Isolation, Structure Elucidation and Evaluation of Anti-inflammatory and Anti-infectious Activities of Fungal Metabolites

Dissertation

Zur Erlangung des akademischen Grades doctor rerum naturalium

(Dr. rer. nat)

Vorgelegt dem Rat der Chemisch-Geowissenschaftlichen Fakultät der

Friedrich-Schiller-Universität Jena

Von Kemami Wangun, Hilaire Vignie

geboren am 08.11.1973 in Bangoua (Kamerun)

Gutachter

1. Prof. Dr. Christian Hertweck
2. Prof. Dr. Rainer Beckert
3. Prof. Dr. Georg Pohnert

Tag der öffenlichen Verteidigung: 25. 10. 2006

an meine Familie

Danksagung

Mein besonder Dank gilt Herrn Prof. Dr. Udo Gräfe für die Überlassung des Themas, Herrn Prof. Dr. C. Hertweck für die ständige Diskussionbereitschaft und die hervorragende Betreuung dieser Arbeit.

Ich danke dem DAAD (Deutscher Akademischer Austausch-diensts) für die Gewährung eines Stipendiums (ein Jahr).

Mein Dank gilt ebenfalls allen Mitarbeitern der Abteilung Biomolekulare Chemie des Leibniz-Institut für Naturstoff-Forschung und Infektionsbiologie-Hans-Knöll-Institut für die interessanten Diskussionen sowie für das freundliche Arbeitklima. Besonders bedanke ich mich bei Herrn Dr. P. Gebhardt, Dr. F. Gollmick, und Herrn Dr. K. Ishida, sowie Herrn Dr. A. Berg und Frau Dr. B. Schlegel für die wertvollen fachlichen Diskussionen.

Ich möchte mich weiterhin bei Frau H. Röhrig, Frau G. Schwinger, Frau A. Perner, Frau Rhein, Frau Klose und Frau U. Zscherpe für die Fermentationen und die Testungen, die Aufnahme von Masse und NMR Spektren und für die sprachliche Unterstützung bedanken.

Nicht zuletzt danke ich allen, die mir eine nicht immer fachliche, aber für das Gelingen dieser Arbeit wichtige Unterstützung leisteten. Ich denke hier insbesondere an Herrn Prof. A. E. Nkengfack, an Herrn Prof. B. Ngadjui, sowie an Herrn Dr. J. R. Fotsing.

List of figures

List of tables

List of abbreviations

3α-HSD:	3α-hydroxysteroid dehydrogenase
COSY:	Correlation Spectroscopy
COX:	Cyclooxygenase
CTA:	*N*-cetyl-*N*,*N*-trimethylammoniumbromide
DMSO:	Demethylsulfoxide
DEPT:	Distortionless Enhancement of Polarization Transfer
ESI-MS:	Electrospray Ionisation Mass Spectroscopy
FPP:	Farnesylpyrophosphate
HMBC:	Heteronuclear Multiple Bond Correlation
HMQC:	Heteronuclear Multiple-Quantum Coherence
HPLC:	High Performance Liquid Chromatography
HR-ESIMS:	High Resolution Electrospray Ionisation Mass Spectroscopy
NMR:	Nuclear Magnetic Resonance
NOESY:	Nuclear Overhauser Effect Spectroscopy
NSAID:	Non-steroidal Anti-inflammatory Drugs
PTLC:	Preparative Thin Layer Chromatography
TLC:	Thin Layer Chromatography
TPA:	12-*O*-tretradecanoyphorbol-1,3-acetate
UA:	Uric acid
XO:	Xanthine oxidase

List of publications

H. V. Kemami Wangun, A. Berg, W. Hertel, A. E. Nkengfack & C. Hertweck
Anti-inflammatory and Anti-hyaluronate Lyase Activities of Lanostanoids from *Piptoporus betulinus*. *J. Antibiot*. 2004, **75**, 755-758.

H. V. Kemami Wangun, H. Dörfelt & C. Hertweck
Nebularic Acids and Nebularilactones, Novel Drimane Sesquiterpenoids from the Fungus *Lepista nebularis*. *Eur. J. Org. Chem*. 2006, **7**, 1643-1646.

H. V. Kemami Wangun, A. Härtl, T.T. Kiet & C. Hertweck
Inotilone and Related Phenylpropanoid Polyketides from *Inonotus* sp. and their Identification as Potent COX and XO Inhibitors. *Org. Biomol. Chem*, **4**, 2006, 2545-2548.

H. V. Kemami Wangun & C. Hertweck
Squarrosidine and related *bis*hispidin derivatives from *Pholiota squarrosa* and *Phillinus pini* and their identification as anti-inflammatory agents, in preparation

H. V. Kemami Wangun & C. Hertweck
Epicoccalone, a High oxygenated coumarin polyketide from *Epicoccum* sp. as serine protease inhibitor, in preparation

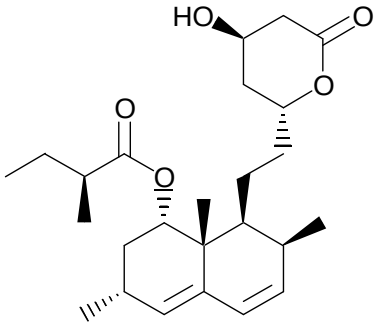
1 Introduction

1.1 Overview

Fungi make an enormous contribution to our life. The role of yeast in the production of alcohol and bread is well known. We consume fungi directly in the form of edible mushrooms and in cheese, which get their characteristic flavour and aroma from the presence of fungi. Fungi are also used for the production of antibiotics and enzymes for use in the food industry. Over the last decades Fungi have been used for the production of recombinant proteins,[1-4] some of which have great therapeutic potential. Although infrequently recognised as important decomposers of organic detritus, Fungi play a significant role in degrading biological matter, such as fallen leaves. In a more negative note some fungi (for example member of the genus *Aspergillus* and *Candida*) are capable of causing serious life threatening infections[5-7] in immuno-compromised patients, and other fungi can be serious environmental contaminants. According to a recent publication less than 5% of fungal species are currently known,[8] suggesting that millions of fungal species and therefore, potentially million of fungal bioactive natural products remain to be discovered.

1.2 Fungi as a source of novel bioactive compounds

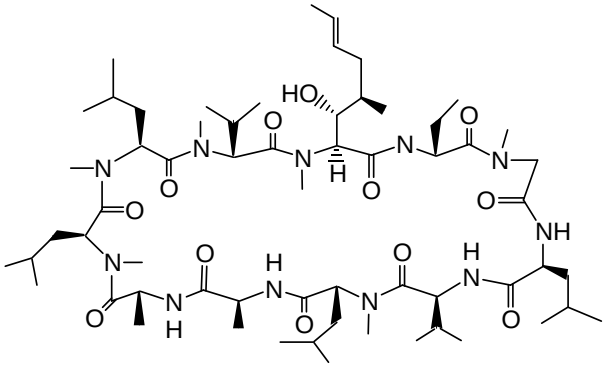
Among all known producers of natural products, microorganism in general and fungi in particular represent a rich source of biologically active metabolites that find Wide-ranging applications in medicine. Moreover, according to a more recent review, of the New Chemical Entities to reach the market as drugs (covering all diseases) during the period 1981-2002, roughly 60% are either natural products, natural product-derived, natural product mimics, or biological (peptides or proteins) of natural origin.[9,10] This is especially evident in the fields of cancer and infectious diseases, where over 60% and 75% of the drugs are of natural origin, respectively. Notable examples of these are from fungal origin. Two examples of useful bioactive fungal metabolites are lovastatin (**1**) and cyclosporin (**2**).



1

Lovastatin was first isolated from the culture broth of *Aspergillus terreus* at Merck & Co. and has been representing the most successful drugs from nature for several years.[11] Later the same compound, but named monacolin K, and related metabolites were isolated from the culture broth of *Monascus ruber*,[12,13] *Penicillium*, and *Aspergillus*,[14] respectively. Lovastatin is an important metabolite because of its activity. It functions by inhibiting the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme of cholesterol biosynthesis (mevalonate pathway)[15,16] with a K_i value 6.4 X 10^{-10}. Therefore, lovastatin (**1**) represents an interesting therapeutic principle for the treatment of hypercholesterolemia. It was introduced in the market by Merck in 1987.

Cyclosporin A (**2**), a cyclic nonribosomal peptide was identified at Sandoz in Switzerland as an antifungal agent isolated from the culture broth of the fungus *Tolypocladium inflatum*, formerly classified as *Trichoderma polysporum*.[17] In the course of more detailed pharmacological studies applying *in vivo* animal models cyclosporin displayed remarkable immunosuppressive properties.[18-22] Only seven years after its first publication cyclosporin was commercialized by Sandoz (now Novartis) under the trade name Sandimmune. Cyclosporin is used in transplantation surgery as a drug that prevents organ rejection, without substantially affecting the immune response causing protection against bacterial infection.



2

In our study we focussed mainly on the discovery of new fungal natural products acting on clinically relevant targets in inflammatory and infectious (microbial and viral) diseases.

1.3 Inflammation

1.3.1 Definition

The symptoms of an inflammation (redness, edema, heat, pain and disturbed tissue function) result from complex pathological processes that include the increase of blood flow and vascular permeability, activation of humoral and cellular defence mechanisms, sensibilization etc. These processes are mediated by a variety of signalling molecules produced by mast cells, macrophages, granulocytes, platelets, lymphocytes, nerve ending, as well as by complement activation factors. The mediators belong to different chemical classes, such as biologically active amines (histamine, serotonin), proteins and peptides (hydrolytic enzymes, cytokines, growth factors, and antibodies), reactive oxygen species (superoxide anion, hydroperoxide, hydroxyl radicals) and lipids (platelet activating factors and prostaglandins).[23-26] These mediators initiate, maintain, aggravate, and modulate the course of a vast number of human disorders. Besides the huge number of proinflammatory players, the complexity of such processes is further increased by the involvement of endogenous antiphlogistics and immunomodulatory mechanisms.

1.3.2 Anti-inflammatory drugs and their mechanism of action

About 3,500 years ago in ancient Egypt, the Ebers papyrus recommended the application of a decoction of the dried leaves of myrtle to the abdomen and back to expel rheumatic pains from the womb. A thousand years later, Hippocrates recommended the juices of the poplar tree for treating eye disease, and those of willow bark to relieve the pain of childbirth and to reduce fever. All of these medicinal remedies contain salicylates. Through the Middle Ages, further use of salicylates were found, such as plasters to treat wound and various other external and internal applications, including the treatment of menstrual pain and the discomfort of dysentery. In China and other parts of Asia, salicylate containing plants have been applied therapeutically. The curative effect of *Salix* and *Spirea* species for example were even known to the early inhabitants of North America and South Africa.[27] Due to these observations acetylsalicylic acid was synthesized guided by the natural

salicylic acid, and nearly 100 years later, aspirin features as the most successful drug worldwide. However, its mechanism of action remained unclear until Ferreira, Moncada and Vane demonstrated in 1971 that aspirin and aspirin-like drugs inhibit the biosynthesis of prostaglandins.[28] The discovery that each and every chemically large group of aspirin-like drugs, so called non-steroidal anti-inflammatory drugs (NSAIDs), all act by inhibiting the enzyme involved in the generation of prostaglandin from arachidonic acid (cyclooxygenase, COX) provided an unifying explanation of their therapeutic actions and firmly established certain prostaglandins as important mediators of inflammatory diseases. Here, it should be noted that arachidonic acid leads not only to COX products, but also to leukotrienes via the 5-lipoxygenase pathway.[29,30] In this context non-steroidal anti-inflammatory drugs such as diclofenac and indomethacin, have emerged as the most commonly used anti-inflammatory agents, including the therapy of rheumatoid arthritis. Many of these drugs target COX-1 rather than COX-2. But due to the side effect including gastrointestinal ulceration and bleeding, renal damage and platelet dysfunction associated only with the inhibition of COX-1,[31-34] the selective inhibition of enzyme subtypes COX-2 has become an important goal. Further works have reported that NSAIDs inhibit 3α-hydroxy steroidehydrogenase (3α-HSD),[35,36] and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced edema.[37,38] More recently kinases were believed to play also a crucial role in the expression and activation of inflammatory mediators.[39]

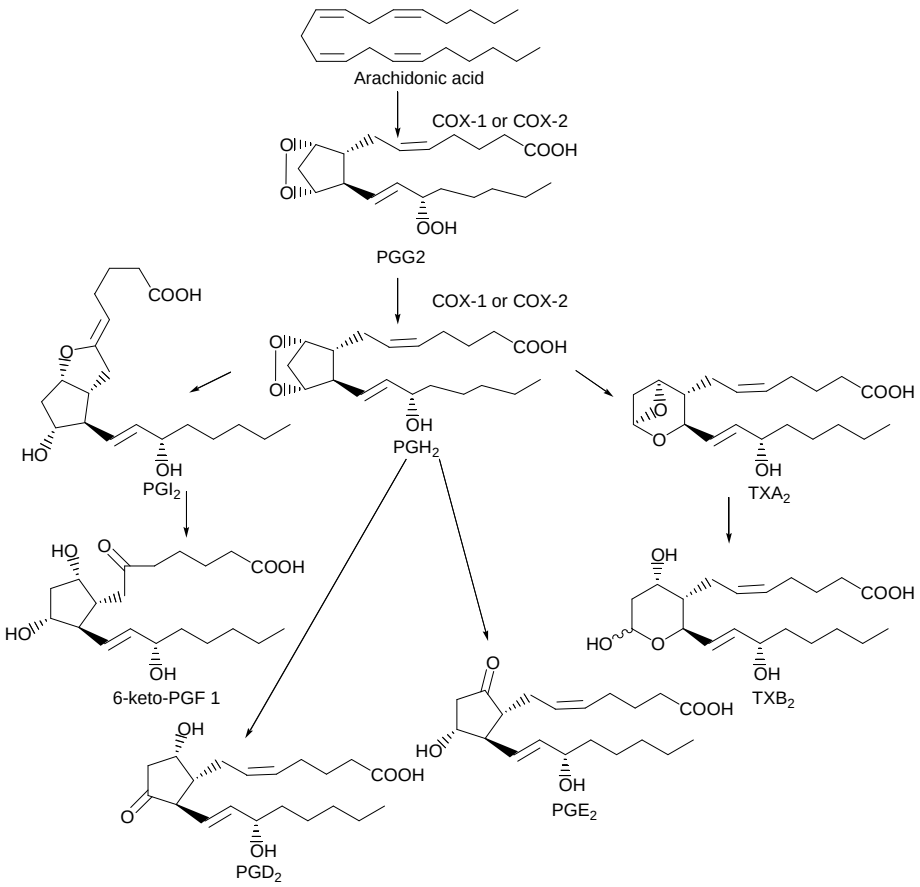


Fig. 1. The arachidonic acid cascade induced by COX

In contrast with rheumatoid arthritis, gouty arthritis is mediated by the crystallisation of uric acid (UA) in the joints.[40,41] Gout can be treated with drugs that either increase the urinary excretion of UA, or with xanthine oxidase (XO) inhibitors that block the terminal step of UA biosynthesis.[42,43] The purine analogue allopurinol is yet the only XO inhibitor in clinical use. Unfortunately, it seems to be associated with an infrequent but severe hypersensitivity.[44] Thus, the search for new potent inhibitors of these enzymes, which could be useful as lead structures for new anti-inflammatory and anti-arthritic therapeutics, plays a pivotal role.

1.3.3 Fungal metabolites as anti-inflammatory agents

To date only a few fungal metabolites including triterpenes from the culture broths of the fungus *Penicillium griseofulvum*,[45] the fruiting body of the fungus *Fomitopsis pinicola*[46] and *Ganoderma colossum*,[47] and cyathane diterpenes from the fruiting body of the fungus *Sarcodon glocaupus*[48] *and Sarcodon scabrosus*[49] have been reported as anti-inflammatory agents. (Fig. 2)

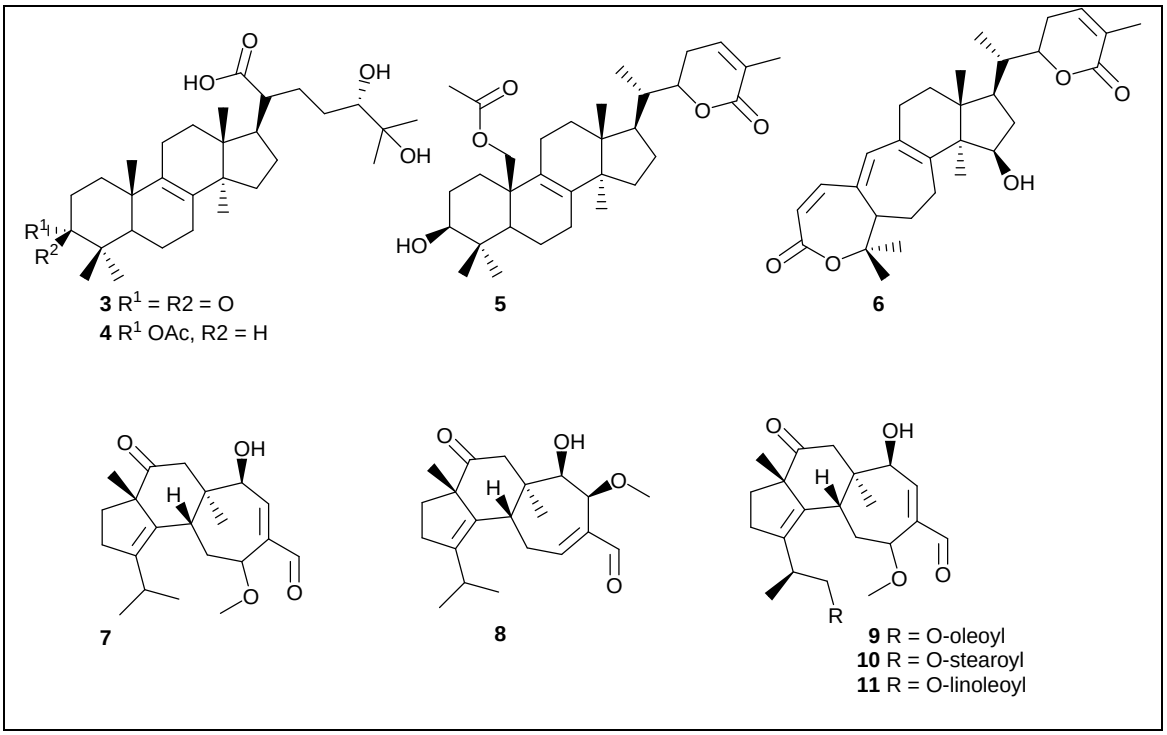


Fig. 2. Selected anti-inflammatory fungal metabolites

All these compounds exhibited their anti-inflammatory activity with diverse mechanisms of action. Fomitopinic acid A (**3**) and B (**4**) from the fruiting body of the fungus *Fomitopsis pinicola* have been reported to inhibit the activities of COX-1 and COX-2 with a significant selectivity for COX-2 for fomitopinic acid A (**3**); Colossolactone A (**5**) and D (**6**) from the fruiting body of the fungus *Ganoderma colossum* inhibited the enzyme 3α-hydroxysteroid dehydrogenase (3α-HSD); and the cyathane diterpenes glaucopine A (**7**), glaucopine B (**8**) from the fruiting body of the fungus *Sarcodon glocaupus*, as well as those isolated from the mushroom *Sarcodon scabrosus*, 19-*O*-oleoylsarcodonin A (**9**), 19-*O*-stearoylsarcodonin A (**10**) and 19-*O*-linoleoylsarcodonin A (**11**) exhibited significant inhibitory activities towards the 12-*0*-tetradecanoylphorbol-13-acetate (TPA)-induced edema of mouse ear.

2 Aim of the study

The aim of this work is to examine selected fungi (filamentous fungi and mushrooms) from special habitats for their metabolic profiles and to evaluate their biological activities (anti-inflammatory, antimicrobial and antiviral, etc.). This includes:

-The selection of strains on the basis of biological pre-screenings.

-The cultivation and the optimisation of the cultivation conditions (nutrient type, pH, temperature and fermentation duration for filamentous fungi) of selected strains and fermentation on a big scale in order to have a large amount of crude extract.

-The isolation and the purification of secondary metabolites from the extracts of microbial fermentation or from those of the fruiting bodies using chromatographic methods (i.e. Sephadex, silica gel column chromatography, PTLC, HPLC etc.).

-The elucidation of the structures of purified metabolites using spectroscopic techniques such as ESI-MS, MS/MS, IR, UV, ^{1}H NMR, ^{13}C NMR, HMBC, COSY, HMQC and NOESY.

-The evaluation of characterised secondary metabolites (known or new) for their inhibitory activity against enzymes involved in inflammation processes such as 3α-hydroxysteroid dehydrogenase (3α-HSD), cyclooxygenase I (COX-1), cyclooxygenase II (COX-II), xanthine oxidase (XO), serine protease (α-chymotrypsin), as well as for their inhibitory activity against growth promotion of various micro-organisms.

3 Results and discussion

3.1 Evaluation and selection of the strains

The selection of strains was based on biological pre-screening (we used 3α-HSD assay for the screening of anti-inflammatory compounds and agar diffusion assay for the pre-screening of antibacterial and antifungal metabolites) and chemical screening using analytical techniques such as TLC, HPLC and HPLC/MS. This procedure led to the selection of eight strains, *Trichoderma crassum* HKI 0471, *Epicoccum sp*. HKI 0470, *Lepista nebularis* HKI 0411, *Aspergillus sp*. HKI 0472, *Inonotus* sp., *Pholiota squarrosa*, *Phellinus pini* DSM 5238 and *Piptoporus betulinus*. The results of pre-screenings are showed in table 1.

Strain	Activity		
	3α-HSD	Antibacterial	Antifungal
Trichoderma crassum HKI 0471	-	++	+++
Epicoccum sp. HKI 0470	++	+	+
Lepista nebularis HKI 0411	-	+++	+++
Aspergillus sp. HKI 0472	++	+	+
Inonotus sp.	+++	+	+
Pholiota squarrosa	+++	+	+
Phellinus pini DSM 5238	+++	+	+
Piptoporus betulinus	+++	++	++

- not active ++ active
+ moderate active +++ strong active

Table 1. Pre-screening's results of selected strains

3.2 Secondary metabolites from selected strains

3.2.1 *Trichoderma crassum*

The fungal genus *Trichoderma* contains cosmopolitan soil-borne fungi, also frequently found on decaying wood,[50] some of which are economically important producer of industrial enzymes[1] and antibiotics or have application as biocontrol agents against plant pathogens. In our effort to isolate some active metabolites from fungal strains, we investigated the species *Trichoderma crassum*, which have not yet been studied.

3.2.1.1 Cultivation, extraction and isolation

Well grown agar cultures of *Trichoderma crassum* HKI 0471 served to inoculate 60 liters of malt medium. The flasks were incubated at 25 ºC during 28 days. The culture broth was filtered and extracted with ethyl acetate. The dark brown crude extract was applied to Sephadex LH-20 and silica gel column chromatography, eluting with a chloroform-methanol gradient. Further purification of fractions using HPLC led to the isolation of the known β-viridin (**13**), α-viridin (**14**), CAF (603) (**19**), 9α-D-arabinofuranoside and cerebroside C (**21**)

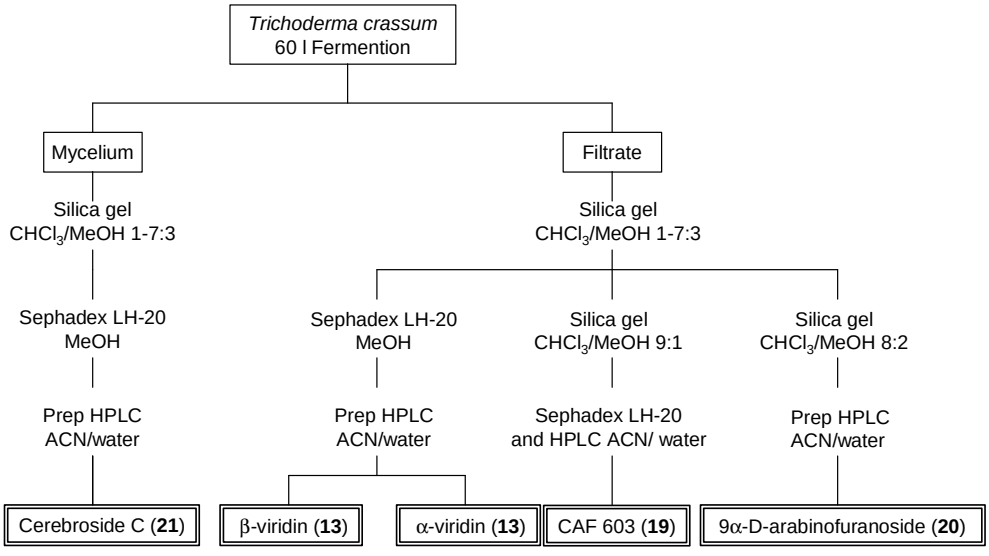
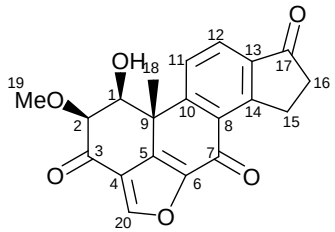


Fig. 3. work up scheme of *Tichoderma crassum* HKI 0471

3.2.1.2 Structure elucidation of β-viridin (**13**)

13 was isolated as a pale yellow powder. The yield was 0.4 mg/l of the culture filtrate of the fungus. On the basis of HR-ESIMS and ^{13}C NMR spectroscopic data, the molecular formula was determined as $C_{20}H_{18}O_6$. DEPT data indicated the presence of two oxymethines. Three ketone functions were indicated by carbon resonances at δ 173.2, 189.0, and 206.3. The structural fragments C(1)HOH-C(2)HOCH$_3$; C(11)H=C(12)H; and C(15)H$_2$-C(16)H$_2$ were deduced through analysis of its ^1H-^1H COSY and HMQC spectra and from the chemical shift considerations. These fragments were connected to other carbon atoms by analysis of long range HMBC correlation as follows: The methoxy methyl protons correlated with C-2 and the H-2 proton correlated with the methoxy carbon, indicating that the methoxy group is attached to C-2. The methyl protons H-18 correlated with C-1, C-5, C-9, and C-10, indicating that the carbon C-9 is attached to C-1, C-5, C-10 and C-18. The connection of C-4 to the three carbons C-3, C-5 and C-20 was revealed by the correlation of H-20 with C-4, C-5 and C-6. The connection of C-13 with the three carbons C12, C14 and C-17 was deduced by the correlation of the aromatic proton H-12 with C-14, C-17, as well as the correlation of the methylene proton H-16 with C-13 and C-17. The aromatic proton H-11 correlated with C-8, C-9 and C-10, indicating the connection of C-10 to C-8, C-9, and C-11. The correlation of the methylene proton H-15 with C-8, C-13 and C-14 established the connection of C-14 to C-8, C-13 and C-15. The most important correlations were the four bond correlations of the aromatic protons H-11 and H-20 with the carbonyl carbon C-7. These correlations unambiguously helped locating one of the carbonyl group at position 7. (Fig. 4) Thus, the structure was found to be that of β-viridin (**13**), which has three chiral centres. The stereochemistry was deduced from the NOESY spectrum, which showed a strong correlation between H-1 and H-2, and a weak correlation between H-1 and H-18. Furthermore, the coupling constant between H-1 and H-2 was measured, and found to be *J* = 5.0 Hz, confirming their *cis*-configuration.



13

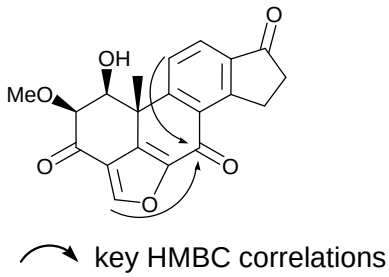
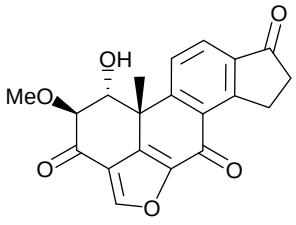


Fig. 4. Structure of β-viridin based on 2D NMR

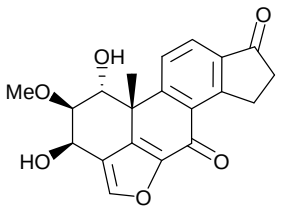
3.2.1.3 Structure elucidation of α-viridin (**14**)

14 was isolated from the same fraction as β-viridin described above. The two metabolites (**13** and **14**) share the same molecular weight (m/z = 353 $[M+H]^+$) The analysis of the spectra of **14** including ^1H NMR, ^{13}C NMR and DEPT 135 spectroscopic data of **14** showed that they share also the same molecular formula ($C_{20}H_{18}O_6$), suggesting that they are isomers. As in the above described molecule, three carbonyl carbons were indicated by ^{13}C NMR spectrum at δ 173.2, 186.9 and 206.3. Therefore, the structure was deduced using NOESY spectrum, which indicated a correlation between the methyl proton H-18 and H-1. Correlations were shown neither between the methyl proton H-18 and H-2 nor between H-1 and H-2. This unambiguously revealed the *trans*-configuration of the protons H-1 and H-2. Supporting information was derived from the coupling constant of these protons, which was found to be *J* = 9.5 Hz. Thus, **14** was identified as the known α-viridin (**14**).

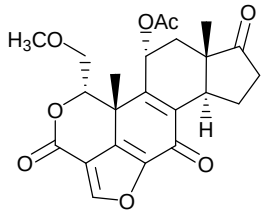


14

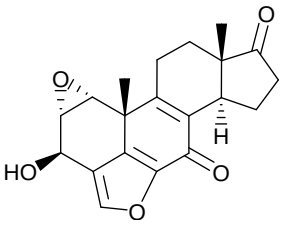
β-Viridin (**13**) and α-viridin (**14**) were first isolated from the culture broth of the fungus *Gliocladium virens*,[51-54] which was incorrectly described as *Trichoderma viride* in the early literature.[55] Their structures were determined by UV, IR, mass spectrometry, and chemical transformations. α- and β-viridin are members of the structurally closely-related class of steroidal furanoids, which include viridiol (**15**), wortmannin (**16**), wortmannolone (**17**) and demethoxy viridiol (**18**).[56]



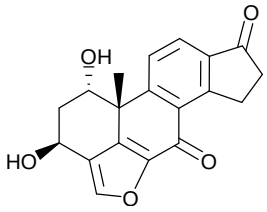
15



16



17

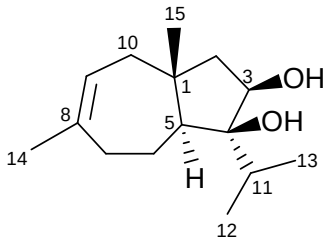


18

β-Viridin (**13**) and α-viridin (**14**) showed significant antifungal activity against *Candida albicans* ATCC 18804 and *Penicillium notatum* JP 36 and antibacterial activity against *Bacillus subtilis* ATCC 6633 with an inhibition of growth of 24, 26 and 25 mm, respectively at a concentration of 1 mg/ml.

3.2.1.4 Structure elucidation of CAF 603 (**19**)

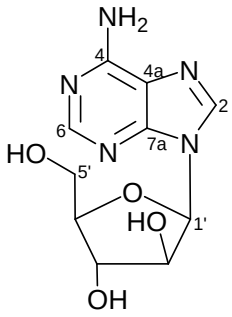
19 was isolated after various chromatography techniques including preparative TLC, CC, sephadex LH-20 and HPLC. On the basis of HR-ESIMS, ^{13}C NMR and DEPT 135 spectroscopic data the molecular formula was found to be $C_{15}H_{26}O_2$. The IR spectrum showed an absorption band at 3383 nm, indicating the presence of hydroxyl groups. In the ^{1}H NMR spectrum of **19** one olefinic proton and one oxymethine proton were visible due to their chemical downfield shift at δ 5.34 and 3.85, respectively. Four methyl protons were also observed at δ 0.85, 0.94, 1.00 and 1.73. Their connections with the rest of the molecule were deduced from long range HMBC experiments. According to the observed HMBC and NOESY correlations, the structure was established to be 8-daucene-3,4-diol, also known as CAF 603 (**19**). This metabolite is related to the carotane sesquiterpenes, and was first isolated by Watanabe et al.[57] **19** shows a strong antifungal activity against *Sporobolomyces salmonicolor* SBUG 549 and moderate activity against *Candida albicans* ATCC 18804 with a diameter of inhibition of 30 and 18 mm, respectively, at a concentration of 1 mg/ml.



19

3.2.1.5 Structure elucidation of adenoside 9α-D-arabinofuranoside (**20**)

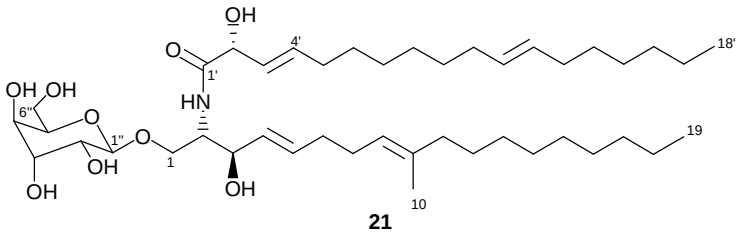
The molecular formula of **20** was found to be $C_{10}H_{13}N_5O_4$ on the basis of HR-ESIMS (m/z = 268 $[M+H]^+$), ^{13}C NMR and DEPT 135 spectroscopic data. ^{13}C NMR suggested the presence of 5 sp^2 carbons and 5 oxygenated sp^3 carbons. The analysis of the long range HMBC and NOESY spectra helped identifying the structure as the known adenoside 9α-D-arabinofuranoside, which has been reported as herbicide[58] and as a potent anti-cancer agent.[59]



20

3.2.1.6 Structure elucidation of cerebroside C (**21**)

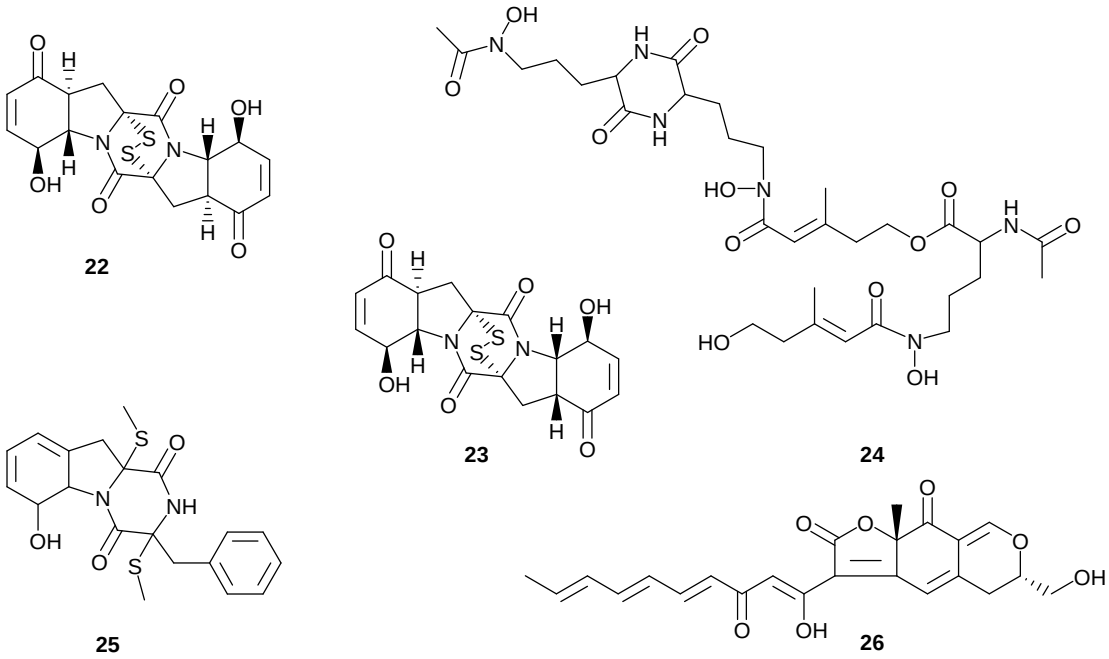
Cerebroside C (**21**) was obtained as a colourless powder. Its molecular formula was found to be $C_{43}H_{79}NO_9$ on the basis of HR-ESIMS (m/z = 776 $[M+Na]^+$), ^{13}C and DEPT 135 NMR spectroscopic data. Its structure was elucidated by comparison of its NMR data with those of cerebroside C already reported in the literature.[60] Cerebrosides have already been isolated from various fungal sources and have been reported as antifungal agents,[60] as stimulants of fruiting body formation in fungi[61] and recently as elicitors of hypersensitive cell death and phytoalexin accumulation in rice plants.[62]



21

3.2.2 *Epicoccum* sp. HKI 0470

The genus *Epicoccum* has already been studied for its natural products content leading to the discovery of epicorazines A (**22**) and B (**23**),[63-65] triornicin (**24**),[66] 3822-B (**25**)[67] and Epicocconone (**26**).[68] All are produced by *Epicoccum nigrum* (*E. purpurascens*), indicating that some members of the genus Epicoccum might have a highly developed and diverse secondary metabolism. To date the investigated strains were isolated from soil samples or from the marine sources such as sediments, wood algae or, as in the case of *Epicoccum*, from marine animals. In the present study the strain *Epicoccum* sp. was isolated from the hat of the mushroom of the fungus *Pholiota squarrosa*.



3.2.2.1 Cultivation and extraction

Epicoccum sp. HKI 0470 strain was first cultivated on solid malt medium (agar plates) at 23^{o} C for 21 days. One part was directly extracted with ethyl acetate, and the other part was used to inoculate 20 l of liquid malt medium as a stand culture. The resulting extracts were chromatographied using open column chromatography on Sephadex and silica gel and reverse-phase HPLC, affording a new phenolic compound, epicoccone B (**29**), a new and highly oxygenated coumarin polyketide, epicoccalone (**30**) and the known epicoccamide (**27**), epicoccine (**28**) and orevactaene (**31**).

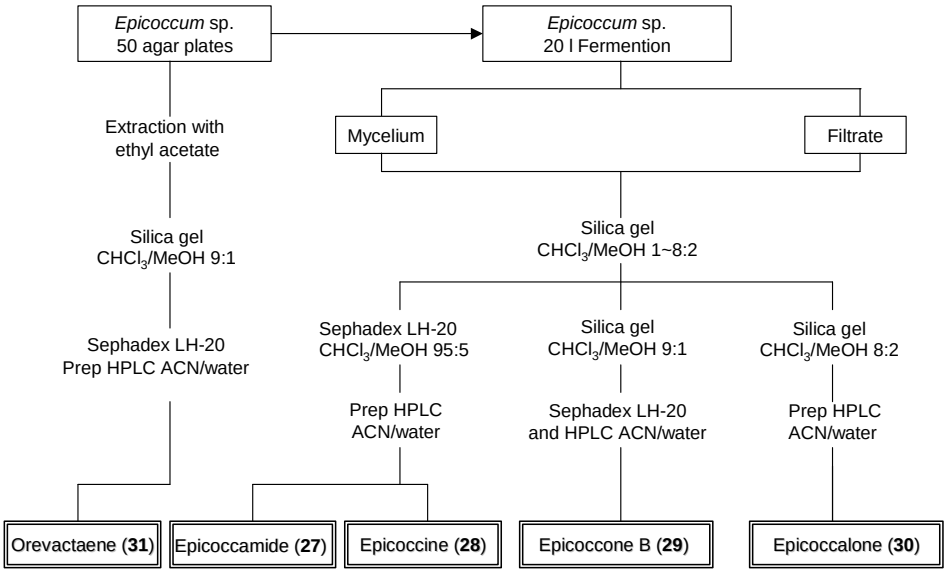


Fig. 5. Work up scheme for *Epicoccum* sp. HKI 0472

3.2.2.2 Structure elucidation of epicoccamide (**27**)

The EIS-MS mass spectrum of **27** showed the molecular ion peak at m/z 580 $[M+Na]^+$. Its molecular formula was determined as $C_{29}H_{51}NO_9$ based on HR-ESIMS and ^{13}C NMR spectroscopic data. The IR spectrum of **27** showed absorption bands for hydroxyl groups at 3367 cm^{-1}, three carbonyl carbons at 1709, 1646, and 1612 cm^{-1}. This observation was confirmed by the ^{13}C NMR spectrum, which indicated carbonyl carbon signals at δ 173.1, 191.9 and 194.5. The ^1H NMR spectrum indicated three methyl protons signals at δ 1.13 (d, *J* = 6.9, H-23), δ 1.34 (d, *J* = 6.9, H-5) and δ 2.92 (s, H-6). An aliphatic chain (14 x CH_2), as well as the glycosyl proton signals were also indicated at δ 1.18 and 3.44 - 4.46, respectively. The long range HMBC correlation observed between the methyl protons δ 1.13 (d, *J* = 6.9, H-23) and C-7 (δ 194.5), C-8 (δ 36.0) and C-9 (δ 33.7), as well as the correlation of the methyl proton signals δ 1.34 (d, *J* = 6.9 Hz, H-5) with C-4 (δ 59.5) and C-3 (δ 191.9), and the correlation of the methyl proton signals δ 2.92 (s, H-6) with C-4 (δ 59.5) and C-1 (δ 173.3) established the structure of the aglycone moiety as a tetramic acid with the long chain CH_2 connected to C-8. This tetramic acid moiety can be presented in three tautomeric forms. (Fig. 6)

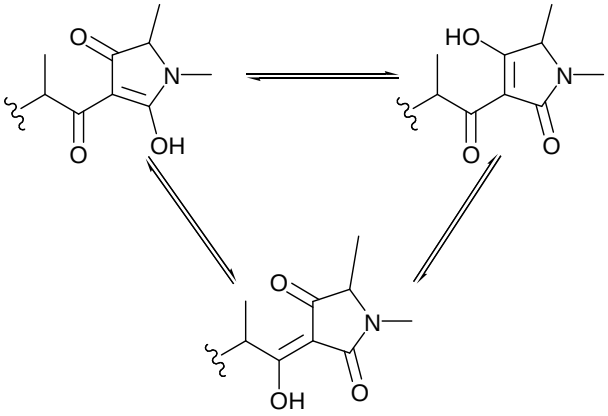
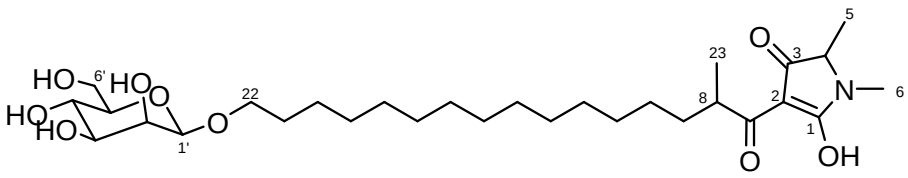


Fig. 6.Tautomeric forms of the tetramic acid part of epicoccamide

The sugar part of the **27** was identified as mannose by comparison of the ^{1}H and ^{13}C NMR data with those reported.[69] While the aglycone part and sugar were identified, it was necessary to determine the stereochemistry of the glycosidic linkage. The coupling constant between the anomeric proton H-1' and the proton H-2' was found to be $J_{H\text{-}1',\ H\text{-}2'}$ = 0 Hz, indicating that **27** contained a β-mannose. Further confirmation of the structure was obtained from the MS/MS mass spectrometry, which clearly revealed the daugther ion peak for aglycone moiety (m/z = 396).



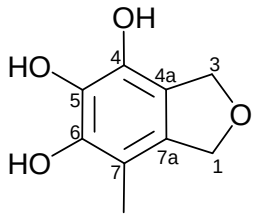
27

Database reaches led to the recently isolated epicoccamide (**27**) from *Epicoccum purpurascens*.[70] Epicoccamide showed weak antibacterial activity against *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus ATCC 511* and moderate antifungal activity against *Sporobolomyces salmonicolor* SBUG 549 at a concentration of 1 mg/ml.

3.2.2.3 Structure elucidation of epicoccine (**28**)

On the basis of HR-ESIMS and ^{13}C NMR data the molecular formula of **28** was determined as $C_9H_{10}O_4$. It was obtained as yellow oil with the yield of 0.1 mg/l of the culture filtrate of the fungus. The IR spectrum of **28** showed absorption bands for hydroxyl groups at 3280 cm^{-1}, and for aromatic rings at 1627 and 1510 cm^{-1}. A signal attributable to methyl protons at δ 1.91 (3H, s, H-H-8), two oxymethylene proton

signals at δ 4.83 (2H, s, H-1)) and δ 4.87 (2H, s, H-3), respectively, as well as three hydroxyl proton signals at δ 8.02 (1H, s), 8.06 (1H, s) and 8.45 (1H, s) were deduced from the ^{1}H NMR spectrum of **28**. The analysis of ^{13}C NMR and DEPT 135 spectra indicated the presence of 6 fully substituted sp^2 carbons and two oxygenated sp^3 carbons. The long range HMBC correlations observed between the methyl protons H-8 (δ 1.91) and C-7 (δ 108.2), C-7a (δ 132.5) and C-6 (δ 143.9), as well as Those observed between the oxymethylene protons and C-4a (δ 115.6), C-7a (δ 128.4), C-4 (δ 137.6), C-5 (δ 132.5) C-6 (δ 143.9) and C-7 (δ 108.2) enabled the elucidation of the structure of **28** as the known 1,3-dihydro-7-methyl-4,5,6-trihydroxy-isobenzofurane (epicoccine) (**28**) from *Apergillus terreus*. It has been reported to be an antioxidant that efficiently inhibits the oxidation of linoleic acid by sardine flesh lipoxygenase (LOX).[71]

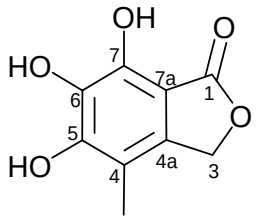


28

3.2.2.4 Structure elucidation of epicoccone B (**29**)

Epicoccone B **29** was obtained as yellow oil with a yield of 0.1mg/l of the culture filtrate of the fungus. The IR spectrum of the metabolite showed absorption bands for hydroxyl groups at 3420 cm^{-1} and 3134 cm^{-1}, for carboxyl groups at 1725 cm^{-1}, and for aromatic rings at 1628, 1515 and 1481 cm^{-1}. On the basis of HR-ESIMS and ^{13}C NMR data, **29** has the molecular formula $C_9H_8O_5$. The ^{1}H NMR spectrum of **29** showed two signals attributable to methyl proton signals at δ 1.96 (1H, s) and to a methylene proton signal at δ 5.09 (1H, s). The remaining three protons were attributable to phenolic exchangeable hydroxyl protons. As in the ^{1}H NMR spectrum of epicoccine (**28**), the absence of the aromatic proton signals in the ^{1}H NMR spectrum of **29** suggested that the aromatic part of its structure is fully substituted. The ^{13}C NMR and DEPT 135 spectra of **29** showed two sp^3 carbon signals (one methyl carbon at δ 10.7 and one oxymethylene carbon at δ 67.6) six quaternary sp^2 carbons, as well as a carboxyl carbon at δ 169.8. The protonated carbons and their corresponding protons and the full connection of compound **29** were established

using HMQC and HMBC experiments. The long range HMBC showed the correlations between the methyl protons H-8 (δ 1.96) and C-4 (δ 109.1), C-4a (δ 138.0), and C-5 (δ 151.1). The correlations between the oxymethylene protons δ 5.09 (2H, s) and C-1 (δ 169.8), C-4 (δ 109.1), C-4a (δ 138.0), C-5 (δ 151.1), C-7 (δ 143.0), and C-7a (δ 102.7) were also visible. Thus, **29** was elucidated as 4-methyl-5,6-7-trihydroxy-1(3*H*)-isobenzofuranone, a new natural product.



29

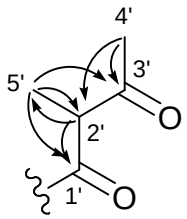
The newly described metabolite named epicoccone B (**29**) is an isomer of epicoccone A[72] in which the methyl carbon is attached to C-7. Further confirmation of the structure of **29** was derived from the NOESY spectrum, which showed a strong correlation between the methyl protons and the oxymethylene protons.

In the anti-inflammatory assay epicoccone B (**29**) exhibited moderate inhibitory activity towards 3α-hydroxysteroid dehydrogenase.

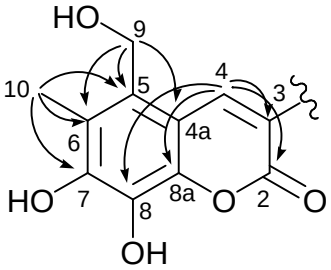
3.2.2.5 Structure elucidation of epicoccalone (**30**)

The ESI-MS mass spectrum of **30** indicated the molecular ion peak at m/z 321 $[M+H]^+$. Its molecular formula was determined as $C_{16}H_{16}O_7$ based on HR-EISMS (found $[M-H]^-$: 319.0814 calcd. for $C_{16}H_{16}O_7$: 319.0818) and the ^{13}C NMR spectra. The IR spectrum of **30** showed absorption bands for hydroxyl groups at 3244 cm^{-1}, for carbonyl groups at 1699 cm^{-1}, and for aromatic rings (1598, 1562 and 1465 cm^{-1}). The ^1H NMR spectrum of **30** revealed three signals attributable to the methyl groups at δ 1.23 (3H, d, *J* = 7.1 Hz), 2.22 (3H, s) and 2.28 (3H, s), one oxymethylene at δ 4.68 (2H, s) and two methine protons including an olefinic proton at δ 4.71 (1H, q) and 8.89 (1H, s), respectively. The remaining three protons were attributable to the phenolic exchangeable hydroxyl protons. Similar to the compounds **28** and **29**, the absence of the aromatic proton signals in the ^1H NMR spectrum suggested that the aromatic part of the structure is fully substituted. The ^{13}C NMR and DEPT 135 spectra of **30** indicated 5 sp^3 carbon signals (three methyl groups, one oxymethylene

and one methine), 8 sp^2 carbon signals including 7 quaternary sp^2 carbon signals (three of which are oxygenated), as well as two carbonyl signals and one carboxyl carbon signal at δ 194.0, 206.2 and 159.2, respectively. The protonated carbons and their corresponding protons and the full connection of **30** were established using HMQC and HMBC experiments. The correlation of the methyl proton H-5' (δ 1.23) with the carbons C-1' (δ 194.0), C-2' (δ 57.3) and C-3' (δ 206.2), the correlation of the proton H-4' (δ 2.28) with the carbons C-2' (δ 57.3), and C-3' (δ 206.2) and the correlation of the proton H-2' (δ 4.71) with the carbon C-1' (δ 194.0), C-3' (δ 206.2) and C-5' (δ 12.1) revealed the 2-methylbutan-1,3-dione as a side chain of the **30**.

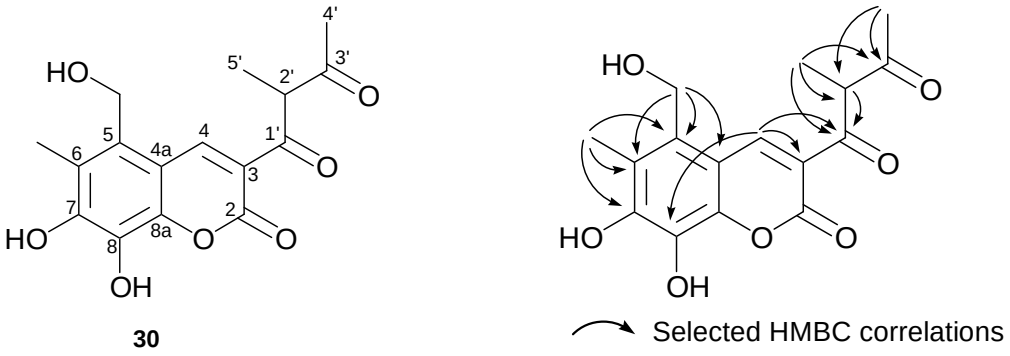


For the identification of the second part of **30**, long range HMBC played an important role. The correlation of the proton of the oxymethylene carbon H-9 (δ 4.68) with C-4a (δ 109.9), C-5 (δ 131.6) and C-6 (δ 122.4) and the correlation of the methyl protons H-10 with C-5 (δ 131.6), C-6 (δ 122.4) and C-7 (δ 151.6) suggested the connection of the oxymethylene carbon C-9 (δ 55,6) and the methyl carbon C-10 (δ 11.3) at the C-5 and C-6, respectively. The most important correlation was that of the olefinic proton H-4 (δ 8.89) with C-2 (δ 159.2), C-3 (δ 116.5) C-5 (δ 131.6) and C-8 (δ 130.2). All these correlations enabled the elucidation of the second part of the molecule as 5-hydroxymethylene-6-methyl-7,8-dihydroxycoumarin.



Due to the correlation observed between the olefinic proton H-4 (δ 8.89) and the carbonyl carbon C-1', it was evident that the two parts are connected at C-3. Thus, **30** was elucidated as 3-(3-methylbutan-1,3-dione)-5-hydroxymethylene-6-methyl-7,8-dihydroxycoumarin, a new natural product named epicoccalone (**30**). Further confirmation of the structure was obtained from the MS/MS spectrum, which showed peak fragments at m/z = 221, 203 and 175 in negative mode. These fragments are the result of the cleavage of the side chain moiety, (m/z = 221) followed by the

elimination of H_2O (m/z = 203) and the loss of the carbonyl of the carboxyl moiety (m/z = 175). (Fig. 11)



30

Fig. 7. Structure of epicoccalone based on 2D NMR

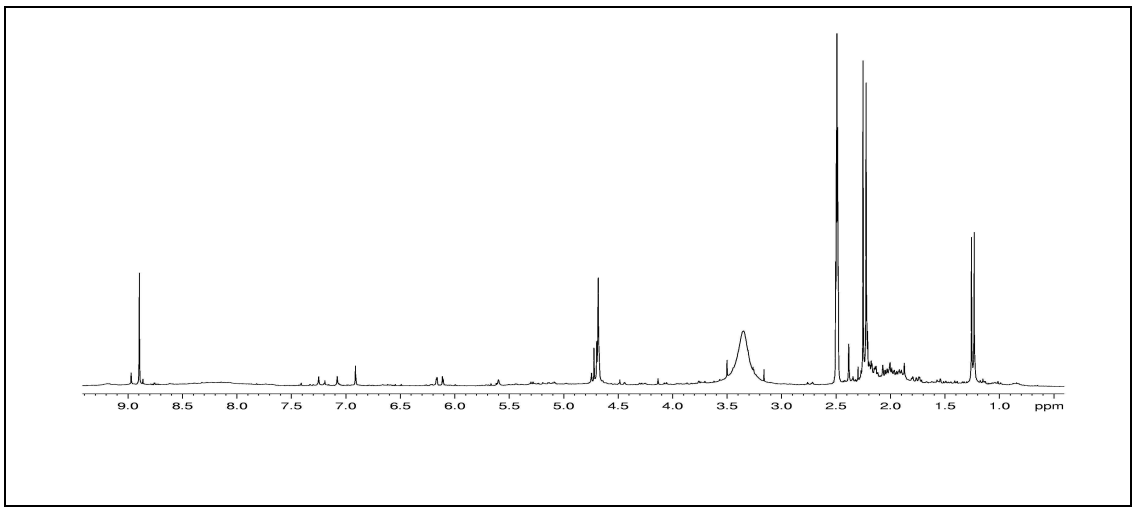


Fig. 8. ^{1}H NMR spectrum of epicoccalone

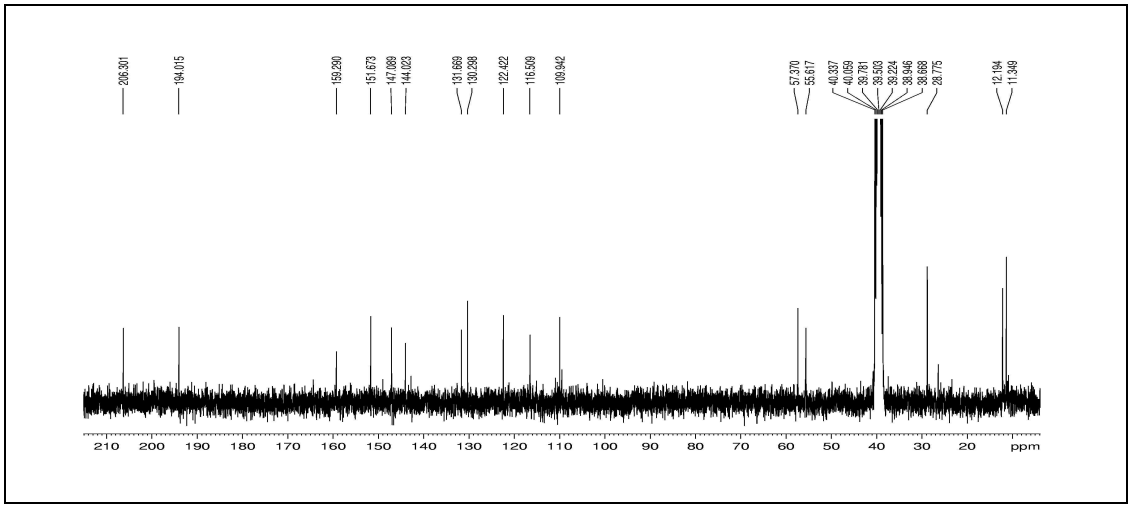


Fig. 9. ^{13}C NMR spectrum of epicoccalone (**30**)

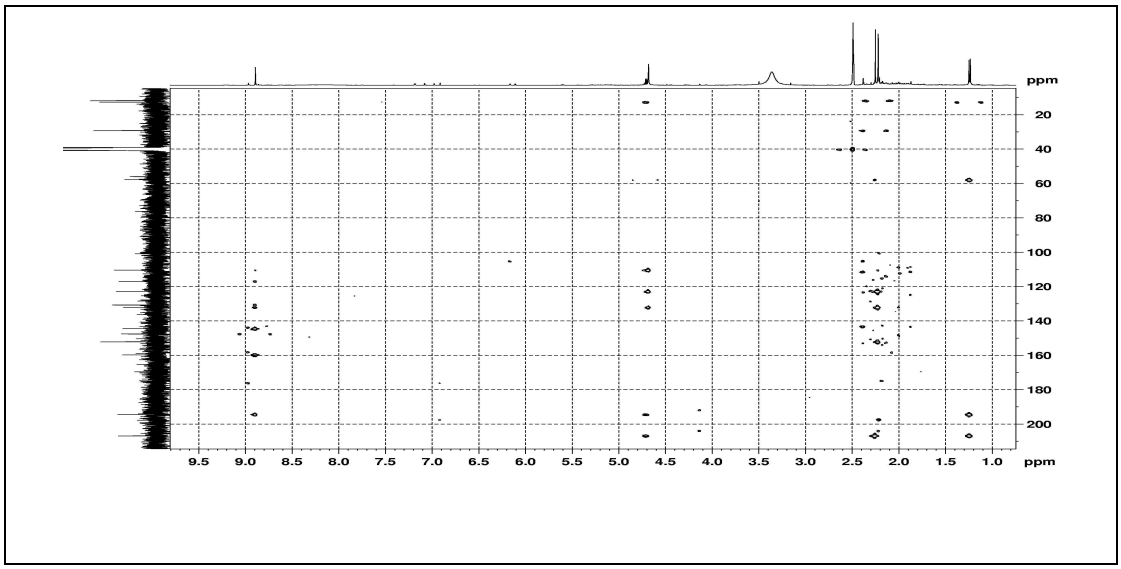


Fig. 10. HMBC spectrum of epicoccalone (**30**)

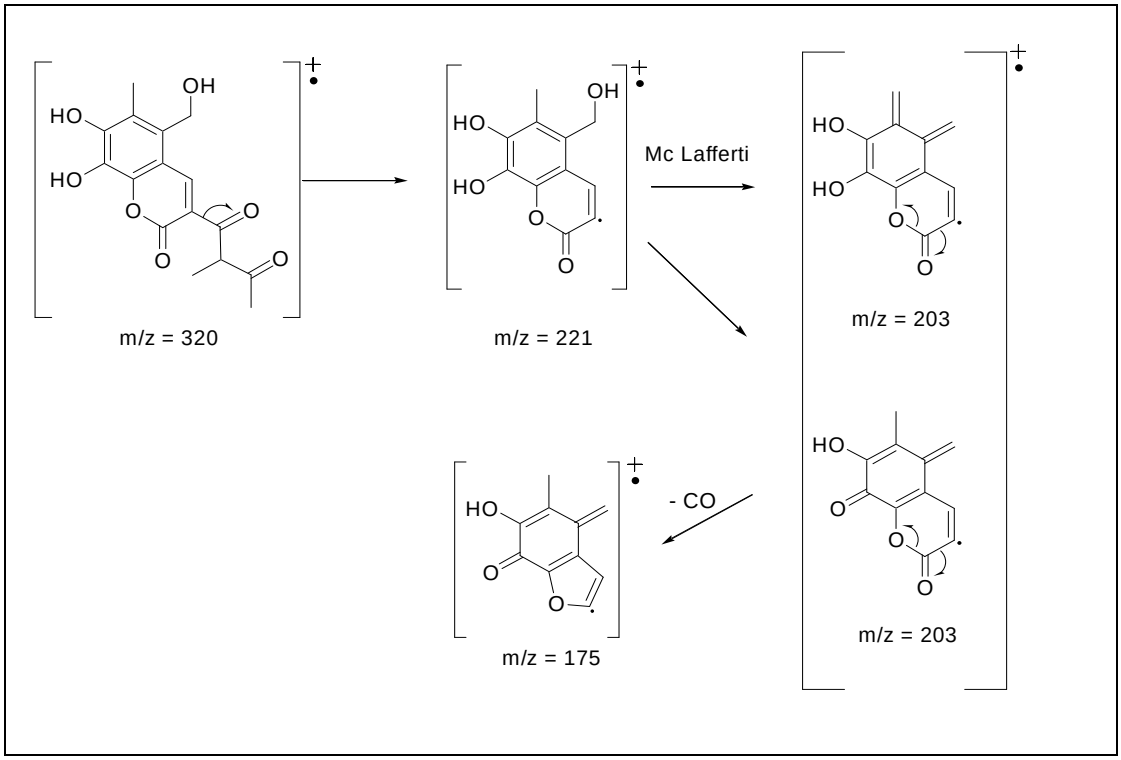


Fig. 11. Fragmentation mechanism of epicoccalone (**30**)

3.2.2.6 Biological activity of epicoccalone

In the antimicrobial assays epicoccalone showed no detectable activity against Gram positive, Gram negative bateria and fungi.

In the enzyme inhibition assays epicoccalone exhibited moderate inhibition activity towards 3α-HSD but proved to be a potent α-chymotrypsin inhibitor with an IC_{50} value of 27 µg/ml (Fig. 12). It should be noted that α-chymotrypsin is a serine protease[73-75] that plays critical roles in several physiological processes including digestion, blood coagulation, complement activation, fibrinolysis and reproduction. However, it is well known that serine proteases are not only a physiological necessity, but can be of potential hazard if present in excessive quantities. Therefore, serine proteases are responsible of vascular clotting and cerebral and coronary disorders. The excessive activity of this enzyme can also cause skin disease, including glomerulonephritis, pancreatitis and other disorders that could be controlled by its inhibition.[76] There is increasing evidence that serine protease inhibitors regulate inflammation by inhibiting the proteolytic activity of serine protease released by leucocytes. They may also contribute directly or indirectly to the defence against invading microorganisms.[77]

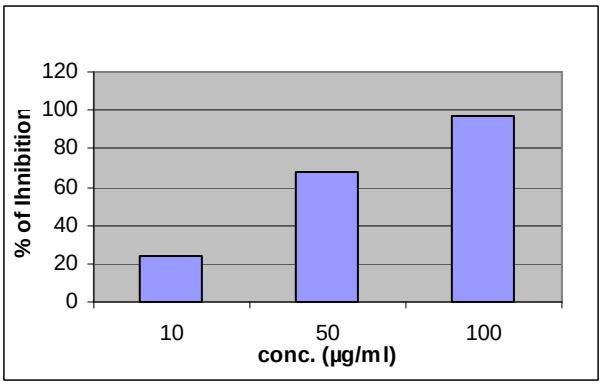
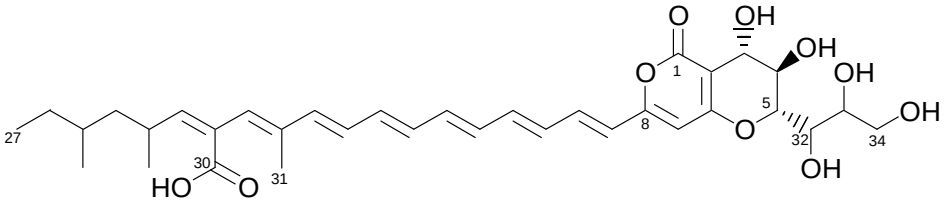


Fig. 12. Inhibition activity of epicoccalone (**30**) towards α-chymotrypsin

3.2.2.7 Structure elucidation of orevactaene (**31**)

Orevactaene (**31**) was isolated as yellow oil. It has the molecular weight of 613 $[M+H]^+$ and the molecular formula of $C_{34}H_{44}O_{10}$ based on HR-ESIMS. The IR spectrum revealed strong absorption band for hydroxyl groups and for a conjugated carbonyl at 3251 and 1671 cm^{-1}, respectively. The UV spectrum showed the absorption maxima at 430 nm, indicating the presence of a polyene. The analysis of

^{13}C and DEPT 135 revealed the presence of 34 carbons, including two carboxyl carbons, 18 sp^2 carbons (1 of which is oxygenated), 14 sp^3 carbons (5 oxymethines, and 1 oxymethylene) and two carboxyl carbons. The presence of carboxyl carbons was confirmed by the ^{13}C NMR data, which showed two signals at δ 162.2 and 169.3. The ^{1}H-^{1}H COSY revealed the alignment H-9/H-10/H-11/H-12/H-13/H-14/H-15/H-16/H-17/H-18, H-22/H-23/H-24/H-25/H-26/H-27 and H-3/H-4/H-5/H-32/H-33/H-34. The long range HMBC (Fig. 13) helped connecting the rest of the molecule. For example the correlation of the proton H-20 (δ 6.11) with C-30 (δ 169.3) and the correlation of the proton H-22 (δ 5.55) with C-30 enabled the connection of the carboxyl carbon at C-21 (δ 131.4). The correlations of H-3 with C-1 (δ 162.2), C-2 and C-6 (δ 167.7) were also visible. Thus, the structure of **31** was determined as shown below. The confirmation of the structure was derived from the MS/MS spectrum, which showed the pseudo molecular ion at m/z 403, 447, 491, and 521 in negative mode. These fragments are result of the cleavage of the hydroxylated side chain moiety, followed by the cleavage of the dihydroxypyranne ring and by the decarboxylation (Fig. 14)



31

The search for this structure in the database revealed the known orevactaene, which has been reported to be a binding inhibitor of HIV-1 Rev protein to the Rev response element (RRE) with an IC_{50} value of 3.6 µM from *Epicoccum nigrum* WC47880.[78]

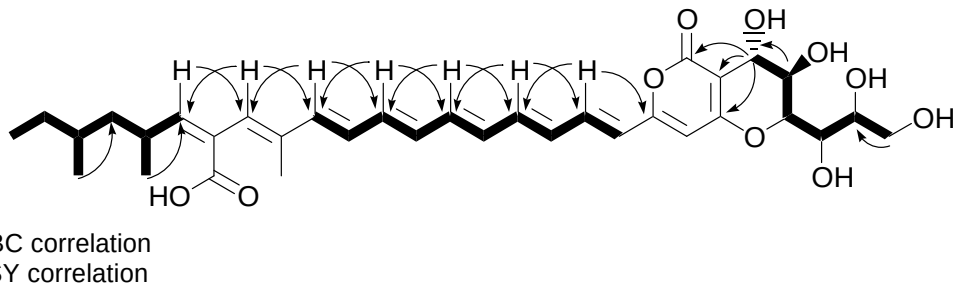


Fig. 13. Structure of orevactaene (**31**) based on 2D NMR

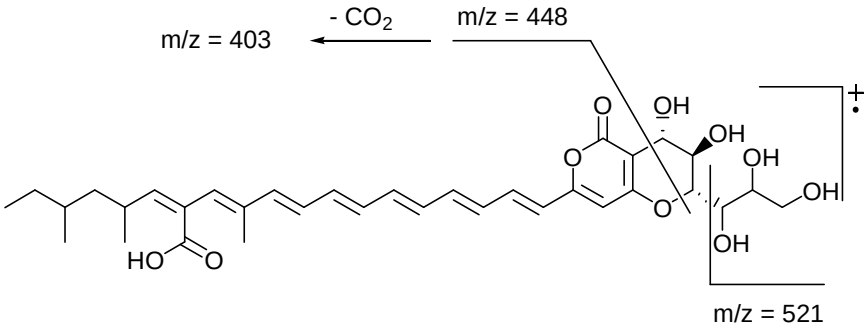


Fig. 14. Fragmentation mechanism of orevactaene based on MS/MS spectrum

3.2.3 *Lepista nebularis*

3.2.3.1 Cultivation and extraction

The strain *Lepista nebularis* HKI 0411 collected in Siberia appeared to produce antimicrobial metabolites as revealed by an initial screening with the crude extract. It was then cultivated under the condition of surface fermentation at 25 ^{o}C in 500 ml Erlenmeyer flasks containing 100 ml of MPG medium as shake culture. After cultivation for 28 days the mycelium cake from culture medium (60 l) was harvested and extracted twice with ethyl acetate and methanol (each 10 l). The culture broth was thoroughly extracted with ethyl acetate and the combined extracts were dried and the solvents were evaporated. The residue (3.2 g) was subjected to column chromatography on Sephadex and silica gel with stepwise $CHCl_3$ and $CHCl_3$/MeOH (9:1, 1:1, v / v) as eluent. Active components were isolated through bioassays guided purification. Final purification was achieved by preparative HPLC, yielding 7 active metabolites, nebularic acid A (**32**), nebularone (**33**), nebularic acid B (**34**), nebularilactone A (**35**), nebularilactone B (**36**), N-acetyltyramine (**46**) and diatretyne (**47**).

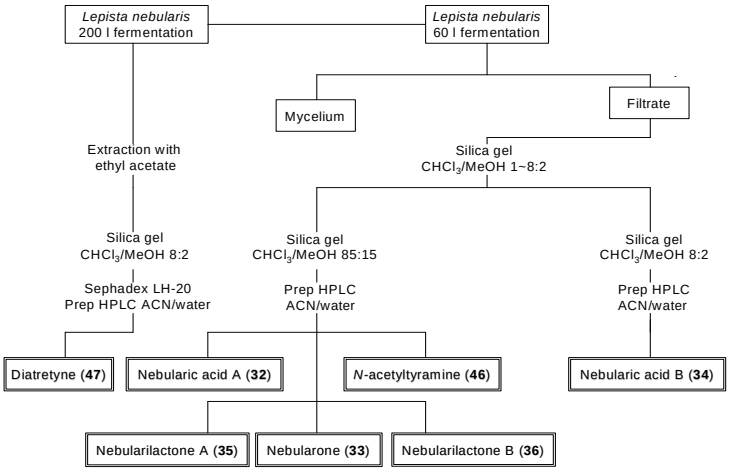
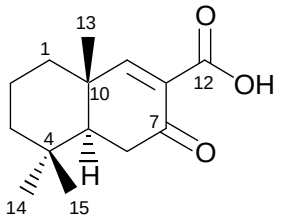


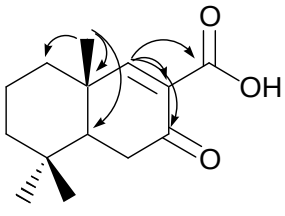
Fig. 15. Work up scheme of *Lepista nebularis* HKI 0411

3.2.3.2 Structure elucidation of nebularic acid A (**32**)

32 was obtained as a white powder. Its molecular formula was determined as $C_{14}H_{20}O_3$ based on HR-ESIMS (found $[M+H]^+$: 237.1480, calcd. for $C_{14}H_{21}O_3$: 237.1485) and ^{13}C NMR spectroscopic data. The ^1H NMR spectrum showed 19 non exchangeable protons including one olefinic proton at δ 8.02 and three methyl protons at δ 0.88, 0.92 and 1.14. Analyses of ^{13}C, DEPT 135 and HMQC NMR spectra of **32** indicated the presence of two methine carbons (one of which is sp^2 hybridized) four methylene carbons, three methyl carbons and three quaternary carbons (one of which is sp^2 hybridized), a carboxyl carbon at δ 164.0 and a carbonyl carbon at δ 204.3. The presence of carboxyl and carbonyl groups was confirmed by the IR spectrum, which showed strong absorption bands at 1742 cm^{-1} and 1632 cm^{-1}. The ^1H-^1H COSY spectrum revealed the coupling system H-1/H-2/H-3, and H-5/H-6. By means of HMBC all connections were fully assigned. The geminal arrangement of the methyl groups C-14 (δ 20.9) and C-15 (δ 32.6) was established by the correlation of H-14 (δ 0.92) and H-15 (δ 0.88) with C-4 (δ 33.0), C-3 (δ 40.7) and C-5 (δ 49.4). Similarly, the correlation of H-13 (δ 1.14) with C-10 (δ 38.3), C-1 (δ 36.8) and C-9 (δ 175.4) revealed the connection of the methyl group C-13 (δ 17.1) with C-10 (δ 38.3). Another important correlation was observed between the olefinic proton H-9 (δ 8.02) and C-8 (δ 123.5), C-7 (δ 204.3) and C-12 (δ 164.0), which helped unambiguously identifying the position of the carboxyl and carbonyl groups. The correlation of H-6 (δ 2.65, 2.50) with C-5, C-7, C-4 and C -8 was also observed. Thus, **32** was identified as 7-oxo-11-nordrim-8-en-12-oic acid (nebularic acid A)



32



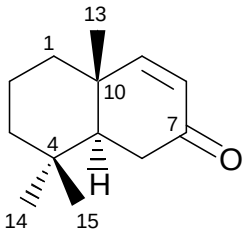
Selected HMBC correlation

Fig. 16. Structure of nebularic acid based on 2D NMR

3.2.3.3 Structure elucidation of nebularone (**33**)

^1H and ^{13}C NMR spectra of **33** were almost identical with those obtained from **32**. However, it appeared from the analyses of ^{13}C, DEPT 135 and HMQC NMR spectra

of **33** that it contained two hydrogenated olefinic carbons. This was confirmed by the ^{1}H NMR spectrum, which indicated the olefinic protons signals at δ 5.72 (d, *J* = 9.8 Hz, H-8), and 6.59 (d, *J* = 9.8 Hz, H-9). Furthermore, the ^{13}C NMR spectrum indicated a carbonyl carbon signal at δ 201.5. No carboxyl carbon signal was detectable, suggesting that **33** was a decarboxylation product of **32**. This finding was strongly supported by mass spectra, as well as IR spectra, which showed a mass difference of 44 units compared to **32**, and an absorption band for carbonyl carbon at 1709 cm^{-1}, respectively. Thus, **33** was elucidated as bisnor-11,12-drim-8-en-7-one named nebularone (**33**).



33

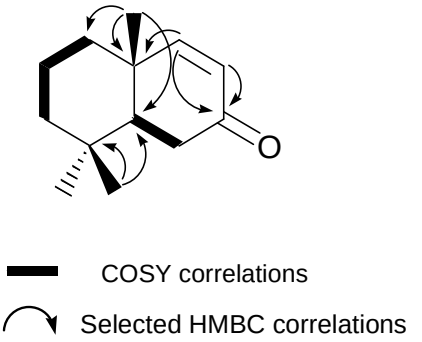


Fig. 17. Structure of nebularone based on 2D NMR

This is the first time that nebularone was isolated as natural product. However, it had already been prepared as an intermediate for the synthesis of the drim-8-en-7-one starting with decalone.[79]

3.2.3.4 Structure elucidation of nebularic acid B (34)

^{1}H and ^{13}C NMR spectra of **34** were almost similar with those obtained for **32** and **33**. However, it appeared from the analyses of ^{13}C, DEPT 135 and HMQC NMR spectra that **34** contained no olefinic carbon. The two olefinic carbons present in **32** were replaced by a methine and by a quaternary sp^{3} carbon. According to their chemical downfield shifts δ 69.5 and 62.2, both of them are oxygenated. Supporting information was obtained from mass spectra, which showed a mass difference of 16 units compared to **32**. The pseudo molecular ion peak obtained by HR-ESIMS accounted for a molecular formula of $C_{14}H_{20}O_4$ (found [M-H]$^{-}$: 251.1262, calcd.: 251.1264). Therefore, **34** was identified as 7-oxo-8,9-epoxy-11-nordriman-12-oic acid

(**34**) (nebularic acid B). The relative configuration of **34** was established using NOESY experiments, which showed a correlation between the methine proton H-5 and the oxymethine proton H-9.

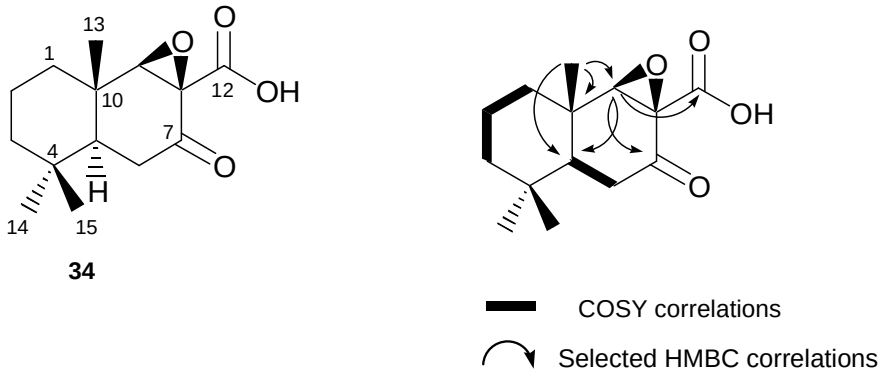
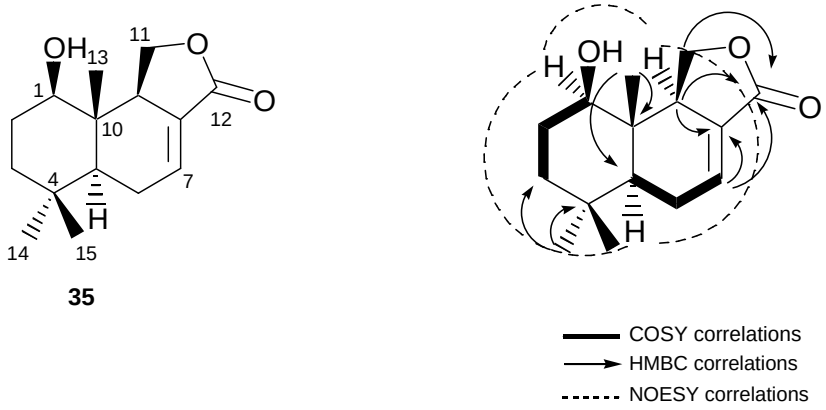


Fig. 18. Structure of nebularic acid B based on 2D NMR

3.2.3.5 Structure elucidation of nebularilactone A (35)

The molecular formula of nebularilactone A (**35**) was determined as $C_{15}H_{22}O_3$ based on HR-ESIMS (found $[M+H]^+$: 251.1642, calcd. for $C_{14}H_{23}O_3$: 251.1650) and ^{13}C NMR spectroscopic data. The ^1H NMR spectrum of **35** showed 22 non exchangeable protons including one olefinic proton at δ 6.85 and three methyl groups at δ 0.78, 0.94 and 1.34. ^{13}C, DEPT and HMQC NMR spectra of **35** indicated the presence of four methine carbons (two of which are sp^2 hybridized and oxygenated according to their chemical downfield shifts δ 79.8 and 135.2, respectively), four methylene carbons (one of which is oxygenated δ 69.2), three methyl carbons, three quaternary carbons (one of which is sp^2 hybridized), and a lactone carbon δ 170.1. The presence of the lactone group was confirmed by the IR spectrum, which indicated a strong absorption band at 1729 cm^{-1}. The ^1H-^1H COSY spectrum revealed the coupling system H-1/H-2/H-3, H-5/H-6/H-7 and H-9/H-11. The other connections were fully assigned by means of the long range HMBC spectrum. The geminal arrangement of the methyl groups C-14 (δ 21.5) and C-15 (δ 32.5), and the connection of the methyl C-13 were established in the same manner as described for compounds **33** and **34.** The lactone ring was connected according to the correlation of H-7 (δ 6.85) with C-8 (δ 127.6), C-9 (δ 50.1) and C-12 (δ 170.1), the correlation of H-9 with C-8, C-11 (δ 69.2) and C-12 and the correlation of the oxymethylene protons H-11 (δ 4.15) with the carbons C-8, C-9 and C-12. The most important correlations were the correlation of H-9 with C-1 (δ 79.8), the correlation of H-13 with C-1 and that of H-1 (δ 3.39) with

C-10 (δ 39.5), C-13, and C-9. These correlations helped identifying the position of the hydroxyl group. The relative sterochemistry was established using NOESY experiments, which indicated correlations between H-1/H-5, H-1/H-9 and H-5/H-9. Thus, **35** was identified as 1β-hydroxydrim-7-en11,12-olide, designated nebularilactone A (**35**).



35

Fig. 19. Structure of nebularilactone A based on 2D NMR

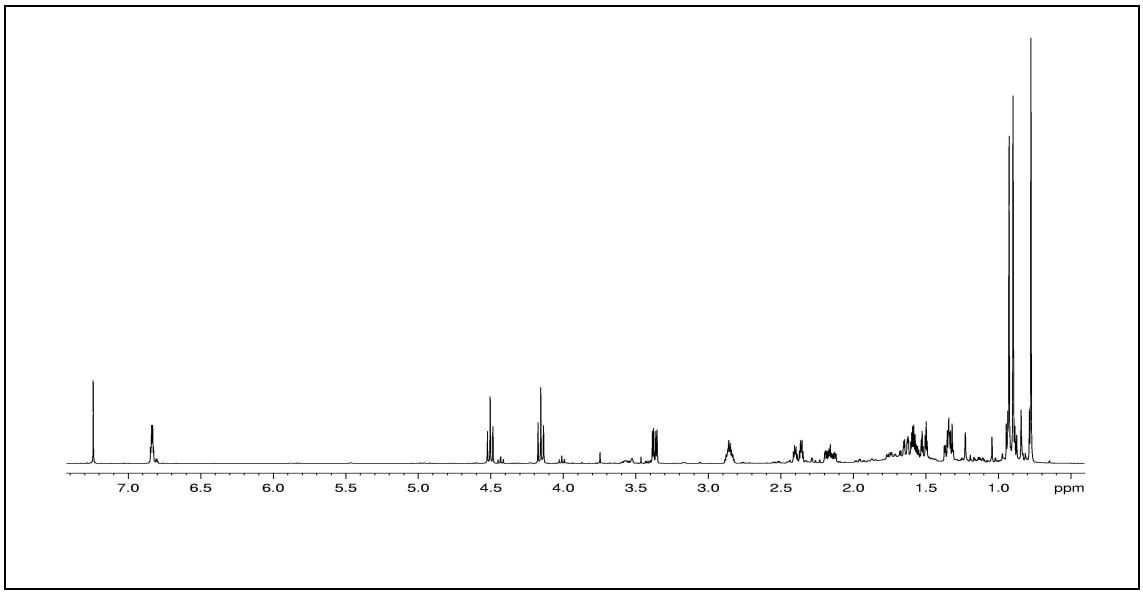


Fig. 20. ^{1}H NMR spectrum ($CDCl_3$) of nebularilactone A (**35**)

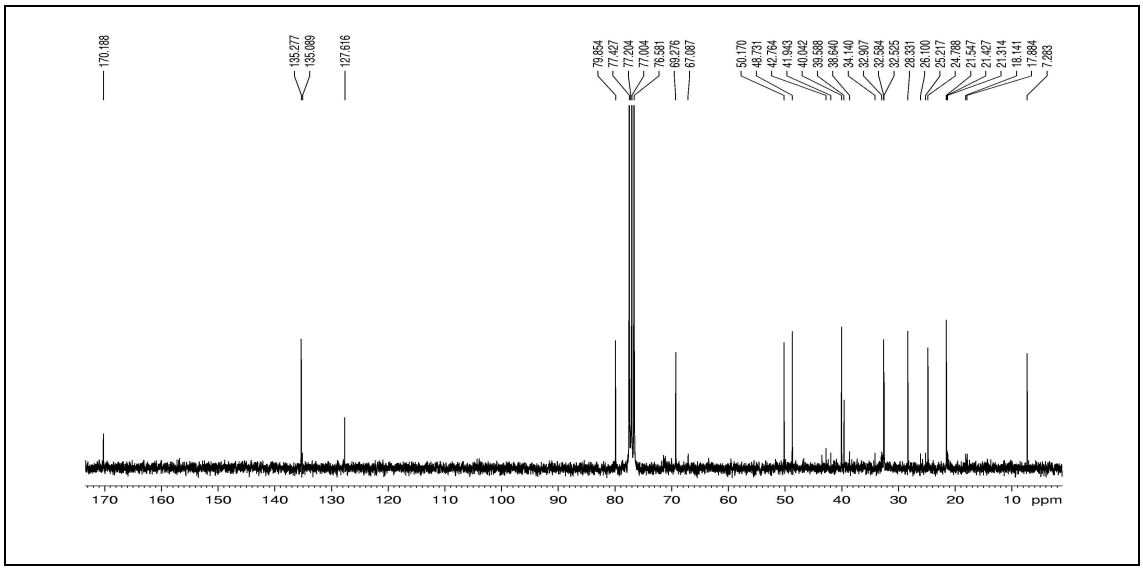


Fig. 21. ^{13}C NMR spectrum ($CDCl_3$) of nebularilactone A (**35**)

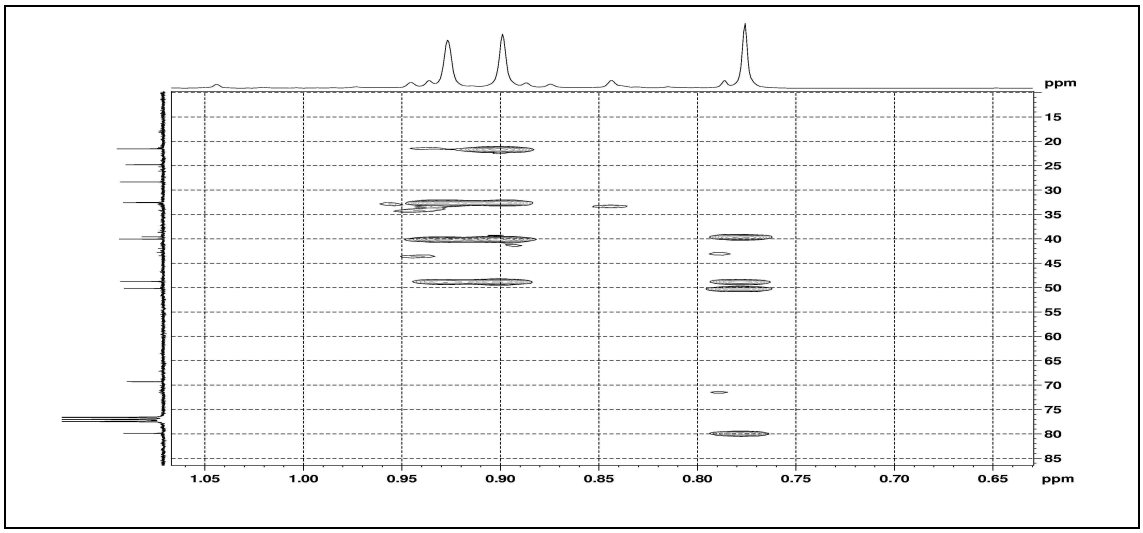
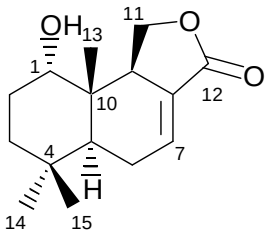


Fig. 22. Key HMBC correlations of nebularilactone A (**35**)

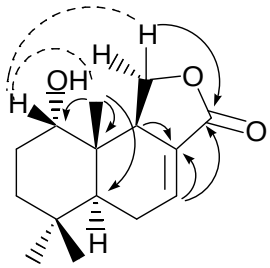
3.2.3.6 Structure elucidation of nebularilactone B (**36**)

36 was obtained as a colourless oil. Its molecular formula was determined as $C_{15}H_{22}O_3$ based on HR-ESIMS (found $[M+H]^+$ 251.1642, calcd. for $C_{14}H_{23}O_3$: 251.1650) and ^{13}C NMR. ^1H, ^{13}C, DEPT 135 and HMBC spectra of **36** were almost identical with those obtained from **35**. However, it appeared from the analyses of NOESY spectrum of **36** that the proton H-1 (δ 3.51) correlated with the methyl protons H-13 (δ 0.78). No correlation was found between the proton H-1, H-5 (δ 1.75)

and H-9 (δ 3.58). Thus, **36** was identified as 1α-hydroxydrim-7-en-11,12-olide (nebularilactone B).



36



HMBC correlations
NOESY correlations

Fig. 23. Structure of nebularilactone B based on 2D NMR

This is the first isolation and full characterization of componds **32**-**36** as natural products. However, it should be noted that the detection of the methyl ester of **32** from the methylated extract of *Lepista glaucocana* has been reported previously.[80] Nebularic acid A appears to be the biogenetic progenitor of nebularone and nebularic acid B, which features an unusual α,β-epoxy moiety for drimane.

3.2.3.7 Model for the biosynthesis of drimane sesquiterpenoids

In analogy to the majority of sesquiterpenoids, the drimane sesquiterpenoids are biosynthesised by an enzyme-mediated solvolysis of the pyrophosphate group of farnesylpyrophosphate (FPP) (**37**),[81-83] which is cyclized and converted to the final products by hydrolysis and oxidation. Therefore, the co-occurrence of drimanes and *nor* drimanes may arise through decarboxylation of drimanic acid from drimenol (**39**). (Fig. 24)

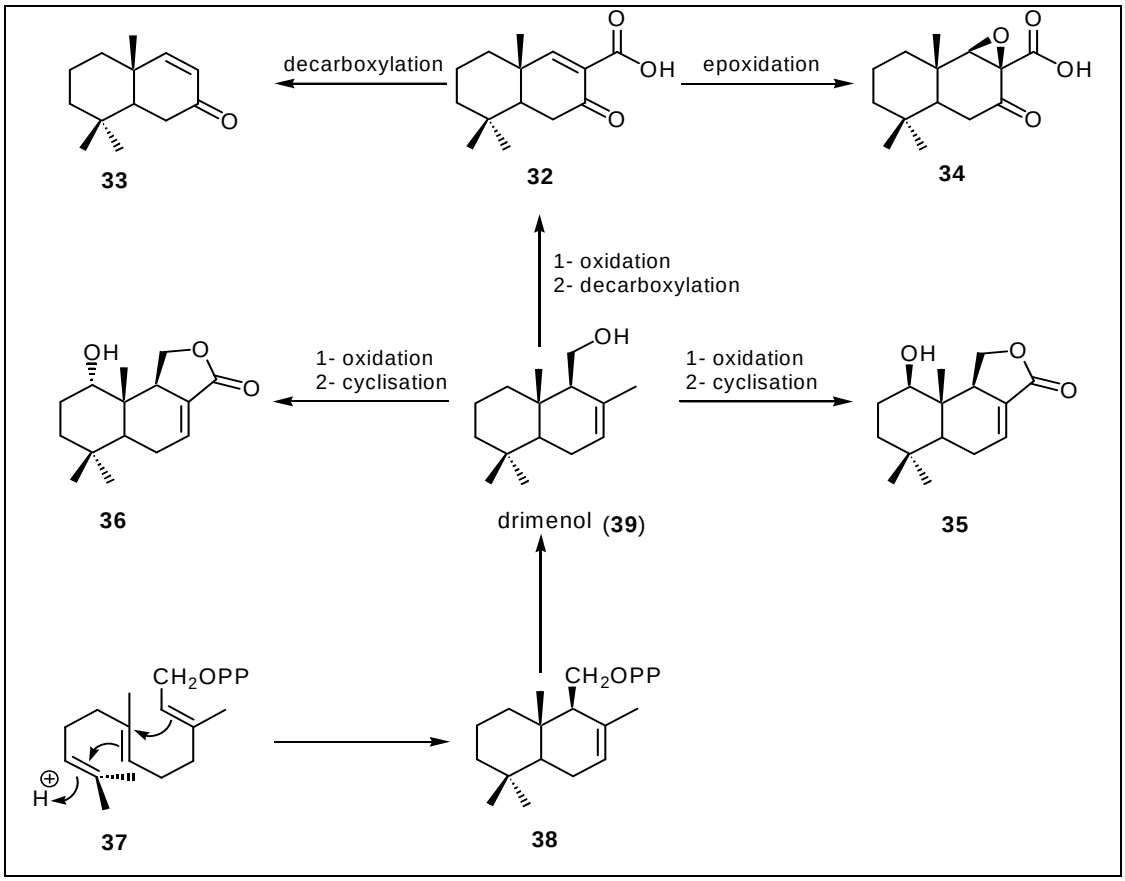


Fig. 24. Proposed biosynthesis of nebularone, nebularic acids and nebularilactones

3.2.3.8 Biological activity of isolated drimanes

Nebularone (**33**), nebularic acids A (**32**) and B (**34**) as well as nebularilactones A (**35**) and B (**36**) have been tested in various bioassays system, including antimicrobial, anti-inflammatory and antiviral assays. Nebularic acid B, nebularilactones A and B exhibited no significant activity. Conversely, nebularic acid A exhibited a strong antiviral activity against coxsackievirus, which has been found to be one of the main causes of certain debilitating or life-threatening diseases such as the inflammation of the heart muscle, the so-called myocarditis[84] with an IC_{50} value of 6 µM. (Fig. 25)

A number of antiviral drimane sesquiterpenoids have already been reported including *epi*-albrassitriol (**40**),[85] which exhibited a moderate activity in *in vitro* tests against influenza A and mixovirus (MIC 44.4 µm/ml), kuehneromycin-A (**41**),[86] which proved to be a non-competitive inhibitor of the reverse transcriptase of avian myeloblastosis virus (IC_{50} 75 µg/ml), and a weak inhibitor for HIV-1 reverse transcriptase (IC_{50} 185 µg/ml) and mniopetals A-D (**41**-**45**),[87] which inhibited the reverse transcriptase of avian myeloblastosis virus and moloney murine leukemia virus with IC_{50} values of 41-77 µM and 1.7-7 µM, respectively.

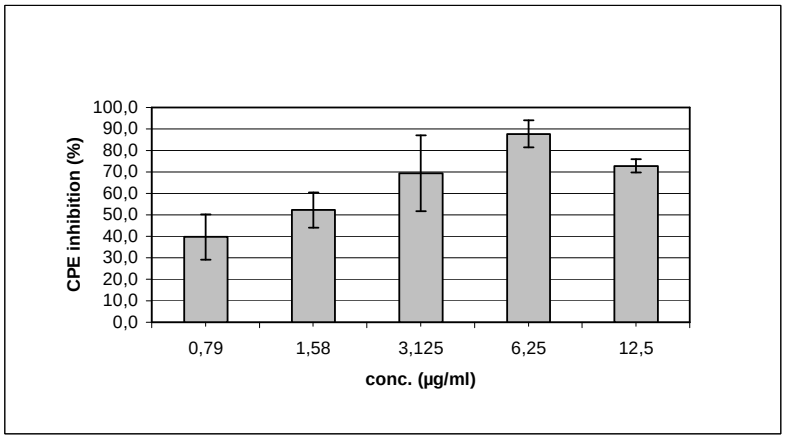
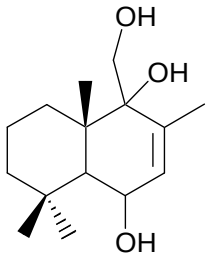
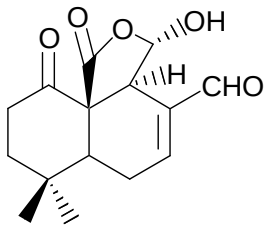


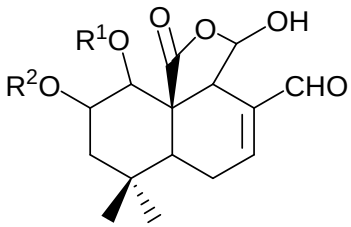
Fig. 25. Inhibition of coxsackievirus B3-induced CPE in HeLa cells of nebularic acid A (**32**)



40



41



42 R^1: H
R^2: *O*-(2R-acetoxydecanoyl)

43 R^1: H
R^2: *O*-(2-hydroxyoctanoyl)

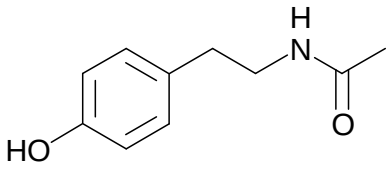
44 R^1: H
R^2: *O*-(2-hydroxydecanoyl)

45 R^1: *O*-(2-hydroxydecanoyl)
R^2: H

3.2.3.9 Structure elucidation of *N*-acetyltyramine (**46**)

The molecular formula of **46** was determined as $C_{10}H_{13}O_2$ based on HR-ESIMS (m/z = 180 $[M+H]^+$). The IR spectrum showed an absorption band for a carboxamide carbon at 1635 cm^{-1}. The ^1H NMR spectrum indicated two proton signals assigned to an AA' BB' spin coupling for a *para*-subtituted phenyl moiety. The analysis of ^{13}C and DEPT 135 NMR spectra revealed the presence of 6 sp^2 carbons and three sp^3 carbons including two methylene carbons. Thus, the structure of **46** was assigned as *N*-acetyltyramine according to the HMBC correlation. This compound was already

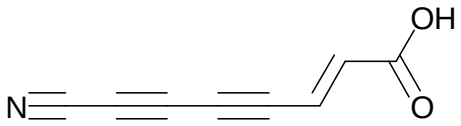
reported from bacterial strains including marine Streptomycetes and *Mycobacterium tuberculosis*.



46

3.2.3.10 Structure elucidation of diatretyne (**47**)

47 was obtained after various bioassay guided chromatographic purification steps including TLC, CC (Silica gel and Sephadex LH-20) and HPLC. Its molecular formula was determined as $C_8H_3NO_2$ based on HR-ESIMS (m/z 144 $[M-H]^-$). The IR spectrum showed an absorption band of a carboxyl carbon at 1685 cm^{-1} and the UV (MeOH) spectrum revealed various maxima (λ_{max} 230, 239, 268, 284, 302, 322 nm), suggesting the presence of a polyine. The ^1H NMR spectrum indicated the presence of two *trans* disubstituted protons at δ 6.60 (d, *J* = 16.2 Hz) and 6.77 (d, *J* = 16.2 Hz). The long range HMBC of these protons with other carbons led to the elucidation of **47** as 7-cyano-hept-2-ene-4,6-diynoic acid (**47**).



47

The search for this structure in the database revealed that **47** is a known antimicrobial compound named diatretyne, which has been reported from the fungus *Clitocybe diatreta*.[88,89]

3.2.3.11 Biological activity of diatretyne (47)

The activity of diatretyne was evaluated in various bioassay test systems including antimicrobial, cytotoxic (HeLa cells) and antiproliferative assays (murine fibroblast cell line L-929 and human leukemia cell line K-562). For all these tests diatretyne was found to be highly active. (table 2, 3 and Fig. 26)

	MIC (µg/ml)					
	MRSA 134/94	*VRSA* 1528	*E.coli ATCC* 25922	*Staphylococcus aureus* 1719/00	*Mycobacterium Vaccae*	*Candida albican*
Diatretyne	1.56	1.56	6.25	1.56	6.25	25
Ciproflox.	25	0.8	0.05	25	0.4	-
Amp.	-	-	-	-	-	0.2

Table 2. MIC value of antimicrobial activity of diatretyne (**47**)

Diatretyne		
Antiproliferative activity		Cytotoxicity
L-929 GI_{50}(µg/ml)	K-562 GI_{50}(µg/ml)	HeLa CC_{50} (CC_{10}) (µg/ml)
2.4	0.8	4.7 (3.6)

Table 3. Antiproliferative and cytotoxic data of diatretyne (**47**)

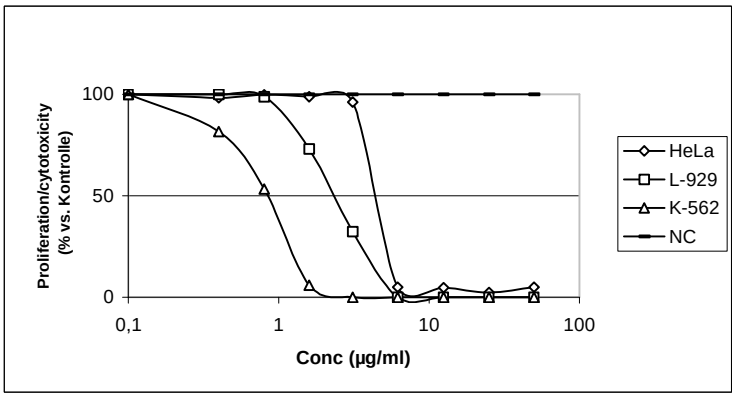


Fig. 26. Antiproliferative and cytotoxic activity of diatretyne (**47**)

3.2.4 *Aspergillus* sp. HKI 0472

Apergillus sp. HKI 0472 was isolated from a soil sample (compost) collected in Bafoussam, a region situated in west Cameroon. It showed a good agreement with the microscopic characteristics of *Aspergillus*. It was deposited in the strain collection of the Leibniz-Institute for Natural Product Research and Infection Biology, HKI, Jena (Germany).

3.2.4.1 Cultivation and extraction

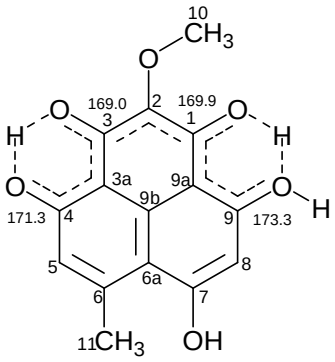
The strain *Aspergillus* sp. was cultivated and extracted in the same way as described above for *Lepista nebularis* to afford a new phenolic metabolite with an unusual conformation, funalenine (**48**) and the known TMC-256C2 and TMC-256A1.

3.2.4.2 Structure elucidation of funalenine (**48**)

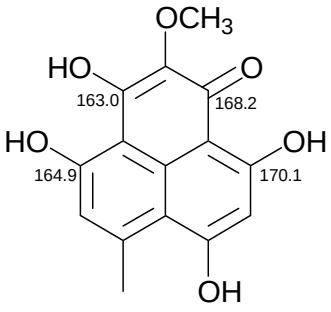
The ^{1}H NMR spectrum of **48** displayed two singlet proton signals at δ 2.68 and 3.67, which are attributable to a methyl and a methoxy groups, respectively. Moreover, two singlet proton signals at δ 18.39 and 18.42 attributed of protons putatively involved in intramolecular resonance assisted hydrogen bonds were visible. The presence of the methyl and methoxy groups was supported by the DEPT spectrum, which showed eleven aromatic quaternary carbon signals in addition to the signals of methyl and methoxy. For the assignment of the other constituting protons and carbons ^{13}C, ^{1}H long range coupled NMR spectra (HMBC) were particulary helpful. The connection of the methoxy residue to C-2 (δ 130.4) was suggested by the correlation of the methoxy protons H-10 (δ 3.67) with C-2 (δ 130.4). The correlation of the protons at δ 18.39 and 18.42 with C-2 (δ 130.4), C-3 (δ 169.0), C-3a (δ 106.9), C-4 (δ 171.3), C-5 (δ 116.4) and C-1 (δ 169.9), C-2, C-8 (δ 98.9), C-9 (δ 173.3), and C-9a (δ 102.7) (Fig. 27), respectively, afforded the alignment C-8, C-9, C-9a, C-1, C-2, C-3, C-3a, C-4, and C-5. This was evidence of strong intramolecular hydrogen bonds in the molecule. The other alignment C-5, C-6 (δ 143.7), C-6a (δ 106.7), C-7 (δ 163.3) and C-8 (δ 98.9) as well as the connection of C-11 (δ 25.0) were suggested as shown in Fig. 27. Supporting evidence was given by the long-range ^{1}H-^{1}H COSY and NOESY between H-5 (δ 6.32) and the methyl protons H-11 (δ 2.68), between 7-OH and H-8 (δ 6.04) and between the hydroxyl proton 9-OH, respectively. Therefore, the structure of **48**, was determined as funalenine (**48**), in which the π -electron in the A, B, C rings are totally delocalized. **48** was found to be a conformer of funalenone (**49**),[90] which was produced by an *Aspergillus niger* strain from a soil sample collected in Funabashi City, Chiba, Japan.[92] They share the same molecular weight and the same molecular formula. The comparison of the ^{13}C NMR data of **48** and **49**, which have been measured using the same solvent (DMSO-d_6) indicated some disagreements, particularly with the C-3 and C-4 signals, which appeared at δ 169.0 and 171.3 for **48**, and δ 163.0 and 164.9 for **49**, respectively. Moreover, other disagreements were found in the IR spectra, which indicated an absorption band at

1584 cm^{-1} for **48** (attributable to the aromatic double bonds) and an absorption band at 1616 cm^{-1} for **49** (attributable to the ketone function). These comparisons indicated that **48** have a weak carbonyl character for the carbon C-1 (δ 169.9), C-3 (δ 169.0), C-4 (δ 171.3) and C-9 (δ 173.3) and therefore, strongly supported the structure of **48.** The tautomer form **49** is stabilized by the intramolecular resonance assisted hydrogen bonds (RAHB). This concept has been introduced by Gilli et al. to explain the observed strong hydrogen bonding in the oxygen analogue.[91,92] In this model a strong hydrogen bonding is possible if a bond allows charge flow through covalent bonds from a donor to the acceptor atom. In addition, it should be noted that intramolecular hydrogen bonds are implicated in the control of the conformation of many biological molecules and are known to contribute to the interactions necessary for efficient molecular recognition.[93,94]

The treatment of **48** with diazomethane afforded only the 7-methoxy derivative, and thus, the great stability of **48** was confirmed.



48



49

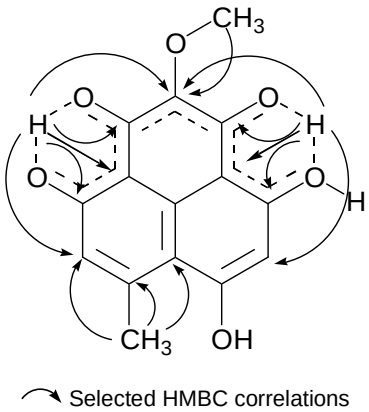


Fig. 27. Structure of funalenine based on 2D NMR

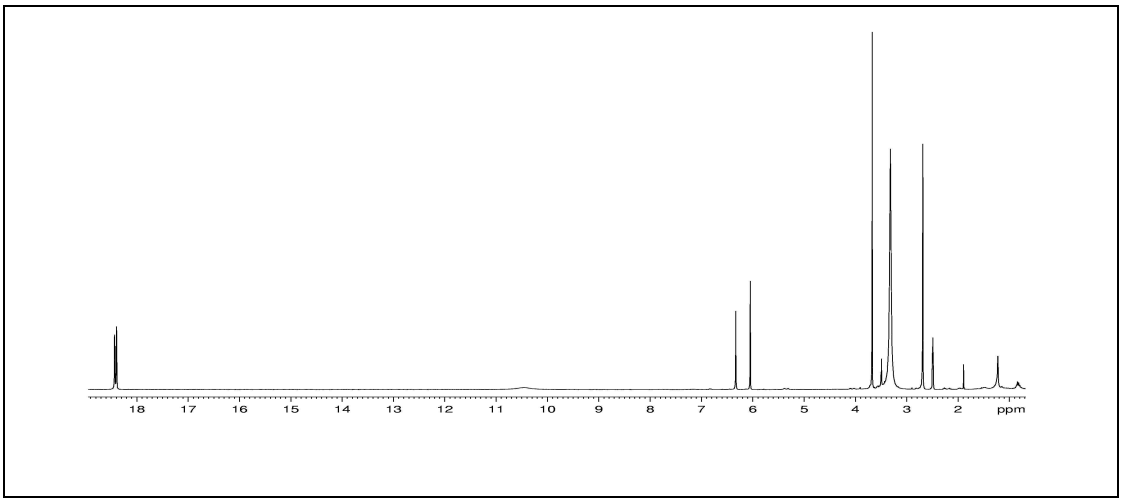


Fig. 28. ^{1}H NMR spectrum (DMSO-d_6) of funalenine (**48**)

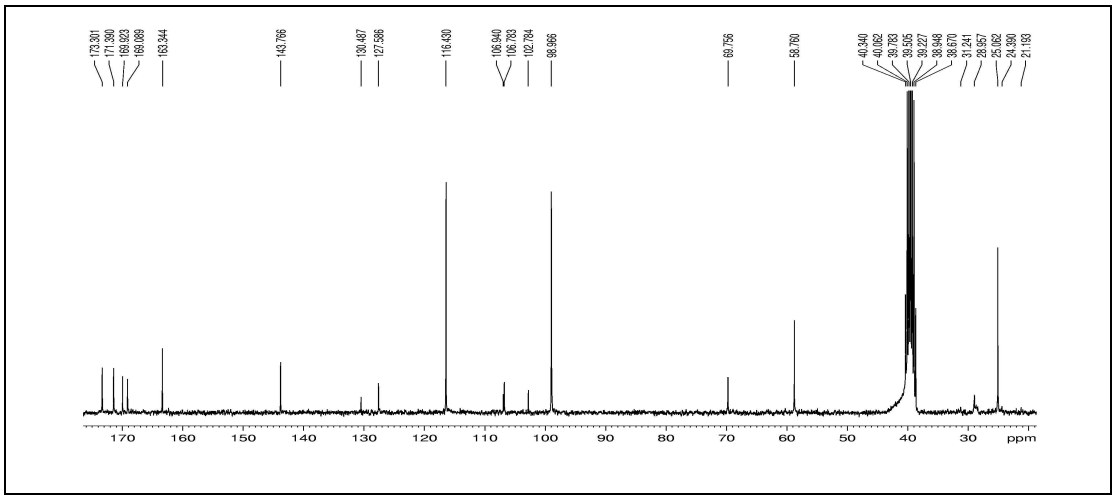


Fig. 29. ^{13}C NMR spectrum (DMSO-d_6) of funalenine (**48**)

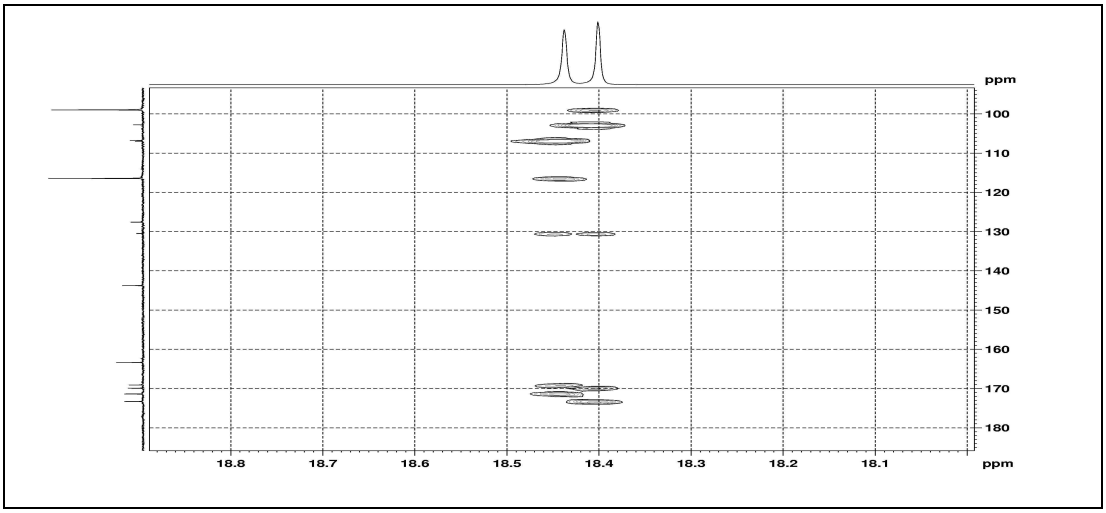


Fig. 30. Key HMBC correlation spectrum of funalenine (**48**)

3.2.4.3 Biological activity of funalenine

As funalenone (**49**), funalenine (**48**) showed no antimicrobial activity against a series of Gram-postive bacteria, Gram-negative bacteria and fungi at concentration >500 µg/ml. However, funalenine (**48**) displayed potent anti-inflammatory activity by inhibiting the enzyme 3α-hydroxysteroid dehydrogenase (Fig. 31), COX-1 and COX-2 with an IC_{50} value of 52 µM, 36 µM and 45 µM, respectively. Indomethacin was used as standard. It should also been noted that fungal phenalenones have been reported recently as inhibitor of HIV-1 integrase.[95]

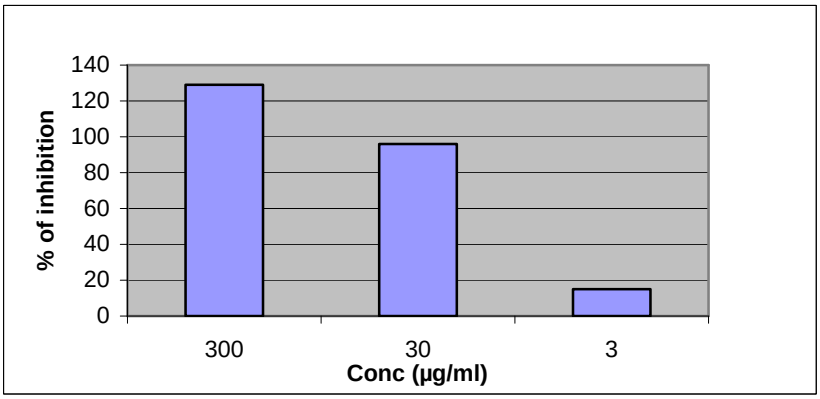
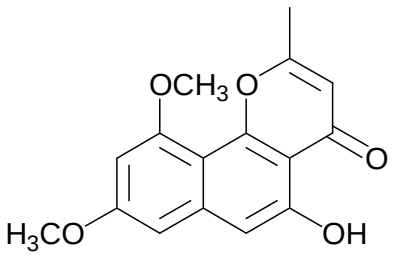


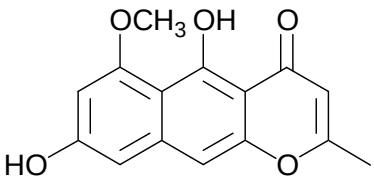
Fig. 31. Inhibition of 3α-HSD by funalenine (**48**)

3.2.4.5 Structure elucidation of TMC-256C2 and TMC-256A1

TMC-256C2 (**50**) and TMC-256A1 (**51**) were identified in comparison with the spectroscopic data of the known TMC-256C2 (asperxanthone) and TMC-256A1 from *Aspergillus niger*,[96] respectively.



50



51

51 is known as an inhibitor of IL-4 driven luciferase and germine Cε mRNA with IC_{50} values of 25 µM and 6.6 µM, respectively.

3.2.5 *Inonotus sp.* (fruiting body)

The fruiting body of *Inonotus* sp. was collected in Vietnam. Its identity was verified by Prof. Thrinh Tam Kiet from the Mycological Research Centre, Hanoi State University, Vietnam, where a specimen was deposited.

3.2.5.1 Extraction and isolation

100 g of fresh fruiting body was cut into small pecies, dried and crushed. The resulting powder was extracted three times with ethanol and $CHCl_3$:MeOH (3 x 2 l, 3 days each). The combined extracts (5 g) of a brown solid was subjected to a pre-screening, which revealed the presence of potent inhibitors of key enzymes involved in inflammatory processes, including 3α-HSD, COX and xanthine oxidase, as well as the presence of some moderate antibacterial components. For the isolation of the active components the extract was subjected in various chromatographic separation techniques. Bioassay-guided separation using open column and preparative HPLC yielded three new phenolic metabolites, inonotic acid methyl ester (**52**), inotilone (**53**), *iso*hispidin (**58**) and the known (*E*)-4-(3,4-dihydroxyphenyl)but-3-en-2-one (**56**) and hispidin (**57**).

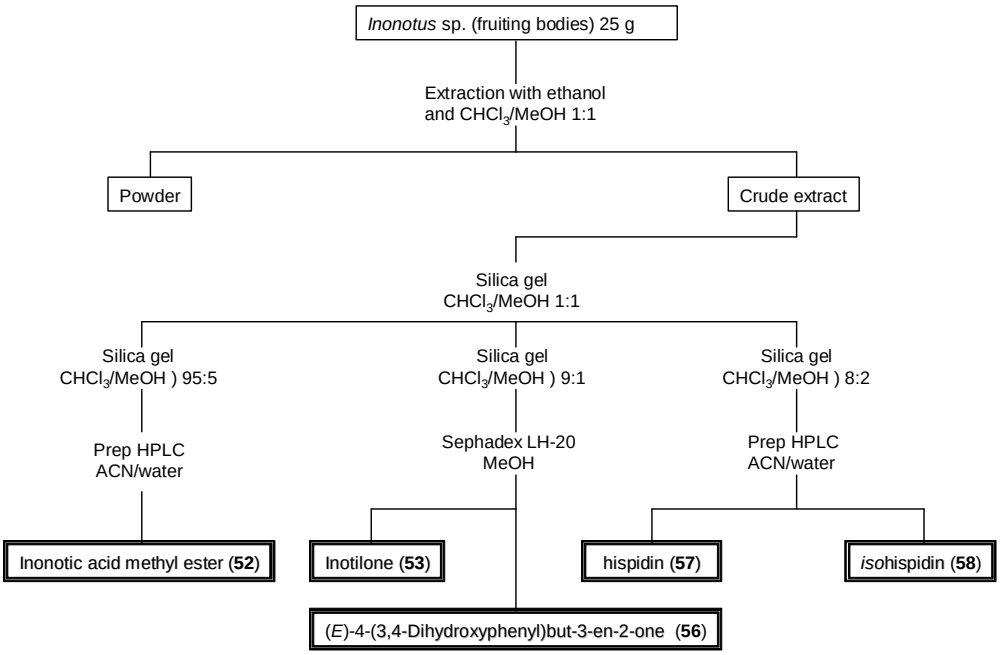
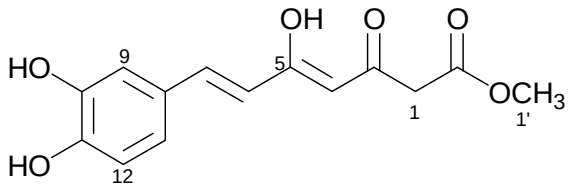


Fig. 32. Work up scheme for the isolation of phenolic components from *Inonotus* sp.

3.2.5.2 Structure elucidation of inonotic acid methyl ester (**52**)

The ESI-MS mass spectrum of **52** showed a molecular ion peak at m/z 277 $[M-H]^-$. Based on HR-EIMS and the ^{13}C NMR spectrum, the molecular formula of **52** was determined as $C_{14}H_{14}O_6$. The IR spectrum showed absorption bands for hydroxyl groups (3183 cm^{-1}), for a conjugated carbonyl (1632 cm^{-1}), for a carboxyl carbon (1733 cm^{-1}), and for aromatic rings (1567, 1513 and 1435 cm^{-1}). The ^1H NMR spectrum showed also signals attributable to the ABX spin coupling system of a trisubstituted phenyl moiety at δ 6.77 (1H, d, *J* = 8.1 Hz, H-12), 7.02 (1H, dd, *J* = 8.2, 1.8 Hz, H-13), 7.07 (1H, d, *J* = 1.8 Hz, H-9), a *trans* disubstituted double bond at δ 7.45 (1H, d, *J* = 15.8 Hz, H-7) and 6.50 (1H, d, *J* = 15.8 Hz, H-6), as well as two exchangeable phenolic hydroxyl protons at δ 9.15 and 9.65, and one chelated proton at δ 15.20. Analyses of ^{13}C, DEPT 135 and HMQC NMR spectra of **52** indicated 14 carbon signals including six sp^2 methines, four quaternary sp^2 carbons (three of which are oxygenated), one methylene carbon (δ 45.6), a methoxy carbon (δ 51.8) a carbonyl carbon (δ 191.8) and a carboxyl carbon (δ 167.9). For the connection of all these carbons HMBC was very helpful. The correlation of H-9 (δ 7.07) with C-7 (δ 141.0), C-8 (δ 126.2), C-10 (δ 145.6), C-11 (δ 148.4), the correlation of H-12 (δ 6.77) with H-8, H-10, H-11, H-13 and the correlation of H-13 (δ 7.02) with C-7, C-8, C-9, C-11 and C-12 revealed an *ortho* substitution of the phenolic hydroxyl protons. Another important correlation was that of the methylene protons with C-1 (δ 167.9), C-3 (δ 191.8) and C-4 (δ 100.3). Therefore, the structure of **52** was assigned as 7-(3,4-dihydroxy-phenyl)-5-hydroxy-3-oxo-hepta-4,6-dienoic acid methyl ester, named inonotic acid methyl ester (**52**).

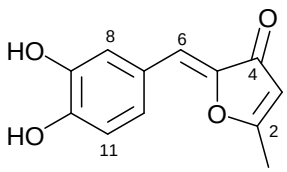


52

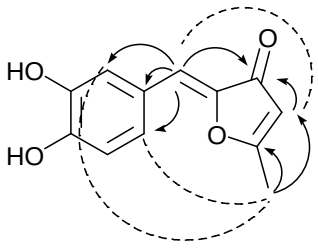
3.2.5.3 Structure elucidation of inotilone (**53**)

53 was obtained as a yellow oil. The IR spectrum of **53** showed absorption bands for hydroxyl groups at 3183 cm^{-1}, for a conjugated carbonyl (1681 cm^{-1}), and for aromatic rings (1588, 1516 and 1434 cm^{-1}). The ESI-MS mass spectrum showed the

molecular ion peak at m/z 217 $[M-H]^-$. Its molecular formula was determined as $C_{12}H_{10}O_4$ based on HR-ESIMS and the ^{13}C NMR spectrum. The ^{1}H NMR spectrum showed signals attributable to the ABX spin coupling system of a trisubstituted phenyl moiety at δ 6.80 (1H, d, *J* = 8.2 Hz, H-11), 7.17 (1H, dd, *J* = 2.0, 8.2 Hz, H-12) and 7.35 (1H, d, *J* = 2 Hz, H-8). Two olefinic protons δ 6.49 (1H, s, H-6), 5.82 (1H, d, *J* = 0.6, H-3) and a methyl proton signal δ 2.39 (3H, s, H-13) were also observed. The remaining two protons were attributable to the phenolic exchangeable hydroxyl protons. The ^{13}C NMR and DEPT135 spectra of **53** showed 11 sp^2 carbon signal including five methines, and three quaternary oxygenated carbons. The presence of the carbonyl moiety was also confirmed by the ^{13}C NMR spectrum, which showed one carbon signal of C-4 (δ 186.6). The protonated carbons and their corresponding protons and the full connection of the structure of **53** was established using HMQC and HMBC experiments, respectively. The correlation of the methyl protons with C-2 (δ 180.4), C-3 (δ 105.4) and the correlation of the olefinic proton (H-3) at δ 5.82 with C-2 (δ 180.4), C-4 (carbonyl moiety) δ 186.6 and C-5 (δ 144.3) unambiguously revealed a disubstituted dihydrofuranone moiety. The correlation of the olefinic proton H-6 (δ 6.49) with C-4 (δ 186.6), C-5 (δ 144.3), C-7 (δ 122.9), C-8 (δ 117.9) and C-12 (δ 124.7) enabled the connection of the dihydrofuranone moiety with the rest of the molecule. The stereochemistry of the C-5 double bond was carried out using molecular modeling and the NOESY spectrum, which showed a correlation between H-1' (δ 6.49) and H-3 (δ 5.82) and the correlation between the protons H-3' (δ 7.35) and H-7' (δ 7.17) with the methyl protons at δ 2.39. Thus, the structure was established as a new 2-(3,4-dihydroxybenzylidene)-5-methyfuran-3-one named inotilone (**53**).



53



Selected HMBC correlations

NOESY correlations

Fig. 33. Structure of inotilone (**53**) based on 2D NMR

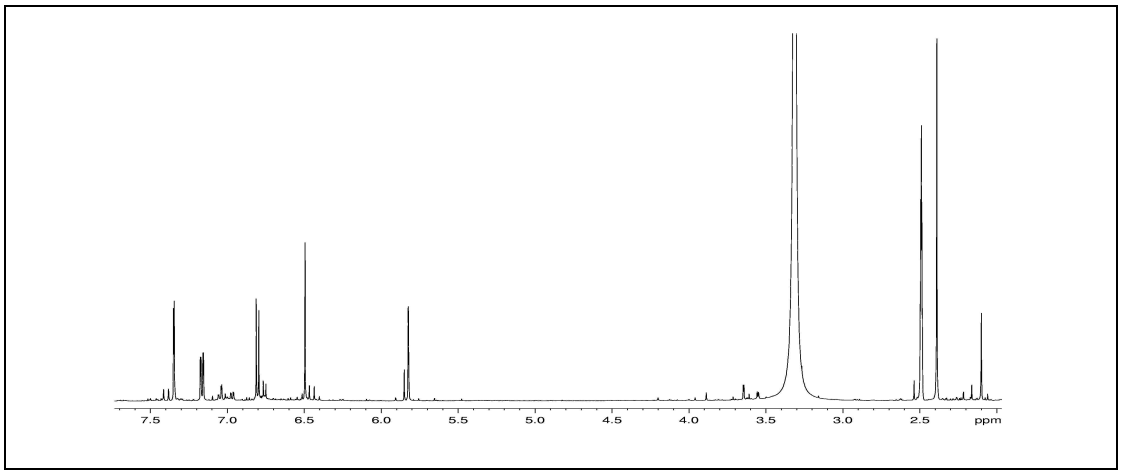


Fig. 34. ^{1}H NMR spectrum (DMSO-d_6) of inotilone (**53**)

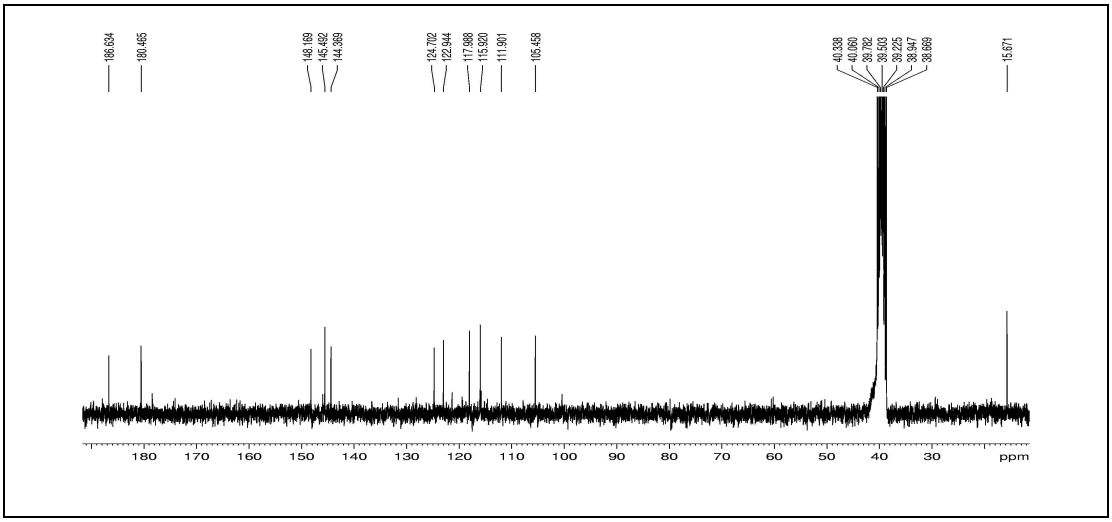


Fig. 35. ^{13}C NMR spectrum (DMSO-d_6) of inotilone (**53**)

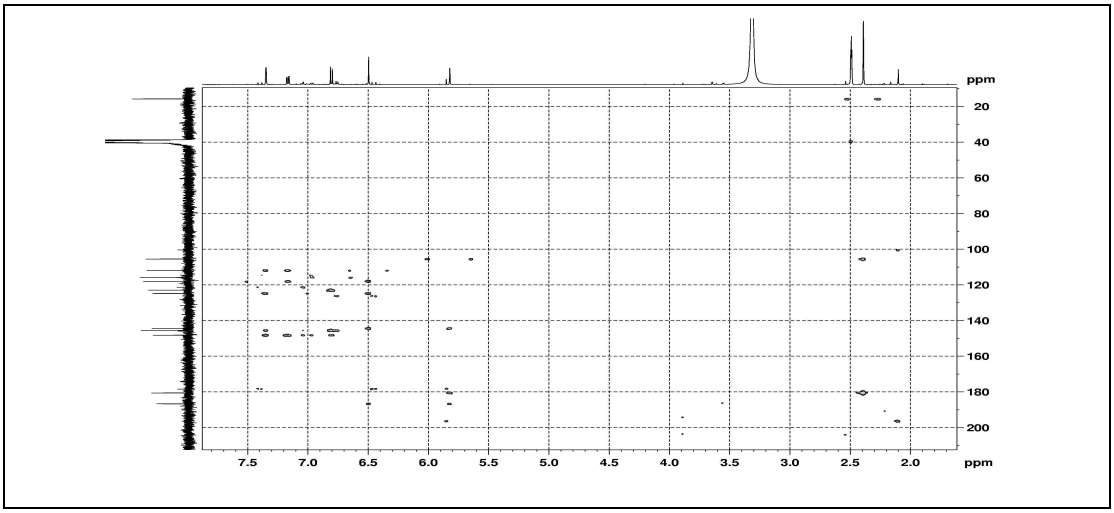
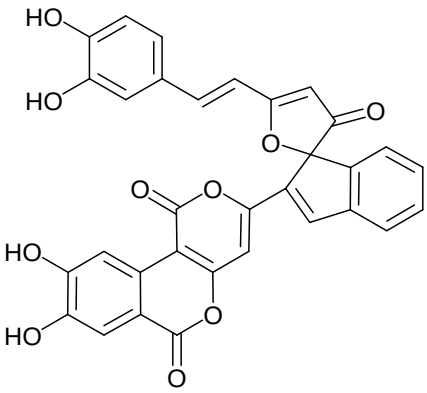
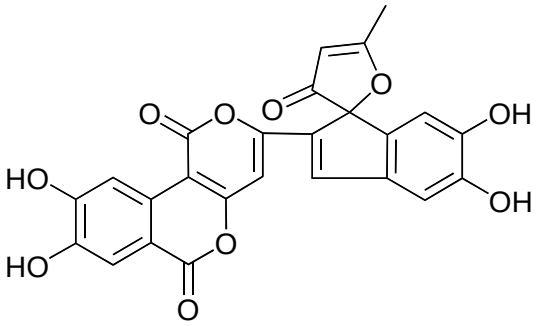


Fig. 36. HMBC spectrum of inotilone (**53**)

Recently, related 5-methyl-3(2*H*)-furanone metabolites including phillygridin G (**54**)[97] and phillygridin E (**55**)[98] have been reported from the fruiting body of *Phellinus igniarius*.



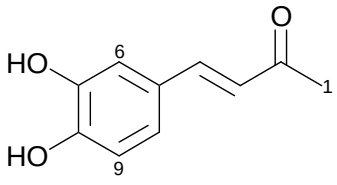
54



55

3.2.5.4 Structure elucidation of (*E*)-4-(3,4-dihydroxyphenyl)but-3en-2-one (**56**)

The molecular formula of **56** was determined as $C_{10}H_{10}O_3$ based on HR-ESIMS and the ^{13}C NMR spectrum. The IR spectrum indicated absorption bands for hydroxyl groups at 3459 cm^{-1}, for a conjugated carbonyl (1597 cm^{-1}) , and for aromatic rings (1596, 1533 and 1442 cm^{-1}). Similar to that of inotilone, the ^1H NMR spectrum of **56** showed signals attributable to the ABX spin coupling system of a trisubstituted phenyl moiety at δ 6.78 (1H, d, *J* = 7.78 Hz, H-19), 6.98 (1H, dd, *J* = 1.9, 8.2 Hz, H-10) and 7.07 (1H, d, *J* = 1.9 Hz, H-8), as well as a *trans* disubstituted double bond at δ 6.53 (1H, d, *J* = 16.1 Hz, H-3) and 7.52 (1H, d, *J* =16.1 Hz, H-4), and a methyl proton signal. Therefore, the structure of **56** was identified as the known (*E*)-4-(3,4-dihydroxyphenyl)but-3-en-2-one (**56**).[98]

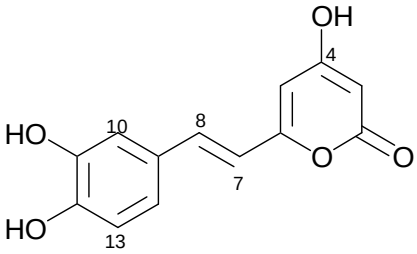


56

3.2.5.5 Structure elucidation of hispidin (**57**)

57, the major product of the mushroom *Inonotus* sp. was obtained as a yellow powder. The IR spectrum indicated absorption bands for hydroxyl groups (3093 cm^{-1}), for a conjugated carbonyl (1650 cm^{-1}), and for aromatic rings (1594, 1544 and 1449 cm^{-1}). The ESI-MS mass spectrum showed the molecular ion peak at m/z 245

[M-H]$^-$. Its molecular formula was determined as $C_{13}H_{10}O_5$ based on HR-ESIMS, ^{13}C NMR and DEPT 135 spectra. The ^1H NMR spectrum indicated signals attributable to the ABX spin coupling system of a trisubstituted phenyl moiety at δ 6.75 (1H, d, *J* = 8.4 Hz, H-13), 6.93 (1H, dd, *J* = 2.1, 8.4 Hz, H-14), 7.01 (1H, d, *J* = 2.1 Hz, H-10), a *trans* di-substituted double bond at δ 6.63 (1H, d, *J* = 15.9 Hz, H-7) and 7.13 (1H, d, *J* = 15.9 Hz, H-8) and three exchangeable phenolic hydroxyl protons at δ 9.02, 9.40 and 11.60. Analyses of ^{13}C, DEPT 135 and HMQC NMR spectra of **57** showed 13 sp^2 carbon signals (four of which are oxygenated), and a carboxyl carbon at δ 162.3. The long range correlation of H-7 (δ 6.63) with C-6 (δ 159.7) and C-5 δ 100.4, the correlation of H-5 (δ 6.14) with C-3 (δ 89.1), C4 (δ 170.3), C-6 (δ 159.7) and C-7 (δ 116.3) and the correlation of H-3 (δ 5.26) with C-2 (δ 162.3), C-4 (δ 170.3) and C-5 (δ 100.4) enabled the identification of **57** as 6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone, also known as metabolite hispidin (**57**), early reported from *Polyporus hispidus*.[99] A variety of activities including antimicrobial and antioxidant activities, as well as the inhibition of protein kinase C (PKC) βI and βII isoforms (IC_{50} 2μM) have been reported for hispidin.[100,101] Recent studies have demonstrated that highly-purified synthetic hispidin is cytotoxic at the range of 10^{-5} to 10^{-7} mol/l towards normal human MRC-5 fibroblasts and towards two human cancer cell lines, SCL-1 (keratinocytes) and capan-1 (pancrealitic duct cells).[102]

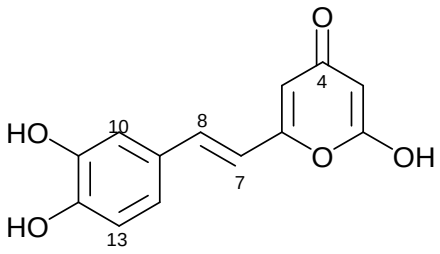


57

3.2.5.6 Structure elucidation of *iso*-hispidin (**58**)

The mass spectrum of **58** exhibited the molecular ion at m/z 245 [M-H]$^-$. Based on the HR-ESIMS the molecular formula was established as $C_{13}H_{10}O_5$. The IR spectrum showed absorption bands for hydroxyl groups (3359 cm^{-1}), for a conjugated carbonyl (1644 cm^{-1}) and for aromatic rings (1590, 1505 and 1414 cm^{-1}). The ^1H NMR spectrum of **58** showed signals similar to those of hispidin (**57**). But compared to those of hispidin their chemical shifts were quite different. This suggested that **58**

was the tautomeric form of **57.** This assumption was confirmed by the ^{13}C NMR spectrum, which showed a signal for a conjugated carbonyl (δ 179.1) attributable to the carbon C-4. Therefore, the structure of **58** was elucidated as the highly stable 6-(3,4-dihydroxystyryl)-2-hydroxy-4-pyrone, designated *iso*-hispidin (**58**).



58

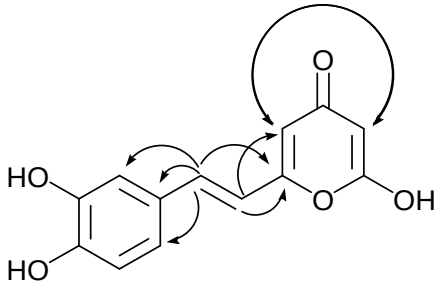


Fig. 37. Structure of *iso*-hispidin based on 2D NMR

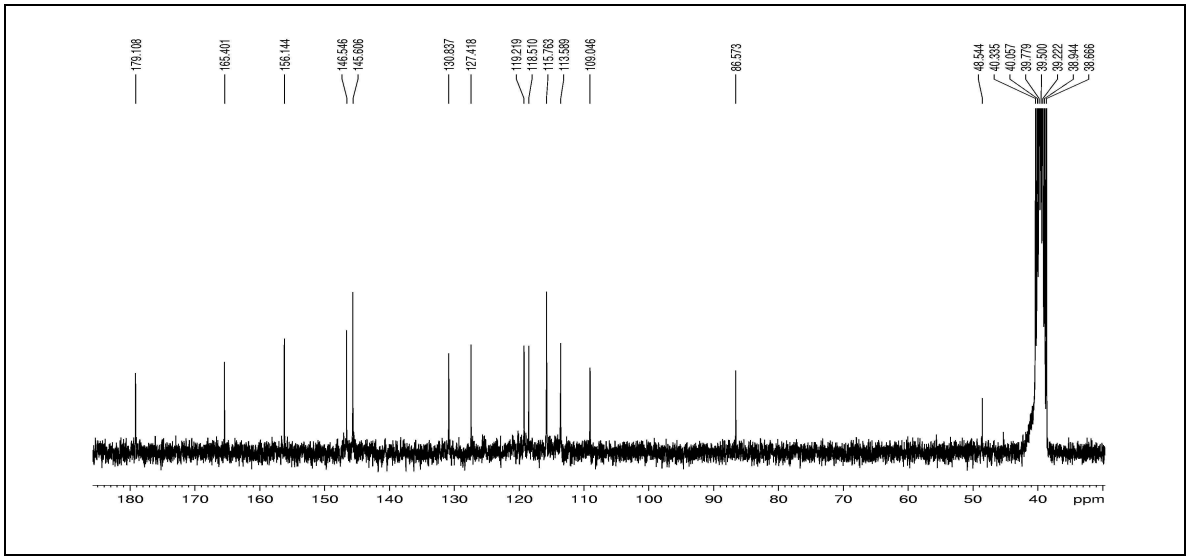


Fig. 38. ^{13}C NMR spectrum (DMSO-d_6) of *iso*-hispidin (**58**)

3.2.5.7 Biogenetic relation of the isolated metabolites from *Inonotus* sp.

The structures of compounds **52**, **53** and **58**, as well as that of the known compounds **56** and **57** suggest that all metabolites share the same biosynthetic origin. All compounds represent linear or cyclized polyketides derived from caffeyl-CoA (**59**). While **56** appears to be a shunt product resulting from a premature release from the polyketide synthase, **52**, **53**, **57** and **58** are the result of two rounds of elongation. The structurally unusual **53** could be the product of a decarboxylation-radical ring closure sequence via the known metabolite hispolon **64**.[103] A related sequence could

be involved in the formation of the tri- and tetrahydroxyaurone aglycones of sulphurein and cernuosides. [104,105]

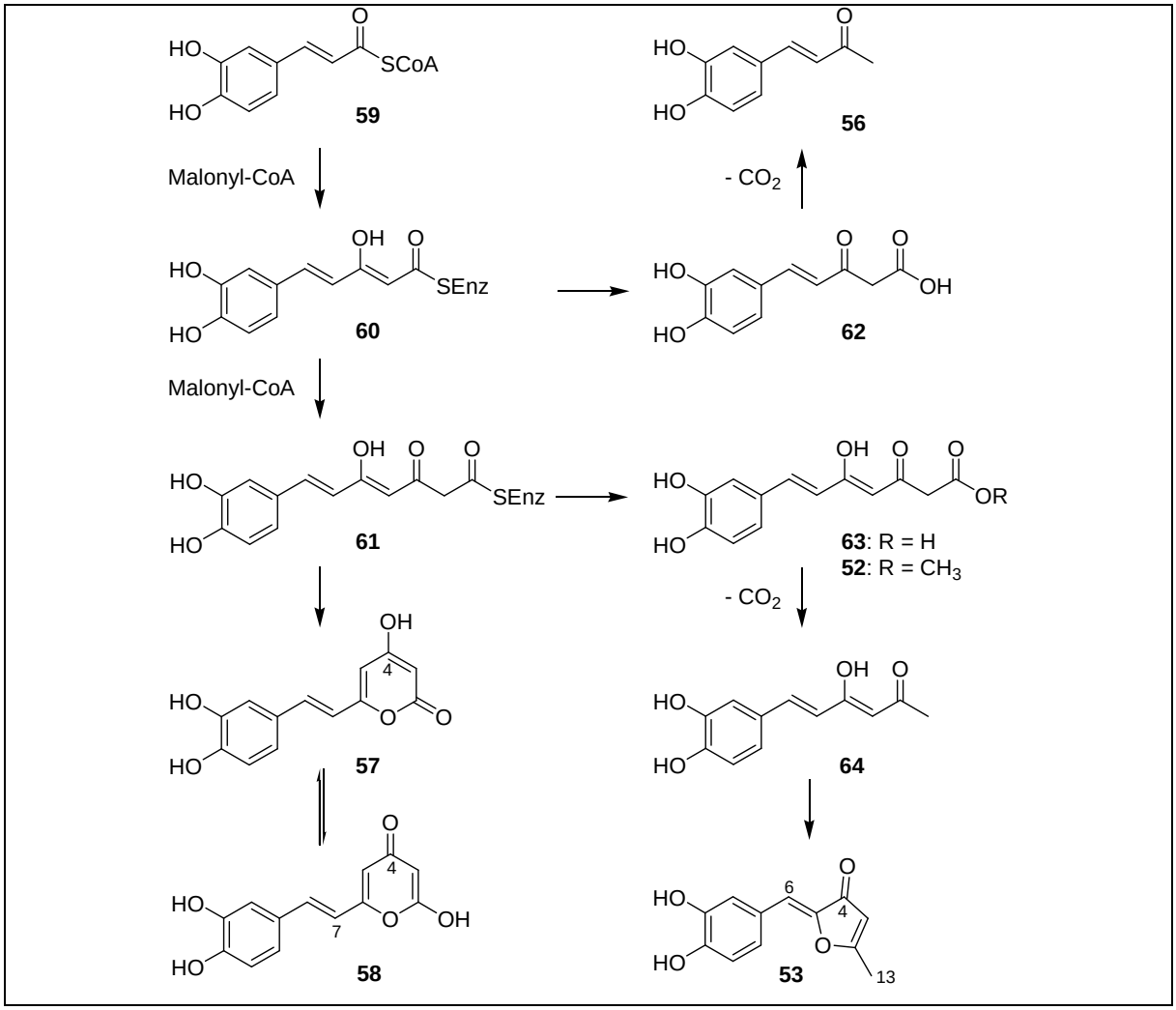


Fig. 39. Structures of *Inonotus* sp. metabolites and model for their biosynthesis

3.2.6 *Pholiota squarrosa*

The fruiting body of *Pholiota squarrosa* was isolated in Jena (Germany) and its taxonomic identification was verified by Dr. Martin Roth, from the Leibniz-Institute for Natural Product and infection biology, HKI, Pilot Plant for Natural Products, where a specimen was deposited. Similar to that of Inonotus sp., the crude extract of the mushroom *Pholiota squarrosa* exhibited significant inhibitory activities against 3α-HSD, COX and XO.

3.2.6.1 Extraction and isolation

The fruiting body of *Pholiota squarrosa* (15 g dry weight) was treated the same way as those of *Inonotus* sp. to afford a related phenolic metabolite, squarrosidine (**65**).

3.2.6.2 Structure elucidation of squarrosidine (**65**)

The molecular formula of **65** was determined as $C_{27}H_{20}O_9$ based on HR-ESIMS and ^{13}C NMR data. The ^{1}H NMR spectrum showed three signals attributable to the characteristic ABX spin coupling system at δ 6.72 (1H, d *J* = 8.1 Hz, H-13), 6.88 (1H, dd *J* = 1.8, 8.1 Hz, H-14), 6.96 (1H, d *J* = 1.8 Hz, H-10). In addition, two signals were assigned to an AA'BB' spin coupling system of a *para*-disubstituted phenyl moiety at δ 6.76 (2H, d, *J* = 8.7 Hz, H-11' and H-13'), 7.43 (2H, d, *J* = 8.7 Hz, H-10' and H-14'). Furthermore, the presence of two *trans*-disubstituted double bonds at δ 6.52 (1H, d, *J* = 15.9 Hz, H-7), 6.97 (1H, d, *J* = 15.9 Hz, H-8), 6.62 (1H, d, *J* = 15.9 Hz, H-7') and 7.06 (1H, d, *J* = 15.9 Hz, H-8') was established.

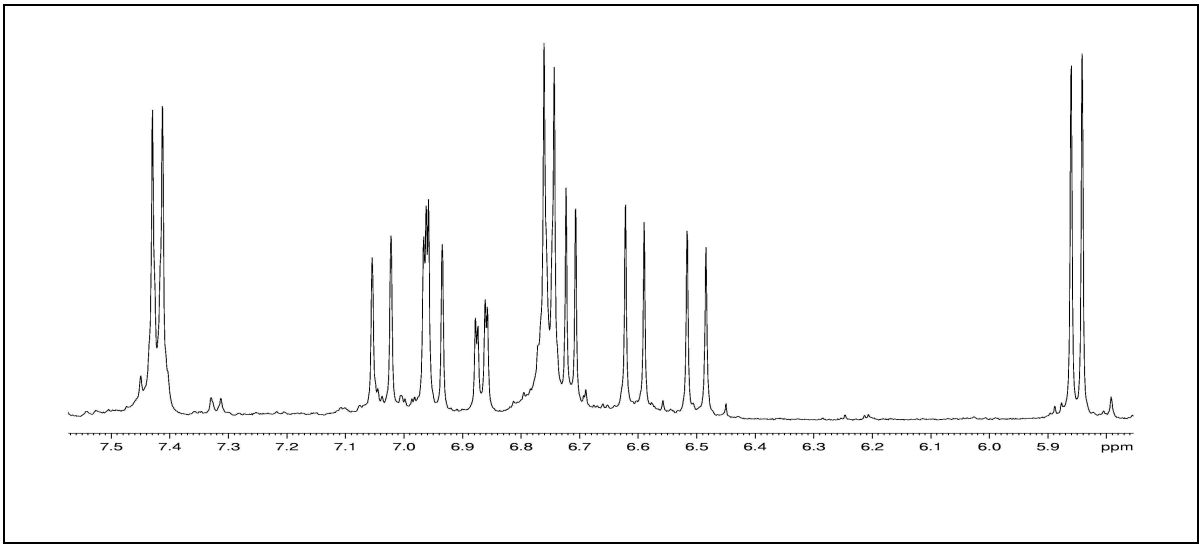
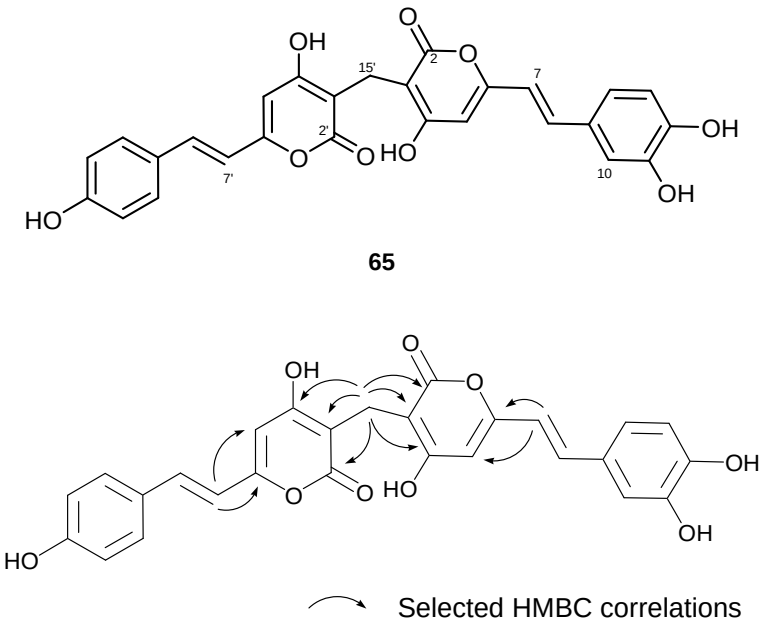


Fig. 40. ^{1}H NMR spectrum (DMSO-d_6) of squarrosidine (**65**)

The ^{13}C NMR and DEPT spectra showed one methylene sp^3 and 26 sp^2 carbon signals including 11 methines, 13 quaternary carbons (7 of which are oxygenated) and two carboxyl carbons. The presence of the carboxyl carbons was confirmed by IR, which showed the absorption band for a conjugated carboxyl carbon at 1661 cm^{-1}. HMQC and HMBC experiments helped resolving the structure (see figure 41). In the HMBC spectrum the correlation of H-10 with C-9, C-11, C-12, and C-14, the correlation of H-13 with C-9, C-11, C-12 and C-14 and the correlation of H-14 with C-

9, C-10, C-12 and C-13 confirmed the presence of the 11,12-dihydroxystyryl moiety. Moreover, the correlation of H-8 with C-6, C-9, C10, and C-14, that of H-7 with C-5, and the three-bond correlations of H-5 with C-3 revealed hispidin (**57**) as a substructure of **65**. This observation was confirmed by comparison of the ^{13}C NMR data with those of **57**. For the connection of the second part of **65**, HMBC was once more very helpful. The correlation of H-10' with C-10' and of H-11' with C-11' established the *para*-hydroxystyryl moiety. Furthermore, the correlation of H-7' with C-6', C-9', C-10', C-14'; the correlation of H-7' with C-5' and the correlation of H-5' with C-4' and C-3' helped identifying bisnoryangonin (**66**) as the second part of the molecule. It is remarkable that bisnoryangonin has been previously reported as a metabolite of *Pholiota squarrosa-adiposa*.[106] HMBC data (correlation of H-15' with C-2, C-2', C-3, C-3' C-4 and C-4') finally revealed that the two parts are connected through the C-15' methylene. Thus, **65** was elucidated as an unprecedented methylene-bridged bisstyrylpyrone derivative designated squarrosidine.



65

Fig. 41. Selected HMBC correlations of squarrosidine (**65**)

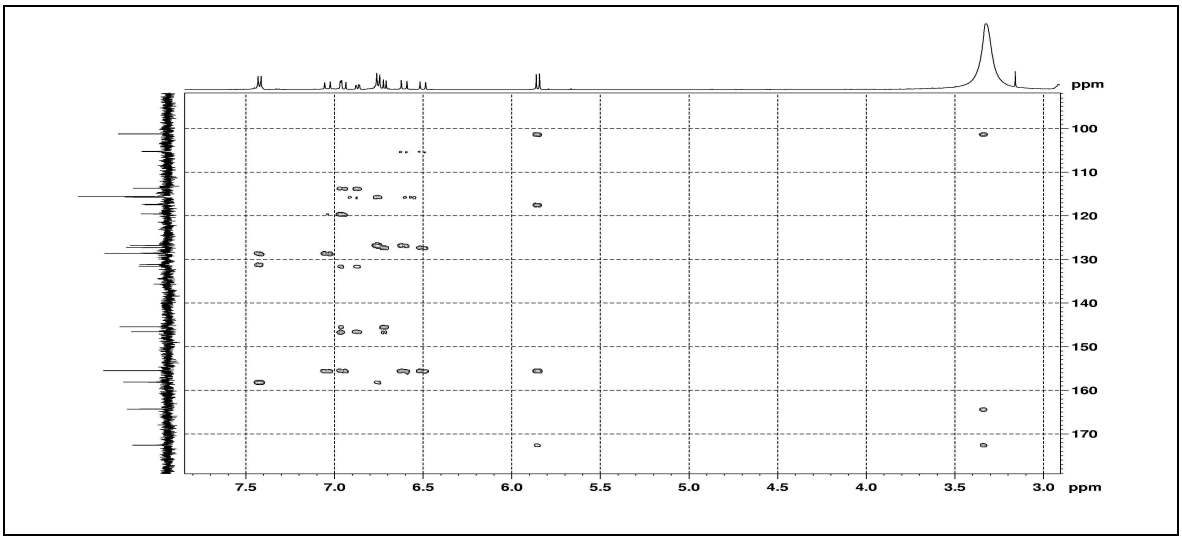


Fig. 42. HMBC spectrum of squarrosidine (**65**)

3.2.6.3 Model for the biosynthesis of squarrosidine

The formation of squarrosidine can be rationalized by the nucleophilic vinylogous addition of the enol of **57** to a tautomeric form of a methylated bisnoryangonin derivative **67** (or *vice versa*). (Fig. 43)

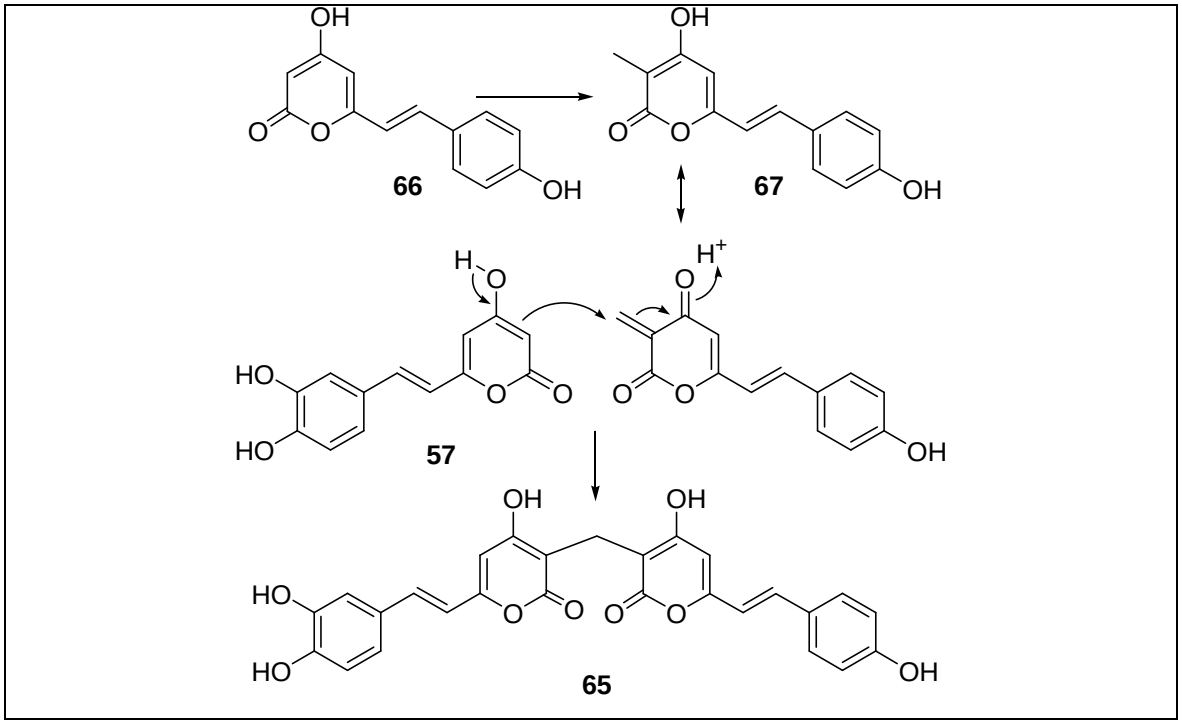
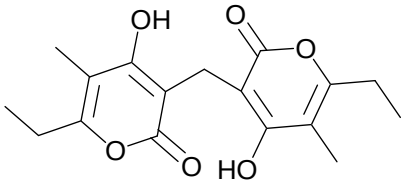
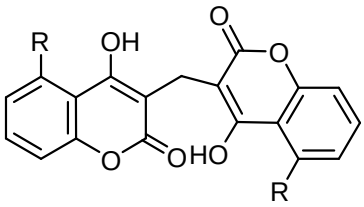


Fig. 43. Biosynthesis of squarrosidine (**65**)

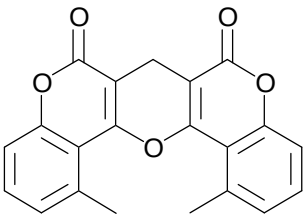
The only known methylene bridged *bis*-pyrone compounds are heliopyrone (**68**),[107] dicoumarol (**69**),[108] gerberinol (**70**),[109] and 1,13-dimethyl-6*H*,7*H*,8*H*-chromeno[3',4':5,6]pyrano[3,2-*c*]chromene-6,8-dione (**71**).[110]



68



69 R = H
70 R = CH_3



71

3.2.7 *Phellinus pini* DSM 5238

Phellinus pini was retrieved from the fungal collection of the Leibniz-Institute for Natural Product Research and Infection Biology, HKI.

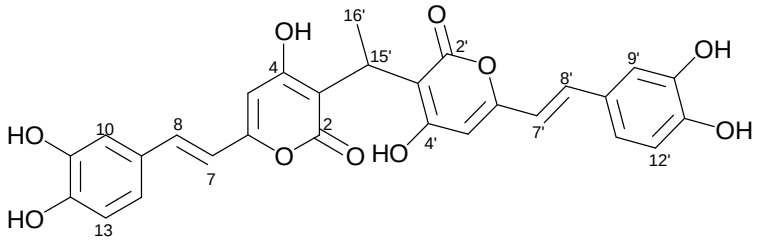
3.2.7.1 Cultivation, extraction and isolation

Well grown agar plates of the *Phellinus pini* DSM 5238 were used to inoculated 30 l of liquid malt medium as a stand culture. The dark brown crude extract from the ethyl acetate extraction of the culture broth exhibited significant inhibitory effect against 3α-HSD, COX and XO. It was therefore, applied on Sephadex and silica gel for the purification of active metabilites, using 300 ml portions of $CHCl_3$, $CHCl_3$/MeOH (9:1), and $CHCl_3$/MeOH (7:3). Further purification led to the isolation of three metabolites, pinillidine (**72**), pinillic acid (**73**), and hypholomine B (**74**). The work up scheme of the extract of the *Phellinus pini* DSM 5238 strain was the same as for *Trichoderma crassum.*

3.2.7.2 Structure elucidation of pillidine (**72**)

72 was obtained as an orange oil The IR spectrum showed absorption bands for hydroxyl groups (3071 cm^{-1}), for conjugated carbonyl (1672 cm^{-1}) and for aromatic rings (1601, 1548 and 1429 cm^{-1}). The mass spectrum exhibited the molecular ion at

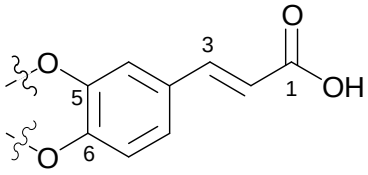
m/z 517 $[M-H]^-$. The HR-ESIMS established the molecular formula as $C_{13}H_{10}O_5$. The ^{1}H NMR spectrum of **72** was similar to that of hispidin (**57**), except for the presence of an additional doublet and quartet signals (δ 1.45, and 4.55, respectively). In the ^{13}C NMR spectrum of **72**, signals were observed at δ 16.5 and 25.5 in addition to those similar to **57**. According to the mass (m/z = 518), it became obvious that **72** represents a *bis*-hispidin derivative. The structure of **72** was then established as *bis*-hispidinylethane. This compound has not yet been described in the literature.



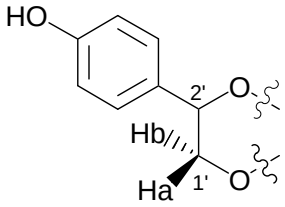
72

3.2.7.3 Structure elucidation of pinillic acid (**73**) and hipholomine B (**74**)

The molecular formula of **73** was determined as $C_{17}H_{14}O_5$ based on HR-ESIMS and ^{13}C NMR data. The ^{1}H NMR spectrum showed three signals attributable to the characteristic ABX spin coupling system at δ 7.18 (1H, d, *J* = 2.0 Hz, H-5), 6.93 (1H, d, *J* = 8.7 Hz, H-8), 7.12 (1H, dd, *J* = 2.0, 8.7 Hz, H-9). In addition, two signals were assigned to an AA'BB' spin coupling system of a *para*-disubstituted phenyl moiety at δ 7.28 (2H, d, *J* = 8.5 Hz, H-4' and H-8'), 6.83 (2H, d, *J* = 8.5 Hz, H-5' and H-7'). Furthermore, the presence of a *trans*-disubstituted double bond at δ 6.34 (1H, d, *J* = 15.9 Hz, H-2), 7.59 (1H, d, *J* = 15.9 Hz, H-3) was also established. The ^{13}C NMR spectrum showed a carboxylic acid carbon signal, as well as 12 sp^2 carbon signals (three of which are oxygenated) and two oxygenated sp^3 carbon including one oxymethine (δ 76.2) and one oxymethylene (δ 70.5). The oxymethylene protons appeared as two diastereotopic protons at δ 4.04 (H-1'a, dd, *J* = 8.8, 11.5 Hz, 1H) and 4.36 (H-1'b, dd, *J* = 2.3, 11.5 Hz, 1H). Moreover, the COSY spectrum revealed the alignment H1'/H2'. These observations were evidence of the presence of an assymetric centre at C-2' (δ 76.2). The HMBC spectrum enabled the elucidation of the final structure as follows: the correlation of H-2 (δ 6-34, d, *J* = 15.9 Hz, 1H) and H-3 (δ 7.59, d, *J* = 15.9 Hz, 1H) with C-1 (δ 170.7), and the correlation of H-3 with C-5 and C-6 helped identifying caffeic acid (substructure 1) as a substructure of **73**.

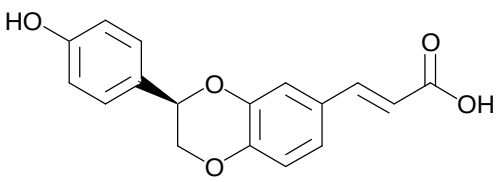


substructure 1



substructure 2

The substructure 2 of **73** was also established according to the correlation of H-2' with H-4'. Finally the observed strong correlation between H1'a and H-7 led to the elucidation of the structure of **73** as that of a new natural product, belonging to the class of lignan secondary metabolites. The relative steochemistry of **73**, designated pinillic acid (**73**), was established using NOESY, which showed correlations between H-1'a and H-8, H-1'a and H1'b and between H-1'a and H-2'.



73

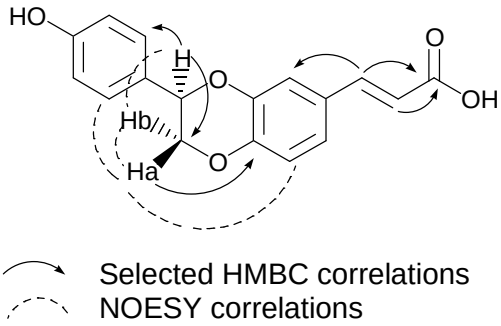


Fig. 44. Structure of pinillic acid based on 2D NMR

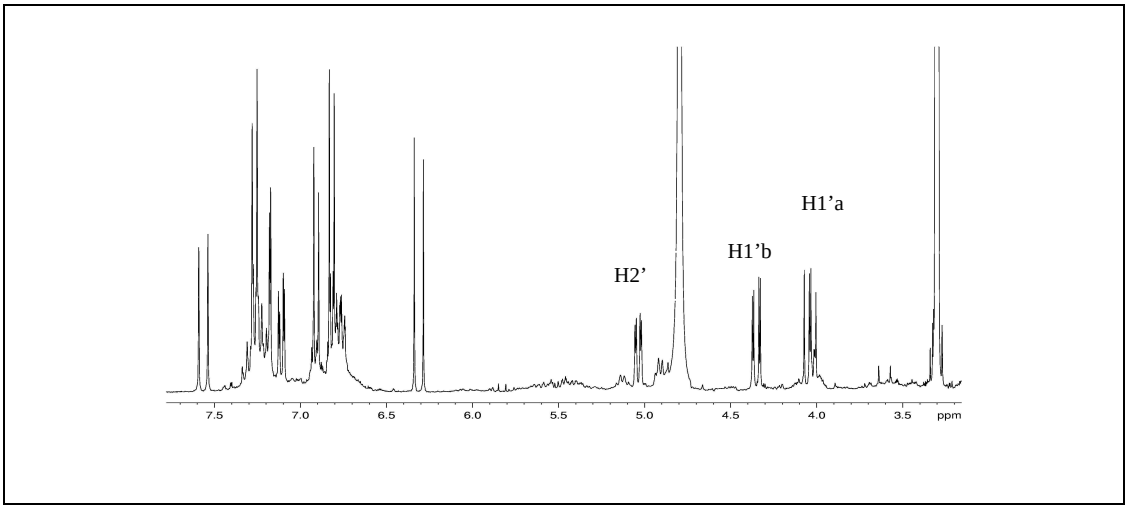


Fig. 45. ^{1}H NMR spectrum (CD_3OD) of pinillic acid (**73**)

Further confirmation of the structure was derived from the MS/MS spectrum, which indicated the peak fragments at m/z = 253, 177, 133 and 119 in negative mode. These fragments are the result of a decarboxylation (m/z = 253) and the cleavage of the dioxane moiety through a retro-Diels-Alder mechanism, (m/z = 177 and 119) followed by the decarboxylation (m/z = 133). (Fig. 46) Related metabolites have been isolated from plant including *Grewia bilamellata*,[111] *Silybum marianum*[112] and *Hibiscus syriacus*.[113]

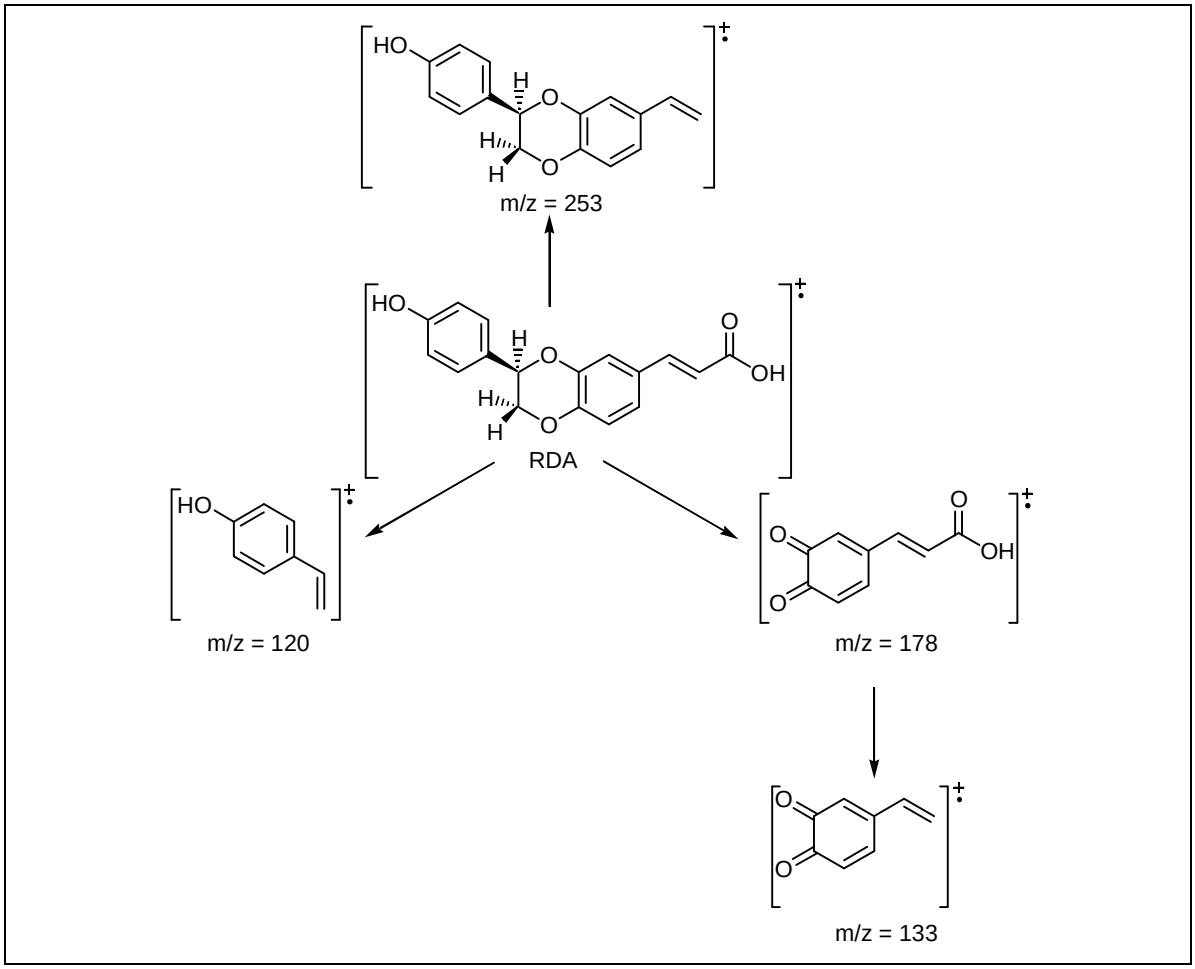


Fig. 46. Fragmentation mechanism of pinillic acid based on MS/MS

3.2.7.4 Model for the biosynthesis of Pinillic acid

The biosynthesis of pinillic acid can be rationalized by a peroxidase catalysed cycloaddition between *p*-hydroxyvinylbenzene and caffeic acid. (Fig. 47)

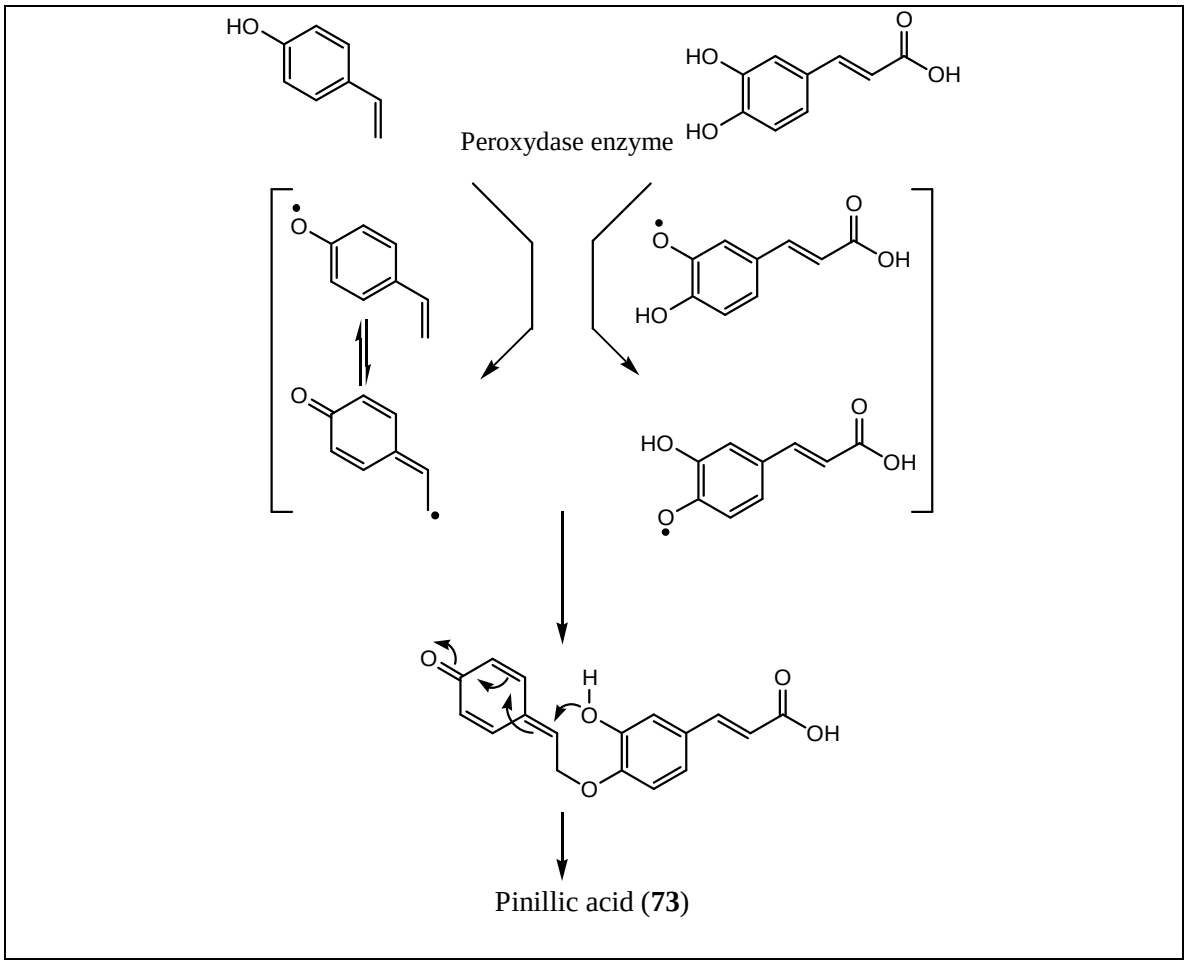
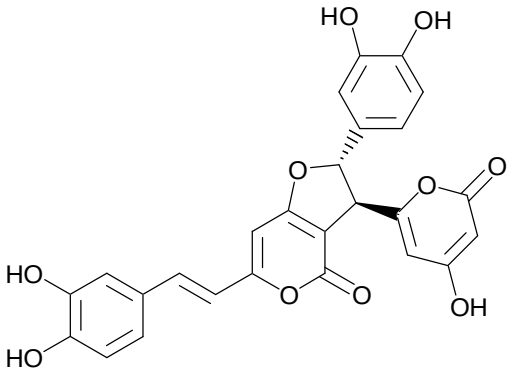


Fig. 47. Proposed biosynthesis of pinillic acid (**73**)

All the spectroscopic data of **74** proved to be that of the diasteroisomer mixture of the known hypholomine B (**74**). [114]



74

3.2.7.5 Biological activity evaluations of the metabolites from *Inonotus* sp. and *Pholiota squarrosa*

All phenolic metabolites from *Inonotus sp*, *Pholiota squarrosa* and *Phellinus pini* were evaluated for their inhibitory activities towards the enzymes 3α-hydroxysteroid

dehydrogenase (3α-HSD), COX-1, COX-2 and XO. Their inhibitory potencies, expressed as IC_{50} values, are shown in table 4 and are compared with those of the standards, indomethacin and allopurinol. The results in the present study demonstrate that phenolic compounds exhibit strong COX inhibitory effects with prevalence for COX-2 in the case of the compounds **52**, **53**, **56**, **57**. Interestingly, hispidin (**57**) and the new inotilone (**53**) selectively inhibit COX-2 at a level comparable with those of the marketed selective inhibitors meloxicam and nimesulide with an IC_{50} ratio (COX-2/COX-1) value of 0.08 μM both. In all cases, except for compound **53**, strong 3α-HSD inhibitory effects were noted, as well as moderate inhibitory effects towards XO, except hispidin (**57**), which exhibited an inhibitory activity at a level comparable with that of the standard allopurinol. As far as the tautomeric compounds **57** and **58** are concerned, it seems that the α-pyronne is more active than the γ-pyrone. In summary, we have isolated and characterized five new phenylpropanoid-derived polyketides with potent COX and XO inhibitory activities from the mushrooms *Inonotus* sp., *Pholiota squarrosa*, and *Phellinus pini* DSM 5238. Apart from their anti-inflammatory activities, the structures of the dihydrofuranone-substituted and dioxane-substituted caffeyl derivative **53** and **73** and the unusual bisstyrylpyrones **65** are most notable.

Compound	**IC_{50} (μM)**				
	3α-HSD	**COX-1**	**COX-2**	**COX-1/COX-2**	**XO**
52	16.1	0.46	0.21	0.4	7.1
53	50.4	0.3	0.03	0.08	9.1
56	8.9	0.03	0.01	0.3	10.1
57	8.1	0.01	0.0008	0.08	4.4
58	12.1	0.05	0.13	2.6	13.8
65	8.1	0.02	0.04	2.0	8.1
72	5.8				5.8
73	-	-	-		-
74	25.5				6.5
Indomethacin	15.8	0.1	6	60	n.a
Allopurinol	n.a	n.a	n.a		4.4

Table 4. Inhibitory activities of **52**, **53**, **56**, **57**, **58**, **65, 72, 73 and 74** towards 3α-HSD, COX-1, COX-2 and XO.

3.2.8 *Piptoporus betulinus* (fruiting body)

The fruiting bodies of *Piptoporus betulinus* were collected in a forest district near Jena (Thuringia, Germany) and deposited in the strain collection of the Leibniz-Institute for Natural Product Research and Infection Biology, HKI.

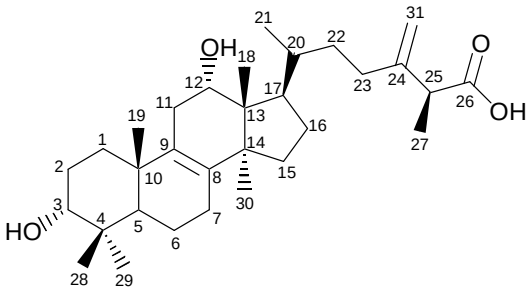
3.2.8.1 Extraction and isolation

The mushroom of the fungus *Piptoporus betulinus* was cut into small pecies, dried and crushed. The resulting powder was extracted three times with ethyl acetate, chloroform and methanol. The ethyl acetate extract of a brown solid, which was found to have a strong anti-inflammatory effect (significant inhibitory activity against 3α-HSD), was subjected to silica gel chromatography and preparative HPLC, affording five lanostanoids-like triterpenes (two of which are new) namely, polyporenic acid A (**75**), acetylpolyporenic acid A (**76**), (25*S*)-(+)-12α-hydroxy-3α-methylcarboxyacetate-24-methyllanosta-8,24(31)-diene-26-oic acid (**77**), (25S.3‘S)-(+)-12α-hydroxy-3α-(3‘-hydroxy-4‘-methoxycarbonyl-3‘-methylbutyryloxy)-24-methyllanosta-8,24(31)-dien-26-oic acid (**78**) and Polyporenic acid C (**79**).

3.2.8.2 Structure elucidation of polyporenic acid A (**75**)

The molecular formula of **75** was determined as $C_{31}H_{50}O_4$ based on HR-ESI-MS. The ^{1}H NMR spectrum of **75** showed 50 non exchangeable protons, including two olefinic protons and seven methyl groups. Analyses of the ^{13}C NMR, DEPT 135 and HMQC spectra of **75** further identified the presence of seven methyl carbons, ten methylene carbons (one of which is sp^2 hybridized), six methine carbons (two of which are oxygenated), six quaternary carbons (two of which are sp^2 hybridized) and a carboxyl carbon δ 178.7. This was also confirmed by the IR spectrum, which showed absorption at 1708 cm^{-1}. The sequences of couplings H15/H16/H17/H20; H21/H20/H22/H23/H31, H6/H7, H1/H2/H3 was obtained by ^{1}H-^{1}H COSY spectra. For the assignment of the other constituting protons and carbons ^{13}C, ^{1}H long range coupled NMR spectra (HMBC) were particularly helpful. The correlation of H-31 (δ 4.89, brs, 2H) with C-24 (δ 151.6) and C-23 (δ 33.2), the correlation of H-25 (δ 3.16, q, *J* = 7.0 Hz, 1H) with C-26 (δ 178.7), C-27 (δ 16.2) , C-23 (δ 33.2) , C-24 (δ 151.6) and C-31 (δ 110.0) and the correlation of H-27 (δ 1.25, d, *J* = 7.0, Hz, 3H) with C-24 (δ 151.6), C-25 (δ 48.3) and C-26 (δ 178.7) were evidence of the connection of the carboxyl carbon in C-25. Another important aspect of the analyses was the observed

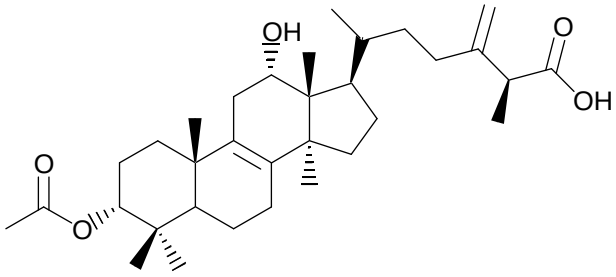
HMBC correlations of H-12 (δ 4.00, d, *J* = 8.1 Hz, 1H) with C-9 (δ 134.7), C-11 (δ 32.9), C-13 (δ 50.7) and C-14 (δ 50.6). These correlations helped identifying the position of the hydroxymethine. **75** was then elucidated as the known polyporenic acid A. The spectral data were found to be in good agreement with those reported.[115,116]



75

3.2.8.3 Structure elucidation of 3α- acetylpolyporenic acid A (**76**)

76 was obtained as a colourless oil $[\alpha]^{22}_{D}$+30^{o} (MeOH; c 0.18). Its formula was determined as $C_{33}H_{52}O_5$ based on HR-ESIMS. The ^{1}H NMR spectrum of **76** was similar to that of polyporenic acid A (**75**) except for the presence of an additional singlet at δ 2.06 (s, 3H). In the ^{13}C NMR spectrum two additional signals were observed at δ 171.0 (carboxyl carbon signal), and 21.3 (methyl carbon signal). The long range HMBC helped connecting these additional carbons by showing a correlation of the methine proton H-3 (δ 4.65, brs, 1H) and the methyl proton H-2'(δ 2.06, s, 3H) with the carboxyl carbon C-1' (δ 171.0). This indicated that the carbon C-3 is connected to an acetate group. Consequently, **76** was identified as 3α-acetylpolyporenic acid A (**76**).



76

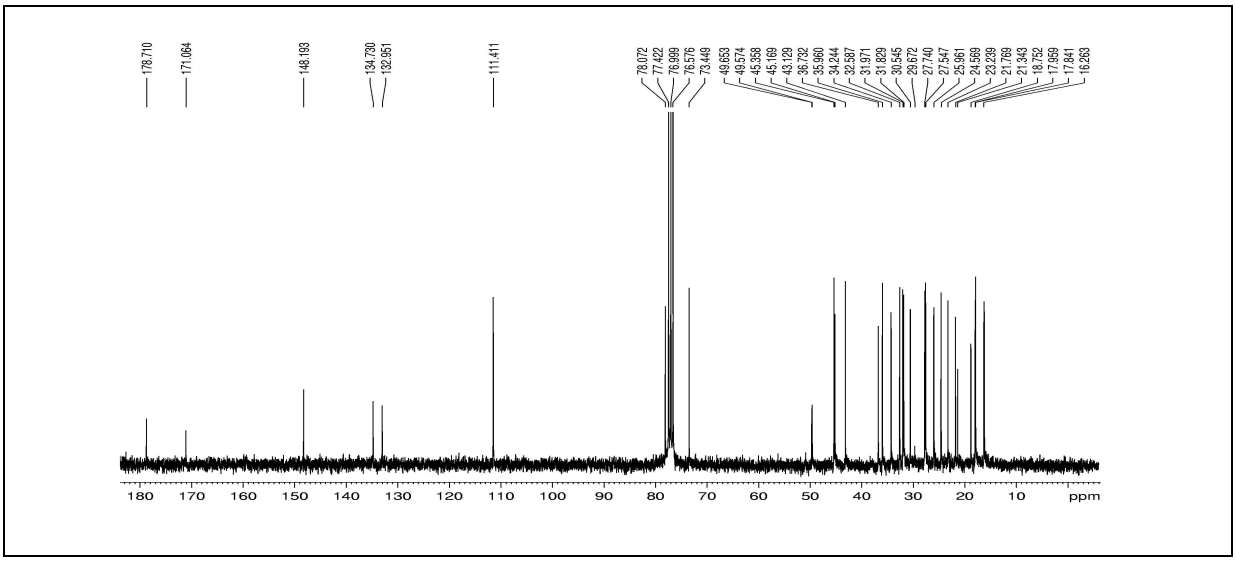
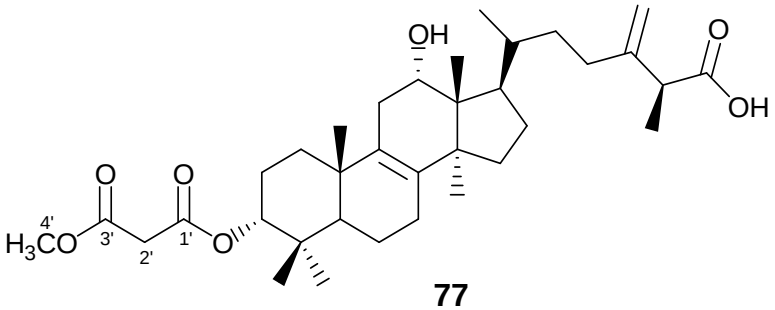


Fig. 48. ^{13}C NMR spectrum ($CDCl_3$) of **76**

3.2.8.4 Structure elucidation of (25*S*)-(+)-12α-hydroxy-3α-methylcarboxyacetate-24-methyllanosta-8,24(31)-diene-26-oic acid (**77**)

In the ^{1}H and ^{13}C NMR spectra of **77**, which was obtained as a colourless oil $[\alpha]^{22}_{D}$+19.2^{o} (MeOH; c, 0.50), all the signals assignable to **75** were almost identical. But the C-3 (δ 79.7) of **77** was substituted by another functional group. The ^{1}H NMR spectrum revealed the presence of two additional singlets at δ 3.39 (s, 2H) and 3.70 (s, 3H). In the ^{13}C NMR spectrum, additional signals were also observed at δ 41.7 (CH_2), 52.3 (CH_3O), 166.0 ppm (C=O) and 167.2 (C=O). The long range HMBC spectrum data helped elucidating the additionnal moiety by showing the correlation of the protons of the additional methylene with the two additional carboxyl carbons, and the correlation of the protons of additional methoxy moiety with the carboxyl carbon at δ 167.2. Thus, the substituent at C-3 (δ 79.7) could be identified as methyl malonate ($-CO_2CH_2CO_2CH_3$), which was supported by the IR spectrum (strong absorption at 1722 cm^{-1}) and MS data (pseudo molecular ion peak at m/z 609.3756 $[M+Na]^{+}$). Consequently, compound **77** was identified as (25*S*)-(+)-12α-hydroxy-3α-methylcarboxyacetate-24-methyllanosta-8,24(31)-diene-26-oic acid (**77**).



77

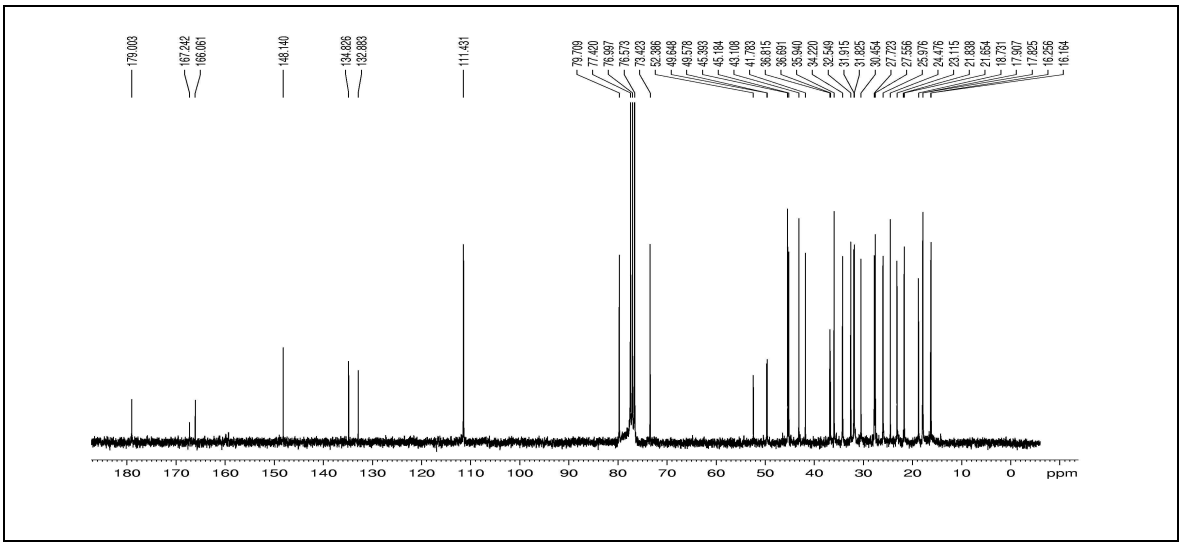
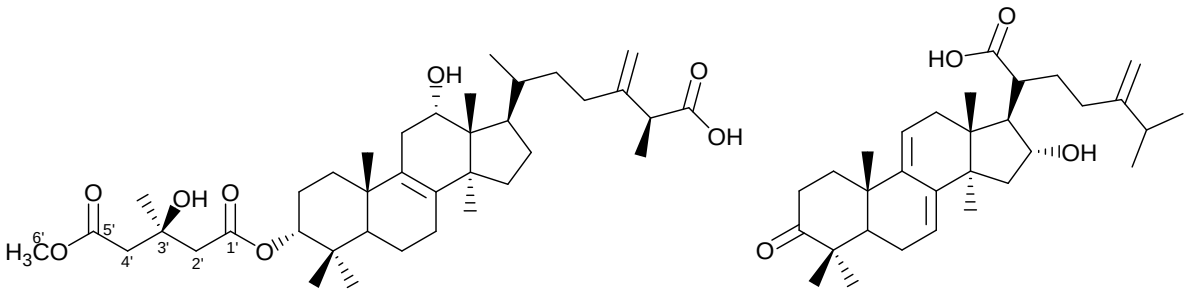


Fig. 49. ^{13}C NMR spectrum ($CDCl_3$) of **77**

This is the first isolation and full characterization of **76** and **77**. However it is important to note that their methyl ester derivatives have been isolated from the methylated extract of *Piptoporus betulinus*.[117]

3.2.8.5 Structure elucidation of (25S.3'S)-(+)-12α-hydroxy-3α-(3'-hydroxy-4'-methoxycarbonyl-3'-methylbutyryloxy)-24-methyllanosta-8,24(31)-dien-26-oic acid (**78**) and Polyporenic acid C (**79**)

Similar to those of **76** and **77**, the ^{1}H and ^{13}C NMR spectra of **78** indicated all signals assignable to polyporenic acid A (**75**). The ESI-MS exhibited a molecular ion peack at m/z = 667 $[M+Na]^+$, suggesting that the C-3 of **78** was substituted by a functional group other than acetate or methyl malonate. A signal due to an oxygen-bearing quaternary carbon was observed at δ 69.7 in the ^{13}C NMR spectrum. Three correlations from the methyl protons at δ 1.39 to the quaternary carbon C-3' (δ 69.7), to the methylene carbon C-2' (δ 45.0) and to the other methylene carbon C-4' (δ 44.8) were observed from the HMBC spectrum. Finally the two additional carbon signals attributable to the carboxyl carbons, enabled the identification of **78** as the *O*-(3-hydroxy-3-methylglutaryl) derivative of **75**: ((25S.3'S)-(+)-12α-hydroxy-3α-(3'-hydroxy-4'-methoxycarbonyl-3'-methylbutyryloxy)-24-methyllanosta-8,24(31)-dien-26-oic acid). The absolute configuration of *O*-(3-hydroxy-3-methylglutaryl)[118] was determined by comparing the optical rotation with literature data. All physicochemical and spectrometric data obtained for compounds **79** proved to be identical with those reported for polyporenic acid C.[118]



78 **79**

3.2.8.6 Biological activity of the isolated metabolites from *Piptoporus betulinus*

3.2.8.6.1 Anti-inflammatory activity

76-**79** were tested for their ability to inhibit the key enzymes involved in the inflammatory processes including COXs and 3α-HSD. While **76**-**79** exhibited only weak inhibition activity of COX-1, they proved to be promising anti-inflammatory agents in the 3α-hydroxysteroid dehydrogenase (3α-HSD)-assay according to the method described by Penning.[119] Most remarkably, **76**, **78** and **79** inhibited 3α-HSD at the level comparable to indomethacin reference and **77** exhibited a stronger activity than that of indomethacin. (Fig. 50 and table 5)

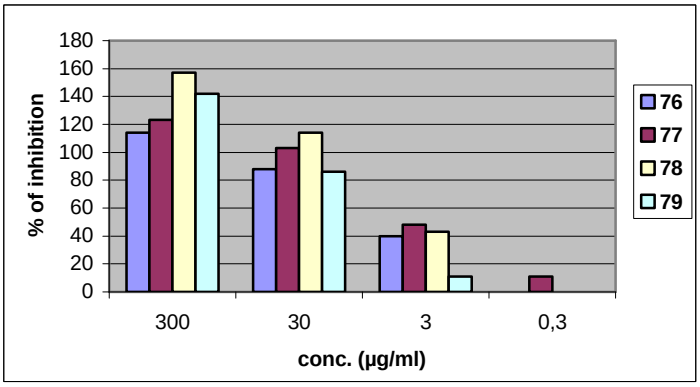


Fig. 50. Inhibition of **76**, **77**, **78** and **79** towards 3α-HSD

	76	**77**	**78**	**79**	indomethacin
IC_{50} (µg/ml)	8.5	4.0	5.5	17.5	6.5

Table 5. Inhibitory activity of **76**-**79** against 3α-HSD (IC_{50} values)

3.2.8.6.2 Anti-hyaluronidase activities

Anti-hyaluronidase activities were measured spectrophotometrically based on the precipitation of undigested hyaluronane by *N*-cetyl-*N*,*N*-trimethylammoniumbromide (CTA) and the measurement as turbidity (E_{600nm}) using the method described by Di Ferrante.[120] Although **76-79** showed no activity against bovine hyaluronidase (Fig. 51 and table 6) they are potent inhibitors of hyaluronate lyase from *Streptococcus agalactiae*. This enzyme acts as surface antigen, which is responsible of the degradation of hyaluronane (hyaluronic acid), the most abundant matrix of the connective tissues, predominantly into disaccharide units. This degradation facilitates the invasion of bacterial pathogens, mostly Gram-positive *Streptococci* into the host tissues.[121,122] Strikingly, the methylated derivatives of **78** and **79** exhibited no inhibition towards the bacterial hyaluronate lyase, and bovine hyaluronidase, suggesting that the carboxyl moieties of **76**-**79** are essential for activity. To date only saccharic acid is known to selectively inhibit bacterial hyaluronate lyase, while not affecting the enzymatic activity of testicular hyaluronidase.[123] Thus, **76**-**79** appear to be a new class of compounds possessing a similar selectivity.

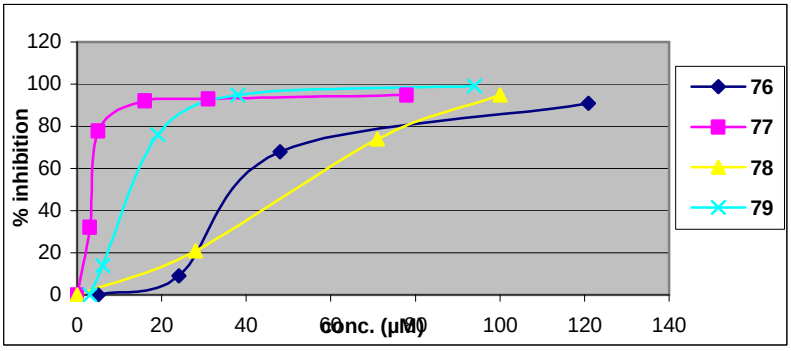


Fig. 51. Inhibition of **76**, **77**, **78** and **79** towards hyaluronat lyase

	76	**77**	**78**	**79**
IC50 (µM)	40.0	3.5	51.0	12.5

Table 6. Inhibitory activity of **76**-**79** against *Streptococcus agalactiae* hyaluronate lyase (IC_{50} values)

4 Summary

The present study is mainly focused on the discovery of new fungal natural products acting on clinically relevant targets in inflammatory and infectious (microbial and viral) diseases.

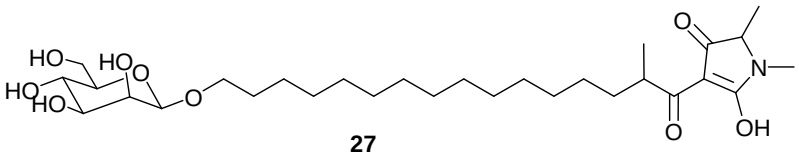
4.1 Anti-inflammatory compounds

Rheumatoid arthritis is a chronic debilitating inflammatory disease that affects up to 1% of the population with a 3:1 prevalence in women over men but has no known cure. While a number of small-molecule drug therapies exist to ease symptoms, they often demonstrate limited efficacy and many adverse side effects. The therapy based on the inhibition of the key enzymes (3α-HSD, COX-1 and COX-2) involved in the biosynthesis of prostaglandins has been shown to be a relatively safe and effective therapy for the treatment of various inflammatory diseases. In contrast with rheumatoid arthritis, gouty arthritis is mediated by the crystallisation of uric acid (UA) in the joints. Its therapies are based either on the increase of the urinary excretion of UA, or on the blockage of the terminal step of the biosynthesis of UA using xanthine oxidase (XO) inhibitors. Allopurinol, the only XO inhibitor in clinical use, seems to be associated with a severe side effect. On the basis of these therapies, it is highly desirable to develop the next generation of small-molecule targeting with these enzymes, which could be useful as lead structures for the new generation of anti-inflammatory and anti-arthritic drugs.

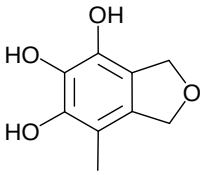
To achieve this goal, six fungal strains, *Epicoccum* sp HKI 0470, *Aspergillus* sp HKI 0472. *Inonotus* sp, *Pholiota squarrosa*, *Phellinus pini* DSM 5238 and *Piptoporus betulinus* were selected based on the pre-screening assay. We used 3α-HSD assay as a pre-screening for anti-inflammatory compounds.

Epicoccum sp. HKI 0470

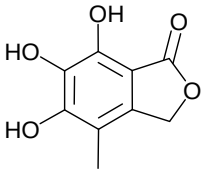
Two new metabolites, epicoccone B (**29**) and epicoccalone (**30**) in addition to three known metabolites, epicoccamide (**27**), epicoccine (**28**) and orevactaene (**31**), were isolated from *Epicoccum* sp HKI 0470.



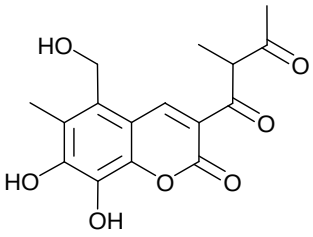
27



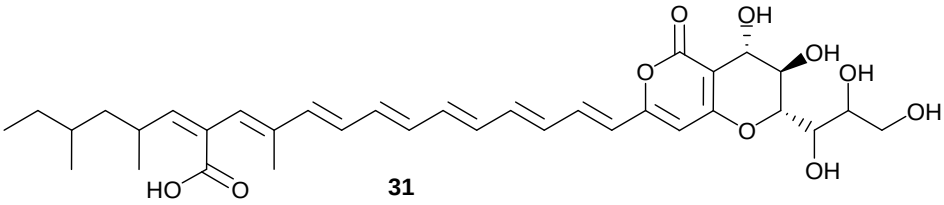
28



29



30

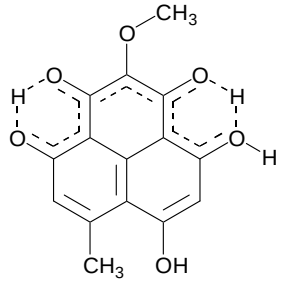


31

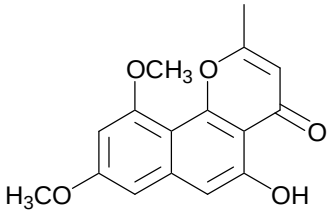
While **29**, **30** and **31** displayed only weak inhibition effect towards 3α-HSD, **30** was identified as a serine protease (α-chymotrypsin) inhibitor. Here it should be noted that there is increasing evidence about possible involvement of the serine protease inhibitors in the regulation of inflammation, host defence against infection, tissue repair and extra cellular matrix synthesis.

***Aspergillus* sp. HKI 0472**

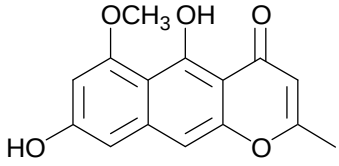
From the strain *Aspergillus* sp. three compounds were isolated, namely funalenine (**48**), TMC-256C2 (**50**) and TMC-256A1 (**51**). While TMC-256C2 (**50)** and TMC-256A1 (**51**) exhibited no anti-inflammatory activity, the new funalenine, with an unusual conformation, proved to be a potent anti-inflammatory drug by inhibiting 3α-HSD, COX-1 and COX-2 with IC_{50} values of 52 μM, 36 μM and 45 μM, respectively.



48



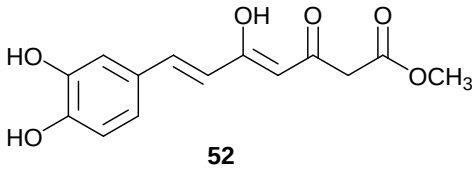
50



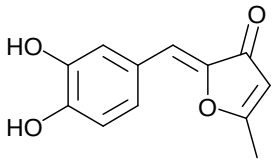
51

***Inonotus* sp. (fruiting body)**

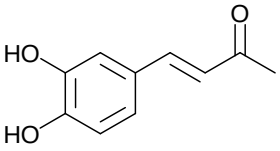
In the pre-screening assay the ethyl acetate extract of mushroom *Inonotus* sp. exhibited a strong anti-inflammatory activity. The bioassay guided purification led to the isolation of 5 compounds, three of which are new, namely inonotic acid methy ester (**52**), inotilone (**53**), and *iso*hispidin (**58**). The known compounds are (*E*)-4-(3,4-dihydroxyphenyl)but-3-en-2-one (**56)** and hispidin (**57**).



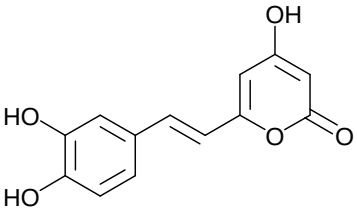
52



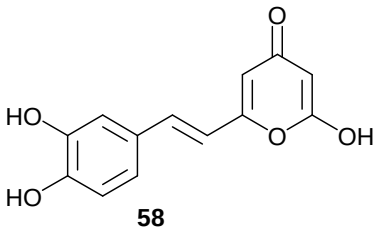
53



56



57

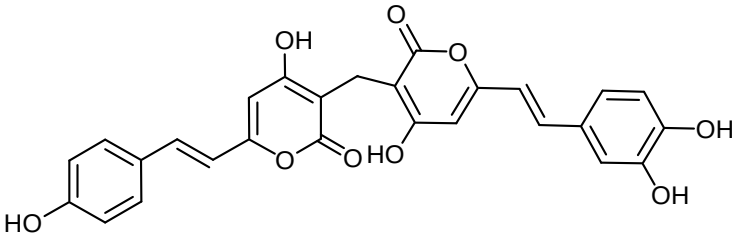


58

All these compounds exhibited strong COX inhibitory effects with prevalence for COX-2 in the case of **52**, **53**, **56** and **57**. It should be highlighted that **53** and **57** selectively inhibited COX-2 at concentrations as low as those of the marketed selective inhibitors meloxicam and nimesulide. In all cases, except for **53**, strong 3α-HSD inhbitory effects were noted, as well as moderate inhibitory effects towards XO, except **57**, which exhibited an inhibitory activity at the level comparable with that of the standard allopurinol.

Pholiota squarrosa (fruiting body)

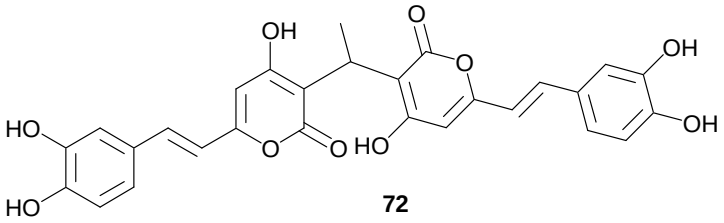
The new compound named squarrosidine (**65**) is the only metabolite we have isolated from the mushroom *Pholiota squarrosa* using bioassay guided separation. It is *a bis*hispidin derivative with an unusual methylene bridge. **65** displayed significant 3α-HSD, COX-1, COX-2 and XO inhibitory effect with an IC_{50} values of 8.1, 0.02, 0.04 and 8.1 μM, respectively. The ratio COX-1/COX-2 value was 2, indicating that **65** is a non-selective COX inhibitor.



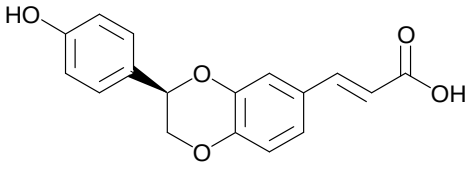
65

***Phellinus pini* DSM 5238**

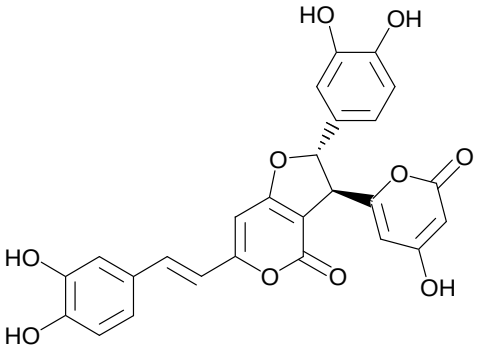
Three compounds were isolated from the filamentous fungus *Phellinus pini* DSM 5238. These are pinillidine (**72**) (a known structure, which has not yet been described in the literature), pinillic acid (**73**) a new lignan metabolite, and the known hypholomine B (**74**). While **73** displayed no anti-inflammatory activity, **72** and **74** exhibited significant inhibitory effects towards 3α-HSD and XO.



72



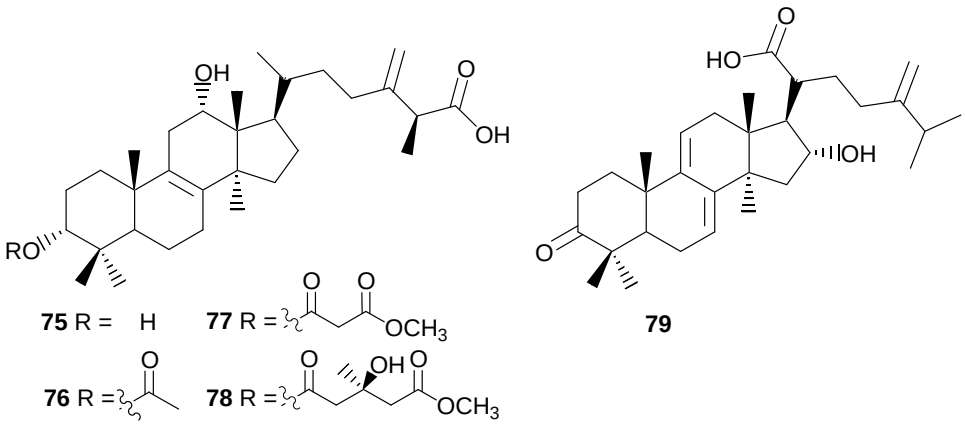
73



74

Piptoporus betulinus (fruiting body)

Bioassay guided fractionation of ethyl acetate extract of the mushroom *Piptopurus betulinus* led to the isolation of 5 lanostanoid-like triterpenes, two of which are new, acetylpolyporenic acid A (**76**) and (25*S*)-(+)-12α-hydroxy-3α-methylcarboxyacetate-24-methyllanosta-8,24(31)-diene-26-oic acid (**77**)). The known compounds are polyporenic acid A (**75**), (25S.3'S)-(+)-12α-hydroxy-3α-(3'-hydroxy-4'-methoxycarbonyl-3'-methylbutyryloxy)-24-methyllanosta-8,24(31)-dien-26-oic acid (**78)** and Polyporenic acid C (**79**). **76** and **77** exhibited remarkable inhibitory effects towards 3α-HSD. Most remarkably **77** exhibited a stronger activity than that of the reference (indomethacin).



In conclusion, the screening for the search for new anti-inflammatory compounds led to the isolation of 22 compounds, 10 of which are new. Among the new compounds 7 proved to be promising anti-inflammatory agents. These are funalenine (**48**), inonotic acid methyl ester (**52**), Inotilone (**53**), *iso*hispidin (**58**), squarrosidine (**65**), acetylpolyporenic acid A (**76**) and (25*S*)-(+)-12α-hydroxy-3α-methylcarboxyacetate-24-methyllanosta-8,24(31)-diene-26-oic acid (**77**). It should be noted that hispidin (**57**), (*E*)-4-(3,4-dihydroxyphenyl)but-3-en-2-one (**56**), pinillidine (**72**) and hypholomine B (**74**) are described here for the first time as anti-inflammatory agents.

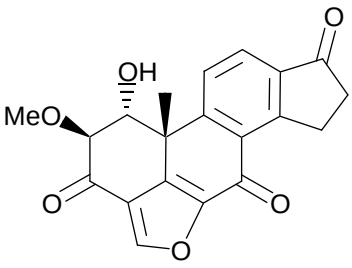
4.2 Anti-infectious compounds

Despite the successes in the treatment of some infectious diseases during the past three decades, the search for new antimicrobial or antiviral drugs remains an area of active investigation. Effective treatment is not available for many infectious diseases. Moreover, the selection of resistant and cross-resistant mutants caused partially by the narrow spectrum of the mechanism of activity, as well as potential toxic side-effects demand the discovery of new drugs.

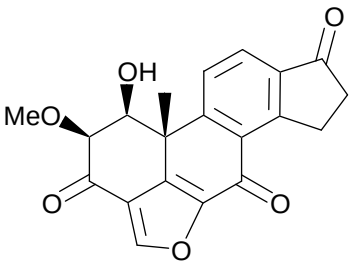
To meet this objective, two strains, *Trichoderma crassum* HKI 0471 and *Lepista nebularis* HKI 0411 were selected based on pre-screening assays on agar plates.

Trichoderma crassum HKI 0471

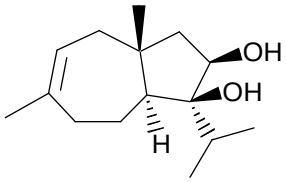
Three known active metabolites, β-viridin (**13**), α-viridin (**14**) and CAF 603 (**19**) were isolated from *Trichoderma crassum* HKI 0471. These metabolites exhibited strong anti-fungal activity against *Candida albicans* ATCC 18804 and *Sporobolomyces salmonicolor* SBUG 549, respectively.



13



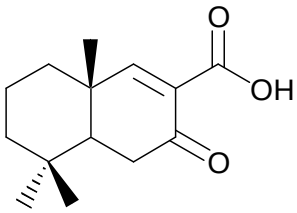
14



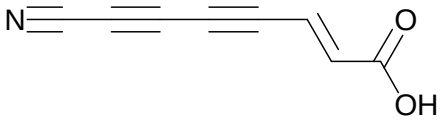
19

Lepista nebularis HKI 0411

In addition to the new antiviral metabolite (nebularic acid A (**32**)), a known strong anti-microbial metabolite (diatretyne (**47**)) was isolated from *Lepista nebularis* HKI 0411.



32



47

32 exhibited a strong anti-virale activity against cosackievirus with an IC_{50} value of 6 μM, and diatretyne (**47**) exhibited significant anti-fungal and bacterial activities against *Candida albicans* ATCC 18804 (MIC = 25 μg/ml) multi-resistant *Streptococcus aureus* (MIC = 1.56 μg/ml) and *Mycobacterium* vaccae (MIC = 6.25 μg/ml). Diatretyne exhibited also cytotoxic and antiproliferative activities.

5 Experimental section

5.1 General experimental procedures

Melting point and decomposition points were adjusted with the Wagner & Munz melting point apparatus and were not corrected.

IR spectra (film) were recorded on a Sattelite FTIR spectrometer equipped with a ATR device and on a JASCO FT/IR-4100 spectrometer equipped with ATR a device.

UV spectra were measured with a Specord 200 Carl Zeiss technology spectrometer (Analytik Jena AG, Jena) and with a HP UVIS (Desaga GmbH, Wiesloch) apparatus for TLC.

Optical rotations were mesured with a Propol Digital Automatic Polarimeter.

^{1}H NMR spectra were recorded on a Bruker Avance 500 DRX spectrometer (Bruker, Kalrlsruhe, Germany) at 300 MHz. Coupling constants (*J*) in Hz. Abbreviations: *s* = singulet, *d* = doublet, *dd* = double doublet, *t* = triplet , *q* = quartet, *m* = multiplet, *brs* = broad.

^{13}C NMR spectra including DEPT were recorded on a Bruker Avance 500 DRX spectrometer (Bruker, Karlsruhe, Germany) at 75 MHz. Chemical shifts were measured relative to tetramethylsilane as standard.

2D-NMR spectra

^{1}H-^{1}H COSY (^{1}H, ^{1}H-correlated spectroscopy), HMBC (Heteronuclear Multiple Bond Connectivity), HMQC (Heteronuclear Multiple Quantum Coherence) and NOESY (Nuclear Overhauser Effect Spectroscopy)

Electrospray Ionisation Mass Spectroscopies (ESI-MS/Tandem-MS (MS/MS) were performed on a VG Quattro with an ASI source (VG Biotech Atrincham, England) apparatus, eluent: MeOH/H_2O = 99:1, rate: 15 µl/min, temperature: 65 ^{o}C.

ESI-MS/Tandem-MS (MS/MS)/MS^{n} were recorded on a Benchtop Mass spectroscopy apparatus Finnigan LCQ (Finnigan, Bremen).

High-Resolution Electrospray Ionisation Mass Spectroscopies (HR-ESIMS) were recorded on a AMD 402 double-focusing mass spectrometer with BE geometry (AMD, Intestra, Harpstedt, Germany).

LC/MS were recorded on a Finnigan TSQ quantum ULTRA AM apparatus equipped with a Finnigan Surveyor PDA detector, a Finnigan Surveyor Autosampler plus, and a Finnigan Surveyor LC pump plus.

High Performance Liquid Chromatography (HPLC).

Intrument-1. Analytical HPLC was recorded on a SHIMADZU (Kyoto, Japan) multiwavelength detector SPD-M 10AVP (DAD), equipped with two pumps type LC-10AT, System controller type: SCL-10AVP mixing chamber, injection valve (type rheodyne) with sample loop 20 μl, hardware: class VP 54.0. Analytical column: 1) Eurochrom 4.6 x 125 mm without pre-column: stationary phase: Hypersil, ODS 120 x 5 μm; 2) Vertex 4.6 x 250 mm, stationary phase: Nucleosil NP 100-C-18, particle size 5 μm.

Intrument-2. Preparative HPLC was performed using a Gilson binary gradient HPLC system equipped with a UV detector (UV/VIS -151), two pumps type Gilson 305 and 306, Gilson 811C dynamic mixer and a Gilson 806 manometric module.

Intrument-3. Preparative HPLC was performed using a Shimadzu, Kyoto, Japan equipped with two pumps type LC-8A, detector: SPD-10A system-controller: SCL-8A, fractions collector: FCV-100B preparative column: Vertex 16 x 250 mm with 16 x 30 mm pre-column, stationary phase: Eurospher C-18 Rp 100 x 5 μm; 2); Vertex 16 x 250 mm with 16 x 30 mm pre column, stationary phase: Nucleosil NP 100-C-18, particle size 5 μm, pore diameter 100 Å (Macherey-Nagel & Co.). HPLC-solvents: acetonitrile/water-azeotrope (83% acetonitrile / 17% water bp. 74-80 ^{o}C) the azeotrope was redistilled with a Fischer LABODEST 107 solvent recycling apparatus.

Lyophilizer drying: All water contained in the fractions was lyophiolized with a Virtus Sentry (The Virtus Company Gardiner, New York 12525) apparatus.

Materials

Thin layer and preparative thin layer chromatographies (TLC and PTLC) were carried out with silica gel 60 F_{254} 20 x 20 cm plates Merck KGaA Darmstadt, Germany.

Column chromatographies (CC) were performed on silica gel (60, Merck; 0.063-0.2 μm) and Sephadex LH-20 (Pharmacia).

Spray reagents

Vanillin/sulphuric acid: 2.5 g vanillin was added to a solution containing 425 ml methanol, 50 ml acetic acid and 25 ml sulphuric acid.

Anisaldehyd/sulphuric acid: 1ml anisaldehyde was added to 100 ml solution containing 85 ml methanol, 14 ml acetic acid and 1 ml sulphuric acid.

Ehrlich's Reagent: 1 g of 4-dimethylaminobenzaldehyde was dissolved in a mixture of 25 ml hydrochloric acid (37%) and 75 ml methanol.

Microbiological materials

Fermenter: 200 l fermenter (B. Braun Biotech International, Melsungen) consisted of a culture container, magnet-couplet propeller stirrer, cooler with thermostat, control unit with pH and antifoam regulation, Control unit: pH regulator, antifoam regulator and air regulator.

Storage of strains: deep-freeze storage in a Dewar vessel, Fa. -Capillaries for deep-freeze storage: diameter 1.75 mm, length 80 mm, Fa. Hirschmann Laborgeräte, Eberstadt.

Laboratory Shaker: ISF-1-w Adolf Kühner AG. Basel

Autoclave: Albert Dargatz Autoclave, volume 119 l, working temperature 121 ^{o}C, working pressure 1.2 kg/cm^{2}.

Antimicrobial assay disc: 9 mm diameter, Schleicher & Schuell No. 321 261.

Culture media: Glucose, bacto peptone, bacto agar, dextrose, soybean, mannit yeast extract and malt extract were from Merck.

Laminar-Flow-Box: Haraeus instrument (Germany)

Ingredients of different media

All the microbiological work was performed under sterile conditions as unsual.

The ingredients of medium were consisted of:

Malt extract, Glucose, Caseine peptone, Merck.

Degreased soybean flour, Henselwerk GmbH.

Yeast extract, Oxoid

Oatmeal (Holo Hafergold), Neuform

BiTek Agar-Agar, Difco

Glucose, AppliChem

Maltose, Fluka

Biomalt, Villa Natura

.

Recepies for media

All the culture media were autoclaved at 1.2 bar and 121 ^{o}C for 35 min

Malt medium (liquid medium) (g/l)	
Malt extract	20
Yeast extract	2
Glucose	10
$(NH_4)_2HPO_4$	0.5
Agar	1
pH 7.0	

Zeolith medium (g/l)	
Glycerine	5
Glucose	30
Peptone	5
NaCl	2

MPG-medium (shake culture)(g/l)	
Glucose	10
Malt extract	20
Soybean flour	2
Yeast extract	1
KH_2PO_4	1
$MgSO_4.7H_2O$	0.5
pH 5.5	

Sbo1-Medium (shaking culture) (g/l)	
Glucose	10
Saccharose	10
Yeast extract	0.8
Caseine peptone	2
Soybean flour	5
$(NH_4)_2HPO_4$	0.5
$CaCO_3$	0.3
$(NH_4)_2SO_4$	5
pH 6.5	

5.2 Microbiological and analytical methods

Storage of strains

All strains (filamentous fungi) were stored in liquid nitrogen for a long time. The strains were inoculated in agar plates with suitable media at 25 ^{o}C.

Pre-screening

The fungal isolates were cultivated in a 1 l scale in 500 ml Erlenmeyer flasks containing 200-300 ml of malt medium, MPG medium, Sbo1, medium, and zeolith medium. The flasks were shacked for about one month after which the entire fermentation broth was freeze-dried and the residues were extracted with ethyl

acetate. The extracts were evaporated to dryness and used for antimicrobial tests at a concentration of 100-1000 µg/ml.

5.3 Biological screening method

5.3.1 Antimicrobial screening

Antimicrobial activities were determined qualitatively by agar diffusion tests according to: Deutsches Arzneibuch,[124] 9th Edition, 1986, 47-48 and 424-430, Deutscher Apotheker Verlag Stuttgart. Test organisms (*Bacillus subtilis* ATCC 6633*, Escherichia coli* SG 458, *Streptococcus aureus* BB 271*, Micrococcus luteus* SG 125 A*, Mycobacterium vaccae* IMET 10670*, Saccharomyces cerevisiae Gi300, Sporobolomyces salmonicolor* SBUG 549*, Candida albicans* ATCC 18804*, Penicillium notatum* JP 36*, Fusarium culmorum* JP 15*, Phoma destructiva* i 1015*, Aspergillus fumigatus* ATCC 46645*, Aspergillus terreus etc.* were suspended in the melted agar medium and poured into Petri dishes. Holes of 9 mm in diameter were cut in the agar and filled with 50 µl of a 100 mg/l or a 1000 mg/l solution of the crude extract. Inhibition zones were measured with a ruler after overnight incubation.

5.3.2 Antiviral screening assay

The antiviral assays were carried out by Dr. Michaela Schmidtke from the institute of virology of the University of Jena.

Material

HeLa cell (Human cervix carzinoma cell line (USA)
Coxsackievirus B3 strain Nancy (Institut for poliomyelitis Moskow Russia)
Minimal essential Medium/Earle (MEM) SIGMA, Deisenhofen
Trypsin Berlinchemie, Berlin
Tricin Serva Heidelberg

Method

Virus stocks of the coxsackie virus B3 strain Nancy (Coxsackie virus B3; Institute of Poliomyelitis and virus Encephalitides, Moscow, Russia) the influenza virus A strain Hong Kong/68 (H3N2; Schaper and Brümmer, Salzgitter, Germany), and the acyclovir- and phosphonoformic acid-sensitive laboratory herpes simplex virus type 1 strain (HSV-1; kindly provided by W. Hampl Universität Ulm, Germany) were prepared in HeLa Ohio, MDCK and GMK cells, respectively. About 50 µl aliquots of

the virus stocks were stored at –70 °C until use. The titre of the virus was determined by plaque assay on the respective monolayers.

Plaque reduction assays

The plaque reduction assays were carried out as described by Schmidtke et al.[125] Briefly, after removal of the cell growth medium, confluent 2-day-old cell monolayer in 6 well plates (Flacon 3046) were inoculated with 1 ml of the respective virus suspension in the test medium (coxsackie virus on HeLa; influenza virus A on MDCK; HSV-1 K1 on GMK cells) containing approximately 100 plaque forming units (pfu) in the absence of serial twofold dilutions of the test compounds. After adsorption at 37 °C for 1 h, the inoculum was aspirated and 2 ml of the respective test medium containing 0.4% agar and the appropriate concentrations of the drugs were added. Each compound concentration was tested in duplicate. The tests were incubated at 37 °C for 48 h (coxsackie virus B3 and influenza virus A or 72 h for HSV-1 K1 until plaques appeared and then fixed and stained with formalin (3% v / v) and ethanol (1.67% v / v) in water overnight. Plaques were counted over a light box after removal of the agar overlay. The average plaque count from two compound treated wells at each concentration was plotted against the average plaque count of three untreated virus-infected wells. The concentration required to reduce the plaque number by 50% (IC_{50}) was calculated from the mean dose-response curves of at least three plaque reduction assays.

5.4 Enzyme inhibition test assays

5.4.1 α-Chymotrypsin inhibitory assay

The α-Chymotrypsin inhibitory assay was carried out by Dr. Keishi Ishida from the Leibniz-Institute for Natural Product Research and Infection Biology, HKI, Biomolecular Chemistry.

Material

Chymotrypsin: α-Chymotrypsin from bovine pancreas (C4129, SIGMA).

N-succinyl-phenylalanine-*p*-nitroanilide (35572, SERVA)

Method

The chymotrypsin inhibitory activity was determined by the method of Cannell et al.[126] Each assay mixture containing 30 µL of 50 mM Tris-HCl buffer (pH 7.6), 50 µl of chymotrypsin solution (150 U/ml in 50 mM Tris-HCl buffer (pH 7.6)) and 20 µl of test solution were added to each cuvette and pre-incubated at 37 ^{o}C for 5 min. Each reaction was started with the addition of 100 µl of substrate solution (*N*-succinyl-phenylalanine-*p*-nitroanilide in Tris-HCl (pH 7.6, 1 mg/ml)). The absorbance of the reaction mixture was immediately measured at 405 nm. The change colour was measured after 30 min incubation at 37 ^{o}C.

5.4.2 3α-Hydroxysteroid dehydrogenase (3α-HSD) inhibition assay

Test principle

3α-HSD from rat liver cytosol can be inhibited by non-steroidal antiphlogistics. 3α-HSD catalyses the reduction of 5β-dihydrocortisone with consumption of NADPH. The NADPH consumption is determined photometrically as a decrease of extinction at 340 nm. Indomethacin and ibuprofen are used as standard compounds.

Material

Aqua dest. (purest)
1 M Phosphatbuffer (pH 6.0)
9 mM NADPH (SIGMA)
5 mM 5β-dihydrocortisone (SIGMA)
UV visible spectrophotometer (UV 160A, Shimadzu, 340 nm)

Procedure

This assay was conducted spectrophotometrically according to the method described by T. M Penning.[119]

5.4.3 Cyclooxygenase inhibition assays

Test Principle

The chemical chemiluminescent COX (ovine) inhibitor screening assay utilizes the heme-catalyzed hydroperoxidase activity of ovine cyclooxygenases to generate luminescence in the presence of a cyclic naphthalene hydrazide and the substrate arachidonic acid.

Material

Assay Buffer (Cyaman Chemical)

Heme (Cyaman Chemical)

COX-1 (ovine) (Cyaman Chemical)

COX-2 (ovine) (Cyaman Chemical)

Arachidonic acid (Cyaman Chemical)

Potassium Hydroxide (Cyaman Chemical)

Chemiluminescent substrate (Cyaman Chemical)

96 well plate (Cyaman Chemical)

Plates cover (Cyaman Chemical)

Method

Background Wells-10 µl of heme, 10 µl of assay buffer, and 10 µl of solvent (DMSO) were added to three wells

100% Initial Activity Wells- 10 µl of heme, 10 µl of enzyme (either COX-1 or COX-2), and 10 µl of solvent were added to three wells.

Inhibitor Wells- 10 µl of heme, 10 µl of enzyme and 10 µl of inhibitor were added to three wells. 200 µl of buffer was then added to all of the used wells. The plate was inserted into the luminometer, which has been dispensed with 10 µl of the chemiluminiscence substrate, immediately 50 µl of arachidonic acid was dispensed to all the using wells. The total volume of this assay was 290 µl in all wells. After inhibition by NSAIDs, the direct residual activity of COX was measured by addition of a proprietary luminescent substrate and arachidonic acid. Light emission starts immediately and is directly proportional to the COX activity in the sample. The chemiluminescent signal was measured over 5 seconds. One unit of COX activity is defined as the amount of enzyme needed to consume 1 nM of oxygen per minute.

5.4.4 Xanthine oxidase assay

The test was conducted according to the method described by Pierce et al.[130]

Test Principle

The superoxide anion radicals can be quantified as relative light units (RLU) by use of the chemiluminescent detector lucigenin. Allopurinol (1,5-dihydro-4*H*-pyrazol[3,4-d]pyrimidin-4-one) inhibits xanthine oxidase, and it is used as a therapeutic agent in the treatment of gout and as standard compound in this assay.

Material

HBSS (10x) (Hank's solution without Ca und Mg; GIBCO BRL)

Aqua dest.

Lucigenin (Sigma Chemical Co.)

Allopurinol (SIGMA Chemical Co.)

Hypoxanthine (SIGMA Chemical Co.)

Xanthine oxidase (SIGMA Chemical Co.)

Procedure

100 µl lucigenin (5×10^{-4} M) was added to a well, followed by 50 µl xanthine oxidase grade III (sigma, 0.1 units/ml) and an appropriate solution of the test compound. After equilibration of the plate at 37 ^{o}C for 15 min, background CL reading were made every 5 mn intervals for 15 min before 50 µl hypoxanthine (Sigma, 10 mg/ml) was added to start the reaction. The results were expressed as the percentage decrease in the total CL produced in the presence of the test compound 30 mn after addition of hypoxanthine to the plate. Chemiluminescence was measured continuously for 10 min and was expressed as relative light unit (RLU) per minute.

3α-HSD, COX and XO assays were performed by Dr. Albert Härtl from the Leibniz-Institute for Natural Product Research and Infection Biology, HKI.

5.4.5 Assay for hyaluronat lyase (*Streptococcus agalactiae*) activity

There are many different assays for hyaluronidase activity based on spectrometric, radiochemical, fluorogenic, enzymimmunological, chemical, physicochemical and zymographic analysis. The simple assay used is based on the precipitation of undegested hyaluronane by *N*-cetyl-*N*-,*N*,*N*-trimethylammoniumbromide and the measurement of turbidity (E_{600nm}). It has been adapted to an automated 96-well-microplate format .[120] The test was performed by Dr. Waltraud Hertel from the Leibniz-Institute of Natural Product and Infection Biology, HKI.

Procedure

The incubation solution containing hyaluronic acid in acetate buffer (pH 6), hyaluronate lyase of a defined activity and the inhibitory solution (1 mg/ml DMSO) was carried out for 30 min at 37 ^{o}C. After incubation, *N*-cetyl-*N*-,*N*,*N*-trimethylammoniumbromide was added. The optical density at 600 nm of the reaction mixture was measured by spectrophotometer.

Inhibitory effects of the test compounds were calculated as follows:

$$\%\text{of inhibition} = \frac{A_{600nm\ sample}}{A_{600nm\ control}} \times 100$$

5.4.6 Antiproliferative and cytotoxic assays [127,128]

The test compounds were dissolved in ethanol (10 mg/ml) before dilution with RPMI 1640. The test compounds were assayed against cell lines K-562 (human chronic myeloid leukemia) and L-929 (mouse fibroblast) for their antiproliferative effects. The adherent cells of L-929 were harvested at the logarithmic growth phase after soft trypsinization, using 0.25% trypsin in PBS containing 0.02% EDTA (Biochrom KG L2163). For each experiment with K-562, L-929, and HeLa approximately 10,000 cells were seeded with 0.1 ml RPMI 1640 (GIBCO BRL 21875-034), containing 25 µg/ml gentamicin sulfate (BioWhittaker 17-528Z), but without HEPES, per well of the 96-well microplates (K-562: NUNC 163320, L-929, HeLa: NUNC 167008).

Cells and culture conditions

Cells of established non-adherent cell lines K-562 (DSM ACC 10) and adherent L-929 (DSM ACC 2) were cultivated in RPMI 1640 medium (GIBCO BRL), supplemented with 25 µg/ml gentamicin sulfate (Cambrex), 10% heat inactivated fetal bovine serum (GIBCO BRL), and GlutaMAX (GIBCO BRL) at 37 ^{o}C in high density polyethylene flasks (NUNC 156340). HeLa (DSM ACC 57) cells were grown in RPMI 1640 culture medium (GIBCO BRL 21875-034) supplemented with 25 µg/ml gentamicin sulfate (BioWhittaker 17-528Z), and 10% heat inactivated fetal bovine serum (GIBCO BRL 10500-064) at 37 ^{o}C in high density polyethylene flasks (NUNC 156340).

Condition of incubation

Cells of L-929, K-562, and HeLa were incubated for 72 hours at 37 ^{o}C under a humidified atmosphere and 5% CO_2.

Methods of evaluation

Suspension cultures of K-562 in microplates were analysed by an electronic cell analyser system (CASY 1 SCHÄRFE, Reutlingen, Germany).

The 0.2 ml content of each well in the microplate was diluted to 1:50 with CASYTON (SCHÄRFE, Reutlingen, Germany) (NaCl: 7.93 g/l; EDTA disodium salt dihydrate: 0.38 g/l; KCl: 0.4 g/l; NaH_2PO_4 x 1 H_2O: 0.22 g/l; NaH_2PO_4 x 2 H_2O: 2.45 g/l; NaF: 0.3 g/l). Every count/ml was automatically calculated from the arithmetic mean of three successive counts of 0.4 ml each. From the dose response curves, the GI_{50} values (concentration which inhibited cell growth by 50%) were calculated with the software for data evaluation CASYSTAT (SCHÄRFE, Reutlingen, Germany). At the same incubation time, the adherent L-929 and HeLa cells were fixed by glutaraldehyde and stained with a 0.05% solution of methylene blue for 15 min. After gently washing the stain was eluted by 0.2 ml of 0.33 N HCl from the wells. The optical densities were measured at 660 nm in SUNRISE microplate reader (TECAN). Comparisons of the different values were performed with software Magellan (TECAN).

5.5 Isolation and identification of metabolites

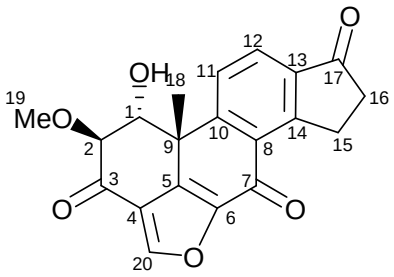
5. 5.1 *Trichoderma crassum* HKI 0471

The strain *Trichoderma crassum* HKI 0471 was obtained from the strain collection of HKI. Fifteen days malt agar-plate cultures were prepared and the well grown agar-plate cultures were used to inoculate malt liquid medium. The surface cultivation was carried out under sterile conditions in 500 ml Erlenmeyer flasks containing 300 ml medium. The whole culture broth was extracted twice by two volumes of ethyl acetate. The residue (10 g) of the evaporated ethyl acetate extract from 20 l of culture was first pre-screened and then applied to a silica gel column in chloroform. Elution was performed with 500 ml portions of $CHCl_3$, $CHCl_3$/MeOH (9:1), and $CHCl_3$/MeOH (7:3). The fractions obtained were separated using Sephadex LH 20 and HLPC on reverse phase column (RP_{18} Spherisorb, 25 mm x 250 mm) using a binary gradient (water/acetonitrile 95:5 to 5:95; 30 min). The individual peaks were collected and purified by the same preparative procedure.

5.5.1.1 Pre-screening

Chemical screening by TLC revealed a remarkable yellow zone. In addition numerous UV absorbing bands were observed. The most of them were coloured violet after have been sprayed with vanillin/sulphuric acid. In the antimicrobial pre-screening the crude extract exhibited strong antifungal activity particularly against *Candida albicans*.

β-Viridin (**13**) ($C_{20}H_{16}O_6$) was obtained as yellow crystals by open column chromatography on Sephadex LH-20 and silica gel using MeOH and $CHCl_3$/MeOH (v / v = 95 : 5) as eluent, respectively. Further purification was achieved by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 19.2 min. Upon spraying with vanillin/sulphuric acid or Emmerie-Engel's reagent a strong greenish- brown spot was obtained on the TLC plate.



Mp: 222~224

$[\alpha]^{20}_D$ -224

ESI-MS: 353 $[M+H]^+$, 407 $[M+MeOH+Na]^+$, 727 $[2M+Na]^+$

UV (MeOH): λ_{max} 240, 302 nm

IR (film) 3276, 3092, 1705, 1673, 1621, 1586, 1529,1122, 1061, 1010, 977 cm^{-1}

^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.26 (d, *J* = 10 Hz, 1H, H-1), 4.15 (d, *J* = 10 Hz, 1H, H-2), 8.61 (d, *J* = 8.3 Hz, 1H, H-11), 7.95 (d, *J* = 8.3 Hz, 1H, H-12), 3.66 (m, H-15), 2.73 (m, H-16), 1.71 (s, H-18), 3.84 (s, H-19), 8.26 (s, H-20)

^{13}C NMR ($CDCl_3$, 75 MHz): δ 75.4 (d, C-1), 85.9 (d, C-2), 189.0 (s, C-3), 122.1 (s, C-4), 142.0 (2, C-5), 146.7 (s, C-6), 173.2 (s, C-7), 137.3 (s, C-8), 41.7 (s, C-9), 154.9 (s, C-10), 128.3 (d, C-11), 127.5 (d, C-12), 129.8 (s, C-13), 155.6 (s, C-14), 27.2 (t, C-15), 36.3 (t, C-16), 206.3 (s, C-17), 28.4 (d, C-18), 61.7 (s, C-19), 148.5 (d, C-20)

α-Viridin (**14**) ($C_{20}H_{16}O_6$) was obtained as yellow crystals by open column chromatography on Sephadex LH 20 and silica gel using MeOH and $CHCl_3$/MeOH (v / v = 95 : 5) as eluent, respectively. Further purification was achieved by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 20.1 min . Upon spraying with vanillin/sulphuric acid or Emmerie-Engel's reagent a strong greenish- brown spot was obtained on the TLC plate.

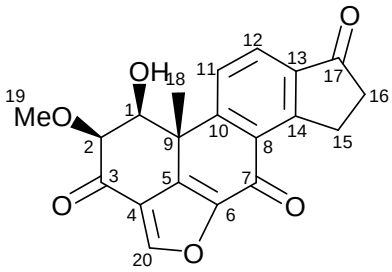
Mp: 140°C

$[\alpha]^{20}_D$ +50

ESI-MS: m/z 353 $[M+H]^+$, 407 $[M+MeOH+Na]^+$, 727 $[2M+Na]^+$

UV (MeOH): λ_{max} 240, 302 nm

IR (film): 3276, 3092, 1705, 1673, 1621, 1586, 1529, 1122, 1061, 1010, 977 cm^{-1}



^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.26 (dd, *J* = 5.0, 10.6 Hz, 1H, H-1), 3.87 (d, *J* = 5.0 Hz, 1H, H-2), 8.64 (d, *J* = 8.2 Hz, 1H, H-11), 7.95 (d, *J* = 8.2 Hz, 1H, H-12), 3.61, 3.85 (m, H-15), 2.72 (m, H-16), 1.66 (s, H-18), 3.73 (s, H-19), 8.28 (s, H-20)

^{13}C NMR ($CDCl_3$, 75 MHz): δ 73.2 (d, C-1), 83.5 (d, C-2), 186.9 (s, C-3), 121.6 (s, C-4), 142.9 (2, C-5), 147.0 (s, C-6), 173.2 (s, C-7), 137.2 (s, C-8), 41.9 (s, C-9), 156.4 (s, C-10), 127.8 (d, C-11), 127.6 (d, C-12), 129.8 (s, C-13), 157.9 (s, C-14), 29.7 (t, C-15), 36.3 (t, C-16), 206.3 (s, C-17), 28.4 (d, C-18), 60.9 (s, C-19), 149.3 (d, C-20)

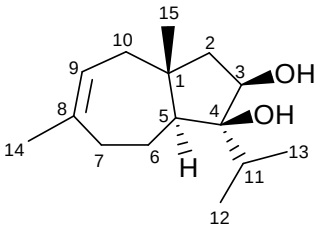
8-Daucene-3,4-diol (CAF 603) ($C_{15}H_{26}O_2$) was obtained as a colourless oil by open column chromatography on silica gel and Sephadex LH-20 using $CHCl_3$/MeOH 95:5 and MeOH as eluent, respectively. Further purification was achieved by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 25.6 min. Upon spraying with vanillin/sulphuric acid a strong blue spot was obtained on the TLC plate, suggesting a terpenoid.

$[\alpha]_D^{20}$ -26

ESI-MS: m/z 261 $[M+Na]^+$, 256, $[M+NH_4]^+$, 499 $[2M+Na]^+$

MS/MS: m/z 221 $[M-H_2O]^+$, 203 $[M-2H_2O]^+$,

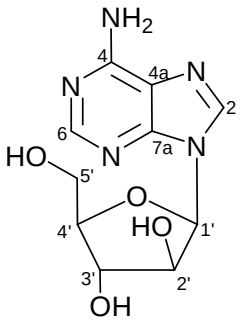
IR (film): 3383, 2951, 1450, 1381, 1317 cm^{-1}



^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.60 (m, 2H, H-2), 4.02 (dd, *J* = 1.4, 7.5 Hz, 1H, H-3), 1.47 (m, 1H, H-5), 1.55 (m, 2H, H-6), 2.06 (m, 2H, H-7), 5.34 (dd, 1H, H-9), 1.75, 1.85 (m, 2H, H-10), 1.80 (m, 1H, H-11), 0.85 (d, *J* = 6.9 Hz, 3H, H-12), 0.96 (d, *J* = 6.9 Hz, 3H, H-13), 1.75 (s, 3H, H-14), 1.00 (s, 3H, H-15)

^{13}C NMR ($CDCl_3$, 75 MHz): δ 42.2 (d, C-1), 50.3 (t, C-2), 72.4 (d, C-3), 84.3 (s, C-4), 58.4 (d, C-5), 20.7 (t, C-6), 34.4 (t, C-7), 139.0 (s, C-8), 122.4 (d, C-9), 42.8 (t, C-10), 35.1 (d, C-11), 17.7 (q, C-13), 17.0 (q, C-12), 27.3 (q, C-14), 21.1 (q, C-15)

Adenine-9α-D-arabinofuranoside ($C_{10}H_{13}N_5O_4$) was obtained as white solid crystal by open column chromatography on silica gel using and $CHCl_3$/MeOH 80:20. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 9.5 min. Upon spraying with vanillin/sulphuric acid a strong red spot was obtained on the TLC plate.



MP: 258~260

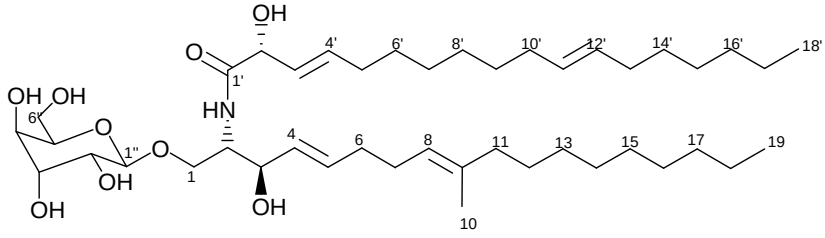
ESI-MS: m/z 268 $[M+H]^+$, 290 $[M+Na]^+$

IR (film): 3315, 3154, 2914, 2845, 1662, 1602, 1570, 1466, 13330, 1299, 1106 cm^{-1}

^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.33 (s, 1H, H-2), 7.25 (s, 2H, NH_2), 8.10 (s, 1H, H-6), 5.86 (d, 1H, *J* = 6.2 Hz, 1H, H-1'), 4.25 (dd, *J* = 5.1, 10.3 Hz, 1H, H-2'), 4.15 (dd, H-3'), 3.95 (dd, 1H, H-4'), 3.60 (s, 2H, H-5'), 5.54 (dd, *J* = 7.6, 2.5 Hz, 1H, OH), 5.47 (d, overlapped, 1H, OH), 5.25 (d, *J* = 4.17 Hz, 1H, OH).

^{13}C NMR (DMSO d_6, 75 MHz): δ 140.3 (s, C-2), 156.3 (s, C-4), 119.5 (s, C-4a), 152.7 (d, C-6), 149.2 (s, C-7a), 88.2 (d, C-1'), 73.8 (d, C-2'), 70.9 (d, C-3'), 86.2 (d, C-4'), 61.9 (t, C-5').

Cerebroside C ($C_{43}H_{79}NO_9$) was obtained as colourless crystals by open column chromatography on $CHCl_3$/MeOH 70:30 using $CHCl_3$/MeOH 70:30 as eluent . Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min). Upon spraying with vanillin/sulphuric acid a strong red spot was obtained on the TLC plate.



Mp: 159~169^{o}C

$[\alpha]_D^{20}$ -6.2^{o}

ESI-MS: m/z 754 $[M+H]^+$, 776 $[M+Na]^+$

IR (film): 3268, 2918, 2849, 1638, 1530, 1466, 1229, 1154, 1082, 1032 cm^{-1}

^{1}H NMR: (CD_3OD, 300 MHz): δ 3.71, 4.1 (m, 2H, H-1), 3.98 (m, 1H, H-2), 3.62 (m, 1H, H-3), 5.71 (m, 1H, H-4), 5.45 (m, *J* = 5.1 Hz, 1H, H-5), 1.96 (m, 2H, H-6), 2.01 (m, 2H, H-7), 5,15 (m, 1H, H-8), 1.52 (s, 3H, H-10), 1.40 (m, overlapped, 2H, H-11-H-17), 0.80 (t, 3H, H-18), 4.41 (d, *J* = 6.2 Hz, 1H, H-2'), 5.73 (m, 1H, H-3'), 5.49 (m, 1H, H-4'), 2.01 (m, 2H, H-5'), 2.01 (m, *J* = 5.1 Hz, 2H H-6'), 1.40 (m, overlapped, 2H, H-11'-H-17'), 0.80 (t, 3H, H-18'), 4.27 (d, *J* = 7.71 Hz,1H, H-1''), 4.10 (m, 1H, H-2''), 3.30 (m, 1H, H-3''), 3.27 (m, 1H, H-4''), 2.85 (m, 5.1, 1H, H-5''), 3.68 (m, 2H, H-6'')

^{13}C NMR (CD_3OD, 75 MHz): δ 69.6 (t, C-1), 54.6 (d, C-2), 72.9 (d, C-3), 134.5 (d, C-4), 131.0 (d, C-5), 33.4 (t, C-6), 33.7 (t, C-7), 124.8 (d, C-8), 136.7(s, C-9), 16.1 (q, C-10), 40.7-23.7 (t, C-11-C-18), 14.4 (q, C-19) δ 175.4 (t, C-1'), 74.1 (d, C-2'), 134.7 (d, C-3'), 129..0 (d, C-4'), 33.4 (t, C-5'), 33.7-23.7 (t, C-6-C-17'), 14.4 (q, C-18'), 104.7 (d, C-1''), 72.9 (d, C-2''), 77.9 (d, C-3''), 71.6 (d, C-4''), 75.0 (d, C-5''), 62.7 (t, C-6'')

5.5.2 *Epiccoccum* sp. HKI 0470

The strain *Epicoccum sp*. HKI 0470 was isolated from the hat of the basidiomycete *Pholiota squarrosa*. Fifteen days malt agar-plate cultures (25^{o}) were prepared and the well grown agar plate cultures were used to inoculate a liquid malt medium. The surface cultivation was carried out under sterile conditions in 500 ml Erlenmeyer flasks containing 300 ml medium. The whole culture broth was extracted twice by two volumes of ethyl acetate. The residue (7.5 g) of the evaporated ethyl acetate extract from 30 l of culture was first pre-screened and then applied to a silica gel column in chloroform. Elution was performed with 300 ml portions of $CHCl_3$, $CHCl_3$/MeOH (9:1), $CHCl_3$/MeOH (7:3) and MeOH. The fractions obtained were separated using Sephadex LH 20 and HLPC on reverse phase column (RP_{18} Spherisorb, 25 mm x 250 mm) using a binary gradient (water/acetonitrile 95:5 to 5:95; 30 min). The individual peaks were collected and purified by the same preparative procedure to

afford the new compounds, epicoccalone and epicoccone B, and the already known compounds, epicoccamide, epicoccine and orevactaene.

5.5.2.1 Pre-screening

Chemical screening by TLC showed a remarkable yellow zone. In addition, numerous UV absorbing bands were observed. The most of them were coloured violet after have been sprayed with vanillin/sulphuric acid. In the biological screening, the crude extract exhibited significant inhibitory activity against 3α-hydroxysteroid dehydrogenase, showing that the extract contained some anti-inflammatory products. Furthermore, the antimicrobial pre-screening of the crude extract exhibited moderate anti-fungal anti-bacterial activity.

Epicoccamide ($C_{29}H_{51}NO_9$) was obtained as colourless oil by open column chromatography on Sephadex LH-20 and silica gel using $CHCl_3$/MeOH 95:5 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 40 min) R_t = 33.8. Upon spraying with vanillin/sulphuric acid brown spot was obtained on the TLC plate.

ESI-MS: m/z 580.4 $[M+Na]^+$, 1137 $[2M+Na]^+$, 556$[M-H]^-$

MS/MS: m/z 557.9, 396.1

$[\alpha]_D^{20}$ -10.3 (c = 0.10 EtOH)

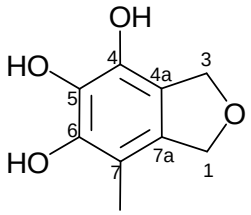
UV (EtOH): λ_{max} 282 nm

IR (film): 3367, 2922, 2852, 1709, 1646, 1612, 1487, 1448, 1339, 1235, 1067 cm^{-1}

^{1}H NMR (CD_3OD, 300 MHz): δ 3.52 (br, 1H, H-4), 1.30 (d, 3H, *J* = 6.9, H-5), 2.91 (brs, 3H, H-6), 3.78 (1H, m), 1.27, 1.72 (m, 2H, H-9), 1.24, 1,36 (m, 2H, H-10-19), 1.42 (m, 2H, H-20), 1.64 (m, 2H, H-21), 3.56 (ddd, 1H, *J* = 2.9, 6.8, 9.8 Hz, H-22), 3.98 (dt, 1H, *J* = 6.8, 6.8, 9.8 Hz, H-22), 1.02 (d, *J* = 6.2 Hz, H-23), 4.55 (brs, 1H, H-1') 3.88 (brs d, *J* = 3.2 Hz, H-2'), 3.48 (dd, 1H, *J* = 3.2, 9.5, H-3'), 3.62 (t, 1H, *J* =9.5, 9.5, H-4'), 3.26 (ddd, *J* = 3.0, 5.6, 9.5 Hz, H-5'), 3.75 (dd, 1H, *J* = 5.6, 11.8 Hz, H-6') 3.94 (dd, 1H, *J* = 3.0, 11.8 Hz, H-6').

^{13}C NMR (CD_3OD, 75 MHz): δ175.3 (s, C-1), 101.6 (s, C-2), 197.7 (s, C-3), 61.8 (d C-4), 16.0 (q, C-5) , 26.5, (q, C-6), 201.1 (s, C-7), 40.8 (d, C-8), 35.0 (t, C-9), 30.8-31.0 (t, C-10-19), 27.2 (t, C-20), 30.8 (t, C-21), 70.6 (t, C-22), 18.0 (q, C-23), 101.7 (d, C-1'), 72.5 (d, C-2'), 75.3 (d, C-3'), 68.5 (d, C-4'), 78.1(d, C-5'), 62.8 (t, C-6').

Epicoccin (**28**) ($C_9H_{10}O_4$) was obtained as colourless crystal by open column chromatography on Sephadex LH-20 and silica gel using $CHCl_3$/MeOH 95:5 as eluent. Further purification was done by HPLC using a binary gradient (water/acetonitrile 95:5 to 5:95; 40 min) R_t = 14.5 min. Upon spraying with vanillin/sulphuric acid or Emmerie-Engel's reagent a strong greenish-brown spot was obtained on the TLC plate.



ESI-MS: m/z 205 $[M+Na]^+$, 387 $[2M+Na]^+$, 181$[M-H]^-$

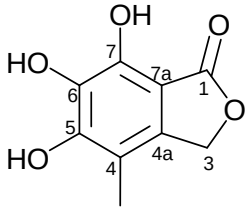
Mp: 205~207 ^oC

UV (MeOH): λ_{max} 248 nm

^{1}H NMR (DMSO-d_6, 300 MHz): δ 4.83 (s. 2H, H-1), 4.87 (s, 2H, H-3), 8.45 (s, brs 1H, OH-4), 8.06 (s, brs 1H, OH-5), 8.02 (s, brs, 1H, OH-6), 1.91 (s, 3H, H-8)

^{13}C NMR (DMSO-d_6, 75 MHz): δ 72.5 (t, C-1), 71.7 (t, C-3), 137.6 (s, C-4), 132.5, (s, C-5), 143.9 (s, C-6), 108.2 (s, C-7), 115.6, (s, C-4a), 128.4 (s, C-7a), 12.1 (q, C-8).

Epicoccone B (**29**) ($C_9H_8O_5$) was obtained as colourless oil by open column chromatography on Sephadex LH-20 and silica gel using $CHCl_3$/MeOH 95:5 as eluent. Further purification was done by HPLC using a binary gradient (water/acetonitrile 9:1 to 5:95; 30 min) R_t = 14 min. Upon spraying with vanillin/sulphuric acid or Emmerie-Engel's reagent a strong greenish- brown spot was obtained on the TLC plate.



ESI-MS: m/z 197 $[M+Na]^+$, 219 $[M+Na]^+$, 195$[M-H]^-$

IR (film): 3420, 3134, 1725, 1628, 1514, 1249, 1104, 879 cm^{-1}

^{1}H NMR (DMSO-d_6, 300 MHz): δ 5.09 (s. 2H, H-3), 1.96 (s, 3H, H-8).

^{13}C NMR (DMSO-d_6, 75 MHz): δ 169.8 (s, C-1), 67.6 (t, C-3), 109.1 (s, C-4), 138.0 (s, C-4a), 151.1 (s, C-5), 132.8 (s, C-6), 143.0 (s, C-7), 102.7 (s, C-7a), 10.7 (t, C-8)

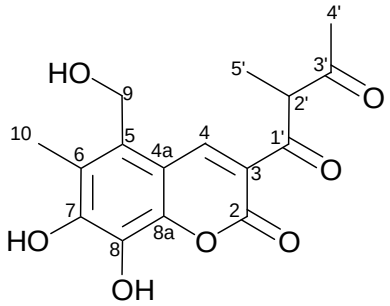
Epicoccalone (**30**) ($C_{16}H_{16}O_7$) was obtained as yellow powder by open column chromatography and sephadex LH 20 and silica gel using $CHCl_3$/MeOH 85:15 as eluent. Further purification was done by HPLC using a binary gradient (water/acetonitrile 8:2 to 5:95; 30 min) R_t = 15.8 min. Upon spraying with vanillin/sulphuric acid or Emmerie-Engel's reagent a strong greenish-brown spot was obtained on the TLC plate.

ESI-MS: m/z 321 $[M+Na]^+$, 343 $[M+Na]^+$ 663 $[2M+Na]^+$, 319 $[M-H]^-$

MS/MS: m/z 221, 203, 175

HR-ESIMS (found $[M-H]^-$; 319.0814 calcd. for $C_{16}H_{61}O_7$: 319.0818)

UV (MeOH): λ_{max} 269, 390, 463 nm

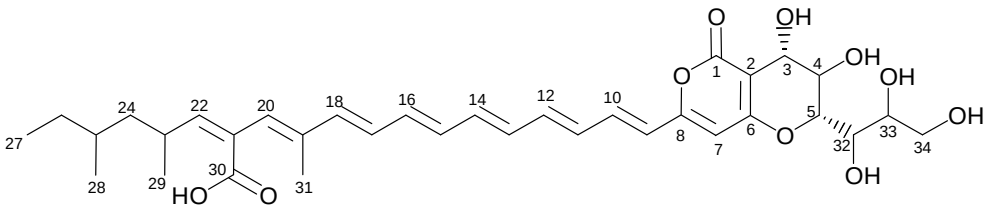


IR (film): 3244, 2925, 1699, 1598, 1562, 1465, 1268, 1197, 1107, 895 cm^{-1}

^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.89 (s. 1H, H-4), 4.68 (s, 2H, H-9), 2.22 (s, 3H, H-10), 4.71 (q, 1H, H-2'), 2. 28 (s, 1H, H-4'), 1.23 (d, *J* = 7.1 Hz 3H, H-5')

^{13}C NMR (DMSO-d_6 75 MHz) δ 159.2 (s, C-2), 116.5 (s, C-3), 147.0 (d, C-4), 131.6 (s, C-5), 109.9 (s, C-5a), 122.4 (s, C-6), 151.6 (s, C-7), 130.2 (s, C-8), 144.0 (s, C-8a), 55.6 (t, C-9), 11.3 (q, C-10), 194.0 (s, C-1'), 57.3 (d, C-2'), 206.2 (s, C-3'), 28.7 (q, C-4'), 12.1 (q, C-5').

Orevactaene (**31**) ($C_{34}H_{44}O_{10}$) was obtained as yellow oil by open column chromatography on Sephadex LH-20 and silica gel using $CHCl_3$/MeOH 9:1 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 40 min) R_t = 25.1 min . Upon spraying with vanillin/sulphuric acid brown spot was obtained on the TLC plate.



ESI-MS: m/z 613 $[M+H]^+$

MS/MS: m/z 521, 447, 403

UV (MeOH): λ_{max} 430, 452 nm

IR (film): 3251, 2957, 2923, 1671, 1539, 1417, 1200, 1134, 1045, 1000 cm^{-1}

^{1}H NMR (DMSO-d_6, 300 MHz) δ 4.23 (d, 1H, *J* = 9.5 Hz H-3), 4.10 (dd, 1H, *J* = 9.5, 9.3 Hz, H-4), 3.27 (dd, 1H *J* = 9.3, 2.8 Hz, H-5), 6.16 (s, 1H, H-7), 6.31 (d, 1H, *J* = 15.2 Hz, H-9), 7.00 (dd, 1H, *J* = 15.2 Hz, 11.4, H-10), 6.43 (dd, 1-H, overlapped, H-11), 6,70 (dd, 1H overlapped, H-12), 6.42 (dd, 1H overlapped, H-13), 6.50 (dd, 1H, overlapped, H-14), 6.40 (dd, overlapped, H-15), 6..48 (dd, 1H, overlapped, H-16), 6.40 (dd, overlapped, H-17), 6.41 (dd, 1H, overlapped, H-18), 6.11 (s, 1H, H-20), 5.55 (d, 1H, 10.3, H-22), 2.88 (m, H-23), 1.08,1.24 (m, 2H, H-24), 1.20 (m, 1H), 1.10, 1.20 (m, 2H, H-26), 0.80 (t, 3H, *J* = 7.6 Hz, H-27), 0.78 (d, 3H, *J* = 7.2 Hz, H-28), 0.96 (d, *J* = 7.1 Hz, 3H, H-29), 8.01 (brs, 1H COOH), 1.79, (s, 3H, H-31), 3.70 (dd, 1H, *J* = 6.2, 2.8 Hz, H-32), 3.37 (m, 1H, overlapped), 3.39, 3.42 (m, 2H, H-34).

^{13}C NMR (DMSO-d_6, 75 MHz): δ 167.6 (s. C-1), 101.4 (s, C-2), 74.5 (d, C-3), 67.9 (d, C-4), 75.3 (d, C-5), 162.1 (s, C-6), 101.5 (d, C-7), 157.6 (s, C-8), 122,7 (d, C-9), 134.6 (d, C-10), 131.8 (d, C-11), 138.7 (d, C-12), 129.0 (d, C-13), 136.4 (d, C-14), 133.1 (d, C-15), 135.5 (d, C-16), 132.8 (d, C-17), 139.1 (d, C-18), 134.7 (s, C-19), 130.6 (d, C-20), 131.4 (s, C-21), 146.7 (d, C-22), 31.5 (d, C-23), 44.1 (t, C-24), 32.2 (d, C-25), 29.7 (t, C-26), 11.3 (q, C-27), 18.9 (q, C-28), 21.4 (q, C-29), 169.6 (s, C-30), 13.3 (q, C-31), 69.1 (d, C-32), 79.3 (d, C-33), 60.9 (t, C-34).

5.5.3 *Lepista nebularis* HKI 0411

The strain *Lepista nebularis* was collected as mycelia culture (derived from the tissue plugs of the fruiting body) in Siberia. The taxonomy of the fungus was verified by Dr. Heinrich Dörfelt of the Institute for Nutrition and Environment, Friedrich-Schiller-University, Jena. A specimen (HKI 0411) was deposited in the fungal collection of the Leibniz-Institute for Natural Product Research and Infection Biology, HKI, Jena, Germany.

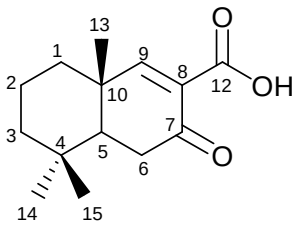
5.5.3.1 Cultivation and extraction

Twenty eight days agar-plate cultures were prepared as inoculum using a medium composed as follows (g/l): malt extract 40, yeast extract 4, agar 15, deionised water , pH 6.0. Agar chips (4-5 cm^2) of the plate cultures were used to inoculate a liquid MPG medium. The surface cultivation was carried out under sterile conditions in 500 ml Erlenmeyer flasks containing 300 ml medium. The whole culture broth was extracted twice by two volumes of ethyl acetate. The residue (5 g) of the evaporated ethyl acetate extract from 60 l of culture was first pre-screened and then applied to a silica gel column in chloroform. Elution was performed with 500 ml portions of $CHCl_3$, $CHCl_3$/MeOH (9:1), and $CHCl_3$/MeOH (7:3). The fractions obtained were then separated using Sephadex LH-20 and HLPC on reverse phase column (RP_8 Spherisorb, 25 mm x 250 mm) using a binary gradient (water/acetonitrile 95:5 to 5:95; 30 min). The individual peaks were collected and purified by the same preparative procedure to afford the new natural compounds: nebularone, nebularic acid A, nebularic acid B, nebularilactone A, nebularilactone B and the known compounds *N*-Acetyltyramine and diatretyne.

5.5.3.2 Pre-screening

Chemical scrennig by TLC indicated a considerable number of compounds. Furthermore numerous UV absorbing bands were observed. The most of them were coloured after have been sprayed with vanillin/sulphuric acid, indicating the presence of terpenoids. In the biological pre-screening, the crude extract exhibited no activity in the enzyme assays, but in the antimicrobial pre-screening a strong antifungal and antibacterial activity particularly against multiresistant *Streptococcus* and *Candida albicans* were observed.

Nebularic acid A (**32**) ($C_{14}H_{20}O_3$) was obtained as colourless oil by open column chromatography on Sephadex LH-20 and Silica gel using $CHCl_3$/MeOH 95:5 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 25.1 min. Upon spraying with vanillin/sulphuric acid a strong violet spot was obtained on the TLC plate, suggesting a terpenoid.

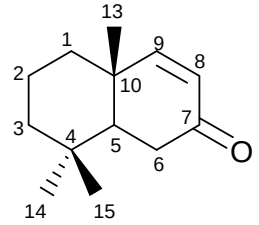


ESI-MS: m/z 237 $[M+H]^+$, 259 $[M+Na]^+$
HR-ESIMS (found $[M+H]^+$; 237.1480 calcd.
for $C_{14}H_{21}O_3$: 237.1485)
UV (MeOH): λ_{max} 246 nm
IR (film): 2925, 2846, 1743, 1633, 1432, 1410, 1258, 1186 cm^{-1}

^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.4-1.8 (m, 2H, H-1), 1.6-1.8 (m, 2H, H-2), 1.1-1.5 (m, 2H, H-3), 1.78 (m,1H, H-5), 2.50-2.65 (dd, *J* = 3.8, 18.0 Hz, 2H, H-6), 8.02 (s, 1H, H-9), 1.14 (s, 3H, H-13), 0.88 (s, 3H, H-14), 0.92 (s, 3H, H-15)

^{13}C NMR ($CDCl_3$, 75 MHz): δ 36.8 (t, C-1), 18.1 (t, C-2), 40.7 (t, C-3), 33.0 (s, C-4), 49.4 (d, C-5), 35.2 (t, C-6), 204.3 (s, C-7), 123.5 (s, C-8), 175.4 (d, C-9), 38.3 (s, C-10), 164.0 (s, C-12), 17.1 (q, C-13), 20.9 (q, C-14), 32.6 (q, C-15)

Nebularone (**33**) ($C_{13}H_{20}O$) was obtained as colourless oil by open column chromatography on Sephadex LH-20 and Silica gel using $CHCl_3$/MeOH 95:5 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 25.5 min. Upon spraying with vanillin/sulphuric acid a strong violet spot was obtained on the TLC plate, suggesting a terpenoid.

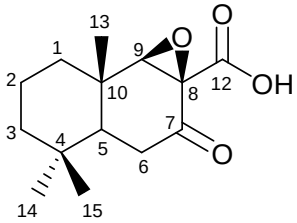


ESI-MS: m/z 193 $[M+H]^+$, 215 $[M+Na]^+$
HR-EISMS (found $[M+H]^+$; 193.1524 calcd.
for $C_{13}H_{21}O$: 193.1587)
UV (MeOH): λ_{max} 246 nm
IR (film): 2925, 2846, 1676, 1457, 1390, 1371, 1259, 1102 cm^{-1}

^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.30-1.61 (m, 2H, H-1), 1.55-1.70 (m, 2H, H-2), 1.21-1.50 (m, 2H, H-3), 1.72 (dd, *J* = 3.7, 13.9, 1H, H-5), 2.35 (m, 2H, H-6), 5.72 (d, *J* = 9.8 Hz, 1H, H-8), 6.59 (d, *J* = 9.8 Hz, 1H, H-9), 1.07 (s, 3H, H-13), 0.87 (s, 3H, H-14), 0.91 (s, 3H, H-15)

^{13}C NMR ($CDCl_3$, 75 MHz) : δ 38.0 (t, C-1), 18.5 (t, C-2), 41.5 (t, C-3), 33.0 (s, C-4), 50.4 (d, C-5), 22.3 (t, C-6), 201.5 (s, C-7), 125.4 (d, C-8), 162.6 (d, C-9), 36.8 (q, C-10), 18.2 (q, C-13), 20.8 (q, C-14), 32.1 (q, C-15)

Nebularic acid B ($C_{14}H_{20}O_4$) was obtained as colourless crystals by open column chromatography on Sephadex LH 20 and silica gel unsing $CHCl_3$/MeOH 95:5 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 22.0. Upon spraying with vanillin/sulphuric acid a strong violet spot was obtained on the TLC plate, suggesting a terpenoid.



ESI-MS: m/z = 253 $[M+H]^+$, 251 $[M-H]^-$
HR-EISMS (found $[M-H]^-$; 251.1262, calcd. for $C_{14}H_{19}O_4$: 251.1264)
UV (MeOH): λ_{max} 246 nm
IR (film): 2925, 2846, 1730, 1633, 1432, 1410, 1258, 1186 cm^{-1}

^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.4-1.8 (m, 2H, H-1), 1.58-1.78 (m, 2H, H-2), 1.22-1.58 (m, 2H, H-3), 1.65 (m,1H, H-5), 2.16-2.35 (dd, *J* = 3.8, 18.0 Hz, 2H, H-6), 3.00 (s, 1H, H-9), 1.13 (s, 3H, H-13), 0.85 (s, 3H, H-14), 0.89 (s, 3H, H-15)

^{13}C NMR ($CDCl_3$, 75 MHz): δ 37.3 (t, C-1), 19.2 (t, C-2), 42.4 (t, C-3), 33.5 (s, C-4), 42.5 (d, C-5), 36.5 (t, C-6), 205.1 (s, C-7), 62.2 (s, C-8), 69.5 (d, C-9), 35.2 (s, C-10), 172.7 (s, C-12) 17.1 (q, C-13), 21.3 (q, C-14), 33.0 (q, C-15)

Nebularilactone A (**35**) ($C_{15}H_{22}O_3$) was obtained as colourless oil by open column chromatography on Sephadex LH-20 and Silica gel using $CHCl_3$/MeOH 95:5 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 20.5. Upon spraying with vanillin/sulphuric acid a strong violet spot was obtained on the TLC plate, suggesting a terpenoid.

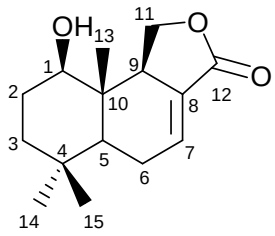
ESI-MS: m/z = 253 $[M+H]^+$, 251 $[M-H]^-$

HR-ESIMS (found $[M-H]^-$; 251.1642 calcd.

for $C_{15}H_{22}O_3$: 251.1650)

UV(MeOH): λ_{max} 246 nm

IR (film): 2925, 2846, 1730, 1430, 1409, 1258, 1186 cm^{-1}



^1H NMR ($CDCl_3$, 300 MHz): δ 3.39 (dd, *J* = 4.7, 10.8 Hz, 1H, H-1), 1.63 (m, 2H, H-2), 1.32-1.52 (m, 2H, H-3), 1.34 (m, 1H, H-5), 2.20-2.40 (m, 2H, H-6), 6.85 (dd, *J* = 3.4, 7.0 Hz, 2H, H-7), 2.82 (s, 1H, H-9), 4.15-4.50 (dt, *J* = 9.6 Hz, 2H, H-11) 0.78 (s, 3H, H-13), 0.94 (s, 3H, H-14), 0.90 (s, 3H, H-15)

^{13}C NMR ($CDCl_3$, 75 MHz): δ 71.4 (d, C-1), 26.0 (t, C-2), 34.1 (t, C-3), 32.7 (s, C-4), 43.4 (d, C-5), 25.1 (t, C-6), 135.2 (s, C-7), 127.5 (s, C-8), 42.7 (d, C-9), 38.2 (s, C-10), 67.1 (s, C-11). 170.3 (s, C-12), 13.6 (q, C-13), 21.3 (q, C-14), 32.8 (q, C-15)

Nebularilactone B (**36)** ($C_{15}H_{22}O_3$) was obtained as clourless oil by open column chromatography on Sephadex LH-20 and Silica gel using $CHCl_3$/MeOH 95:5 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 20.59. Upon spraying with vanillin/sulphuric acid a strong violet spot was obtained on the TLC plate, suggesting a terpenoid

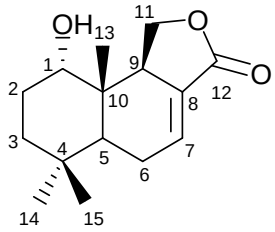
ESI-MS: m/z = 253 $[M+H]^+$, 251 $[M-H]^-$

HR-ESIMS (found $[M-H]^-$; 251.1642 calcd.

for $C_{15}H_{22}O_3$: 251.1650)

UV (MeOH): λ_{max} 246 nm

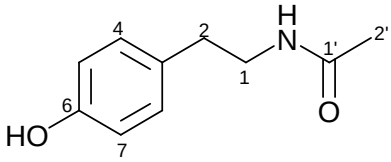
IR (film): 2925, 2846, 1730, 1430, 1409, 1258, 1186 cm^{-1}



^1H NMR ($CDCl_3$, 300 MHz) δ 3.51 (brs, *J* = 4.8, 10.9 Hz, 1H, H-1), 1.59-1.98 (m, 2H, H-2), 1.26-1.76 (m, 2H, H-3), 1.75 (m, 1H, H-5), 2.15-2.43 (m, 2H, H-6), 6.85 (dd, 1H, H-7), 3.58 (m, 1H, H-9), 4.05-4.4.48 (dt, *J* = 9.4 Hz, 2H, H-11), 0.78 (s, 3H, H-13), 0.93 (s, 3H, H-14), 0.94 (s, 3H, H-15)

^{13}C NMR ($CDCl_3$, 75 MHz) δ 79.8 (d, C-1), 28.3 (t, C-2), 40.0 (t, C-3), 32.5 (s, C-4), 48.7 (d, C-5), 24.7 (t, C-6), 135.2 (s, C-7), 127.6 (s, C-8), 50.1 (d, C-9), 39.5 (s, C-10), 69.2 (s, C-11) 170.1 (s, C-12), 7.28 (q, C-13), 21.5 (q, C-14), 32.5 (q, C-15)

***N*-Acetyltyramine** (**46**) ($C_{10}H_{13}NO_2$) was obtained as a clourless powder by open column chromatography on Sephadex LH-20 and Silica gel using $CHCl_3$/MeOH 70:30 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 13.1 min. Upon spraying with vanillin/sulphuric acid a greenish- brown spot was obtained on the TLC plate.



ESI-MS: 180 $[M+H]^+$, 202 $[M+Na]^+$

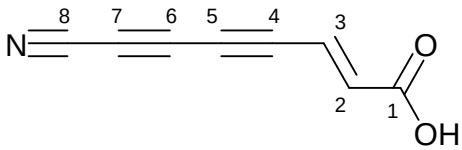
UV (MeOH): λ_{max} 214, 276 nm

IR (film): 3329, 2933, 1629, 1594, 1566, 1514, 1361, 1245 cm^{-1}

^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.14(m, 2H, H-1), 2.50 (m, 2H, H-2), 6.93 (d, *J* = 8.3, 1H, H-4), 6.66 (d, *J* = 8.3 Hz, 1H, H-5), 6.66 (d, *J* = 8.3 Hz, 1H, H-7), 6.93 (d, *J* = 8.3, 1H, H-8), 1.76 (s, 3H, H-2'), 7.84 (brs, 1H, NH)

^{13}C NMR ($CDCl_3$, 75 MHz): δ 40.5 (t, C-1), 34.3 (t, C-2), 129.4 (s, C-3), 115.0 (d, C-4), 129.3 (d, C-5), 155.6 (s, C-6), 129.3 (d, C-7), 115.0 (d, C-8), 168.9 (s, C-1'), 22.5 (q, C-2')

Diatretyne (**47**) ($C_8H_3NO_2$) was obtained as yellowish needles by open column chromatography on Sephadex LH 20 and Silica gel using $CHCl_3$/MeOH 85:15 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 19.6 min . Upon spraying with vanillin/sulphuric acid no spot was obtained on the TLC plate. At 254 nm it absorbed light very strongly.



ESI-MS: 144 $[M-H]^-$

UV (MeOH): λ_{max} 230, 239, 268, 284, 302, 322 nm

IR (film): 1684, 1609, 1419, 1319, 1263, 1209, 965, 908 cm^{-1}

^{1}H NMR (DMSO-d_6, 300 MHz): δ 6.66 (d, *J* = 16.1 Hz, 1H, H-2), 6.83 (d, *J* = 16.1 Hz, 1H, H-3)

^{13}C NMR (DMSO-d_6, 75 MHz): δ 167.0 (s, C-1), 140.5 (d, C-2), 121.2 (d, C-3), 78.0 (s, C-4), 78.5 (s, C-5), 66.7 (s, C-6), 56.9 (s, C-7), 105.4 (s, C-8)

5.5.4 *Aspergillus* sp. HKI 0472

The strain was isolated from a soil sample (compost) collected in Bafoussam, a region situated in west of Cameroon and showed to have a good agreement with the misroscopic features of *Aspergillus* sp. It was deposited in the strain collection of the Leibniz-Institute for Natural product Research and Infection Biology, HKI, Jena Germany, under the number HKI 0472.

5.5.4.1 Fermentation

the producer strain *Aspergillus sp.* was cultivated as surface cultures at 25^{o}C in 500 ml Erlemeyer bottles containing 100 ml liquid malt medium. Each bottle was inoculated with a 1 cm^2 area of a 20 days agar culture. After 28 days of cultivation at 25 ^{o}C, the mycelium cake was harvested from 20 l of culture and extracted twice with 10 l of ethyl acetate. The culture broth was extracted three times with 10 l of ethyl acetate. The combined extracts were dried and evaporated. The residue (3.2 g) was subjected to silica gel chromatography (silica gel 60, Merck, 0,063~0.1 mm, column 4 x 60 cm), using stepwise $CHCl_3$, and $CHCl_3$–MeOH (9:1, 8:2, 1:1, v / v) as eluents. Final purification, which was achieved by preparative HPLC (Sperisorb ODS-2 RP_{18}, 5 µm (Promochem), 250 x 25 mm, acetonitrile/0.1% TFA (83:17 v / v), 10 ml/minute, UV-detection 210 nm) yielded 20 mg of funalenine and 10 mg and 15 mg of the known TMC-256A1 and asperxanthone, respectively.

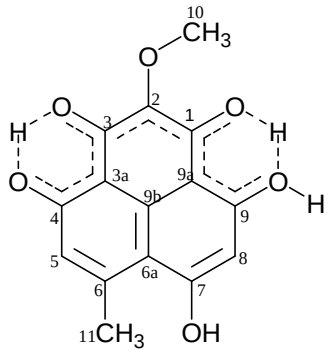
5.5.4.2 Pre-screening

Chemical screening by TLC revealed a considerable number of compounds. Furthermore numerous UV absorbing bands were observed, and most of the compounds were coloured greenish-brown after been sprayed with Emmerie-Engel's reagent, indicating the presence of phenolic compounds. In the biological screening, the crude extract exhibited a remarkable activity towards 3α-hydroxysteroid

dehydrogenase, suggesting the presence of anti-inflammatory compounds. In the antimicrobial pre-screening no significant activity was detected.

Funalenine (**48**) ($C_{15}H_{12}O_6$) was obtained as yellow crystals by open column chromatography on Sephadex LH-20 and Silica gel using $CHCl_3$/MeOH 70:30 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 20.1 min. Upon spraying with vanillin/sulphuric acid or Emmerie-Engel's reagent a strong greenish- brown spot was obtained on the TLC plate.

ESI-MS: m/z 289 $[M+H]^+$, 287 $[M-H]^-$
HR-ESIMS (found $[M-H]^-$; 289.0709 calcd.
for $C_{15}H_{11}O_6$: 287.09590)
UV (MeOH): λ_{max} 214, 282, 364, 412 nm
IR (film): 3192, 2923, 1586, 1380, 1349, 1267, 1147, 1028, 900, 822 cm^{-1}

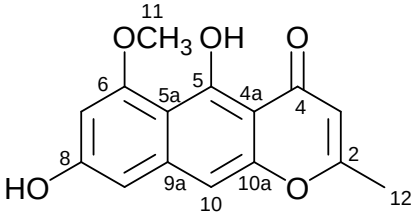


^{1}H NMR (DMSO-d_6, 300 MHz): δ 6.32 (s, 1H, H-5), 6.04 (s, 1H, H-8), 3.67 (s, 3H, H-10), 2.68 (s, 3H, H-11), 18.39 (s, 1H, OH), 18.42 (s, 1H, OH)

^{13}C NMR (DMSO-d_6, 75 MHz): δ 169.9 (s, C-1), 130.4 (s, C-2), 169.0 (s, C-3), 106.9 (s, C-3a), 171.3 (s, C-4), 116.4 (d, C-5), 143.7 (s, C-6), 106.7 (s, C-6a), 163.3 (s, C-7), 98.9 (d, C-8), 173.3 (s, C-9), 102.7 (d, C-9a), 127.5 (s, C-9b) 58.7 (q, C-10), 25.0 (q, C-11)

TMC-256A1 (**50**) ($C_{15}H_{12}O_5$) was obtained as yellow crystals by open column chromatography on Sephadex LH-20 and Silica gel using $CHCl_3$/MeOH 70:30 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 21.5. Upon spraying with vanillin/ sulphuric acid or Emmerie-Engel's reagent a strong yellow spot was obtained on the TLC plate.

ESI-MS: m/z 273 $[M+H]^+$, 271 $[M-H]^-$
UV (MeOH): λ_{max} 220, 270, 410 nm
IR (film): 3100, 1607, 1571, 1430, 1359, 1271, 1113, 1084, 856 cm^{-1}

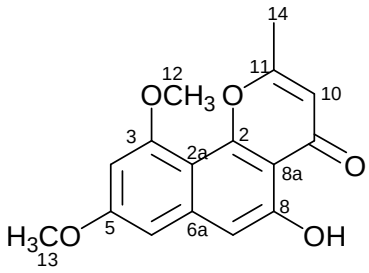


^{1}H NMR (DMSO-d_6, 300 MHz): δ 6.11 (s, 1H, H-3), 6.45 (d, *J* = 1.9 Hz, 1H, H-7) 6.61 (d, *J* = 1.9 Hz, 3H, H-9), 6.99 (s, 1H, H-10), 2.34 (s, 3H, H-11), 14.8 (s, 1H, OH)

^{13}C NMR (DMSO-d_6, 75 MHz): δ 168.3(s, C-2), 106.5 (d, C-3), 183.5 (s, C-4) 102.8 (s, C-4a), 162.2 (s, C-5), 106.5 (s, C-5a), 160.5 (s, C-6), 97.4 (d, C-7), 159.9 (s, C-8), 101.0 (d, C-9), 140.8 (s, C-9a), 99.7 (d, C-10), 152.4 (s, C-10a), 55.5 (q, C-11), 20.0 (q, C-12)

Asperxanthone (Flavasperone; TMC-256C2; Norrubrofusarin dimethyl ether) (**51**) ($C_{16}H_{14}O_5$) was obtained as a yellow powder by open column chromatography on Silica gel using $CHCl_3$/MeOH 90:10 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 27.6. Upon spraying with vanillin/ sulphuric acid a greenish- brown spot was obtained on the TLC plate.

ESI-MS: 287 $[M+H]^+$, 285$[M-H]^-$
UV (MeOH): λ_{max} 240, 282, 367 nm
IR (film): 1672, 1617, 1577, 1525, 1459, 1436, 1388, 1329, 1210, 876, 824 cm^{-1}



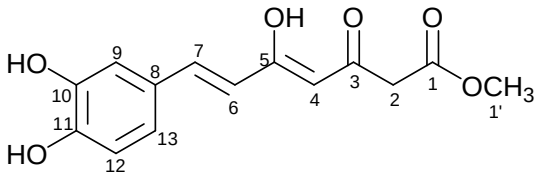
^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.35 (d, *J* = 1.9 Hz, 1H, H-4), 6.52 (d, *J* = 1.9 Hz, 1H, H-6) 6.90 (s, 1H, H-7), 6.27 (s, 1H, H-10), 3.92 (s, 3H, H-11), 3.88 (s, 3H, H-12), 2.45 (s, 3H, H-14)

^{13}C NMR ($CDCl_3$, 75 MHz): δ 155.7 (s, C-2), 104.8 (s, C-2a), 159.0 (s, C-3), 96.9 (d, C-4) 161.4 (s, C-5), 97.9 (d, C-6), 141.1(s, C-6a), 105.7 (d, C-7), 156.5 (s, C-8), 108.7 (s, C-8a), 182.8 (s, C-9), 110.1 (d, C-10), 166.5 (s, C-11), 55.8 (q, C-12), 55.4 (q, C-13), 20.4 (q, C-14)

5.5.5 *Inonotus sp.* (fruiting body)

The fruiting body of *inonotus* sp. was collected in Vietnam. Its identification was verified by Prof. Trinh Tam Kiet from the Mycological Research Centre, Hanoi State University, 334 Nguyen Trai Street, Hanoi, Vietnam, where a specimen was deposited. 500 g of fresh fruiting bodies were cut in to small pecies, dried and crushed. The resulting powder was extracted three times with ethanol, chloroform / methanol (3 x 2l 3 days each). The combined extract (3 g) of a brown solid, which was found to have a strong anti-inflammatory effect in the 3α-hydroxysteroid dehydrogenase (3α-HSD) assay, was subjected to silica gel chromatography (silica gel 60, Merck, 0,063~0.1 mm, column 4 x 60 cm), using stepwise $CHCl_3$-MeOH (9:1, 8:2, 1:1 v / v) as eluents. Bioassay-guided fractionation and monitoring by mass spectrometry led to the detection of six active substances with m/z = 217, 291, 244, 244 and 177 in the negative mode. The yields were, 20 mg of inonotic acid methylester, , 4 mg of inotilone, 500 mg of hispidin A, 15 mg of *iso*-hispidin and 6 mg of (*E*)-4-(3,4-dihydroxyphenyl)but-3en-2-one.

Inonotic acid methylester (**52**) ($C_{14}H_{14}O_6$) was obtained as yellow oil by open column chromatography on Sephadex LH-20 and Silica gel using $CHCl_3$/MeOH 95:5 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 20.5 min. Upon spraying with vanillin/ sulphuric acid or Emmerie-Engel's reagent a strong greenish- brown spot was obtained on the TLC plate.



ESI-MS: m/z 279 $[M+H]^+$, 277 $[M-H]^-$

HR-ESIMS (found $[M-H]^-$; 277.0682 calcd. for $C_{14}H_{13}O_6$: 277.0707)

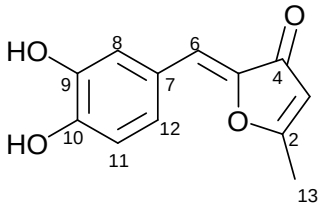
UV (MeOH): λ_{max} 261, 380 nm

IR (film): 3094, 1733, 1632, 1567, 1513, 1435, 1282, 1022, 974 cm^{-1}

^1H NMR (DMSO-d_6, 300 MHz): δ 3.55 (s, 2H, H-2), 5.91 (s, 1H, H-4), 6.50 (d, *J* = 15.8 Hz, 1H, H-6), 7.45 (d, *J* = 15.8 Hz, 1H, H-7), 7.07 (d, *J* = 1.8 Hz, 1H, H-9), 6.77 (d, *J* = 8.1 Hz, 1H, H-12), 7.02 (dd, *J* = 1.8, 8.1 Hz, 1H, H-13), 3.65 (s, 3H, H-1')

^{13}C NMR (DMSO-d_6, 75 MHz): δ 167.9 (s, C-1), 45.6 (t, C-2), 191.8 (s, C-3), 100.3 (d, C-4), 178.3 (s, C-5), 118.6 (d, C-6), 141.0 (d, C-7), 126.2 (s, C-8), 114.7 (d, C-9), 145.6 (s, C-10), 148.4 (s, C-11), 115.7 (d, C-12), 121.5 (s, C-13), 51.8 (q, C-1')

Inotilone (**53**) ($C_{12}H_{10}O_4$) was obtained as yellow oil by open column chromatography on Sephadex LH 20 and silica gel using $CHCl_3$/MeOH 90:10 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 16 min. Upon spraying with vanillin/ sulphiric acid or Emmerie-Engel's reagent a strong greenish- brown spot was obtained on the TLC plate.

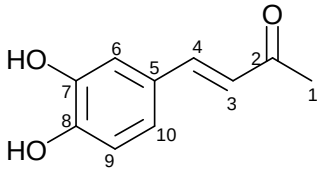


ESI-MS: m/ z 219 $[M+H]^+$, 217 $[M-H]^-$
HR-ESIMS (found $[M-H]^-$; 217.0495 calcd. for $C_{12}H_9O_4$: 217.0495)
UV (MeOH): λ_{max} 264, 312, 378 nm
IR (film): 3184, 1682, 1588, 1435, 1287, 1014, 951 cm^{-1}

^{1}H NMR (DMSO-d_6, 300 MHz): δ 5.82 (s, 1H, H-4), 6.49 (s, 1H, H-6), 7.35 (d, *J* = 2.0 Hz, 1H, H-8), 6.80 (d, *J* = 8.2 Hz, 1H, H-11), 7.17 (dd, *J* = 2.0, 8.2 Hz, 1H, H-12), 2.39 (s, 3H, H-13)

^{13}C NMR (DMSO-d_6, 75 MHz): δ 180.4 (s, C-2), 105.4 (d, C-3), 186.6 (s, C-4), 144.3 (s, C-5) 111.9 (d, C-6), 122.9 (s, C-7), 117.9 (d, C-8), 145.4 (s, C-9), 148.1 (s, C-10), 115.9 (d, C-11), 124.7 (s, C-12), 15.6 (d, C-13)

***(E)*-4-(3,4-dihydroxyphenyl)but-3en-2-one** (**56**) ($C_{10}H_{10}O_3$) was obtained as yellow crystals by open column chromatography on Sephadex LH-20 and Siliica gel using $CHCl_3$/MeOH 80:20 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 15.0 min. Upon spraying with vanillin/ sulphuric acid or Emmerie-Engel's reagent a strong greenish-brown spot was obtained on the TLC plate.

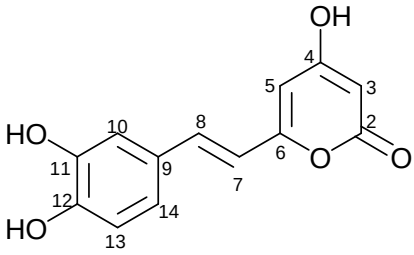


ESI-MS: m/ z 179 $[M+H]^+$, 177 $[M-H]^-$
UV (MeOH): λ_{max} 248, 361, nm
IR (film): 3459 1597, 1533, 1442, 1357, 1276, 1110, 964, 803 cm^{-1}

^{1}H NMR (CD_3OD, 300 MHz): δ 2.32 (s, 3H, H-1), 6.53(d, *J* = 16.1 Hz, 1H, H-3), 7.52 (d, *J* = 16.1 Hz, 1H, H-4), 7.07 (d, *J* = 1.9 Hz, 1H, H-6), 6.78 (d, *J* = 8.2 Hz, 1H, H-9), 6.98 (dd, *J* = 1.9, 8.2 Hz, 1H, H-10)

Hispidin (**57**) ($C_{13}H_{10}O_5$) was obtained as yellow crystals by open column chromatography on Sephadex LH-20 and silica gel using $CHCl_3$/MeOH 80:20 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 15.9 min. Upon spraying with vanillin/sulphuric acid or Emmerie-Engel's reagent a strong greenish- brown spot was obtained on the TLC plate.

ESI-MS: m/ z 245 $[M+H]^+$, 244 $[M-H]^-$
UV (MeOH): λ_{max} 258 and 379 nm
IR (film): 3093, 1594, 1544, 1373, 1284, 1260, 1150, 1015 and 957 cm^{-1}

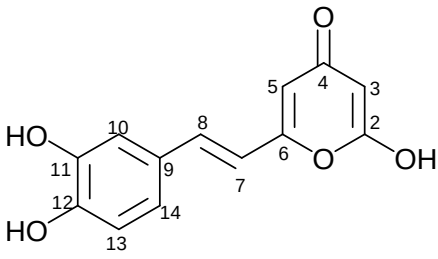


^{1}H NMR (DMSO-d_6, 300 MHz): δ 5.26 (d, *J* = 2.1 Hz, 1H, H-3), 6.14 (d, *J* = 2.1 Hz, 1H, H-5), 6.63 (d, *J* = 15.9 Hz, 1H, H-7), 7.13 (d, *J* = 15.9 Hz, 1H, H-8), 7.01 (d, *J* = 2.1 Hz, 1H, H-10), 6.75 (d, *J* = 8.4 Hz, 1H, H-13), 6.93 (dd, *J* = 2.1, 8.4 Hz, 1H, H-14)

^{13}C NMR (DMSO-d_6, 75 MHz): δ 162.3 (s, C-2), 89.1 (d, C-3), 170.3 (s, C-4), 100.4 (d, C-5), 159.7 (s, C-6), 116.3 (d, C-7), 134.5 (d, C-8), 126.7 (s, C-9), 114.0 (d, C-10), 145.5 (s, C-11), 147.3 (s, C-12), 115.7 (d, C-13), 120.2 (d, C-14)

***iso*-Hispidin** (**58**) ($C_{13}H_{10}O_5$) was obtained as yellow crystals by open column chromatography on Sephadex LH-20 and Silica gel using $CHCl_3$/MeOH 80:20 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 14.0 min. Upon spraying with vanillin/sulphuric acid or Emmerie-Engel's reagent a strong greenish- brown spot was obtained on the TLC plate.

ESI-MS: m/z 245 $[M+H]^+$, 244 $[M-H]^-$
HR-ESIMS (found $[M-H]^-$; 245.0464 calcd. for $C_{15}H_{15}O_6$: 245.0445)
UV (MeOH): λ_{max} 248, 361 nm



IR (film): 3095, 1649, 1590, 1494, 1411, 1276, 1202, 1050, 1000 cm^{-1}

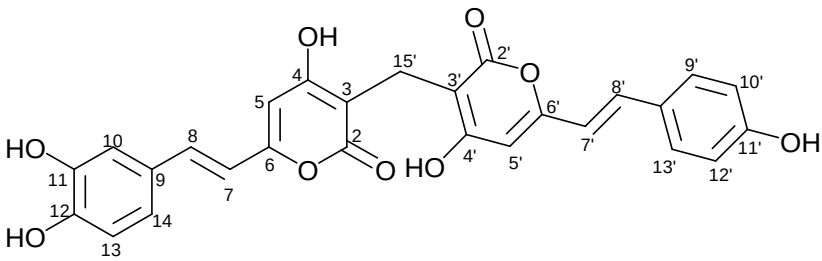
^{1}H NMR (DMSO-d_6, 300 MHz): δ 4.42 (d, *J* = 1.2 Hz, 1H, H-3), 5.59 (d, *J* = 1.2 Hz, 1H, H-5), 6.12 (d, *J* = 15.8 Hz, 1H, H-7), 6.87 (d, *J* = 15.8 Hz, 1H, H-8), 6.94 (d, *J* = 1.5 Hz, 1H, H-10), 6.70 (d, *J* = 8.1 Hz, 1H, H-13), 6.82 (dd, *J* = 1.5, 8.1 Hz, 1H, H-14)

^{13}C NMR (DMSO-d_6, 75 MHz): δ 165.4 (s, C-2), 86.5 (d, C-3), 179.1 (s, C-4), 109.0 (d, C-5), 156.1 (s, C-6), 118.5 (d, C-7), 130.8 (d, C-8), 127.4 (s, C-9), 113.5 (d, C-10), 145.6 (s, C-11), 146.5 (s, C-12), 115.7 (d, C-13), 119.2 (d, C-14)

5.5.6 *Pholiota squarrosa* (fruiting body)

The fruiting body of *Pholiota squarrosa* was collected in Jena (Germany) and its identification was verified by Dr. Martin Roth, from the Leibniz-Institute for Natural Product and Infection Biology, HKI, Pilot Plant for Natural Products, where a specimen was deposited. 25 g of fresh fruiting bodies were cut in to small pieces, dried and crushed. The resulting powder was extracted three times, chloroform /methanol: 1:1 (3 x 2 l 3 days each). The combined extract (2.5 g) of a brown solid, which was found to have a strong anti-inflammatory effect in the 3α-hydroxysteroid dehydrogenase (3α-HSD) assay, was subjected to silica gel chromatography (silica gel 60, Merck, 0,063~0.1 mm, column 4 x 60 cm), using stepwise $CHCl_3$-MeOH (9:1, 8:2, 1:1 v / v) as eluents. Bioassay-guided fractionation and monitoring by mass spectrometry led to the detection of the unique active substance with m/z = 487 in the negative mode. The yield was 4 mg of squarrosidine.

Squarrosidine (**65**) ($C_{27}H_{20}O_9$) was obtained as yellow oil by open column chromatography on Sephadex LH-20 and silica gel using $CHCl_3$/MeOH 85:15 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 23.5. Upon spraying with vanillin/ sulphuric acid or Emmerie-Engel's reagent a strong greenish-brown spot was obtained on the TLC plate.



ESI-MS:m/z 489 $[M+H]^+$, 487 $[M-H]^-$

HR-ESIMS (found $[M-H]^-$; 487.1065 calcd.

for $C_{27}H_{19}O_9$: 487.1024)

UV (MeOH): λ_{max} 219, 258, 369 nm

IR (film): 3204, 1661, 1601, 1556, 1513, 1414, 1273, 1022, 987 cm^{-1}

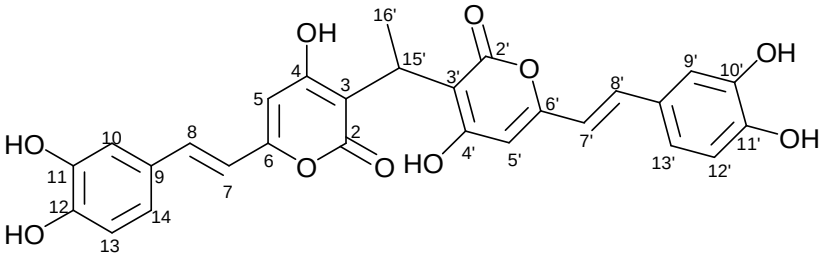
^1H NMR (DMSO-d_6, 300 MHz): δ 5.85 (s, 1H, H-5), 6.52 (d, *J* = 15.9 Hz, 1H, H-7), 6.97 (d, *J* = 15.9 Hz, 1H, H-8), 6.96 (d, *J* = 1.8 Hz, 1H, H-10), 6.72 (d, *J* = 8.1 Hz, 1H, H-13), 6.88 (dd, *J* = 1.8, 8.1 Hz, 1H, H-14), 5.84 (s, 1H, H-5'), 6.62 (d, *J* = 15.9 Hz 1H, H-7'), 7.06 (d, *J* = 15.9 Hz, 1H, H-8'), 7.43 (d, *J* = 8.7 Hz, 1H, H-10'), 6.76 (d, *J* = 8.7 Hz, 1H, H-13'), , 7.43 (d, *J* = 8.7 Hz, 1H, H-14'), 3.33 (s, 1H, H-1'')

^{13}C NMR (DMSO-d_6, 75 MHz): δ 164.4 (s, C-2), 101.2 (s, C-3), 172.5 (s, C-4), 105.2 (s, C-5), 155.4 (s, C-6), 117.3 (d, C-7), 131.5 (d, C-8), 127.2 (s, C-9), 113.6 (d, C-10), 146.5 (s, C-11), 145.4 (s, C-12), 115.6 (d, C-13), 119.5 (d, C-14), 164.2 (s, C-2'), 101.2 (s, C-3'), 172.5 (d, C-4'), 105.2 (d, C-5'), 155.4 (s, C-6'), 117.4 (d, C-7'), 131.1 (d, C-8'), 126.7 (s, C-9'), 128.5 (d, C-10'), 115.6 (d, C-11'), 158.1 (s, C-12'), 115.6 (d, C-13'), 128.5 (d, C-14'), 18.9 (s, C-1'')

5.5.7 *Phellinus pini* DSM 5238

The fungus was cultivated under the condition of surface fermentation at 25 ^oC in 500 ml Erlenmeyer flasks containing 300 ml liquid malt medium. After cultivation for 28 days at 25 ^oC the mycelium cake from the culture medium (40 l) was extracted twice with ethyl acetate (25 l) and methanol (each 10 l). The culture broth was thoroughly extracted with ethyl acetate. The organic layer was combined and concentrated under reduced pressure. The resulting dark brown oil was washed with *n*-hexane to remove fatty acid and the residue was dissolved in a small volume of $CHCl_3$ and subjected to column chromatography (silica gel 60, Merk, 0.063 - 0.1 mm, column 4 x 60 cm), using stepwise $CHCl_3$ and $CHCl_3$-MeOH (9:1, 1:1, *v/v*) as eluents. Active components were isolated on the basis of bioassays. Final purification was achieved by preparative HPLC using a Spherisorb ODS-2 Rp_{18} column (250 x 25 mm, 5 µm, Promochem, and acetonitrile-H_2O (83:17, v / v) as eluent (flow rate 10 ml/min, UV-detection at 300 nm), yielding pinillidine (8 mg), pinillic acid (4 mg), hypholomine B (7 mg) and hispidin (100 mg).

Pinillidine (**72**) ($C_{28}H_{22}O_{10}$) was obtained as yellow oil by open column chromatography on Sephadex LH-20 ans Silica gel using $CHCl_3$/MeOH 80:20 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 22.3 min. Upon spraying with vanillin/sulphuric acid or Emmerie-Engel's reagent a strong greenish- brown spot was obtained on the TLC plate.



ESI-MS: m/z 517 $[M+H]^-$

UV (MeOH): λ_{max} 256, 388 nm

IR (film): 3189, 2921, 1649, 1584, 1557, 1494, 1375 cm^{-1}

^{1}H NMR (DMSO-d_6, 300 MHz): δ 6.13 (s, 1H, H-5), 6.64 (d, *J* = 16.0 Hz, 1H, H-7), 7.16 (d, *J* = 16.0 Hz, 1H, H-8), 7.01 (d, *J* = 1.9 Hz, 1H, H-10), 6.74 (d, *J* = 8.3 Hz, 1H, H-13), 6.93 (dd, 1.9, *J* = 8.3, 8.4 Hz, 1H, H-14), δ 6.13 (s, 1H, H-5'), 6.64 (d, *J* = 16.0 Hz, 1H, H-7'), 7.16 (d, *J* = 16.0 Hz, 1H, H-8'), 7.01 (d, *J* = 1.9 Hz, 1H, H-10'), 6.74 (d, *J* = 8.3 Hz, 1H, H-13'), 6.93 (dd, *J* = 1.9, 8.3 Hz, 1H, H-14'), 4.55 (q, 1H, H-15'), 1.45 (d, *J*= 7.5, 3H, H-16')

^{13}C NMR (DMSO-d_6, 75 MHz): δ 164.2 (s, C-2), 105.2 (d, C-3), 166.5 (s, C-4), 105.2 (d, C-5), 156.6 (s, C-6), 116.1 (d, C-7), 133.7 (d, C-8), 126.8 (s, C-9), 114.0 (d, C-10), 145.5 (s, C-11), 147.1 (s, C-12), 115.7 (d, C-13), 120.1 (d, C-14), 164.2 (s, C-2'), 105.2 (d, C-3'), 166-5 (s, C-4'), 105.2 (d, C-5'), 156.6 (s, C-6'), 116.1 (d, C-7'), 133.7 (d, C-8'), 126.8 (s, C-9'), 114.0 (d, C-10'), 145.5 (s, C-11'), 147.1 (s, C-12'), 115.7 (d, C-13'), 120.1 (d, C-14') 25.5 (d, C-15'), 16.5 (q, C-16')

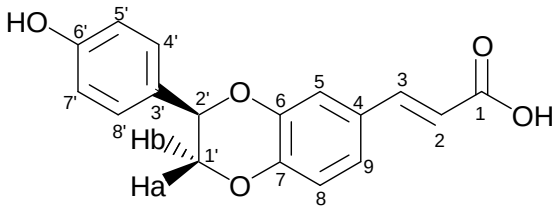
Pinillic acid (**73**) ($C_{17}H_{14}O_5$) was obtained as yellow powder by open column chromatography on Sephadex LH-20 and Silica gel using $CHCl_3$/MeOH 80:20 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t 21.3 min. Upon spraying with Emmerie-Engel's reagent a strong greenish- brown spot was obtained on the TLC plate.

ESI-MS: m/z 297 $[M-H]^-$

HR-ESIMS (found $[M-H]^-$; 297.0755 calcd. for $C_{17}H_{13}O_5$: 297.0758)

UV (MeOH): λ_{max} 230, 290, 330 nm

IR (film): 3198, 2925, 1681, 1613, 1581, 1507, 1436, 1170, 815 cm^{-1}



^{1}H NMR (CD_3OD, 300 MHz): δ 6.34 (d, *J* = 15.9 Hz, 1H, H-2), 7.59 (d, *J* = 15.9 Hz, 1H, H-3), 7.18 (d, *J* = 2.0 Hz, 1H, H-5), 6.93 (d, *J* = 8.7 Hz, 1H, H-8), 7.12 (d, *J* = 2.0, 8.7 Hz, 1H, H-9), 4.04 (dd, *J* = 8.8, 11.5 Hz, 1H, H-a1'), 4.36 (dd, *J* = 2.3, 11.5 Hz, 1H, H-b1'), 5.04 (dd, *J* = 2.3, 8.8 Hz, 1H, H-2'), 7.28 (d, *J* = 8.5 Hz, 1H, H-4'), 6.83 (d, 8.5, 1H, H-5'), 6.83 (d, *J* = 8.5 Hz, 1H, H-7'), 7.28 (d, *J* = 8.5 Hz, 1H, H-8')

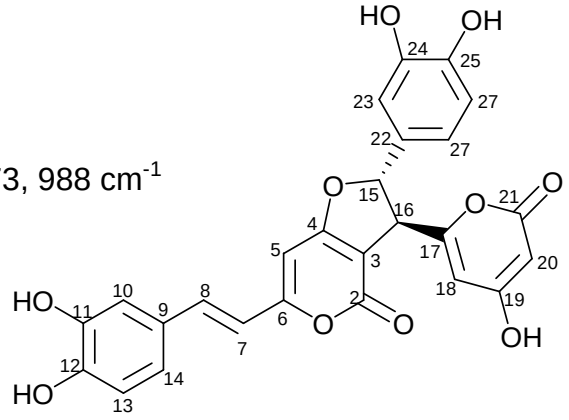
^{13}C NMR (CD_3OD, 75 MHz): δ 170.7 (s, C-1), 117.4 (d, C-2), 146.0 (d, C-3), 129.4 (s, C-4), 117.6 (d, C-5), 145.7 (s, C-6), 146.7 (s, C-7), 118.4 (d, C-8), 123.1 (d, C-9), 70.5 (t, C-1'), 76.2 (d, C-2'), 128.5 (s, C-3'), 129.1 (d, C-4'), 116.4 (d, C-5'), 159.1 (s, C-6'), 116.4 (d, C-7'), 129.1 (d, C-8')

Hypholomine B (**75**) ($C_{26}H_{18}O_{10}$) was obtained as yellow oil by open column chromatography on Sephadex LH-20 and silca gel using $CHCl_3$/MeOH 70:30 eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 30 min) R_t = 18.3. Upon spraying with vanillin/sulphuric acid or Emmerie-Engel's reagent a strong greenish- brown spot was attained on the TLC plate.

ESI-MS: m/z 489 $[M+H]^-$, 979 $[2M-H]^-$

UV (MeOH): λ_{max} 256, 388 nm

IR (film): 3070, 1602, 1549, 1430, 1284, 1173, 988 cm^{-1}



^{1}H NMR (DMSO-d_6, 300 MHz): δ 6.59 (s, 1H, H-5), 6.98 (d, *J* = 15.9 Hz, 1H, H-7), 7.29 (d, *J* = 15.9 Hz, 1H, H-8), 7.05 (d, *J* = 1.9 Hz, 1H, H-10), 6.73 (d, *J* = 8.3 Hz, 1H, H-13), 6.98 (dd, *J* = 1.9, 8.3 Hz, 2H, H-14), 5.81 (d, *J* = 6.0 Hz, 1H, H-15), 4.29

(d, *J* = 6.1 Hz, 1H, H-16), 6.09 (d, *J* = 2.0 Hz, 1H, H-18), 5.29 (d, *J* = 2.0, 1H, H-20), 6.92 (d, *J* = 8.3 Hz, 1H, H-22), 6.77 (d, *J* = 8.3 Hz, 1H, H-25), 6.88 (d, *J* = 2.0, 8.3 Hz, 1H, H-26)

^{13}C NMR (DMSO-d_6, 75 MHz): δ 162.0 (s, C-2), 98.1 (s, C-3), 171.4 (s, C-4), 94.7 (d, C-5), 163.2 (s, C-6), 138.2 (d, C-7), 136.2 (d, C-8), 126.5 (d, C-9), 114.1 (d, C-10), 145.6 (s, C-11), 147.8 (s, C-12), 115.5 (d, C-13), 120.8 (d, C-14), 90.4 (d, C-15), 50.9 (d, C-16), 158.9 (d, C-17), 101.2 (d, C-18), 170.0 (s, C-19), 89.3 (s, C-20), 163.4 (s, C-21), 129.5 (d, C-22), 117.3 (d, C-23), 145.5 (s, C-24), 146.3 (d, C-25), 115.8 (d, C-26), 121.6 (d, C-27)

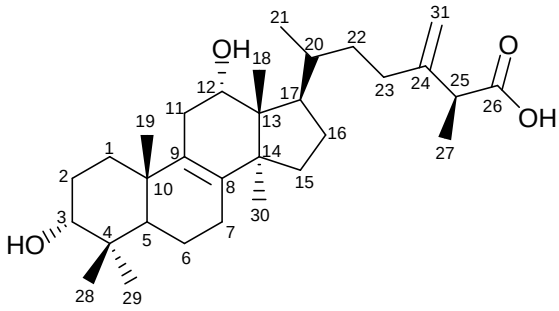
5.5.8 *Piptoporus betulinus* (fruiting body)

The fruiting body of *Piptoporus betulinus* was isolated from the forest district near Jena (Thuringia, Germany). One kilogram of fresh fruiting bodies was cut into small pieces, dried and crushed. The resulting powder was extracted three times with ethylacetate, chloroform and methanol (3 x 2 l 3 days each). The ethyl acetate extract (3 g) of a brown solid, which was found to have a strong anti-inflammatory effect was subjected to silica gel chromatography (silica gel 60, Merck, 0,063~0.1mm, column 4 x 60 cm), using stepwise $CHCl_3$-MeOH (9:1, 8:2, 1:1 v / v) as eluents. Bioassay-guided fractionation using a 3α-hydroxysteroid dehydrogenase (3α-HSD) assay and monitoring by mass spectrometry led to the detection of four active substances with m/z = 529, 587, 645, 483 in positive mode.

Yield: 10 mg of polyporenic acid A, 8 mg of 3α-acetylpolyporenic acid A, 15 mg of (25S)-(+)-12αhydroxy-3α-methylcarboxyacetate-24-methyllanosta-8,24(31)-diene-26-oic acid, 40 mg of (25S3'S)-(+)-12α-hydroxy-3α-(3'-hydroxy-4'methoxycarbonyl-3'-methylbutyryloxy)-24-methyllanosta-8,24(31)-dien-26-oic acid and 30 mg of polyporenic acid C.

Polyporenic acid A (**75**) ($C_{31}H_{50}O_4$) was obtained as colourless oil by open column chromatography on silca gel using $CHCl_3$/MeOH 80:20 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 50 min) R_t = 23.9. Upon spraying with vanillin/sulphuric acid a violet spot was obtained on the TLC plate, suggesting a terpernoid.

$[\alpha]_D^{20}$ +69 (MeOH, c = 1 mg/ml)
ESI-MS: m/z 487 $[M+H]^+$, 509 $[M+Na]^+$
UV (MeOH): λ_{max} 264 nm
IR (film): 3400, 2932, 1706, 1570, 1456, 1373, 1204, 1056, 890 cm^{-1}

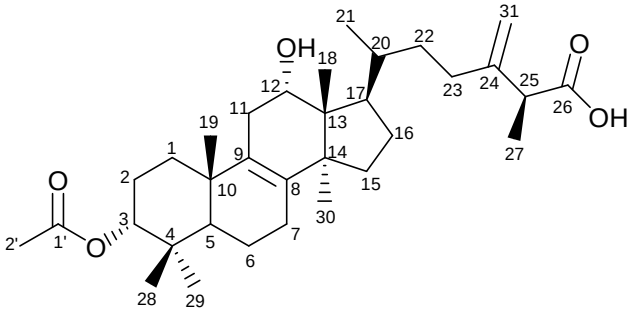


^{1}H NMR (CD_3OD, 300 MHz): δ 1.48-167 (m, 2H, H-1), 1.61-1.69 (m, 2H, H-2), 4.65 (brs, *J* = 7.2 Hz, 1H, H-3), 1.49-1.61 (m, 1H, H-5), 1.49 (m, 2H, H-6), 2.02 (m, 2H, H-7), 2.03 (m, 2H, H-11), 4.00 (d, *J* = 8.1 Hz, 1H, H-12), 2.25 (m, 2H, H-15), 1.35-2.10 (m, 2H, H-16), 2.02 (m, 1H, H-17), 0.66 (s, 3H, H-18), 1.01 (s, 3H, H-19), 1.40 (m, 1H, H-20), 1.06 (d, *J* = 6.5 Hz, 3H, H-21), 1.24-1.65 (m, 2H, H-22), 2.02 (m, 2H, H-23), 3.16 (q, *J* = 7.0 Hz, 1H, H-25), 1.25 (d, *J* = 7.0 Hz, 3H, H-27), 0.88 (s, 3H, H-28), 0.96 (s, 3H, H-29), 1.10 (s, 3H, H-30), 4.89 (brs, 2H, H-31)

^{13}C NMR (CD_3OD, 75 MHz): δ 31.2 (t, C-1), 26.7 (t, C-2), 76.6 (d, C-3), 38.5 (s, C-4), 45.4 (d, C-5), 19.2 (t, C-6), 27.2 (t, C-7), 135.9 (s, C-8), 134.7(s, C-9), 37.9 (s, C-10), 34.3 (t, C-11), 73.7 (d, C-12), 50.7 (s, C-13), 50.6 (s, C-14), 33.1 (t, C-15), 28.9 (t, C-16), 43.9 (d, C-17), 16.9 (q, C-18), 19.3 (q, C-19), 37.6 (d, C-20), 18.0 (q, C-21), 35.6 (t, C-22), 33.2 (t, C-23), 151.6 (s, C-24), 48.3 (d, C-25), 178.7 (s, C-26), 17.1 (q, C-27), 22.7 (q, C-28), 28.9 (q, C-29), 25.1 (q, C-30), 110.0 (t, C-31)

3α-Acetylpolyporenic acid A (**76**) ($C_{33}H_{52}O_5$) was obtained as colourless oil by open column chromatography on silca gel using $CHCl_3$/MeOH 85:15 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 50 min) R_t = 33.6 min. Upon spraying with vanillin/sulphuric acid a violet spot was obtained on the TLC plate, suggesting a terpernoid.

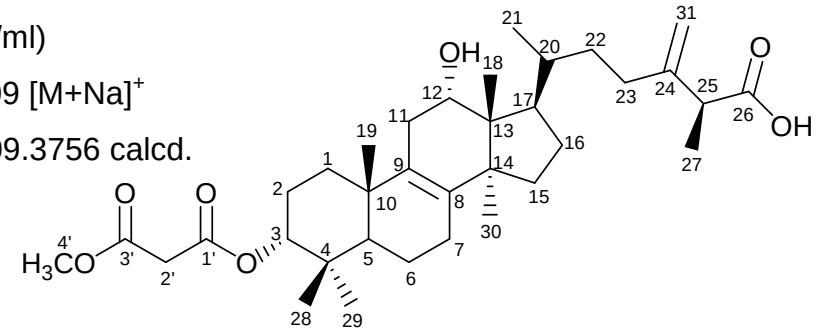
$[\alpha]_D^{20}$ +30 (MeOH, c = 1 mg/ml)
ESI-MS: m/z 529 $[M+H]^+$, 551 $[M+Na]^+$
HR-ESIMS (found $[M+Na]^+$; 551. calcd. for $C_{33}H_{52}O_5$ Na: 3701551.4288)
UV (MeOH): λ_{max} 264 nm
IR (film): 2939, 1709, 1457, 1374, 1245, 1181, 1010, 892 cm^{-1}



^{1}H NMR ($CDCl_3$, 300Hz): δ 1.49 (m, 2H, H-1), 1.61-1.69 (m, 2H, H-2), 4.65 (brs, *J* = 7.2, 1H, H-3), 1.49-1.61 (m, 1H, H-5), 1.49 (m, 2H, H-6), 2.02 (m, 2H, H-7), 2.09-2.63 (m, 2H, H-11), 4.00 (d, *J* = 8.1 Hz, 1H, H-12), 1.19-1.67 (m, 2H, H-15), 1.35-2.10 (m, 2H, H-16), 2.02 (m, 1H, H-17), 0.60 (s, 3H, H-18), 0.96 (s, 3H, H-19), 1.40 (m, *J* = 7.2 Hz, 1H, H-20), 1.00 (d, *J* = 6.5 Hz, 3H, H-21), 1.24-1.65 (m, 2H, H-22), 2.02 (m, 2H, H-23), 3.16 (q, *J* = 7.0, 1H, H-25), 1.30 (d, *J* = 7.0 Hz, 3H, H-27), 0.85 (s, 3H, H-28), 0.90 (s, 3H, H-29), 1.00 (s, 3H, H-30), 4.93-4.97 (brs, 2H, H-31), 2.06 (s, 3H, H-2')

^{13}C NMR ($CDCl_3$, 75 MHz): δ 30.5 (t, C-1), 23.2 (t, C-2), 78.0 (d, C-3), 36.7 (s, C-4), 45.1 (d, C-5), 17.9 (t, C-6), 25.9 (t, C-7), 134.7 (s, C-8), 132.9 (s, C-9), 36.7 (s, C-10), 32.5 (t, C-11), 73.4 (d, C-12), 49.5 (s, C-13), 49.6 (s, C-14), 31.9 (t, C-15), 27.7 (t, C-16), 43.1 (d, C-17), 16.1 (q, C-18), 18.7 (q, C-19), 35.9 (d, C-20), 17.8 (q, C-21), 34.2 (t, C-22), 31.8 (t, C-23), 148.1 (s, C-24), 45.1 (d, C-25), 178.7 (s, C-26), 16.2 (q, C-27), 21.7 (q, C-28), 27.5 (q, C-29), 24.5 (q, C-30), 111.0 (t, C-31), 171.0 (s, C-1'), 21.3 (s, C-2')

(25S)-(+)-12α-hydroxy-3α-methylcarboxyacetate-24-methyllanosta-8,24(31)-diene-26-oic acid (**77**) ($C_{35}H_{54}O_7$) was obtained as colourless oil by open column chromatography on silca gel using $CHCl_3$/MeOH 85:15 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 50 min) R_t = 33.8 min. Upon spraying with vanillin/sulphuric acid a violet spot was obtained on the TLC plate, suggesting a terpernoid.



$[\alpha]_D^{20}$ +19 (MeOH, c = 1 mg/ml)
ESI-MS: m/z 587 $[M+H]^+$, 609 $[M+Na]^+$
HR-ESIMS (found $[M-H]^-$; 609.3756 calcd. for $C_{35}H_{54}O_7Na$: 609.4446)
UV (MeOH): λ_{max} 264 nm
IR (film): 2940, 1731, 1456, 1374, 1152, 1017, 974, 753 cm^{-1}

^{1}H NMR ($CDCl_3$, 300Hz): δ 1.50 (m, 2H, H-1), 1.62-1.90 (m, 2H, H-2), 4.70 (brs, *J* = 7.2 Hz, 1H, H-3), 1.44 (m, 1H, H-5), 1.44-1.60 (m, 2H, H-6), 2.01 (m, 2H, H-7), 2.10-

2.60 (m, 2H, H-11), 4.00 (d, *J* = 8.0 Hz, 1H, H-12), 1.19-1.65 (m, J = 6.9 Hz, 2H, H-15), 1.37-1.65 (m, 2H, H-16), 2.01 (m, 1H, H-17), 0.60 (s, 3H, H-18), 0.97 (s, 3H, H-19), 1.40 (m, *J* = 7.2 Hz, 1H, H-20), 1.00 (d, *J* = 6.5 Hz, 3H, H-21), 1.24-1.65 (m, 2H, H-22), 2.01-2.20 (m, 2H, H-23), 3.15 (q, *J* = 7.0 Hz, 1H, H-25), 1.32 (d, *J* = 7.0 Hz, 3H, H-27), 0.92 (s, 3H, H-28), 0.86 (s, 3H, H-29), 1.07 (s, 3H, H-30), 4.93-4.97 (brs, *J* = 6.9 Hz, 2H, H-31), 3.38 (s, 3H, H-2'), 3.70 (s, 3H, H-4')

^{13}C NMR ($CDCl_3$, 75 MHz): δ 30.4 (t, C-1), 23.1 (t, C-2), 79.7 (d, C-3), 36.8 (s, C-4), 45.4 (d, C-5), 17.9 (t, C-6), 25.9 (t, C-7), 134.8 (s, C-8), 132.8 (s, C-9), 36.6 (s, C-10), 32.5 (t, C-11), 73.4 (d, C-12), 49.5 (s, C-13), 49.6 (s, C-14), 31.9 (t, C-15), 27.7 (t, C-16), 43.1 (d, C-17), 16.1 (q, C-18), 18.7 (q, C-19), 35.9 (d, C-20), 17.8 (q, C-21), 34.2 (t, C-22), 31.8 (t, C-23), 148.1 (s, C-24), 45.1 (d, C-25), 179.0 (s, C-26), 16.2 (q, C-27), 21.6 (q, C-28), 27.5 (q, C-29), 24.4 (q, C-30), 111.4 (t, C-31), 166.0 (s, C-1'), 41.7 (s, C-2'), 167.2 (s, C-3'), 52.3 (s, C-4')

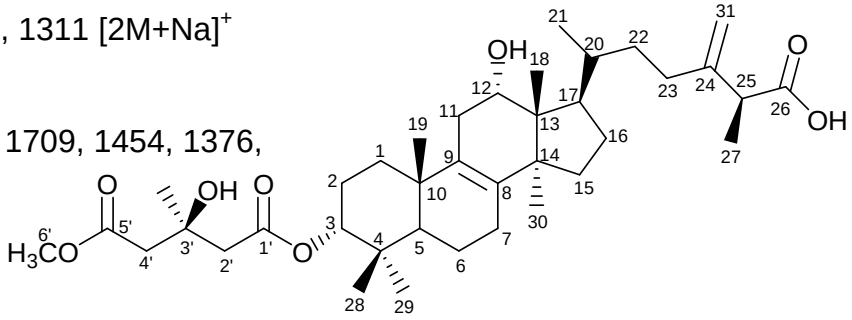
(25S.3'S)-(+)-12α-hydroxy-3α-(3'-hydroxy-4'methoxycarbonyl-3'-methylbutyryloxy)-24-methyllanosta-8,24(31)-dien-26-oic acid (**78**) ($C_{38}H_{60}O_8$) was obtained as a colourless oil by open column chromatography on silca gel using $CHCl_3$/MeOH 90:10 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 50 min) R_t = 45.2. Upon spraying with vanillin/sulphuric acid a violet spot was obtained on the TLC plate, suggesting a terpernoid.

$[\alpha]_D^{20}$ +17 (MeOH, c = 1 mg/ml)

ESI-MS: m/z 667 $[M+Na]^+$, 1311 $[2M+Na]^+$

UV (MeOH): 264 nm

IR (film): λ_{max} 3377, 2938, 1709, 1454, 1376, 1202, 1023 cm^{-1}



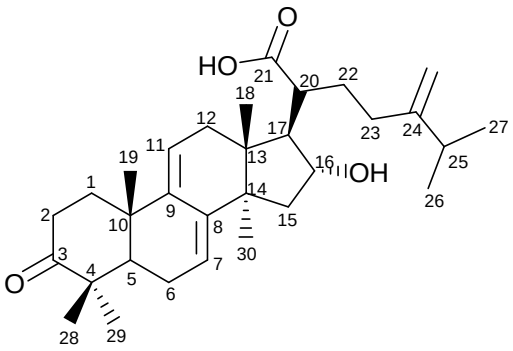
^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.50 (m, 2H, H-1), 1.62-1.90 (m, 2H, H-2), 4.70 (brs, *J* = 7.2 Hz, 1H, H-3), 1.44 (m, 1H, H-5), 1.44-1.60 (m, 2H, H-6), 2.01 (m, 2H, H-7), 2.10-2.60 (m, 2H, H-11), 4.00 (d, *J* = 8.0 Hz, 1H, H-12), 1.19-1.65 (m, 2H, H-15),

1.37-1.65 (m, 2H, H-16), 2.01 (m, 1H, H-17), 0.60 (s, 3H, H-18), 0.97 (s, 3H, H-19), 1.40 (m, 1H, H-20), 1.00 (d, *J* = 6.5 Hz, 3H, H-21), 1.24-1.65 (m, 2H, H-22), 2.01-2.20 (m, 2H, H-23), 3.15 (q, *J* = 7.0 Hz, 1H, H-25), 1.32 (d, 7.0 Hz, 3H, H-27), 0.92 (s, 3H, H-28), 0.86 (s, 3H, H-29), 1.07 (s, 3H, H-30), 4.93-4.97 (brs, 2H, H-31), 2.6-2.7 (dd, overlapped, 2H, H-2'), 1.34 (s, 3H, H-4'), 2.6-2.7 (dd, overlapped, 2H, H-5'), 3.69 (s, 3H, H-7')

^{13}C NMR ($CDCl_3$, 75 MHz): δ 30.5 (t, C-1), 23.3 (t, C-2), 78.6 (d, C-3), 36.7 (s, C-4), 45.4 (d, C-5), 17.9 (t, C-6), 25.9 (t, C-7), 134.8 (s, C-8), 132.8 (s, C-9), 36.6 (s, C-10), 32.7 (t, C-11), 73.2 (d, C-12), 49.6 (s, C-13), 49.6 (s, C-14), 31.9 (t, C-15), 27.7 (t, C-16), 43.0 (d, C-17), 16.2 (q, C-18), 18.7 (q, C-19), 36.0 (d, C-20), 17.8 (q, C-21), 34.2 (t, C-22), 31.8 (t, C-23), 148.8 (s, C-24), 45.0 (d, C-25), 179.1 (s, C-26), 16.2 (q, C-27), 21.7 (q, C-28), 27.3 (q, C-29), 24.5 (q, C-30), 110.8 (t, C-31), 172.0 (s, C-1'), 45.0 (s, C-2'), 69.7 (s, C-3'), 27.3 (s, C-4'), 44.8 (s, C-5'), 171.8 (t, C-6'), 51.7 (s, C-7'),

Polyporenic acid C (**79**) ($C_{31}H_{46}O_4$) was obtained as a colourless powder by open column chromatography on silca gel using $CHCl_3$/MeOH 85:15 as eluent. Further purification was done by HPLC using gradient (water/acetonitrile 95:5 to 5:95; 50 min) R_t = 27.5 min. Upon spraying with vanillin/sulphuric acid a violet spot was obtained on the TLC plate, suggesting a terpernoid.

$[\alpha]_D^{20}$ –6 (MeOH, c = 1 mg/ml)
ESI-MS: m/ z 483 $[M+H]^+$, 987 $[2M+H]^+$
IR (film): 3402, 2965, 2932, 1717, 1678, 1450, 1252, 1109, 1016, 883 cm^{-1}



^{1}H NMR (DMSO-d_6, 300 MHz): δ 1.60 (m, 2H, H-1), 2.20-2.8 (m, 2H, H-2), 1.41 (dd, *J* = 4.1, 11.4 Hz, 1H, H-5), 2.01-2.20 (m, 2H, H-6), 5.47(brs, 1H, H-7), 5.40 (brs, 1H, H-11), 1.77-2.07 (m, 2H, H-12), 1.37-2.02 (m, 2H, H-15), 3.90 (brs, 1H, H-16), 1.88 (m, 1H, H-17), 0.56 (s, 3H, H-18), 1.10 (s, 3H, H-19), 2.23 (m, 1H, H-20), 1.60-1.95 (m, 2H, H-22), 1.94 (m, 2H, H-23), 2.20 (m, 1H, H-25), 0.98 (d, *J* = 6.7 Hz, 3H, H-26), 0.98 (d, *J* = 6.7 Hz, 3H, H-27), 0.95 (s, 3H, H-28), 0.95 (s, 3H, H-29), 1.07 (s, 3H, H-30), 4.70 (d, *J* = 18.3 Hz, 2H, H-31)

^{13}C NMR (DMSO-d_6, 75 MHz): δ 35.9 (t, C-1), 34.2 (t, C-2), 215.0 (s C-3), 46.7 (s, C-4), 50.3 (d, C-5), 23.0 (t, C-6), 119.9 (d, C-7), 141.9 (s, C-8), 144.0 (s, C-9), 36.7 (s, C-10), 116.5 (d, C-11), 35.1 (t, C-12), 43.6 (s, C-13), 48.1 (s, C-14), 43.1 (t, C-15), 75.0 (d, C-16), 55.9 (d, C-17), 16.1 (q, C-18), 21.9 (q, C-19), 46.7 (d, C-20), 176.9 (s, C-21), 29.9 (t, C-22), 31.8 (t, C-23), 154.9 (s, C-24), 33.1 (d, C-25), 21.61 (q, C-26), 21.6 (q, C-27), 25.3 (q, C-28), 25.6 (q, C-29), 21.5 (q, C-30), 106.6 (t, C-31)

6 References

(1) Keranen, S.; Penttila, M. *Curr. Opin. Biotechnol.* **1995**, *6*, 534-537.

(2) Marx, F. *Appl. Microbiol. Biotechnol.* **2004**, *65*, 133-142.

(3) Archer, D. B.; Jeenes, D. J.; Mackenzie, D. A. *Antonie Van Leeuwenhoek* **1994**, *65*, 245-250.

(4) Durand, H.; Clanet, M.; Tiraby, G. *Enzyme Microbiol. Technol.* **1988**, *10*, 341-345.

(5) Kwon-Chung, K. J. *Mycopathologia* **1987**, *100*, 113.

(6) Denning, D. W. *Clin. Infect. Dis.* **1998**, *26*, 781-805.

(7) Alexander, B. D. *Curr. Opin. Infect. Dis.* **2002**, *15*, 583-589.

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

(9) Cragg, G. M.; Newman, D. J.; Snader, K. M. *J. Nat. Prod.* **1997**, *60*, 52-60.

(10) Newman, D. J.; Cragg, G. M.; Snader, K. M. *J. Nat. Prod.* **2003**, *66*, 1022-1037.

(11) Grabley, S.; Thiericke, R. *Drug discovery from nature*, 1999, P13.

(12) Endo, A.; Monacolin, K. *J. Antibiot.* **1979**, *32*, 852-854.

(13) Endo, A. *J. Antibiot.* **1980**, *33*, 334-6.

(14) Endo, A. *J. Med. Chem.* **1985**, *28*, 401-405.

(15) Alberts, A. W.; Chen, J.; Kuron, G.; Hunt, V.; Huff, J.; Hoffman, C.; Rothrock, J.; Lopez, M.; Joshua, H.; Harris, E.; Patchett, A.; Monaghan, R.; Currie, S.; Stapley, E.; Albers-Schonberg, G.; Hensens, O.; Hirshfield, J.; Hoogsteen, K.; Liesch, J.; Springer, J. *Proc. Natl. Acad. Sci.* **1980**, *77*, 3957-3961.

(16) Slater, E. E.; MacDonald, J. S. *Drugs* **1988**, *36*, 72-82.

(17) Dreyfuss, M.; Harri, E.; Hofmann, H.; Kobel, H.; Pache, W.; Tscherter, H. *Eur. J. Appl. Microbiol.* **1976**, *3*, 125-133.

(18) Ruegger, A.; Kuhn, M.; Lichti, H.; Loosli, H. R.; Huguenin, R.; Quiquerez, C.; von Wartburg, A. *Helv. Chim. Acta.* **1976**, *59*, 1075-1092.

(19) Borel, J. F. *Transplant. Proc.* **1980**, *12*, 233.

(20) Borel, J. F.; Wiesinger, D. *Br. J. Pharmacol.* **1979**, *66*, 66P-67P.

(21) Borel, J. F.; Feurer, C.; Magnee, C.; Stahelin, H. *Immunol.* **1977**, *32*, 1017-1025.

(22) Calne, R. Y.; White, D. J. *IRCS Med. Sci.* **1977**, *5*, 595.

(23) Hume, D. A.; Fairlie, D. P. *Curr. Med. Chem.* **2005**, *12*, 2925-2929.

(24) Henson, P. M. *Nat. Immunol.* **2005**, *6*, 1179-1181.

(25) Rennard, S. I. *Proc. Am. Thorac. Soc.* **2004**, *1*, 282-287.

(26) Safayhi, H.; Sailer, E. R. *Planta. Med.* **1997**, *63*, 487-493.

(27) Vane, J. R.; Botting, R. M. *Inflamm. Res.* **1998**, *47*, S78-87.

(28) Ferreira, S. H.; Moncada, S.; Vane, J. R. *Nat. New. Biol.* **1971**, *231*, 237-239.

(29) Chen, X. S.; Sheller, J. R.; Johnson, E. N.; Funk, C. D. *Nature* **1994**, *372*, 179-182.

(30) Funk, C. D. *Science* **2001**, *294*, 1871-1875.

(31) Langenbach, R.; Morham, S. G.; Tiano, H. F.; Loftin, C. D.; Ghanayem, B. I.; Chulada, P. C.; Mahler, J. F.; Lee, C. A.; Goulding, E. H.; Kluckman, K. D.; Kim, H. S.; Smithies, O. *Cell* **1995**, *83*, 483-492.

(32) Morham, S. G.; Langenbach, R.; Loftin, C. D.; Tiano, H. F.; Vouloumanos, N.; Jennette, J. C.; Mahler, J. F.; Kluckman, K. D.; Ledford, A.; Lee, C. A.; Smithies, O. *Cell* **1995**, *83*, 473-482.

(33) Dinchuk, J. E.; Car, B. D.; Focht, R. J.; Johnston, J. J.; Jaffee, B. D.; Covington, M. B.; Contel, N. R.; Eng, V. M.; Collins, R. J.; Czerniak, P. M.; et al. *Nature* **1995**, *378*, 406-409.

(34) Vane, J. R.; Bakhle, Y. S.; Botting, R. M. *Annu. Rev. Pharmacol. Toxicol.* **1998**, *38*, 97-120.

(35) Pawlowski, J.; Huizinga, M.; Penning, T. M. *Agents Actions* **1991**, *34*, 289-293.

(36) Penning, T. M.; Sharp, R. B.; Krieger, N. R. *J. Biol. Chem.* **1985**, *260*, 15266-15272.

(37) Yasukawa, K.; Ikeya, Y.; Mitsuhashi, H.; Iwasaki, M.; Aburada, M.; Nakagawa, S.; Takeuchi, M.; Takido, M. *Oncology* **1992**, *49*, 68-71.

(38) Yasukawa, K.; Takido, M.; Takeuchi, M.; Sato, Y.; Nitta, K.; Nakagawa, S. *Chem. Pharm. Bull.* **1990**, *38*, 774-776.

(39) Adcock, I. M.; Chung, K. F.; Caramori, G.; Ito, K. *Eur. J. Pharmacol.* **2006**, *533*, 118-32.

(40) Dalbeth, N.; Haskard, D. O. *Rheumatology* **2005**, *44*, 1090-1096.

(41) Choi, H. K.; Mount, D., B.,; Reginato, A. M. *Ann. Intern. Med.* **2005**, *143*, 499-516.

(42) Rastelli, G.; Costantino, L.; Albasini, A. *J. Am. Chem. Soc.* **1997**, *119*, 3007-3016.

(43) Ishibuchi, S.; Morimoto, H.; Oe, T.; Ikebe, T.; Inoue, H.; Fukunari, A.; Kamezawa, M.; Yamada, I.; Naka, Y. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 879-882.

(44) Hande, K. R.; Noone, R. M.; Stone, W. J. *Am. J. Med.* **1984**, *76*, 47-56.

(45) Erkel, G.; Rether, J.; Anke, T.; Sterner, O. *J. Antibiot.* **2003**, *56*, 337-343.

(46) Yoshikawa, K.; Inoue, M.; Matsumoto, Y.; Sakakibara, C.; Miyataka, H.; Matsumoto, H.; Arihara, S. *J. Nat. Prod.* **2005**, *68*, 69-73.

(47) Kleinwachter, P.; Anh, N.; Kiet, T. T.; Schlegel, B.; Dahse, H. M.; Hartl, A.; Grafe, U. *J. Nat. Prod.* **2001**, *64*, 236-239.

(48) Curini, M.; Maltese, F.; Marcotullio, M. C.; Menghini, L.; Pagiotti, R.; Rosati, O.; Altinier, G.; Tubaro, A. *Planta. Med.* **2005**, *71*, 194-196.

(49) Kamo, T.; Imura, Y.; Hagio, T.; Makabe, H.; Shibata, H.; Hirota, M. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 1362-1365.

(50) Druzhinina, I.; Kubicek, C. P. *J. Zhejiang. Univ. Sci. B* **2005**, *6*, 100-12.

(51) Grove, J. F.; Moffatt, J. S.; Vischer, E. B. *J. Chem. Soc.* **1965**, *33*, 3803-3811.

(52) Grove, J. F.; McCloskey, P.; Moffatt, J. S. *J. Chem. Soc.* **1966**, 743-747.

(53) Blight, M. M.; Coppen, J. J. W.; Grove, J. F. *Chem. Comm.* **1968**, 1117-1118.

(54) Hanson, J. R. *Nat. Prod. Rep.* **1995**, *12*, 381-384.

(55) Nakahara, K.; Yoshida, K.; Ito, T.; Suzaki, K.; Kudo, A. *Arch. Phytopathol. Plant. Protect.* **2000**, *33*, 519-527.

(56) Wipf, P.; Halter, R. J. *Org. Biomol. Chem.* **2005**, *3*, 2053-2061.

(57) Watanabe, N.; Yamagishi, M.; Mizutani, T.; Kondoh, H.; Omura, S.; Hanada, K.; Kushida, K. *J. Nat. Prod.* **1990**, *53*, 1176-1181.

(58) Isaac, B. G.; Ayer, S. W.; Letendre, L. J.; Stonard, R. J. *J. Antibiot.* **1991**, *44*, 729-732.

(59) Lee, W. W.; Benitez, A.; Goodman, L.; Baker, B. R. *J. Am. Chem. Soc.* **1960**, *82*, 2648-2649.

(60) Sitrin, R. D.; Chan, G.; Dingerdissen, J.; DeBrosse, C.; Mehta, R.; Roberts, G.; Rottschaefer, S.; Staiger, D.; Valenta, J.; Snader, K. M.; Stedman, R. J.; Hoover, J. R. E. *J. Antibiot.* **1988**, *41*, 469-480.

(61) Kawai, G.; Ikeda, Y.; Tubaki, K. *Agri. Biol. Chem.* **1985**, *49*, 2137-2146.

(62) Koga, J.; Yamauchi, T.; Shimura, M.; Ogawa, N.; Oshima. K.; Umemura, K.; Kikuchi, M.; Ogasawara, N. *J. Biol. Chem.* **1998**, *273*, 31985-31991.

(63) Kleinwachter, P.; Dahse, H. M.; Luhmann, U.; Schlegel, B.; Dornberger, K. *J. Antibiot.* **2001**, *54*, 521-525.

(64) Deffieux, G.; Baute, M. A.; Baute, R.; Filleau, M. J. *J. Antibiot.* **1978**, *31*, 1102-1105.

(65) Deffieux, G.; Filleau, M. J.; Baute, R. *J. Antibiot.* **1978**, *31*, 1106-1109.

(66) Frederick, C. B.; Bentley, M. D.; Shive, W. *Biochemistry* **1981**, *20*, 2436-2438.

(67) Mizogami, K.; Mikami, A.; Okazaki, T.; Hanada, K. In *Japan patent*, 1991.

(68) Bell, P. J.; Karuso, P. *J. Am. Chem. Soc.* **2003**, *125*, 9304-5.

(69) Agrawal, P. K. *Phytochemistry* **1992**, *31*, 3307-3330.

(70) Wright, A. D.; Osterhage, C.; König, G. M. *Org. Biomol. Chem.* **2003**, *1*, 507-510.

(71) Ishikawa, Y.; Ito, T.; Lee, K. H. *J. Jpn. Oil Chem.* **1996**, *45*, 1321-1325.

(72) Abdel-Lateff, A.; Fisch, K. M.; Wright, A. D.; König, G. M. *Planta Med.* **2003**, *69*, 821-834.

(73) Babine, R. E.; Bender, S. L. *Chem. Rev.* **1997**, *97*, 1359-1472.

(74) Rawlings, N. D.; Barrett, A. J. *Methods Enzymol.* **1994**, *244*, 19-61.

(75) Barrett, A. J.; Rawlings, N. D. *Arch. Biochem. Biophys.* **1995**, *318*, 247-250.

(76) Craik, M. S.; Debouck, S. *In perspectives in Drug Discovery and Design, McKerrow, J.H., James, M.N.G., eds., Escom: Leiden* **1995**, *2*, 1-125.

(77) Hiemstra, P. S. *Biochem. Soc. Trans.* **2002**, *30*, 116-120.

(78) 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.* **1997**, *7*, 2295-2298.

(79) Banerjee, A. K.; Correa, J. A.; Laya-Mino, M. *J. Chem. Soc.* **1998**, *11*, 710-711.

(80) Errington, S. G.; Farell, I. W.; Halsall, T. G.; Hearn, M. T. W.; Jones, E. R. H.; Thaller, V. *J. Chem. Res.* **1987**, 47.

(81) Fontana, A.; Tramice, A.; Cutignano, A.; D'Ippolito, G.; Gavagnin, M.; Cimino, G. *J. Org. Chem.* **2003**, *68*, 2405-2409.

(82) Fontana, A.; Villani, G.; Cimino, G. *Tetrahedron Lett.* **2000**, *41*, 2429-2433.

(83) Kubanek, J. M. *Thesis, UMI, Ann Habor* **1998**.

(84) Kim, K. S.; Hufnagel, G.; Chapman, N. M.; Tracy, S. *Rev. Med. Virol.* **2001**, *11*, 355-68.

(85) Grabley, S.; Thiericke, R.; Zerlin, M.; Gohrt, A.; Philipps, S.; Zeeck, A. *J. Antibiot.* **1996**, *49*, 593-595.

(86) Erkel, G.; Lorenzen, K.; Anke, T.; Velten, R.; Gimenez, A.; Steglich, W. *Z. Naturforsch.* **1995**, *50*, 1-9.

(87) Velten, R.; Klostermeyer, D.; Steffan, B.; Steglich, W.; Kuschel, A.; Anke, T. *J. Antibiot.* **1994**, *47*, 1017-1024.

(88) Anchel, M. *Arch. Biochem. Biophys.* **1958**, *78*, 100-110.

(89) Heatley, N. G.; Stephenson, J. S. *Nature* **1957**, *179*, 1078.

(90) Inokoshi, J.; Shiomi, K.; Masuma, R.; Tanaka, H.; Yamada, H.; Omura, S. *J. Antibiot.* **1999**, *52*, 1095-1100.

(91) Gilli, G.; Bellucci, F.; Ferretti, V.; Bertolasi, V. *J. Am. Chem. Soc.* **1989**, *111*, 1023-1028.

(92) Bertolasi, V.; Gilli, P.; Ferretti, V.; Gilli, G. *J. Am. Chem. Soc.* **1991**, *113*, 4917-4925.

(93) Folmer, B. J. B.; Sijbesma, R. P.; Kooijmann, H.; Spek, A. L.; Meijer, E. W. *J. Am. Chem. Soc.* **1999**, *121*, 9001-9007.

(94) Fores, M.; Duran, M.; Sala, M. J. *J. Phys. Chem. A* **1999**, *103*, 4525-4532.

(95) Shiomi, K.; Matsui, R.; Isozaki, M.; Chiba, H.; Sugai, T.; Yamaguchi, Y.; Masuma, R.; Tomoda, H.; Chiba, T.; Yan, H.; Kitamura, Y.; Sugiura, W.; Omura, S.; Tanaka, H. *J. Antibiot.* **2005**, *58*, 65-68.

(96) Sakurai, M.; Kohno, J.; Yamamoto, K.; Okuda, T.; Nishio, M.; Kawano, K.; Ohnuki, T. *J. Antibiot.* **2002**, *55*, 685-692.

(97) Wang, Y.; Mo, S.-Y.; Wang, S.-J.; Li, S.; Yang, Y.-C.; Shi, J.-G. *Org. Lett.* **2005**, *7*, 1675-1678.

(98) Mo, S.; Wang, S.; Zhou, G.; Yang, Y.; Li, Y.; Chen, X.; Shi, J. *J. Nat. Prod.* **2004**, *67*, 823-828.

(99) Edwards, R. L.; Lewis, D. G.; Wilson, D. V. *J. Chem. Soc.* **1961**, 4995-5002.

(100) Park, I. H.; Chung, S. K.; Lee, K. B.; Yoo, Y. C.; Kim, S. K.; Kim, G. S.; Song, K. S. *Arch. Pharm. Res.* **2004**, *27*, 615-618.

(101) Goldfine, H.; Wadsworth, S. J.; Johnston, N. C. *Infect. Immun.* **2000**, *68*, 5735-5741.

(102) Gonindard, C.; Bergonzi, C.; Denier, C.; Sergheraert, C.; Klaebe, A.; Chavant, L.; Hollande, E. *Cell. Biol. Toxicol.* **1997**, *13*, 141-153.

(103) Ali, A. A. N.; Jansen, R.; Pilgrim, H.; Liberra, K.; Lindequist, U. *Phytochemistry* **1996**, *41*, 927-929.

(104) Shimokoriyama, M.; Hattori, S. *J. Am. Chem. Soc.* **1953**, *75*, 1900-1904.

(105) Seikel, M. K.; Geissman, T. A. *J. Am. Chem. Soc.* **1950**, *72*, 5725-5730.

(106) Brady, L. R.; Benedict, R. G. *J. Pharm. Sci.* **1972**, *61*, 318.

(107) Opitz, L.; Hänsel, R. *Tetrahedron Lett.* **1970**, *11*, 3369-3370.

(108) Sengupta, P.; Sen, M.; Karuri, P.; Wenkert, E.; Halls, T. D. J. *J. Ind. Chem. Soc.* **1985**, *62*, 916.

(109) Stahmann, M. A.; Huebner, C. F.; Link, K. P. *J. Biol. Chem.* **1941**, *138*, 513-527.

(110) Choudhary, M. I.; Lannang, A. M.; Anjum, S.; Fun, H. K.; Lontsi, D.; Tangmouo, J. G. *Acta. Cryst.* **2006**, *62*, O116-O118.

(111) Ma, C.; Zhang, H. J.; Tan, G. T.; Hung, N. V.; Cuong, N. M.; Soejarto, D. D.; Fong, H. H. *J. Nat. Prod.* **2006**, *69*, 346-350.

(112) Samu, Z.; Nyiredy, S.; Baitz-Gacs, E.; Varga, Z.; Kurtan, T.; Dinya, Z.; Antus, S. *Chem. Biodivers.* **2004**, *1*, 1668-1677.

(113) Yun, B.-S.; Lee, I.-K.; Ryoo, I.-J.; Yoo, I.-D. *J. Nat. Prod.* **2001**, *64*, 1238-1240.

(114) Fiasson, J.-L.; Gluchoff-Fiasson, K.; Steglich, W. *Chem. Ber.* **1977**, *110*, 1047-1057.

(115) Curtis, R. G.; Sir Heilbron, I.; Jones, E. R. H.; Woods, G. F. *J. Chem. Soc.* **1953**, 457-464.

(116) King, T. J.; Smith, R. W.; Begley, M. J.; Goad, L. J. *Tetrahedron Lett.* **1984**, *25*, 3489-3492.

(117) Bryce, T. A.; Campbell, I. M. *Tetrahedron Lett.* **1967**, *23*, 3427-3434.

(118) Kamo, T.; Asanoma, M.; Shibata, H.; Hirota, M. *J. Nat. Prod.* **2003**, *66*, 1104-1106.

(119) Penning, T. M. *J. Pharm. Sci.* **1985**, *74*, 651-654.

(120) Di Ferrante, N. *J. Biol. Chem.* **1956**, *220*, 303-306.

(121) MD Sohai, A.; Vinod, B. *Curr. Sci.* **2004**, *86*, 285-295.

(122) Stephen, J.; Kenneth, B.; Songlin, L.; Mark, J. *Glycobiology* **2001**, *11*, 297-304.

(123) Okorukwu, O. N.; Vercruysse, K. P. *J. Enzyme. Inhib. Med. Chem.* **2003**, *18*, 377-382.

(124) *Deutscher Apotheker Verlag Stuttgart* **1986**, 47-48, 424-430.

(125) Schmidtke, M.; Schnittler, U.; Jahn, B.; Dahse, H.; Stelzner, A. *J. Virol. Methods* **2001**, *95*, 133-143.

(126) Cannell, R. J.; Kellam, S. J.; Owsianka, A. M.; Walker, J. M. *Planta Med.* **1988**, *54*, 10-14.

(127) Westfall, B. B. *Natl. Cancer Inst. Monogr.* **1962**, *7*, 147-158.

(128) Groth, T.; Falck, P.; Miethke, R.-R. *ATLA* **1995**, *23*, 790-799.

Zusammenfassung

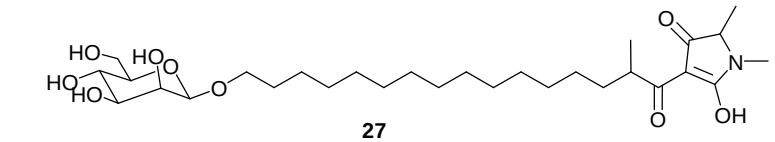
Die vorliegende Arbeit hatte zum Ziel neue natürliche Pilzsekundärmetabolite aufzufinden, die auf klinisch relevante Targets (Entzündungen und Infektionen) und/oder gegen Mikroben- bzw. Viren wirken.

Entzündungshemmende Verbindungen

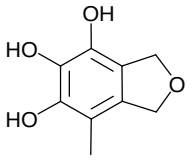
Gelenkrheumatismus ist eine chronische Entzündungskrankheit, die bis zu 1% der Bevölkerung betrifft, mit einer Häufigkeit von 3:1 bei Frauen mehr als bei Männern auftritt und keine bekannte Therapie hat. Es gibt zwar bereits eine Anzahl von Wirkstoffen mit kleinem Molekulargewicht zur Behandlung der Symptome, diese zeigen aber nur begrenzte Wirkung und viele Nebenwirkungen. Die auf der Hemmung der Schlüsselenzyme (3α-Hydroxysteroid dehydrogenase (3α-HSD), Cyclooxygenase I (COX-1) and Cyclooxygenase II (COX-2)) basierende Therapie, die auf die Biosynthese der Prostaglandine wirkt, hat sich als relativ sicher und wirksam bei zahlreichen Entzündungskrankheiten erwiesen. Im Gegensatz zum Gelenkrheumatismus entsteht Gichtrheumatismus durch die Kristallisierung der Harnsäure (UA) in den Gelenken. Die Therapien dieser Form von Rheuma basieren entweder auf der Zunahme der Harnausscheidung der UA oder auf der Blockierung der letzten Biosyntheseschritte von UA durch Xanthin-Oxidase-Hemmungen (XO-Hemmung). Der einzige klinisch verwendbare XO-Inhibitor Allopurinol verursacht schwere Nebenwirkungen. Deswegen ist es erstrebenswert, eine spezielle, auf diese Enzyme gerichtete Wirkstoffe (mit kleinem Molekulargewicht) zu entwickeln, die als Leitstruktur für neue Entzündungsmittel und Rheumamedikamente dienen können. Um dieses Ziel zu erreichen, wurden nach einer 3α-HSD-Analyse sechs Pilzstämme, *Epicoccum* sp HKI 0470, *Aspergillus* sp HKI 0472, *Inonotus* sp, *Pholiota squarrosa*, *Phellinus pini* DSM 5238 und *Piptoporus, betulinus*, ausgewählt.

Epicoccum sp. HKI 0470

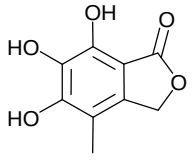
Neben den bereits bekannten Verbindungen Epicoccamide (**27**), Epicoccine (**28**) und Orevactaene (**31**), wurden aus *Epicoccum* sp. HKI 0470 zwei neue Metabolite Epicoccone B (**29**) and Epicoccalone (**30**) isoliert.



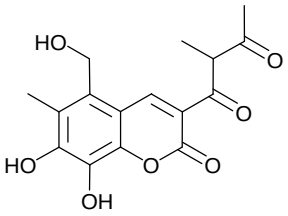
27



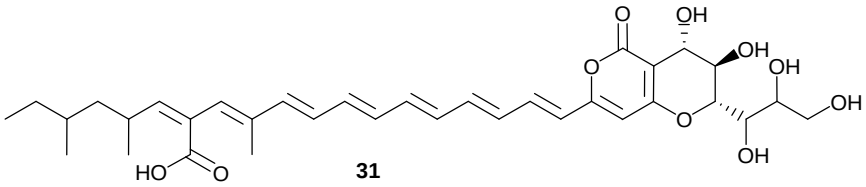
28



29



30

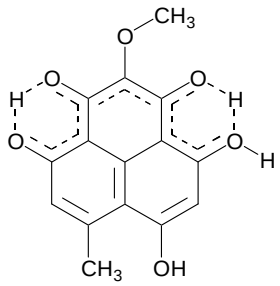


31

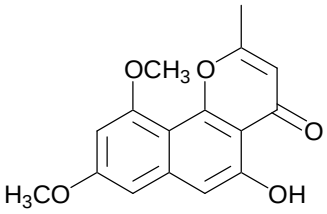
Während **29, 30** und **31** nur geringe Hemmungswirkung auf die 3α-HSD zeigten, wurde bei **30** Serin-Protease-Hemmung (α-Chymotrypsin) beobachtet. Es muss an dieser Stelle festgestellt werden, dass es einen Zusammenhang zwischen der Serin-Protease-Hemmung, der Regulierung der Entzündung, dem Aufbau der Abwehrkräfte im Falle einer Infektion der Wiederherstellung des Gewebes und der Synthese zusätzlicher Kernzellen gibt.

***Aspergillus* sp. HKI 0472**

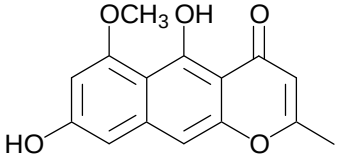
Aus dem Stamm *Aspergillus* sp. HKI 0472 wurden drei Verbindungen isoliert: Funalenine (**48**), TMC-256C2 (**50**) und TMC-256A1 (**51**). Während TMC-256C2 (**50)** und TMC-256A1 (**51**) keine entzündungshemmende Wirkung zeigten, erwies sich das neue Funalenin mit dem ungewöhnlichen Aufbau als wirksames entzündungshemmendes Mittel. Es hemmt 3α-HSD, COX-1 und COX-2 mit den IC_{50} Werten von 52 µM bzw. 36 µM und 45 µM.



48



50

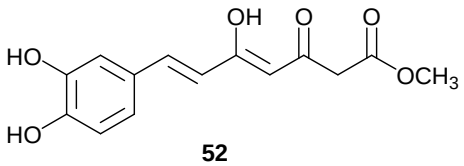


51

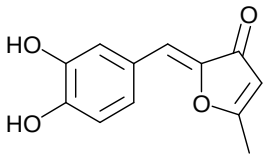
.

***Inonotus* sp.** (Fruchtkörper)

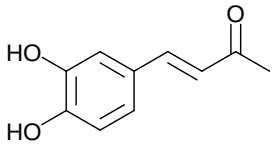
Beim Vorscreening zeigte der Essigester Extrakt der Pilzart *Inonotus* sp. eine starke entzündungshemmende Wirkung. Die Bereinigung durch Bioanalyse führte zu der Isolierung von 5 Verbindungen von denen drei neu waren: Inonotic säuremethylester ester (**52**), Inotilon (**53**), und *Iso*hispidin (**58**). Die bereits bekannten Verbindungen sind (*E*)-4-(3,4-Dihydroxyphenyl)but-3-en-2-one (**56)** und Hispidin (**57**).



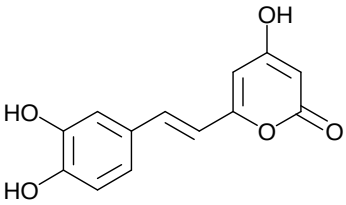
52



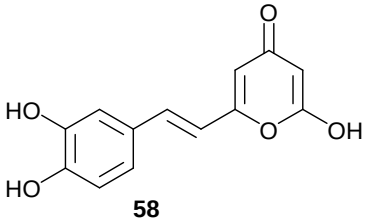
53



56



57

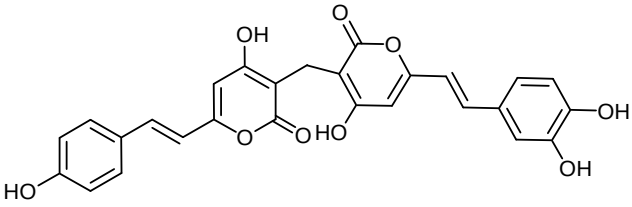


58

Alle diese Verbindungen zeigten eine starke COX-Hemmungswirkung speziell bei COX-2. Es soll hier hervorgehoben werden, dass **53** und **57** selektiv das COX-2-Enzym mit Konzentrationen hemmen, die genauso niedrig sind, wie die der vermarkteten selektiven Inhibitoren Meloxicam und Nimesulide. In allen Fällen bis auf **53** wurde eine starke 3α-HSD-Hemmungswirkung festgestellt, sowie eine schwache Inhibition von XO, bis auf **57**, wo eine Hemmung festgestellt wurde, die mit der des Standards Allopurinol vergleichbar ist.

Pholiota squarrosa (Fruchtkörper)

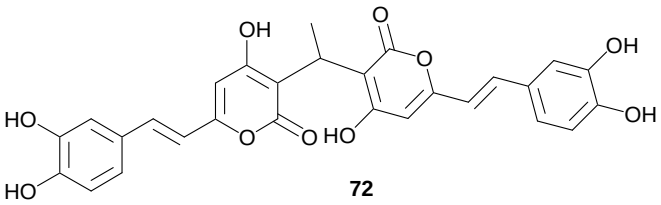
Die neue Verbindung mit dem Namen Squarrosidin (**65**) ist der einzige Metabolit, der aus dem Pilz *Pholiota squarrosa* anhand der von der Bioaktivität geleiteten Chromatographie isoliert werden konnte. Die Verbindung ist ein *Bis*hispidin-Derivat mit einer seltenen Methylenbrücke. **65** zeigt starke 3α-HSD, COX-1, COX-2 und XO-Hemmungswirkung mit einem $IC1_{50}$ Wert von 8.1, 0.02, 0.04 bzw. 8.1 μM.



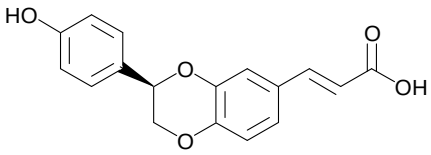
65

***Phellinus pini* DSM 5238**

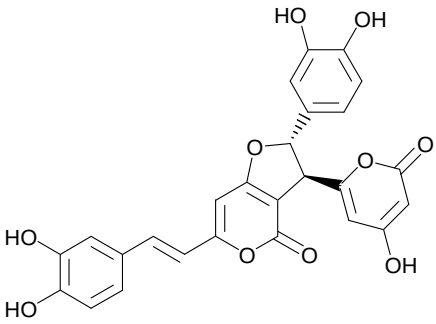
Drei Verbindungen wurden aus dem filamentösen Pilz *Phellinus pini* DSM 5238 isoliert. Es sind Pinillidin (**72**) (eine bekannte Struktur, deren analytische Daten aber in der Fachliteratur noch nicht vorliegen), Pinillic säure (**73**), ein neuer Metabolit, und das bereits bekannte Hypholomin B (**74**). Während **73** keine entzündungshemmende Wirkung zeigte, stellen **72** und **74** potente Inhibitoren der 3α-HSD und XO. dar.



72



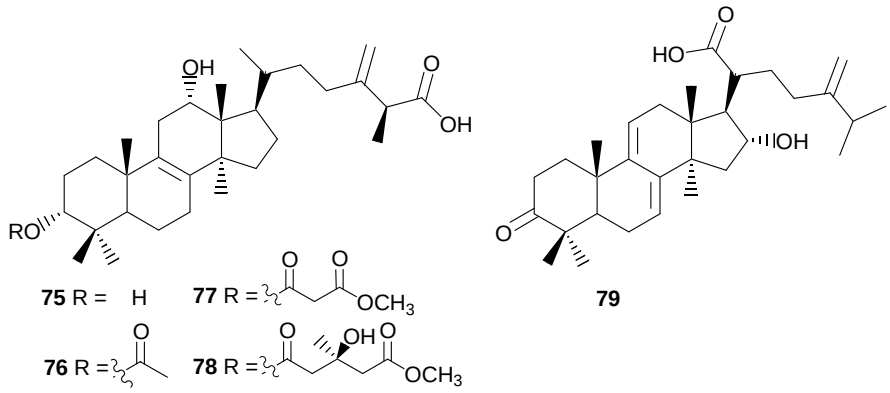
73



74

Piptoporus betulinus (Fruchtkörper)

Die Fraktionierung des Essigester Extrakts des *Piptoporus betulinus* führte zur Isolierung von 5 Lanostanoid-artigen Triterpenen, von denen zwei neu sind: Acetylpolyporenicsäure A (**76**) und (25*S*)-(+)-12α-Hydroxy-3α-methylcarboxyacetat-24-methyllanosta-8,24(31)-dien-26-oik säure acid (**77**)). Die bereits bekannten Verbindungen sind Polyporenicsäure A (**75**), (25S.3'S)-(+)-12α-Hydroxy-3α-(3'-hydroxy-4'-methoxycarbonyl-3'-methylbutyryloxy)-24-methyllanosta-8,24(31)-dien-26-oik säure (**78)** und Polyporenic säure C (**79**). **76** und **77** zeigten bemerkenswerte Hemmungswirkungen bei 3α-HSD. Bemerkenswert ist die Tatsache, dass **76** eine noch stärkere Wirkung zeigte als das Referenzmittel (Indomethacin).



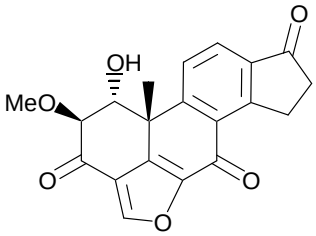
Die vorliegende Untersuchungsreihe, die zum Zweck hatte, neue entzündungshemmende Verbindungen zu finden, führte zur Isolierung von 22 Verbindungen, von denen 10 neu sind. Von diesen Verbindungen erwiesen sich 7 als vielversprechende entzündungshemmende Wirkstoffe: Funalenine (**48**), Inonotic säuremethyester (**52**), Inotilon (**53**), *Iso*hispidin (**58**), Squarrosidin (**65**), Acetylpolyporenic säure A (**76**) und (25*S*)-(+)-12α-Hydroxy-3α-methylcarboxyacetat-24-methyllanosta-8,24(31)-dien-26-oik säure (**77**). Zudem wurden Hispidin (**57**), (*E*)-4-(3,4-dihydroxyphenyl)but-3-en-2-one (**56**), Pinillidine (**72**) und Hypholomine B (**74**) zum ersten Mal als entzündungshemmende Wirkstoffe beschrieben.

Infektionshemmende Verbidungen

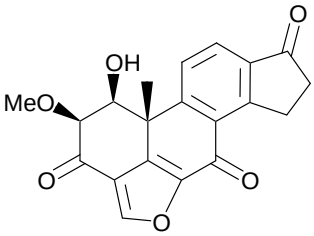
Trotz der Erfolge bei der Behandlung einiger Infektionskrankheiten bleibt die Suche nach neuen Arzneimitteln gegen Viren und Mikroben ein wichtiges Gebiet, da es für viele Infektionskrankheiten keine wirksame Behandlung gibt. Das auftreten von resistenten Mutanten erfordert die Entdeckung neuer Arzneimittel. Um neue antibakterielle Verbindungen zu identifizieren, wurden zwei Pilzarten *Trichoderma crassum* HKI 0471 und *Lepista nebularis* HKI 0411 mittels antifungalen und antibakteriellen Vorscreenings ausgewählt.

Trichoderma crassum HKI 0471

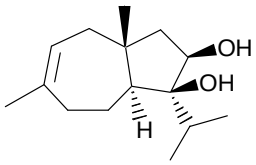
Drei bereits bekannte Metabolite, β-Viridin (**13**), α-Viridin (**14**) und CAF 603 (**19**) wurden aus *Trichoderma crassum* HKI 0471 isoliert. Diese Metabolite zeigten eine starke antifungale Wirkung gegen *Candida albicans* ATCC 18804 und *Sporobolomyces salmonicolor* SBUG 549.



13



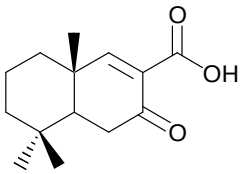
14



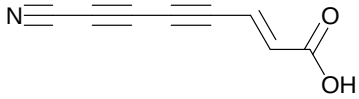
19

Lepista nebularis HKI 0411

Neben zu der neuen antiviralen Metabolite (Nebularic säure A (**32**)), wurden auch starke antimikrobielle Metabolite (Diatretyne (**47**)) aus *Lepista nebularis* (HKI 0411) isoliert.



32



47

32 zeigt eine starke Wirkung gegen Viren und den Cosackievirus mit einem IC_{50} Wert von 6 μM, und Diatretyne (**47**) zeigt eine deutliche Wirkung gegen Pilze und bakterielle Entwicklung bei *Candida albicans* ATCC 18804 (MIC = 25 μg/ml), multi-resistente *Streptococcus aureus* (MIC = 1.56 μg/ml) und *Mycobacterium vaccae* (MIC = 6.25 μg/ml). Diatretyn zeigt auch zytotoxische und antiproliferative Wirkung.

Lebenslauf

Name:	Kemami Wangun, Hilaire Vignie
Geburtsdatum:	08. 11. 1973
Geburtsort:	Bangoua (Kamerun)
Familienstand:	Verheiratet

Ausbildung

09. 1985 – 06. 1989	Mittelschule am „Lycee de Dschang“ (Kamerun)
Abschluss	BEPC (Brevet d’ Etudes du Premier Cycle)
09. 1989 – 06. 1992	Gymnasium an den „ Lycee de Dschang“
Abschluss	Abitur mit Hauptfächern Mathematik und Naturwissenschaft (Baccalaureat D)
10. 1992 – 09. 1999	Studium der Chemie an der Universität von Jaunde I (Kamerun)
1996	„Bachelor“ an der Universität von Jaunde I (Kamerun)
1998	„Master’s Degree“ der organischen Chemie an der Universität von Jaunde I (Kamerun) Thema: Beitrag zur chemischen Untersuchung einer kamerunischen Heilpflanze: *Bridelia micrantha* (Euphorbiaceae)
1999	„First Year Doctorate Degree“ der organischen Chemie an der Universität von Jaunde I (Kamerun)
Seit April 2003	Doktorarbeit am Leibniz-Institut für Naturstoff-Forschung und Infektionsbiologie-Hans-Knöll-Institut, Biomolekulare Chemie

Beruf

10. 1998 – 06. 2002	Assistententätigkeit am Institut für Organische Chemie der Universität von Jaunde I (Kamerun)

Selbständigkeitserklärung:

Ich erkläre, dass ich die vorliegende Arbeit selbständig und unter Verwendung der angegebenen Literatur, Hilfsmittel und Quellen angefertigt habe. Die Arbeit wurde weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Jena, 20. 06. 2006 Kemami Wangun, Hilaire Vignie