2.5 Optical rotation 19
3.2.6 Melting point 19
3.2.7 X-ray analysis 19
19

3.3 Origin and taxonomy of algal samples 19
3.3.1 Fungal strains for chemical investigation 19

3.3.2 Isolation of endophytic fungi from egyptian algae 20

3.4 Biological testing 21
3.4.1 Agar diffusion assays 21
3.4.2 Tyrosin kinase inhibitory activity 21
3.4.3 HIV-1 reverse transcriptase inhibitory activity 22

3.4.4 Antioxidative activity 22
3.4.4.1 Thiobarbituric acid reactive substances method 22
3.4.4.2 α,α-Diphenyl-β-picrylhydrazyl radical scavenging effects 23

3.4.5 Cytotoxicity 23
3.4.5.1 Human cancer lines 23
3.4.5.2 Brine shrimp lethality bioassay 23

3.4.6 Recombinant yeast estrogenic assay 24

3.5 Chemicals 24
3.5.1 Media components 24
3.5.2 Solvents 25

4 Description of partial projects 26

4.1 Endophytic marine fungal strains; isolation, selection, mass cultivation and biological activities 26

4.2 New antioxidant hydroquinone derivatives from the algicolous marine fungus *Acremonium* sp. 30

4.3 Two new xanthone derivatives from the algicolous marine fungus *Wardomyces anomalus* 49

4.4 A new antioxidant isobenzofuranone derivative from the algicolous marine fungus *Epicoccum* sp. 57

4.5 Novel sorbicillinoid derivatives from the marine fungus *Trichoderma viride* associated with the Caribbean sponge *Agelas dispar* 66

4.6 Secondary metabolites from marine fungi 85
4.6.1 *Alternaria* sp. 85
4.6.2 *Sporormiella* sp. 86
4.6.3 *Myrioconium* sp. 87

5 Summary **89**

6 Discussion and future prospects **90**

7 References **102**

8 Appendix **108**

8.1 Secondary metabolites isolated during this study 108

8.2 ^{1}H NMR spectra of selected compounds 111

1 Introduction

1.1 Marine microorganisms

Marine microorganisms, particularly fungi and bacteria, have provided new incentives for marine natural products research over the past 15 years, and also continue to be the subject of vigorous chemical investigation (Liberra & Lindequist, 1995; Faulkner, 2001; Blunt *et al.*, 2003). The diversity of secondary metabolites reported during the recent decade is fascinating. This highlights the importance of marine microorganisms as a source of natural products (König & Wright, 1996; Pietra, 1997; Faulkner, 2000 a and b).

The marine environment comprises nearly three quarters of the earth's surface, and can be considered a soup of essentially all imaginable types of microbes (König & Wright, 1999). They may occur suspended, on living or inanimate surfaces as epibionts, or as symbionts. Microorganisms play important roles in all the major elemental cycles in the oceans (Hawksworth, 1991), and are intimately involved in ecological phenomena, e.g. biofouling, settlement, and metamorphosis. The marine environment is unique in terms of its specific composition in both organic and inorganic substances, as well as temperature ranges, and pressure conditions. Ecological niches e.g. deep-sea hydrothermal vents, mangrove forests, algae, sponge, and fish provide habitats for the evaluation of specific microorganisms (Kohlmeyer, 1979).

The difficulties associated with the collection of marine macroorganisms as well as the inadequate amount of the bioactive substance isolated (Proksch *et al.*, 2002), motivated many research groups to investigate the microbes associated with them, or those found directly in the water column and/or marine sediments (König & Wright, 1996). There are some advantages obvious when investigating microbes as compared to macroorganisms. These include; e.g. biotechnological fermentations with different parameters are possible without ecological exploitation, reisolation of the compounds is possible after large scale cultivation of the microorganism, and microorganisms are easier manipulated genetically. On this basis, marine microbes become a central topic for many groups investigating natural products with the aim of finding pharmaceutical drugs or compounds useful for agriculture (Osterhage, 2001).

An endophyte (Tan and Zou, 2001) is a bacterium (including actinomycetes) or fungal micro-organism, which spends the whole or part of its life cycle colonizing inter- and/or intra-cellular space inside the healthy tissues of the host plants, typically causing no apparent symptoms of disease. Often endophytes remain asymptomatic for many years and only

become parasitic when their hosts are stressed. For the isolation of endophytes it is important that collected algal material looks healthy, it must not have any visual symptoms of disease (Osterhage Dissertation, 2001).

1.2 Marine fungi

Marine microbes, particularly fungi, have recently been utilized as a new source of novel bioactive secondary metabolites (Biabani & Laatsch, 1998; König & Wright, 1999). This study is thus devoted to the investigation of some marine fungal strains, derived from algae or sponges, and aims at finding new natural products with biological activity and/or novel chemical structures.

The kingdom of fungi is the second largest group after insects, and widely distributed in nature. Fungi occur in Antarctic ice, tropical, and temperate regions. They inhabit soils, the surface of mountain rocks, and seawater (Feofilova, 2001). Fungi parasitize plants, protozoa, fishes, insects, and mammals. The generally accepted estimate of the number of the fungal species on Earth is a conservative 1.5 million (Hawksworth & Rossman, 1997). Although it is uncertain exactly how many fungal species are already known, one can be reasonably sure that it is in the range of 72,000 to 100,000. This implies that the fungi known today do not exceed 5% of all existing species. Evidently, the majority of fungi inhabiting the world have not yet been described. This implies that fungi represent an enormous source for natural products with diverse chemical structures and activities (Hawksworth, 1991, 1997).

Fungi from marine habitats are separated into obligate and facultative marine species, the former being restricted to the marine environment and the latter occurring also in freshwater or terrestrial localities, or both (Kohlmeyer, 1974). In 1991 Kohlmeyer & Volkmann-Kohlmeyer listed 321 filamentous higher marine fungi. Their key included 255 Ascomycetes, 60 mitosporic fungi, and only six Basidiomycetes. The isolation of a fungal strain from a marine sample does not prove that this fungus is actively living in the marine environment. It is always possible to isolate a terrestrial fungus being a contaminant in the marine habitat. Possibly such a fungal isolate was dormant in the form of spores or hyphen fragments until the surrounding conditions in the laboratory became favorable for germination and growth. Most fungi isolated from marine samples are not proven to be obligate or facultative marine. Thus, the more general expression marine-derived fungi is used (Kohlmeyer & Kohlmeyer, 1979).

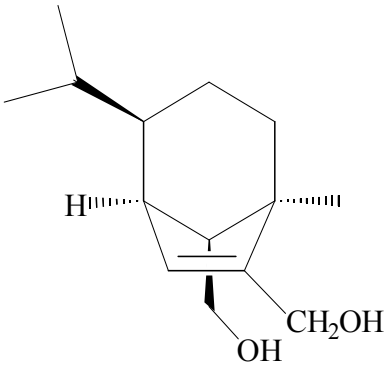
1.2.1 Secondary metabolites of algae-derived fungi

Special ecological niches in the marine environment have been observed as interesting for many natural products chemists. Such niches are marine microorganisms associated with macroorganisms e.g. algae, sponges. Most kinds of macroorganisms e.g. algae, sponges, corals, tunicates, and fish have extensively been investigated for their natural products content (Faulkner, 2001). These macroorganisms also serve as hosts for microorganisms and were recently considered as an important source of natural products. The current project focused on fungi associated with marine algae and sponges. Natural products in these association may have an ecological purpose. In many cases the natural function of secondary metabolites is, however unknown (König & Wright, 1996).

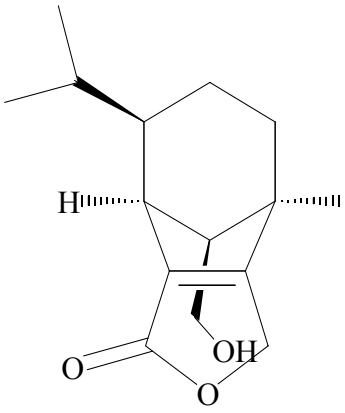
There are several reviews dealing with marine fungal metabolites (Faulkner, 2001; Pietra, 1997). They covered the isolated metabolites till 2000. In addition to this reviews, Dr. Claudia Osterhage, a prior Ph. D. student in our group, listed the metabolites isolated from algicolous fungi for the period from 1993 till May 2000. For these reasons only the metabolites isolated from algicolous fungi after May 2000 up to May 2003 are listed in Table 1. In Figure 1 the chemical structures of the compounds are shown (drawn in an alphabetical order).

Table 1. Metabolites reported from algae-derived fungi (May 2000-May 2003).

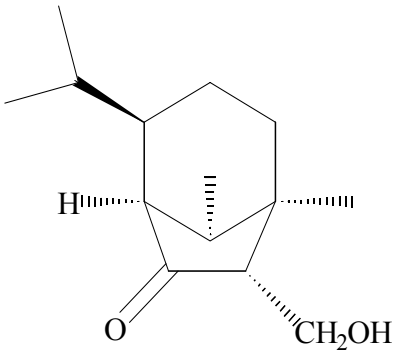
Algal species investigated	**Fungus isolated & investigated**	**Metabolite (s) isolated**	**Literature**
Ceratodictyon spongiosum	KO63	Dictyonamides A and B	Komatsu *et al*. (2001)
Enteromorpha intestinalis	*Penicillium* sp.	Penochalasins D-H	Iwamoto *et al*. (2001)
Rosenvingea sp.	*Pestalotia* sp.	Pestalone	Cueto *et al*. (2001)
Sargassum tortile	*Leptosphaeria* sp.	Leptosins M, M_1, N, and N_1	Yamada *et al*. (2002)
Fucus spiralis	*Phoma tropica*	5-Hydroxyramulosin	Osterhage *et al*. (2002)
Liagora viscida	*Drechslera dematioidea*	Isosativenetriol, Drechslerines A and B, 9-Hydroxyhelminthosporol, Drechslerines C-G, Sativene epoxide	Osterhage *et al*. (2002)
Carpopeltis cornea	*Aspergillus parasiticus*	Parasitenone	Son *et al.* (2002)
Codium fragile	*Fusarium* sp.	Tetrapeptide JM47	Jiang *et al*. (2002)
Gracilaria verrucosa	Unidentified	N_b-Acetyltryptamine	Li *et al*. (2003)



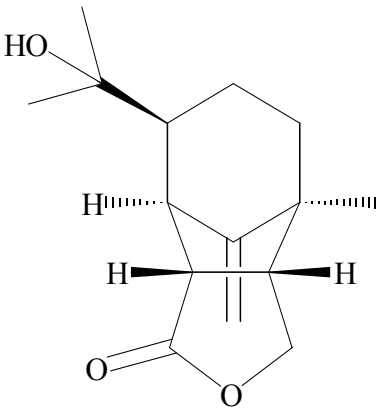
Drechslerine A



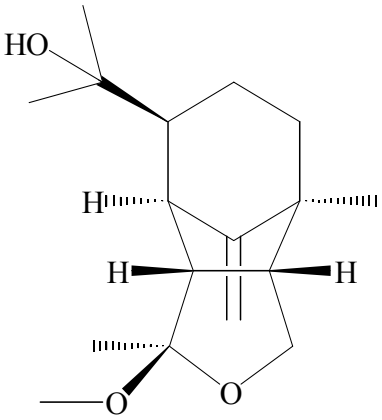
Drechslerine B



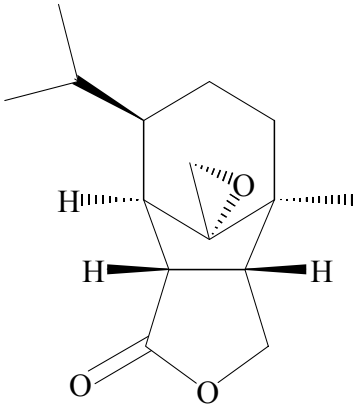
Drechslerine C



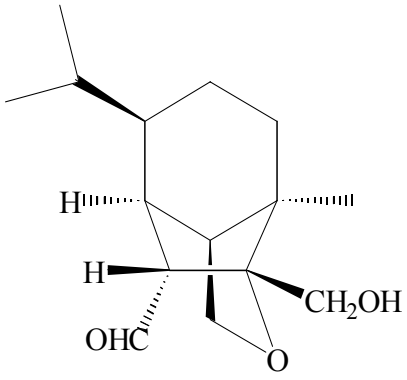
Drechslerine D



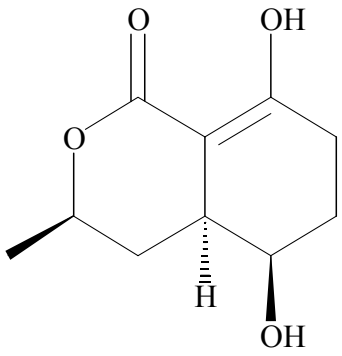
Drechslerine E



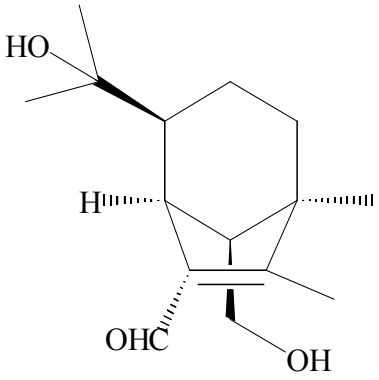
Drechslerine F



Drechslerine G



5-Hydroxyramulosin



5-Hydroxyhelminthosporol

Figure 1: Metabolites from algae-derived fungi.

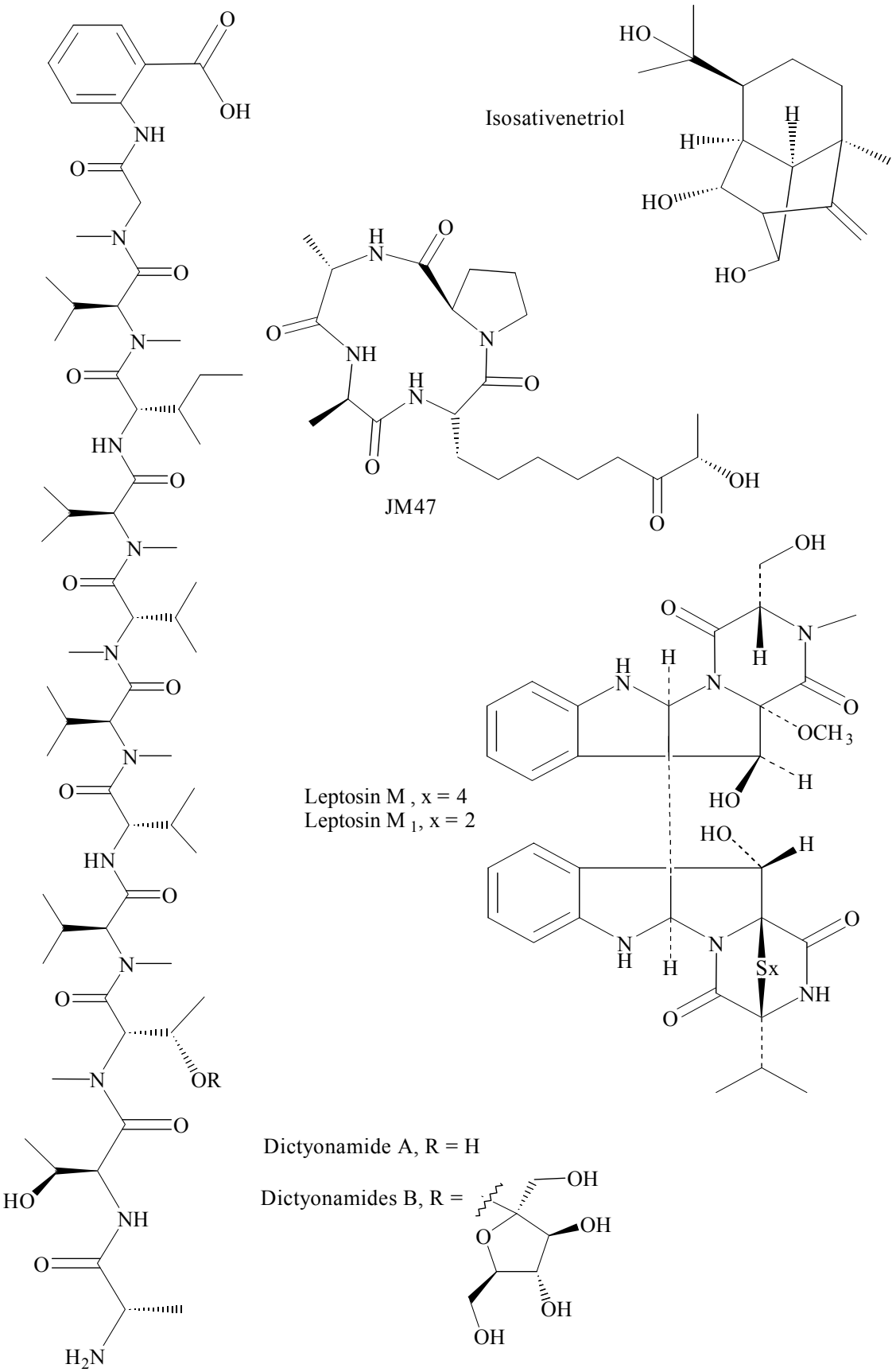
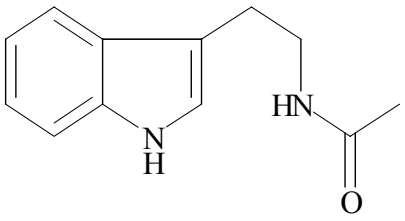
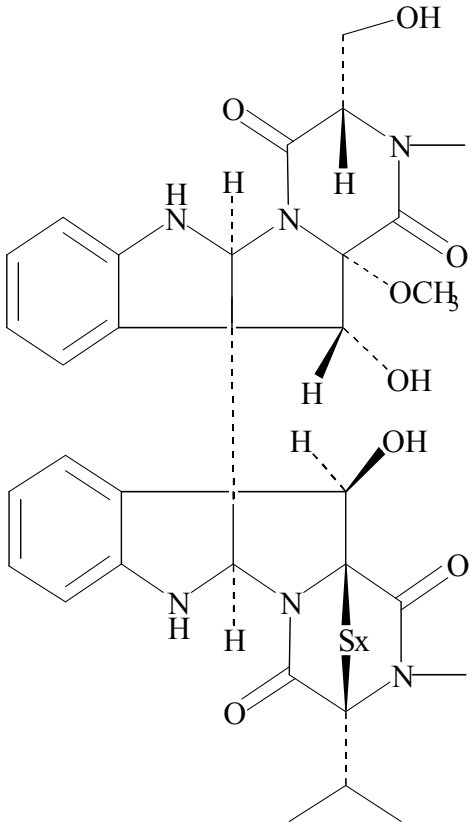


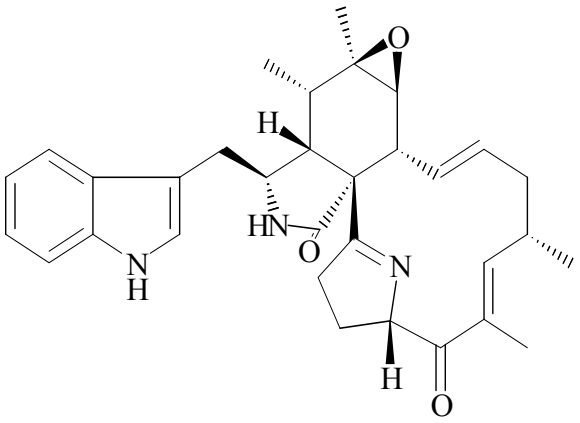
Figure 1 (continued): Metabolites from algae-derived fungi.



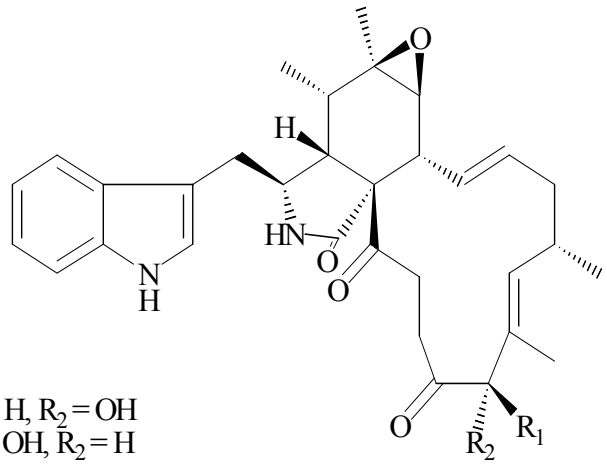
N_b-acetyltryptamine



Leptosin N, x = 4
Leptosin N$_1$, x = 3

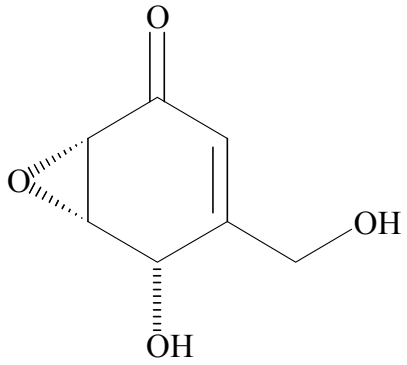


Penochalasin D

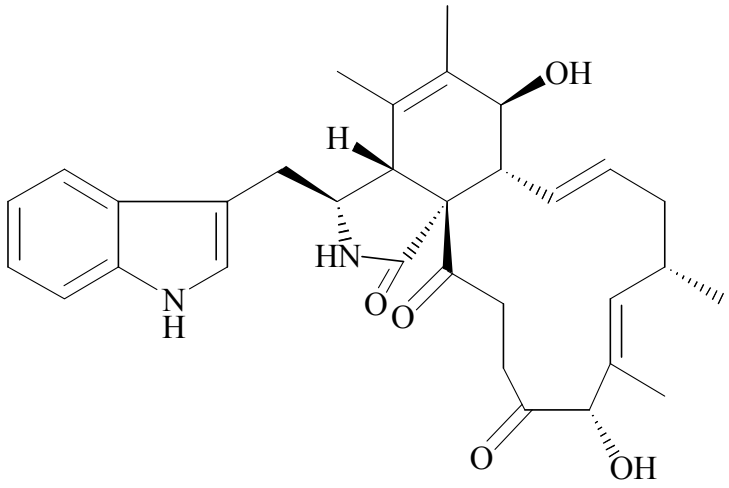


Penochalasin E, R_1 = H, R_2 = OH
Penochalasin F, R_1 = OH, R_2 = H

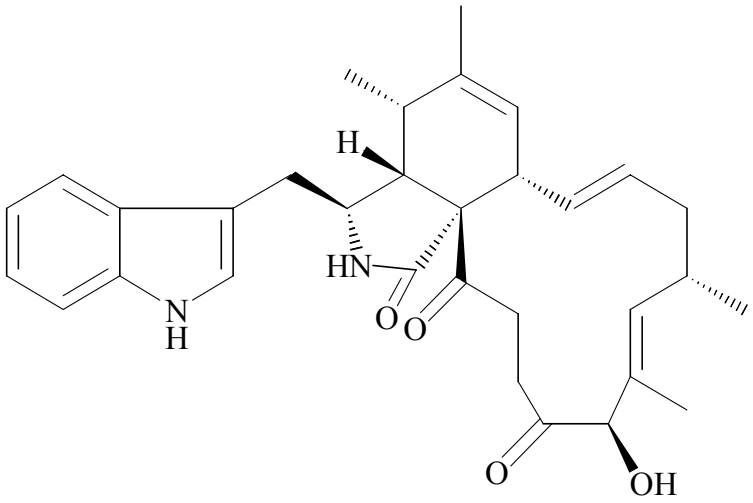
Figure 1 (continued): Metabolites from algae-derived fungi.



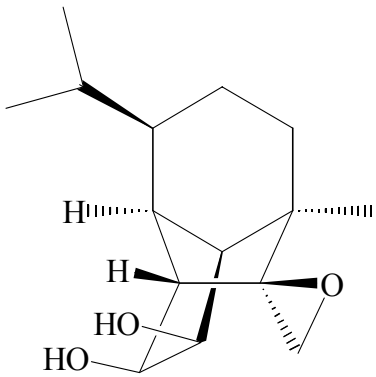
Parasitenone



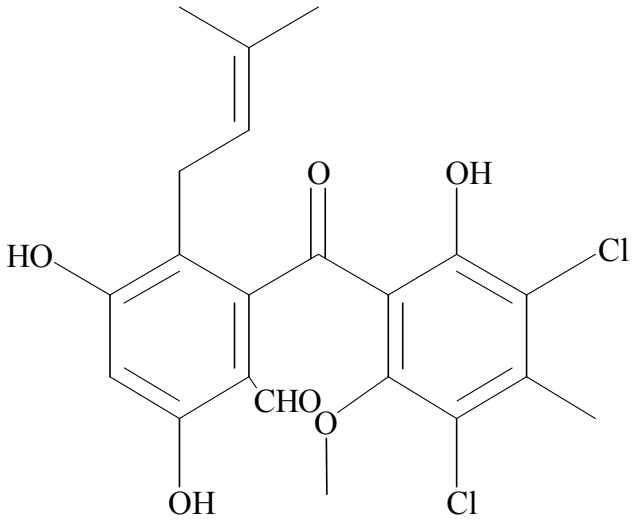
Penochalasin H



Penochalasin G



Sativene epoxide



Pestalone

Fgure 1 (continued): Metabolites from algae-derived fungi.

1.2.2 Secondary metabolites from sponge-derived fungi

As some marine microorganisms survive under extreme environmental conditions, it can be expected that they may have evolved to biosynthesize biologically active chemical compounds (Brauers *et al*., 2000). They often live in symbiosis with soft bodied filter feeding invertebrates such as sponges, and their bioactive metabolites may be interpreted as chemical mediated defense mechanisms for protecting their host organism from environmental dangers such as predation (Proksch *et al*., 2002). The association between sponge and microorganisms probably dates back to 500 million years ago (Friedrich *et al*., 1999). The relationship between marine invertebrate (sponge) and marine microorganisms (fungi or bacteria) may be manifold, it can serve as food or just live inside it. Thus, this relation is complex and far from being understood (Proksch *et al*., 2002, Steinert *et al*., 2000). The relationship between host sponges and fungi is still unknown; only marine Ascomycetes of the genus *Koralionastes* have been reported to be in some way associated with crustaceous sponges (Kohlmeyer & Volkmann-Kohlmeyer, 1991).

As sponges are filter-feeding organisms, which take up the spores and mycelium fragments from seawater, the development of these fungi under laboratory conditions does not mean that they are active inside the sponge (Höller *et al*., 2000; Biabani *et al*., 1998). The isolation of a fungus from a marine organism also does not indicate whether the fungus is obligate or facultative marine (Proksch *et al*., 2002).

As mentioned before, there are valuable reviews dealing with marine fungi (Liberra & Lindequist, 1995; Pietra, 1997; Faulkner, 2001; Blunt *et al.*, 2003). In addition to these reviews, Dr. Ulrich Höller, a former Ph. D. student in our group, listed the metabolites isolated from sponges-derived fungi in the time between 1993 and 1998. For these reasones only the metabolites which were isolated after this date are listed in Table 2 (1998-May 2003). In Figure 2 the chemical structures of the compounds are shown (drawn in an alphabetical order).

Table 2. Metabolites reported from sponge-derived fungi (2000-2002).

Sponge species investigated	Fungus isolated & Investigated	Metabolite (s) isolated	Literature
Callyspongia aerizusa	*Drechslera hawaiiensis*	Speciferol A; Butoxyl-spiciferin	Edrada *et al*. (2000)
	Cladosporium herbarum	Pandangolide 3 and 4; Acetyl sumiki's acid	Jadulco *et al.* (2001)
		Herbarin A and B	Jadulco *et al* (2002)
Unidentified	Unidentified	Iso-cladospolide B; seco-Patulolide C; Pandangolide 1and 2	Smith *et al*. (2000)
Hyrtios proteus	*Aspergillus niger*	Asperic acid	Varoglu *et al*. (2000)
Aplysina aerophoba	*Microsphaeropsis* sp.	10-Hydroxy-18-methoxyl betaenone; 10-Hydroxy-18-*N*-2-naphthyl-N-phenylamino betaenone; three 1,3,6,8-Tetra- hydroxyl-anthroquinone congeneres	Brauers *et al*. (2000)
	Hortaea werneckii	Hortein	Brauers *et al*. (2001)
	Microsphaeropsis sp.	Microsphaerones A and B	Wang *et al*. (2002)
Porifera sp.	*Emericella variecolor*	Varitriol; Varioxirane, Dihydroterrein; Varixanthone	Malmstrøm *et al*. (2002)
Xestospongia exigua	*Penicillium cf. montanense*	Xestodecalactones A-C	Edrada *et al*. (2002)
	Aspergillus versicolor	Aspergiones A-F	Lin *et al*. (2003)
Niphates olemda	*Curvularia lunata*	Lunatin	Jadulco *et al*. (2002)

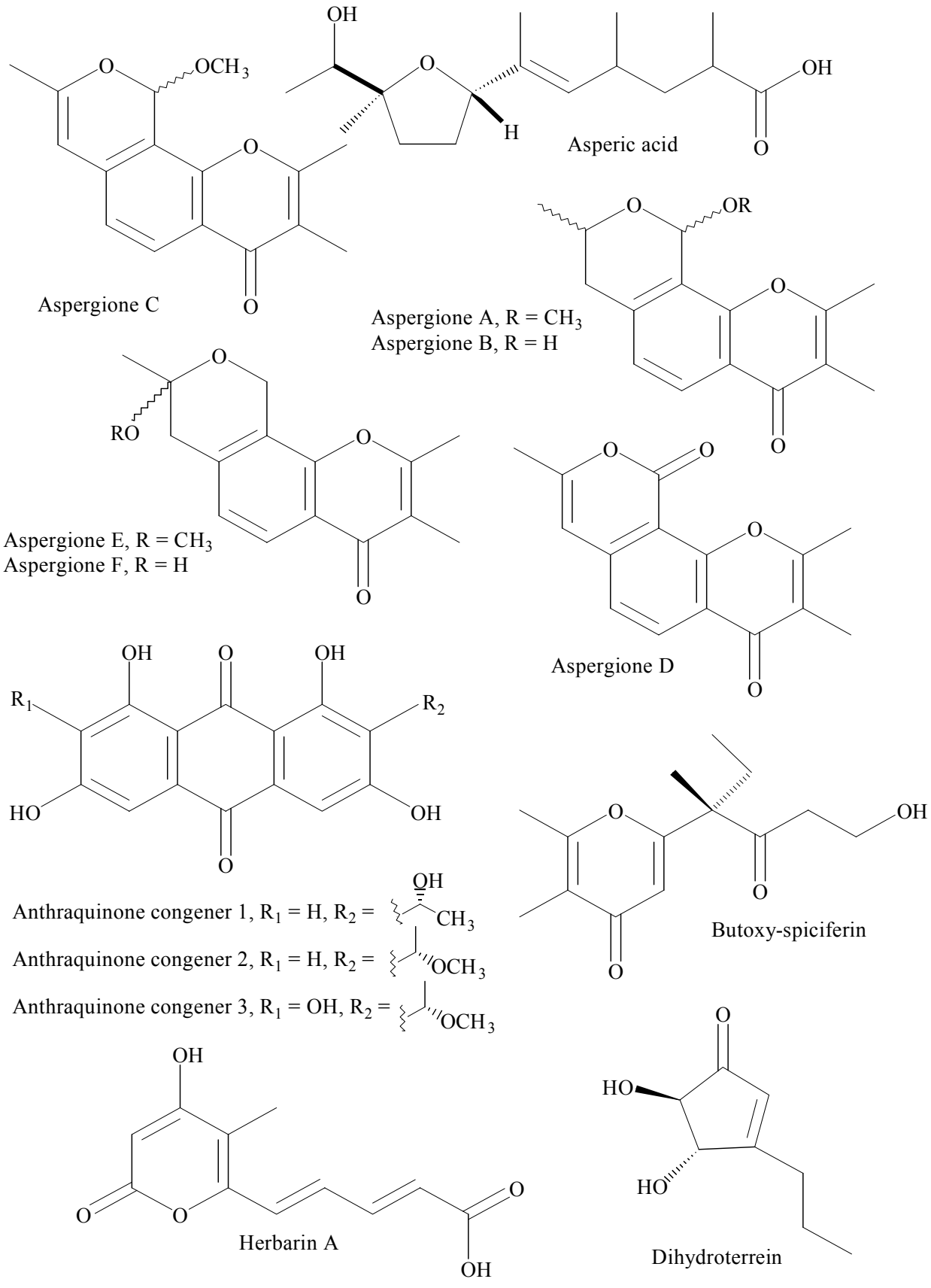
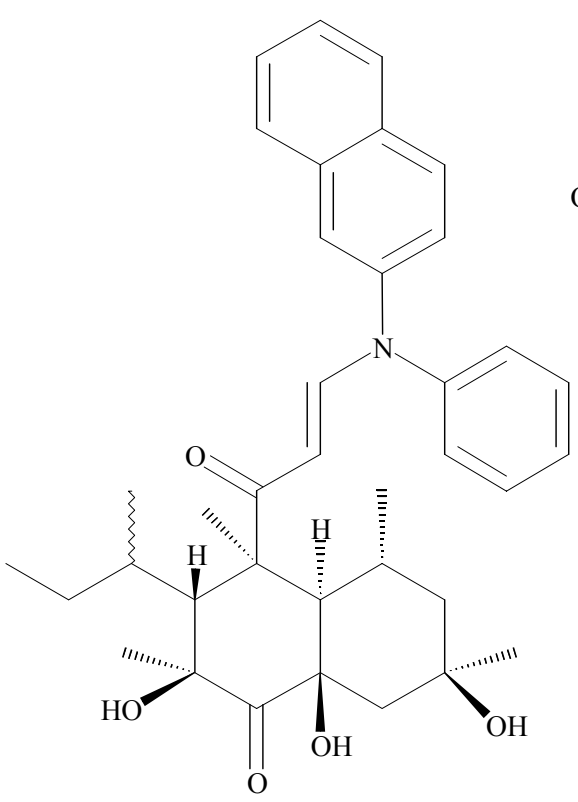
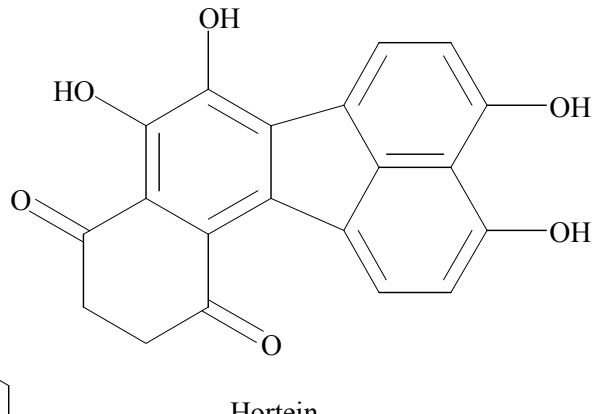


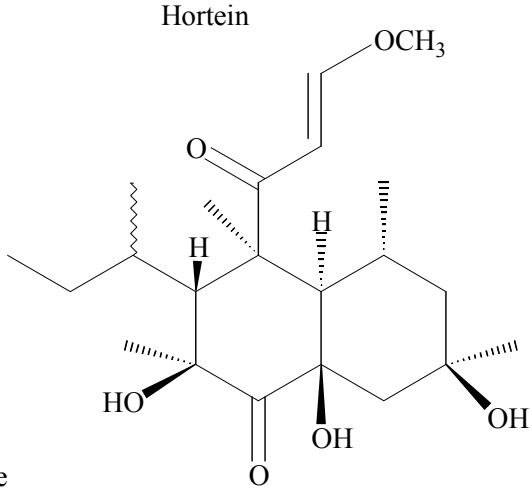
Figure 2: Metabolites from sponge-derived fungi.



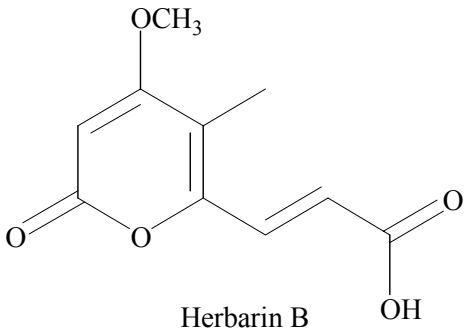
10-Hydroxy-18-N-naphthyl-N-phenylaminobetaenone



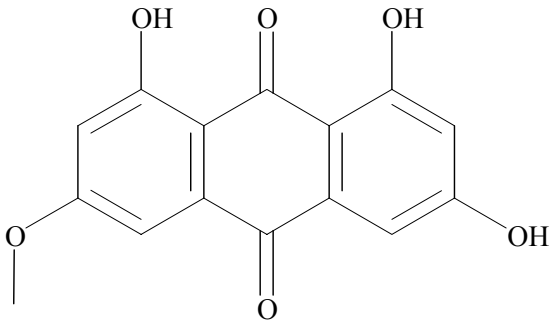
Hortein



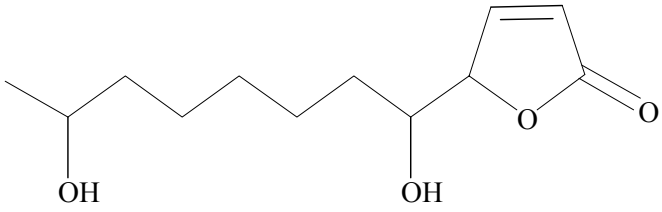
10-Hydroxy-18-methoxylbetaenone



Herbarin B



Lunatin



iso-Cladaspolide B

Figure 2 (continued): Metabolites from sponge-derived fungi.

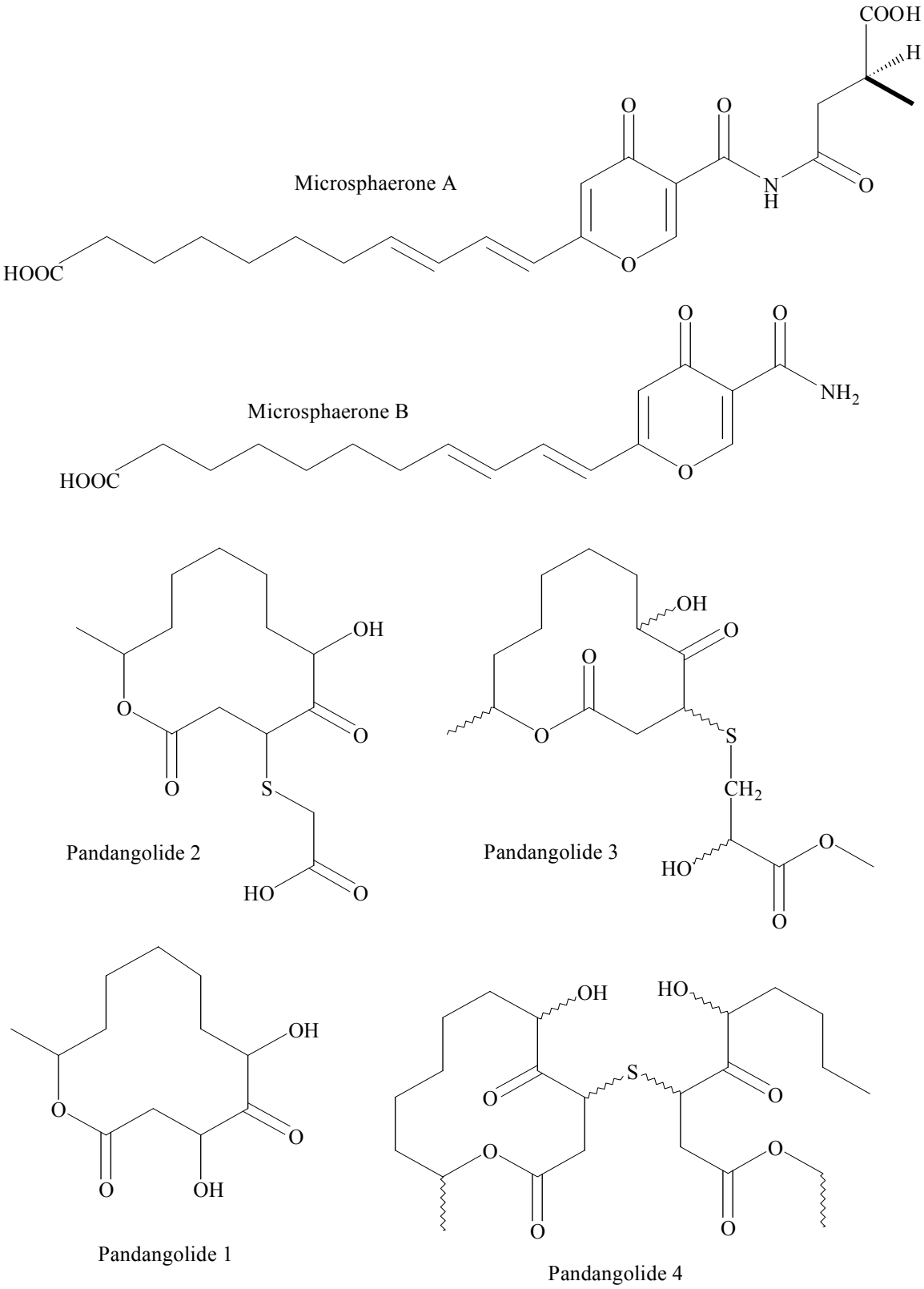
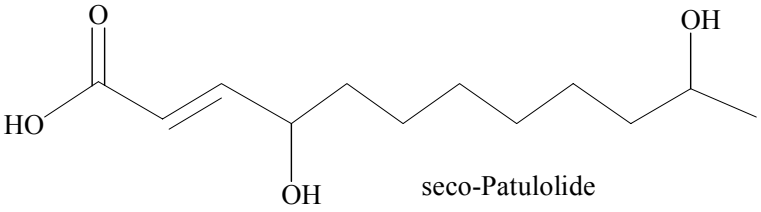
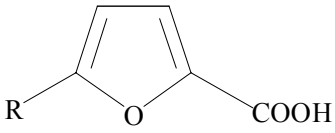


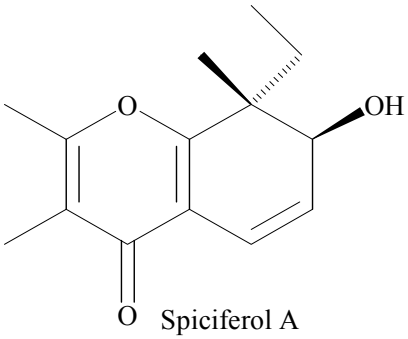
Figure 2 (continued): Metabolites from sponge-derived fungi.



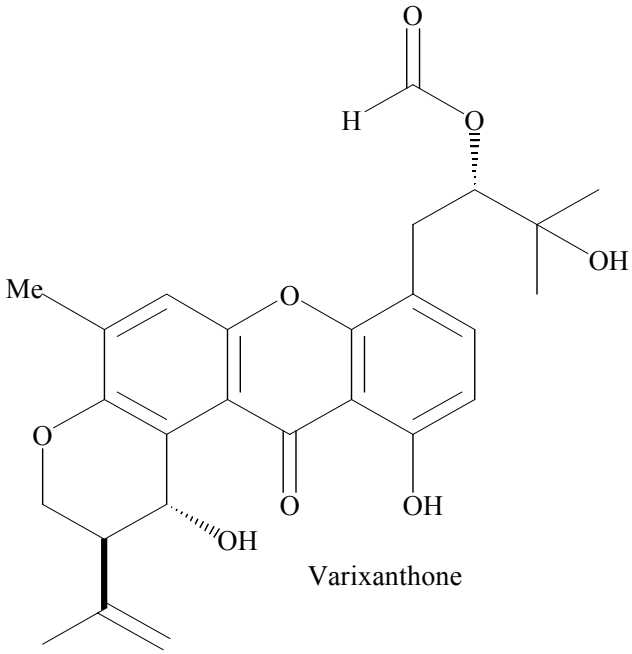
seco-Patulolide



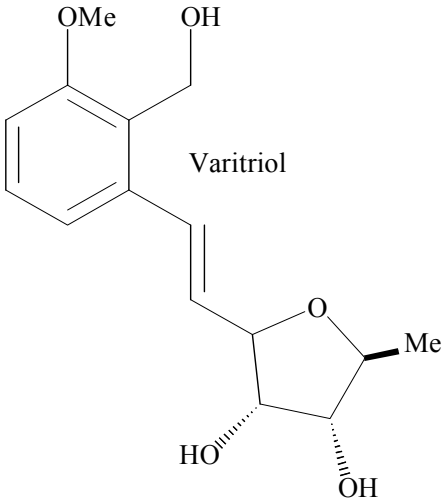
Sumiki's acid acetyl ester
R = CH_2OCOCH_3



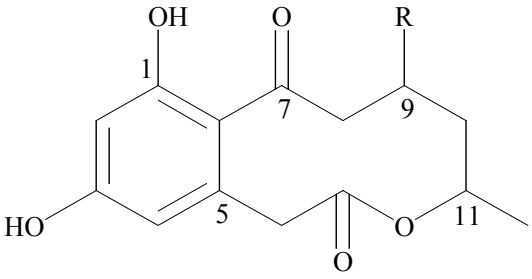
Spiciferol A



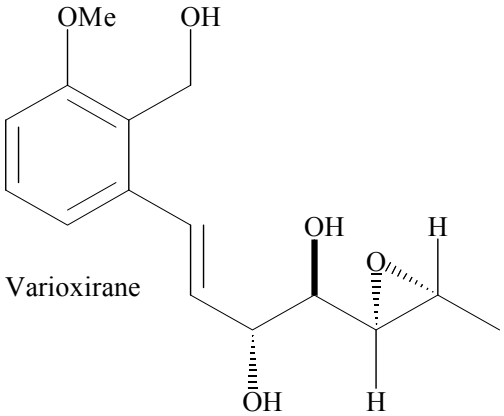
Varixanthone



Varitriol



Xestodecalactone A, R = H
Xestodecalactone B, R = OH, 9, 11-cis
Xestodecalactone C, R = OH, 9, 11-trans



Varioxirane

Figure 2 (continued): Metabolites from sponge-derived fungi.

2 Scope of the present study

The main objective of the present study was the isolation of new and preferably biologically active secondary metabolites from marine microorganisms, especially marine-derived fungi. Marine fungi were obtained from macroalgae and to a lesser extent from sponges.

2.1 Biological and chemical screening of fungal extracts

After taxonomical identification of fungal strains, isolates belonging to typical marine genera were selected for further investigation. Small scale cultivation and extraction enabled biological activity testing. These tests mainly consisted of agar diffusion assays for antibacterial, antifungal and antialgal activity. An additional investigation by ^{1}H NMR was used for chemical characterisation of the fungal extract. The results obtained with these methods were used for the selection of strains for detailed chemical investigation.

2.2 Chemical investigation of fungal strains

Large-scale cultivation, extraction and separation using diverse chromatographic methods, mainly HPLC, was to be employed, in order to isolate pure compounds. Structure elucidation was performed using mainly 1D NMR and 2D NMR techniques, and by physical characterisation of the isolated metabolites.

2.3 Pharmacological potential of isolated pure compounds

Each pure compound obtained during this study was to be tested in as many different bioassays as possible. Especially antimicrobial, antioxidant and enzyme inhibitory activities were to be evaluated with the help of collaborating scientists.

3 Materials and methods

The special materials and methods used during this study are described in detail in the "Description of partial projects" chapter as they are of specific interest for the respective study. This chapter describes the general materials and methods, which were used in many or all partial projects in order to avoid repetitions.

3.1 Chromatography

3.1.1 Thin layer chromatography (TLC)

TLC was carried out using either TLC aluminium sheets silica gel 60 F_{254} (Merck 5554) or pre-coated TLC plates SIL RP-18W / UV 254 (Macherey-Nagel). Standard chromatograms of fungal extracts were prepared by applying 20 µL solution (5 mg/mL) to a silica gel TLC plate and developing it with DCM/MeOH (95/5 v/v) under saturated conditions. Chromatograms were detected under UV light (254 and 366 nm), and with vanillin-H_2SO_4 reagent (1g vanillin dissolved in 100 ml H_2SO_4, heated at 100 °C after spraying).

3.1.2 Vacuum-liquid chromatography (VLC)

Sorbents for VLC were either silica gel 60 (0.063-0.200 mm, Merck 7734; Merck 7739; normal-phase) or Polyprep 60-50 C18 (Machery-Nagel 71150; reversed-phase). Columns were filled with the appropriate sorbent soaked with petroleum ether or MeOH. Before applying the sample, the column was equilibrated with the first designated eluent.

3.1.3 High performance liquid chromatography (HPLC) and detectors

HPLC was carried out either using a Merck-Hitachi system equipped with an L-6200A Intelligent Pump, a L-4500A diode array detector, a D-6000A interface with D-7000 HSM software, a Rheodyne 7725i injection system, or a system equipped with a Waters associates chromatography pump, a Knauer Differential refractometer, a Rheodyne 7725i injection system and a Linseis L 200 E recorder. If not stated otherwise, the system with differential refractometer as detector was used. Columns used were either a Knauer Spherisorb S ODS 2 (5 µm, 250 x 8 mm), a Merck LiChrospher Si 60 (5 µm, 250 x 7 mm), or similar columns. Typical flow rates were 2.0 - 3.0 mL/min, or 1.0 mL/min in special cases (see partial project chapter). All solvents were distilled prior to use. The eluents were degassed under reduced pressure. If not stated otherwise, samples were diluted in the eluent to yield solutions of 20-40

mg/mL. Injected amounts usually equalled 5-10 mg of extract/run.

3.1.3.1 HPLC analysis of *Trichoderma viride* ethyl acetate extract

The HPLC analysis was performed on a Waters apparatus (four solvent delivery systems model 600 controller pump, automatic sample injector model 717, and photodiode array detector model 996, using RP-HPLC column (XTerra RP-18, 5 µm, 250 x 4.6 mm, Waters). Data acquisition and quantification were performed with Millennium 32 version 3.1 software (Waters). The mobile phase was gradient elution from 70:30 H_2O:MeOH to 30:70 H_2O:MeOH, for 60 min; then to MeOH, for 40 min; followed by 10 min MeOH, finally, return back to 70:30 H_2O:MeOH in 5 min. The injecting volume of the ethyl acetate extract was 25 µl of 2 mg/mL (methanol). All the compounds were detected at λ_{max} 360 nm and 300 nm. Before each elution the column calibrated for 20 minuts.

3.2 Structure elucidation

Structures were elucidated mainly using 1D and 2D NMR techniques and various MS methods. If necessary, additional parameters such as optical rotation, UV and IR properties were determined. Identity of isolated compounds with compounds reported in literature was judged, if not stated otherwise, based on ^1H and ^{13}C NMR spectroscopic data, and specific optical rotation. Literature searches were done using Chapman & Hall Natural Products on CD-ROM (Buckingham, 2001), and Beilstein on-line databases. Structures were designated as new, if they could not be found in Beilstein database and Chemical Abstracts.

3.2.1 NMR spectroscopy

^1H NMR spectra of extracts and pure compounds as a purity check were recorded at the Pharmaceutical Biology Institute, Bonn, on Bruker Avance 300 DPX spectrometer operating at 300 MHz or a Bruker Avance 500 DRX spectrometer operating at 500 MHz (with $(CD_3)_2CO$, or MeOH-d_4, or C_6D_6, or $CDCl_3$ as solvent). ^1H NMR spectra of pure compounds and all other NMR measurements were performed at Pharmaceutical Biology Institute on Bruker DPX-300, or at Pharmaceutical Chemistry Institute on Bruker DRX-500 NMR spectrometer operating at 300, or 500 MHz (^1H), and 75.5, or 125 MHz (^{13}C), respectively. Spectra of pure compounds were processed using Bruker ^1D WIN-NMR or ^2D WIN-NMR software. They were calibrated using solvent signals (^{13}C: $CDCl_3$ 77.0 ppm, CD_3OD 49.0 ppm, C_6D_6 128.0 ppm, $(CD_3)_2CO$ 29.8 ppm) or a signal of the portion of the partly or not deuterated solvent (^1H: $CHCl_3$ in $CDCl_3$: 7.26 ppm, CH_3OH in CD_3OD: 3.35 ppm, C_6H_6 in C_6D_6: 7.20, $(CH_3)_2CO$ in $(CD_3)_2CO$; 2.04 ppm). Multiplicity for ^{13}C was deduced from DEPT

experiments; s = C, d = CH, t = CH_2, q = CH_3. Structural assignments were based on spectra resulting from one or more of the following NMR experiments: ^1H, ^{13}C, DEPT, ^1H-^1H COSY, ^1H-^{13}C direct correlation (HMQC), ^1H-^{13}C long-range correlation (HMBC), 2D NOESY and ^1H difference NOE.

3.2.2 Mass spectrometry

Mass spectral measurements were performed by Dr. G. Eckardt, Ms. C. Sondag and Ms. K. Peters (All Department of Chemistry, University of Bonn) using a Kratos MS 50 (EI), Kratos Concept 1H (FAB) and a Finnigan MAT 95 (EI, ESI) spectrometer.

The GC-MS analysis was carried out on a Perkin-Elmer (Auto system XL) gas chromatograph equipped and coupled to a Perkin-Elmer Turbomass spectrometer with a 30 m x 0.32 mm N931-6023 Pe-1 (film thickness of 0.25 μm) capillary column. The instrument was set to an initial temperature of 50 °C, and maintained at this temperature for 10 min. At the end of this period the oven was heated at 10°/min up to 250°C, and kept there for 10 min. Injection port temperature was 220°C, He flow rate was 2 mL/min, and samples were injected in splitless mode. Mass spectral scan range was 35-650 Da.

LC-ESIMS experiments were carried out on API 2000, Triple Quadrupole LC-MS/MS, Applied Biosystems/MDS Sciex equipped with Agilent 1100 Serie incl. DAD. The column was Macherey-Nagel Nucleodure 100-5 C18 HPLC column, 2mm x 125 mm. The solvents were (A) 2 mmol aqueous NH_4Ac and (B) 2 mmol NH_4Ac of MeOH, employing gradient elution from 9:1 H_2O:MeOH to MeOH in 20 min, followed by 10 min MeOH, 0.25 mL/min, 10 μL injecting volume of 1mg/mL of the total extract. Following are the MS parameters: ionization mode, positive and negative mode; scan range, 100-700 amu; scan rate, 0.5 scan/sec; and cone voltage, 20 eV.

3.2.3 UV measurements

UV spectra were recorded a Perkin-Elmer Lambda 40 with a UV WinLab software, using 1.000 cm quartz cells (Institute for Pharmaceutical Biology, University Bonn).

3.2.4 IR spectroscopy

IR spectra were recorded a Perkin-Elmer spectrometer, Spectrum BX Instruments with FT-IR Paragon 1000 PC software, at the Institute for Pharmaceutical Biology, University of Bonn.

3.2.5 Optical rotation

Optical rotations were measured by using a Jasco DIP 140 Polarimeter (Institute for Pharmaceutical Biology, University of Bonn). The tested samples were dissolved in the proper solvent (e.g. MeOH or $CHCl_3$). Then transferred into specific cell (10.0 cm) and using a Monochromatic Sodium D-line light by 589 nm at room temperature (22-23°C).

3.2.6 Melting point

Melting points were measured by Leitz 350 Microscope Heating Stage and are not corrected.

3.2.7 X-ray analysis

The single crystal X-ray analysis of 2-(1-methylethylidene) pentanedioic acid (**5**) was performed by Prof. Dr. P. G. Jones, Institute for Inorganic and Analytical Chemistry, TU Braunschweig. Crystals were grown from MeOH.

3.3 Origin and taxonomy of fungal samples

3.3.1 Fungal strains for chemical investigation

All chemically investigated fungi, in this study, were obtained from fungal culture collection of Professor G. M. König (Pharmaceutical Biology Institute, University Bonn), and were isolated by former Ph. D. student Dr. Ulrich Höller and Claudia Osterhage.

Acremonium sp., closely related to *A. roseogriseum* (S. B. Saksena) W. (Hypocreace, Ascomycota, M 1-11-1, 506) was isolated from the brown alga *Cladostephus spongius* (Hudson) C. Agardh, collected at the Spanish coast (Moraira, Mediterranean Sea).

Alternaria sp. (Pleosporaceae, Ascomycota, M7-19, 539), isolated from red alga, *Jania rubens* L. (Corallinaceae), collect from water around Island of Morira, Mediterranean sea.

Epicoccum sp. (Tuberculaiaceae, Hyphomycetes, N1-12, 353), was isolated from the brown alga *Fucus vesiculosus* L., collected at the German coast (Tönning, North Sea).

Myrioconium sp. (Coelomycetes Ascomycota, Cux 3-10, 547), isolated from *Fucus vesiculosus*. L CUX-3 (Fucaceae), was collected from water around the Cuxhaven, North Sea.

Sporormiella sp. (Anamorph, Ascomycota, G 2-1, 493), isolated from brown alga *Stypopodium sp.* (Stypocaulaceae).

Trichoderma viride Persoon in Römer (Hypocreaceae, Ascomycota, 193E61, 54), was isolated from the sponge *Agelas dispar* J (Family Agelasidae, order Agelasida, CT193 E), collected by divers from the waters around the Caribbean Island of Dominica.

Wardomyces anomalus Brooks & Hansford (Microascaceae, Ascomycota, OS4T3-2-1, 406), was isolated from the green alga *Enteromorpha* sp. (Ulvaceae) collected around Fehmarn island in the Baltic Sea.

3.3.2 Isolation of endophytic fungi from egyptian algae

All algal samples were stored in sterile ASW supplied with 250 mg/L benzylpenicillin and 250 mg/L streptomycin sulphate at 4 °C for a week until examination. Algal samples were sliced aseptically into small pieces and placed in EtOH (70 %) or sodium hypochlorite solution (3.5 %), rinsed three times with sterile DW to remove the EtOH, and placed on isolation media. Surface sterilization time was optimised for each alga before placing cubes onto isolation medium. For the inoculation of algal samples medium I was used containing antibiotics (250 mg/L benzylpenicillin and 250 mg/L streptomycin sulphate). Samples were incubated at RT (Approx. 20 °C). Fungal colonies were transferred to one or more of the media for identification. These included media II, III. The preservation and maintenance of stock cultures were carried out in test tubes using medium 1V, then kept at 4 °C.

Medium 1: Standard nutrient agar (SNA): KH_2PO_4 1 g/L, KNO_3 1 g/L, $MgSO_4 \times 7\ H_2O$ 0.5 g/L, KCl 0.5 g/L, glucose $\times\ H_2O$ 0.2 g/L, sucrose 0.2 g/L, agar 20 g/L.

Medium II: Glucose peptone yeast extract agar (GPY): glucose $\times\ H_2O$ 1 g/L, peptone from soymeal 0.5 g/L, yeast extract 0.1 g/L, agar 15 g/L, ASW IL.

Medium III: Biomalt 20 g/L, agar 20 g/L and ASW 1 L.

Medium 1V: Biomalt 50 g/L, agar 20 g/L and ASW 1 L.

3.4 Biological testing

3.4.1 Agar diffusion assays

Agar diffusion assays were carried out in principle according to Schulz *et al.* (1995). Test organisms were the bacteria *Bacillus megaterium* de Bary (gram positive) and *Escherichia coli* (Migula) Castellani & Chambers (gram negative), the fungi *Microbotryum violaceum* (Pers.) Roussel (Ustomycetes), *Mycotypha microspora* Fenner (Zygomycetes), and *Eurotium repens* Corda (Ascomycetes), and the alga *Chlorella fusca* Shih Krauss (Chlorophyceae).

Sample solutions contained 5 mg/mL extract or 1 mg/mL pure compound. Samples were prepared by taking 50 µL of each solution and pipetting it onto a sterile antibiotic filter disk (Schleicher & Schuell 2668), which was then placed onto the appropriate agar medium and sprayed with a suspension of the test organism. Growth media, preparation of spraying suspensions, and conditions of incubation were as employed by Schulz *et al*. (1995). The radii of the resultant zones of inhibition were measured from the edge of the filter disks. For extracts, a growth inhibition zone or complete inhibition zone ≥3 mm and /or a complete inhibition ≥1 mm were regarded as a positive result; growth inhibition: growth of the appropriate test organism was significantly inhibited compared to a negative control; complete inhibition: no growth at all in the appropriate zone.

3.4.2 Tyrosine kinase inhibitory activity

The DMSO sample solution of the appropriate extract/pure compound was diluted with H_2O (1:1 v/v) to yield corresponding sample solutions (1 mg/mL). TK inhibitory activity was determined using a commercial test kit (Tyrosine Kinase Assay Kit, non-radioactively, Boehringer Mannheim, Cat. No. 1 534 513), modified by Dr. G. F. Matthée (Dissertation, 1999). Assays were carried out by Christine Klemke using T cell tyrosine kinase $p56^{lck}$ (Upstate Biotechnology). Sample solutions were incubated with 1 µM TK substrate II, biotin-labelled (Boehringer Mannheim), 1 mM ATP, 10 mM $MgCl_2$, 1 U TK $p56^{lck}$, 20 µL dilution buffer, and 20 µL assay buffer for 1 h at 30 °C. The resultant concentration of the extract/compound in the test mixture was 200 µg/mL. Dilution buffer (pH 7.0) contained 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 10 % glycerol, and 0.1 % ethylphenolpoly(ethylene-glycolether)$_n$ (NP-40). Assay buffer (pH 7.5) contained 250 mM Tris, 25 mM NaF, 2.5 mM EDTA-Na2, 4.0 mM [ethylenebis (oxyethylene-nitrilo)] tetraacetic acid (EGTA), 5 mM DTT, and 150 µM Na_3VO_4. The enzyme activity was determined as described in the TK Assay Kit, except the measuring wavelength was 415 nm instead of 405

nm. In each test series 3 mM piceatannol (Boehringer Mannheim) was included as a positive control. Samples which reduced the enzyme activity to 60 % or less relative to a negative control were regarded as active.

3.4.3 HIV-1 reverse transcriptase inhibitory activity

The assay was performed by Mr. C. Dreikorn according to a protocol established by Dr. G. F. Kirsch (2000). DMSO standard solutions of the appropriate extracts/compounds were diluted with lysis buffer (1:10 v/v) to yield corresponding sample solutions. HIV-1 RT inhibitory activity was measured using 20 µL of the sample solution, 20 µL lysis buffer, and 20 µL reaction mixture which were then incubated for 1 h at 37 °C. The resultant concentration of the extract in the test mixture was 66 µg/mL. Lysis buffer (pH 7.8) contained 1 ng recombinant HIV-1 RT (Boehringer Mannheim), 50 mM Tris, 80 mM KCl, 2.5 mM dithiothreitol (DTT), 0.75 mM ethylenediamintetraacetic acid (EDTA), and 0.5 % Triton-X100. The reaction mixture contained template/primer hybrid poly(A)*oligo(dT)15 (600A260 nm/mL), 8 µM thymidine-5'- triphosphate (dTTP), 8 µ M digoxigenin- and biotin-labeled-2'-deoxy-(uridine-5'- triphosphate) (dUTP), 40 µ M TrisHCl, 230 mM KCl, 24 mM $MgCl_2$, and 8 mM DTT (all Boehringer Mannheim). Enzyme activity was measured following the ELISA protocol described by Eberle & Seibl (1992) at λ = 415 nm versus λ = 490 nm using an ELISA reader (Rainbow, SLT Labinstruments Deutschland GmbH). As a positive control, 10 µ M of phosphonoformic acid (Sigma) was included in each test series. Extracts which reduced the enzyme activity to 80 % or less relative to a negative control were regarded as active.

3.4.4 Antioxidative activity

3.4.4.1 Thiobarbituric acid reactive substances method (TBARS assay)

The method used was adapted from Wallin *et al*. (1993) and modified by K. M. Fisch as follows: Assays were performed in flat bottom polystyrene 96 well microtiter plates. The final volume of the reaction mixture in the oxidation step of linolenic acid methyl ester was 70 µL/well. The solution was made of 40 µL of 50 mM phosphate buffer (pH = 7.2), 10 µL of 10 % (w/v) sodium dodecyl sulphate, 5 µL of sample in ethanol (1 mg/mL and 0.2 mg/mL), 5 µL linolenic acid methyl ester 70 mM in ethanol, and finally 10 µL of 0.08 % (w/v) $FeSO_4.7H_2O$ in 0.025 M HCl. Plates were placed in a thermomixer and incubated at 50 °C and 500 rpm for 30 min. At the end of this period 5 µL butylated hydroxytoluene (BHT) 15 mM in ethanol was added immediately so as to prevent further oxidation. To each well 20 µL of 50 % (w/v)

trichloroacetic acid and 40 µL of 0.88 % (w/v) thiobarbituric acid, dissolved in 0.3 % (w/v) NaOH, were added. The plate was then covered with a micromat and incubated at 60 °C and 500 rpm for a further 30 min. At the end of this period the plate was maintained at room temperature for 4 min and the absorbances then determined at 532 nm, less the background absorbance at 600 nm, using a SLT Spectral Rainbow microtiter plate reader.

3.4.4.2 α,α-Diphenyl-ß-picrylhydrazyl (DPPH) radical scavenging effects

Assays were performed in flat bottom polystyrene 96 well microtiter plates. The DPPH radical scavenging effects of the total extract and compounds were performed by using a modified previously established methodology (Blois *et al*., 1958 and Amarowicz *et* al., 2000). To 100 µL of each sample (1 mg/mL) in EtOH 25 µL DPPH (1 mM) in EtOH was added. The resultant mixture was briefly shaken and maintained at room temperature, in the dark for 30 min. At the end of this period the absorbance of the mixture was measured at 517 nm, using a SLT Spectral Rainbow microtiter plate reader.

3.4.5 Cytotoxicity

3.4.5.1 Human cancer cell lines

The cytotoxicity was carried out at the Institute for Experimental Onkology in Freiburg, through the cooperation with Prof. Dr. Heinz-Herbert Fiebig. Ten human cancer lines were tested i.e. three lung carcinoma (LCL H460, LXFL 529L, and LXFA 629L), two breast cancer (MACL MCF7, and MAXF 401NL) two melanoma (MEXF 462NL and MEXF 486L), two kidney cancer (RXF 944L and RXF 486L) and a uterine cancer (UXF 1138L). The assays were performed as previously published (Grever *et al.*, 992).

3.4.5.2 Brine shrimp lethality bioassay

Brine shrimps (*Artemia salina*) is a simple convenient general bioassay and also indicative for cytotoxicity (Meyer *et al*., 1982; Solis *et al*., 1993). The brine shrimp eggs were hatched in artificial seawater (ASW). 40 mg/L of the eggs supplemented with 6 mg/L dried yeast and oxygenated with aquarium pump for 48 hours in room temperature (22-25°C). 100 µL of the sample solution (1 mg/mL) transferred into sterile microtiter plate. The plate left till evaporated over night. Then 150 µL of the *A. salina* culture medium together with a few *A. salina* larvae was added, followed by 150 µL water. For each sample four replicate were performed. After 24 and 48 hours the plates were examined under a binocular microscope and

the numbers of dead (non-motile) nauplii in each well were counted aginst the negative control.

Culture medium for *A. salina*: 2.4 g Tris, 25.7 g NaCl, 4.7 $MgCl_2$ (presolved), 0.7 g KCl, 0.2 g Na_2CO_3 (presolved), 6.4 g $MgSO_4$ (presolved), 1.1 g $CaCl_2$ (presolved), 1000 mL aqua bidest, adjusted to pH 7.1, stored at 4 °C or 10.0 g spezial artemia salt (Dohse aquaristik Bonn), 1000 mL aqua bidest.

Culture: 0.3 g Artemia cysts/400mL culture medium (RT) in a 1 L beaker covered with gas was ventilated under oxygen for 48 h.

Collecting: The adult were collected by using a brine-shrimp sieve (Dohse aquaristik Bonn).

3.4.6 Recombinant yeast estrogenic assay

The assay was performed by Mr. M. Nett (Pharmaceutical Biology Institute, University of Bonn). Details procedure and medium components of this assay have been previously described (Routledge and Sumpter, 1996). In brief, yeast cells transfected with the human estrogen recptor (ERα) gene, together with expression plasmids, were incubated in medium containing the extract or pure compounds, in addition to the chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG). Upon binding the active ligand, the estrogen-occupied receptor intertacts with transcription factors and other transcriptional componenta to modulate gene transcription. This causes expression of the reporter gen Lac-Z and the enzyme produced (galactosidase) is secreted into the medium, which causes the CPRG to change into red product that can be measured by absorbance at λ_{max} 540 nm.

3.5 Chemicals

3.5.1 Media components:

Agar (Fluka 05040)
Biomalt (Villa Natura, Kirn)
Malt extract (Merck 5391)
Peptone from casein, tryptic digest (Fluka 70172)
Peptone from meat, enzymatic digest (Fluka 70175)
Peptone from Soya meal, papain-digested (Merck 7212)
Yeast extract (Fluka 70161)

Benzylpenicillin (Fluka 13750)
Streptomycin sulphate (Fluka 85880)
Water used was de-ionised using a Millipore (milli-Q®) system.

All other chemicals/components were research grade, and if not stated otherwise in the text, supplied by Merck.

3.5.2 Solvents:

EtOH for UV-measurements was from Merck (Uvasol®980). All other solvents were research grade and supplied by Merck, except n-BuOH (Roth). Water used was deionised using a Millipore (milli-Q®) system. Acetone, $CHCl_3$, DCM, EtOAc, hexane, cyclohexane, MeOH and petroleum ether were distilled prior to use.
Acetone-d_6 (Chemotrade 97509, 99.8 % D).
Methanol-d_4 (ICB 0844-25, 99.8 % D).
Benzene-d_6 Chemotrade, 99.87 % D).

4 Description of partial projects

4.1 Endophytic marine fungi; isolation, selection, mass cultivation and biological activities

Isolation of endophytic fungi

One of the tasks within this project was the isolation of endophytic fungi from marine algae in order to investigate them for the production of new and/or biological active metabolites during the current study. Some of the marine fungal strains examined, were isolated from algae or sponges by former Ph. D. students of our research group, i.e. Dr. Ulrich Höller and Dr. Claudia Osterhage.

From 11 egyptian algal samples, a total of 14 fungal strains were isolated (Table 3). Six of them were identified as belonging to the genus *Aspergillus* and *Penicillium*. Since they might be pathogenic they are excluded from any further studies. The remaining eight strains are not yet identified. Table 3 lists the algae, the collection site and number of the fungal strains isolated.

Table 3. Algal samples from Egypt as a source of endophytic fungi.

No.	Alga name	Source of algal samples	Identified fungi	Unidentified fungi
1	*Laurencia papilosa*[a]	Suez Canal, Ismailia	*Aspergillus* sp.	2
2	*Gelidium* sp.	Port Said	-	-
3	*Sarconium* sp.	Suez Canal, Ismailia	-	-
4	*Jania* sp.	Suez Canal, Ismailia	-	-
5	*Ulva* sp.	Suez Canal, Ismailia	*Aspergillus* sp.	1
6	*Acanthophora* sp.	Suez Canal, Ismailia	*Aspergillus* sp.	1
7	*Enteromorpha* sp.	Suez Canal, Ismailia	*Penicillium* sp.	-
8	*Enteromorpha* sp.	Port Said	*Penicillium* sp.	1
9	*Hypnea valentiae*	Suez Canal, Ismailia	-	2
10	*Hypnea musciformis*	Suez Canal, Ismailia	*Aspergillus* sp.	1
11	*Ulva* sp.	Suez Canal, Ismailia	-	-

[a]All algae collected from a deepth at 0-1 meter (September 2002).

Selection of strains for further investigations of their secondary metabolites, example *Trichoderma viride*

The selection of strains for mass cultivation was based on the biological activity of the extract, and/or secondary metabolites detected in ^{1}H NMR spectra, especially from signals present in the δ 5-9 region. In some cases, the total extract was subjected to further analysis employing HPLC-DAD and/or LC-ESIMS with the aim to confirm the ^{1}H NMR data.

As an example the HPLC-DAD and LC-ESIMS investigation of the ethylacetate extract of the fungus *Trichoderma viride* shall be discussed in this chapter. In all cases, a literature survey was performed to obtain information on published compounds from relevant genera, which revealed the ability of the selected strain might be producing variable structures.

The ethylacetate extract of *T. viride* was analysed on reversed phase HPLC-DAD. A H_2O/MeOH gradient from 70/30 to 0/100 H_2O/MeOH on RP-18 used for this purpose. Thus, eleven peaks in the total extract of *T. viride* were detected at 360 nm (Fig. 3a), and also at 300 nm. The latter had partially different retention times (Fig. 3b) and thus, are different substances. Studying the UV spectra indicated that peaks 5, 6 and 9 possess a similar chromophore and comparable molecular weights as detected by LC-ESIMS. UV maxima and Rt (min) of *Trichoderma* extract: 213/280 at 42.5, 212/264/366 at 43.8, 212/363 at 50.5, 212/363 at 52.9, 212/366 at 56.3, 212/255/363 at 57.5, 213/298/369 at 76.7, 213/366 at 87.3, 212/297/374 at 88.9, 212/362 at 91.2, 213/272/306/406 at 92.7.

T. viride extract was also analysed by LC-ESIMS (positive and negative mode), and eleven peaks were detected. LC-MS spectra allowed to deduce the molecular weight of the metabolites produced by *T. viride.* The positive mode ESIMS analysis led to detection of seven peaks [m/z/ Rt (min)] 265/8.6, 251/9.1, 225/15.0, 497/16.5, 499/19.1, 497/21.1, and 497/21.5. The negative mode ESIMS analysis led to the detection of ten peaks [m/z/ Rt (min)]: 263/8.6, 249/9.1, 359/13.5, 393/16.7, 495/16.5, 407/16.9, 407/18.1, 497/19.1, 495/ 21.1, and 495/21.5. Subtraction of the peaks detected by the two modes from each other showed that eleven individual peaks were detected.

The results obtained from HPLC-DAD analysis were in a good agreement with those obtained from the LC-ESIMS analysis. A similar number of peaks was detected. UV maxima of some detected peaks indicated that those compounds had a similar chromophore, where others differed, selected spectra are shown in Figure 3C.

Biological activity of the fungal strains

All extracts were subjected to brine shrimp lethality and antibacterial, antifungal and antialgal assays. Few of them were tested for their antioxidant properties. The bioactivities will be disscussed in the individual partial projects.

The combination of HPLC-DAD/MS results with those obtained from the ^{1}H NMR spectrum and the biological assays, facilitated the selection of the seven fungal strains for chemical investigation. These fungi represented different genera. Six of them are mitosporic fungi i.e. *Acremonium* sp., *Wardomyces anomalus,*, *Epicoccum* sp., *Trichoderma viride*, *Alternaria* sp. and *Myrioconium* sp., and one is a fungus belonging to the Ascomycetes i.e. *Sporormiella* sp. (Table 4).

Table 4. Selected fungi for chemical investigation.

C. No[a]	**Code**	**Name**
54	193E61	*Trichoderma viride*
353	N1-12	*Epicoccum* sp.
406	OS4T3-2-1	*Wardomyces anomalus*
493	G 2-1	*Sporormiella* sp.
506	M 1-11-1	*Acremonium* sp.
539	M7-19	*Alternaria* sp.
547	Cux 3-10	*Myrioconium* sp.

[a]This number refers to the fungal collection (Pharmaceutical Biology Institute, Bonn).

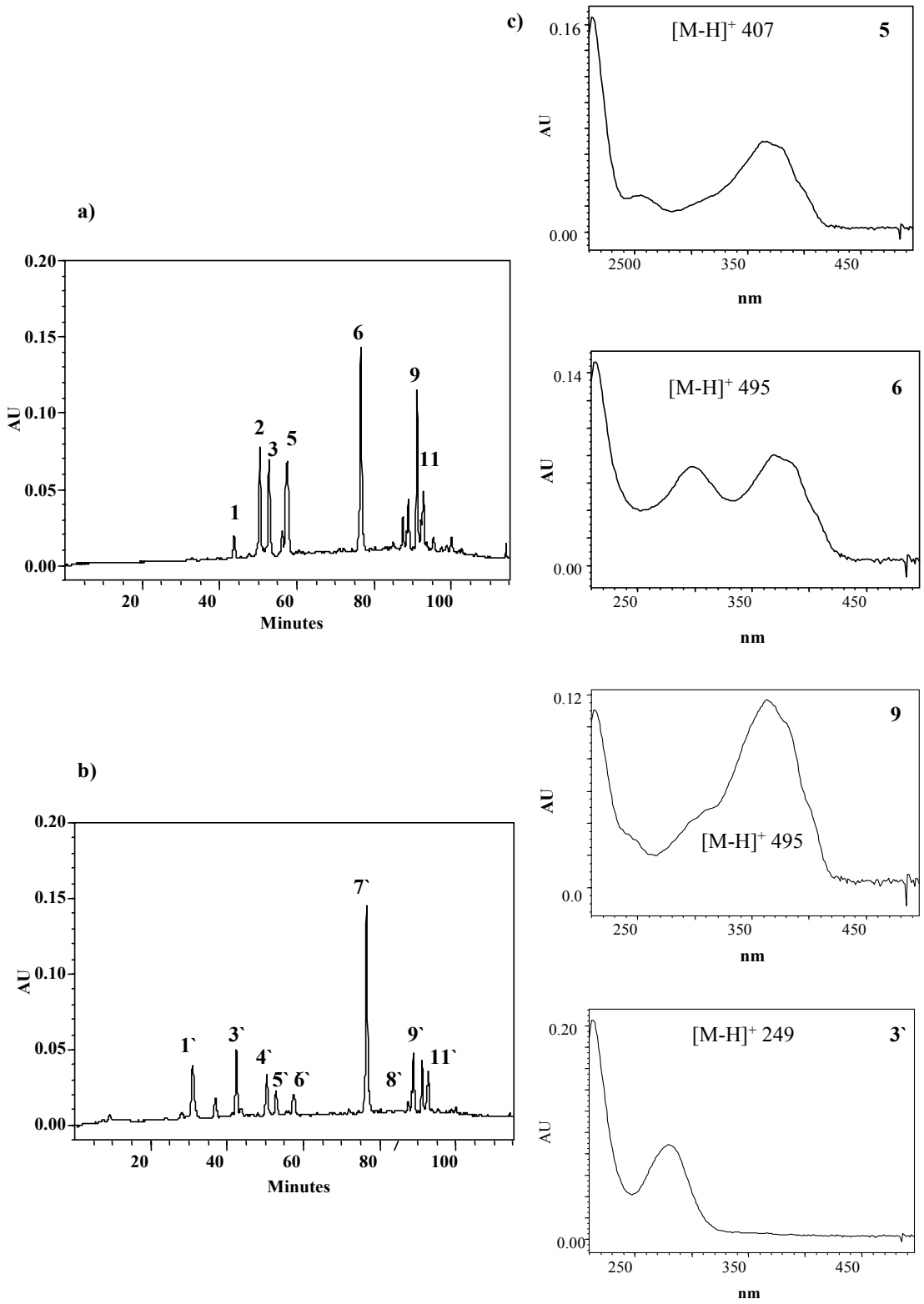


Figure 3. HPLC-chromatograms of the total extract of *T. viride.* a) UV-detection at 360 nm and b) UV-detection at 300 nm. Panel (c) shows selected UV spectra (range of 200-500 nm) and results of ESIMS $[M-H]^+$. HPLC with RP-18 (5 µm, 250 x 8 mm i.d, Knauer), and gradient elution from H_2O/MeOH (70/30) to MeOH (100) in 115 min, 2 ml/min.

4.2 New antioxidant hydroquinone derivatives from the algicolous marine fungus *Acremonium* sp.

Abstract

A marine fungal isolate, identified as *Acremonium* sp., was mass cultivated and found to produce two novel hydroquinone derivatives, 7-isopropenylbicyclo[4.2.0]octa-1,3,5-triene-2,5-diol (**1**) and 7-isopropenylbicyclo-[4.2.0]octa-1,3,5-triene-2,5-diol-5-β-D-gluopyranoside (**2**). Compound **1** and its glucoside **2** possess a most unusual ring system. The new natural products (3*R**,4*S**)-3,4-dihydroxy-7-methyl-3,4-dihydro-1(2*H*)-naphthalenone (**3**) and (3*S**, 4*S**)-3,4-dihydroxy-7-methyl-3,4-dihydro-1(2*H*)-naphthalenone (**4**) were obtained as a 1:0.84 mixture. 2-(1-Methylethylidene) pentanedioic acid (**5**) was isolated for the first time as a natural product and its structure proven by X-ray analysis. In addition to these compounds an inseparable mixture of three new isomeric compounds, pentanedioic acid 2-(1-methyl-ethylidene)-5-methyl ester (**6**), pentanedioic acid 2-(1-methylethylidene)-1-methyl ester (**7**), and pentanedioic acid 2-(1-methylethenyl)-5-methyl ester (**8**), was also obtained. Isolated together with the new compounds were three known hydroquinone derivatives, **9**-**11**. The structures of all compounds were determined by interpretation of their spectroscopic data (1D and 2D NMR, MS, UV, and IR). Each isolate was tested for its antioxidant properties, and compounds **1** and **9**-**11** were found to have significant activity.

Introduction

Marine microorganisms, particularly marine fungi, have recently gained prominence as an important source of biologically active secondary metabolites (König & Wright, 1996). Among marine fungi, those living in association with marine algae are a particularly promising source of novel natural products due to the special ecological niche in which they exist. The association between algae and fungi appears to be highly developed since nearly one third of all higher marine fungi described are so-called algicolous or algae- associated organisms (Kohlmyeyer & Kohlmeyer, 1979). Recently, marine derived fungi have yielded some unique biologically active metabolites, such as ascosalipyrrolidinone and ascosalipyrone (Osterhage *et al.*, 2000), microsphaeropsisin (Höller *et al.*, 1999), and mactanamide (Lorenz *et al.*,1998), further supporting the idea that these organisms are truly valuable producers of potential therapeutic agents.

Natural antioxidants (Yen *et al.*, 1996) are known to be produced by *Penicillium roquefortii* (Hayashi *et al.*, 1995), *Aspergillus candidus* (Yen *et al.*, 1999, 2001), *Mortierella* sp. (Hirota *et al.*, 1997), and *Emericella falconensis* (Takahashi *et al.*, 2000) and by fungi of the genus *Acremonium* (Teshima *et al.*, 1991). *Acremonium* sp. (Hypocreace, Ascomycetes), are noted for their secondary metabolite content, with around 132 compounds having been reported from fungi of this genus, including the halymecins (Chen *et al.*, 1996), oxepinamides A-C (Belofsky *et al.*, 2000), acremolactone (Sassa *et al.*, 1998), and orbuticin (Roy *et al.*, 1996).

Materials and methods

Isolation and taxonomy of the fungal strain

Algal material (*Cladostephus spongius,* Cladostephaceae, M 1) was collected by divers from waters around Spain (Moraira, Mediterranean Sea). After sterilization with 70 % ethanol, algal samples were rinsed with sterile water and pressed onto agar plates to detect any residual fungal spores on the surface. Sterilized algae were then cut into pieces and placed on agar plates containing isolation medium: 15 g/L agar, 1 L seawater from the sample-collecting site, benzyl penicillin and streptomycin sulphate (250 mg/L). Fungal colonies growing out of the algal tissue were transferred onto medium for sporulation: 1.0 g/L glucose, 0.1 g/L yeast extract, 0.5 g/L peptone from meat, enzymatic digest, 15 g/L agar, 1 L artificial seawater, the pH was adjusted at 8, in order to enable taxonomy of the isolates.

Cultivation

The fungus *Acremonium* sp. (Hypocreace, Ascomycetes, M 1-11-1, 506) was cultivated at room temperature for two months in 7 L (14 Fernbach flasks) of solid biomalt agar containing 50 g biomalt, 15 g agar, and 1 L ASW. Each Fernbach flask was inoculated with 10 ml of 10-day-old cultures (room temperature) grown in biomalt media without agar.

Biological activity, see chapter 3.4.

Extraction and isolation

The mass cultivated fungus, mycelia and medium, was diluted with water (100 mL/L) and homogenized using an Ultra-turrax model T25 at 8000 min^{-1}, the resulting mixture was exhaustively extracted with EtOAc (3 x 10 L) to yield 4.5 g of a highly viscous brownish black gum. The extract (4.3 g) was fractionated by VLC (Si gel 60, 0.063-0.200 mm, Merck) employing gradient elution from petroleum ether to EtOAc to MeOH, to yield 8 fractions (Pet.ether:EtOAc:MeOH, 1:0:0; 5:5:0; 2.5:7.5:0; 0:1:0; 0:7.5:2.5; 0:5:5; 0:2.5:7.5; 0:0:1, each 200 mL) (47.6, 433, 1050, 1114, 99, 600, 385 and 41 mg, respectively). VLC fraction 3 (1.05 g) was further fractionated by VLC using RP-18 material (Polyprep 60-50 C18, Machery-Nagel 71150), and gradient elution from H_2O to MeOH, to yield 15 fractions (H_2O: MeOH, 1:0; 1:0; 9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; 1:9; 0:1; 0:1; 0:1; 0:1, each in 50 mL), which were combined, based on ^{1}H NMR spectral similarities, to give nine fractions (20, 42.8, 236, 60, 10, 48, 8, 10, 2 mg, respectively). Fraction 3 appeared most promising based on several ^{1}H NMR resonances in the δ 6-9 region, and was further fractionated by normal phase HPLC (Eurospher Si, 5 µm, 250 x 8 i.d., Knauer) with cyclohexane:acetone, 8:2, in 30 min, 3 mL/min, as eluent to yield six compounds; **1** (t_R = 10 min, 14 mg or 2 mg/L), **6-8** (t_R = 8 min, 14 mg or 2 mg/L), **9** (t_R = 13 min, 8 mg or 1.1 mg/L), and **10** (t_R =13 min, 10 mg or 1.4 mg/L). VLC Fraction 4 (1.1 g) was also further fractionated by VLC using normal phase silica and gradient elution from n-hexane to EtOAc to MeOH, to yield 15 fractions (n-hexane:EtOAc:MeOH, 1:0:0; 1:0:0; 9:1:0; 8:2:0; 7:3:0; 6.4:0; 5:5:0; 4:6:0; 3:7:0; 2:8:0; 1:9:0; 0:1:0; 0:5:5; 0:0:1; 0:0:1, 50 mL each), which were combined, again based on their ^{1}H NMR spectral data, into 6 fractions (5.0, 85.5, 116.4, 5.1, 195.6, 3.2 mg, respectively). Of these, fraction 5 was purified using HPLC with RP-18 (Eurospher-100, 5 µm, 250 x 8 mm i.d., Knauer), and gradient elution from H_2O:MeOH, 3:1 to MeOH in 25 min, 2 mL/min, to yield **11** (t_R = 11 min, 25 mg or 3.5 mg/L). From fraction 4 crystals of **5** (6 mg or 0.85 mg/L) were obtained by recrystallisation from CH_2Cl_2:$(CH_3)_2CO$ (3:7); the mother liquor was purified by

RP-18 HPLC employing gradient elution from H_2O:MeCN, 4:1 to MeCN in 40 min, 2 mL/min, to yield **3** and **4** (t_R =15 min, 2 mg or 0.28 mg/L) and **10** (t_R = 17 min, 3 mg, 0.42 mg/mL). VLC fraction 6 (0.6 g) was further fractionated by VLC over normal phase (230-400 mesh ASTM, Merck), 15 fractions were collected as mentioned above (50 mL each), and combined into 7 pools (15, 185, 100, 35, 117, 66, 10 mg, respectively), according to ^{1}H NMR spectral data. Pool 3 (0.1 g) was the most promising one and fractionated over normal phase HPLC (Eurospher Si, 5µ, 250 x 8 i.d., Knauer) using a gradient from CH_2Cl_2 to MeOH, in 25 min, 2 mL/min, to afford **2** (t_R = 17 , 20 mg, 2.8 mg/L).

Acid hydrolysis of 2

A solution of **2** (5 mg in 1 mL EtOH) and 10 mL of 7 % HCl:EtOH (3:7) was refluxed for 4 hours. At the end of this period the mixture was extracted with EtOAc, and the aqueous layer neutralised with 7 % KOH. After evaporation of the solvent, the residue was extracted with C_5H_5N. HPLC purification of the C_5H_5N extract (Lichrosorb-100 NH_2, 5 µ, 250 x 8 i.d., Knauer), using MeCN:H_2O 8:2 as eluent yielded glucose (1 mg), $[\alpha]^{23}_D$ = +20.7° (*c* 0.08, H_2O), lit. (Merck index online) $[\alpha]^{23}_D$ = +18.7° - +52.7° (*c* 10.0, H_2O).

GC-MS analysis of 3 and 4

This analysis was carried out on a Perkin-Elmer (Auto system XL) gas chromatograph coupled with a Perkin-Elmer Turbomass spectrometer using a 30 m x 0.32 mm N931-6023 Pe-1 (film thickness of 0.25 µm) capillary column. The instrument was set to an initial temperature of 50 °C and maintained at this temperature for 10 min. At the end of this period the oven was heated at 10°/min up to 250°C, and kept there for 10 min. Injection port temperature was 220°C, He flow rate was 2 mL/min, and samples were injected in the splitless mode. Mass spectral scan range was 35-650 DA. The GC of **3** and **4** showed two peaks. Retention times, relative intensities (%) and EIMS of the relevant peaks were as follows: Peak A, 21.4 min, 100, EIMS m/z (% rel int), 192 [M^+] (<1), 174 (77), 159 (5), 145 (11), 119 (32), 90 (18); Peak B, 24.2 min, 84, EIMS m/z (% rel int), 192 [M^+] (<1), 174 (77), 159 (5), 148 (46), 145 (13), 119 (100), 90 (20).

GC-MS analysis of 6-8

The mixture of compounds **6-8** was analysed by GC-MS in an identical manner to the analysis performed with **3** and **4**. Retention times, relative intensities and EIMS of the relevant peaks were: Peak A (19.4 min, 92), EIMS *m/z* (% rel int), 186 (1), 168 (50), 140 (100

), 81 (90), 67 (100); Peak B (19.8 min, 72); EIMS *m/z* $[M]^+$ (% rel int), 186 $[M^+]$ (3), 168 (10), 140 (33), 81 (40), 67 (100) Peak C (20.2 min, 100) EIMS *m/z* (% rel int), 186 $[M^+]$ (2), 168 (77), 140 (100), 81 (60), 67 (100).

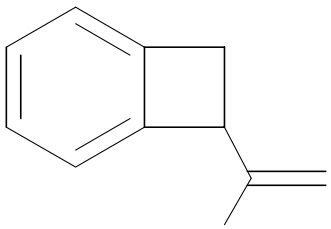
X-ray structure analysis of compound 5

2-(1-Methylethylidene)-pentanedioic acid (**5**) was recrystallised from MeOH to yield transparent prismatic crystals. A crystal 0.34 x 0.15 x 0.11 mm^3 was selected for crystallographic measurement. It has, molecular formaula = $C_9H_{12}O_4$, molecular mass = 172.18 amu, crystal system = triclinic, space group P(11-1), unit cell dimensions a = 5.0439 Å, b = 7.2708 Å, c = 12.3363(16), α = 89.527(3)°, β = 84.456(3)°, γ = 74.754(3)°, volume = 434.37(10) $Å^3$, Z = 2, density = 1.316 mg/m^3, *F*(000) = 184, absorption = 0.106 μ, λ (Mo K α) = 0.71073 Å. The intensity data were collected at 133 K to q(max) 30° on a Bruker SMART 1000 CCD diffractometer. A total of 5047 reflections were recorded, of which 2495 [R(int) = 0.0282] were unique. The structure was solved by direct methods and refined by full matrix least-squares methods on F^2 using the SHELXL97 program (Sheldrick`s program). Acidic hydrogens were refined freely, methyls as rigid groups, methylene hydrogens with a riding model. Final R indices: R1 [$I > 2\sigma(I)$] = 0.042, wR2 (all reflections) = 0.124; goodness of fit 1.047 (these data have been deposited at the Cambridge crystallographic data center).The molecule is shown in Fig. 5. Its dimensions may be regarded as normal. The molecular conformation is illustrated by the torsion angles C2-C3-C4-C5 177.3(1), C3-C4-C5-O3 177.6(1)° (antiperiplanar) and C1-C2-C3-C4 85.0(1), C6-C2-C3-C4 93.2(1)° (orthogonal). The molecules are connected by hydrogen bonds of the "carboxylic acid dimer" type [O3-H03···O4' and O1-H01...O2'] across inversion centres to form chains of molecules parallel to [11-1] (Fig. 6).

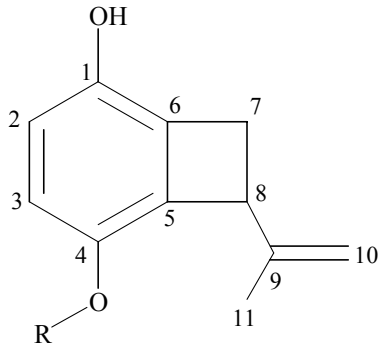
Results and discussion

The fungus *Acremonium* sp. closely related to *A. roseogriseum* (S. B. Saksena) W. Gams based on morphological characteristics was isolated from tissues of the brown alga *Cladostephus spongius* (Hudson) C. Agardh. The fungus was cultivated on a solid biomalt medium with added artificial sea salt. Successive fractionation of the EtOAc extract by vacuum liquid chromatography (VLC) over silica followed by normal (NP), and reversed phase (RP-18) HPLC yielded compounds **1**-**11**. In the current partial project, the isolation and structure elucidation of two novel hydroquinone derivatives (**1** and **2**), two new dihydronaphthalenones (**3** and **4**), four new acyclic carboxylic acid derivatives (**5**-**8**), and three known fungal metabolites (**9**-**11**) are reported (Tanaka *et al.*, 1999).

The molecular formula of **1** was deduced by accurate mass measurement to be $C_{11}H_{12}O_2$. In the ^{13}C NMR spectrum of **1**, eleven resonances, attributable to 1 x CH_3, 2 x CH_2, 3 x CH, and 5 x C groups (Table 5), were evident. It was also clear from these data that since four of the six degrees of unsaturation within **1** were due to multiple bonds (4 x C═C), the molecule was bicyclic. The ^1H and ^{13}C NMR data enabled all but two hydrogen atoms of **1** to be accounted for, thus, it was evident that the remaining two must be present in OH groups, a deduction supported by IR (ν_{max} 3314 cm^{-1}), and ^1H NMR (δ 7.64 brs, CD_3OD exchangeable) data. Also evident from the ^1H and ^{13}C NMR data of **1** were resonances consistent with the presence of a tetrasubstituted aromatic ring [δ 6.51 (d, *J* = 8.8, H-2); 6.54 (d, *J* = 8.8, H-3)], a deduction supported by the maximum at 286 nm in the UV spectrum of **1**, an allylic methyl, an exomethylene, a benzylic methylene, and an allylic benzylic methine group. Accounting for all ^1H and ^{13}C NMR resonances associated with C-H one-bond interactions and from cross peaks seen in the ^1H-^{13}C 2D NMR shift-correlated (HMQC) spectrum of **1**, it was possible to deduce the planar structure of **1** by interpretation of its ^1H-^1H COSY and HMBC (Figure 4) spectra. From the ^1H-^1H COSY spectrum of **1**, it was evident that H_2-7 coupled to H-8, and that H_3-11 had an allylic coupling to H_2-10. Cross peaks in the HMBC spectrum of **1** between H_2-10 and C-8, C-9 and C-11, between H_2-7 and C-2, C-5 and C-6, and between H-8 and C-3, C-4, C-5 and C-6 enabled Fragment 1 to be developed. The similarity in the ^{13}C NMR chemical shifts of the resonances associated with C-1 and C-4 (δ 145.9 s, 145.8 s), and C-2 and C-3 (116.0 d, 115.9 d) showed the aromatic ring of **1** to be pseudo-symmetrically substituted, as shown in **1**. The absolute stereochemistry at C-8 was not determined. For **1**, a new hydroquinone derivative, the trivial name acremonin A is proposed.



Fragment 1



1 R = H
2 R = ß-D-glucopyranose

The positive FABMS and NMR data of **2** showed it to have the molecular formula $C_{17}H_{22}O_7$. Comparison of the ^1H and ^{13}C NMR data of **2** with those of **1**, and with the ^{13}C NMR data of arbutin (Table 5), revealed it to be a glucopyranose derivative of **1**. The chemical shift and

coupling constant [4.81 d (J = 7.7 Hz)], attributable to the anomeric proton of the glucose showed it to be β-linked to the rest of the molecule. Acid hydrolysis of **2** yielded free glucose, to which the D-configuration was assigned on the basis of its optical rotation (see experimental section). The point of attachment of the sugar to the aglycone was concluded to be at C-4 based on the deshielded nature of the resonances associated with C-3, C-4, C-5, and that of H-3, relative to the equivalent resonances found in **1**. This conclusion was supported by cross peaks in the NOESY spectrum of **2** between H-1' and H-8, and one of the exomethylene protons. For **2** the trivial name acremonin A glucoside is proposed.

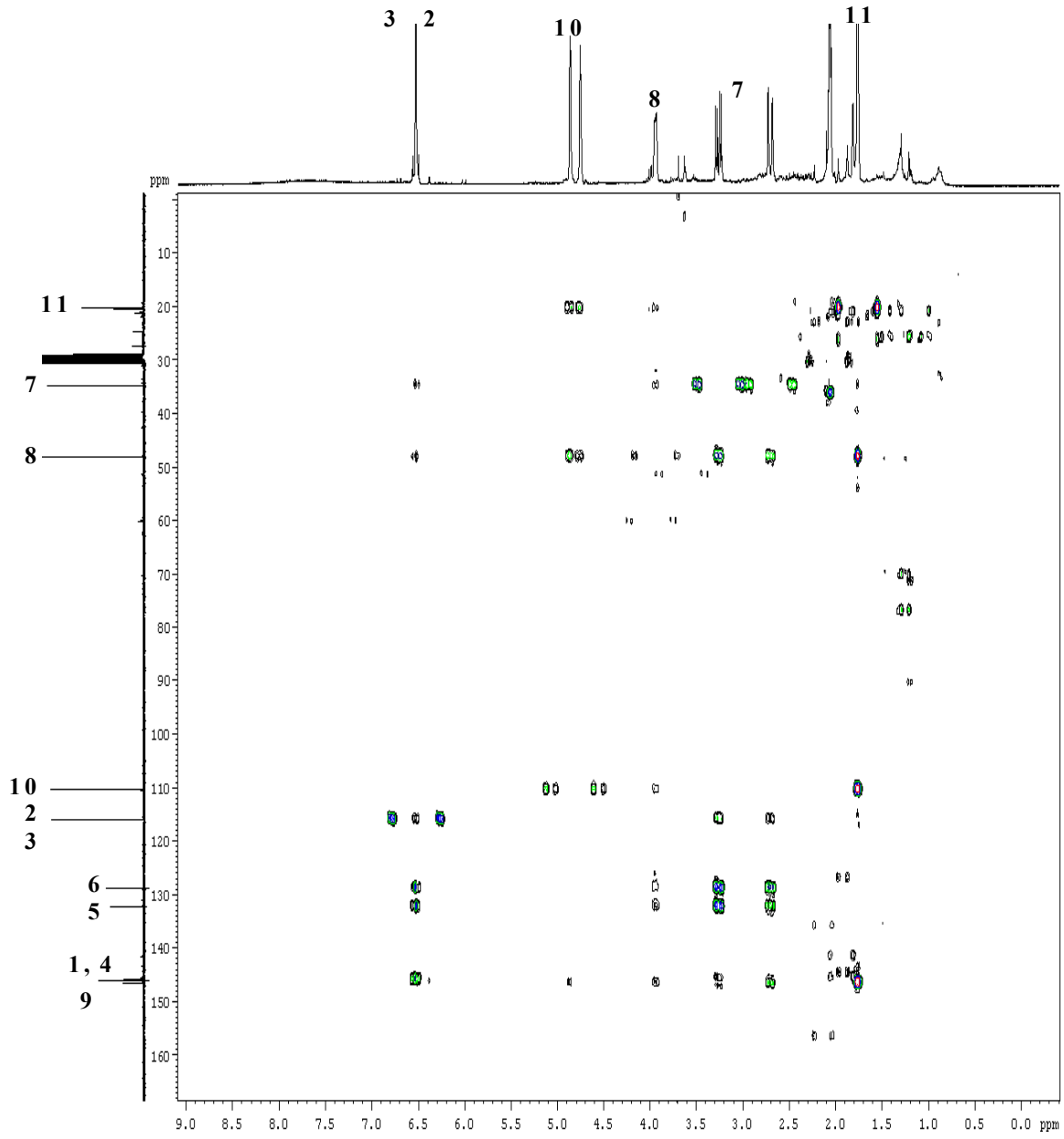
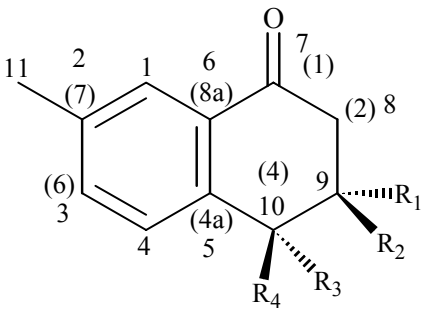


Figure 4. HMBC NMR spectrum of acremonin A (**1**), ^{1}H [C_3D_6O, 300 MHz] and ^{13}C NMR [C_3D_6O, 75.5 MHz].

Table 5. ^{1}H [$(CD_3)_2CO$, 300 MHz] and ^{13}C NMR [$(CD_3)_2CO$, 75.5 MHz] spectral data for compounds **1** and **2**,[a] and ^{13}C NMR data for arbutin

	1			Arbutin[b]	**2**		
P.	δ ^{13}C	δ ^{1}H	HMBC	δ ^{13}C	δ ^{13}C	δ ^{1}H	HMBC
1	145.9 (s)[c,]*			153.7 (s)	146.6 (s)*		
2	116.0 (d)	6.51 (1H, d, 8.8[d])	C-1, C-4, C-6	119.3 (d)	116.1 (d)	6.57 (1H, d, 8.8)	C-1, C-4, C-6
3	115.9 (d)	6.54 (1H, d, 8.8)	C-1, C-4, C-5, C-8	116.6 (d)	117.8 (d)	6.74 (1H, d, 8.8)	C-1, C-4, C-5, C-8
4	145.8 (s)*			152.4 (s)	147.9 (s)*		
5	132.3 (s)			116.6 (d)	134.4 (s)		
6	128.8 (s)			119.3 (d)	128.9 (s)		
7	34.7 (t)	3.26 (1H, dd, 13.2, 5.5)	C-1, C-2, C-5, C-6, C-8, C-9		34.5 (t)	3.28 (1H, dd, 13.2, 5.5)	C-1, C-2, C-5, C-6, C-8, C-9
		2.70 (1H, dd, 13.2, 2.6)				2.77 (1H, dd, 13.2, 2.6)	
8	48.9 (d)	3.94 (1H, brd, 5.5)	C-3, C-5, C-6, C-7, C-9, C-10, C-11		49.1 (d)	4.06 (1H, dd, 2.6, 5.5)	C-5, C-6, C-7, C-9, C-10, C-11
9	146.4 (s)				146.4 (s)		
10	110.4 (t)	4.76 (1H, d, 0.7)	C-8, C-9, C-11		111.6 (t)	4.78 (1H, d, 1.5)	C-8, C-9, C-11
		4.86 (1H, d, 0.7)	C-8, C-9, C-11			4.92 (1H, d, 1.5)	
11	20.3 (q)	1.76 (3H, s)	C-8, C-9, C-10		19.7 (q)	1.74 (3H, s)	C-8, C-9, C-10
OH-1		7.64 (1H, brs)				8.10 (1H, s)	
OH-4		7.64 (1H, brs)					
1′				103.6 (d)	102.7(d)	4.81 (1H, d, 7.7)	C-3′
2′				74.9 (d)	74.9 (d)	3.39 (1H, m)	C-3′, C-4′
3′				77.9 (d)	77.8 (d)	3.39 (1H, m)	C-2′, C-4′
4′				71.4 (d)	71.3 (d)	3.45 (1H, m)	C-2′, C-3′
5′				78.0 (d)	77.9 (d)	3.36 (1H, m)	C-2′, C-3′, C-4′
6′				62.6 (t)	62.8 (t)	3.70 (1H, m), 3.81 (1H, m)	C-5′, C-4′

[a]All assignments are based on 1D and 2D NMR measurements (HMBC, HMQC, COSY). [b]Data obtained by measuring an authentic sample. [c]Implied multiplicities as determined by DEPT (C = s, CH = d, CH_2 = t, CH_3 = q). [d]*J* in Hz. * Assignments may be interchanged.



Numbers in brackets are those used for the systematic naming of 3 and 4.

3 $R_1 = R_3 = H$; $R_2 = R_4 = OH$
4 $R_2 = R_3 = H$; $R_1 = R_4 = OH$

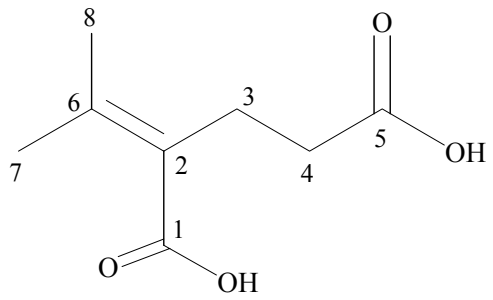
The stereoisomers **3** and **4** (**3**/**4**), which proved to be inseparable by HPLC, analysed for $C_{11}H_{12}O_3$ by accurate mass measurement. The ^{13}C NMR spectrum of the mixture contained 11 carbon resonances for each compound that were attributable in each case to 1 x CH_3, 1 x CH_2, 5 x CH, and 4 x C groups (Table 6). It was also evident from these data that four of the six degrees of unsaturation within the molecules were due to multiple bonds, 3 x C═C, and 1 x C═O; the molecules are thus bicyclic. As the ^{13}C NMR and ^1H NMR spectral data accounted for all but two of the hydrogen atoms within **3** and **4**, it was evident that the remaining two (in each compound) must be present as parts of OH groups. In the ^1H NMR spectrum of **3**/**4** the presence of three aromatic protons that were *meta* (δ 7.68), *ortho* and *meta* (δ 7.43, 7.45), and *ortho* coupled (δ 7.55, 7.60), respectively, revealed the presence of a 1, 3, 4 substituted aromatic ring in each molecule. The UV data of **3**/**4** (λ_{max} 251 and 296 nm) supported the presence of this group and showed it to be further conjugated with the keto-function (δ 196.4 s and 196.2 s). HMBC correlations between the resonance of H_3-11 and those of C-1, C-2, C-3, and C-6, showed the methyl group to reside at C-2, leaving the keto-function to be bonded to either C-5 or C-6. Further HMBC correlation, this time between the resonance for H-4 and those for C-2, C-3, C-5, C-6, and C-10, and the resonance for H-1 and those of C-2, C-3, C-7, and C-11, clearly revealed C-6 to bond with C-7, and C-5 to bond with C-10. From the ^1H-^1H COSY spectrum of **3**/**4** it was evident that H-10 coupled with H-9, which further coupled with H_2-8, leaving C-8 to bond directly with the keto-function, and in so doing complete the planar structure of **3**/**4**. The ^1H-^1H coupling constant associated with the resonances for H-9 in **3**/**4** show H-9 in **4** to have an axial orientation, as must H-10. In **3**, the coupling constants associated with the resonance for H-9 are 2.9, 3.4, and 6.5 Hz, and show this proton to have no diaxial coupling interactions, it must thus be equatorial. Accordingly, $J_{H\text{-}9,\ H\text{-}10}$ in **3** is noticeably smaller, 2.9 Hz, indicating it now to have and axial-equatorial interaction with H-9. The two new natural products **3** and **4** are best named systematically as (3*R**,4*S**) 3,4-dihydroxy-7-methyl-3,4-dihydro-1(2*H*)-naphthalenone and (3*S**,4*S**) 3,4-dihydroxy-7-methyl-3,4-dihydro-1(2*H*)-naphthalenone, respectively.

Table 6. ^{1}H [$(CD_3)_2CO$, 300 MHz] and ^{13}C NMR [$(CD_3)_2CO$, 75.5 MHz] spectral data for compounds **3** and **4**[a]

	3		**4**		**3** and **4**
	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H	HMBC
1	135.2 (d)[b]	7.68 (1H, brs)	135.4 (d)	7.68 (1H, brs)	C-2, C-3, C-7, C-11
2	142.2 (s)		142.7 (s)		
3	126.6 (d)	7.43 (1H, dd, 7.7, 1.5[c])	126.7 (d)	7.45 (1H, d, 7.7, 1.5)	
4	129.4 (d)	7.55 (1H, d, 7.7)	128.9 (d)	7.60 (1H, d, 7.7)	C-6, C-2, C-3, C-5, C-10
5	138.0 (s)		138.1 (s)		
6	132.2 (s)		132.2 (s)		
7	196.4 (s)		196.2 (s)		
8	44.3 (t)	2.79 (1H, dd, 16.7, 3.4) 2.86 (1H, dd, 16.7, 6.5)	44.9 (t)	2.60 (1H, dd, 16.7, 9.3) 2.95 (1H, dd, 16.7, 4.1)	C-6, C-7, C-9, C-10
9	70.7 (d)	4.35 (1H, ddd, 2.9, 3.4, 6.5)	72.4 (d)	4.05 (1H, ddd, 9.3, 7.0, 4.1)	C-7, C-8
10	70.5 (d)	4.92 (1H, d, 2.9)	73.4 (d)	4.68 (1H, d, 7.0)	C-4, C-8
11	20.9 (q)	2.38 (3H, s)	21.0 (q)	2.38 (3H, s)	C-1, C-2, C-3, C-6

[a]All assignments are based on 1D and 2D measurements (HMBC, HMQC, and COSY).
[b]Implied multiplicities as determined by DEPT (C = s, CH = d, CH_2 = t, CH_3 = q). [c]*J* in Hz.

FABMS and NMR analysis of **5** showed it to have the molecular formula $C_8H_{12}O_4$. Its ^{13}C NMR spectrum contained 8 carbon resonances attributable to 4 x C, 2 x CH_2, 2 x CH_3 groups (Table 7). It was also evident from these data that the three degrees of unsaturation within **5** were due to a C═C double bond, and two carbonyl groups as parts of either ester or carboxyl functions, one of which was α, β-unsaturated; the molecule was acyclic. The ^{1}H and ^{13}C NMR data enable all but two of the hydrogen atoms within **5** to be assigned to directly bonded C atoms. The two remaining hydrogens were therefore present as parts of two carboxylic functions, a deduction that was supported by the δ 10.80 (2H) ^{1}H NMR resonance, and the IR data (2922 cm^{-1}), of **5**. With all ^{1}H and ^{13}C NMR resonances assigned in the HMQC spectrum of **5**, it was possible to deduce the structure of **5** by interpretation of its ^{1}H-^{1}H COSY and HMBC spectra. Compound **5** was crystalline X-ray



5

diffraction studies were undertaken, the results of which are shown in Figure 5 and 6, and in the experimental section. As such, **5** is not a new chemical entity since it is known synthetically (Fleury *et al.*, 1963; Puterbaugh *et al.*, 1962). The current report is, however, the first of it from a natural source, and with complete spectroscopic and X-ray data.

Table 7. ^{1}H [$(CD_3)_2CO$, 300 MHz] and ^{13}C NMR [$(CD_3)_2CO$, 75.5 MHz] spectral data for compound **5**[a]

Position	δ ^{13}C	δ ^{1}H	HMBC
1	170.0 (s)[b]		
2	126.2 (s)		
3	26.1 (t)	2.40 (2H, m)	C-2, C-4, C-5
4	33.6 (t)	2.60 (2H, m)	C-1, C-2, C-3, C-5, C-6
5	174.2 (s)		
6	145.2 (s)		
7	22.2 (q)	1.86 (3H, s)	C-1, C-2, C-3, C-6
8	23.1 (q)	1.86 (3H, s)	C-1, C-2, C-3, C-6
2 OH		10.80 (2H, brs)	

[a]All assignments are based on 1D and 2D measurements (HMBC, HMQC, COSY).
[b]Implied multiplicities were determined by DEPT (C = s, CH = d, CH_2 = t, CH_3 = q).

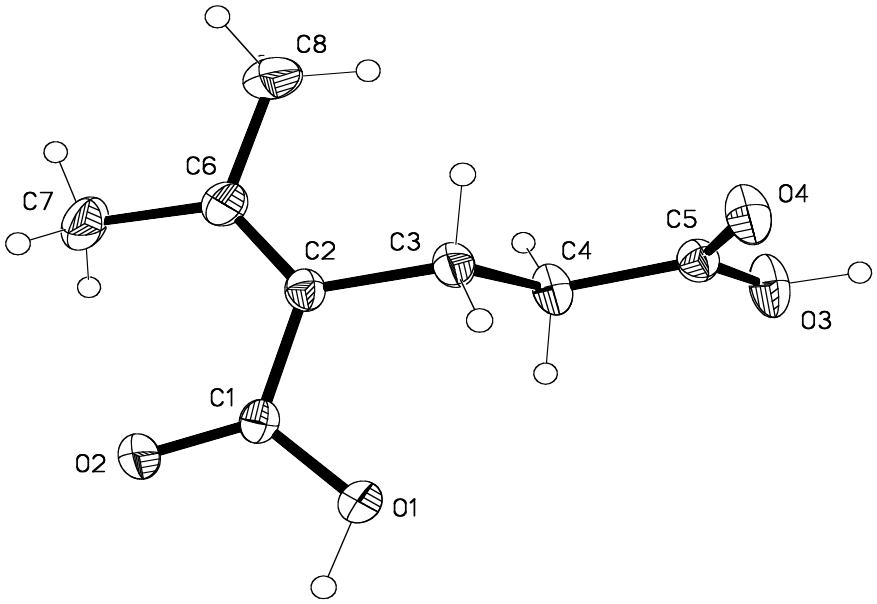


Figure 5. Structure of compound **5** in the crystal. Ellipsoids represent 50% probability levels.

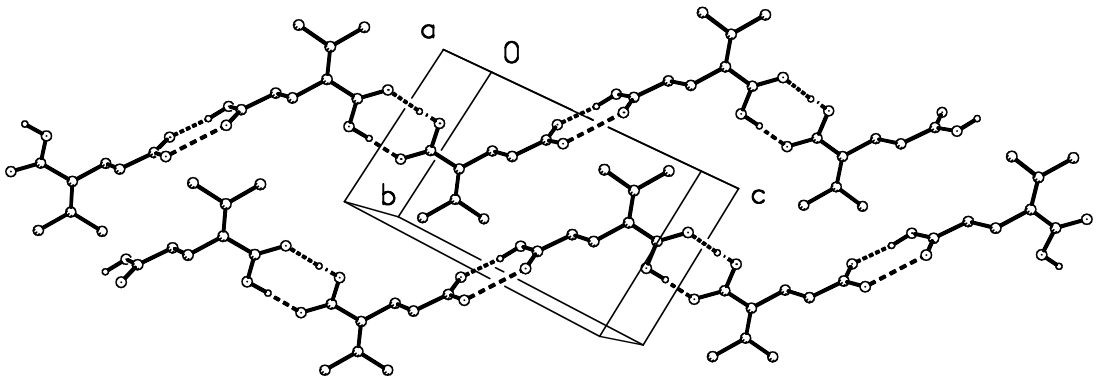
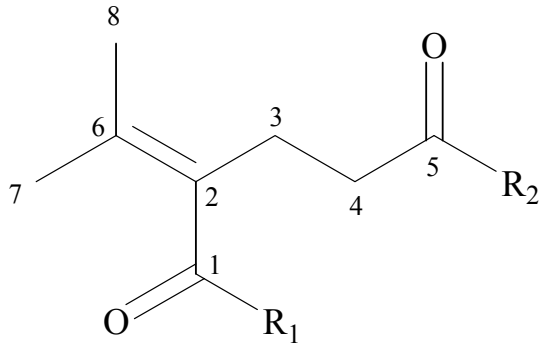


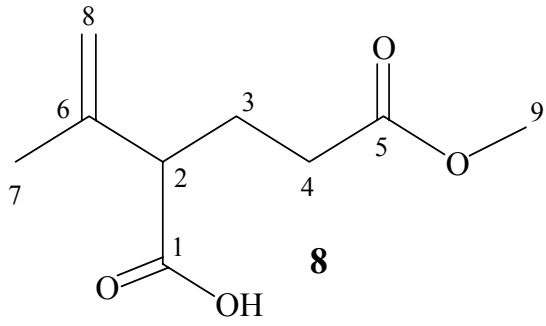
Figure 6. Packing diagram of compound **5**, showing the chains of molecules associated by hydrogen bonds (dashed). Only those hydrogen atoms involved in hydrogen bonding are shown. Radii are arbitrary.

Compounds **6**-**8** are structural isomers that proved to be inseparable by HPLC. GC-MS analysis and accurate mass measurement of the mixture **6**/**8** showed the three compounds present to have the identical molecular formula $C_9H_{14}O_4$. A comparison of the MS and NMR spectral data of **6** and **7** with those of **5** (Tables 7 and 8), indicated **6** and **7** to be monomethoxyl derivatives of **5**. In each case the position of the methoxyl group was established from HMBC data. For **6**, an HMBC correlation between the resonance for H_3-9 and that of C-5, clearly made the C-9 methyl group part of a methyl ester function and revealed the molecule to be pentanedioic acid, 2-(1-methylethylidene)-5-methyl ester. In the case of **7**, the diagnostic HMBC correlation was between H_3-9 and C-1,and showed the molecule to be pentanedioic acid, 2-(1-methyl- ethylidene)-1-methyl ester.



6 $R_1 = OH$, $R_2 = OCH_3$
7 $R_1 = OCH_3$, $R_2 = OH$

Even though the molecular formula of **8** ($C_9H_{14}O_4$) was identical to those of **6** and **7** the NMR data were notably different (Table 8). As for **6** and **7**, the ^{13}C NMR spectrum contained nine carbon resonances attributable to 1 x CH_3, 1 x OCH_3, 3 x CH_2, 1 x CH, and 3 x C, groups. It was also evident from these data that one of the three degrees of unsaturation within **8** was present as an exomethylene group, in contrast to the endo-carbon-carbon double bond found in **6** and **7**, and that the two carbonyl functions present in **5**-**7** were also to be found in **8**. Interpretation of the HMQC, ^1H-^1H COSY and HMBC spectral data of **8** enabled its planar structure to be deduced. Thus, cross peaks in the ^1H-^1H COSY spectrum of **8** showed H_3-7 to long-range couple with H_2-8 and H-2, H-2 also coupled with H_2-3, which further coupled with H_2-4, clearly characterizing the C-8 to C-4 part of the molecule. The HMBC spectral data of **8**, showed correlations between the resonances for H_2-8 and those for C-1, C-2, C-6, and C-7; between that for H-2 and those for C-1, C-3, C-6, C-7, and C-8; and between those for H_2-3 and C-2, C-4, C-5 and C-6, and between that for H_3-9 and that for C-5. These data revealed C-1 to bond with C-2, C-4 with C-5, and the methoxyl group to be part of the methyl ester function at C-5. Accordingly, **8** is most appropriately named pentanedioic acid, 2-(1-methylethenyl)-5-methyl ester.



8

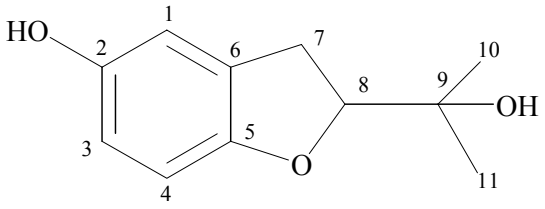
Table 8. ^{1}H (C_6D_6, 500 MHz) and ^{13}C NMR (C_6D_6, 125.5 MHz) spectral data for compounds **6-8**[a]

	6			**7**			**8**		
Position	δ ^{13}C	δ ^{1}H	HMBC	δ ^{13}C	δ ^{1}H	HMBC	δ ^{13}C	δ ^{1}H	HMBC
1	174.2 (s)[b]			168.7 (s)			179.0 (s)		
2	125.3 (s)			125.3 (s)			52.1 (d)	3.08 (1H, dd, 7.6)	C-1, C-3, C-4, C-7, C-9
3	25.6 (t)	2.70 (2H, t, 7.9[c])	C-1, C-2, C-4, C-6	25.3 (t)	2.65 (2H, t, 7.9)	C-1, C-2, C-4, C-6	25.2 (t)	2.18 (1H, m)	C-2, C-4, C-6
								1.90 (1H, m)	C-2, C-4, C-6
4	33.6 (t)	2.41 (2H, t, 7.9)	C-3, C-5	33.5 (t)	2.40 (2H, t, 7.9)	C-3, C-5	31.6 (t)	2.15 (2H, m)	C-2, C-3, C-5
5	172.8 (s)			179.2 (s)			172.9 (s)		
6	150.2 (s)			145.4 (s)			141.6 (s)		
7	23.1 (q)	1.97 (3H, s)	C-2, C-6	23.1 (q)	1.94 (3H, s)	C-2, C-6	20.0 (q)	1.56 (3H, s)	C-1, C-2, C-6, C-8
8	22.8 (q)	1.51 (3H, s)	C-2, C-6	21.8 (q)	1.49 (3H, s)	C-2, C-6	115.0 (t)	4.88 (1H, s)	C-1, C-2, C-6, C-7
								4.83 (1H, s)	C-1, C-2, C-6, C-7
9	51.1 (q)	3.36 (3H, s)	C-5	50.7 (q)	3.42 (3H, s)	C-1	51.0 (q)	3.37 (3H, s)	C-5
OH		8.53 (1H, brs)			8.53 (1H, brs)			8.53 (1H, brs)	

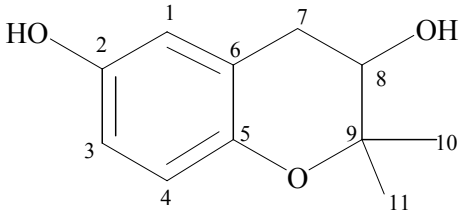
[a]All assignments are based on 1D and 2D NMR measurements (HMBC, HMQC, COSY).

[b]Implied multiplicities as determined by DEPT (C = s, CH = d, CH_2 = t, CH_3 = q). [c]*J* in Hz.

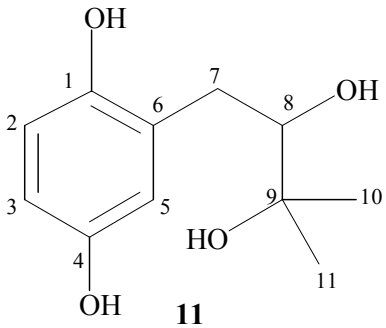
2-(1-Hydroxy-1-methyl)-2,3-dihydrobenzofuran-5-ol, (**9**), 2-dimethyl-chroman-3,6-diol (**10**), and 2-(3-dihydroxy-3-methylbutyl)-benzene-1,4-diol (**11**) were published from *Acremonium murorum* as inhibitor of the magnesium-dependent sphingomyelinase enzyme. Thus, they have anti-inflamatory activity with IC_{50} >200, >200, 7.5 µg/mL, respectively (Tanaka *et al.*, 1999). Compound **11** showed potent activity as comparing with compounds **9** and **10**. It was evident that compound **11** has more free hydroxyl groups which might be required for the enzyme inhibiton.



9



10



11

The most unusual bicyclo[4.2.0]octa-1,3,5-triene ring system found in **1** and **2**, can be generated synthetically by the action of UV light (254 nm) on a butane solution of 3-methyl-1,2-dihydronaphthalene (3-MDHN) (Figure 7) (Duguid *et al.*, 1991); the mode of cyclization used in this *Acremonium* sp., is, however, not known.

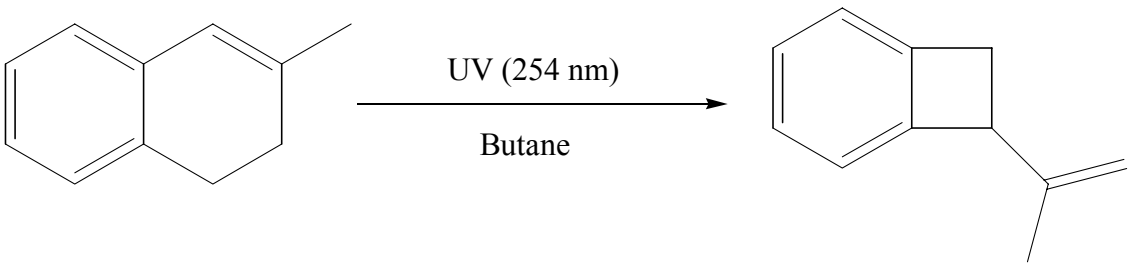


Figure 7. Photolytic generation of bicyclo[4.2.0]octa-1,3,5-triene (BOT) containing compounds from 3-methyl-1,2-dihydronaphthalene (3-MDHN).

Compounds **1**, **2**, **5**, **9**, and **10** were tested in ELISA based assays for their HIV-1 reverse transcriptase and tyrosine kinase $p56^{lck}$ inhibitory activities, and found to be inactive. The antioxidative properties of all compounds were assessed using DPPH radical, and TBARS assays. The results of these assays are shown in Tables 9 and 10, and Figure 8. Compounds **1**, **2**, and **9-11** have significant DPPH radical scavenging effects (85.5, 17.5, 85.8, 72.9, 90.2 %, respectively, at 25.0 µg/mL), with **1**, **9**, **10**, and **11** being also able to inhibit peroxidation of linolenic acid (35.5, 15.9, 9.2, and 16.6 %, respectively, at 37.0 µg/mL) (Figure 8). The results of the assays were not totally surprising since it is well known that the phenolic compounds have antioxidant properties (Aoyama *et al.*, 1982), the activity being dependent on the number and the location of the hydroxyl groups.

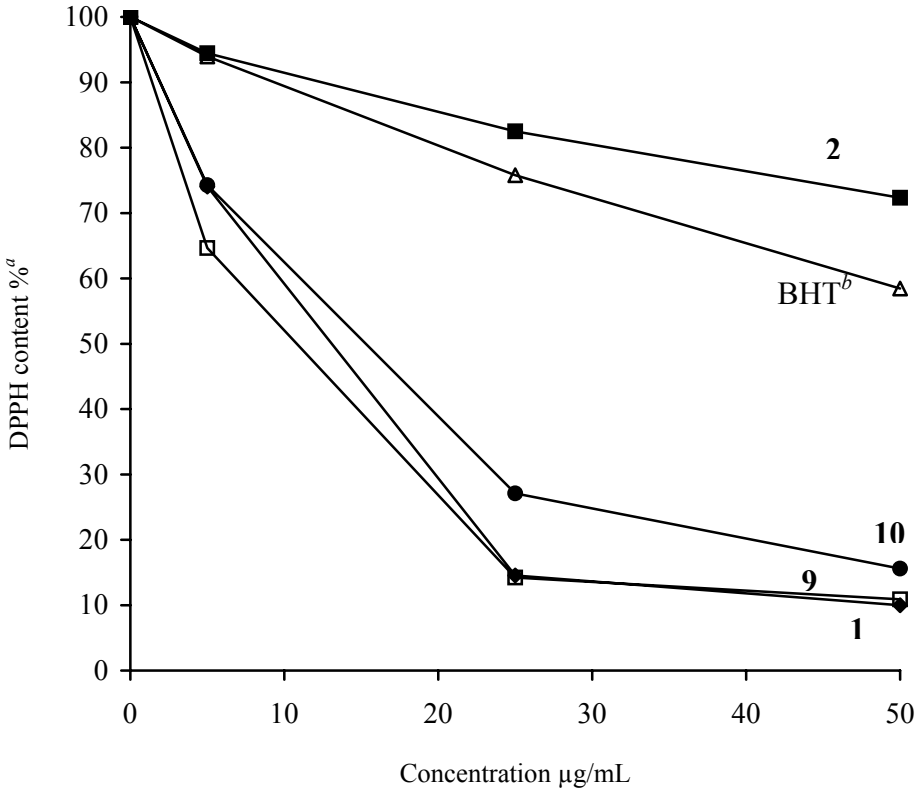


Figure 8. DPPH[c] radical scavenging activity of compounds **1**, **2**, **9** and **10**
[a]Remaining concentration of DPPH radical. [b]BHT = Butylated hydroxytoluene.
[c]DPPH = α,α -Diphenyl-β-picrylhydrazy1.

Table 9. Antioxidative effects (TBARS Assay)[a] of the EtOAc extract of *Acremonium* sp. and compounds **1-11**

	% Inhibition[a]	
Item Tested	7.4	37.0
EtOAc Extract	-27.2	-55.7
1	13.9	35.5
2	-10.6	-10.6
3 and **4**	-22.5	-18.5
5	-9.0	-18.1
6-8	-8.6	-16.3
9	-11.8	15.9
10	-9.8	9.2
11	2.0	16.6
BHT[c]	36.2	43.9
Arbutin	-27.6	-25.2

[a]TBARS = Thiobarbituric acid method. [b]% Inhibition = 100-(A sample[d] - A sample blank) x 100 / (A control - A blank). [c]BHT = Butylated hydroxytoluene. [d]A = Absorbance of test blank and control solutions are measured at both 532 and 600 nm. [e]Concentrations in µg/mL

Table 10. DPPH[a] radical scavenging effects of the EtOAc extract of *Acremonium* sp. and compounds **1-11**

	% Scavenging[b]				
Item Tested	5[c]	25	50	100	500
EtOAc Extract	nt[d]	nt	2.0	4.8	29.3
1	26.0	85.5	90.0	90.0	90.1
2	5.6	17.5	27.7	43.0	79.9
3 and **4**	nt	nt	-1.6	2.8	12.1
5	nt	nt	-0.5	3.8	15.3
6-8	nt	nt	3.4	10.3	35.1
9	35.3	85.8	89.1	90.2	90.4
10	25.7	72.9	85.1	89.1	89.9
11	nt	90.2	90.7	89.9	82.9
BHT[e]	6.0	24.2	41.1	63.8	89.8
Arbutin	1.9	18.3	23.5	52.6	78.6

[a]DPPH = α, α,-Diphenyl-β-picrylhydrazyl. [b]Scavenging % = 100-(A sample[f] x 100 / A control[f]). [c]Concentrations in µg/mL. [d]nt = not tested. [e]BHT = Butylated hydroxytoluene. [f]Absorbance of sample and control measured at 517 nm.

Acremonin A (**1**), 7-isopropenyl-bicyclo[4.2.0]octa-1,3,5-triene-2,5-diol was isolated as yellowish brown viscous oil (14.0 mg). $[\alpha]^{23}_{D}$ = + 93.0° (*c* 1.4, $[CH_3]_2CO$); UV λ_{max} (MeOH) (log ε) 204.9 (4.4), 286 (3.6) nm; IR (film) ν_{max} 3314, 2931, 1495, 1453, 1239 cm^{-1}; ^{1}H and ^{13}C NMR data (Table 5); EIMS *m/z* (% rel int) 176 [M^+] (100), 147 (33), 115 (23), 91 (20); HREIMS *m/z* 176.0837, calcd for $C_{11}H_{12}O_2$ 176.0837.

Acremonin A glucoside (**2**), 7-isopropenyl-bicyclo[4.2.0]octa-1, 3, 5-triene-2, 5-diol, 5-ß-D-glucopyranoside was obtained as amorphous powder (20.0 mg). $[\alpha]^{23}_{D}$ = +4.3° (*c* 2.0, $[CH_3]_2CO$); UV λ_{max} (MeOH) (log ε) 204.9 (4.3), 280 (3.2) nm; IR (film) ν_{max} 3330, 2921, 1490, 1448, 1242 cm^{-1}; ^{1}H and ^{13}C NMR data see (Table 5); FABMS, *m/z* 361 [M+Na]$^+$.

(3*R**,4*S**)-3, 4-Dihydroxy-7-methyl-3, 4-dihydro-1(2*H*)-naphthalenone (**3**) and (3*S**,4*S**)-3, 4-dihydroxy-7-methyl-3, 4-dihydro-1(2*H*)-naphthalenone (**4**) were obtained as amorphous white powdered mixture (2.0 mg). UV λ_{max} (MeOH) (log ε) 251 (3.6), 296 (2.9) nm; ^{1}H and ^{13}C NMR data (Table 6); EIMS *m/z* (% rel int) 192 [M^+] (10), 174 (30), 148 (50), 119 (100); HREIMS *m/z* 192.0799, calcd for $C_{11}H_{12}O_3$ 192.0786.

2-(1-Methylethylidene)-pentanedioic acid (**5**) was obtained as transparent crystalline solid (6 mg). M.p. 131-132°; $[\alpha]^{23}_{D}$ = +4.4° (*c* 0.8, $[CH_3]_2CO$); UV λ_{max} (MeOH) (log ε) 221 (3.8) nm; IR (film) ν_{max} 2922, 2604, 1688, 1655, 1615, 1411 cm^{-1}; ^{1}H and ^{13}C NMR data (Table 7). FABMS *m/z* 173 [M+H]$^+$.

Pentanedioic acid, 2-(1-methylethylidene)-5-methyl ester (**6**), pentanedioic acid, 2-(1-methylethylidene)-1-methyl ester (**7**), and pentanedioic acid, 2-(1-methylethenyl)-5-methyl ester (**8**) was obtained as brownish viscous oil (14 mg). $[\alpha]^{23}_{D}$ = -28.4° (*c* 0.4, MeOH), based on 8 being approximately one third of the mixture; UV λ_{max} (MeOH) (log ε) 220 (3.6) nm, based on **6** and **7** being approximately two thirds of the mixture; IR (film) ν_{max} 2922, 2359, 1735, 1720, 1436, 1216 cm^{-1}; ^{1}H and ^{13}C NMR data (Table 8); EIMS data, see GC-MS Analysis of Compounds **6-8**; HREIMS *m/z* 186.0891 (all three compounds have the identical exact mass), calcd for $C_9H_{14}O_4$ 186.0892.

2-(1-Hydroxy-1-methyl)-2,3-dihydrobenzofuran-5-ol (**9**) was obtained as transparent crystals (8 mg). $[\alpha]^{23}_{D}$ = +13.0° (*c* 0.25, $[CH_3]_2CO$), lit. (Tanaka, *et al.*, 1999) $[\alpha]^{23}_{D}$ = +49.8° (*c* 1.0, MeOH); ^{1}H NMR (300 MHz, $[CD_3]_2CO$) δ 6.65 (1H, dd, *J* = 0.7, 2.2 Hz, H-1), 6.49 (1H, dd, *J* = 2.2, 8.8 Hz, H-3), 6.46 (1H, d, *J* = 8.8 Hz, H-4), 3.03 (1H, dd. *J* = 9.5, 16.1 Hz, H-7), 3.16 (1H, dd, *J* = 8.8, 16.1 Hz, H-7), 4.5 (1H, dd, *J* = 8.8, 9.5 Hz, H-8), 1.17 (3H, s. H_3-10), 1.20 (3H, s, H_3-10), 3.60 (1H, s, OH-9), 7.73 (1H, brs, OH-2); ^{13}C NMR (75.5 MHz$[CD_3]_2CO$) δ

112.8 (d, C-1), 152.0 (s, C-2), 114.3 (d, C-3), 109.3 (d, C-4), 154.1 (s, C-5), 129.2 (s, C-6), 31.6 (t, C-7), 90.0 (d, C-8), 71.4 (s, C-9), 25.5 (q, CH_3, C-10), 26.1 (q, CH_3, C-11); EIMS *m/z* (% rel int), 194 [M^+] (50), 161 (32), 136 (100), 123 (20), 107 (20).

2,2-Dimethyl-chroman-3,6-diol (**10**) was obtained as transparent crystalline solid (13 mg). M.p. 175°C; $[\alpha]^{23}_D$ = -41.0° (*c* 0.25, MeOH), lit. (Tanaka, *et al.*, 1999) $[\alpha]^{23}_D$ = -20.4° (*c* 1.0, MeOH). ^{1}H NMR (300 MHz, $[CD_3]_2CO$) δ 6.56 (1H, d, *J* = 1.8 Hz, H-1), 6.52 (1H, m, H-3), 6.52 (1H, m, H-4), 2.90 (1H, dd, *J* = 4.6, 16.8 Hz, H-7), 2.63 (1H, dd, *J* = 8.4, 16.8 Hz, H-7), 3.73 (1H, d, *J* = 8.4 Hz, H-8), 1.18 (3H, s, H_3-10), 1.30 (3H, s, H_3-10), 4.10 (1H, d, *J* = 4.7, Hz, OH-8), 7.70 (1H, s, OH-2), ^{13}C NMR (75.5 MHz, $[CD_3]_2CO$) δ 116.1 (d, C-1), 147.0 (s, C-2), 117.1 (d, C-3), 115.1 (d, C-4), 151.4 (s, C-5), 121.1 (s, C-6), 32.4 (t, C-7), 70.1 (d, C-8), 77.1 (s, C-9), 20.1 (q, CH_3) 26.1 (q, CH_3); EIMS *m/z* (% rel int), 194 [M^+] (100), 161 (35), 136 (35), 123 (100), 71 (60).

2-(3-Dihydroxy-3-methylbutyl)-benzene-1,4-diol (**11**) was obtained as light brown oil (25 mg). $[\alpha]^{23}_D$ = +15.8° (*c* 0.15, MeOH); lit. (Tanaka, *et al.*, 1999) $[\alpha]^{23}_D$ = +36.0° (*c* 0.2, MeOH). ^{1}H NMR (300 MHz, $[CD_3]_2CO$), δ 6.56 (1H, m, H-2), 6.56 (1H, m, H-2), 6.49 (1H, dd, *J* = 2.9, 0.3 Hz, H-5), 2.58 (1H, dd, *J* = 9.9, 14.0 Hz, H-7), 2.71 (1H, dd, *J* = 1.9, 14.0 Hz, H-7), 3.55 (1H, ddd, *J* = 1.9, 9.9, 14.0 Hz, H-8), 1.18 (3H, s, H_3-10), 1.18 (3H, s, H_3-10), 4.60 (1H, brs, OH-9), 7.65 (1H, brs, OH-4), 8.20 (1H, brs, OH-1), 4,79 (1H, brs, OH-8); ^{13}C NMR (75.5 MHz, $[CD_3]_2CO$) δ 148.8 (s, C-1), 117.0 (d, C-2), 116.5 (d, C-3), 150.3 (s, C-4), 113.7 (d, C-5), 128.0 (s, C-6), 33.6 (t, C-7), 80.4 (d, C-8), 72.0 (s, C-9), 24.6 (q, CH_3-10), 24.5 (q, CH_3-11).

4.3 Two new xanthone derivatives from the algicolous marine fungus *Wardomyces anomalus*

Abstract

A marine fungal isolate, identified as *Wardomyces anomalus*, was cultivated and found to produce two new xanthone derivatives, 2,3,6,8-tetrahydroxy-1-methylxanthone (**12**), and 2,3,4,6,8-pentahydroxy-1-methylxanthone (**13**), in addition to the known xanthone derivative 3,6,8-trihydroxy-1-methylxanthone (**14**), and the known fungal metabolite, 5-(hydroxyl-methyl)-2-furanocarboxylic acid (**15**). The structures of all compounds were determined based on extensive spectroscopic measurements (1D and 2D NMR, MS, UV, and IR). Compounds **12** and **14** showed significant antioxidant activities. The total extract and **12**, **14** and **15** were shown to be inhibitors of $p56^{lck}$ tyrosine kinase.

Introduction

Xanthone derivatives are widespread in nature, commonly occurring in a number of higher plant families, and fungi (Peres *et al.*, 2000; Morel *et al.*, 2000). Some fungal species are well known as sources of xanthone derivatives e.g., *Penicillium raistrickii* (Belofsky *et al.*, 1998), *Phomopsis* sp. (Isaka *et al.*, 2001), *Actinoplanes* sp. (Chu *et al.*, *1997*), *Ascodesmis sphaerospora* (Hein *et al.*, 1998), and *Humicola* sp. (Tabata *et al.*, 1996). Xanthones are known to have a variety of biological activities e.g., antimicrobial, antitubercular, antitumor, and antiviral (Peres *et al.*, 2000; Morel *et al.*, 2000).

In continuation of our projects aimed at finding new natural products with biological activity and/or novel chemical structures from marine-derived fungi (Abdel-Lateff *et al.*, 2002), *Wardomyces anomalus* Brooks & Hansford, isolated from the green alga *Enteromorpha* sp. collected around Fehmarn island in the Baltic Sea, was investigated. The crude extract showed antimicrobial effects towards *Microbotryum violaceum* and *Eurotium repens,* inhibition of HIV-1 reverse transcriptase (HIV-1-RT) (65.4 % at 66 µg/mL), and inhibition of $p56^{lck}$ tyrosine kinase (TK) (98 % at 200 µg/mL). Radical scavenging activity was observed using α,α-diphenyl-β-picrylhydrazyl (DPPH) as indicator (71.9 % at 500 µg/mL). An extensive computer survey indicated there to be no prior investigation of *W. anomalus*. On this basis, the fungus was further investigated in order to identify the natural products responsible for this activity.

Materials and methods

Isolation and taxonomy of the fungal strain

After sterilization with 70 % ethanol, algal (*Enteromorpha* sp., Ulvaceae, OS4T) samples were rinsed with sterile water and pressed onto agar plates to detect any residual fungal spores on the surface of algae. Sterilized algae were then cut into pieces and placed on agar plates containing isolation medium: 15 g/L agar, and 1 L seawater from the sample collecting site, plus antibiotics benzyl penicillin and streptomycin sulfate (250 mg/L). Fungal colonies growing out of the algal tissue were transferred to medium for sporulation (1.0 g glucose, 0.1 g yeast extract, 0.5 g peptone from meat, enzymatic digest, 15 g of agar, and 1 L artificial sea water, pH 8) in order to enable taxonomy of the isolates.

Cultivation

The Fungus *W. anomalus* (Microascaceae, Ascomycetes, OS4T-3-2-1, 406), was cultivated on 100 mL of media, composed of 50 g biomalt, 15 g agar, and 1 L ASW.

Biological activity, see chapter 3.4.

Extraction and isolation

Cultivated *W. anomalus* mycelia and medium were diluted with water (100 mL/L) and homogenized using an Ultra Turrax model T25 at 8000 min^{-1}. The resulting mixture was exhaustively extracted with EtOAc (3 x 0.5 L) to yield 66 mg of highly viscous brownish black oil. The EtOAc extract was fractionated over a Bakerbond SPE column (octadecyl C-18, 40 µm, APD, 60 Å, J. T. Baker, USA), gradient elution H_2O to MeOH, and three fractions were collected, 20 mL of each: Fraction 1 (16.4 mg, 160.4 mg/L) was identified as **15**. Fraction 2 (20 mg) was subjected to HPLC (Eurospher-100 C-18, 5 µm, 250 x 8 mm ID, Knauer), eluted with H_2O:MeOH 8:2, and yielded **13** (1 mg, 10 mg/L). Fraction 3 (26.6 mg) was subjected to reversed (RP-18) phase HPLC, eluted with H_2O:MeOH 4:6, and yielded **12** (6 mg, 60 mg/L), and **14** (2 mg, 20 mg/L).

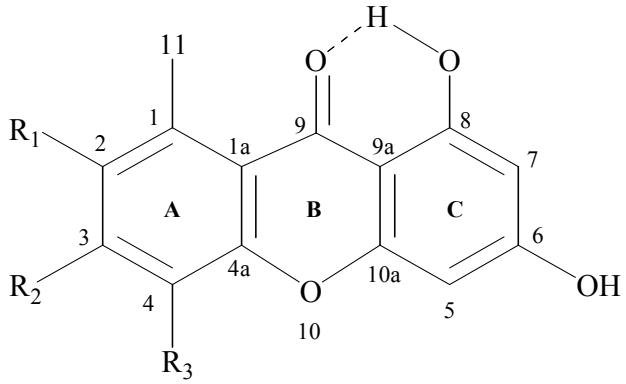
Results and discussion

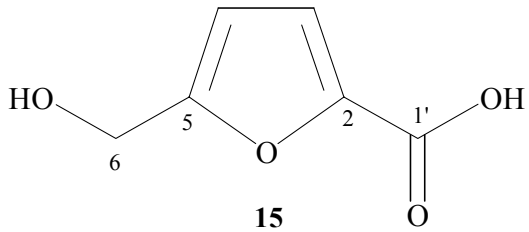
Compound **12** has the molecular formula $C_{14}H_{10}O_6$ as established by high resolution mass measurement. The ^{13}C NMR spectrum of **12** contained 14 carbon resonances attributable to 10 x C, 3 x CH, and 1 x CH_3 groups (Table 11). It was also evident from these data that seven of the ten elements of unsaturation within **12** were present as C=C double bonds, and a carbonyl group (this deduction being supported by an IR absorption at ν_{max} 1646 cm^{-1}), the molecule was thus tricyclic. As the ^{13}C and ^{1}H NMR spectral data enabled all but four of the hydrogen atoms within **12** to be accounted for, it was evident that the remaining four must be present as OH groups, a deduction that was supported by IR data (ν_{max} 3275 cm^{-1}). UV maxima at 234, 258, 311, 358 nm suggested a xanthone nucleus (Morel *et al.*, 2000). The ^{1}H NMR spectral data revealed the presence of three aromatic protons, two of them (H-5 and H-7) with a meta coupling, the third (H-4) one was a singlet, an aryl methyl group (11-CH_3), and a hydrogen-bonded phenolic OH (8-OH). After association of all protons with directly bonded carbons via a 2D NMR (HMQC) spectral measurement, it was possible to deduce the substitution pattern of the xanthone nucleus from HMBC correlations. Thus, HMBC correlations were seen between H-5 and C-6, C-7, C-9 (weak), C-9a, and C-10a, between H-7 and C-5, C-6, C-9 (weak) and C-9a, between H-4 and C-1 (weak), C-1a, C-2, C-3, C-4a, and C-9 (weak), and also between H_3-11, and C-1, C-1a, C-2, C-4a (weak), and C-9 (weak). The

HMBC correlations associated with H_3-11 clearly showed it to reside at C-1. As the 8-OH proton formed a strong hydrogen bond with oxygen, it was likely that this occurred with the oxygen of the C-9 carbonyl group. From HMBC correlations as described previously, it was evident that the C-6 resonated at δ 165.1, and thus was hydroxylated. The ^{13}C NMR chemical shifts of all of the A-ring carbons were then found to be most consistent with the remaining OH groups to be positioned at C-2 and C-3. Further analysis of the ^{1}H and ^{13}C NMR spectral data showed resonances at δ 6.78, and δ 100.9 which supported the location of the remaining aromatic proton at C-4. Compound **12**, for which the trivial name anomalin A is suggested, is a new xanthone derivative that is a structural isomer of **16**, a compound from *Penicillium raistrickii* (Belofsky *et al.*, 1998).

The molecular formula of compound **13** was shown to be $C_{14}H_{10}O_7$ based on HRMS. Comparison of ^{1}H NMR and MS data with those of **12**, revealed **13** to contain a phenolic group more than **12**. This hydroxyl group replaced the aromatic proton H-4, a deduction supported by ^{1}H NMR spectral data which showed the absence of the signal at 6.78 ppm, s, (H-4 in **12**). Compound **13**, for which the trivial name anomalin B is suggested, is a new xanthone derivative.

3,6,8-Trihydroxy-1-methylxanthone (**14**) was identified by complet spectroscopic measuring of 1D and 2D NMR, MS, UV, and IR. Eventually, it comfirmed by comparison with the published data (Harris *et al.*, 1976). 5-(Hydroxymethyl)-2-furanocarboxylic acid (**15**) was identified by comparison of the spectroscopic data with published values (Turner *et al.*, 1977).





15

12 $R_1 = R_2 = OH$, $R_3 = H$
13 $R_1 = R_2 = R_3 = OH$
14 $R_1 = R_3 = H$, $R_2 = OH$
16 $R_1 = H$, $R_2 = R_3 = OH$

Table 11. ^{1}H and ^{13}C NMR spectral data in acetone-d_6 at 300/75.5 MHz for compounds **12-14**[a]

	12			**13**		**14**	
No.	δ C[b]	δ H[c]	HMBC[d]	δ C[f]	δ H[c]	δ C[b]	δ H[c]
1	125.6 (s)			111.9		144.4 (s)	
1a	112.5 (s)			118.0		113.0 (s)	
2	141.8 (s)			142.8		116.9 (d)	6.70 br s
3	152.7 (s)			n[g]		158.1 (s)	
4	100.9 (d)	6.78 s	C-1 (w)[h], C-1a, C-2, C-3, C-4a, C-9 (w)	n[g]		101.5 (d)	6.71 br s
4a	153.5 (s)			n[g]		160.3 (s)	
5	93.6 (d)	6.26 d (2.2)	C-6, C-7, C-9 (w), C-9a, C-10a	93.9	6.31 d (2.1)	94.0 (d)	6.31 d (1.8)
6	165.1 (s)			165.5		164.9 (s)[e]	
7	98.4 (d)	6.15 d (2.2)	C-5, C-6, C-9 (w), C-9a	98.6	6.16 d (2.1)	98.7 (d)	6.17 d (1.8)
8	164.8 (s)			165.6		163.6 (s)[e]	
9	183.5 (s)			183.0		183.0 (s)	
9a	103.8 (s)			104.9		103.8 (s)	
10a	158.0 (s)			158.4		165.5 (s)[e]	
11	13.9 (q)	2.76 s	C-1, C-1a, C-2, C-3 (w), C-4 (w), C-4a (w), C-9 (w)	13.3	2.70 s	23.4 (q)	2.77 s
2-OH		8.97 br s			7.75 s[e]		
3-OH		8.97 br s			8.80 s[e]		9.79 br s[e]
4-OH					9.20 s[e]		
6-OH		8.97 br s			9.54 s[e]		9.71 br s[e]
8-OH		13.58 s	C-8		13.62 s		13.44 s

[a]All assignments are based on 1D and 2D measurements (HMBC, HMQC). [b]Multiplicities were determined by DEPT. [c]δ/ppm, *J* in Hz. [d]Correlations were reported from proton resonances to carbon resonance. [e]Tentative assignment. [f]Data were obtained indirectly from a HMBC spectrum. [g]Not observed in HMBC spectrum. [h]w =weak signal.

The antioxidative properties of all compounds, except **13**, were assessed using DPPH radical and TBARS assays (see material and methods). The results of these assays are shown in Table 12. Compounds **12** and **15** have significant DPPH radical scavenging effects (94.7 and 30.7 %, respectively, at 25.0 µg/mL), and are also able to inhibit peroxidation of linolenic acid (17.0 and 2.6 %, respectively, at 7.4 µg/mL). ELISA based bioassays with HIV-1-RT and TK $p56^{lck}$ allowed determination of enzyme inhibitory activities. The total extract and **12**, **13** and **15** had significant TK $p56^{lck}$ enzyme inhibitor activity (100% enzyme inhibition at 200 µg/mL) (Table 13). Finally, only minor antimicrobial activity was observed for **12**, **14**, and **15** in agar diffusion assays.

Table 12. Antioxidative effects (TBARS Assay)[a] and DPPH[b] radical scavenging effects of the total extract and compounds **12**, **14** and **15**.

	% of Inhibition[c]		% Scavenging effect of DPPH[f]			
Comp. No.	7.4[h]	37.0	25	50	100	500
Extract	-29.2	-50.2	7.8	11.3	20.2	71.9
12	17.0	37.0	94.7	94.8	95.2	95.4
14	-7.4	-11.1	6.2	12.9	25.3	90.6
15	2.6	7.4	30.7	53.4	87.7	97.0
BHT[d]	42.8	48.5	73.4	83.1	89.1	87.8

[a]TBARS = Thiobarbituric acid reactive substances.

[b]DPPH = α,α -Diphenyl-β-picrylhydrazyl

[c]% Inhibition = 100-(A sample[e] - A sample blank[e]) x 100 / (A control - A blank).

[d]BHT = Butylated hydroxytoluene. [e]A = Absorbance of test blank and control solutions are measured at both 532 and 600 nm.

[f]Scavenging % = 100-(A sample[g] x 100 / A control[g]).

[g]Absorbance of sample and control measured at 517 nm.

[h]Concentration in µg/mL

Table 13. Activity of the extract and compounds towards HIV-1 reverse transcriptase, tyrosine kinase p56^{lck} and antimicrobial activity.

Assay	Extract	**12**	**14**	**15**
Tyrosine kinase[a,c,d]	2	0	0	0
HIV-1-RT [a,b,d]	35.6	82.2	82.9	98.3
Bacteria[e]	not active	not active	1mm for *B.m.*	not active
Fungi[f]	3 mm[g] for *M.v.* 2 mm for *E.r.*	1 mm for *M.v.*	1 mm for *E.r.*	not active
Chlorella fusca	1 mm	not active	not active	not active

[a]Percentage of enzyme activity observed relative to negative control (100% HIV-1-RT or TK p56^{lck}).
[b]Reduction of enzyme activity to 80 % or less was regarded as a significant inhibition. Foscarnet (10 μM, rest activity of HIV-1-RT 13%) was used as a positive control.
[c]Reduction of enzyme activity to 40 % or less was regarded as a significant inhibition. Piceatannol (3 mM, rest activity of 2%) was used as a positive control.
[d]Sample concentration for HIV-1-RT test 66 μg/ml, and for TK p56^{lck} test 200 μg/mL.
[e]Tested against *Escherichia coli* (*E. c.*) and *Bacillus megaterium* (*B. m*).[h]
[f]Tested against *Microbotryum violaceum* (*M. v.*), *Eurotium repens* (*E. r.*), *Mycotypha microspora* (*M.m.*).[h]
[g]The radii of the resultant zone of inhibition was measured from the edge of the filter disk.
[h]The concentration of the extract and pure compound is 250 and 50 μg/disk respectively.

Anomalin A (**12**), 2,3,6,8-tetrahydroxy-1-methylxanthone was obtained as yellowish brown amorphous powder (6 mg). UV λ_{max} (MeOH) (log ε) 234 (4.86), 258 (4.86), 311 (4.72), 358 (4.50) nm; IR (film) 3275, 2838, 1646, 1456, 1292 cm^{-1}, ^{1}H and ^{13}C NMR spectral data (Table 11). EIMS *m/z* (% rel int) 274 [M^{+}] (100), 245 (28), 217 (7), 137 (7), 69 (5), accurate mass *m/z* 274.0465, calculated for $C_{14}H_{10}O_6$ 274.04774.

Anomalin B (**13**), 2,3,4,6,8-pentahydroxy-1-methylxanthone, was obtained as yellowish brown amorphous powder (1 mg). UV λ_{max} (MeOH) (log ε) 258 (4.70), 327 (4.48), 370 (sh), nm; IR (film) 3260, 2925, 1647, 1507, 1275 cm^{-1}, ^{1}H NMR spectral data (Table 11). EIMS *m/*z (% rel int) 290 [M^{+}] (30), 248 (25), 190 (100), 161 (35), accurate mass *m/z* 290.0422, calculated for $C_{14}H_{10}O_7$ 290.0426.

3,6,8-Trihydroxy-1-methylxanthone (**14**) was obtained as yellowish brown amorphous powder (2 mg). Lit. (Harris *et al.*, 1976), UV λ_{max} (MeOH) (log ε) 238 (4.72), 310 (4.60) nm; IR (film) 3260, 2925, 1647, 1507, 1275 cm^{-1}; ^1H NMR (300 MHz, $[CD_3]_2CO$), δ 6.71 (1H, br s, H-4), δ 6.70 (1H, br s, H-2), 6.31 (1H, d, *J* = 1.8, H-5), 6.171 (1H, d, *J* = 1.8, H-7), 2.77 (3H, s, H_3-11), 13.44 (1H, s, OH-8); 9.79 (1H, br s, OH-3), 9.71 (1H, br s, OH-6), 9.79 (1H, d, *J* = 1.8, OH-3); ^{13}C NMR (75.5 MHz, $(CD_3)_2CO$) δ 183.0 (s, C-9), 165.5 (s, C-10a), 164.9 (s, C-6), 163.6 (s, C-8), 160.3 (s, C-4a), 158.1 (s, C-3), 144.4 (s, C-1), 116.0 (d, C-2), 113.0 (s, C-1a), 103.8 (s, C-1a), 101.5 (d, C-4), 98.7 (d, C-7), 94.0 (d, C-5), 23.4 (q, C-11). EIMS *m/z* (% rel int) 258 $[M^+]$ (100), 229 (10), 69 (5), accurate mass *m/z* 258.0524, calculated for $C_{14}H_{10}O_5$ 258.0528.

5-(Hydroxymethyl)-2-furanocarboxylic acid (**15**) was obtained as brown oil (16.4 mg). Lit. (Turner, 1977), ^1H NMR (300 MHz, $[CD_3]_2CO$), δ 7.12 (1H, d, *J* = 3.5, H-4), δ 6.44 (1H, d, *J* = 3.5, H-3), 4.52 (2H, s, H-6); ^{13}C NMR (75.5 MHz, $[CD_3]_2CO$) δ 162.5 (s, C-1`), 160.6 (s, C-2), 145.9 (d, C-5), 119.5 (d, C-4), 110.2 (s, C-3), 57.8 (t, C-6). EIMS *m/z* 142, calculated for $C_6H_6O_4$ 142.0266.

4.4 A new antioxidant isobenzofuranone derivative from the algicolous marine fungus *Epicoccum* sp.

Abstract

The fungus *Epicoccum* sp., was isolated from the marine brown alga *Fucus vesiculosus*. After cultivation the fungus was investigated for its secondary metabolite content, and found to contain the new natural product 4,5,6-trihydroxy-7-methylphthalide (epicoccone, **17**), together with 5-(acetoxymethyl)-2-furanocarboxylic acid (**18**), 2-furanocarboxylic acid (**19**), 5-(hydroxymethyl)-2-furanocarboxylic acid (**15**), (-)-(3*R*,4*S*)-4-hydroxymellein (**20**), and (-)-(3*R*)-5-hydroxymellein (**21**). The structures of all compounds were determined by interpretation of their spectroscopic data (1D and 2D NMR, MS, UV, optical rotation and IR). Each isolate was tested for its antioxidative properties, and compound **17** was found to be potently active.

Introduction

Fungi of the genus *Epicoccum* are commonly associated with beach wrack material (Kohlmeyer *et al.*, 1979). Only a few members of this genus of trestrial origin have been studied for their secondary metabolite content e.g. epicorazines A and B (Baute *et al.*, 1976, 1978), triornicin (Frederick *et al.,* 1981), orevactaene (Shu *et al.*, 1997), all being produced by *E. nigrum* (= *E. purpurascens*) and the ability of *Epicoccum* sp. to produce biologically active secondary metabolites is not yet investigated.

During our investigation dealing with isolation and structure elcidation of novel bioactive secondary metabolites of fungi associated with algae, the obligate marine fungus *Epicoccum* sp. was isolated from the brown alga *Fucus vesiculosus* L., collected at the German coast (Tönning, North Sea). After successive fractionation of the EtOAc extract yielded a new potent isobenzofuranone (phthalide) derivative. Isobenzofuranone (phthalide) derivatives like convolvulanic acid A and B (Tsantrizos *et al.*, 1992), and 3-butyl-7-hydroxyphthalide (Makino *et al.*, 1998), were formerly isolated from fungi. This group of natural products is, however also known from higher plants, e.g. 3-ethyl-7-hydroxy phthalide (Kameoka *et al.*, 1975).

Materials and methods

Isolation and taxonomy of the fungal strain

Algal material was collected from the North Sea coast (Tönning, Germany), and identified as *Fucus vesiculosus* L. (Fucaceae, N 1). After sterilisation with 70 % ethanol, algal samples were rinsed with sterile water and pressed onto agar plates to detect any residual fungal spores on their surface. Sterilised algae were then cut into pieces and placed on agar plates containing isolation medium: 15 g/L agar, 1 L seawater from the sample collecting site, benzyl penicillin and streptomycin sulphate (250 mg/L). Fungal colonies growing out of the algal tissue were transferred onto medium for sporulation: 1.0 g/L glucose, 0.1 g/L yeast extract, 0.5 g/L peptone from meat, enzymatic digest, 15 g/L agar, 1 L artificial sea water, the pH was adjusted to 8 in order to enable taxonomy of the isolates.

Cultivation

The fungus *Epicoccum* sp. (Hyphomycetes, Tuberculaiaceae, N 1-12, 353) was cultivated at room temperature for two months in 5 L (20 Fernbach flasks) of solid biomalt agar containing

50 g/L biomalt, 15 g/L agar in ASW. Each Fernbach flask was inoculated with 10 mL of a 10-day-old culture (room temperature) grown in biomalt media without agar.

Biological activity, see chapter 3.4.

Extraction and isolation

Mycelia and medium of the cultivated fungus were diluted with water (100 mL/L), homogenized using an Ika Ultra-turax model T25 at 8000 rpm for 2 min, the resulting mixture exhaustively extracted with EtOAc (3 x 8 L) and filtered. The filtrate was evaporated under reduced prssure to yield 1.7 g of a highly viscous dark redish gum. This extract was fractionated by VLC (2.5 x 20 cm, RP-18, 30 g) employing gradient elution from H_2O to MeOH, to yield 6 fractions (H_2O:MeOH, 1:0; 8:2; 6:4; 4:6; 2:8; 0:1, each 100 mL). ^{1}H NMR identified fractions 1, 4 and 5 as promising for further fractionation. VLC fraction 1 (0.286 g) was fractionated on a Sep Pack column (RP-18) using gradient elution from H_2O to MeOH, to yield 6 fractions (H_2O:MeOH, 1:0; 8:2; 6:4; 4:6; 2:8; 0:1, each 20 mL). SPE fraction 1 was further fractionated by RP-18 HPLC (Eurospher-100, 5 µm, 250 x 8 mm, Knauer), employing gradient elution from H_2O to H_2O:MeOH (1:1) in 30 min, 2 mL/min, to yield **17** (semi-pure), **18**, and a mixture of **19** and **15** (t_R = 27 min, 2.0 mg/L; t_R = 12 min, 0.8 mg/L; t_R = 6 min, 1 mg/L, respectively). Compound **17** was finally purified by gradient elution with 4:1 H_2O:MeOH to 3:7 H_2O:MeOH in 25 min, 2 mL/min. VLC fraction 4 and 5 were pooled, based on similarities of ^{1}H NMR spectral data, and further fractionated by SPE (RP-18), employing gradient elution from H_2O to MeOH, to yield 8 fractions (H_2O:MeOH, 1:0; 8.5:1.5; 7:3; 5.5:4.5; 4:6; 2.5:7.5; 1:9; 0:1, each 20 mL), which were combined, based on their ^{1}H NMR spectral data, into 4 pools. Of these, pool 4 was purified using RP-18 (Eurospher-100, 5 µm, 250 x 8 mm, Knauer) HPLC, and gradient elution from 7:3 H_2O:MeOH to 4:6 H_2O:MeOH in 30 min, 2 mL/min, to yield **20** (t_R = 23 min, 0.3 mg/L) and **21** (t_R = 27 min, 0.3 mg/L).

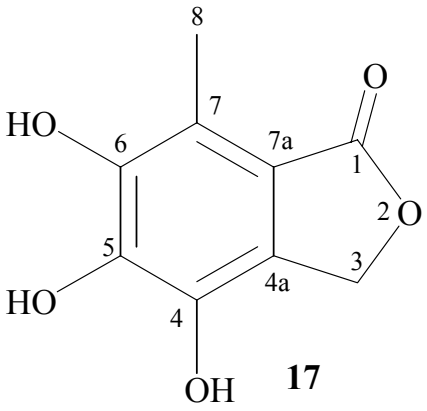
GC-MS analysis of 19 and 15

TMS derivatives were made by dissolving 1 mg of the compound mixture (**19** and **15**) in 100 µL of TFA. Then 0.9 mL of MSTFA (N-methyl-N-trimethylsilyl-trifluroacetamid; WGA GmbH 370520) was added dropwise, and the reaction mixture was left at room temperature for 1 hour. The GC-MS analysis was carried out on a A 30 m x 0.32 mm N931-6023 Pe-1 (film thickness of 0.25 µm) capillary column. The instrument was set to an initial temperature of 50 °C, and maintained at this temperature for 10 min. At the end of this period the oven was heated at 10°/min up to 250°C, and kept there for 10 min. Injection port temperature was

220°C, He flow rate was 2 mL/min, and samples were injected in splitless mode. Mass spectral scan range was 35-650 Da. The chromatogram of the mixture contained two peaks, A and B. Retention times, relative intensities (%, B being 100 %) and EIMS data of the relevant peaks were; Peak A (**19**), 14.8 min, 30, EIMS *m/z* (% rel int), 184 [M^++TMS] (10), 169 (40), 125 (100); Peak B (**15**), 21.3 min, 100, EIMS *m/z* (% rel int), 286 [M^++2xTMS] (<1), 271 (90), 170 (5), 147 (100), 123 (70), 73 (80).

Results and discussion

In the present study the isolation and structure elucidation and biological activity of secondary metabolites obtained from *Epicoccum* sp. (Hyphomycetes, Tuberculaiaceae) cultivated on a solid biomalt medium with added artificial sea salt. Successive fractionation of the EtOAc extract by vacuum liquid chromatography (VLC) over reversed phase silica (RP-18) followed by reversed phase (RP-18) HPLC yielded compounds **17**-**21**, all of which are highly oxygenated.



17

Negative mode electrospray ionisation mass spectrometric (ESIMS) and ^{1}H and ^{13}C NMR analyses of **17** showed it to have the molecular formula $C_9H_8O_5$. In the ^{13}C NMR spectrum of **17** nine resonances attributable to one methyl group, one methylene group, and seven quarternary carbons (Table 14), were evident. It was also clear from these data that four of the six elements of unsaturation within **17** were present as multiple bonds (3 x C=C) and a carbonyl group, a deduction supported by the IR absorption at 1681 cm^{-1}; the molecule was thus bicyclic. As the ^{1}H and ^{13}C NMR data enabled all but three of the hydrogen atoms within **17** to be accounted for, it was evident that the remaining three must be present as parts of OH groups, a deduction that was also supported by the IR data (ν_{max} 3333 cm^{-1}). Also evident from the ^{1}H and ^{13}C NMR data of **17** were resonances consistent with the presence of a fully substituted aromatic ring, a conclusion supported by the UV maximum 273 nm, an allylic methyl, and a benzylic methylene group. After association of all ^{1}H and ^{13}C NMR resonances from cross peaks seen in the ^{1}H-^{13}C 2D NMR shift-correlated (HMQC) spectrum of **17** interpretation of its HMBC spectral data led directly to the structure of **17**. The HMBC spectrum of **17** contained cross peaks between the resonance of H_3-8 and those of C-1, C-5, C-6, C-7 and C-7a, and between H_2-3 and C-1, C-4, C-4a, C-5, and C-7a. These data enabled CH_3-8 to be positioned at C-7 and the carbonyl group of the furanone at C-7a, leaving C-3 to bond with C-4a. X-ray crystallographic analysis

of **17** confirmed these deductions. The quality of the X-ray data was, however, not of publishable quality due to fact that the crystallisation solvent, MeOH, was disordered throughout the crystal. For **17**, a new phthalide derivative, the trivial name epicoccone is proposed.

Table 14. ^{1}H [CD_3OD, 300 MHz] ,^{13}C NMR [CD_3OD, 75.5 MHz] and HMBC data for compound **17**[a]

Position	δ ^{13}C	δ ^{1}H	HMBC
1	174.6 (s)[b]		
3	68.0 (t)	5.16 (2H, brs)	C-1, C-4, C-4a, C-5, C-7a
4	138.1 (s)		
4a	127.9 (s)		
5	141.5 (s)		
6	146.3 (s)		
7	118.3 (s)		
7a	113.9 (s)		
8	9.7 (q)	2.45 (3H, s)	C-1, C-5, C-6, C-7, C-7a

[a]All assignments are based on 1D and 2D measurements (HMBC, HMQC).
[b]Implied multiplicities were determined by DEPT (C = s, CH = d, CH_2 = t, CH_3 = q).

Together with the new compound **17**, 5-(acetoxymethyl)-2-furanocarboxylic acid (**18**)**,** 2-furanocarboxylic acid (**19**), and 5-(hydroxymethyl)-2-furanocarboxylic acid (**15**), (-)-(3*R*,4*S*)-4-Hydroxymellein (**20**), and (-)-(3*R*)-5-Hydroxymellein (**21**) were also isolated from the EtOAc extract of *Epicoccum* sp. They were identified by comparison of their ^{1}H and ^{13}C NMR data and optical rotations with published values (Jadulco *et al.*, 2001; Teleman *et al.*, 1996; Devys *et al.*, 1994 and 1992). The 5-(acetoxymethyl)-2-furanocarboxylic acid (**18**) was first isolated from a culture of *Cladosporium herbarum*, a fungal isolated from marine sponge. The Sumiki's acid (**15**) and 2-furanocarboxylic acid (**19**) are known fungal metabolites originally isolated from *Penicillium italicum* and Pyricularia grisea (Turner *et al.*, 1989). The remaining two compounds (**20** and **21**) are mellein derivative, well known fungal metabolites. The (-)-(3*R*,4*S*)-4-hydroxymellein (**20**) and (-)-(3*R*)-5-hydroxymellein (**21**) were isolated from *Septoria nodorum* Berk (Phaeosphaeria), a common wheat parasite (Devys *et al.*, 1992 and 1994).

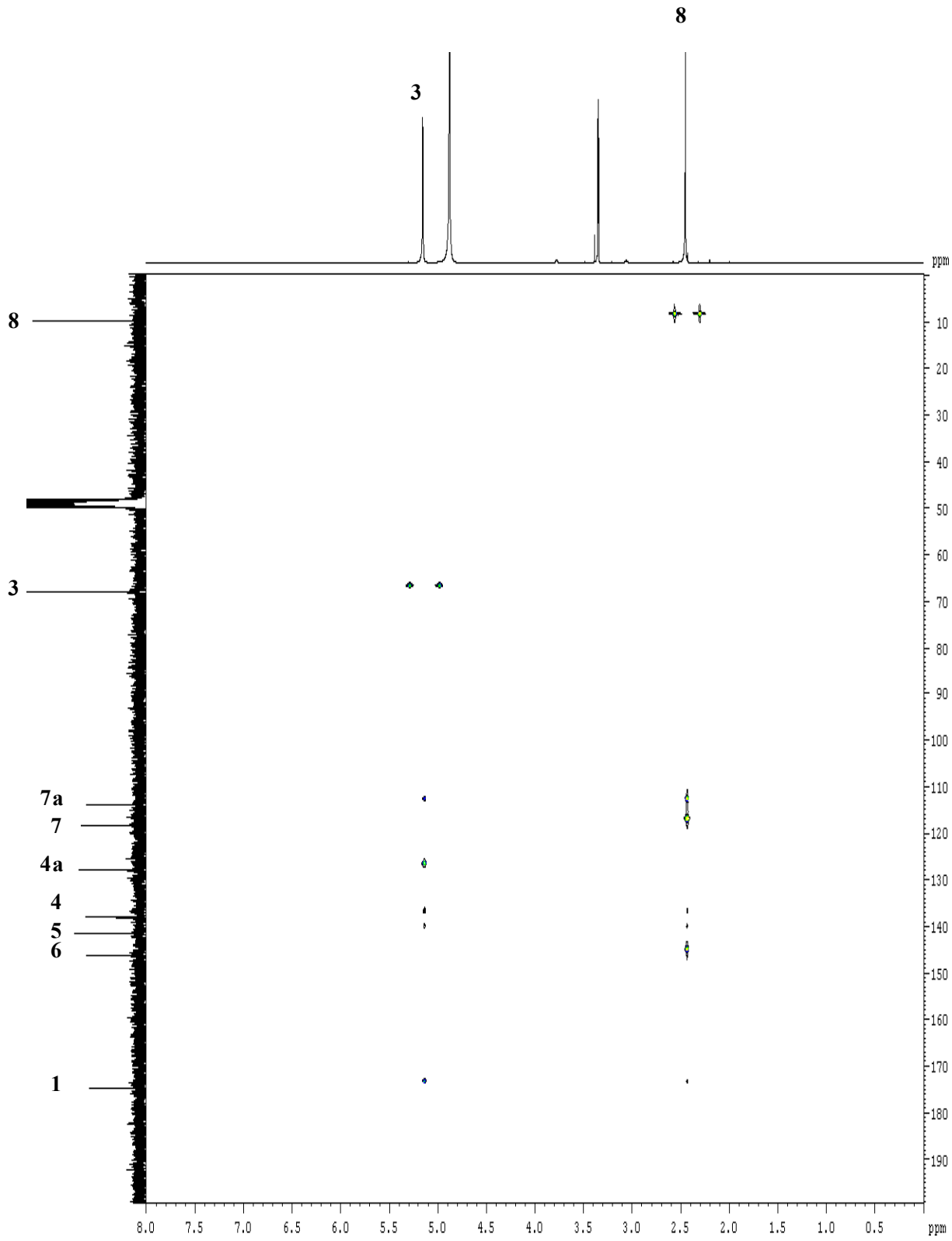
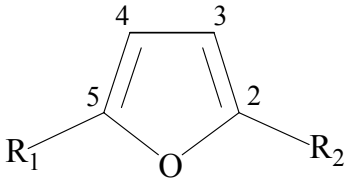
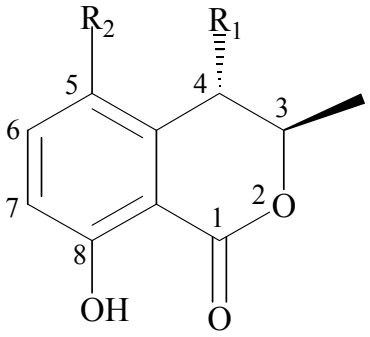


Figure 9. HMBC NMR spectrum of epicoccone (**17**), ^{1}H [CD_3OD, 300 MHz] and ^{13}C NMR [CD_3OD, 75.5 MHz].



18 R_1 = COOH, R_2 = CH_2OAc
19 R_1 = H, R_2 = COOH
15 R_1 = CH_2OH, R_2 = COOH



20 R_1 = OH, R_2 = H
21 R_1 = H, R_2 = OH

The antioxidative properties of all compounds were assessed using DPPH radical scavenging and the TBARS assays. The results of these assays are shown in Tables 15 and 16, and reveal the EtOAc extract, **17** and **21** to have significant DPPH radical scavenging effects (16.8, 95.2, and 22.4 %, respectively, at 25.0 µg/mL), with **17**, **20** and **21** also being able to inhibit peroxidation of linolenic acid (62.1, 18.4, and 30.4 %, respectively, at 37.0 µg/mL) in the TBARS assay. In both of the aforementioned assays, **17** still has significant effects at levels between 1-5 µg/mL. The observed antioxidative activities correlate with the number of phenolic hydroxyl groups of the relevant structures. Thus compound **17**, containing three hydroxyl groups showed the most prominent activity, followed by compound **21** with a hydroquinone moiety. Compound **20** with only one phenol group is the least active metabolite.

Table 15. Antioxidative effects (TBARS assay)[a] of the EtOAc extract of *Epicoccum* sp. and compounds **17**-**21**.

	% Inhibition[b]		
Item tested	3.7[e]	7.4	37.0
EtOAc Extract	-0.1	-2.1	0.1
17	21.3	31.7	62.1
18	4.6	-0.4	2.3
19 and **15**	9.1	7.4	14.4
20	9.5	10.7	18.4
21	14.1	15.2	30.4
BHT[c]	54.2	63.8	73.9

[a]TBARS = Thiobarbituric acid method. [b]% Inhibition = 100-(A[d] sample-A sample blank) x 100/(A control - A blank). [c]BHT = Butylated hydroxytoluene. [d]A = Absorbance measured at 532 less the background Absorbande at 600 nm. [e]Concentration in µg/mL.

Table 16. DPPH[a] radical scavenging effects of the EtOAc extract of *Epicoccum* sp. and compounds **17**-**21**

	% Scavenging[b]						
Item tested	1.0[c]	2.5	5.0	10.0	25.0	50.0	100.0
EtOAc Extract	1.8	0.7	2.5	5.5	16.8	28.5	43.4
17	10.9	28.2	46.2	83.7	95.2	94.9	94.7
18	nt[d]	nt	nt	nt	5.9	10.6	22.2
19 and **15**	nt	nt	nt	nt	1.8	1.7	3.1
20	nt	nt	nt	nt	5.7	9.6	15.9
21	nt	nt	nt	nt	22.4	28.4	35.8
BHT[e]	-0.7	1.7	3.3	8.2	18.6	36.1	56.8

[a]DPPH = α,α-Diphenyl-β-picrylhydrazyl. [b]Scavenging % = 100-(A sample[f] x 100/A control[f]). [c]Concentrations in µg/mL. [d]nt = not tested. [e]BHT = Butylated hydroxytoluene. [f]Absorbance of sample and control measured at 517 nm.

Epicoccone (**17**), 4,5,6-trihydroxy-7-methylphthalide was obtained as white transparent crystalline solid (10.0 mg). M. p.: 249-250 °C; UV λ_{max} MeOH (log ε) 217 nm (4.1), 273 nm (3.6); IR (film) ν_{max} 3333, 2359, 1681, 1608, 1520, 1471,1273 cm^{-1}; ^{1}H and ^{13}C NMR data (Table 14); ESIMS *m/z* 195 [M-H].

5-(Acetoxymethyl)-2-furanocarboxylic acid (**18**) was obtained as amorphous powder (4.0 mg). UV λ_{max} MeOH (log ε) 217 nm (4.6), 247 nm (3.7); IR (film) ν_{max} 3252, 1737, 1700, 1573, 1530, 1364, 1231 cm^{-1}; Lit (Jadulco *et al*., 2001), ^{1}H NMR (300 MHz, CD_3OD) δ 7.00 (1H, d, *J* = 2.2 Hz, H-3), 6.55 (1H, d, *J* = 2.2, H-4), 5.10 (2H, s, H_2-6), 2.11 (3H, s, H_3-8); ^{13}C NMR (75.5 MHz CD_3OD) δ 152.6 (s, C-2), 116.2 (d, C-3), 112.9 (d, C-4), 152.56 (s, C-5), 59.2 (t, C-6), 172.2 (s, C-7), 20.6 (q, C-8); EIMS m/z (% rel int), 184 [M^+] (23), 142 (100), 125 (45), 97 (30), 79 (66); HREIMS m/z 184.0370 (calculated for $C_8H_8O_5$ 184.0369).

2-Furanocarboxylic acid (**19**), and 5-(hydroxymethyl)-2-furanocarboxylic acid (**15**) were obtained as an oily mixture (5.0 mg). IR (film) ν_{max} 3251, 1568, 1532, 1482, 1390, 1366, 1213 cm^{-1}; Lit (Jadulco *et al.,* 2001), ^{1}H and ^{13}C NMR data for **15** was mentioned in chapter 4.3. For **19**, Lit (Teleman *et al.*, 1996), ^{1}H NMR (300 MHz, CD_3OD) δ 7.39 (1H, br m, H-5), 6.80 (1H, br m, H-3), 6.35 (1H, br m, H-4); ^{13}C NMR (75 MHz, CD_3OD) δ 157.1 (s, C-1`), 146.3 (s, C-2), 146.0 (d, C-5); 116.9 (d, C-4), 110.8 (d, C-3); EIMS (see GC-MS analysis).

(-)-(3*R*,4*S*)-4-Hydroxymellein (**20**) was obtained as amorphous solid (1.5 mg). $[\alpha]^{23}_{D}$ = -26.3° (*c* 0.05, $CHCl_3$), lit. (Devys *et al.*, 1992). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.60 (2H, brs, H-3 and 4), 6.99 (1H, d, *J* = 7.7, H-5), 7.53 (1H, dd, *J* = 7.7, 6.6 Hz, H-6), 7.02 (1H, d, *J* = 6.6 Hz, H-7); EIMS m/z (% rel int), 194 [M^+] (50), 150 (95), 121 (100), 94 (18), 65 (23). HREIMS *m/z* 194.0578 (calculated for $C_{10}H_{10}O_4$ 194.0578).

(-)-(3*R*)-5-Hydroxymellein (**21**) was obtained as amorphous powder (1.5 mg). $[\alpha]^{23}_{D}$ = -19.8° (*c* 0.05, CH_2Cl_2), lit. (Devys, *et al.*, 1994). $[\alpha]^{23}_{D}$ = -72 (*c* 1650 mg %). ^{1}H NMR (300 MHz, DMSO) δ 4.70 (1H, m, H-3), 3.07 (1H, dd, *J* = 17.2, 2.9 H-4), 2.65 (1H, m, H-4), 10.36 (1H, s, OH-5), 6.72 (1H, d, *J* = 9.15, H-6), 7.06(1H, d, *J* = 9.15, H-7); EIMS m/z (% rel int), 194 [M^+] (50), 176 (35), 165 (20), 149 (50), 121 (20), 111 (4), 97 (50), 121 (20), 83 (70), 69(7), 57 (100). HREIMS *m/z* 194.0578 (calculated for $C_{10}H_{10}O_4$ 194.0576).

4.5 Novel sorbicillinoid derivatives from the marine fungus *Trichoderma viride* associated with the Caribbean sponge *Agelas dispar*

Abstract

The fungus *Trichoderma viride* isolated from the Caribbean sponge *Agelas dispar*, collected on Dominica, was mass cultivated and found to produce four novel sorbicillinoid polyketide derivatives, trichodermanone A-D (**22**-**25**) and the new natural product 6-(4-hydroxy-1-pentenyl)-4-methoxy-3-methyl-2*H*-pyran-2-one (trichopyranone, **26**). In addition to these compounds two known hexaketide derivatives, epoxysorbicillinol (**27**), vertinolide (**28**) and three known dodecaketides trichodimerol (**29**), bislongiquinolide (trichotetronine, **30**), and bisvertinol (**31**), were isolated. Finally, a known fungal metabolite, 2-furancarboxylic acid (**19**) was obtained. The structures of all compounds were determined by interpretation of their spectroscopic data (1D and 2D NMR, MS, UV and IR). Total extract and compounds **27**-**30** have DPPH radical savenging effects (12.5, 43.8, 15.5, 37.2, and 23.8 % respectively at 230 μmol/L), with **27**-**29** being also able to inhibit peroxidation of linolenic acid (17.8, 5.2 and 19.9, respectively at 164 μmol/L). Compound **27** showed moderate HIV-1 reverse transcriptase inhibitory activity (63.8 % at 200 μg/mL). All compounds and the total extract except **19** were tested for their estrogenic, antitumor and antimicrobial effects and no significant activities were observed.

Introduction

The fungal genus *Trichoderma* (Persoon 1821) is known for its ubiquitous occurrence (Andrade *et al.*, 1992). The taxonomical classification of these fungi, however is difficult. In the last decade considerable interest has been shown in the use of *Trichoderma* sp. as biological control (Cooney *et al.*, 1999). *Trichoderma* sp. are noted for their secondary metabolite content, to date around 500 compounds have been reported from fungi belonging to this genus, including koniginin G (Culter *et al.*, 1999), trichocaranes A-D (Macías *et al.*, 2000), pentenocins A and B (Matsumoto *et al.*, 1999).

Vertinoid (sorbicillinoid) polyketides are a growing and diverse class of structurally novel natural products, and are endowed with a broad range of biological activities. They are hexaketide-derived metabolites. Included within this class are more than fourteen compounds with a C_{14} skeleton, and also dimers with C_{28} (Sperry *et al.*, 1998). For instance, sorbicillin was the first member belonging to the simple vertinoids (C_{14}), and is considered as the hypothetical biosynthetic precursor of epoxysorbicillinol, a yellow pigment obtained from *Trichoderma longibrachiatum* (Sperry *et al.*, 1998). The bisorbicillinoids are the dimeric form of sorbicillin-derived compounds such as bisorbibutenolide, trichodimerol, bisorbicillinolide and bisvertinol. This class of metabolites is restricted to a few genera including *Trichoderma*, *Verticillium, Acremonium,* and *Penicillium* (Abe *et al.*, 1999; Andrade *et al.,*1996; Trifonov *et al.,* 1982, 1983, and 1986). The unusual and complex molecular architecture of these compounds motivated researchers to study their biological activities. Some of them exhibit antioxidant properties e.g. bisorbicillinol, bisorbibutenolide and bisorbicillinolide (Abe *et al*., 2000). The oxidized form of bisvertinol represents the first inhibitor of β-6-glucan biosynthesis, thus, it is considered as a potential antifungal agent (Nicolaou *et al.*, 2000). Trichodimerol, originally isolated from *Trichoderma* sp., showed significant inhibitory activity against lipopolysaccharide induced production of tumor necrosis factor alpha (TNF-α) in human monocytes and thus, represents a new lead for the treatment of septic shock (Abe *et al*., 1998 and 1999; Nicolaou *et al.*, 2000).

On this basis, we continued our projects aimed at finding new natural products with biological activity and/or novel chemical structures from marine-derived fungi (Abdel-Lateff *et al*., 2002 and 2003a and b). The current secondary metabolite investigation was undertaken with the marine fungus *Trichoderma viride* (Hypocreaceae, Ascomycota), isolated from the Caribbean sponge *Agelas dispar* J. (order Agelasida, family Agelasidae), collected from waters around the Island of Dominica. The fungus was cultivated on a solid glucose biomalt medium with added

artificial sea salt. Successive fractionation of the EtOAc extract by vacuum liquid chromatography (VLC) over normal phase silica followed by reversed phase (RP-18) HPLC yielded four novel sorbicillinoid polyketide derivatives trichodermanone A-D (**22**-**25**) and a new pyrone derivative (Trichopyranone, **26**). In addition to these compounds two known hexaketide derivatives epoxysorbicillinol (**27**) (Sperry *et al.*, 1998), vertinolide (**28**) (Trifonov *et al.*, 1982), and three known dodecaketides trichodimerol (**29**) (Andrade *et al.*, 1992; Abe *et al.*, 1998) bislongiquinolide (Trichotetronine, **30**) (Sperry *et al.*, 1998), and bisvertinol (**31**) (Trifonov *et al.*, 1986), were isolated. Finally, a known fungal metabolite, 2-furancarboxylic acid (**19**) (Corey *et al.*, 1958), was also obtained.

Materials and methods

Isolation and taxonomy of the fungal strain

The sponge *Agelas dispa* J. was collected using Scuba in 1993 from the waters around the Caribbean Island of Dominica. The sponge was identified by Dr. R. Desqueroux-Faundez, Musee d'Historie Naturelle, Geneve. The fungus was isolated by inoculating small pieces of its inner tissue on cellulose (10 g/L), yeast extract (1 g/L), benzylpenicillin (250 mg/L), streptomycin sulphate (250 mg/L), agar (15 g/L), and ASW (800 mL/L).

Cultivation

The fungus *Trichoderma viride* (Hypocreaceae, Ascomycota, 193E61, 54), was cultivated at room temperature for two months in 5 L (20 Fern Bach flasks) of solid glucose biomalt agar containing biomalt (50 g/L, Villa Natura Gesundheitsprodukte GmbH, Germany), glucose (10 g/L, J. T. Baker), agar (15 g/L, Fluka Chemie AG) in ASW.

Biological activity, see chapter 3. 4.

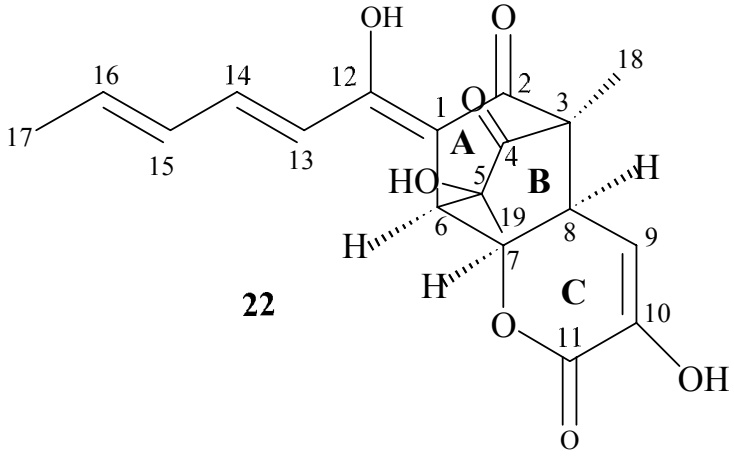
Extraction and isolation

Mycelia and medium were diluted with water (100 mL/L), homogenized using an Ika Ultra-turax T25 at 8000 rpm for 2 min and the resulting mixture exhaustively was extracted with EtOAc (3 x 8 L) and filtered. The filtrate was evaporated under reduced pressure to yield 5 g of a highly viscous yellowish brown gum. The extract was fractionated by Vaccum Liquid Chromatography (VLC) (2.5 x 20 cm, Si gel 60, 120 g, Merck 7739), employing gradient elution from petroleum ether to acetone, to yield 5 fractions (petroleum ether:acetone, 1:0;7.5:

2.5; 5:5; 2.5:7.5; 0:1, each 200 mL). According to the differences in composition detected by ^{1}H NMR spectral data, it was evident that fractions 2 (0.53 g) and 3 (1.2 g) were promising for further fractionation. VLC fraction 3 was fractionated on normal phase silica for TLC (5-40 μm, Merck 7739) by VLC using gradient elution from petroleum ether to EtOAc, to yield 11 fractions (petroleum ether:EtOAc, 1:0; 9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; 1:9; 0:1, each 100 mL). Fractions 3.4-3.7 appeared very promising for further fractionation based on several low field ^{1}H NMR resonances in the δ 6-8 region. Fraction 3.4 was purified by RP-18 HPLC (Eurospher-100, 5 μm, 250 x 8 mm ID, Knauer), employing gradient elution from 9:1 H_2O:MeOH to MeOH in 45 min, 2 mL/min, to yield **19**, **27**, **28**, semi-pure **29**, (t_R = 14 min, 1 mg; t_R = 29 min, 6 mg; t_R = 32 min, 4 mg; t_R = 41 min, 4 mg, respectively). Compound **30** was purified by gradient elution from 1:1 H_2O:MeOH to MeOH in 45 min, 2 mL/min (t_R = 29 min, 4 mg). Fraction 3.5 was purified by RP-18 HPLC (Eurospher-100, 5 μm, 250 x 8 mm ID, Knauer), employing gradient elution from 6:4 H_2O:MeOH to MeOH in 60 min, 2 mL/min, to yield additive amount of **30** (t_R = 39 min, 30 mg). Fraction 3.6 was purified by RP-18 HPLC (Eurospher-100, 5 μm, 250 x 8 mm ID, Knauer), employing gradient elution from 6:4 H_2O:MeOH to MeOH in 60 min, 2 mL/min, to yield five peaks (3.6.3, 3.6.4, 3.6.5, 3.6.6, 3.6.7), (t_R = 15 min, 2.5 mg; t_R = 25 min, 20 mg; t_R = 26 min, 18 mg; t_R = 29 min, 16 mg; t_R = 35 min, 3 mg, respectively). The first peak 3.6.3 was identified as **26**. HPLC peak 3.6.4 was purified using RP-HPLC (XTerra RP-18, 5 μm, 250 x 4.6 mm, Waters), employing gradient elution from 65:35 H_2O:MeOH to MeOH, in 35 min, 1 mL/min, to yield **25** (t_R = 21 min, 10 mg), and **22** (t_R = 25 min, 2 mg). HPLC peak 3.6.5 purified using RP-18 HPLC employing gradient elution from 65:35 H_2O:MeOH to 2:8 H_2O:MeOH, in 35 min, 1 mL/min, to yield compound **24** (t_R = 22 min, 4 mg), and **25** (t_R = 21 min, 1 mg). HPLC peak 3.6.6 was purified using RP-18 HPLC employing gradient elution from 7:3 H_2O:MeOH to 3:7 H_2O:MeOH in 30 min, 1 mL/min, yielded **23** (t_R = 23.5 min, 4 mg). Fraction 3.7 was purified by RP-18 HPLC (Eurospher-100, 5 μm, 250 x 8 mm ID, Knauer), employing gradient elution from 55:45 H_2O:MeOH to MeOH in 60 min, 2 mL/min, to yield additive amount of **25** (t_R = 23 min, 10 mg). VLC fraction 2 (0.56 g) was fractionated by normal phase VLC using gradient elution from petroleum ether to EtOAc to yield 5 fractions (petroleum ether:EtOAc, 1:0; 7.5:2.5; 5:5; 2.5:7.5; 0:1, each 200 mL). Fractions 2.3-2.5 appeared very interesting for further fractionation based on several low field ^{1}H NMR resonances in the δ 6-8 region. Fraction 2.3 was purified by RP-18 HPLC (Eurospher-100, 5 μm, 250 x 8 mm ID, Knauer), employing gradient elution from 5:5 H_2O:MeOH to MeOH in 45 min, 2 mL/min, to yield **29** (t_R = 43 min, 10 mg). Fraction 2.4 was purified by RP-18 HPLC (Eurospher-100, 5 μm, 250 x 8 mm ID, Knauer), employing gradient elution from 7:3 H_2O:MeOH to MeOH in 50 min, 2 mL/min, to yield **23**, **24**, and semi-pure **31** (t_R = 29 min, 2 mg; t_R = 32 min, 2 mg; t_R = 44 min, 5 mg, respectively). Compound **31** was finally purified by gradient elution from 25:75 H_2O:

MeOH to MeOH in 30 min, 2 mL/min (t_R = 14 min, 2 mg). Fraction 2.5 was purified by RP-18 HPLC (Eurospher-100, 5 µm, 250 x 8 mm ID, Knauer), using gradient elution from 6:4 H_2O:MeOH to MeOH in 50 min, 2 mL/min, to yield **28** and **30** (t_R = 22.5 min, 2 mg; 36 min, 5 mg, respectively).

Results and discussion



Negative and positive mode electrospray ionization mass spectrometry (ESIMS), EIMS and ^1H and ^{13}C NMR analysis of compound **22** showed it to have the molecular formula $C_{19}H_{20}O_7$. Its ^{13}C NMR spectra (^1H decoupled and DEPT) showed that seven of the ten elements of unsaturation, as indicated by the molecular formula of **22**, could be attributed to four carbon-carbon double bonds and three carbonyl groups (Table 17); the molecule is thus tricyclic. The ^1H and ^{13}C NMR spectral data established the presence of three sp^3-hyberdized methine groups, five sp^2-hyberdized methine groups, an allylic methyl group, two methyl groups attached to quaternary carbons and eight quaternary carbons (Table 17 and 18). These data also showed that all but three of the hydrogen atoms were bonded directly to carbons, indicating the remaining three to be present as part of hydroxyl functions, a deduction supported by IR data (ν_{max} 3384 cm^{-1}). All ^1H and ^{13}C NMR resonances were assigned using C-H one-bond couplings (HMQC), and from cross peaks seen in the long-range ^1H-^{13}C NMR shift-correlated 2D NMR measurement (HMBC) of **22**. It was possible to deduce the planar structure of **22** by interpretation of its ^1H-^1H COSY and ^1H-^{13}C HMBC spectra. From the ^1H-^1H COSY NMR spectrum of **22**, ^1H-^1H spin couplings between H_3-17 and H-16 and H-15, and correlations between H-13 and H-14 and H-15 were observed. Diagnostic long-range ^1H-^{13}C HMBC correlations between the resonance of H_3–17 to those of C-15 and C-16, between the resonance of H-13 to C-1, C-12 and C-15, and also correlations between H-14 and C-12 and C-16 were evident. These couplings established the C-C bonds from C-1 to C-17, and the presence of a sorbyl side chain (Fragment 1, Figure 10). HMBC long-range correlations observed between the resonances of H-6 to those of C-1, C-2, C-4, and C-5, correlations between H_3-19 and C-4, C-5 and C-6, also between H_3-18 and C-2, and C-4, enabled ring A to be established as shown in

Figure 10. HMBC correlations between H-6 and C-12, and between H-13 and C-1, gave further proof for the sorbyl side chain to be attached to C-1, a deduction also supported by NOESY correlation between H-6 and H-13. The ^{1}H-^{1}H COSY spectrum of **22** showed ^{1}H-^{1}H spin couplings between H-8 and H-7, which further couples with H-6. Long-range C-H correlations, this time observed between the resonances of H-8 to C-2, C-3, C-4 and C-6, between H-7 and both C-1 and C-3, and also correlations observed between H-6 and both C-7 and C-8, indicated C-8 to bond directly to both C-3 and C-7, which irectly bonded to C-6, and thus, ring B within **22**, is completed (Figure 10). From ^{13}C NMR spectral data, it was clear that C-7 (δ 80.9, d) has to be attached to oxygen, and that the carbonyl group C-11 (δ 161.0, s) is part of lactone, a deduction supported by IR data (ν_{max} 1729 cm $^{-1}$). C-9 has to be attached to C-8 because of ^{1}H-^{1}H COSY correlations observed between H-8 and H-9, and HMBC correlations observed between H-9 and C-8. A HMBC couplings between H-9 and C-10 and C-11 enabled the C-C bonds from C-9 to C-11. From these data, ring C was established. Remaining to be incorporated into the planar structure were three hydroxyl functions, which were attached to C-5 (δ 74.5, s), C-10 (δ 151.7, s), and C-12 (δ 170.9, s) as deduced on the basis of ^{13}C NMR chemical shifts. ^{1}H-^{1}H NMR coupling constants for H-8 (10.6, 2.9 Hz) and H-7 (10.6, 3.7 Hz) indicated both to be pseudo-axial and either to have a dihedral angle of 180° or alternatively close to 0°. Diagnostic NOESY correlations between H-8 and H-7 indicated the cis junction between ring B and C. Thus, the relative stereochemistry at C-7 and C-8 has to be 3*S**, 7*S**, respectively. As the proton located at C-6 has a ^{1}H-^{1}H coupling constant of 3.7 Hz it had to be in an equatorial orientation and consequently, the CH_3-18 has an equatorial orientation. As NOE observed between H-6 and H-13 is only possible if H-6 is equatorial and α positioned which also defines CH_3-18 as being α. Own data do not allow to correlate the relative stereochemistry between H-6/CH_3-18 on one side and that of H-7 and H-8 on the other side. The stereochemistry of C-5 could not be solved yet. Diagnostic NOESY correlations between H-15 and H_3–17, and between H-15 and H-13, and between H-16 and H-14, together with typical ^{1}H-^{1}H NMR coupling constants for H-13 (δ 6.55, d, J = 15.0), H-14 (δ 7.29, dd, J = 15.0, 11.0), H-15 (δ 6.44, dd, J = 15.0, 11.0) and H-16 (δ 6.26, dq, J = 15.0, 7.0) established double bonds $\Delta^{13,14}$ and $\Delta^{15,16}$ to have the *E* geometry (Table 17). Thus the relative stereochemistry of the side chain in compound **22** is best described as being 1*Z*, 13*E*, 15*E*. For **22**, a new sorbicillinoid derivative, the trivial name trichodermanone A is proposed.

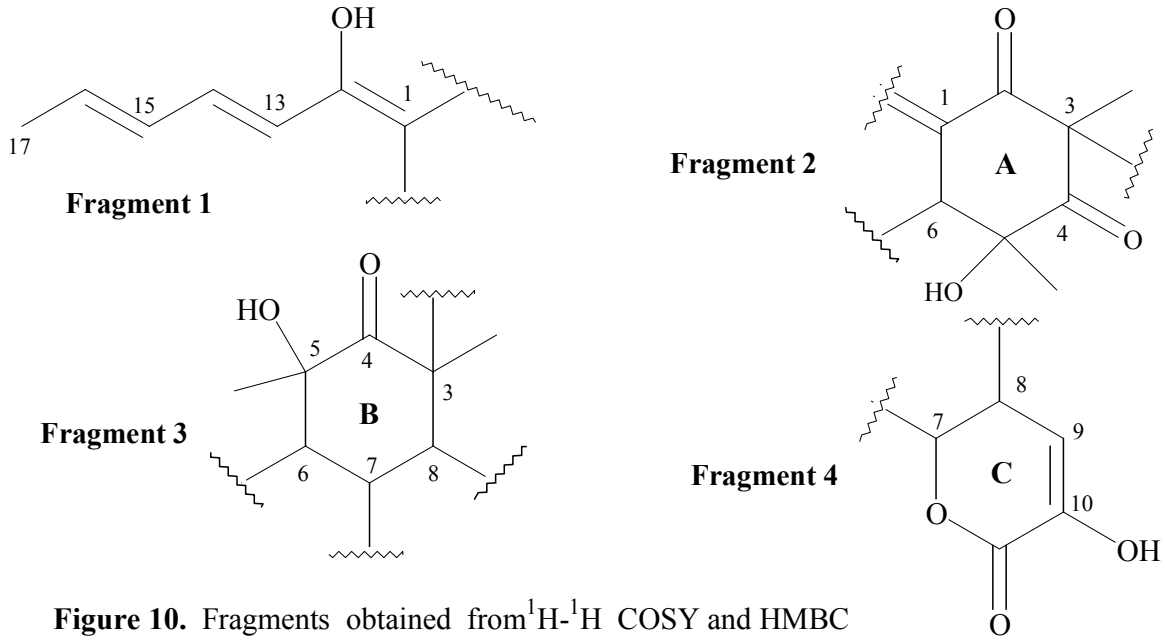
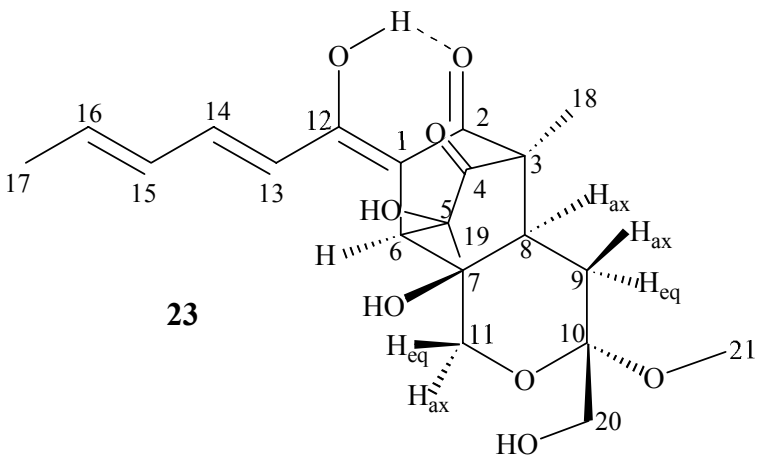


Figure 10. Fragments obtained from ^{1}H-^{1}H COSY and HMBC spectra of **22**

Compound **23** was deduced to have the molecular formula $C_{21}H_{28}O_8$ by interpretation of the NMR and EIMS data, also taking into account the accurate mass of the fragment ion at *m/z* 376.1519 $[M\text{-}CH_4O]^+$. Its ^{13}C NMR spectrum (^{1}H decoupled and DEPT) showed that five of the eight elements of unsaturation, could be attributed to three carbon-carbon double bonds and two carbonyl groups (Table 17); the molecule is thus tricyclic. The ^{1}H and ^{13}C NMR spectra showed the presence of two sp^3-hyberdized methine groups, four sp^2-hyberdized methine groups, three methylene groups (two of them attached to oxygen), an allylic methyl group, two methyl groups attached to quaternary carbons, one of which attached to oxygen, a methoxyl group, and eight quaternary carbons (Tables 17 and 18). The ^{1}H and ^{13}C NMR data enabled all but four hydrogen atoms of **23** to be accounted for; thus it was evident that the remaining four are present as hydroxyl functions, a deduction supported by IR data (ν_{max} 3315 cm^{-1}). From extensive comparison of ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, and HMBC NMR spectral data of **23** with those of **22**, it was evident that the two compounds were in a good agreement concerning the sorbyl unit, and rings A and B. The differences between the two compounds are evident from the NMR spectral data of



23

ring C. After assignment of all protons to their directly bonded carbons via a one-bond ^{1}H-^{13}C NMR shift correlated 2D NMR measurement (HMQC), it was possible to deduce from the ^{1}H-^{1}H COSY spectrum of **23**, a ^{1}H-^{1}H spin system from H-8 to H_2-9, this meant C-8 has to be bonded directly to C-9. The downfield ^{13}C NMR chemical shifts of C-10 (δ 110.6, s) and C-11 (δ 69.8, t) indicated C-10 to be attached to two oxygen atoms and C-11 attached to one oxygen atom. Long-range correlations observed between H-11 (δ 3.71, d, J = 11.0) and both C-7 and C-8 showed C-11 to be attached directly to C-7. C-9 has to be linked to C-10, because of HMBC correlations observed between H_2-9 and C-10. The methoxyl group (C-21) resided at C-10 based on the diagnostic long-range correlation observed between H_3-21 and C-10. HMBC correlations between H-20 (δ 3.81, d, J = 11.0) and C-9 and also between H-20 (δ 3.81, br d, J = 11.0) and C-10, led to the linkage between C-10 and C-20. The connection between C-10 and C-11 via an ether bridge and therefore, the completion of ring C followed by deduction. Remaining to be assigned were four hydroxyl groups. ^{13}C NMR chemical shifts of C-5 (δ 74.8, s), C-7 (δ 92.7, s), C-12 (δ 167.8, s), and C-20 (δ 62.8, t), indicated each carbon to bear a hydroxyl function. As the 12-OH proton formed a strong hydrogen bond with oxygen, it was likely that this occurred with the oxygen of the C-2 carbonyl group. The spin-spin coupling between H-8 (2.57, dd, J = 10.6, 7.7) and H-9 (1.82, dd, J = 13.5, 10.6) indicated the two protons had axial-axial interaction. The 2D NOESY and selective gradient NOE spectra were useful for the relative stereochemistry of **23**. The mutual NOE enhancements observed upon irradiation of the H-11 signal at δ 4.35 and of H-8 at δ 2.57 indicated the two protons had axial orientation, and that they are present on the same side of the molecule. Irradiation of CH_3-21 at δ 2.94 gave enhancement of H_{ax}-11 (δ 4.35), therefore, CH_3-21 was also axial orientation, and had α-orientation. As the chair conformation of ring C is established based on the NOESY and selective NOE correlations between H_{ax}-11, H_{ax}-8, and CH_3-21, the junction of the ring B and C had to be trans and thus, the hydroxyl group at C-7 was β. A molecular modeling calculation of **23** was done indicating the distance between H_{eq}-11 and the CH_3-19 was more than 4.06 Å. This result was consistent with the absence of NOESY correlation between CH_3-19 and H_{eq}-11. Irradiation of H-6 at δ 3.39 gave enhancement of H-11 (δ 3.71) but no effect was observed to H_{ax}-11, and indicated H-6 and H-11 (δ 3.71) had equatorial orientation. These results were validated by a 2D NOESY measurement. Irradiation of CH_3-18 caused enhancement of H_{eq}-9 (δ 2.72) and also H_{ax}-8 and indicated the CH_3-18 to have an equatorial orientation. The relative stereochemistry of **23** has to be 3*S**, 6*S**, 7*R**, 8*R**, 10*S**, 1*Z*, 13*E*, 15*E*, however the centers C-6 and C-3 cannot be related in a relative sense to C-7 and C-8. The configuration at C-5 stays undetermined. For **23**, a new sorbicillinoid derivative, the trivial name trichodermanone B, is proposed.

Table 17. ^{1}H, HMBC and NOESY NMR [a] spectral data for compounds **22** and **23**

	22			**23**		
Position	δ^{1}H	HMBC[b]	NOESY	δ^{1}H	HMBC	NOESY
6	3.74 ($1H_{eq}$, d, 3.7) [d]	C-1, C-2, C-4, C-5, C-7, C-8, C-12	H-13, H_3-19	3.39 (1H, s) [d]	C-1, C-2, C-4, C-5, C-7, C-8, C-12	H-13, H_3-19, H_{eq}-9
7	5.57 (1H, dd, 10.6, 3.7)	C-1, C-3	H-6, H-8			
8	3.58 (1H, dd, 10.6, 2.9)	C-2, C-3, C-4, C-6, C-9	H-9, H-7, H_3-18	2.57 ($1H_{ax}$, dd, 10.6, 7.7)	C-2, C-3, C-4, C-9, C-11	H_{eq}-9, H_{ax}-11, H_3-18
9	5.75 (1H, d, 2.9)	C-7, C-8, C-10, C-11	H-8, H_3-18	1.82 ($1H_{ax}$, d, 13.5,10.6)	C-3, C-7, C-8, C-10	H_{ax}-8, H_3-18
				2.72 ($1H_{eq}$, d, 13.5, 7.7)	C-3, C-7, C-8, C-10	
11				3.71 ($1H_{eq}$, d, 11.0)	C-7, C-8	H_{eq}-6, H_{ax}-11
				4.35 ($1H_{ax}$, br d, 11.0)		H_{ax}-8, H_{eq}-11,CH_3-21
13	6.55 (1H, d, 15.0)	C-1, C-12, C-15	H-6, H-15	6.56 (1H, d, 15.0)	C-12, C-14, C-15	H_{eq}-6, H-15
14	7.29 (1H, dd, 15.0, 11.0)	C-12, C-16	H-16	7.22 (1H, dd, 15.0, 11.0)	C-12, C-13, C-16	H-16
15	6.44 (1H, dd, 15.0, 11.0)	C-16, C-17	H-13, H_3-17	6.40 (1H, dd, 15.0, 11.0)	C-16, C-17	H-13, H_3-17
16	6.26 (1H, dq, 15.0, 7.0)	C-15, C-17	H-14, H_3-17	6.18 (1H, dq 15.0, 7.0)	C-14, C-15, C-17	H-14
17	1.88 (3H, d, 7.0)	C-15, C-16	H-15, H-16	1.88 (3H, d, 7.0)	C-15, C-16	H-15
18	1.19 (3H, s)	C-2, C-3, C-4, C-8	H-8, H-9	1.13 (3H, s)	C-2, C-3, C-4, C-8	H_{ax}-8, H_{eq}-9, H_2-20
19	1.25 (3H, s)	C-4, C-5, C-6	H-6	1.17 (3H, s)	C-4, C-5, C-6	H_{eq}-6
20				3.81 ($1H_{\alpha}$, d, 11.0)	C-9, C-10	H_{eq}-11, H_3-21
				3.50 ($1H_{\beta}$, br d, 11.0)		
21				2.94 (3H, s)	C-10	H_{α}-20, H_{β}-20, H_{ax}-11
OH-5	n[C]			5.15 (1H, br s)	C-5, C-6	
OH-7	n[C]			4.61 (1H, br s)		
OH-10	n[C]					
OH-12	n[C]			14.20 (1H, br s)		
OH-20	n[C]			4.70 (1H, br s)		

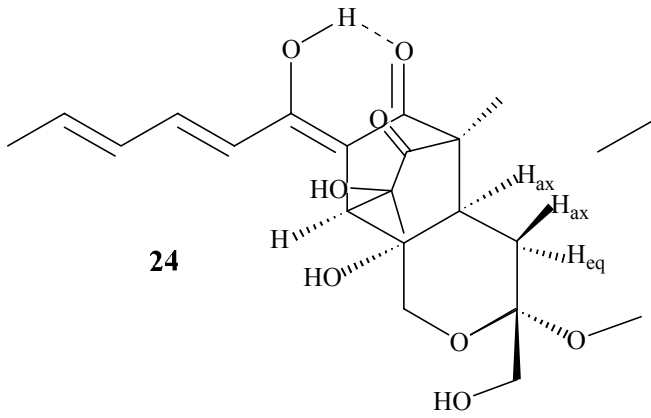
[a]All 2D NMR spectra were measured at 500 MHz, except HMBC was measured at 300 MHz using $(CD_3)_2CO$. [b]Long-range correlation from proton to carbon. [C]The signal was not observed. [d]*J* in Hz.

Table 18. ^{13}C NMR [$(CD_3)_2CO$, 75.5 MHz], spectral data for compounds **22**-**25**

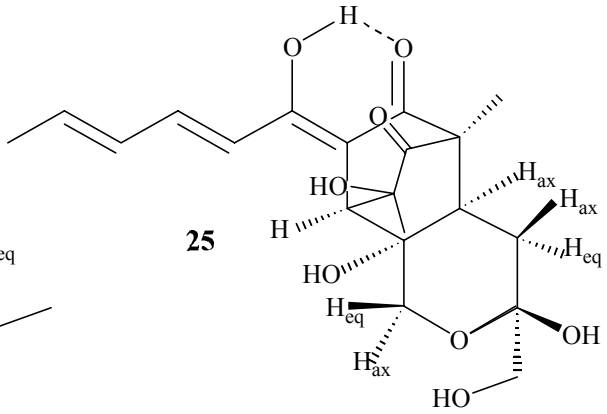
Position	**22**	**23**	**24**	**25**
1	107.8 (s) [a]	112.3 (s)	111.5 (s)	111.6 (s)
2	198.8 (s)	199.0 (s)	200.2 (s)	200.0 (s)
3	64.7 (s)	64.2 (s)	64.6 (s)	67.4 (s)
4	202.3 (s)	210.9 (s)	210.9 (s)	211.1(s)
5	74.5 (s)	74.8 (s)	74.8 (s)	74.8 (s)
6	47.6 (d)	49.1 (d)	49.2 (d)	49.5 (d)
7	80.9 (d)	92.7 (s)	90.3 (s)	90.3 (s)
8	53.0 (d)	50.2 (d)	49.8 (d)	50.0 (d)
9	109.6 (d)	38.1 (t)	41.0 (t)	40.3 (t)
10	151.7 (s)	110.6 (s)	111.0 (s)	107.5 (s)
11	161.0 (s)	69.8 (t)	68.5 (t)	68.3 (t)
12	170.9 (s)	167.8 (s)	169.0 (s)	168.9 (s)
13	120.2 (d)	120.4 (d)	120.1 (d)	120.1 (d)
14	143.4 (d)	141.2 (d)	142.2 (d)	142.1 (d)
15	132.6 (d)	132.1 (d)	132.1 (d)	132.0 (d)
16	140.1 (d)	138.7 (d)	139.7 (d)	139.3 (d)
17	19.2 (q)	18.8 (q)	18.8 (q)	18.8 (q)
18	10.6 (q)	10.9 (q)	11.0 (q)	11.0 (q)
19	23.9 (q)	27.1 (q)	26.8 (q)	27.0 (q)
20		62.8 (t)	62.2 (t)	64.5 (t)
21		48.8 (q)	48.9 (q)	

[a]Implied multiplicities as determined by DEPT (C = s, CH = d, CH_2 = t, CH_3 = q).

Compound **24** analyzed for $C_{21}H_{28}O_8$ by ESIMS and EIMS. The molecular formula was confirmed by HREIMS measurement. The 1D and 2D NMR spectral data of **3** were similar to some extent to those of **23**. It was clear from these data that compound **24** is a stereo-isomer of **23** (see Table 17 and 19). Careful examination of ^{13}C NMR spectral data of **24** and **23** indicated the differences between the two data sets arise from the chemical shift of C-7, which is at δ 90.3, s for **24**, and at δ 92.7, s, for **23**. Thus, the configuration of C-7 changed from *R** in **23** to *S** in **24** based on the deshielded nature of the resonance associated with C-7 in **23** (Dekebo *et al.*, 2002). ^1H-^1H NMR coupling constants for H-8 (δ 2.56, dd, J = 9.9, 7.3 Hz) and H-9 (δ 2.17, dd, J = 13.7, 9.9 Hz) indicated the orientation of the two protons had to be axial-axial orientation. 2D NOESY NMR and selective gradient NOE spectra demonstrated all correlations in **24** were in a good agreement with those observed in **23**. The relative stereochemistry of the rest of **24** is as described in **23**. The configuration at C-5 stays undetermined. For **24**, a new sorbicillinoid derivative, the trivial name trichodermanone C is proposed.



24



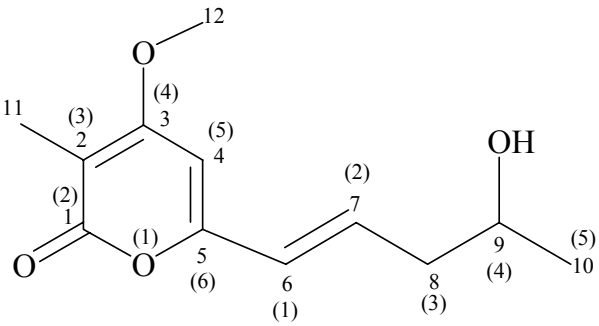
25

Compound **25** has the molecular formula $C_{20}H_{25}O_8$ as deduced by HREIMS measurement. Comparison of the mass spectral data of **25** with those of **24** revealed **25**, to have a methyl group less than **24**. From ^{13}C NMR spectral data of **25**, the absence of the C-21 methyl group was evident. 2D NOESY and selective gradient NOE measurements indicated compound **25** to have the same relative stereochemistry as described in **24** except at C-10. Irradiation of H_{ax}-11 at δ 4.3 caused enhancement of H_2-20. Thus, the configuration at C-10 changed from *S** in **24** to *R** in **25**. For **25**, a new sorbicillinoid derivative, the trivial name trichodermanone D is proposed.

Table 19. ^{1}H, HMBC and NOESY NMR[a] spectral data for compounds **24** and **25**

	24			**25**		
Position	$\delta^{1}H$	HMBC	NOESY	$\delta^{1}H$	HMBC	NOESY
6	3.51($1H_{eq}$, s)[d]	C-1, C-2, C-4, C-5, C-7, C-8, C-12	H-13, H_3-19	3.39 (1H, s)[d]	C-1, C-2, C-4, C-5, C-7, C-8, C-12	H-13, H_3-19, H_{ax}-11, H_{eq}-11
8	2.56 ($1H_{ax}$, dd, 9.9, 7.3)	C-3, C-9, C-11, C-18	H_{eq}-9, H_2-11, H_3-18	2.74 ($1H_{ax}$, dd, 9.9, 7.0)	C-1, C-3	H_{ax}-11,H_{eq}-11, H_3-8, H_{ax}-9
9	1.69 ($1H_{eq}$, dd, 13.9, 7.3)	C-3, C-8, C-10, C-20	H_3-18, H_{α}-20	1.76 ($1H_{ax}$, dd, 13.9, 7.0)		H_{eq}-9, H_{ax}-8, H_3-18
	2.17 ($1H_{ax}$, dd, 13.9, 9.9)	C-3, C-8, C-10	H_{ax}-9, H_{ax}-8	2.12 ($1H_{eq}$, dd, 13.9, 7.0)	C-2, C-3, C-4, C-6, C-9	H_{ax}-8, H_{ax}-9, H_3-18
11	3.91 (2H, br m)	C-6, C-7	H_{ax}-8, H_3-21	3.78 ($1H_{eq}$, d, 11.7)		H_{eq}-6, H_{ax}-11
				4.30 ($1H_{ax}$, d, 11.7)	C-7, C-8, C-10, C-11	H_{ax}-8, H_{eq}-11, H-20
13	6.44 (1H, d, 15.0)	C-12, C-15, C-16	H-15, H-6	6.47 (1H, d, 15.0)		H-15, H-6
14	7.25 (1H, dd, 15.0, 11.0)	C-12, C-15, C-16	H-16	7.26 (1H, dd, 15.0, 11.0)	C-1, C-12, C-15	H-16
15	6.39 (1H, dd, 15.0, 11.0)	C-16	H-13, H_3-17	6.42 (1H, dd, 15.0, 11.0)	C-12, C-16	H-13, H_3-17
16	6.23 (1H, dq, 15.0, 7.0)	C-14, C-15, C-17	H-14	6.26 (1H, dq, 15.0, 7.0)	C-16, C-17	H-14
17	1.86 (3H, d, 7.0)	C-13, C-15, C-16	H-15	1.87 (1H, d, 7.0)	C-15, C-17	H-15
18	1.08 (3H, s)	C-2, C-3, C-4, C-8	H_{ax}-8, H_{ax}-9	1.11 (3H, s)	C-13, C-15, C-16	H_{ax}-8, H_{ax}-9, H_{eq}-9
19	1.14 (3H, s)	C-4, C-5, C-6	H_{eq}-6, H_2-11	1.16 (3H, s)	C-2, C-3, C-4, C-8	H_{eq}-6, H_{ax}-9
20	3.01 ($1H_a$, d, 11.0)	C-10	H_{ax}-9	3.26 ($1H_{\alpha}$, d, 11.0)		H_{ax}-11
	3.85 ($1H_b$, d, 11.0)		H_3-21, H_{α}-20	3.32 ($1H_{\beta}$, d, 11.0)	C-10	
21	3.20 (3H, s)		H_2-11, H_{β}-20			
OH-5	5.70 (1H, br s)			5.45 (1H, br s)[b]		
OH-7	4.06 (1H, br s)			5.15 (1H, br s)[b]		
OH-10						
OH-12	14.00 (1H, br s)			14.00 (1H, br s)		
OH-20	3.55 (1H, m)[b]			4.65 (1H, br s)[b]		

[a]All 2D NMR were measured in 500 MHz, except HMBC was measure in 300 MHz using $(CD_3)_2CO$. [b]Long-range correlation from proton to carbon. [C]The signal was not observed. [d]*J* in Hz



The numbers in brackets are used for systematic naming

26

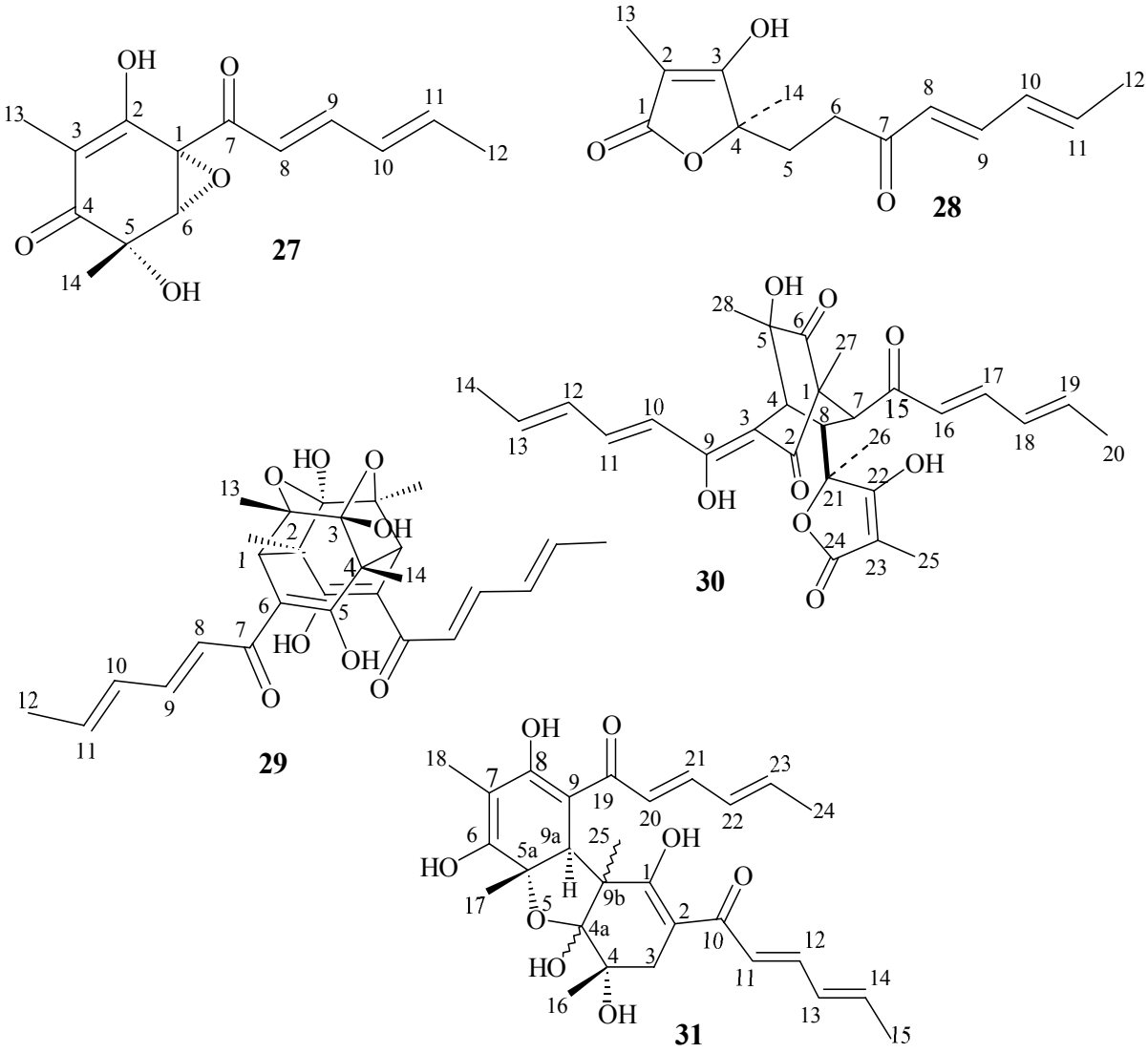
The structure elucidation of compound **26** commenced when the molecular formula of $C_{12}H_{16}O_4$ was established by positive mode electrospray ionisation mass spectrometric (ESIMS), EIMS and NMR spectral data. Finally, the HREIMS measurement validated these data. In the ^{13}C NMR spectrum of **26**, 12 resonances attributable to two methyl groups, a methylene group, three sp^2-hyberdized methine groups, an aliphatic sp^3-hyberdized methine group, a methoxyl group and four quaternary carbons, were evident (Table 20). It was clear from these data that four of five degrees of unsaturation within **26** were due to multiple bonds, three carbon-carbon double bonds and a carbonyl group, thus, the molecule had to be monocyclic. The ^1H and ^{13}C NMR spectral data enabled all but one hydrogen atom of **26** to be accounted for; thus, it was evident that the remaining proton must be present in hydroxyl function, a deduction supported by IR data (γ_{max} 3384 cm^{-1}). Also evident from the ^1H and ^{13}C NMR data of **26** were resonances consistent with the presence of a trisubstituted pyran-2-one ring, a deduction supported by a maximum absorption at 278 nm in the UV spectrum. After association of all ^1H and ^{13}C NMR resonances, associated with C-H one-bond interactions, from cross peaks seen in the ^1H-^{13}C 2D NMR shift-correlated (HMQC) spectrum of **26**, it was possible to deduce the planar structure of **26** by interpretation of its ^1H-^1H COSY and ^1H-^{13}C HMBC spectra. From the ^1H-^1H COSY spectrum of **26**, a ^1H-^1H spin system from H_3-10 to H-6 was evident. Diagnostic cross peaks in the HMBC spectrum were observed between H_3-10 and both C-9 and C-8, between H_2-8 and C-7; the combination of these spectral data (COSY and HMBC) enabled the side chain to be elucidated. Because of characteristic long-range correlations observed between H-4 and C-2, C-3, C-5 and C-6, the side chain has to be attached to the pyranone ring through the bond between C-5 and C-6. Characteristic long-range correlation this time observed between H_3-12 and C-3, led to conclude on the methoxyl group to be attached to C-3. Diagnostic long-range ^1H-^{13}C HMBC correlations observed from the resonance of H_3-11 to those of C-1, C-2, C-3 and C-4, enabled to be position the methyl group at C-2. Remaining to be incorporated into the planar structure is the hydroxyl group. On the basis of ^{13}C NMR chemical shift of C-9 (δ 68.2, d), the hydroxyl function was resided at C-9. The absolute configuration at C-9 was not determined. For **26**, a new pyranone derivative, the trivial name trichopyranone is proposed.

Table 20. ^{1}H [$(CD_3)_2CO$, 300 MHz] and ^{13}C NMR [$(CD_3)_2CO$, 75.5 MHz], spectral data for compound **26**[a]

Position	δ ^{13}C[b]	δ ^{1}H[c]	HMBC
1	165.1 (s)		
2	103.3 (s)		
3	168.6 (s)		
4	96.9 (d)	6.44 (1H, s)	C-2, C-3, C-5, C-6
5	158.7 (s)		
6	126.1 (d)	6.20 (1H, d, 15.7)	
7	137.0 (d)	6.65 (1H, dd, 15.7, 7.7)	
8	44.5 (t)	2.35 (2H, dd, 7.7, 2.9)	
9	68.2 (d)	3.91 (1H, dq, 5.9, 2.9)	
10	25.2 (q)	1.16 (3H, d, 5.9)	C-8, C-9
11	10.8 (q)	1.84 (3H, s)	C-1, C-2, C-3, C-4
12	57.8 (q)	3.95 (3H, s)	C-3
OH-9		3.98 (1H, m)[d]	

[a]All assignments are based on 1D and 2D measurements (HMBC, HMQC, COSY). [b]Implied multiplicities were determined by DEPT (C = s, CH = d, CH_2 = t, CH_3 = q). [c]*J* in Hz. [d]Tentative assignment.

All known compounds were identified by comparison of their spectroscopic data and optical rotations with published values. Epoxysorbicillinol (**27**) was first isolated as a yellow pigment from sponge-derived fungus *Trichoderma longibrachiatum* (Sperry *et al*., 1998). Vertinolide (**28**) was obtained from laboratory contaminant fungus *Verticillium intertextum* and its structure was established by X-ray analysis (Trifonov *et al.,* 1982). Trichodimerol (**29**) was firstly isolated form *Trichoderma logibrachiatum* (Andrade *et al*., 1992). Trichodimerol had DPPH radical scavenging effects (ED_{50} 53.7 μM) comparing with reference substance BHT and α-tocopherol (ED_{50} 27.0 and 22.0 μM, repectively). Finally, compound **29** showed significant inhibitory activity against lipopolysaccharide induced production of tumor necrosis factor alpha (TNF-α) in human monocytes (Nicolaou *et al.*, 2000). Bislongiquinolide (trichotetronine) (**30**) was isolated from *Trichoderma longibrachiatum* as amorphous yellow powder and its structure clearly composed from two sorbyl units and a tetronic acid moiety (Sperry *et al*., 1998). Bisvertinol (**31**) a bisorbicillinoide derivative, was first obtained from *Verticillium intertextum* as a yellow amorphous powder (Trifonov *et al*., 1986). 2-Furancarboxylic acid (**19**) was discussed previously (Chapter 4.4).



The total extract and all compounds except **19**, were tested in ELISA based assays for their HIV-1 reverse transcriptase inhibitory activity, and found to be inactive except **27**, which showed moderate activity (63.8 % at 200 μg/mL). The antimicrobial activities were measured using agar diffusion assays and no significant activity was found. All compounds and total extract were tested for their estrogenic effect and no activity was observed. The antioxidative properties of the total extract and all compounds except **31** and **19** were assessed using DPPH radical and TBARS assays. The results of these assays are shown in Tables 21 and 22, and are in a good agreement with the published data (Abe *et al*., 1998a and b). The antioxidant activity of the known compound **27** is published for the first time. The total extract and all compounds except **19**, were tested for cytotoxicity against ten human cancer cell lines, and no significant activity was observed, except weak activity of the total extract.

Table 21. DPPH[a] radical scavenging effects of the EtOAc extract of *Trichoderma viride* and compounds **22-30**

	%Scavenging[b]					
Item Tested	6[c]	12	23	46	115	230
EtOAc Extract	-2.3	-0.8	-0.6	3.2	7.3	12.5
22	-0.9	0.1	8.5	11.0	11.2	8.6
23	-1.1	-0.8	0.0	-1.3	1.0	1.3
24	-0.8	-3.0	0.7	1.6	3.1	3.4
25	-0.6	0.1	-1.4	0.8	2.1	4.7
26	-0.9	1.0	-0.9	-0.8	1.0	3.7
27	1.8	3.5	8.6	20.3	30.2	43.8
28	-1.5	-2.0	1.9	7.4	10.1	15.5
29	-0.1	2.6	4.8	13.3	23.0	37.2
30	0.4	1.3	4.0	9.1	16.7	23.8
BHT[d]	1.0	4.2	4.0	9.2	17.3	32.8
Vit E	10.9	19.4	38.2	94.0	94.3	94.0

[a]DPPH = α,α-Diphenyl-β-picrylhydrazyl. [b]Scavenging % = 100-(A sample[e] x 100/A control[e]). [c]Concentrations in μmol/L. [d]BHT = Butylated hydroxytoluene. [f]Absorbance of sample and control measured at 517 nm.

Table 22. Antioxidative effects (TBARS Assay)[a] of the EtOAc extract of *Trichoderma* sp. and compounds **22-30**

	% Inhibition[b]				
Item Tested	8[f]	16	33	82	164
EtOAc Extract	6.4	5.0	1.5	-4.4	-5.3
22	2.4	1.4	-3.0	-10.1	-7.2
23	0.7	4.6	7.6	6.8	6.0
24	7.1	9.4	6.3	3.8	5.5
25	6.2	6.4	6.0	4.7	6.2
26	1.9	1.2	3.2	2.2	1.6
27	14.4	11.9	11.7	13.7	17.8
28	3.2	4.6	4.3	3.6	5.2
29	9.1	11.4	9.7	12.1	19.9
30	6.8	3.3	0.9	-2.8	-0.1
BHT[c]	20.3	34.5	50.8	63.9	69.2
Vit. E	12.7	30.9	67.4	70.0	73.6

[a]TBARS = Thiobarbituric acid method. [b]% Inhibition = 100-(A sample[d] - A sample blank) x 100 / (A control – A blank). [c]BHT = Butylated hydroxytoluene. [d]A = Absorbance of test blank and control solutions are measured at both 532 and 600 nm. [f]Concentrations in μmol/L

Trichodermanone A (**22**) was isolated as yellowish viscous oil (2 mg); $[\alpha]^{22}_D$ = + 51.5° (*c* 0.08, MeOH); UV λ_{max} (MeOH) (log ε) 360 (4.3), 250 (4.2), 204 (4.1) nm; IR (film) ν_{max} 3384, 2923, 2359, 1729, 1599, 1404 cm^{-1}; ^1H and ^{13}C NMR data (Table 17 and 18); ESIMS: positive ion *m/z* 361 $[M+H]^+$ and *m/z* 378 $[M+H_2O]^+$; negative ion *m/z* 359 $[M-H]^+$; EIMS *m/z* (% rel int), 361 $[M+H]^+$ (10), 289 (10), 279 (25), 235 (10), 207 (10), 193 (20), 179 (20), 167 (40), 137 (30), 125 (37), 111 (50), 97 (65), 83 (55), 69 (50); calcd for $C_{19}H_{20}O_7$ *m/z* 360.1209.

Trichodermanone B (**23**) was isolated as yellowish viscous oil (4 mg); $[\alpha]^{22}_D$ = + 203° (*c* 0.24, MeOH); UV λ_{max} (MeOH) (log ε) 360 (4.6), 248 (4.2), 203 (4.2) nm; IR (film) ν_{max} 3315, 2920, 2851, 1732, 1630, 1602, 1565, 1455, 1381 cm^{-1}; ^1H and ^{13}C NMR data (Table 17 and 18); EIMS *m/z* (% rel int), 408 $[M^+]$ (10), 376 (100), 101 (80), 95 (40), 59 (90); HREIMS *m/z* 376.1519 $[M-CH_4O]^+$ calcd for $C_{20}H_{24}O_7$ *m/z* 376.1522.

Trichodermanone C (**24**) was isolated as yellowish viscous oil (6 mg); $[\alpha]^{22}_D$ = + 251.5° (*c* 0.2, MeOH); UV λ_{max} (MeOH) (log ε) 358 (4.7), 248 (4.2), 202 (4.4.) nm; IR (film) ν_{max} 3393, 2938, 1733, 1630, 1600, 1561, 1383 cm^{-1}; ^1H and ^{13}C NMR data (Table 18 and 19); ESIMS: negative ion *m/z* 407 $[M-H]^+$; positive ion *m/z* 426 $[M+H_2O]^+$; EIMS *m/z* (% rel int), 408 $[M^+]$ (10), 376 (60), 111 (30), 95 (100); HREIMS *m/z* 408.1780 calcd for $C_{21}H_{28}O_8$ *m/z* 408.1783.

Trichodermanone D (**25**) was isolated as yellowish viscous oil (21 mg); $[\alpha]^{22}_D$ = + 265.7° (*c* 0.5, MeOH); UV λ_{max} (MeOH) (log ε) 360 (4.3), 248 (3.8) nm; IR (film) ν_{max} 3330, 2937, 1731, 1627, 1597, 1556, 1380 cm^{-1}; ^1H and ^{13}C NMR data (Table 18 and 19); EIMS *m/z* (% rel int), 394 $[M^+]$ (20), 376 (100), 305 (22), 287 (20), 101 (37), 95 (65), 59 (37); HREIMS *m/z* 394.1625, calcd for $C_{20}H_{25}O_8$ *m/z* 394.1628.

Trichopyranone, 6-(4-hydroxy-1-pentenyl)-4-methoxy-3-methyl-2*H*-pyran-2-one (**26**) was isolated as yellowish viscous oil (2.5 mg); $[\alpha]^{22}_D$ = - 10.3° (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ε) 334 (3.5), 278 (3.5), 227 (4.0) nm; IR (film) ν_{max} 3384, 2930, 2360, 1676, 1549, 1458, 1258, 1144 cm^{-1}; ^1H and ^{13}C NMR data (Table 20); ESIMS: positive ion *m/z* 225 $[M+H]^+$, EIMS *m/z* (% rel int), 224 $[M^+]$ (25), 180 (100), 152 (25), 139 (20), 125 (30), 111 (60), 83 (45), 71 (55), 57 (65); HREIMS *m/z* 224.1046 calcd for $C_{12}H_{16}O_4$ *m/z* 224.1049.

Epoxysorbicillinol (**27**) was isolated as yellowish brown viscous oil (6 mg); $[\alpha]^{22}_D$ = + 85.5° (*c* 0.2, MeOH); Lit. (Sperry *et al.*, 1998) $[\alpha]_D$ = + 75° (*c* 0.15, MeOH); UV λ_{max} (MeOH) (log ε) 288 (4.3) nm; IR (film) ν_{max} 3354, 2934, 1625, 1585, 1381, 1215, 1015 cm^{-1}; ^1H NMR (300 MHz, $[CD_3]_2CO$) δ 7.25 (1H, dd, *J* = 15.0, 9.9 Hz, H-9), 6.37 (1H, d, *J* = 15.0 Hz, H-8), 6.32-6.28 (2H, m, H-11, H-10), 3.71 (1H, s, H-6), 1.88 (1H, d, *J* = 5.5 Hz, H_3-12), 1.67 (3H, s, H_3-

13), 1.55 (3H, s, H_3-14); ^{13}C NMR (75.5 MHz, $[CD_3]_2CO$) δ 192.5 (s, C-7), 186.4 (s, C-4), 172.5 (s, C-2), 145.5 (d, C-9), 142.9 (d, C-11), 131.2 (d, C-10), 124.7 (d, C-8), 107.2 (s, C-3), 70.4 (s, C-5), 63.1 (s, C-1), 61.9 (d, C-6), 26.3 (q, C-14), 18.9 (q, C-12), 7.9 (q, C-13); EIMS *m/z* (% rel int), 264 [M^+] (12), 249 (20), 221 (20), 127 (22), 95 (100), calcd for $C_{14}H_{16}O_5$ *m/z* 264.0998.

Vertinolide (**28**) was isolated as yellowish brown viscous oil (6 mg); $[\alpha]^{22}_D$ = -11.1° (*c* 0.1, $CHCl_3$); UV λ_{max} (MeOH) (log ε) 262 (4.0), 230 (sh), nm; IR (film) ν_{max} 3404, 2928, 2360, 1694, 1632, 1597, 1230, 1173 cm^{-1}; ^{1}H NMR (300 MHz, $[CD_3]_2CO$); δ 7.17 (1H, dd, *J* = 15.0, 9.9 Hz, H-9), 6.30-6.24 (2H, d, *J* = 15.0 Hz, H-10 and H-11), 6.09 (1H, d, *J* = 15.0 Hz, H-8), 2.57 (2H, m, H-6), 2.44 (2H, m, H-5), 1.89 (3H, d, *J* = 5.4 Hz, H_3-12), 1.68 (3H, s, H_3-14); 1.48 (3H, s, H_3-13), ^{13}C NMR (75.5 MHz, $[CD_3]_2CO$) δ 198.9 (s, C-7), 176.5 (s, C-3), 173.6 (s, C-1), 143.3 (d, C-9), 140.7 (d, C-11), 131.2 (d, C-10), 128.4 (d, C-8), 96.6 (s, C-2), 82.3 (s, C-4), 34.7 (t, C-6), 31.6 (t, C-5), 23.5 (q, C-14), 18.7 (q, C-12), 6.3 (q, C-13); EIMS *m/z* (% rel int), 250 [M^+] (10), 237 (14), 108 (25), 99 (44), 95 (100), calcd for $C_{14}H_{18}O_4$ *m/z* 250.1205.

Trichodimerol (**29**) was isolated as yellow amorphous powder (10 mg); $[\alpha]^{23}_D$ = -480° (*c* 0.1, MeOH), Lit (Nicolaou *et al*., 2000) (film) ν_{max} 3431, 2924, 1737, 1613, 1553, 1263 cm^{-1}; ^{1}H NMR (300 MHz, $[CD_3]_2CO$); δ 7.27 (2H, dd, *J* = 15.0, 11.0 Hz, H-9, H-9`), 6.50–6.40 (4H, m, H-10, H-10`, H-11, H-11`), 6.25 (2H, d, *J* = 15.0 Hz, H-8, H-8`), 3.12 (2H, s, H-1, H-1`), 1.88 (6H, d, *J* = 7.0 Hz, $H_3$-12, $H_3$-12`), 1.40 (6H, s, H_3-14, H_3-14`), 1.32 (6H, s, $H_3$-13, $H_3$-13`); ^{13}C NMR (75.5 MHz, $[CD_3]_2CO$) δ 200.2 (s, C-5, C-5`), 177.5 (s, C-7, C-7`), 144.7 (d, C-9, C-9`), 140.1 (d, C-11, C-11`), 133.3 (d, C-10, C-10`), 121.3 (d, C-8, C-8`), 105.9 (s, C-3, C-3`), 103.5 (s, C-6, C-6`), 81.1 (s, C-2, C-2`), 60.9 (s, C-4, C-4`), 56.1 (d, C-1, C-1`), 21.3 (q, C-13, C-13`), 19.2 (q, C-12, C-12`), 18.9 (q, C-14, C-14`) ; ESIMS: positive ion *m/z* 497 $[M+H]^+$, negative ion *m/z* 495 $[M-H]^+$, calcd for $C_{28}H_{32}O_8$ *m/z* 496.2097.

Bislongiquinolide (trichotetronine) (**30**) was isolated as amorphous yellow powder (39 mg); $[\alpha]^{23}_D$ = +137° (*c* 0.1, MeOH), Lit. (Sperry *et al.*, 1998) $[\alpha]^{23}_D$ = +134° (*c* 0.07, MeOH); UV λ_{max} (MeOH) (log ε) 370 (4.9), 292 (4.9), 263 (4.8) nm; IR (film) ν_{max} 3417, 2932, 1730, 1707, 1658, 1621, 1585, 1561, 1375, 1312, 1230, 1063 cm^{-1}; ^{1}H NMR (300 MHz, $[CD_3]_2CO$); δ 7.30 (2H, dd, *J* = 15.0, 11.0 Hz, H-11, H-17), 6.57-6.20 (6H, m, H-10, H-12, H-13, H-16, H-18, H-19), 3.37 (1H, m, H-4), 3.30 (1H, br d, *J* = 7.0 Hz, H-8), 3.21 (1H, d, *J* = 7.0 Hz, H-7), 1.89 (6H, d, *J* = 6.5 Hz, H_3-14, H_3-20), 1.49 (3H, s, H_3-25), 1.42 (3H, s, H_3-26), 1.17 (3H, s, H_3-28), 0.99 (3H, s, H_3-27); ^{13}C NMR (75.5 MHz, $[CD_3]_2CO$); δ 210.1 (s, C-6), 202.3 (s, C-15), 197.5 (s, C-2), 178.8 (s, C-22), 176.6 (s, C-24), 169.9 (s, C-9), 147.8 (d, C-17), 145.1 (d, C-19), 143.9 (d, C-11), 140.8 (d, C-13), 132.3 (d, C-18), 131.5 (d, C-12), 128.4

(d, C-16), 119.5 (d, C-10), 110.0 (s, C-3), 97.9 (s, C-23), 84.4 (s, C-21), 75.8 (s, C-5), 63.5 (s, C-1), 53.5 (d, C-7), 43.9 (d, C-8), 43.4 (d, C-4), 23.2 (q, C-26), 24.1 (q, C-27), 19.1 (q, C-20), 19.0 (q, C-14), 11.3 (q, C-28), 6.5 (q, C-25); EIMS *m/z* (% rel int), 496 [M^+] (10), 453 (10), 361 (60), 247 (20), 95 (100), calcd for $C_{28}H_{32}O_8$ *m/z* 496.2097.

Bisvertinol (**31**) was isolated amorphous yellow powder (2 mg); $[\alpha]^{23}_D$ = -690° (*c* 0.1, MeOH), $[\alpha]^{23}_D$ = -940° (*c* 0.1, $CHCl_3$), Lit. (Trifonov *et al*., 1986) $[\alpha]^{20}_D$ = -1467° (*c* 0.05, $CHCl_3$); UV λ_{max} (MeOH) (log ε) 363 (4.7), 299 (4.6), 274 (sh), 240 (sh) nm; IR (film) ν_{max} 3375, 2921, 2852, 1726, 1613, 1556, 1457 cm^{-1}; ^1H NMR (300 MHz, $[CD_3]_2CO$); δ 7.22 (2H, dd, *J* = 15.0, 11.0 Hz, H-12, H-21), 6.60 (2H, d, *J* = 15.0 Hz, H-11, H-20), 6.40-6.10 (4H, m, H-23, H-22, H-14, H-13), 3.72 (1H, s, H-9a), 2.77 (1H, d, *J* = 15.0 Hz, H-3), 2.55 (1H, d, *J* = 15.0 Hz, H-3), 1.89 (6H, m, H_3-24, H_3-15), 1.44 (3H, s, H_3-25), 1.41 (3H, s, H_3-18), 1.30 (3H, s, H_3-17), 1.17 (3H, s, H_3-16); ^{13}C NMR (75.5 MHz, $[CD_3]_2CO$), δ 194.3 (s, C-10), 193.0 (s, C-19), 180.3 (s, C-1), 170.5 (s, C-8), 167.5 (s, C-6), 143.0 (d, C-12, C-21), 140.8 (d, C-13, C-22), 137.6 (d, C-14), 137.2 (d, C-23), 122.1 (d, C-11, C-20), 111.0 (s, C-7), 107.0 (s, C-9), 105.1 (s, C-2), 102.6 (s, C-4a), 79.8 (s, C-5a), 74.0 (s, C-4), 59.8 (s, C-9b), 54.9 (d, C-9a), 37.2 (t, C-3), 26.6 (q, C-25), 23.1 (q, C-16), 21.8 (q, C-17), 20.0-19.8 (q, C-24, C-15), 8.0 (q, C-18); ESIMS: positive ion *m/z* 499 $[M+H]^+$, negative ion *m/z* 497 $[M-H]^+$, calcd for $C_{28}H_{34}O_8$ *m/z* 498.2254.

2-Furancarboxylic acid (**19**) was isolated as amorphous powder (1mg) and identified by comparison of the ^1H NMR data with those previously published (Chapter 4.4).

4.6 Secondary metabolites from marine fungi

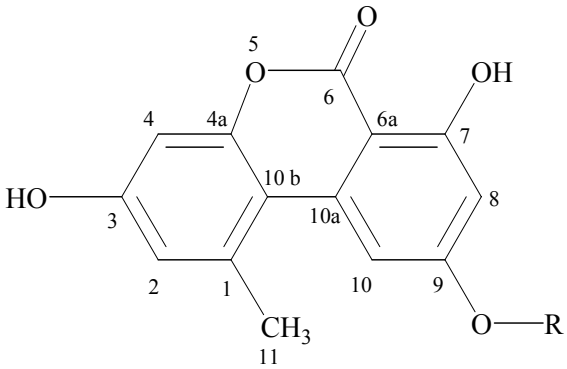
4.6.1 *Alternaria* sp.

Fungi of genus *Alternaria* occur wide spread as a parasite on cultivated plants; they were also found on numerous kinds of organic material under damp conditions, and were also isolated from marine sources (Freeman, 1965). The genus of *Alternaria* is known as a source of secondary metabolites; to date more than 161 compounds have been reported from fungi belonging to this genus, e.g. djalonenol, dibenzo-α-pyrone-djalonensone (Onocha *et al.*, 1995) and zinniol (Gamboa-Angulo *et al.*, 2000). In the preliminary screening for antimicrobial activity using agar diffusion assays, the extract of the mitosporic fungus *Alternaria* sp. (Pleosporaceae, Ascomycota, M 7-19, 539), isolated from the red alga, *Jania rubens* L. (Corallinaceae, M 7), collected from water around the Island of Morira, Mediterranean sea, showed prominent antimicrobial activity (biomalt medium, inhibition zone against *Mycotypha microspora* 0.4 cm, *Eurotium repens* 0.5 cm, *Microbotryum violaceum* 0.5 cm at 250 µg/disc). On this basis and with regard to the low field of ^{1}H NMR signals of the total extract. *Alternaria* sp. was selected for chemical investigation.

Mycelia and 10 L of solid biomalt medium were diluted with water (100 mL/L), homogenized using the Ultra-turax T25 at 8000 rpm^{-1}. The resulting mixture exhaustively extracted with EtOAc (3 x 8 L) and filtered. The filtrate was evaporated under reduced pressure to yield 2.0 g of a highly viscous dark reddish gum. The EtOAc extract was fractionated by VLC (2.5 x 20 cm, Si gel 60, 0.063-0.200 mm, 40 g, Merck) normal-phase silica (gradient pet.ether-EtOAc) to give 10 fractions (pet.ether:EtOAc, 1:0; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; 1:9; 0:1, each 100 mL). According to ^{1}H NMR spectral data, fractions 2 and 3, were interesting for further fractionation. VLC fraction 2 was subjected for NP (Eurospher Si, 5 µm, 250 x 8 ID, Knauer) HPLC using 85:15 pet.ether:acetone, to yield **32** (t_R = 13 min, 0.25 mg/L, 2.5 mg). VLC fraction 3 was subjected for precipitation trials, by dissolving the fraction in acetone and preciptates by CH_2Cl_2, left on the bench few minuts. Turbidity was appeared followed by precipitation of **33** (0.3 mg/L, 3 mg). The remaining fractions appeared not interesting based on ^{1}H NMR spectral data.

The dibenzopyrone derivatives (alternariol **32** and alternariol monomethyl ether **33**) were formally isolated from the mycelium of *A. tenuis* and recorded as the first dibenzo-α-pyrone derivatives (Freeman *et al*, 1965).

Alternariol, 3,7,9-trihydroxy-1-methyl-6*H*-benzo[c]chromen-6-one (**32**), was isolated as white amorphous powder (3 mg). ^{1}H NMR (300 MHz, $[CD_3]_2CO$); δ 7.34 (1H, d, *J* = 1.0, H-8), 6.78 (1H, d, *J* = 1.5 Hz, H-2), 6.68 (1H, d, *J* = 1.5 Hz, H-4), 6.43 (1H, d, *J* = 1.0 Hz, H-10), 2.74 (3H, s, CH_3-11); ^{13}C NMR (75.5 MHz, $[CD_3]_2CO$) δ 166.3 (s, C-9), 166.2 (s, C-6), 166.0 (s, C-7), 159.4 (s, C-3), 154.2 (s, C-4a), 139.7 (s, C-1), 139.6 (s, C-10a), 118.5 (d, C-2), 110.8 (s, C-10b), 105.3 (d, C-4), 102.8 (d, C-8), 102.0 (d, C-10), 99.3 (s, C-6a), 25.8 (q, CH_3-11); EIMS *m/z* (% rel int) 258.1 $[M]^+$ (100), 69 (20), calcd 258.0528 for $C_{14}H_{10}O_5$.



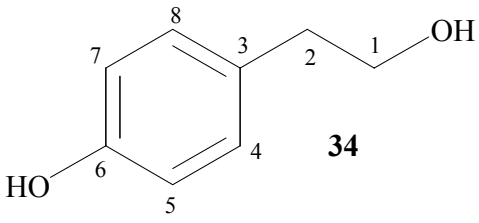
32 R = H
33 R = CH_3

Alternariol monomethyl ether, 3,7-dihydroxy-9-methoxy-1-methyl-6-*H*-benzo[c]chromen-6-one (**33**), was isolated as white amorphous powder (2.5 mg). ^{1}H NMR (300 MHz, $[CD_3]_2CO$); δ 7.34 (1H, d, *J* = 2.2, H-8), 6.83 (1H, d, *J* = 2.6 Hz, H-2), 6.83 (1H, d, *J* = 2.6 Hz, H-4), 6.65 (1H, d, *J* = 2.2 Hz, H-10), 4.01 (3H, s, OCH_3), 2.98 (3H, s, CH_3-11); ^{13}C NMR (75.5 MHz, $[CD_3]_2CO$) δ 167.5 (d, C-9), 166.1 (s, C-6), 165.9 (s, C-7), 159.5 (s, C-3), 154.1 (s, C-4a), 139.6 (s, C-1), 139.1 (s, C-10a), 118.5 (d, C-2), 110.5 (s, C-10b), 104.5 (d, C-4), 102.7 (d, C-8), 99.9 (s, C-6a), 104.5 (d, C-10), 56.2 (q, C-12, OCH_3), 25.6 (q, C-11); EIMS *m/z* (% rel int) 272.1 $[M]^+$ (100), 69 (30), calcd 272.0685 for $C_{15}H_{12}O_5$.

4.6.2 *Sporormiella* sp.

Sporormiella species are well known as coprophilous fungi, mainly found in dung and also isolated from marine sources (Osterhage dissertation, 2001). The genus *Sporormiella* is noted for its ability to produce bioactive secondary metabolites, e.g. similins A and B isolated from *S. similes* (Weber *et al.*, 1992), australifungin reported from *S. australis* (Mandala *et al.*, 1995), terezines A-D isolated from *S. teretispora* (Wang *et al.*, 1995) and sporovexins A-C from *S. vexans* (JS 306) (Soman *et al.*, 1999). In the preliminary screening for antimicrobial activity using agar diffusion assay, the extract of the fungus *Sporormiella* sp. isolated from the brown alga *Stypopodium* sp. (Stypocaulaceae) showed prominent antimicrobial activity (biomalt medium, inhibition zone against *Microbotryum violaceum* 0.4 cm at 250 µg/disc). The fungus *Sporormiella* (Ascomycetes, G 2-1, 493) was cultivated at room temperature for two months in 5 L (20 Erlenmayer flasks) of liquid biomalt containing 50 g biomalt and 1 L distilled water. Each Fernbach flask was inoculated with 10 ml of 10-day-old cultures (room temperature) grown under the same medium. The mycelia and media were extracted by

EtOAc (3 x 10L). The total extract (0.75 g, brown viscous oil) was fractionated by Vaccum Liquid Chromatography (2.5 x 20 cm, Polyprep 60-50 C18, 30 g, Macherey-Nagel 71150; reversed-phase), employing gradient elution from H_2O to MeOH, to yield 4 fractions (H_2O:MeOH, 7.5:2.5; 5:5; 2.5:7.5; 0:1; each 200 mL) (177.6, 249.8, 110.0, 28.5 mg, respectively,). After ^{1}H NMR spectral data analysis, resulted in fraction 1 was interesting for further fractionation. VLC fraction 1 was fractionated by using reversed phase VLC, employing gradient elution from H_2O to MeOH, to yield 9 fractions (H_2O:MeOH, 9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 3:7; 2:8, each 100 mL) (37.0, 66.8, 8.4, 6.3, 7.4, 6.6, 3.6, 6.4 mg, respectively). VLC Fractions 14 (6.3 mg) identified as 2-furancarboxylic acid (**19**) (Chapter 4.4). VLC Fractions 15 and 16 (13 mg) were pooled together, and purified by isocratic RP-18 HPLC using 90:10 H_2O: MeOH, in 35 min, 2 mL/min, to yield *p*-hydroxyphenylethanol (**34**) (t_R = 16.5 min, 0.6 mg/L, 3 mg), which, has been previously repoted from various fungi (Turner and Aldridge, 1983).



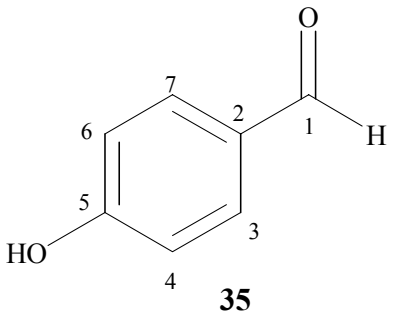
34

p-Hydroxyphenylethanol (**34**) was isolated as colourless oil (3 mg); ^{1}H NMR (300 MHz, CD_3OD) δ 7.00 (2H, d, J = 8.6 Hz, H-4, H-8), 6.70 (2H, t, J = 8.6 Hz, H-5, H-7), 3.65 (2H, t, J = 7.2 Hz, H_2-1), 2.68 (2H, t, J = 7.2 Hz, H_2-2); ^{13}C NMR (75.5 MHz, CD_3OD) δ 157.2 (s, C-6), 131.0 (s, C-3), 130.9 (d, C-4, C-8), 116.1 (d, C-5, C-7), 64.6 (t, C-1), 39.4 (t, C-2); ESIMS: negative ion *m/z* 137.0 $[M-H]^+$, calcd for $C_8H_{10}O_2$ *m/z* 138.0681.

4.6.3 *Myrioconium* sp.

In the preliminary screening for antimicrobial activity using agar diffusion assay, the ethyl acetate extract of a mitosporic fungus *Myrioconium* sp. isolated from *Fucus vesiculosus.*L CUX-3 (Fucaceae), collected by divers around Cuxhaven, North Sea, showed prominent antimicrobial activity (biomalt medium, inhibition zone against *Eurotium repens* 1.2 cm, *Microbotryum violaceum* total inhibition zone at 250 µg/disc). A literature survey was carried out and indicated that there is no publication on secondary metabolites from this genus. The ^{1}H NMR spectrum of the total extract showed signals in the δ 6-9 region. On this basis *Myrioconium* sp. was selected for chemical investigation. The fungus *Myrioconium* sp. (Mitosporic, Cux 3-10, 547) was cultivated at room temperature for two months in 10 L (20 Erlenmayer flasks) of liquid biomalt containing 50 g biomalt and 1 L distilled water. Each Fernbach flask was inoculated with 10 ml of 10 day-old cultures (room temperature) grown in biomalt media without agar.

The mycelia and medium were extracted with EtOAc (3 x 15 L). The total extract (2.95 g, brown viscous oil) was fractionated by VLC (2.5 x 20 cm, Si gel 60, 0.063-0.200 mm, 40 g, Merck), employing gradient elution from pet.ether/EtOAc/MeOH, to yield 8 fractions (pet.ether:EtOAc:MeOH, 7.5:2.5:0; 5:5:0; 2.5: 7.5:0; 0:1:0; 0:7.5:2.5; 0:5:5; 0:2.5:7.5; 0:0:1; each 100 mL) (0.8, 6.0, 1525.0, 75.8, 58.8, 27.4, 136.8, and 94.5 mg, respectively). ^{1}H NMR spectral data analysis, showed fraction 4 to be interesting for further fractionation. NP-HPLC with cyclohexane:EtOAc, 8:2, 2 mL/min, in 30 mints to yield *p*-hydroxybenzaldehyde (**35**) (t_R = 9 min, 0.1 mg/L, 1 mg). This compound is known since longe time ago and well known as microorganisms metabolites (Ayer *et al.*, 1993).



35

p-Hydroxybenzaldehyde (**35**) was isolated as amorphous white powder (1 mg). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.91 (1H, s, H-1), 7.84 (2H, d, *J* = 8.3 Hz, H-3, H-7), 6.96 (2H, d, *J* = 8.3 Hz, H-4, H-6); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 190.8 (s, C-1), 160.9 (s, C-5), 132.4 (d, C-3, C-7), 129.8 (s, C-2), 115.9 (d, C-4, C-6); EIMS *m/z* (% rel int) 122.0 $[M]^+$ (80), 121.0 $[M-H]^+$ (100), 93 $[M-CO]^+$ (45), 65 (30), calcd 122.0368 $C_7H_6O_2$.

5 Summary

The objective of this thesis was the investigation of the secondary metabolites produced by selected fungal strains isolated from the marine environment, and the assessment of their biological activity. Predominantly the antioxidant potential of the isolated compounds was assessed. In addition the isolation of endophytic fungi from Egyptian marine algae was performed.

Extracts of seven fungal strains, including six algal-derived and one sponge-derived fungus, were investigated chemically. This investigation resulted in the isolation and structure elucidation of 34 pure compounds representing different structural sub-classes within the polyketides i.e. hydroquinones, naphthalenones, xanthones, isobenzofuranones, pyrones, furanones and vertinoide/sorbicillinoid derivatives. Sixteen compounds (**1**-**8**, **12**, **13**, **17**, and **22**-**26**) proved to be new natural products.

The structurally unusual hydroquinone derivative acremonin A (**1**) and acremonin A glucoside (**2**) were isolated from *Acremonium* sp. and proved to be a new structural type of natural products. Compound **1** has significant antioxidant activity. Additionally, two naphthalenone stereoisomers (**3** and **4**) were obtained from this fungus. Compound **5** was isolated for the first time as a natural product and its structure was proven by x-ray analysis. In addition to these compounds an inseparable mixture of three new isomeric compounds (**6**-**8**) was analysed. Two new xanthone derivatives (**12** and **13**) were isolated from the rare fungal strain *Wardomyces anomalus*. These compounds showed an interesting new oxygenation pattern. Compound **12** has significant DPPH radical scavenging effects, and is also able to inhibit peroxidation of linolenic acid. Significant TK $p56^{lck}$ enzyme inhibitor activity is also observed for **12**. From *Epicoccum* sp., a new isobenzofuranone derivative (**17**) was isolated. Epicoccone (**17**) has potent antioxidant activity.The sponge-derived fungus *Trichoderma viride* contained four unprecedented and most structurally unusual sorbicillinoid derivatives (**22**-**25**), together with a new pyrone derivative (**26**).

To date reports concerning antioxidant metabolites from marine fungi are very rare. This study demonstrates that marine fungi are an excellent source for the discovery of novel antioxidant derivatives. Besides the prominent biological activity the diversity of structures obtained during this project is a further motivation for the investigation of marine-derived fungi.

6 Discussion and future aspects

The main objective of this study was the investigation of some marine-derived fungi aiming at finding new natural products with biological activity and/or novel chemical structures. Both, sponge- and algae-derived fungi are a prolific source of highly diverse secondary metabolites (Koenig & Wright, 1996; Pietra, 1997; Biabani, 1998; Faulkner 2001). The current project mainly deals with strains, isolated by Dr. Höller (Dissertation, 1999) and Dr. Osterhage (Dissertation, 2001). As aforementioned the selection of the fungal species for further investigation was predominantly based on the bioactivity of the total extract and/or the presence of several low field ^{1}H NMR resonances (δ 5-8 region) in the NMR spectrum of the extract. On this basis, seven fungal strains of different genera were selected for large-scale cultivation. Six of them were mitosporic fungi i.e. *Acremonium* sp., *Wardomyces anomalus*, *Epicoccum* sp., *Trichoderma viride*, *Alternaria* sp., and *Myrioconium* sp., and one was a fungus belonging to the Ascomycetes i.e. *Sporormiella* sp. From their extracts, 34 secondary metabolites were obtained. Sixteen compounds, (**1**-**8**, **12**, **13**, **17**, and **22**-**26**) were obtained as new natural products. In addition to these compounds, 18 known secondary metabolites were isolated.

The experimental work throughout this thesis revealed that most of the isolated metabolites possess antioxidant effects. This activity can be attributed to the presence of phenolic moieties. Especially, acremonin A (**1**), 2-(1-hydroxy-1-methyl)-2,3-dihydrobenzofuran-5-ol (**9**), 2,2-dimethyl-chroman-3,6-diol (**10**), 2-(3-dihydroxy-3-methylbutyl)-benzene-1,4-diol (**11**), anomalin A (**12**) and epicoccone (**17**) had potent antioxidant activities.

The compounds isolated in this study all belong to the polyketides and can be classified according to their structural features into seven sub-classes: hydroquinones, naphthalenones, xanthones, isobenzofuranones, pyrones, furanones and vertinoide/sorbicillinoid derivatives. This diversity of structures highlights the biosynthetic capability of marine derived fungi. Thus, a discussion of hypothetical biosynthetic pathways for the metabolites obtained seems worthwhile.

Hydroquinone derivatives

Five structurally related compounds (**1**, **2**, and **9**-**11**) were obtained from the extract of the alga-derived fungus *Acremonium* sp. Two of them, 7-isopropenyl bicyclo[4.2.0] octa-1,3,5-triene-2,5-diol (**1**), and 7-isopropenylbicyclo[4.2.0]octa-1,3,5-triene-2,5-diol-5-ß-D-glucopyranoside (**2**) possess a most unusual ring system, observed for the first time in natural products. Biosynthetically, **1**, **2** and **9**-**11** are probably related (Fig. 11). All of these compounds possess

an aromatic moiety, possibly of polyketide origin, substituted with a hemiterpene unit. Different cyclization reactions would then yield the three types shown.

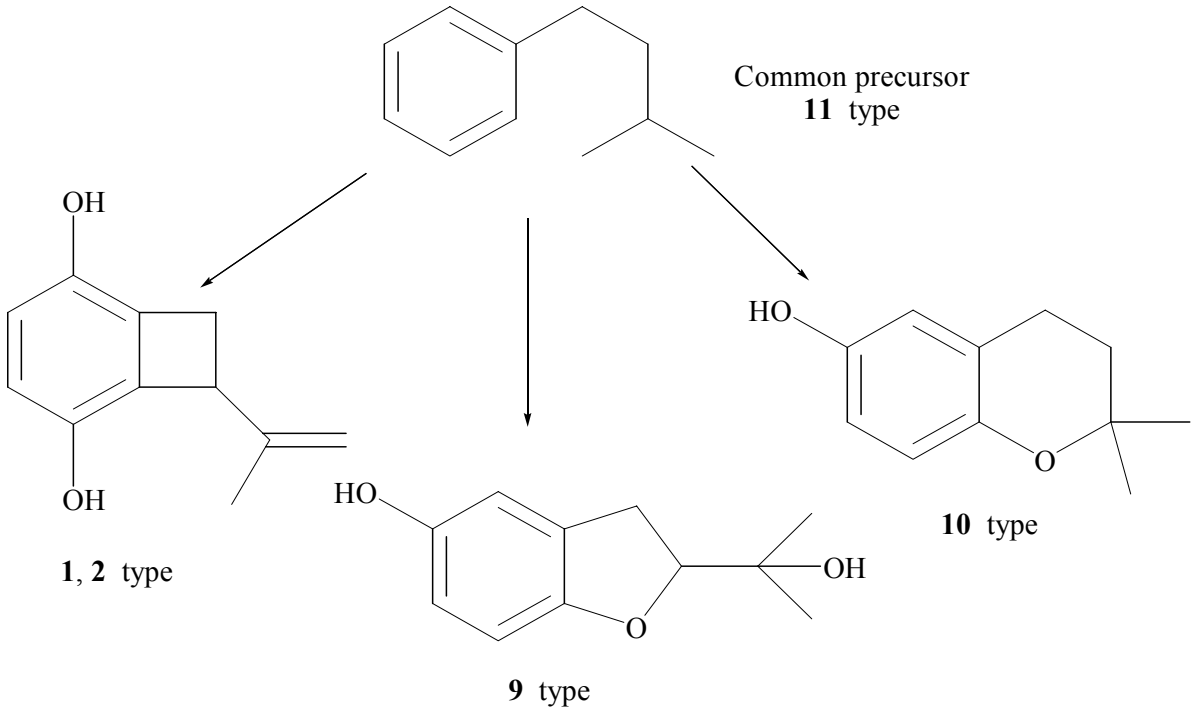


Figure 11. Proposed biosynthetic pathway for compounds **1**, **2** and **9-11**.

Naphthalenone

Two new natural products (3*R**,4*S**)-3,4-dihydroxy-7-methyl-3,4-dihydro-1(2*H*) naphthalene-one (**3**), and (3*S**,4*S**)-3,4-dihydroxy-7-methyl-3,4-dihydro-1(2*H*)-naphthalenone (**4**) were obtained as a 1:0.84 inseparable mixture from *Acremonium* sp. This class of natural products was previously reported from both higher plants and fungi (Talapatra *et al.*, 1988). For instance, 4,8-dihydroxy- tetralone was first isolated from a fungus of the genus *Scytalidium* (Findlay & Kwan, 1973). The naphthalenone skeletone possibly originates from one acetate and four malonate units condensed head to tail through aldol condensation followed by cyclization, aromatization and hydroxylation. Methylation could occur after cyclization as drawn in Figure 12, or the methyl group might originate from a methylmalonyl-CoA unit.

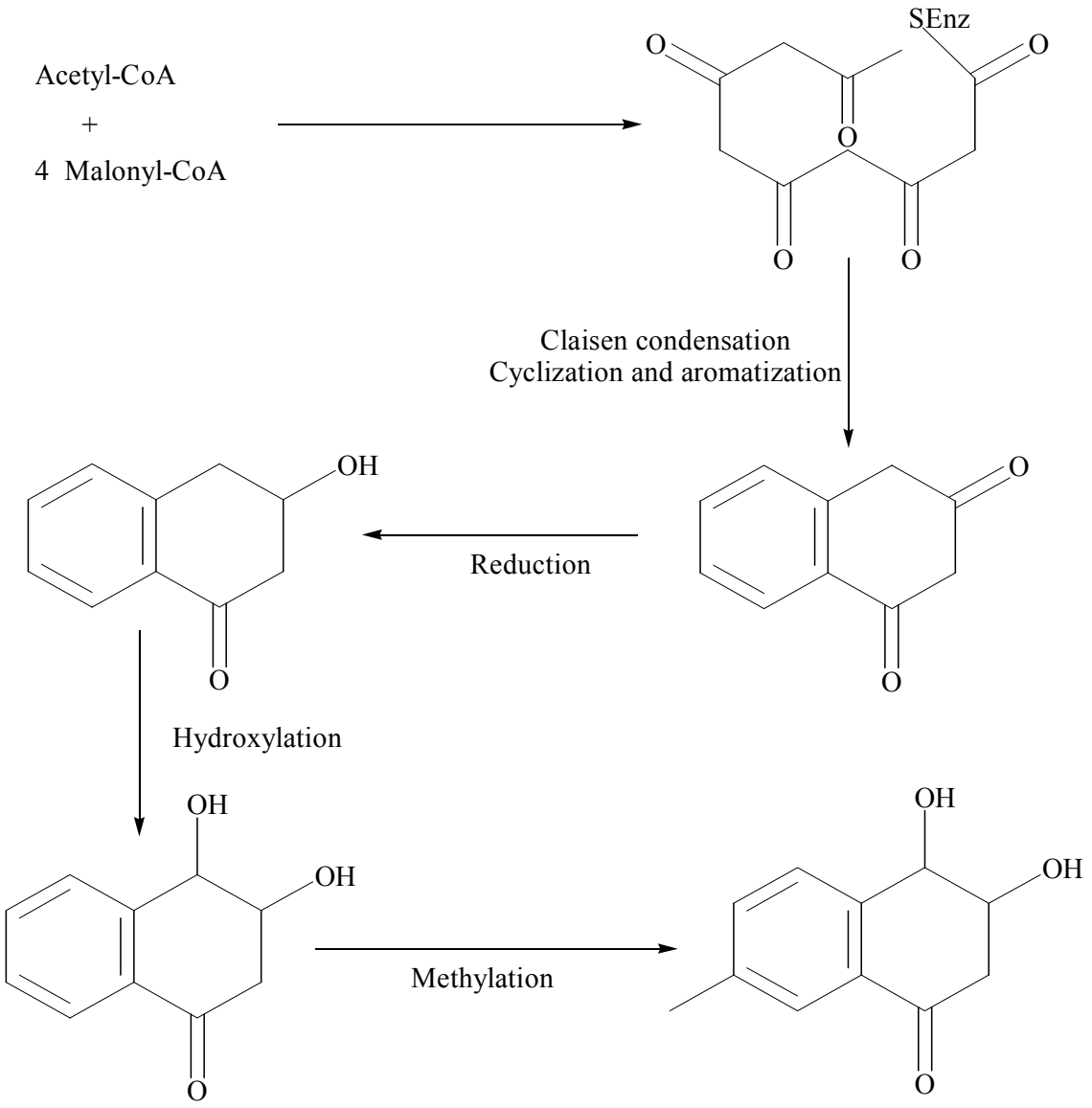


Figure 12. Proposed biosynthetic pathway for naphthalenone derivatives **3** and **4**.

Xanthone derivatives

Three xanthone derivatives (**12-14**) were isolated from the fungus *Wardomyces anomalus*, two of them (**12** and **13**) proved to be new natural products. The third one (**14**) was previously isolated from the lichen *Lecanora reuteri* (Harris *et al*., 1976). Experiments with labelled precursors indicated that the xanthone nucleus is usually produced through the acetate pathway (Hill *et al.*, 1982). In Figure 13 the proposed biosynthetic pathway for compounds **12-14** is shown. This illustrates again the unusual hydroxylation pattern of **12** and **13**.

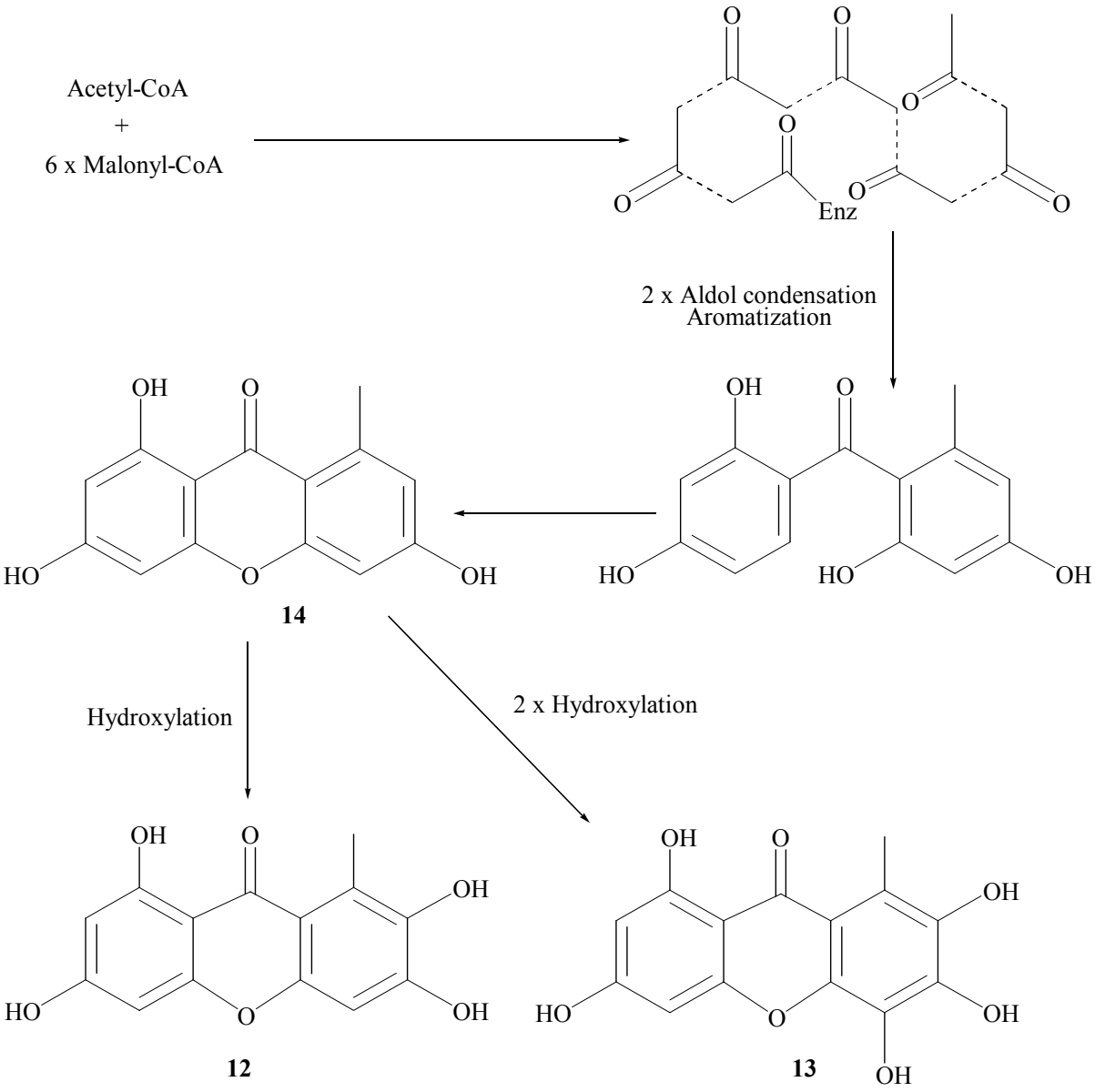


Figure 13. Proposed biosynthetic pathway for compounds **12-14**

Isobenzofuranone derivative

Epicoccone (**17**) is an unique isobenzofuranone derivative and may originate from the condensation of one acetate and three malonate units (Figure 14). Aldol condensation followed by aromatization might be the key steps in this biosynthesis. The presence of the phenolic group activates the ortho positions so as to allow two further hydroxyl groups to be positioned. Methylation leads to the intermediate (**17a**). Oxidation of methyl group, followed by lactonization may give a isobenzofuranone (**17**) (Dewick, 2002).

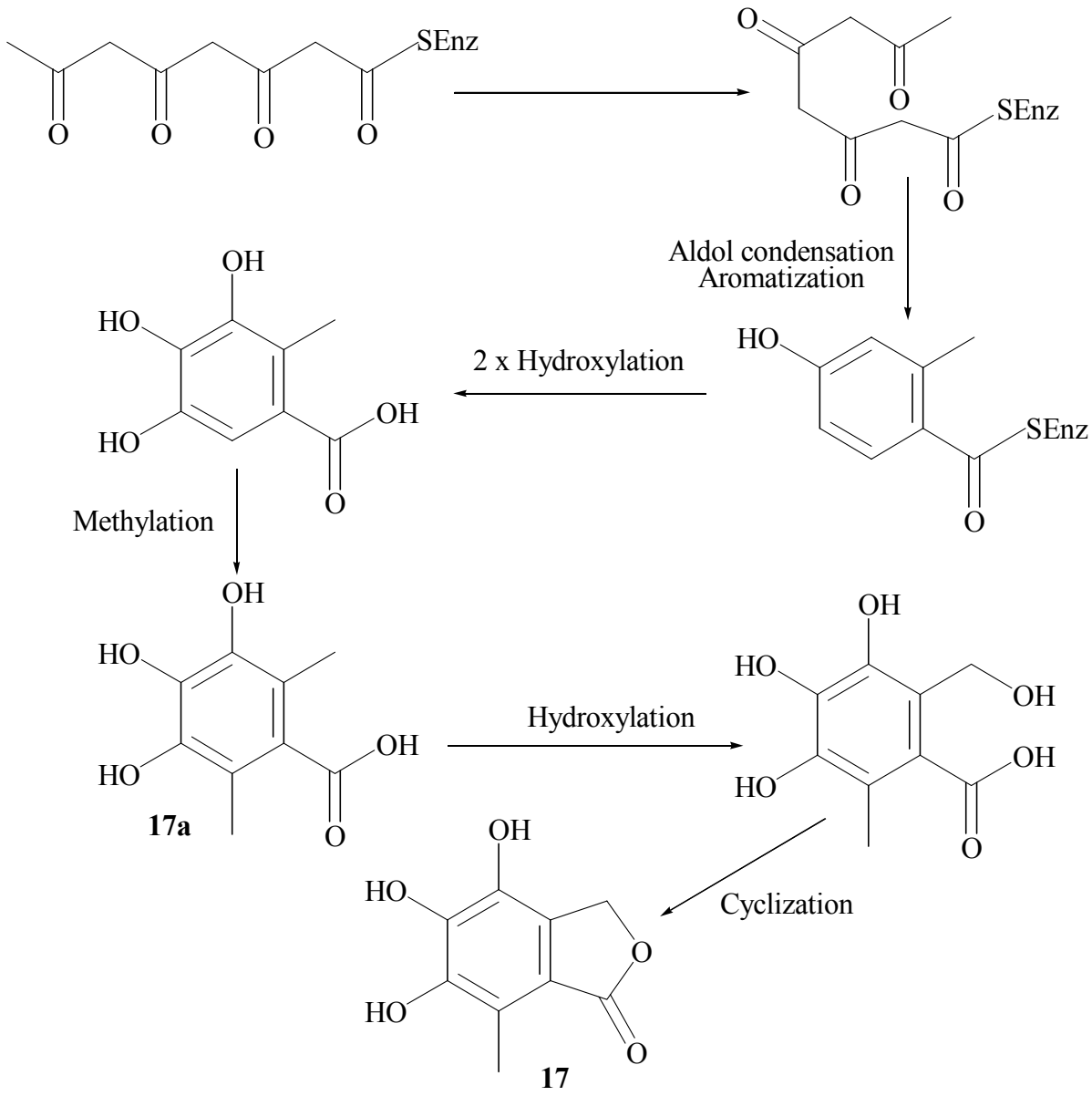


Figure 14. Proposed biosynthetic pathway for compound **17**

Mellein derivatives

Pentaketides of the mellein-type are well known as fungal metabolites, often with prominent biological, e.g. antimicrobial, activity. The mellein derivative (-)-(3*R*,4*S*)-4-hydroxymellein (**20**) and (-)-(3*R*)-5-hydroxymellein (**21**) obtained in this study were first isolated from *Septoria nordorum* (Devys *et al*., 1992), *Lasidioplodia theobromae* (Aldrige *et al*., 1971) and *Sporormia affinis* (McGahren & Mitcher, 1986). A hypothetical biosynthetic pathway shown in Figure 15.

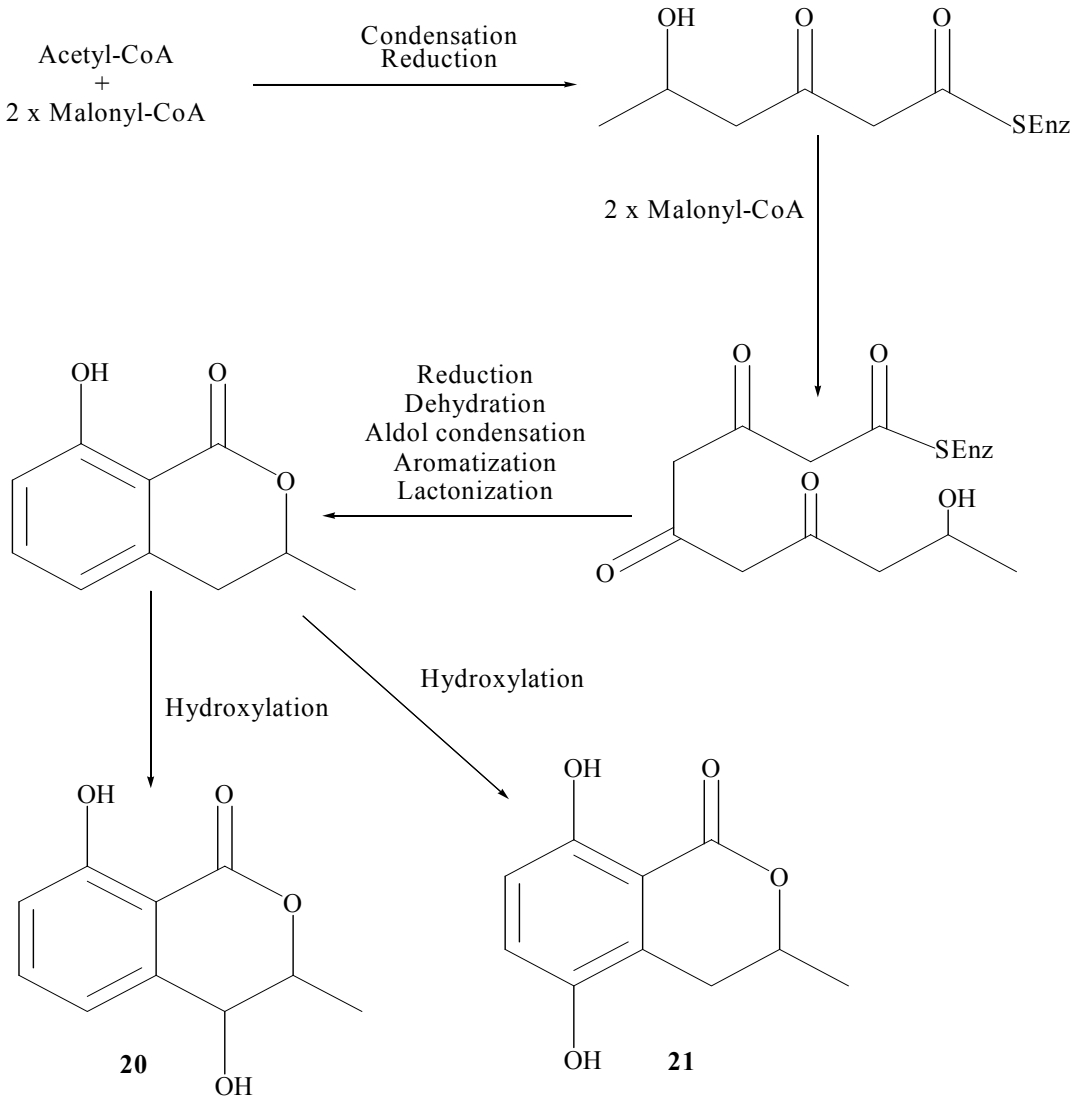


Figure 15. Proposed biosynthetic pathway for compounds **20** and **21**

Vertinoide derivatives

The pathway of vertinoid biosynthesis is shown in Figure 16. It is an extension of the suggestion of Trifonov *et al*. (1986). Their illustration based on the sorbicillinoid derivative, bisvertinol (**31**), which may be produced from two sorbicillin units (Figure 17). Experimental evidence came from Abe *et al*. (2000) who showed that sorbicillinol, which originated from sorbicillin, is the precursor for trichodimerol (**29**) and bisvertinol (**31**). Bisloniquinolide (**30**) was produced by reaction of the intermediate (**28A**) with sorbicillinol (Sperry *et al*., 1998). The intermediate **28A**, was a precursor for vertinolide (**28**), might orignate from sorbicillinol in a

similar way as penicillic acid from orsellinic acid. Our suggestion for **28** is thus based on analogies to the biosynthesis of penicillic acid from orsellinic acid (Figure 17). Penicillic acid was reported as a fungal metabolite from *Penicillium cyclopium*, and biosynthetically produced via the ring fission of orsellinic acid as in Figure 17 (Dewick, 2002). It is possible that compounds **22**-**25** were produced through a Diels-Alder cycloaddition of **A** to **B** followed by oxidation and decarboxylation to yield **22**, or by cyclization to give compounds **23**-**25** (Figure 18).

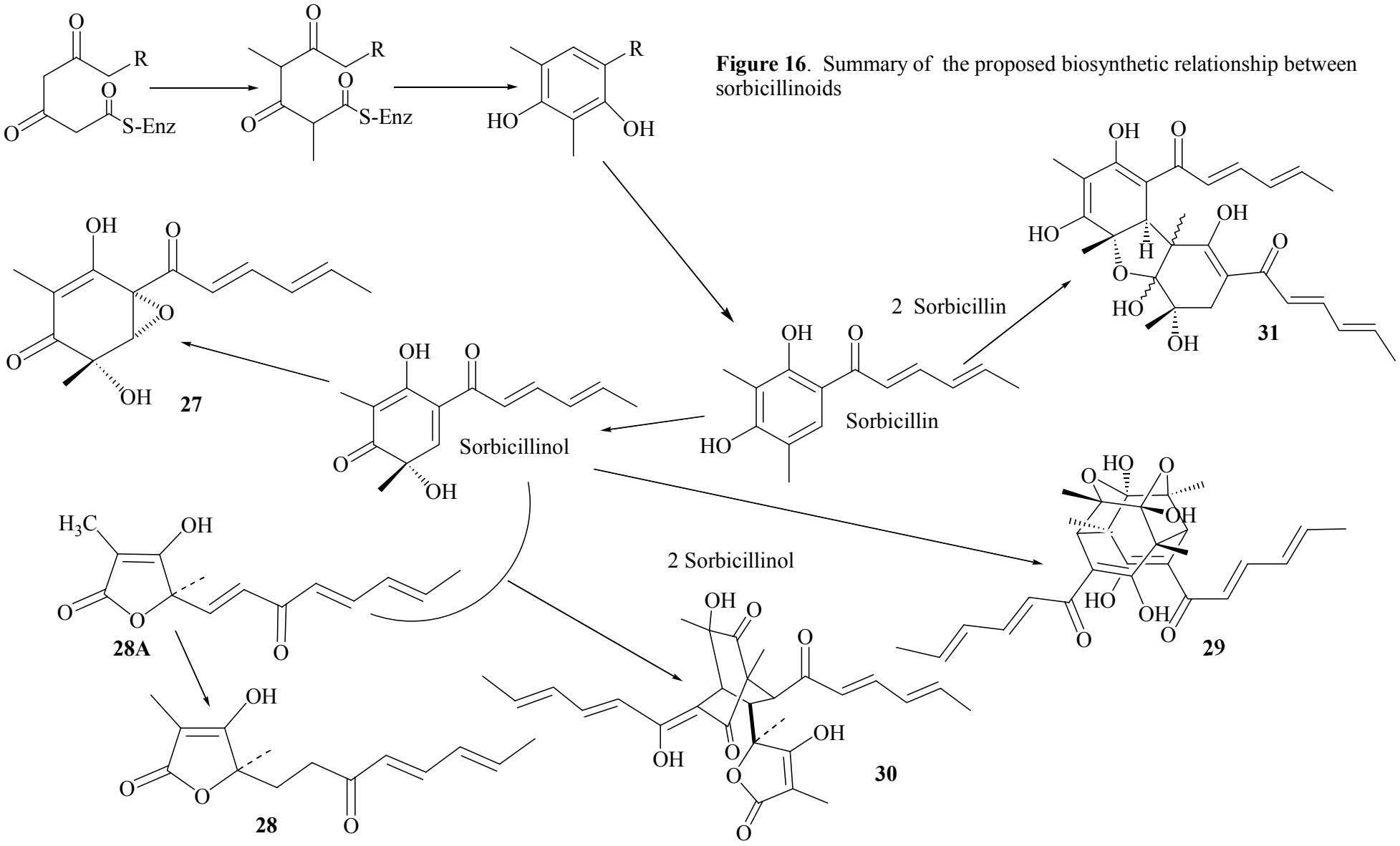


Figure 16. Summary of the proposed biosynthetic relationship between sorbicillinoids

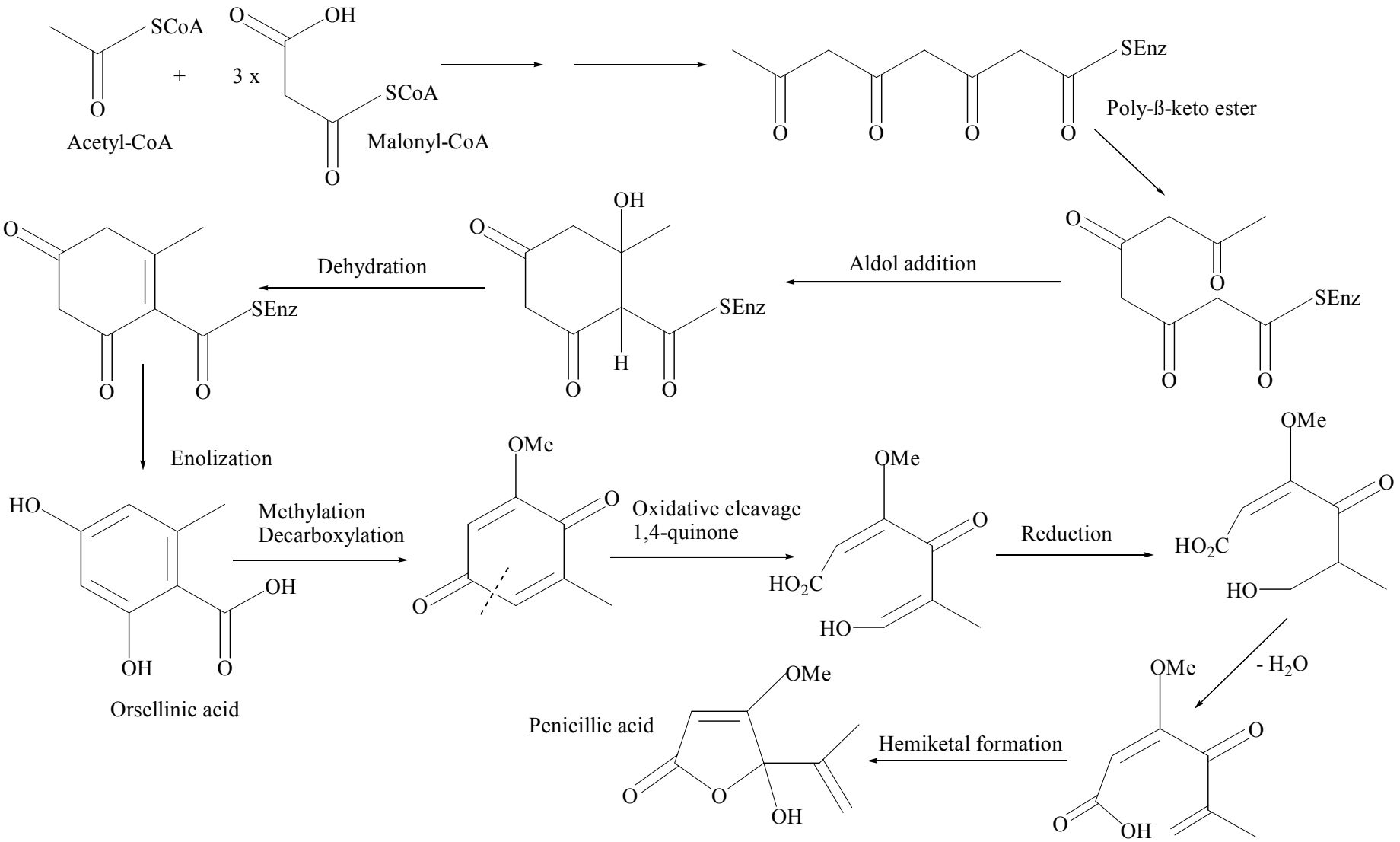


Figure 17. Proposed biosynthetic pathway for penicillic acid.

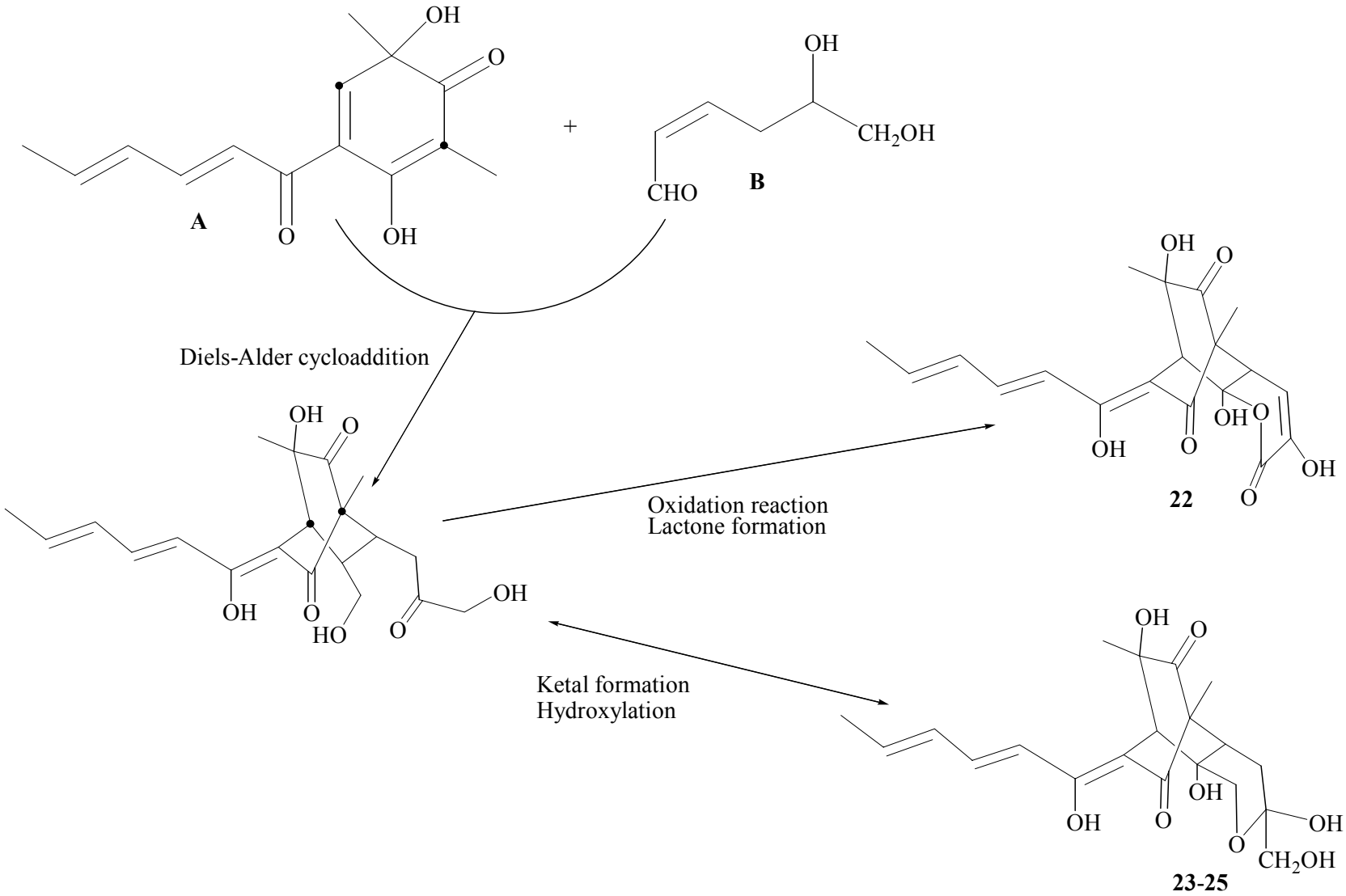


Figure 18. Proposed biosynthetic pathway of compounds **22-25**.

Dibenzopyrone derivatives

Thomas (1961) studied the chemical degradation of labeled alternariol (**32**) derived from sodium [1-^{14}C] acetate and demonstrated the biosynthesis to involve a head to tail condensation of one acetate and six malonate units. This indicated that the two compounds (**32**-**33**) originated from the polyketide pathway through the condensation of the acetyl-CoA and malonyl-CoA as shown in Figure 19.

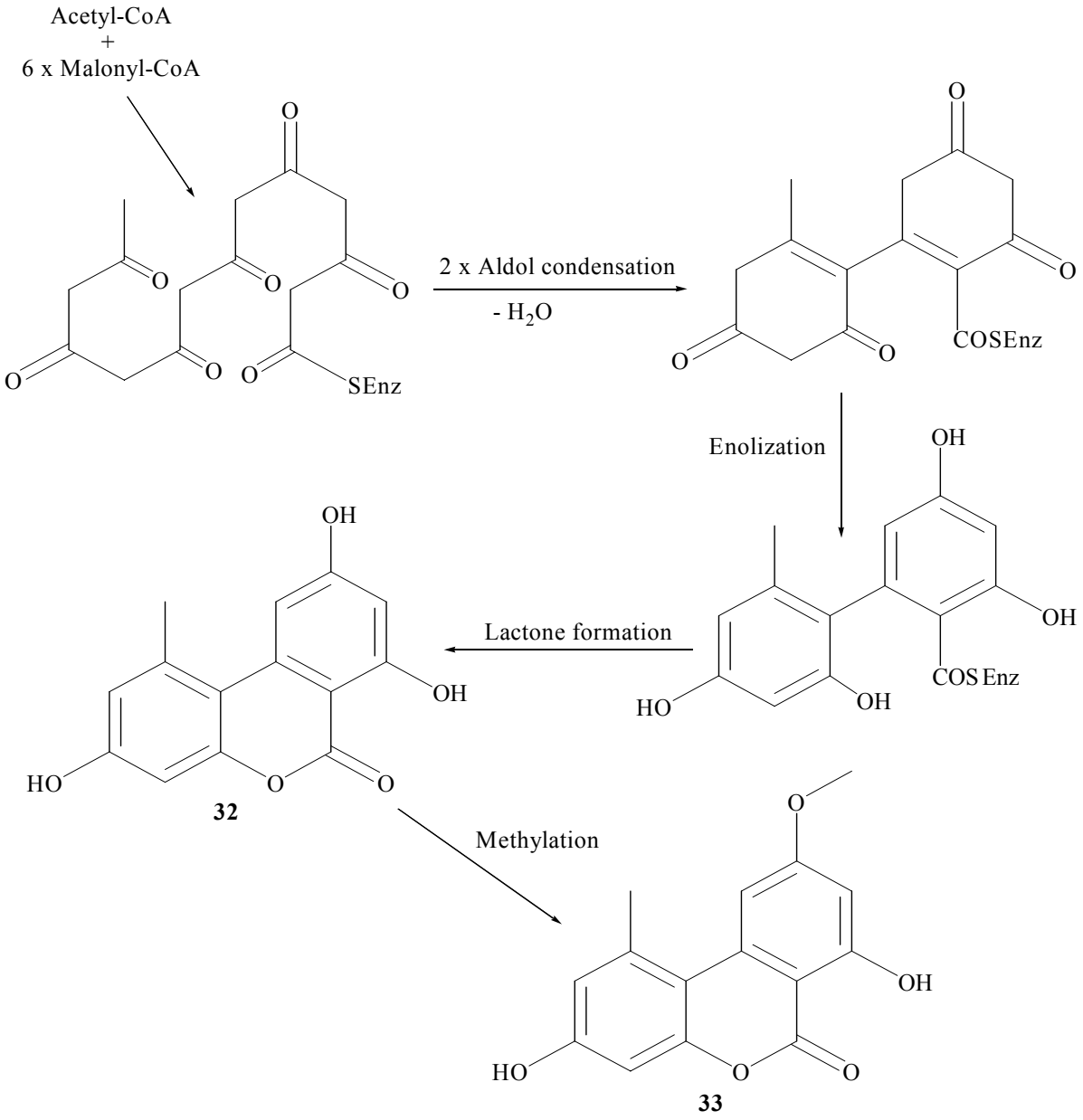


Figure 19. Proposed biosynthetic pathway of alternariol (**32**) and alternariol monomethyl ether (**33**).

The next step of the current project would be the investigation of the biosynthetic pathways based on the hypothesis illustrated, using labelled (^{13}C or ^{2}H or ^{18}O) precursors. The most interesting compounds would be the trichodermanones A-D (**22**-**25**), as well as the other polyketide compounds (**26**-**31**) produced by *Trichoderma viride* since they represent most unusual novel structures. Once the biosynthetic pathway is established, molecular biologic experiments might be worthwhile for the isolation and identification of the genclusters responsible for the polyketide production.

Secondary metabolites isolated during this study represented different structural classes of natural product. This indicates that marine-derived fungi have a wide range of biosynthetic capabilities for the production of novel structures. Within this study the polyketides produced by *Trichoderma viride* possess the most unusual structural skeletons and these compounds might have still undiscovered biological activities. Thus, the application of OSMAC (One Strain-Many Compounds) concept may be worthy in this case (Bode *et al*., 2000, 2002). OSMAC is carried out by using different media and/or different cultivation parameters such as UV-light irradiation, pH, temperature, and oxygen. This could yield further new metabolites.

7 References and notes

Abdel-Lateff, A.; König, G. M.; Fisch, K. M.; Höller, U.; Wright, A. D. *J. Nat. Prod*. **2002**, 65, 1605-1611.

Abdel-Lateff, A.; König, G. M.; Wright, A. D. *J. Nat. Prod*. **2003**, *66*, 706-708.

Abdel-Lateff, A.; Fisch, K. M.; König, G. M.; Wright, A. D. *Planta Med.* **2003**, *69*, 831-834.

Abe, N.; Murata, T.; Hirota, A. *Biosci. Biotechnol. Biochem*. **1998a**, *62*, 2120-2126.

Abe, N.; Murata, T.; Hirota, A. *Biosci. Biotechnol. Biochem.* **1998b**, *62*, 661-666.

Abe, N.; Murata, T.; Yamamoto, K.; Hirota, A. *Tetrahedron Lett.* **1999**, *40*, 5203-5206.

Abe, N.; Sugimoto, O.; Tanji, K.-I.; Hirota, A. *J. Am. Chem. Soc*. **2000**, *122*, 12606-12607.

Aldrige, D. C.; Giles, D.; Turner, W. B. *J. Chem. Soc.* **1971**, 3888-3891.

Amarowicz. R.; Naczk, M.; Shahidi, F. *J. Agric. Food Chem*. **2000**, *48*, 2755-2759.

Andrade, R.; Ayer, W. A.; Mebe, P. P. *Can. J. Chem*. **1992**, *70*, 2526-2535.

Andrade, R.; Ayer, W. A.; Trifonov, L. S. *Can. J. Chem*. **1996**, *74*, 371-379.

Aoyama, T.; Nakakita, Y.; Nakagawa, M.; Sakai, H. *Agric. Biol. Chem*. **1982**, *46*, 2369-2371.

Ayer, W. A.; Trifonov, L. S. *J. Nat. Prod*. **1993**, *56*, 85-90.

Baute, M. A.; Deffieux, G.; Baute, R.; Neveu, A. *J. Antibiot*. **1978**, *11*, 1099-1101.

Baute, R.; Deffieux, G.; Baute, M. A.; Filleau, M. A.; Neveu, A.; *Tetrahedron Lett.* **1976**, 3943-3944.

Belofsky, G. N.; Gloer, K. B.; Gloer, J. B.; Wicklow D. T.; Dowd, P. F. *J. Nat. Prod.* **1998**, *61*, 1115-1119.

Belofsky, G. N.; Anguera, M.; Jensen, P. R.; Fenical, W.; Köck, M. *Chem. Eur. J.* **2000**, *6*, 1355-1360.

Biabani, M. A. F.; Laatsch, H. *J. Prakt. Chem.* **1998**, *340*, 589-607.

Blois, M. S. *Nature* **1958**, 181, 1199-1200.

Blunt, J. W.; Copp, B. R.; Munro, M. H. G.; Northcote, P. T.; Prinsep, M. R. *Nat. Prod. Rep.* **2003**, *20*, 1–48

Bode, H. B.; Bethe, B.; Höfs, R.; Zeeck, A. *Eur. J. Biochem.* **2002**, *3,* 619-627.

Bode, H. B.; Walker, M.; Zeeck, A. *Eur. J. Org. Chem.* **2000**, 1451-1456.

Brauers, G.; Ebel, R.; Edrada, R. A; Wray, V.; Berg, A.; Gräfe, U.; Proksch, P. *J. Nat. Prod.* **2001**, *64*, 651-652.

Brauers, G.; Edrada, R. A.; Ebel, R.; Proksch, P.; Wray, V.; Berg, A.; Gräfe, U.; Schächtele, C.; Totzke, F.; Finkenzeller, G.; Marme, D.; Kraus, J.; Münchbach, M.; Michel, M.; Bringmann, G.; Schaumann, K. *J. Nat. Prod.* **2000**, *63*, 739-745.

Buckingham, J. D. (ed.) *Chemical dictionaries on CD-ROM. Natural Products. Version 7.1* **2001**.

Chen, C.; Imamura, N.; Nishijima, M.; Adachi, K.; Sakai, M.; Sano, H. *J. Antibiot*. **1996**, *49*, 998-1005.

Chu, M.; Truumees, I.; Mierzwa, R.; Terracciano, J.; Patel, M.; Loebenberg, D.; Kaminski, J. J.; Das, P.; Puar, M. S. *J. Nat. Prod*. **1997**, *60*, 525-528.

Cooney, J. M.; Lauren, D. R. *J. Nat. Prod.* **1999**, *62*, 681-683.

Corey, E. J.; Slomp, G.; Dev, S.; Tobinaga, S.; Glazier, E. R. *J. Am. Chem. Soc*. **1958**, *80*, 1204-1206.

Cueto, M.; Jensen, P. R.; Kauffman, C.; Fenicle, W.; Lobkovsky, E.; Clardy, J. *J. Nat. Prod*. **2001**, *64*, 1444-1446.

Culter, H. G.; Culter, S. J.; Ross, S. A.; El Sayed, K.; Dugan, F. M.; Barlett, M. G.; Hill, A. A.; Hill, R. A.; Parker, S R. *J. Nat. Prod*. **1999**, *62*, 137-139.

Dekebo, A.; Lang, M.; Polborn, K.; Dagne, E.; Steglich, W. *J. Nat. Prod*. **2002**, *65*, 1252-1257.

Dewick P. M. *Medicinal Natural Products, a biosynthetic approach.* WILEY Press, London, **2002**.

Devys, M.; Barbier, M.; Bousquet, J.-F.; Kollmann, A. *Phytochemistry* **1994**, *35*, 825-826.

Devys, M.; Barbier, M.; Bousquet, J.-F.; Kollmann, A. *Z. Naturforsch.* **1992***, 47c,* 779-781.

Duguid, R. J.; Morrison, H . *J. Am. Chem. Soci.* **1991**, *113*, 1271-1281.

Edrada, R. A.; Heubes, M.; Brauers, G.; Wray, V.; Berg, A.; Gräfe, U.; Wohlfarth, M.; Mühlbacher, J.; Schumann, K.; Sudarsono; Bringmann, G.; Proksch, P. *J. Nat. Prod.* **2002**, *65*, 1598-1604.

Edrada, R. A.; Wray, V.; Berg, A.; Gräfe, U.; Sudarsono, Brauers, G.; Proksch, P. *Z. Naturforsch.* **2000**, *55c*, 218-221.

Eberle, J.; Seibl, R. *J. Virolog*. *Meth*. **1992**, *40*, 347-356.

Faulkner, D. *J. Nat. Prod. Rep.* **2000a**, *17*, 1-6.

Faulkner, D. *J. Nat. Prod. Rep.* **2000b**, *17*, 7-55.

Faulkner, D. *J. Nat. Prod. Rep.* **2001**, *18*, 1-49.

Feofilova, E. P. *Appl. Biochem. Microbiol*. **2001**, *37*, 124-137.

Fleury, J. P.; Lichtenberger, A. L. J. *Bull. Soc. Chim. France*, **1963**, 565-570.

Frederick, C. B.; Szaniszlo, P. J.; Vickrey, P. E.; Bentley, M, D.; Shive, W. *Biochem. J.* **1981**, *20*, 2436-2436.

Freeman, G. G. *Phytochemistry* **1965**, *5*, 719-725.

Friedrich, A. B.; Merkert, H.; Fendert, T.; Hacker, J.; Proksch, P.; Hentschel, U. *Marine Biology* **1999**, *134*, 461-470.

Gamboa-Angulo, M. M.; Alejos-Gonzále, F.; Esculanta-Erose, F.; García-Sosa, k.; Delagado-Lamas, G.; Peña-Rodríguez, L. M. *J. Nat. Prod*. **2000**, *63*, 1117-1120.

Grever, M. R.; Schepartz, S. A.; Chabner, B. A. *Semin*. *Oncol*. **1992**, *19*, 622-638.

Harris, C. M.; Roberson, J. S.; Harris, T. M. *J. Am. Chem. Soc*. **1976**, *98*, 5380-5386.

Hawksworth D. L. *Mycol. Res.* **1991**, *95*, 641-655.

Hawksworth D. L.; Rossman A. Y. *Phytopathology* **1997**, *87*, 888-891.

Hayashi, K.; Suzuki, K.; Kawaguchi, M.; Nakajima, T.; Suzuki, T.; Numata, M.; Nakamura, T. *Biosci. Biotech. Biochem*. **1995**, *59*, 319-320.

Hein, S. M.; Gloer, J. B.; Koster, B.; Malloch, D. *J. Nat. Prod*. **1998**, *61*, 1566-1567.

Hirota, A.; Morimitsu, Y.; Hojo, H. *Biosci. Biotech. Biochem*. **1997**, *61*, 647-650.

Hill, J. G.; Nakashima, T. T.; Vederas, J. C. *J. Am. Chem. Soc*. **1982**, *104*,1745-1748.

Höller, U.; König, G. M.; Wright A. D. *J. Nat. Prod*. **1999**, *62*, 114-118.

Höller, U.; Wright A. D.; Matthée, G. F.; König, G. M.; Draeger, S.; Aust, H.-J.; Schulz, B. *Mycol. Res*. **2000**, *104*, 1354-1365.

Isaka, M.; Jaturapat, A.; Rukseree, K.; Danwisetkanjana, K.; Tanticharoen, M.; Thebtaranonth , Y. *J. Nat. Prod*. **2001**, *64*, 1015-1018.

Iwamoto, C.; Yamada, T.; Ito, Y.; Minoura, K.; Numata, A. *Tetrahedron* **2001**, *57*, 2997-3004.

Jadulco, R.; Brauers, G.; Edrad, R. A.; Ebel, R.; Wray, V.; Sudarsono; Proksch, P. *J. Nat. Prod.* **2002**, 65, 730-733.

Jadulco, R.; Proksch, P.; Wray, V.; Sudarsono; Berg, A.; Gräfe, U. *J Nat Prod*, **2001**, *64*, 527-530.

Jiang, Z.; Barret, M.-O.; Boyd, K. G.; Adams, D. R.; Boyd, A. S. F.; Burgess, J. G. *Phytochemistry* **2002**, *60*, 33-38.

Kameoka, H.; Miyazawa, M.; Haze, K. *Phytochemistry*, **1975**, *14*, 1676-1677.

Kirsch, G.; König, G. M.; Wright, A. D.; Kaminsky R. *J. Nat. Prod*. **2000**, *63*, 825-829.

Kohlmeyer, J.; Kohlmeyer, E. In *Marine Mycology, The higher fungi; Academic press*; New York, San Francisco, London, **1979**; pp, 54-69.

Kohlmeyer, J. *Veröff. Inst. Meeresforsch. Bremerh.* **1974**, *5*, 339-356.

Kohlmeyer, J.; Volkmann-Kohlmeyer, B. *Bot. Marina* **1991**, *34*, 1-61.

Komatsu, K.; Shigemori, H.; Kobayashi, J. *J. Org. Chem.* **2001**, *66*, 6189-6192.

König, G. M.; Wright, A. D. *Planta Med*. **1996**, *62*, 193-211.

König, G. M.; Wright, A. D. *Trends in marine biotechnology*. In: *Drug Discovery from Nature*. Grabley, S.; Thiericke, R. editors, Springer Verlag. Berlin **1999**. P. 180-187.

Li, Y.; Li, X. F.; Kim, D. S.; Choi, H. D.; Son, B. W. *Arch. Pharm. Res*. **2003**, *26*, 21-23.

Liberra, K.; Lindequist, U.; *Pharmazie* **1995**, *50*, 583-588.

Lin, W.; Brauers, G.; Ebel, R.; Wray, V.; Berg, A.; Sudarsono; Proksch, P. *J. Nat. Prod.* **2003**, *66*, 57-61.

Lorenz, P.; Jensen, P. R.; Fenical, W. *Nat. Prod. Lett.* **1998**, *12*, 55-60.

Macías, F. A.; Varela, R. M.; Simonet, A. M.; Culter, H. G.; Culter, S. J.; Eden, M. A.; Hill, R. A. *J. Nat. Prod*. **2000**, *63*, 1197-1200.

Makino, M.; Endoh, T.; Ogawa, Y.; Watanabe, K.; Fujimoto, Y. *Heterocycles* **1998**, *48*, 931-1934.

Malmstrøm, J.; Christophersen, C.; Barrero, A. F.; Oltra, J. E.; Justicia, J.; Rosales, A. *J. Nat. Prod*. **2002**, *65*, 364-367.

Mandala, S. M..; Thornton, R. A.; Frommer, B. R.; Curotto, J. E.; Rasidsky, W.; Kurtz, M. B.; Giacobbe, R. A.; Bills, G. F.; Cabello, M. A.; Martin, I.; Pelaez, F.; Harris, G. H. *J. Antibiot.* **1995**, *48*, 349-356.

Matsumoto, T.; Ishiyama, A.; Yamaguchi, Y.; Masuma, R.; Ui, H.; Shiomi, K.; Yamada, H.; Ōmura, S. *J. Antibiot.* **1999**, *52*, 754-757.

McGahren, W. *J. Am. Chem. Soc*. **1969**, *91*, 157

Merck Index, www.ulb.uni-bonn.de/Cdroms/datenbanken.htm, monographs number 4467.

Molecular Modeling of compounds **22-25** were calculated by conformation search (grid scan) using a MMFF force field as implemented in the Cerius^2 4.0 (MSI) molecular modeling software package. The models were further refined with 1500 iterations of minimisation. Calculations were performed using a Silicon Graphics O2 workstation (Irix 6.5.6).

Morel, C.; Séraphin, D.; Oger, J.- M.; Litaudon, M.; Sévenet, T.; Richomme, P.; Bruneton, J. *J. Nat. Prod*. **2000**, *63*, 1471-1474.

Meyer, B. N.; Ferrigni, N. R.; Putnam, J. E.; Jacobsen, L. B.; Nichols, D. E.; McLaughlin, J. L. *Planta Med*. **1982**, *45*, 31-34.

Matthée, G. F. Dissertation at Gemeinsame Naturwissenschaftlichen Fakultät, Technischen Universität Carolo-Wilhelmina, Braunschweig, Deutschland, **1999**.

Nicolaou, K. C.; Vassilikogiannakis, G.; Simonsen, K. B.; Baran, P. S.; Zhong, Y.-L.; Vidali, V. P.; Pitsinos, E. N.; Couladouros, E. A. *J. Am. Chem. Soc*. **2000**, *122*, 3071-3079.

Osterhage, C.; Kaminsky, R.; König, G. M.; Wright, A. D. *J. Org. Chem*. **2000**, 6412-6417.

Osterhage, C.; König, G. M.; Höller, U.; Wright, A. D. *J. Nat. Prod*. **2002**, *65*, 306-313.

Osterhage, C.; König, G. M.; Jones, P. G.; Wright, A. D. *Planta med.* **2002**, *68*, 1052-1054.

Osterhage, C. Dissertation at Gemeinsame Naturwissenschaftlichen Fakultät, Technischen Universität Carolo-Wilhelmina, Braunschweig, Deutschland, **2001**.

Onocha, P. A.; Okorie, D. A.; Connolly, J. D. Roycroft, D. D. *Phytochemistry* **1995**, *40*, 1183-1189.

Peres, V.; Nagem, T. J.; Oliveira, F. F. *Phytochemistry* **2000**, *55*, 683-710.

Pietra, F. *Nat. Prod. Rep.* **1997**, *14*, 453-464.

Proksch, P.; Edrad, R. A.; Ebel, R. *Appl. Microbiol, Biotechnol.* **2002**, *59*, 125-134.

Puterbaugh, W. H. *J. Org. Chem*. **1962**, *27*, 4010-4015.

Routledge, E. J.; Sumpter, J. P. *Environ. Toxicol. Chem.* **1996**, *15*, 241-248.

Roy, K.; Chatterjee, S.; Deshmukh, S. K.; Vijayakumar, E. K. S.; Ganguli, B. N.; Fehlhaber, H.-W. *J. Antibiot*. **1996**, *49*,1186-1187.

Sassa, T.; Kinoshita, H.; Nukina, M.; Sugiyama, T. *J. Antibiot*. **1998**, *51*, 967-969.

Schulz, B.; Suker, J.; Aust, H. J.; Krohn, K.; Ludewig, K.; Jones, P. G.; Döring, D. *Mycol. Res*. **1995**, *99*, 1007-1015.

Sheldrick, G. M. SHELX97 (*SHELXS and SHELXL*). *Manual of Programs for the Refinement of Crystal Structures*; University of Göttingen, Germany, 1997.

Shu, Y.-Z.;, Ye, Q.; Li, H.; Kadow, K. F.; Hussain, R. A.; Huang, S.; Gustavson, D. R.; Lowe, S. E.; Chang, L.-P.; Pirnik, D. M.; Kodukula, K. *Bioorg. Med. Chem. Lett. Bio* **1997**, *7*, 2295-2298.

Soman, A. G.; Gloer, J. B.; Koster, B.; Malloch, D. *J. Nat. Prod.* **1999**, 62, 659-661.

Smith, C. J.; Abbanat, D.; Bernan, V. S.; Maiese, W. M.; Greenstein, M.; Jompa, J.; Tahir, A.; Ireland, C. M. *J. Nat. Prod*. **2000**, *63*, 142-145.

Son, B. W.; Choi, J. S.; Kim, J. C.; Nam, K. W.; Kim, D.-S.; Chung, H. Y.; Kang, J. S.; Choi, H. D. *J. Nat. Prod.* **2002**, *65*, 794-795.

Solis, P. N.; Wright, C. W.; Anderson, M. M.; Gupta, M. P., Phillipson, J. D. *Planta Med*. **1993**, *59*, 250-252.

Sperry, S.; Samuels, G. J.; Crews, P. *J. Org. Chem.* **1998**, *63*, 10011-10014.

Steinert, M.; Hentschel, U.; Hacker, J. *Naturwissenschaften* **2000**, *87*, 1-11.

Tabata, N.; Tomoda, H.; Iwai, Y.; Ōmura, S. *J. Antibiot.* **1996**, *49*, 267-271.

Talapatra, S. K.; Karmacharya, B.; De, S. C.; Talapatra, B. *Phytochemistry*, **1988**, *27*, 3929-3932.

Tan, R. X.; Zou, W. X.; *Nat. Prod. Rep*. **2001**, *18*, 448-459.

Takahashi, N.; Tamagawa, K.; Kawai, K.; Fukui, T. *Biol. Pharm. Bull*. **2000**, *23*, 989-994.

Tanaka, M.; Nara, F.; Yamasato, Y.; Ono, Y.; Ogita, T. *J. Antibiot*. **1999**, *52*, 827-830.

Teleman, A.; Hausalo, T.; Tenkanen, M.; Vuorinen. T. *Carbohydr. Res.* **1996**, *280*, 197-208.

Thomas, R. *J. Biochem.* **1961**, *80*, 234-240.

Teshima, Y.; Shin-Ya, K.; Shimazu, A.; Furihata, K.; Chul, H. S.; Furihata, K.; Hayakawa, Y.; Nagai, K.; Seto, H. *J. Antibiot.* **1991**, *44*, 685-687.

Trifonov, L. S.; Bieri, J. H.; Prewo, R.; Dreiding, A. S.; Hoesch, L.; Rast, D. M. *Tetrahedron* **1983**, *39*, 4243-4256.

Trifonov, L. S.; Hilpert, H.; Floersheim, P.; Dreiding, A. S.; Rast, D. M.; Skrivanova, R.; Hoesch, L. *Tetrahedron* **1986**, *42*, 3157-3179.

Trifonov, L.; Bieri, J. H.; Prewo, R.; Dreiding, A. S.; Rast, D. M.; Hoesch, L. *Tetrahedron* **1982**, *38*, 397-403.

Tsantrizos, Y. S.; Ogilive, K. K.; Watson, A. K. *Can. J. Chem.* **1992**, *70*, 2276-2284.

Turner, W. *Fungal Metabolites* 2; Academic Press: London, **1977**; p 26.

Varoglu, M.; Crews, P. *J. Nat. Prod.* **2000**, *63*, 41-43.

Wallin, B.; Rosengren, B.; Shertzer, H. G.; Camejo, G. *Anal. Biochem.* **1993**, *208*, 10-15.

Wang, C.-Y.; Wang, B.-G..; Brauers, G.; Guan, H.-S.; Procksch, P.; Ebel, R. *J. Nat. Prod.* **2002**, *65*, 772-775.

Wang, Y.; Gloer, J. B. *J. Nat. Prod.* **1995**, *58*, 93-99.

Wegner, C.; Schwibbe, M.; König, G. M.; Wright A. D. *Phytochem. Anal*. **2000**, *11*, 1-7.

Wessels, M.; König, G. M.; Wright A. D. *J. Nat. Prod.* **1999**, *62*, 927-930.

Weber, H. A.; Swenson, D. C.; Gloer, J. B. *Tetrahedron Lett.* **1992**, *33*, 1157-1160.

Westenburg, H. E.; Lee, K.-J.; Lee, S. K.; Fong, H. H. S.; Breemen, R. B.; Pezzuto, J. M.; Kinghorn, A. D. *J. Nat. Prod.* **2000**, 63, 1696-1698.

Yamada, T.; Iwamoto, C.; Yamagaki, N.; Yamanouchi, T.; Minoura, K.; Yamori, T.; Uehara, Y.; Andoh, T.; Umemura, K.; Numata, A. *Tetrahedron* **2002**, *58*, 479-487.

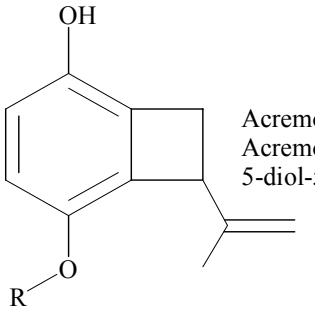
Yan, X.; Chuda, Y.; Suzuki, M.; Nagata, T. *Biosci, Biotechnol. Biochem*. **1999**, *63*, 605-607.

Yen, G.-C., Lee, C.-A. *J. Food Prot*. **1996**, *59*, 1327-1330.

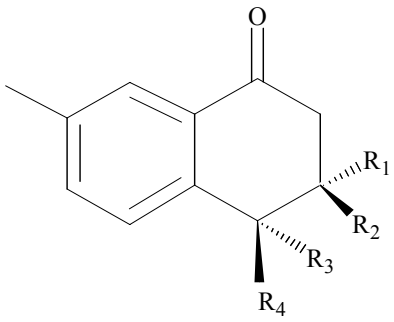
Yen, G.-C.; Chang, Y.-C. *J. Food Prot*. **1999**, *62*, 657-661.

Yen, G. C.; Lee, C. E. *J. Sci. Food Agric*. **1997**, *75*, 326-332.

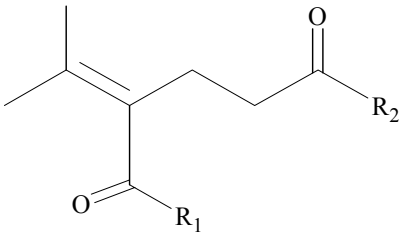
Yen, G.-C.; Chang, Y.-C.; Sheu, F.; Chiang, H.-C. *J. Agric. Food Chem*. **2001**, *49*, 1426-1431.



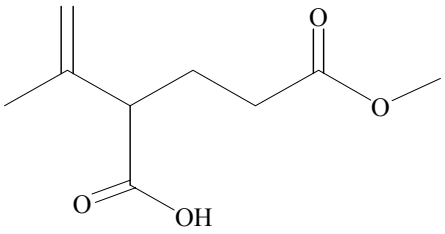
Acremonin A (**1**) 7-isopropenylbicyclo[4.2.0]octa-1,3,5-triene-2, 5-diol, R = H
Acremonin A glucoside (**2**) 7-isopropenylbicyclo-[4.2.0]octa-1,3,5-triene-2, 5-diol-5- ß-D-gluopyranoside, R = ß-D-glucopyranose



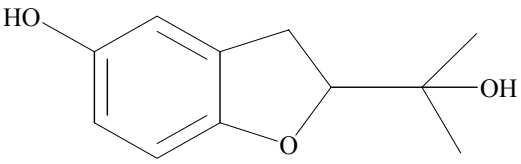
(3*R**,4*S**)-3,4-dihydroxy-7-methyl-3,4-dihydro-1(2*H*)-naphthalenone (**3**)
$R_1 = R_3 = H$; $R_2 = R_4 = OH$
(3*S**,4*S**)-3,4-dihydroxy-7-methyl-3,4-dihydro-1(2*H*)-naphthalenone (**4**)
$R_2 = R_3 = H$; $R_1 = R_4 = OH$



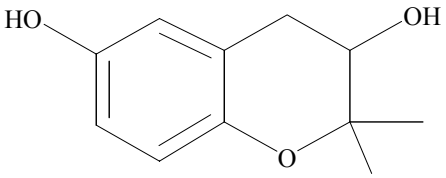
2-(1-Methylethylidene)pentanedioic acid (**5**)
$R_1 = OH$, $R_2 = OH$
pentanedioic acid 2-(1-methylethylidene)-5-methyl ester (**6**)
$R_1 = OH$, $R_2 = OCH_3$
pentanedioic acid 2-(1-methylethylidene)-1-methyl ester (**7**)
$R_1 = OCH_3$, $R_2 = OH$



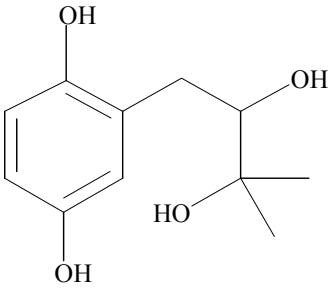
Pentanedioic acid 2-(1- methylethenyl)-5-methyl ester (**8**)



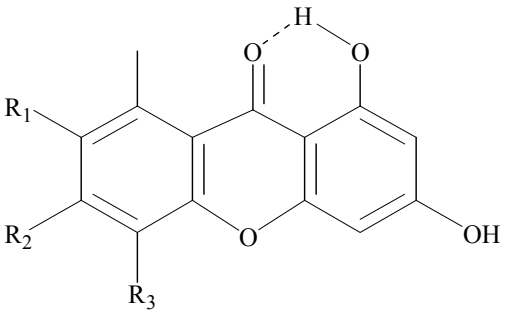
2-(1-Hydroxy-1-methyl)-2,3-dihydrobenzofuran-5-ol (**9**)



2, 2-Dimethyl-chroman-3, 6-diol (**10**)

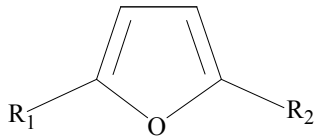


2-(3-Dihydroxy-3-methylbutyl)-benzene-1, 4-diol (**11**)

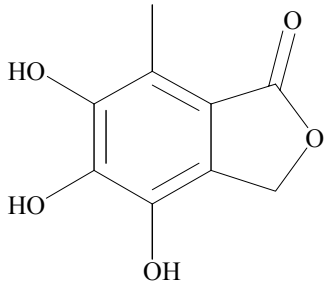


Anomalin A (**12**) 2,3,6,8-tetrahydroxy-1-ethylxanthone
$R_1 = R_2 = OH$, $R_3 = H$
Anomalin B (**13**) 2,3,4,6,8-pentahydroxy-1- methylxanthone
$R_1 = R_2 = R_3 = OH$
3,6,8-Trihydroxy-1-methylxanthone (**14**) $R_1 = R_3 = H$, $R_2 = OH$

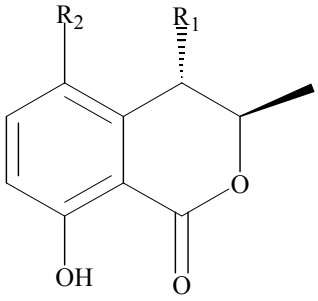
Figure 20. Metabolites **1-14** isolated during this study.



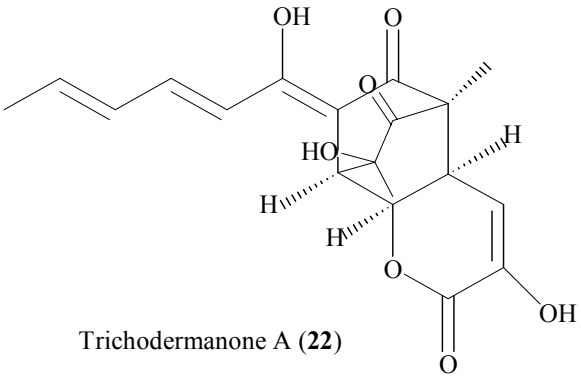
5-(Hydroxymethyl)-2-furanocarboxylic acid
$R_1 = CH_2OH$, $R_2 = COOH$(**15**)
5-(Acetoxymethyl)-2-furanocarboxylic acid
$R_1 = COOH$, $R_2 = CH_2OAc$(**18**)
2-Furanocarboxylic acid
$R_1 = H$, $R_2 = COOH$ (**19**)



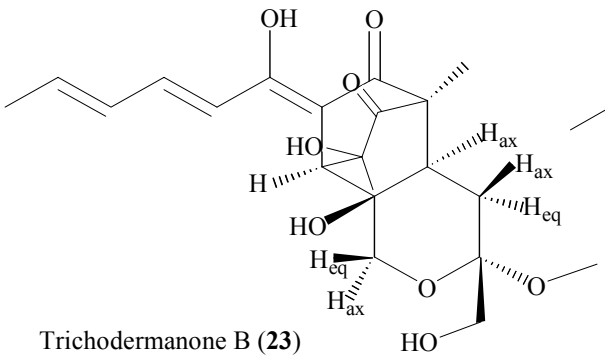
Epicoccone, 4,5,6-trihydroxy-7-methylphthalide (**17**)



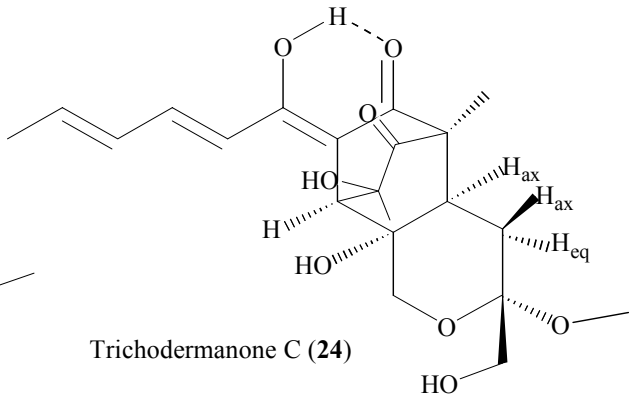
R_1	R_2	
OH	H	(-)-(3R,4S)-4-Hydroxymellein (**20**)
H	OH	(-)-(3R)-5-Hydroxymellein (**21**)



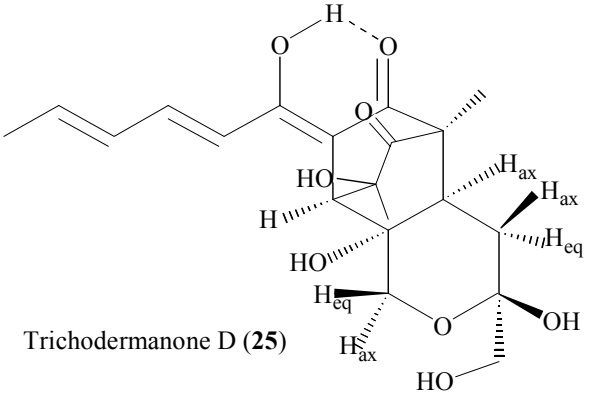
Trichodermanone A (**22**)



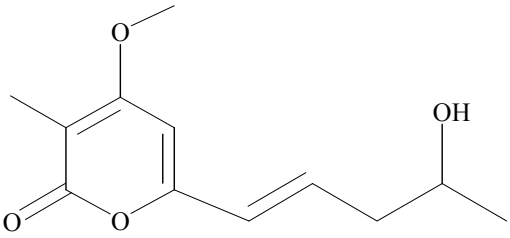
Trichodermanone B (**23**)



Trichodermanone C (**24**)

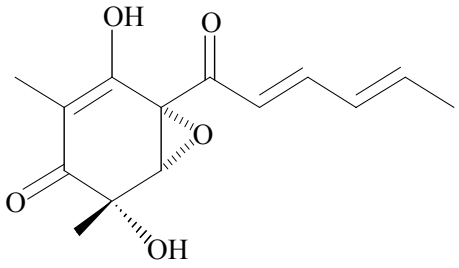


Trichodermanone D (**25**)

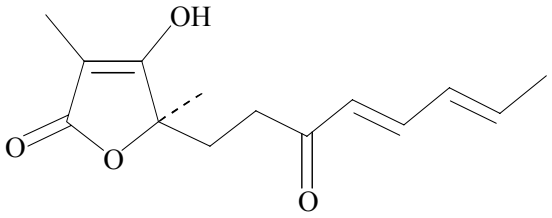


6-(4-Hydroxy-1-pentenyl)-4-methoxy-3-methyl-2H-pyran-2-one (**26**).

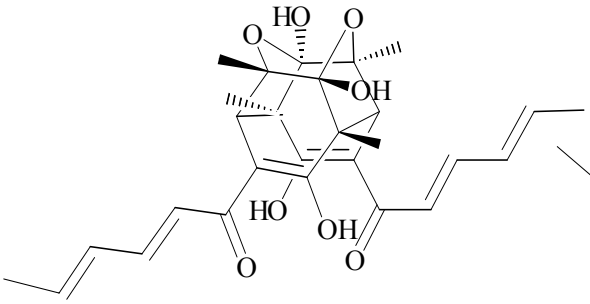
Figure 21. Metabolites **15** and **17**-**26** isolated during this study.



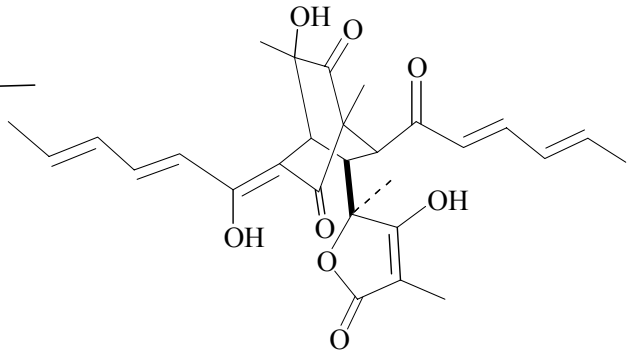
Epoxysorbicillinol (**27**)



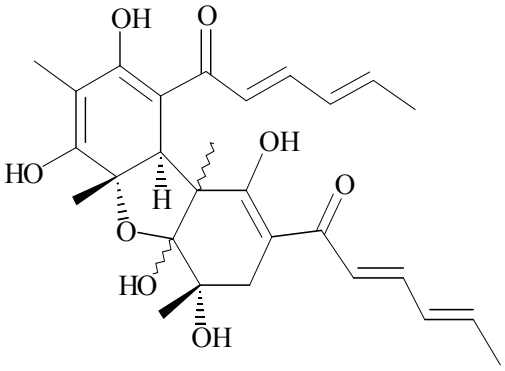
Vertinolide (**28**)



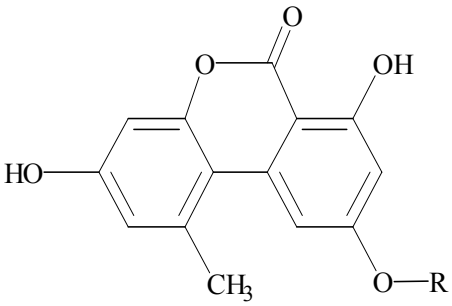
Trichodimerol (**29**)



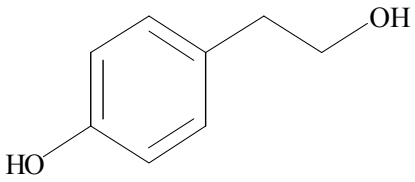
Bislongiquinolide (trichotetronine) (**30**)



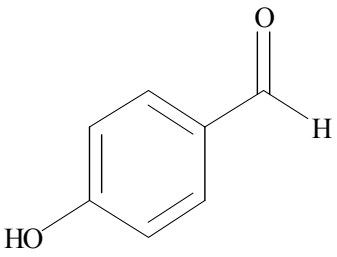
Bisvertinol (**31**)



Alternariol, R = H (**32**)
Alternariol monomethyl ether, R = CH_3 (**33**)



p-Hydroxyphenylethanol (**34**)



p-Hydroxybenzaldehyde (**35**)

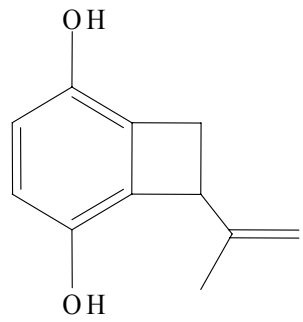
Figure 22. Metabolites **27-35** isolated during this study.

8.2 ^{1}H NMR spectra of selected compounds

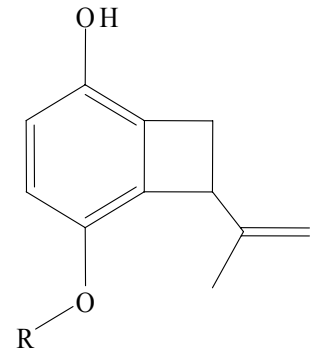
page

Acremonin A (**1**) 112
Acremonin A glucoside (**2**) 112
(3R*,4S*)-3,4-Dihydroxy-7-methyl-3,4-dihydro-1(2*H*)-naphthalenone (**3**) 113
(3S*,4S*)-3,4-Dihydroxy-7-methyl-3,4-dihydro-1(2*H*)-naphthalenone (**4**) 113
2-(1-Methylethylidene)-pentanedioic acid (**5**) 113
Pentanedioic acid, 2-(1-methylethylidene)-5-methyl ester (**6**) 114
Pentanedioic acid, 2-(1-methylethylidene)-1-methyl ester (**7**) 114
Pentanedioic acid, 2-(1-methyl ethenyl)-5-methyl ester (**8**) 114
2-(1-Hydroxy-1-methyl)-2, 3-dihydrobenzofuran-5-ol (**9**) 114
2,2-Dimethyl-chroman-3,6-diol (**10**) 115
2-(3-Dihydroxy-3-methylbutyl)-benzene-1,4-diol (**11**) 115
Anomalin A (**12**) 116
Epicoccone, 4,5,6-trihydroxy-7-methylphthalide (**17**) 116
5-(Acetoxymethyl)-2-furanocarboxylic acid (**18**) 117
(-)-(3R,4S)-4-Hydroxymellein (**20**) 117
(-)-(3R)-5-Hydroxymellein (**21**) 118
Trichodermanone A (**22**) 118
Trichodermanone B (**23**) 119
Trichodermanone C (**24**) 119
Trichodermanone D (**25**) 120
Trichopyranone (**26**) 120
Epoxysorbicillinol (**27**) 121
Vertinolide (**28**) 121
Trichodimerol (**29**) 122
Bislongiquinolide (trichotetronine) (**30**) 122
Bisvertinol (**31**) 123
Alternariol monomethy ether (**33**) 123

Acremonin A, 7-isopropenyl-bicyclo[4.2.0]octa-1,3,5-triene-2, 5-diol (**1**)
300 MHz, $(CD_3)_2CO$

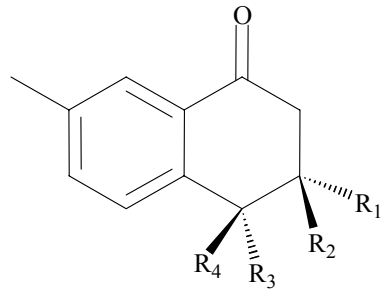


Acremonin A glucoside, 7-isopropenyl-bicyclo[4.2.0]octa-1,3,5-triene-2,5-diol, 5-ß-D-glucopyranoside (**2**)
300 MHz, $(CD_3)_2CO$



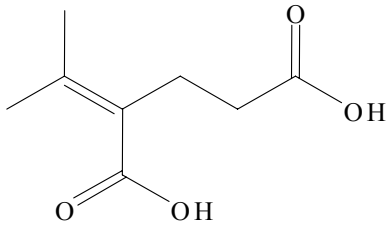
R =ß-D-glucopyranose

(3*R**,4*S**)-3,4-Dihydroxy-7-methyl-3,4-dihydro-1(2*H*)-naphthalenone (**3**)
(3*S**,4*S**)-3,4-Dihydroxy-7-methyl-3,4-dihydro-1(2*H*)-naphthalenone (**4**)
300 MHz, $(CD_3)_2CO$

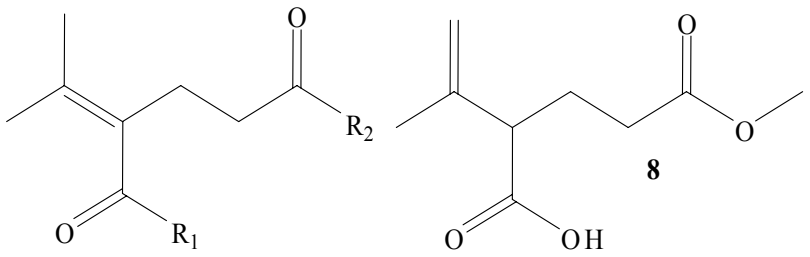


3 $R_1 = R_3 = H$; $R_2 = R_4 = OH$
4 $R_2 = R_3 = H$; $R_1 = R_4 = OH$

2-(1-Methylethylidene)-pentanedioic acid (**5**)
300 MHz,$(CD_3)_2CO$



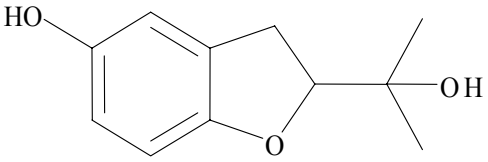
Pentanedioic acid, 2-(1-methylethylidene)-5-methyl ester (**6**)
Pentanedioic acid, 2-(1-methylethylidene)-1-methyl ester (**7**)
Pentanedioic acid, 2-(1-methyl ethenyl)-5-methyl ester (**8**)
300 MHz, $(CD_3)_2CO$



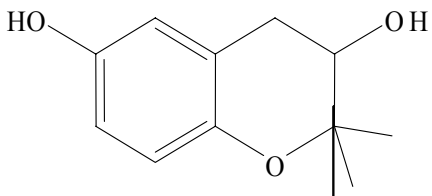
8

6 R_1 = OH, R_2 = OCH_3
7 R_1 = OCH_3, R_2 = OH

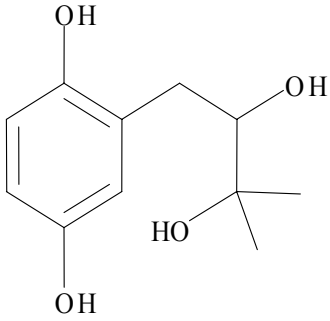
2-(1-Hydroxy-1-methyl)-2,3-dihydrobenzofuran-5-ol (**9**)
300 MHz, $(CD_3)_2CO$



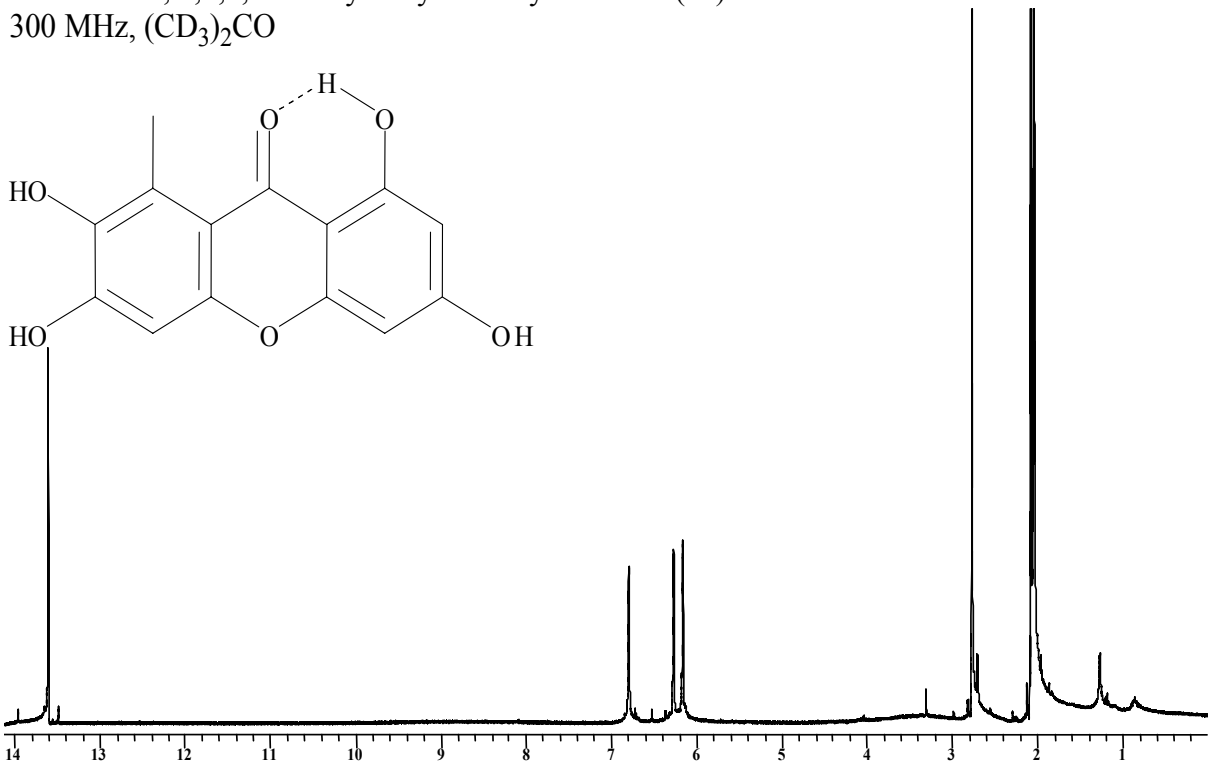
2,2-Dimethyl-chroman-3,6-diol (**10**)
300 MHz, $(CD_3)_2CO$



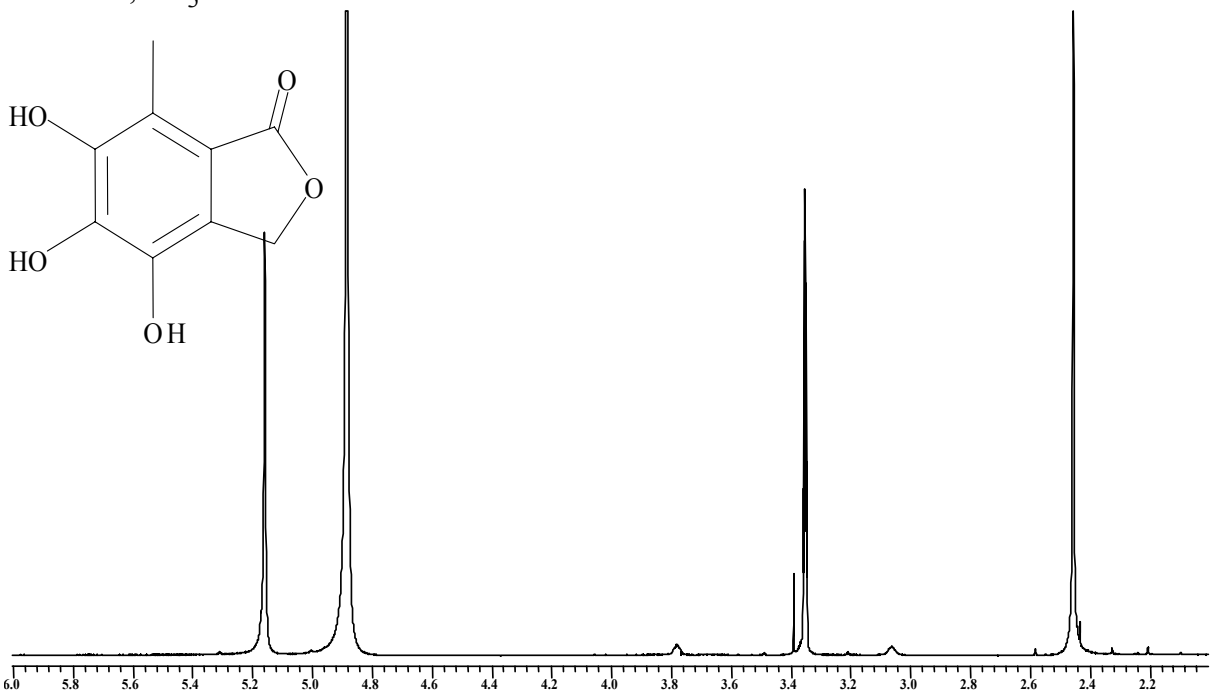
2-(3-Dihydroxy-3-methylbutyl)-benzene-1,4-diol (**11**)
300 MHz, $(CD_3)_2CO$



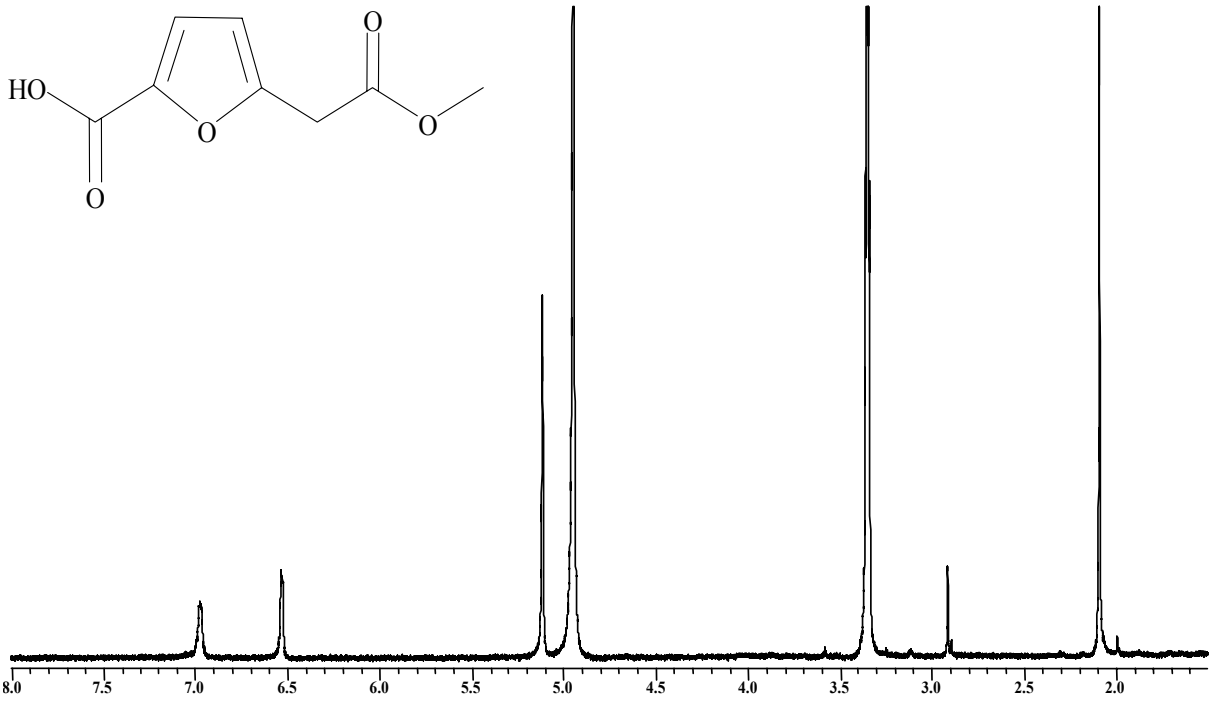
Anomalin A, 2,3,6,8-tetrahydroxy-1-methylxanthone (**12**)
300 MHz, $(CD_3)_2CO$



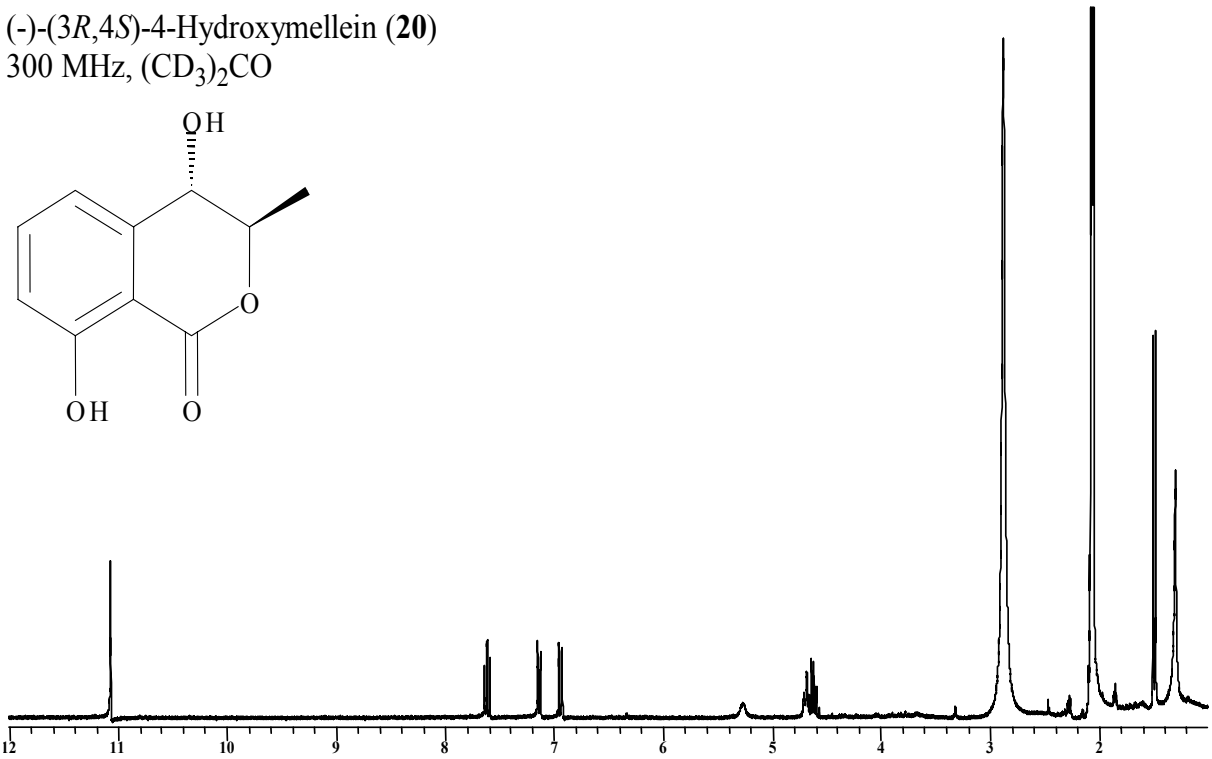
Epicoccone, 4,5,6-trihydroxy-7-methylphthalide (**17**)
300 MHz, CD_3OD



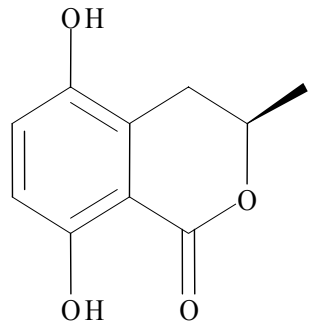
5-(Acetoxymethyl)-2-furanocarboxylic acid (**18**)
300 MHz, CD_3OD



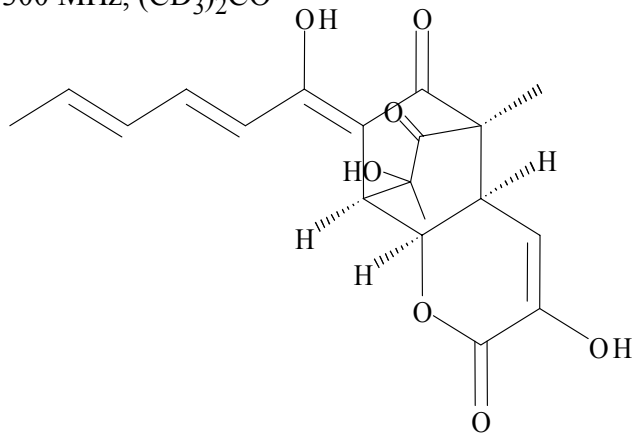
(-)-(3*R*,4*S*)-4-Hydroxymellein (**20**)
300 MHz, $(CD_3)_2CO$



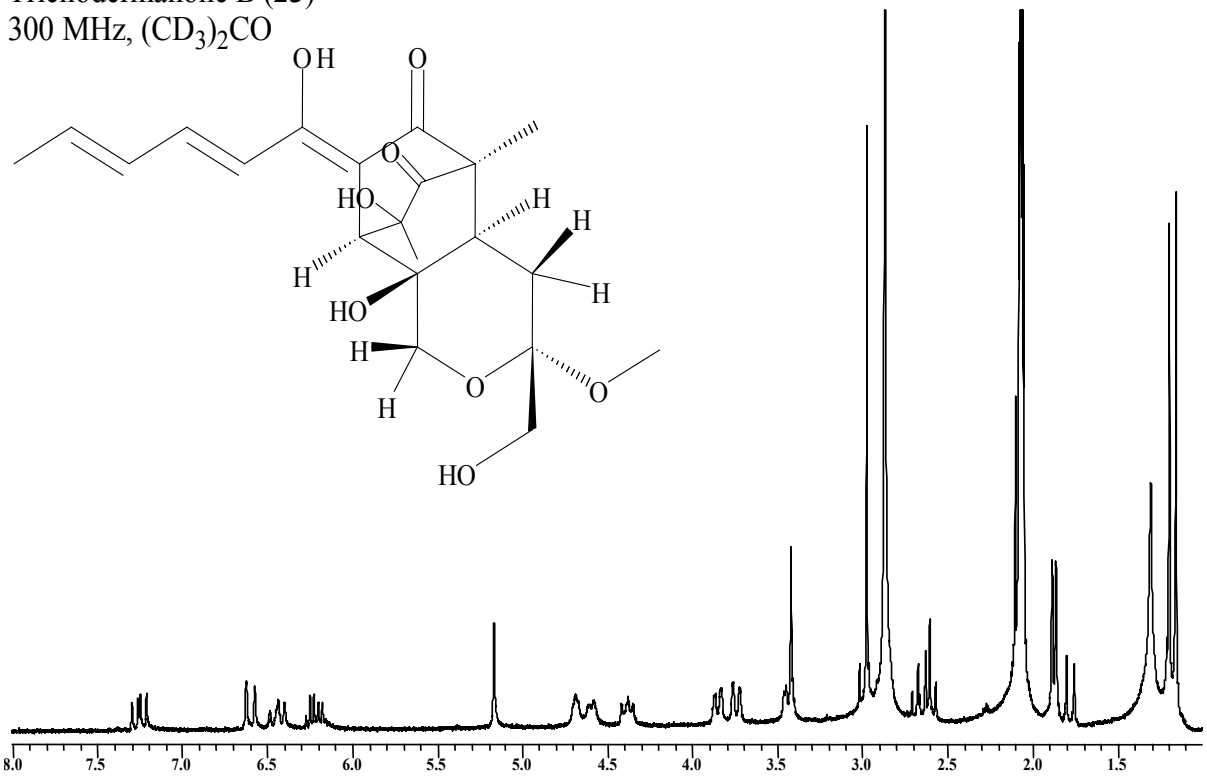
(-)-(3*R*)-5-Hydroxymellein (**21**)
300 MHz, $(CD_3)_2CO$



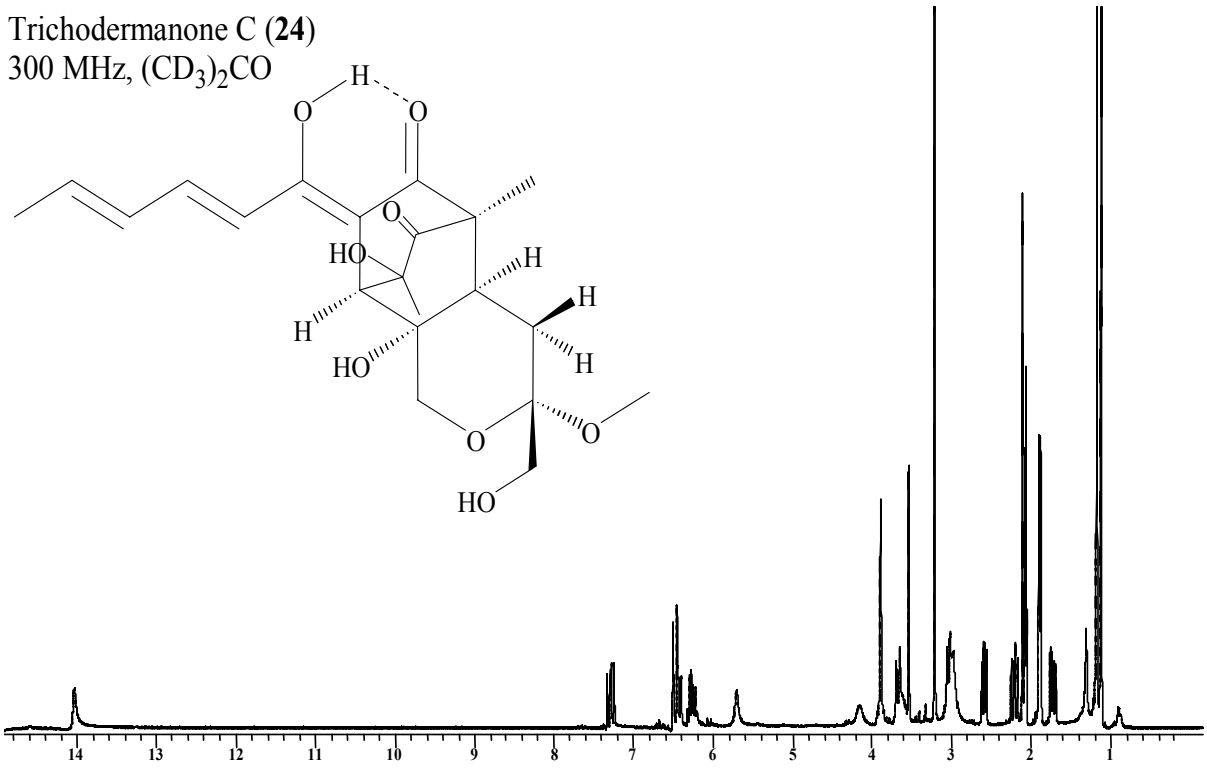
Trichodermanone A (**22**)
300 MHz, $(CD_3)_2CO$



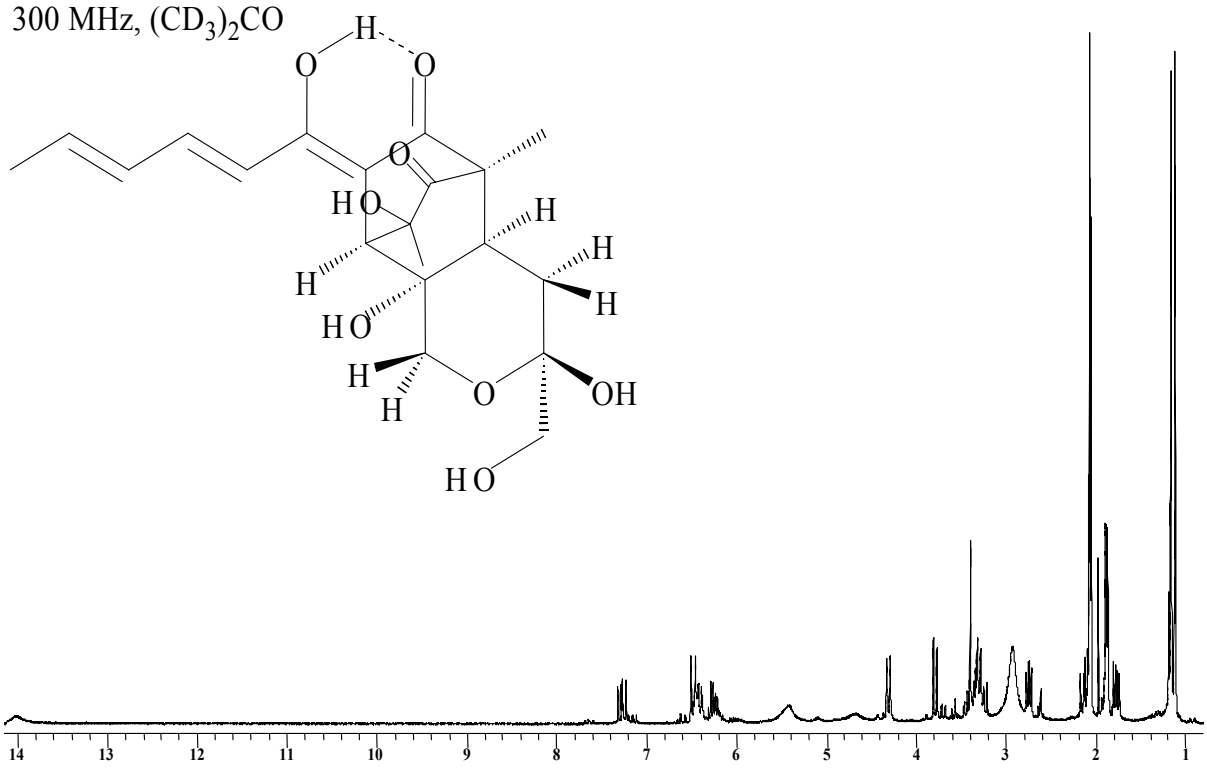
Trichodermanone B (**23**)
300 MHz, $(CD_3)_2CO$



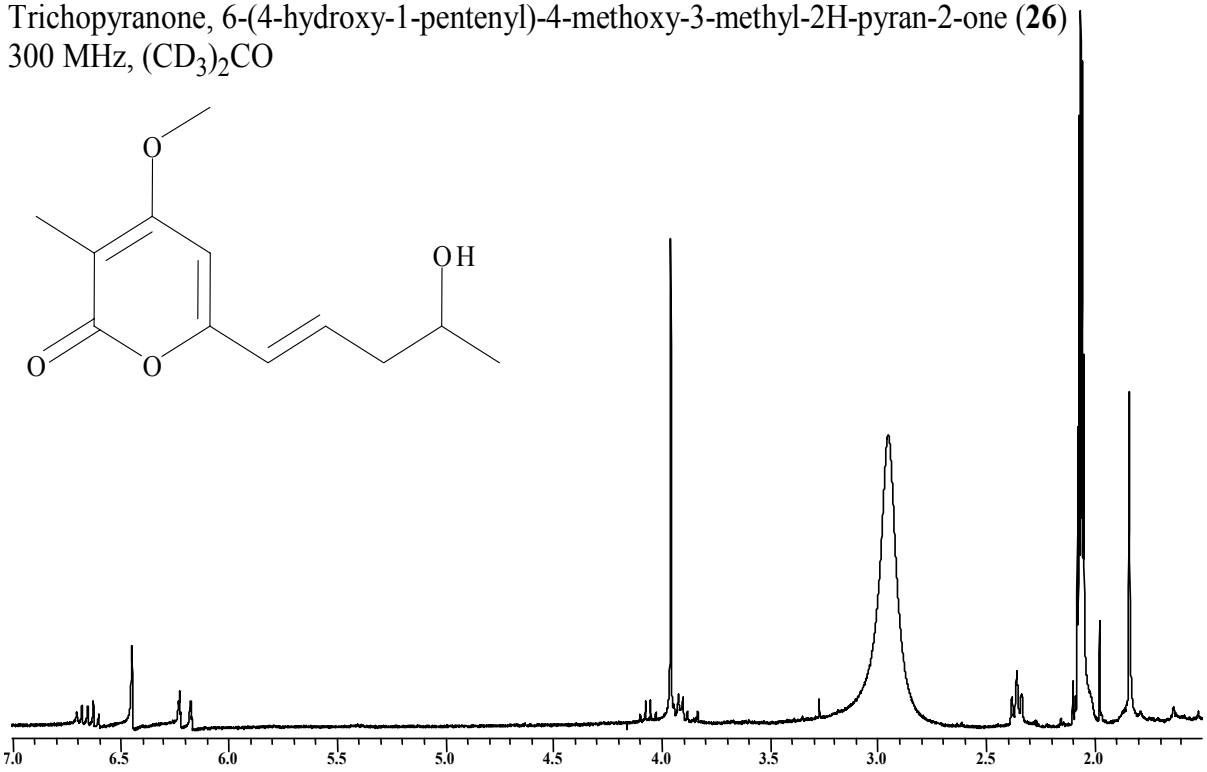
Trichodermanone C (**24**)
300 MHz, $(CD_3)_2CO$



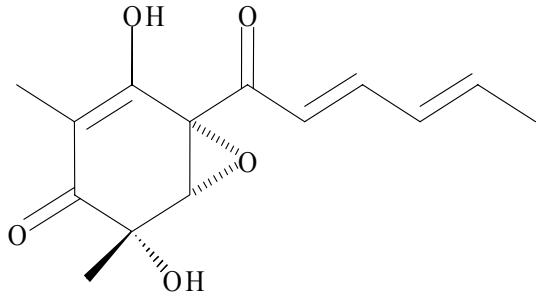
Trichodermanone D (**25**)
300 MHz, $(CD_3)_2CO$



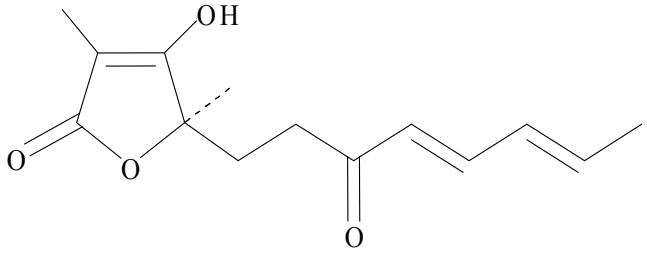
Trichopyranone, 6-(4-hydroxy-1-pentenyl)-4-methoxy-3-methyl-2H-pyran-2-one (**26**)
300 MHz, $(CD_3)_2CO$



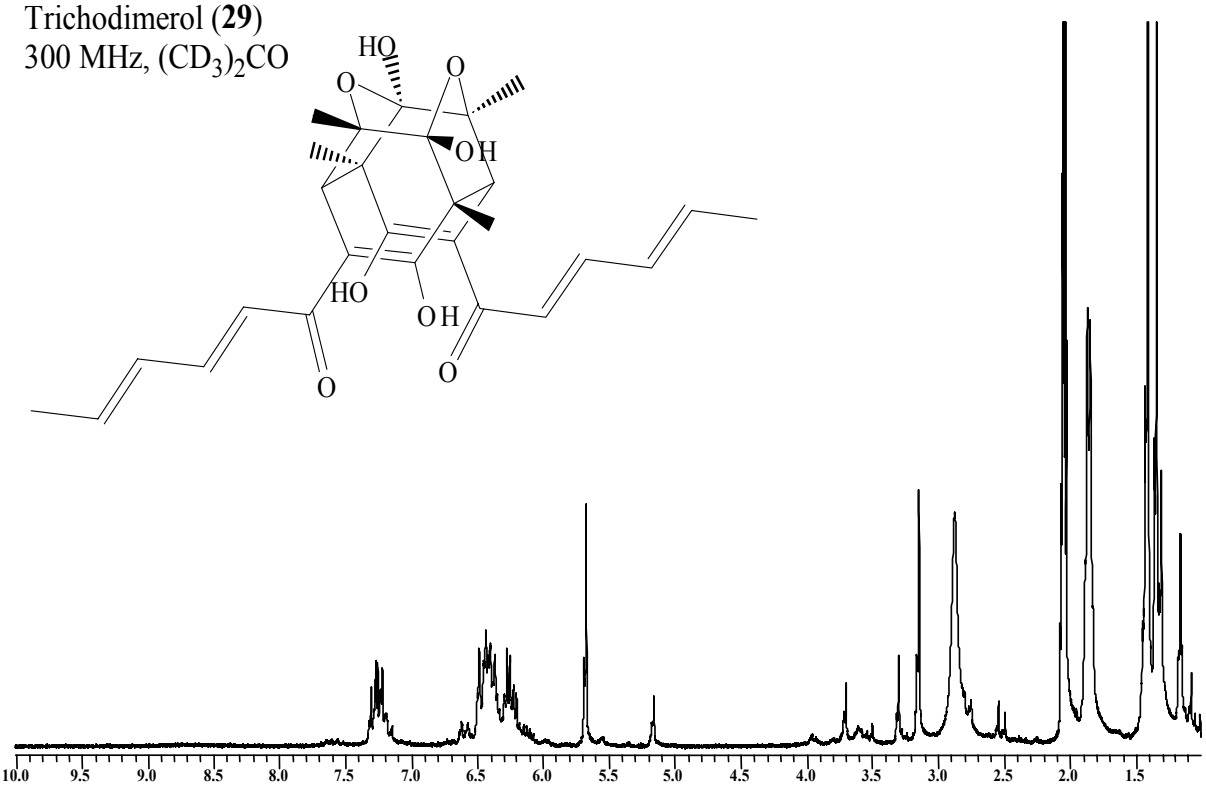
Epoxysorbicillinol (**27**)
300 MHz, $(CD_3)_2CO$



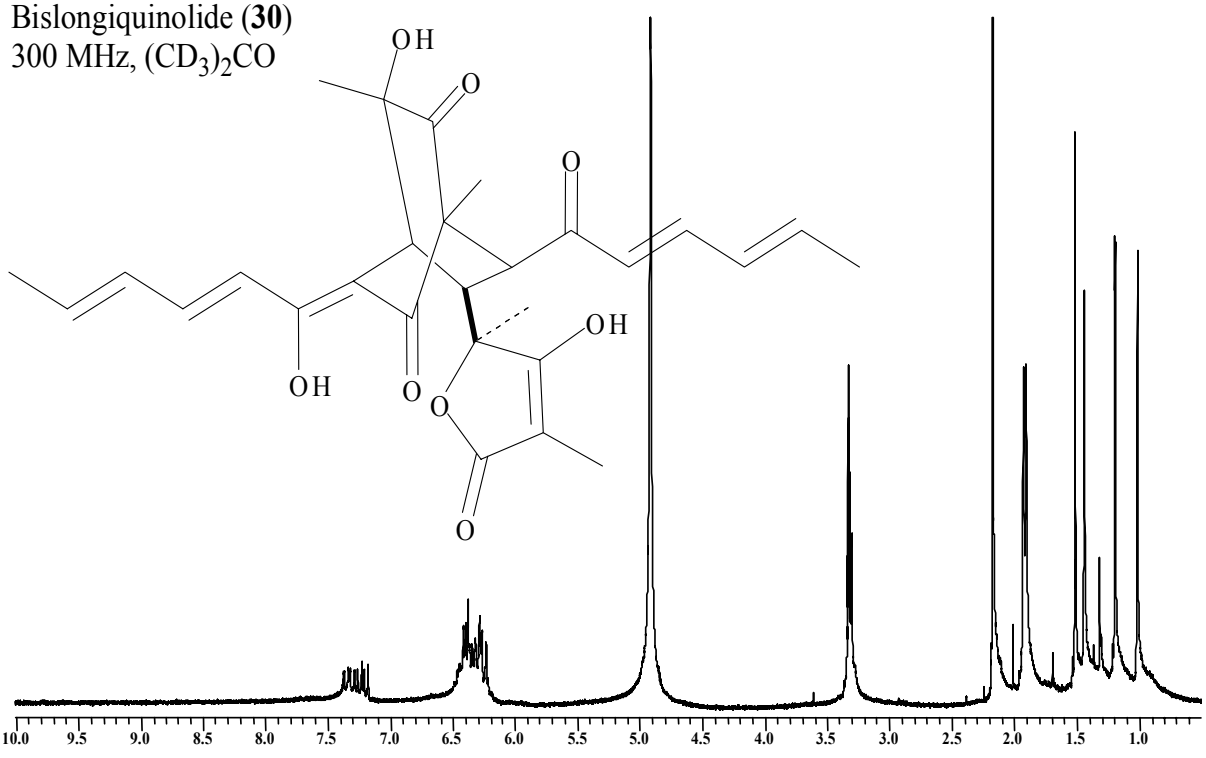
Vertinolide (**28**)
300 MHz, $(CD_3)_2CO$



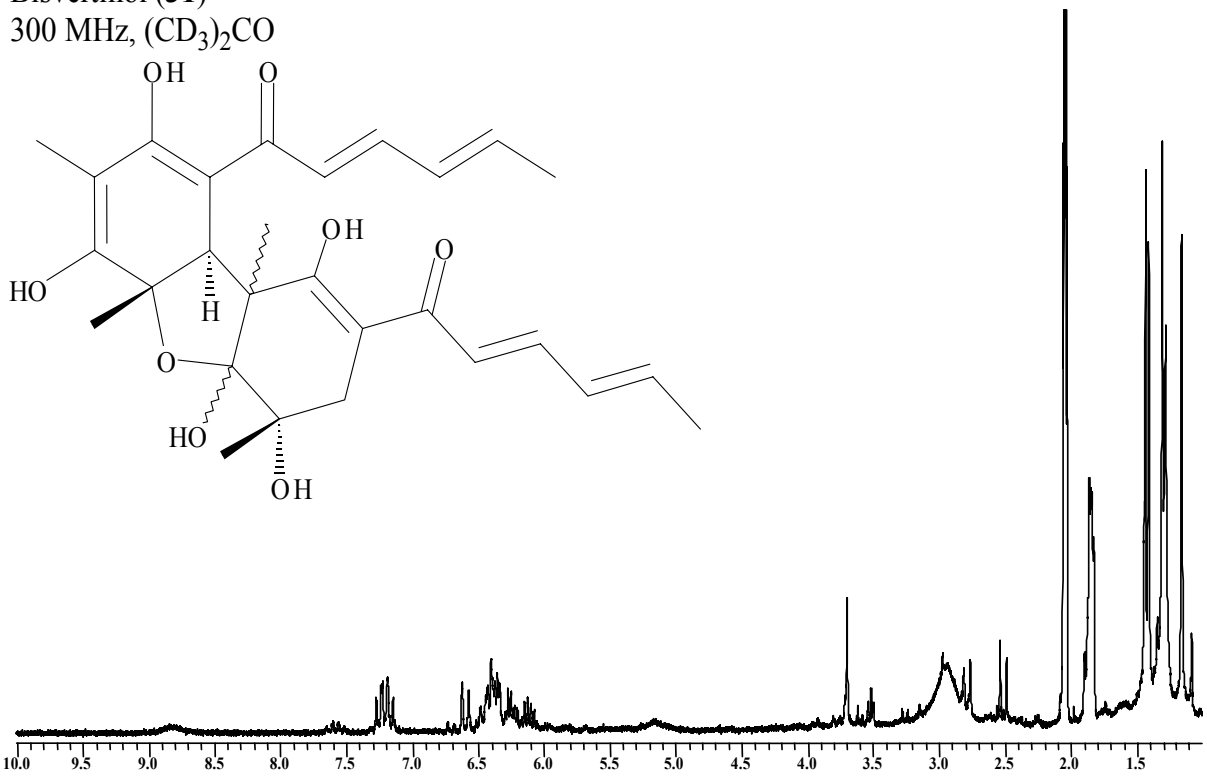
Trichodimerol (**29**)
300 MHz, $(CD_3)_2CO$



Bislongiquinolide (**30**)
300 MHz, $(CD_3)_2CO$



Bisvertinol (**31**)
300 MHz, $(CD_3)_2CO$



Alternariol monomethy ether (**33**)
300 MHz, $(CD_3)_2CO$