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Indole Alkaloids as Potential Leads in Drug Discovery and Further Secondary Metabolites from Terrestrial and Marine Bacteria

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Dissertation

Indole Alkaloids as Potential Leads in Drug Discovery and Further Secondary Metabolites from Terrestrial and Marine Bacteria

Dissertation

zur Erlangung des Doktorgrades
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vorgelegt von

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aus

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D7

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Die vorliegende Arbeit wurde in der Zeit von Oktober 2007 bis Oktober 2010 im Institut für Organische Chemie der Georg-August-Universität zu Göttingen unter der Leitung von Herrn Prof. Dr. H. Laatsch angefertigt.

Herrn Prof. Dr. H. Laatsch danke ich für die Möglichkeit zur Durchführung dieser Arbeit sowie die ständige Bereitschaft, auftretende Probleme zu diskutieren.

For my parents

Table of Content

1 Introduction

Since ages nature has been satisfying fundamental needs of individuals in the form of medicines for the cure of a wide spectrum of diseases. Plants have been playing a leading role in the improvement of sophisticated conventional medicine systems. The best-known evidence is the Egyptian medical text “Papyrus Ebers”, dating back to 2900 BCE (Before Christian Era). There are records regarding the application of plant derived substances in Mesopotamia from around 2600 BCE, and many are still used today for the treatment of illnesses ranging from coughs and colds to parasitic infections and inflammation.[1] Thorough documentation of the Chinese manual Bencao Gangmu (Materia Medica) over the years,[2] have come up with the first record dating from about 1100 BCE (Wu Shi Er Bing Fang, containing 52 prescriptions), being followed by works such as the Shennong Herbal (100 BCE; 365 drugs) and the Tang Herbal (659 CE; 850 drugs). Likewise, documentation of the Indian Ayurvedic system dates from before 1000 BCE (Charaka; Sushruta and Samhitas with 341 and 516 drugs respectively).[3, 4]

In the western world, the Greeks and Romans made substantial inputs to the consistent growth of the use of herbal drugs. Dioscorides, a Greek physician (100 CE), meticulously collected, stored, and made use of medicinal herbs during his expeditions with Roman armies. Galen (130-200 CE), a practitioner and teacher of pharmacy and medicine in Rome, was renowned for his complex medicines and formulas used in composing drugs. The preservation of much of the Greco-Roman expertise during the Dark and Middle Ages (5th-12th centuries) may be attributed to the Arabs. This information could have helped the Arabs to comprise the use of their own resources, together with Chinese and Indian herbs unknown to the Greco-Roman world.

1.1 Marine habitat as a source of natural products

The application of plant-derived secondary metabolites as drugs has approached us as an inheritance of folk medicine based on herbal treatments[5,6]. Some familiar illustrations are the anti-malarial drug quinine obtained from fever-tree (*Cinchona officinalis)* bark, the analgesics codeine and morphine isolated from opium poppy (*Papaver somniferum*) latex, and the hepatoprotective substance silymarin isolated from the milk thistle (*Silybum marianum*)[6,7]. Natural products isolated from marine organisms have also been shown to have a great potential in drug discovery[8]. With the oceans covering 70% of the Earth's surface, and with the incomparableness of the environmental circumstances present in the deep sea, it is simply comprehensible why the ocean can be regarded as a very promising source of natural drugs. The marine habitat has been investigated for novel antibiotics over the past four decades, becoming a highly significant and affluent source of effective molecules and drug leads reported to possess a wide scope of activities. Alkaloids comprise one of the biggest categories of natural products, which are synthesized by terrestrial and marine organisms on all progressive stages. Alkaloids are mostly present in an organism as a combination featuring several main and further minor compounds of similar biosynthetic origin and varying barely in functional groups. These set of compounds has in fact emerged to resist against predators, and as a consequence alkaloids are frequently highly powerful and toxic molecules[9].

Marine invertebrates have demonstrated to be an excellent resource of active molecules, one of the highly potent groups being indole alkaloids. While many of these marine alkaloids are very similar to the endogenous amines (serotonin, dopamine, or histamine), their expected resemblance to a range of neurological actions and substantial impact on animal behavior is nearly uninvestigated.

Marine bacteria and other marine microorganisms develop unique metabolic and physiological abilities. These abilities enable them to survive in extreme habitats and to produce compounds that might not be produced by their terrestrial counterparts. Since 1990, the number of bioactive metabolites from marine bacteria has exponentially increased (Fig. 1)[10].

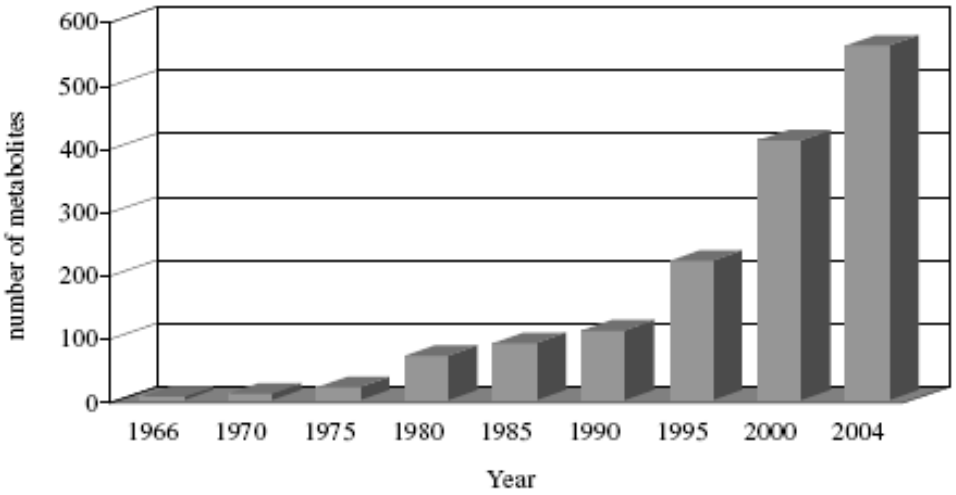
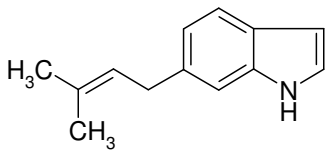


Figure 1: Annual increase in the number of marine bacterial metabolites, according to AntiBase[11].

1.2 Indoles as a potential target used in drug discovery

Indole alkaloids, their activity, synthesis, and possible application as drugs have been previously described in a few articles.[12] 6-Prenylindole (**1**) has been isolated from *Streptomyces* sp. TP-A0595[13] and the structure was determined by comparison with the synthesized sample, prepared from 4-bromo-2-nitrotoluene using a Leimgruber-Batcho reaction[14] and palladium-catalysed prenylation with 1,1-dimethylallyl alcohol.[15] 6-Prenylindole (**1**) inhibited *in vitro* the growth of *Alternaria brassicicola* TP-F0423 and *Fusarium oxysporum* f. sp. tulipae TU-4-2. (-)-Diolmycin A2 (**2**), isolated from a fermentation broth of *Streptomyces* sp. WK-2955,[16] has activity against coccidiosis, which is an infection of the intestinal tract caused by a single cell parasite. The free radical scavenger, carbazostatin (**3**) isolated from *Streptomyces chromofuscus*,[17] shows a strong inhibition of the lipid peroxidation induced by free radicals.



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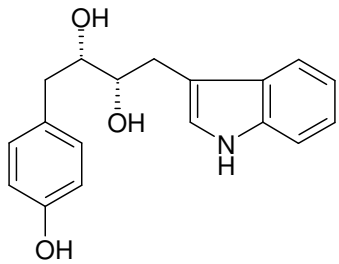

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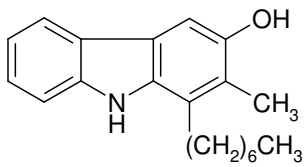
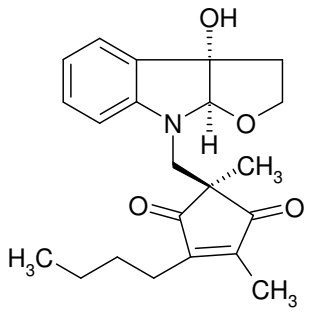
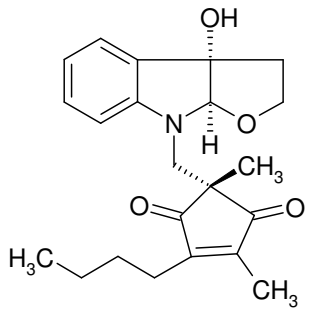


3

Madindolines A (**4a**) and B (**4b**) are metabolites isolated from *Streptomyces nitrosporeus* K93-0711.[18] Madinoline A and B showed inhibition of IL-6- and IL-11-initiated bone resorption and IL-6-driven proliferation[19].

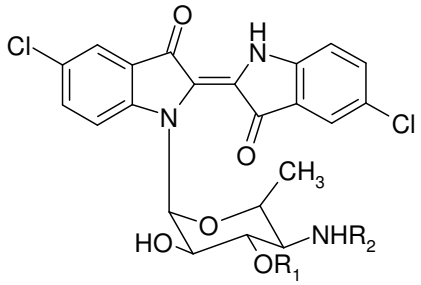


4a



4b

Three new derivatives of 5,5'-dichloroindigo, akashins A (**5a**), B (**5b**), and C (**5c**), have been isolated from the terrestrial *Streptomyces* sp. GW 48/1497.[20] Although indigo itself shows no biological activity, akashins A, B, and C are active against various human tumor cell lines with IC_{50} values of about 2.8 μg/ml .

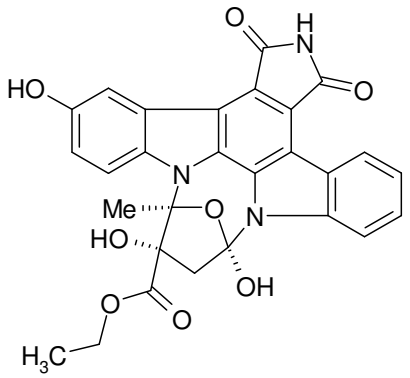


5a: R^1 = H, R^2 = H

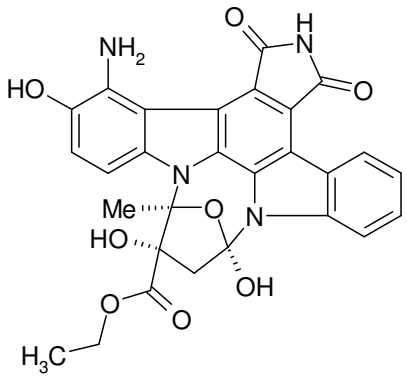
5b: R^1 = H, R^2 = COMe

5c: $R^1 = R^2 = CH(OH)CH_3CH_2CH_3$

Two novel indolocarbazole antibiotics, (-)-indocarbazostatin (**6a**) and (-)-indocarbazostatin B (**6b**), have been isolated from a culture broth of a *Streptomyces* sp. as inhibitors of NGF-induced neuronal differentiation in rat pheochromocytoma PC12 cells.[21] Compounds **6a** and **6b** inhibited NGF induced neurite outgrowth from PC12 cells at 6 nM and 24 nM, respectively.

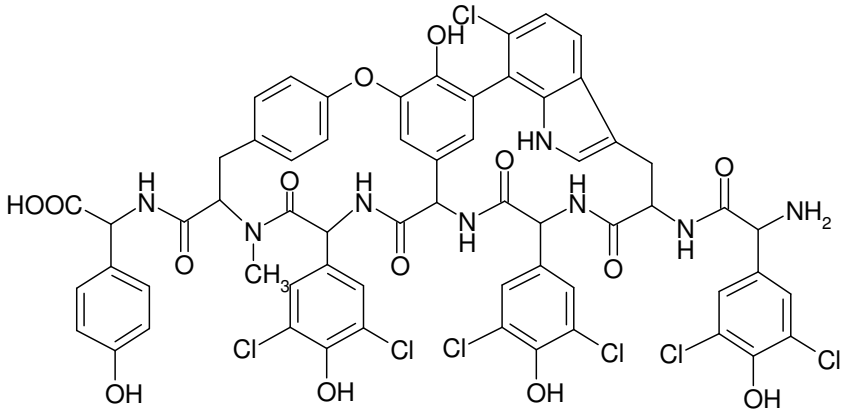


6a



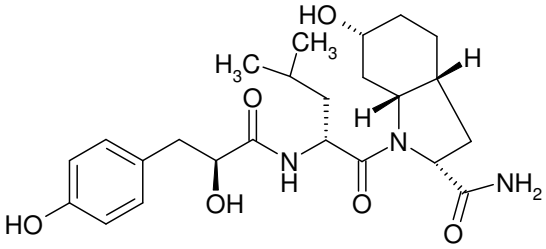
6b

The vancomycin-related condensed aromatic peptide, SCH 212394 (**7**), has been isolated from fermentation broth of a streptomycete.[22] SCH 212394 (**7**) showed an IC_{50} of 1.2 μM in the CD28 assay in the presence of fetal bovine serum (FBS) and 0.07 μM in its absence. It also showed an IC_{50} of 0.13 μM in the CD4-*gp*120 binding in the absence of FBS and an IC_{50} of 8.9 μM in the complement assay.



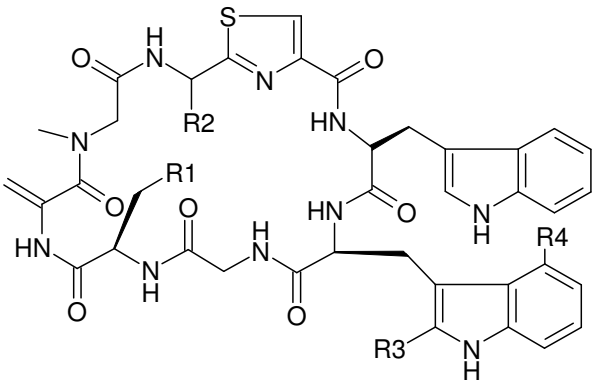
7

The novel protease inhibitor, aeruginosin EI461 (**8**), has been isolated from a natural bloom of the cyanobacterium *Microcystis aeruginosa*.[23] Aeruginosin EI461 differs from the known aeruginosins in the relative and absolute stereochemistry of the 6-hydroxy substituent on the octahydroindole ring and inhibited the activity of the serine protease trypsin by 15% at a concentration of 45.5 μg/ml.



8

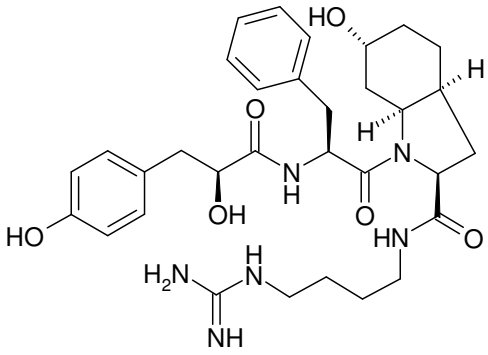
A group of cyclic octapeptides, argyrins A–H (**9a–h**) has been isolated from the culture broth of the myxobacterium *Archangium gephyra*.[24] Compounds **9a** and **9b** contain 2-(1-aminoethyl)thiazol-4-carboxylic acid and the unusual amino acid, 4'-methoxytryptophan. In **9c** and **9d**, the latter is replaced by 4'-methoxy-2'-methyltryptophan. The antibiotics A21459 A and B,[25] are proved to be identical with **9b** and **9a** , respectively, so their structures should be revised with respect to 4'-methoxytryptophan. Argyrin B (**9b**) was found to be a potent inhibitor of T cell independent antibody formation by murine B cells and strongly inhibited the two-way murine mixed lymphocyte reaction. All angyrins had slight antibiotic activity, especially against *Pseudomonas* sp., and inhibited growth of mammalian cell cultures.[26]



9a: R^1 = H, R^2 = Me, R^3 = H, R^4 = OMe
9b: R^1 = Me, R^2 = Me, R^3 = H, R^4 = OMe
9c: R^1 = H, R^2 = Me, R^3 = Me, R^4 = OMe
9d: R^1 = Me, R^2 = Me, R^3 = Me, R^4 = OMe
9e: R^1 = H, R^2 = Me, R^3 = R^4 = H
9f: R^1 = H, R^2 = CH_2OH, R^3 = H, R^4 = OMe
9g: R^1 = Me, R^2 = CH_2OH, R^3 = H, R^4 = OMe
9h: R^1 = R^2 = R^3 = H, R^4 = OMe

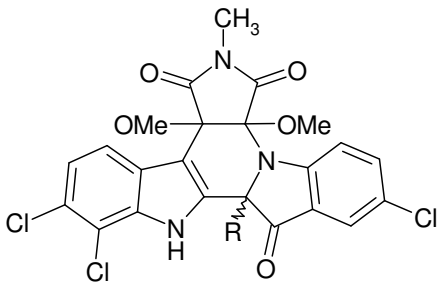
Microcin SF608 (**10**) has been isolated from a nontoxic strain of the cyanobacterium Microcystis *aeruginosa* and inhibited trypsin with an IC_{50} of 0.5 μg/ml.[27]. In

addition, the conformational analysis of L-Choi (L-Choi was used as abbreviation for (2S,3aS,6R,7aS)-2-carbamoyl-6-hydroxy-octahydroindole-1-carboxylic acid amide) containing peptides was performed using NMR spectroscopy to examine the *cis-trans* isomer equilibrium of the L-Phe-L-Choi bond.

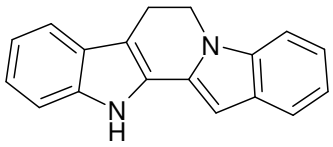


10

Moyopomycin A (**11a**) and B (**11b**) are bis-indole natural products with the rare pentacyclic 12*H*-pyrido[1,2-a:3,4-b']diindole system[28] (**12**). In the last few years, a variety of indolecarbazoles has been isolated from natural sources,[29] and because of their diverse structures and different biological activities, they became a very interesting research topic. Indolo[3,2-*a*]carbazoles or indolo[3,2-*c*]carbazoles like the natural BE-54017 (**13**) have structures closely related to **11a** and **11b**, and were also isolated from *Streptomyces* sp.[30] Also included are homofascaplysin A, B and C isolated from the Fijian sponge *Fascaplysinopsis reticulata*[31] and later synthesized by Gribble *et al.*, [32] and fascaplysin (**14**) isolated as quaternary salt from Fijian sponge *Fascaplysinopsis* sp. Berquist [33] was the first member of this group and was also synthesized by Gribble *et al.*[34] Fascaplysin (**14**) and Homofascaplysin are reported to be exclusively from marine origin.[35] Recently some brominated fascaplysins have been isolated and their cytotoxicities were reported.[35]



11a R = OH **11b** R = OMe



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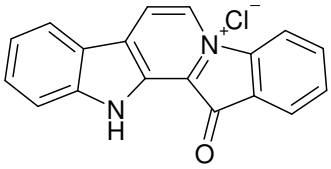
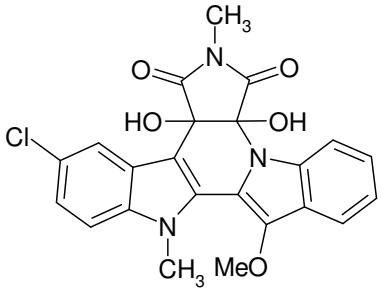
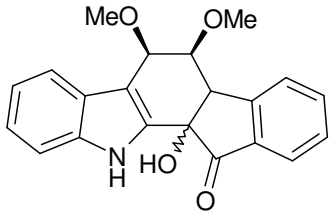


14

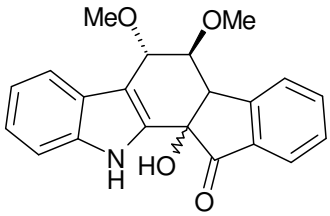


13

Fascaplysin (**14**) is reported to inhibit the growth of microbes such as *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Saccharomyces cerevisiae*. It also shows strong activity against the murine leukaemia L1210 [33], and recently it has been demonstrated that fascaplysin interferes with the elements of the cell cycle machinery by inhibiting the cycle-dependent kinase 4 (cdk4) [36] and by interacting with DNA [37]. Further reports on the reactivity of **14** delivered a mixture of two stereoisomers [37] **15a** and **15b**. Compound BE-54017 (**13**) showed also activity against P388 murine leukaemia cells. [30]



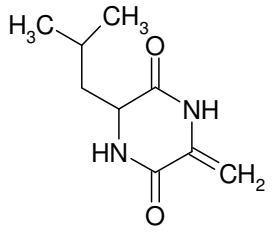
15a



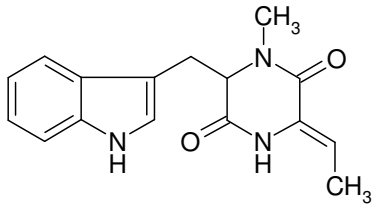
15b

A screening of crude extracts of North Sea bacteria using the agar diffusion method yielded inhibition zones of 15-25 mm diameter on a variety of test organisms, while highly bioactive strains gave inhibition diameters of up to 50 mm. Tests with brine shrimps and human cell lines in screens for antitumor activity gave surprisingly

positive results on the nanogram scale (Hel 3, Hel 38). They also exhibited high leishmaniacidal and antimalarial activities.[38]



16



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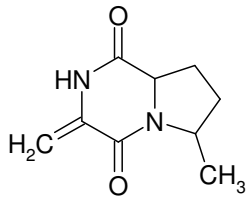
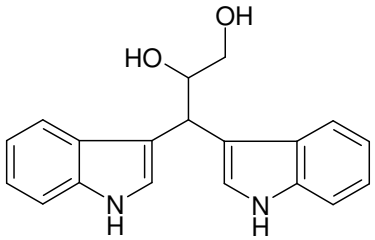


18

Reports from the literature showed that only some of the publications have covered the small number of metabolites derived from North Sea bacteria. The *α,β*-unsaturated diketopiperazine **16** was produced by the North Sea strain Bio39[39]. The same metabolite has been isolated from a *Penicillium* sp.[40] Pronounced antitumor activity was observed for the compounds of **17** and **18**, which had been isolated from *Streptomyces spectabilis*[41,42]



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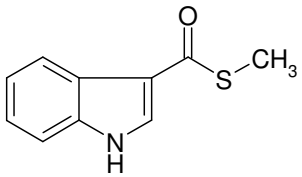
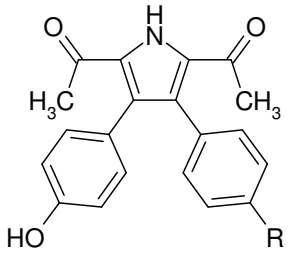
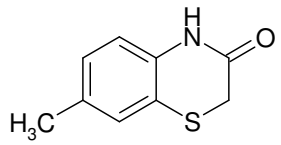


20

The extracts of strain Hel45 contained the diketopiperazines *cyclo*(phenyl-prolyl) and *cyclo*(tyrosyl-prolyl). However, they are dominated by large amounts of unsubstituted indole, the known dimer 3-(3,3'-diindolyl)-propane-1,2-diol (**19**)[43] and various new oligomeric indole derivatives[44]. The lipid phase of Hel45 delivered additionally *N*-(2-hydroxyethyl)-11-octadecenoic acid amide, 17-methyl-16-octadecenoic acid[45], and indole-3-carboxylic acid thiomethyl ester (**20**).



21a: R = H,
21b: R = OH



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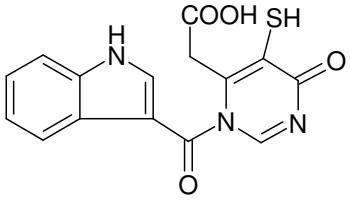

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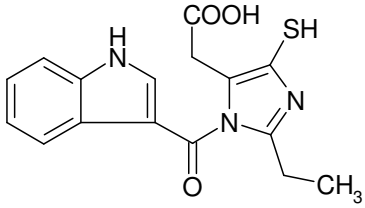

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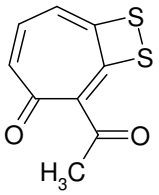
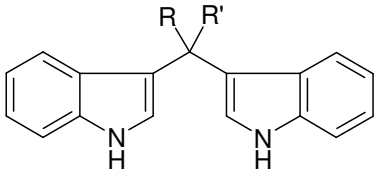
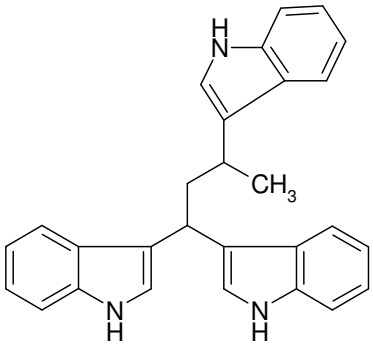


25

3-(4'-Hydroxyphenyl)-4-phenylpyrrole-2,5-dicarboxylic acid (**21a**), 3,4-di(4'-hydroxyphenyl)-pyrrole-2,5-dicarboxylic acid (**21b**) and 7-hydroxy-2*H*-benzo-[1,4]thiazin-3-one (**22**) were isolated by Zeeck and co-workers[46] from the culture broth of the North Sea strain RK377 fermented on MB medium with artificial sea-water. Two new imidazole and pyrimidin derivatives, namely glusun I (**23**) and glusun II (**24**), were isolated from the same strain on SJ medium. Continuing these investigations, the same group has isolated tropodithietic acid (**29**), a novel carboxylic tropone skeleton connected with a four-membered disulphide ring system, from a North Sea strain T5. The compound exhibited antibacterial, antifungal and antitumor activities. The structure of tropodithietic acid (**25**) was elucidated by X-ray analysis and spectroscopic data.

More than 1000 alkaloids with indole skeleton have been reported from microorganisms[47]. One third of theses compounds are peptides with masses beyond 600 Dalton, where the indole is tryptoptophan-derived.





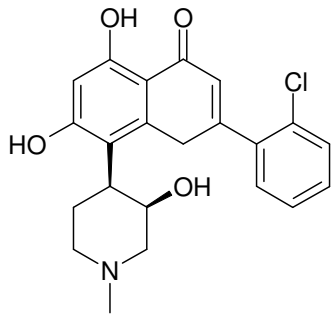
26: R = Ac, R' = Me
28: R = H, R' = H
29: R = 3-indolyl, R' = H

27

The strain Bio249 was isolated from a biofilm grown on a glass plate in the North Sea and taxonomically classified as closely related to *Vibrio parahaemolyticus*, and investigated recently by Veluri[44] in our research group. Two new indole alkaloids, namely 3,3-bis(3-indolyl)-butane-2-one (**26**) and 1,1,3-tris(3-indolyl)butane (**27**) have been isolated from strain Bio249. Additionally, the plant metabolite arundine[48] (**28**), 1,1,1-tris(3-indolyl)methane[49] (**29**) (previously known from synthesis) and several other metabolites have been found.

1.2.1 Natural products and their analogues used as anticancer agents

Flavopiridol (**30**) is a synthetic compound based on the natural product rohitukine (**31**) isolated from *Dysoxylum binectariferum*. Flavopiridol showed inhibition of cyclin-dependent kinases (the regulators of the G2 to M transition in the cell cycle) and it entered into phase I and phase II of clinical trials against a broad range of tumors.[50] It also showed to be an inducing agent in the transcription process, having a potent inhibition of CDK-7 and -9, the kinases primarily responsible for promoting RNAP II (RNA polymerase II) activity.



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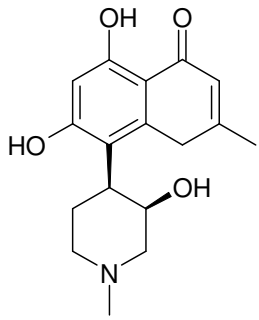
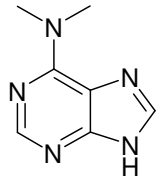


31

The search for purine-derived analogues was inspired by inhibition of cyclin-dependent kinase 1 (CDK1)/ cyclin B by 6-dimethylaminopurine (6-DMAP) (**32**) and isopentenyladenine (**33**) which were isolated from *Castanea* species. Olomucine (**34**) isolated from the cotyledons of the radish, represented efficacy (IC_{50} 7 μM) and selectivity for cyclin dependent kinases (CDKs) and, to some extent, MAP kinases, by direct competition with ATP. Olomucine had been synthesized,[51] but showed no significant kinase inhibition due to fact of binding excessively with ATP. This led to the further development of this series using combinatorial chemistry techniques giving roscovitine (**35**), purvalanol A (**36**) and purvalanol B (**37**). Olomucine and roscovitine are very potent inhibitors of CDK-7 and -9 like flavopiridol. The purvalanols demonstrated potency with IC_{50} values in the 4-40 nM range, compared to 450 nM for roscovitine.[52] The *R*-isomer of roscovitine is presently under investigation in phase II under the auspices of Cyclacel Pharmaceuticals (United Kingdom) with reports of clinical trials in Europe. (*R*)-Roscovitine is being used with cytotoxins and is considered for sequential treatment of signal transduction inhibitors (SIT are drugs

that may prevent the ability of cancer cells to multiply quickly and invade other tissues).



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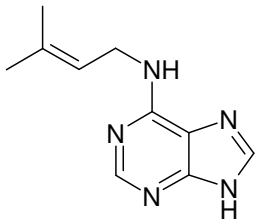

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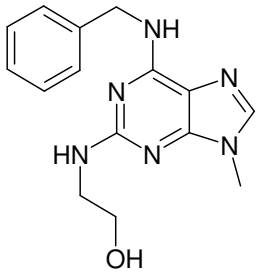

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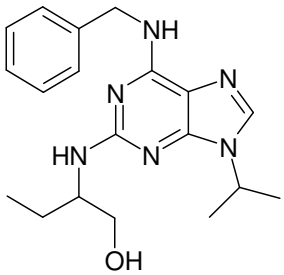

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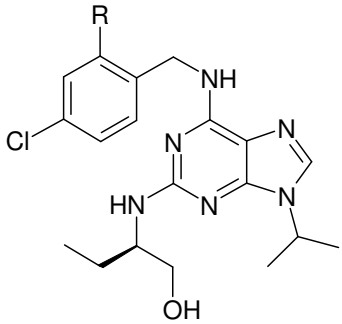
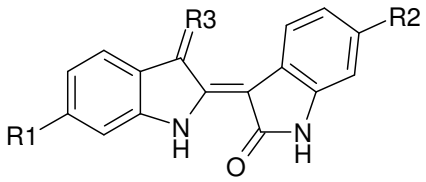
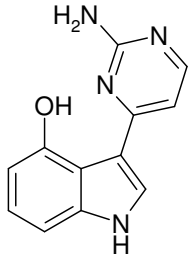


36 R = H

37 R = COOH

Although indigo and the indirubin (**38**) are plant products, they have also been isolated from a number of marine mollusks belonging to the *Muricidae* family of gastropods,[53] natural or recombinant bacteria,[54] and human urine.[55] The indirubins were recognized as being inhibitors of several CDKs and are potent inhibitors of glycogen synthase kinase-3 (GSK-3).[56] 6-Bromoindirubin (**39**), first isolated from the mollusk *Hexaplex trunculus*,[57] and its chemically modified oxime derivative BIO (**40**), demonstrated large specificity versus CDK1/cyclin B and/or CDK/p25, as well as significantly greater specificity against a wide range of other kinases. Among other natural products with indirubin-like kinase inhibitory activities are the meridianins (e.g., meridianin A; **41**), a group of halogenated indole derivatives that are closely related to the base structures of the psammopemmins [e.g., psammopemmin A (**42**)] and discodermindole (**43**). The psammopemmins and discodermindol were isolated from sponges, whereas the meridianins were isolated from the ascidian *Aplidium meridianum*.[58]



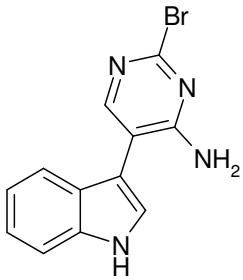


38 R^1 = H, R^2 = H, R_3 = O

39 R^1 = H, R^2 = Br, R_3 = O

40 R_1 = H, R_2 = Br, R_3 = NOH

41



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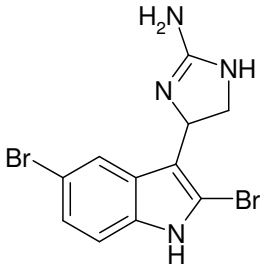
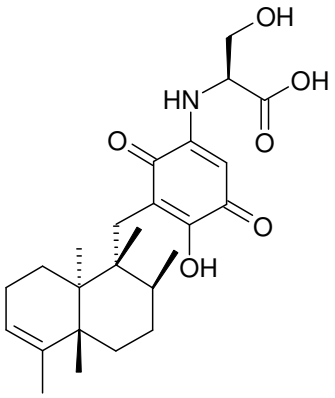


43

Nakijiquinone C (**45**), isolated from a marine sponge by Kobayashi *et al.*[59], showed inhibition of epidermal growth factor receptor (EGFR), c-ErbB2, and protein kinase C (PKC), in addition to having cytotoxic activity against L1210 and KB cell lines.[59] In a screening program, new analogues based on the nakijiquinone C backbone were prepared for testing against a battery of kinases with similar protein domain folds. Seven new inhibitors with low micromolar activity *in vitro* were obtained, including one VEGFR-2 inhibitor (**46**) and four inhibitors of Tie-2 kinase (**47**-**51**). Further investigation on kinase inhibitors led to the discovery of the first natural product inhibitor of Tie-2 kinase [60] (**48**) from the plant *Acacia aulacocarpa*, and demonstrated the activity of synthetic pyrrolo[2,3-*d*]pyrimidines as inhibitors of the same class of kinases.[61-64] The details of the models used, the chemistry leading to the nakijiquinone-based compounds, and the ribbon structures of the kinase domain of the insulin receptor, with the corresponding homology domains of the as yet uncrystallized VEGFR-2 and *Tie-2*, have been fully reviewed.[65,66]



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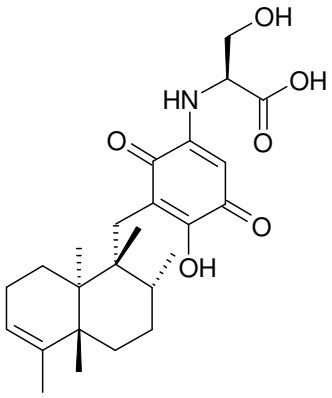

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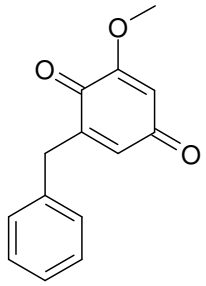

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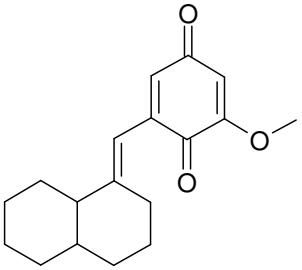

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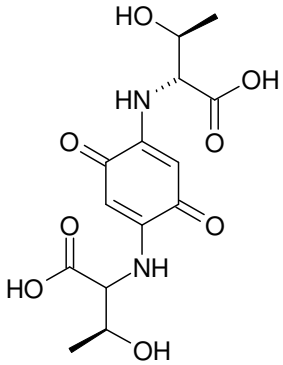

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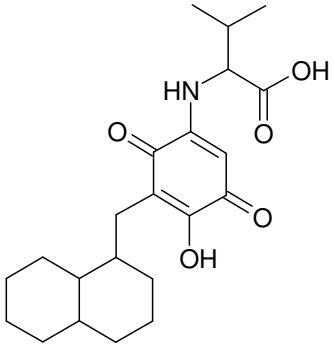

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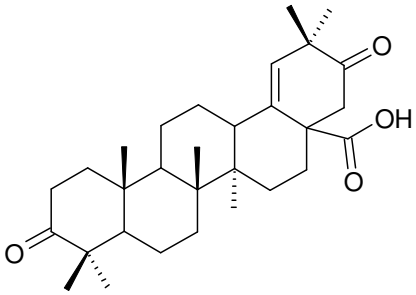


48

In chart 1, the drugs were classified as N (an unmodified natural product); ND (a modified natural product); S (a synthetic compound with no natural product conception); S/NM (a synthetic compound showing competitive inhibition of the natural product substrate); S* (a synthetic compound with a natural product pharmacophore); and S*/NM (a synthetic compound with a natural product pharmacophore showing competitive inhibition of the natural product substrate). The chart covers the

period from January 1981 to the middle of October 2008 and includes 1024 new chemical entities, with an increase of 50 small molecules in the two years. From given data, the majority of the compounds are formally synthetic (67%), but the analysis indicates that 18% of these correspond to the S* and S*/NM classes (Natural product pharmacophore) and 13% fall into the S/NM class. Thus, as with the 2007 analysis, the proportion of truly synthetic (i.e., devoid of natural product inspiration and coded as S) is still at 37%. In considering disease categories, 68.3% of anti-infectives (antibacterials, antifungal, antiparasitic, and antiviral) were classified as naturally derived or inspired (N; ND; S*; S*/NM; S/NM), while in the cancer treatment area, 79.8% were in these categories, with the figure dropping to 62.9% if the S/NM category is excluded.

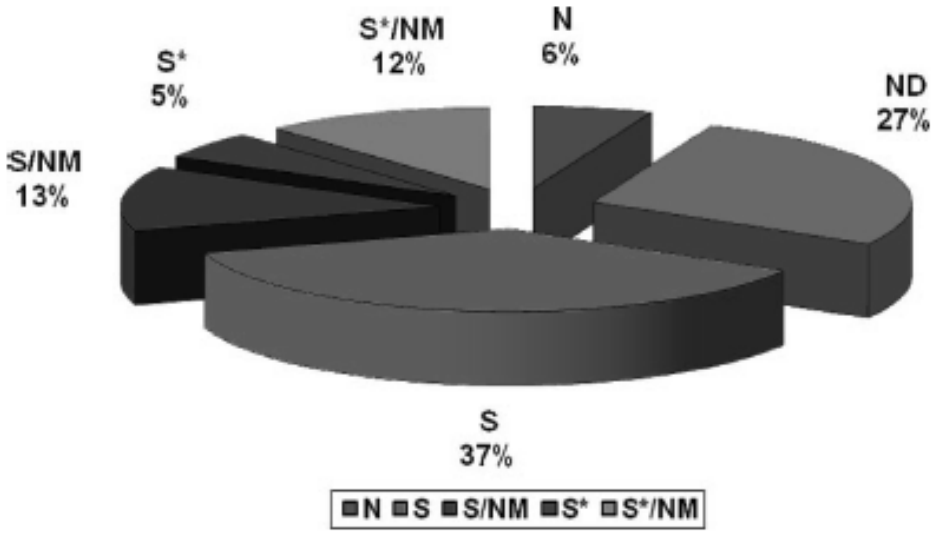


Chart 1. Small Molecule New Chemical Entities 01/1981-10/2008, by source (*N*) 1024)[67]

N (an unmodified natural product);
ND (a modified natural product);
S (a synthetic compound with no natural product conception);
S/NM (a synthetic compound showing competitive inhibition of the natural product substrate);
S* (a synthetic compound with a natural product pharmacophore);
S*/NM (a synthetic compound with a natural product pharmacophore showing competitive inhibition of the natural product substrate)

1.3 Aim of the present investigation

The aim of the present investigation was the isolation, purification and structure elucidation of secondary metabolites with biological activity from marine and terrestrial microorganisms. In order to attain this goal, a series of sequential chemical (HPLC-MS) and biological screening processes had to be undertaken. For the isolation and purification of the different constituents of crude extracts obtained from the fermentation, various chromatographic methods (silica gel, Sephadex LH-20, PTLC etc.) had to be used. Various spectroscopic methods (NMR, MS, IR, UV, X-ray crystal analysis if possible) are utilized for the structure elucidation in addition to databases (AntiBase, Dictionary of Natural Products and Chemicals Abstracts), for rapid dereplication. Fractions and pure compounds are submitted for different bioassays (i.e. antimicrobial test, antifungal, brine shrimp test) to explore new bioactivities against targets.

After selecting the strains based on the chemical and biological screening assays, the fermentation on a large scale should be performed to isolate the metabolites.

2 General Techniques

2.1 Collection of strains

The strains for this research project were obtained via cooperations with various microbiological groups.

- All the marine *Streptomyces* sp. with numbers beginning with "B" were isolated and taxonomically identified by E. Helmke from the Alfred-Wegner Institute for Polar and Marine Research (Bremerhaven, Germany). They were cultivated on M_2^+ medium (= M_2 medium + seawater). The Antarctic marine bacterium sp. T262 was also isolated by E. Helmke.
- All terrestrial *Streptomyces* spp. with numbers "Ank-xxx" were isolated and taxonomically identified by H. Anke, IBWF, Kaiserslautern. The strains were cultivated on M_2 medium.

2.2 Working up of selected strains

The general working up procedure of the investigated strains can be summarized in the following steps (Figure 2). The strains were evaluated first through chemical and biological screening. The interesting strains were then cultivated at large scale and after isolation of the metabolites, structure elucidations were performed.

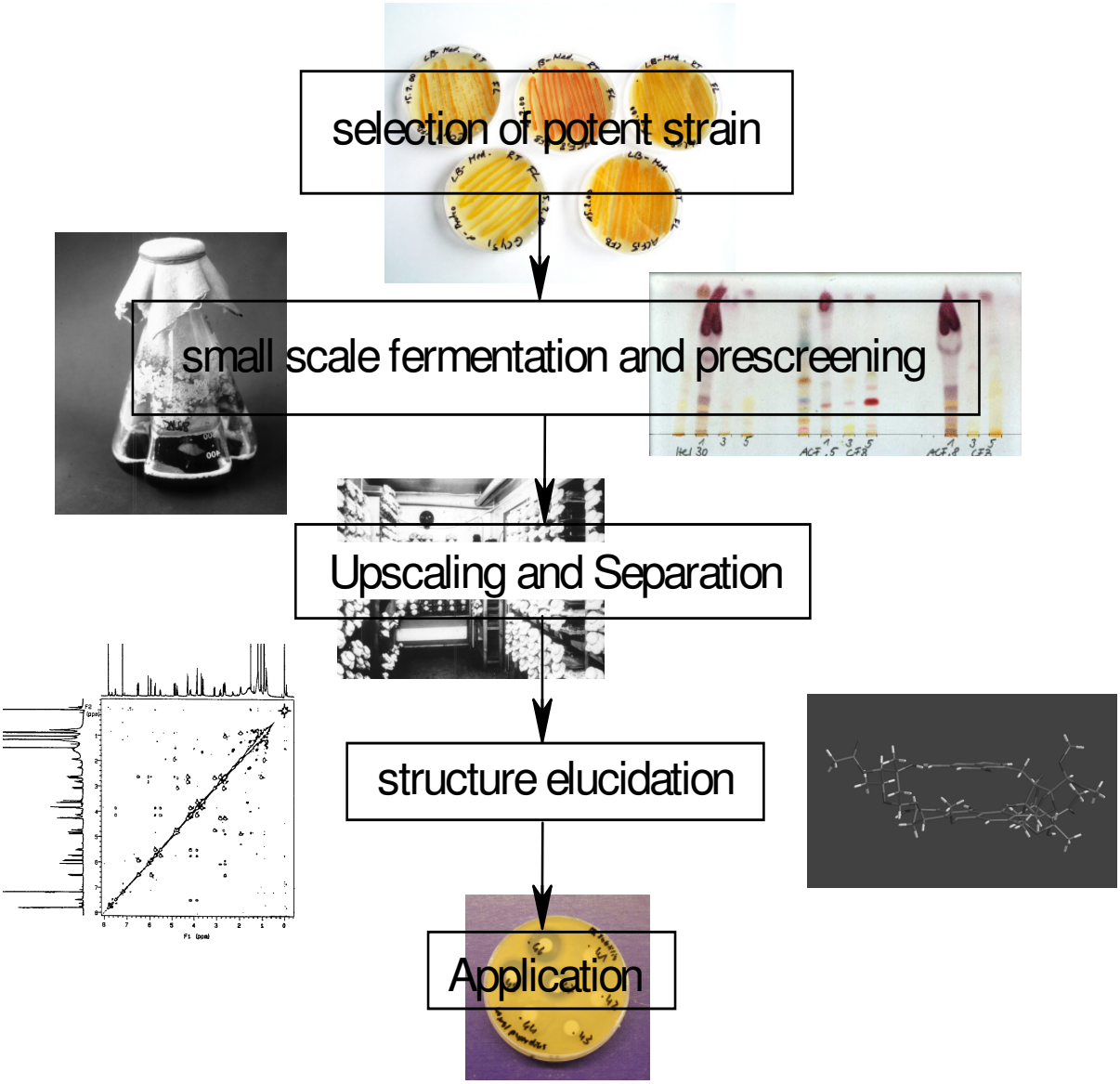


Figure 2: The concept for isolation of natural products

2.3 Primary screening

Due to the highly diverse metabolic capabilities of the available bacterial strains, it is essential to select effective strains either on the basis of their biological activity, or for their production of new natural products. This is done by means of a pre-screening. Hence well-grown 1-L shaker cultures were freeze-dried and the resulting residue was extracted with ethyl acetate (3 times), and the solution evaporated under vacuum at 40 °C. To evaluate the antibiotic activity of the extracts, they were subjected to agar diffusion tests using the bacteria *Escherichia coli*, *Streptomyces viridochromogenes* (Tü57), *Bacillus subtilis, Staphylococcus aureus*, the fungi *Mucor miehei* (Tü284), *Candida albicans*, and the micro-algae *Chlorella vulgaris*, *Chlorella sorokiniana,* and *Scenedesmus subspicatus* as test organisms. In parallel, the cytotoxic activity was evaluated against brine shrimps (*Artemia salina*). The extracts were also chemically screened by TLC, using UV absorption or fluorescence and spray reagents, as well as by HPLC analysis to dereplicate known compounds and to avoid unwanted strains. Chemical and biological screenings complement each other very well: The sensitivity of biological methods is much higher than that of the chemical analysis and can detect even traces, whereas the chemical screening targets on novel skeletons even if they do not show bioactivity. The strains that produce interesting metabolites are subjected to the scale-up cultivation.

2.4 Large-scale cultivation and extraction

Most of the strains are producing the metabolites in a small quantity; large-scale fermentation is therefore necessary to get an adequate amount of products. The procedure includes stepwise 1) the preparation of initial agar culture of the producing organism and 2) the inoculation of a 2-liter liquid culture and then 3) scale-up to provide up to 50 litres of culture broth. The fermentation may be carried out in shaking flasks or in a fermentor. After harvesting, the culture broth is filtered through a filter press by adding Celite. Prolonged storage of extracts in ethyl acetate at room temperature can lead to degradation of the compounds and lower overall yields. The extracts should be therefore evaporated as soon as possible, and it is strongly recommended to store the residues at the coldest temperature possible to minimize degradation of compounds. Adsorption on XAD resin is another efficient extraction method for obtaining the crude extracts. For this purpose, the water phase of the culture

filtrate is passed at a suitable flow rate through a glass column containing XAD resin. The compounds are eluted from XAD usually with methanol or a methanol/water gradient. Extraction with XAD is better than the alternatively used ethyl acetate extraction to remove sugars and other inorganic compounds. Highly polar water-soluble compounds can be obtained if lipophilic interactions are possible, good recovery rates are obtained, and it is easy to recover and purify the resin for further use. The isolation procedures depend mainly on the polarity of the compounds of interest (which can be determined by thin layer chromatography with eluents of varying polarity). There are two preliminary separation systems, which are commonly suitable for most metabolites:

Flash chromatography of the extract on silica gel using a stepwise gradient of dichloromethane/methanol or ethyl acetate/cyclohexane. This system classifies the fractions depending on their polarity. Disadvantage is the contact with silica gel, as this may rearrange, oxidize, cleave or even destroy metabolites.

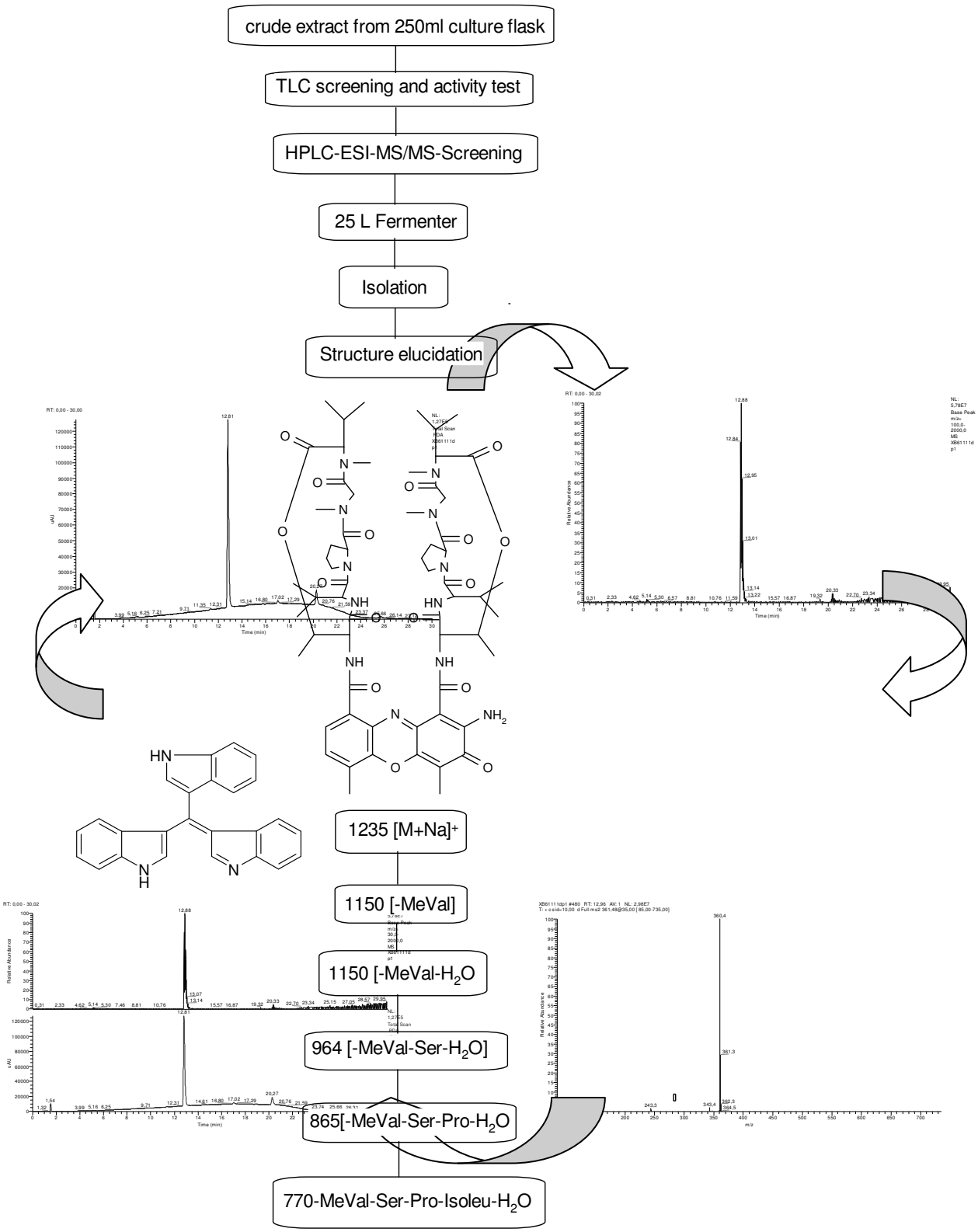
Size-exclusion chromatography using Sephadex LH-20: The separation is based on the molecular weight. Sephadex does not have the former disadvantages and the recovery rate for the compounds is also higher. The afforded fractions are monitored by TLC to decide the next isolation steps, which may be PTLC, silica gel column chromatography, or again Sephadex LH-20.

2.5 Dereplication

Despite of the existence of modern methods, the isolation and structural elucidation of natural compounds is a time-consuming and expensive process. The *dereplication* is therefore an important step with the aim to distinguish between known compounds and unknowns, and consequently allowing excluding the known compounds at an earlier stage. The principle of this method is to compare data fragments of mixtures or pure metabolites with suitable literature data. By comparing the HPLC-MS data and retention times with the reference data collection, their identification is performed. This method needs sample amounts in traces and affords reliable results, if authentic samples had been available to measure the reference data. UV data and MS fragmentation patterns are also useful to identify unknown metabolites, if these show similar chromophores or fragmentation patterns as known analogues.

Presently, ESI MS/MS spectra of more than 1000 of the most frequently isolated substances are included in our database of natural products. Our results have shown that already known natural products can be identified easily even from crude extracts obtained from bacterial broths. Application of this method is a very valuable tool to make the process of finding new biological and pharmacological active compounds more efficient. As it will never be possible to collect a complete sample set and to measure all experimental data under identical conditions, reference values also from the literature have to be used. If NMR data are selected, results from 1D measurements can be translated into substructures, which then will be used for a database search. In this case, normally sufficiently pure samples are required. Databases with the NMR or UV data and a variety of other molecular descriptors are available[68]. The most comprehensive data collection of natural compounds is the Dictionary of Natural Products (DNP), which compiles metabolites from all natural sources, including plants. Our own data collection (AntiBase) is, however, more appropriate for the dereplication of microbial products, as the identification depending on structural features and spectroscopic data is more comprehensive, faster and more reliable. In the case of new compounds, a database search is also helpful because novel skeletons are rare and usually related compounds, which are already known, are easily revealed by a database search, thus identifying at least the compound class. Finally, the Chemical Abstracts, the most comprehensive bank of chemical information worldwide, is used for a final confirmation that a given structure is new. Sub-structure searches with small fragments are not possible here for technical reasons.

The combination of liquid chromatography with detection methods such as NMR spectroscopy (HPLC NMR) and tandem mass spectrometry (HPLC-MS/MS) has recently led to new strategies by which biological matrices, e.g., crude plant extracts[69] or extracts from marine organisms[70], are screened to obtain as much information as possible about known constituents even with a minimum amount of material. As most compounds of interest are thermally labile, HPLC-ESI MS/MS would be the method of choice to identify known molecules from multi-component mixtures with high selectivity and sensitivity[71].



Schematic representation of Natural product isolation from Microorganism Dereplication by molecular ion

3 Marine *Streptomyces* spp.

3.1 Marine *Streptomyces* sp. B7380

In the primary screening, the ethyl acetate extract of the terrestrial *Streptomyces* sp. isolate B7380 showed interesting characteristics during TLC: It showed five middle polar, under UV blue fluorescent bands, which displayed a yellow colour reaction with anisaldehyde/sulphuric acid and became green with Ehrlich's reagent. Moreover, the extract showed a moderate activity against *Bacillus subtilis* and *Escherichia coli*, and strong activity against *Streptomyces viridochromogenes* (Tü 57); however, no activity was found against fungi, yeasts or microalgae. HPLC/MS of the crude extract showed halogen isotopes as for bromine, and on investigation of this strain four new bromine-containing metabolites were obtained. Bromine containing compounds are rare in bacteria, although bromide is a ubiquitous constituent of seawater. TLC-directed work-up of the water extract by silica gel column chromatography and size exclusion chromatography resulted in four fractions. Fraction I was subjected to column chromatography to give 4-hydroxy-10-methyl-11-oxo-dodec-2-en-1,4-olide (**52**). Fraction II was purified using silica gel column chromatography followed by PTLC and Sephadex LH-20 to give 5-(6-hydroxy-6-methyloctyl)-furan-2(5H)-one (**53**), the isatin derivative 3-hydroxy-3-(2-oxopropyl)indolin-2-one (**58**) and a new bromine containing compound, 2-(2-amino-5-bromophenyl)-N,N-dimethyl-2-oxo-acetamide (**54**). Fraction III was also purified using the same chromatographic techniques, and 5-(6,7-dihydroxy-6-methyloctyl)-furan-2(5H)-one (**55**), 5-bromo-3-hydroxy-3-(2-oxobutyl)-indolin-2-one (**56**) and 5-bromo-3-hydroxy-3-(3-oxobutan-2-yl)indolin-2-one (**57**) were isolated. Fraction IV gave 5-bromo-3-hydroxy-3-(4-methyl-2-oxopentyl)-indolin-2-one (**65**) and 3,5-dihydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one (**64**).

Butenolides **52**, **53** and **55** are α,β-unsaturated lactones, which are often encountered in fungi, bacteria, and gorgonians, to name a few. Their saturated analogues act as signalling substances in bacteria[72] and enhance e.g. spore formation of *Streptomycetes* or induce metabolite production.[73] Based on the spectroscopic data, a substructure search in AntiBase delivered these compounds which had been previously isolated in our group by Mukku[74]. The isatin derivative 3-hydroxy-3-(2-

oxopropyl)indolin-2-one[75] (**58**) is reported to show cytotoxic activity against L1210 and Jurkat cells with 50-100 μg/ml.

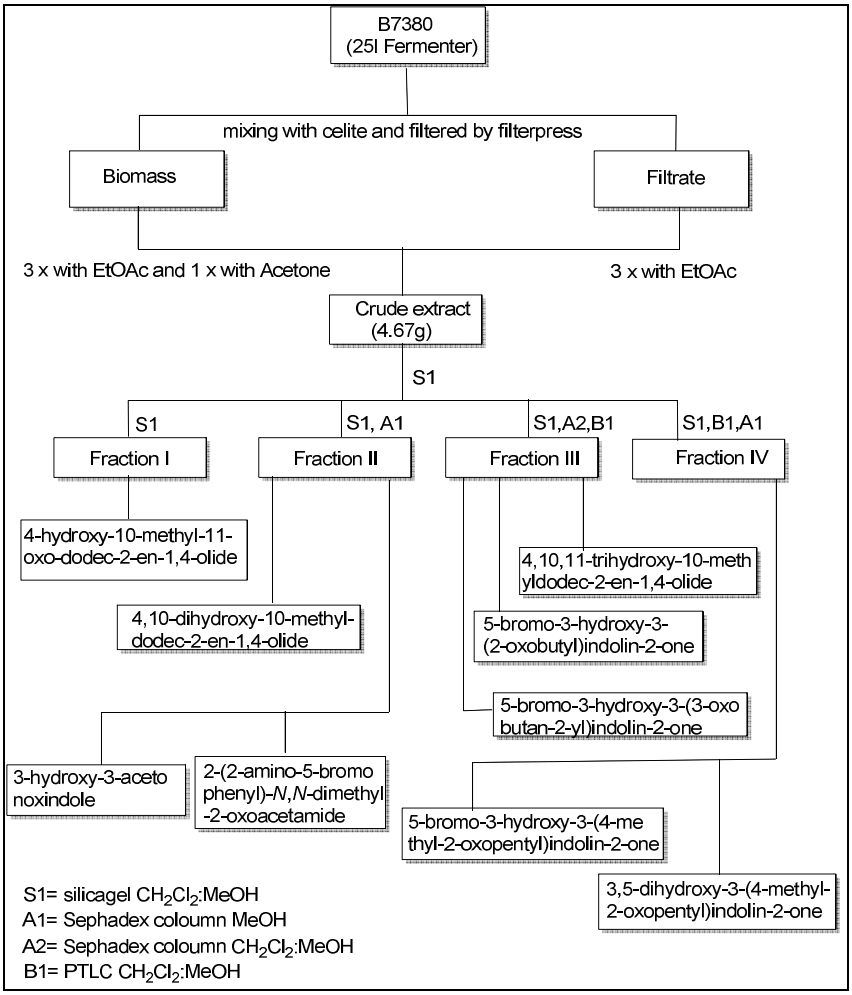
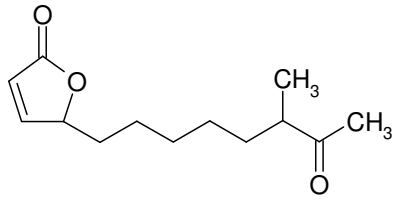


Figure 3: Working up scheme of the Marine *Streptomyces* sp. isolate B7380

3.1.1 4-Hydroxy-10-methyl-11-oxo-dodec-2-en-1,4-olide

Compound **52** gave a reddish pink colour reaction with anisaldehyde/sulphuric acid after heating, but was not UV absorbing. The ^{1}H NMR spectrum of this compound exhibited the butenolide moiety: In the aliphatic region, the spectrum revealed a methyl doublet at δ 1.06, the methyl singlet of an acetyl group at δ 2.13, overlapping multiplet signals between δ 1.79 and 1.36 for five methylene groups and a methine multiplet at δ 2.56, which was possibly attached to an sp^2 carbon. ESIMS revealed the molecular ion peak at *m/z* 224. By a search in AntiBase using the above

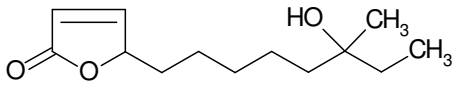
spectroscopic data, the isolated compound was assigned as 4-hydroxy-10-methyl-11-oxo-dodec-2-en-1,4-olide (**52**). The structure was further confirmed by comparison with the literature data and authentic spectra.



52

3.1.2 4,10-Dihydroxy-10-methyl-dodec-2-en-1,4-olide

Sub-fraction II showed no UV absorbing bands in the nonpolar region but turned to violet and red with anisaldehyde/sulphuric acid on heating. The constituent was isolated as colourless oil by silica gel column chromatography followed by RP-18 column separation. The ^{1}H NMR spectrum of **53** showed three signals at δ 7.45 (dd), 6.11 (dd) and 5.05 (m), which are typical for protons H-2, 3, and 4 of the butenolide moiety. In the aliphatic region, one methyl doublet at δ 1.08, one methyl singlet at δ 1.12 and the multiplet of six methylene groups between δ 2.03 and δ 1.54 were observed. A search in AntiBase using these data identified this compound as 4,10-dihydroxy-10-methyl-dodec-2-en-1,4-olide (**53**).

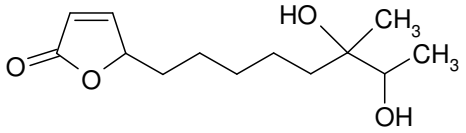


53

3.1.3 4,10,11-Trihydroxy-10-methyldodec-2-en-1,4-olide

Purification of sub-fraction VI on Sephadex LH-20 followed by RP-18 delivered the oily colourless compound **55**, which showed a violet colouration with anisaldehyde/sulphuric acid on TLC. The ^{1}H NMR spectrum showed very close similarity to that of **53**. Two oxymethine signals at δ 5.05 (m, H-4) and.3.64 (q, H-11) were present, but the two geminal dimethyl groups in **53** were replaced by one methyl triplet

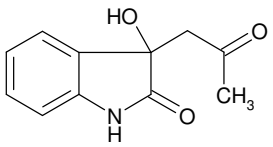
at δ 1.11 and one methyl singlet at δ 1.15. A literature search resulted in the identification of compound **55** as 4,10,11-trihydroxy-10-methyldodec-2-en-1,4-olide. The structure of **55** was further confirmed by comparing with the authentic spectra as well as literature data.[76] Compound **55** was isolated in our group by Fotso from the marine-derived *Streptomyces* spp. Mei35 and LR4612.[76,77]



55

3.1.4 3-Hydroxy-3-acetonyloxindole

Compound **58** was isolated as a UV absorbing colourless solid, which gave on TLC a violet colouration with anisaldehyde/sulphuric acid and a green colour reaction with Ehrlich's reagent. The compound showed a peak at *m/z* = 205 in ESI spectrometry. The ^{1}H NMR spectrum displayed aromatic signals at δ = 7.23, 7.16, 6.90, and 6.77 with a pattern typical for an *ortho*-disubstituted benzene ring. In addition, signals with a large coupling constant were observed in the aliphatic region at δ 3.25 and 3.98 (AB, J = 16.9 Hz, 2H, 8-H), which were attributed to a geminal coupling and indicated the presence of an oxygenated diastereotopic methylene group. A search in AntiBase with the available information gave 3-hydroxy-3-acetonyloxindole.



58

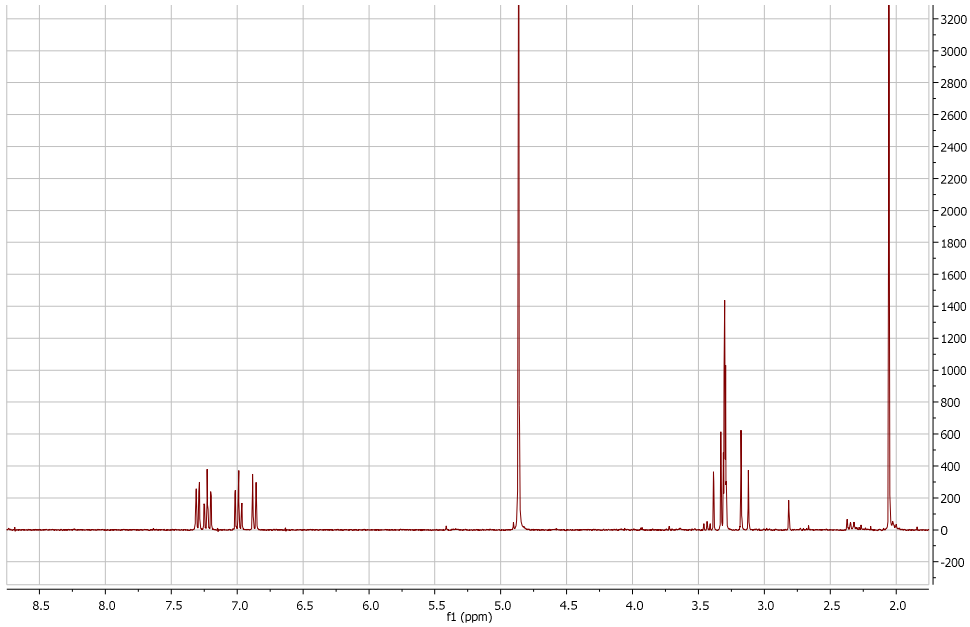


Figure 4: ^{1}H NMR spectrum (CD_3OD, 300 MHz) of 3-hydroxy-3-(2-oxopropyl)indolin-2-one (**58**)

3-Hydroxy-3-acetonyloxindole (**58**) is obviously the aldol adduct of acetone to isatin, and it is indeed formed from the latter just on standing at room temperature in acetone solution. It is therefore a well-known synthetic product, but had also been found in nature before by the group of Fenical and in our group[127] as a natural product.

3.1.5 2-(2-Amino-5-bromophenyl)-N,N-dimethyl-2-oxoacetamide

Compound **54** was isolated from fraction IV as middle polar yellow UV absorbing solid. The compound showed a weak colour change to yellow on treatment with anisaldehyde/sulphuric acid spray reagent on TLC. The presence of bromine was confirmed by the isotope peaks on ESI-MS at 293 and 295 $[M+Na]^+$; HRESIMS confirmed the molecular formula as $C_{10}H_{11}N_2O_2Br$. The ^{1}H NMR spectrum of **54** displayed a 1H doublet (2.6 Hz) at δ 6.78 together with two *ortho*-coupled aromatic proton signals at δ 7.38 and δ 7.36, indicating an aromatic 1,3,4-substitution pattern. In the aliphatic region, two methyl singlets at δ 2.95 and 3.08 suggested N-methylation. Dereplication using the aromatic substitution pattern and the high-resolution mass gave no hit in AntiBase, pointing to a new metabolite. The structure was finally derived from HMBC correlations as shown in Figure 6 and Table 1.

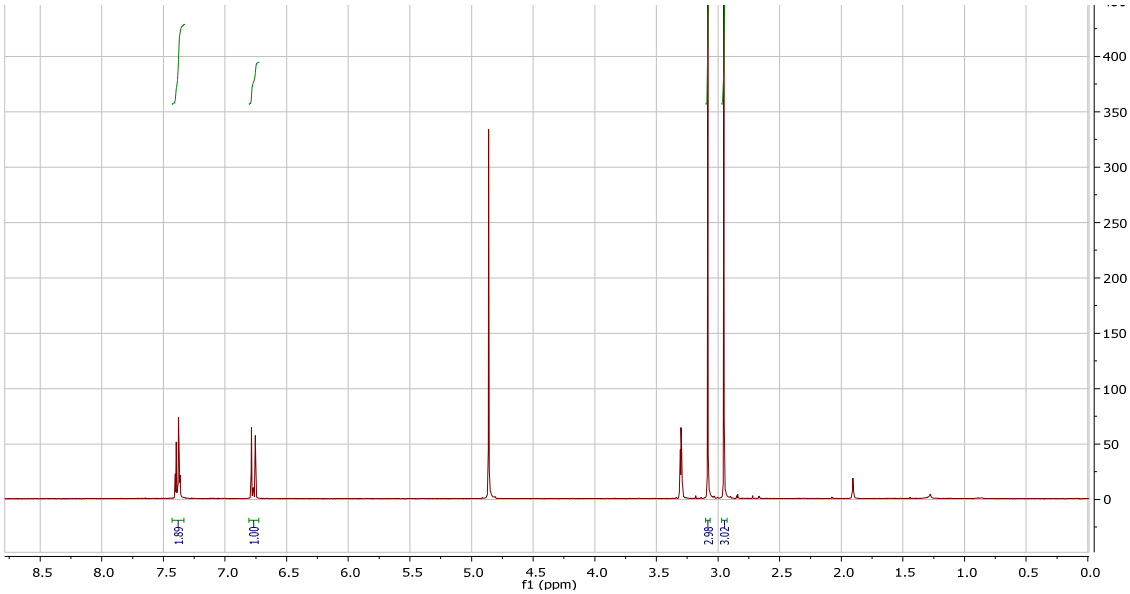


Figure 5: ^{1}H NMR spectrum (CD_3OD, 300 MHz) of 2-(2-amino-5-bromophenyl)-N,N-dimethyl-2-oxoacetamide **(54)**

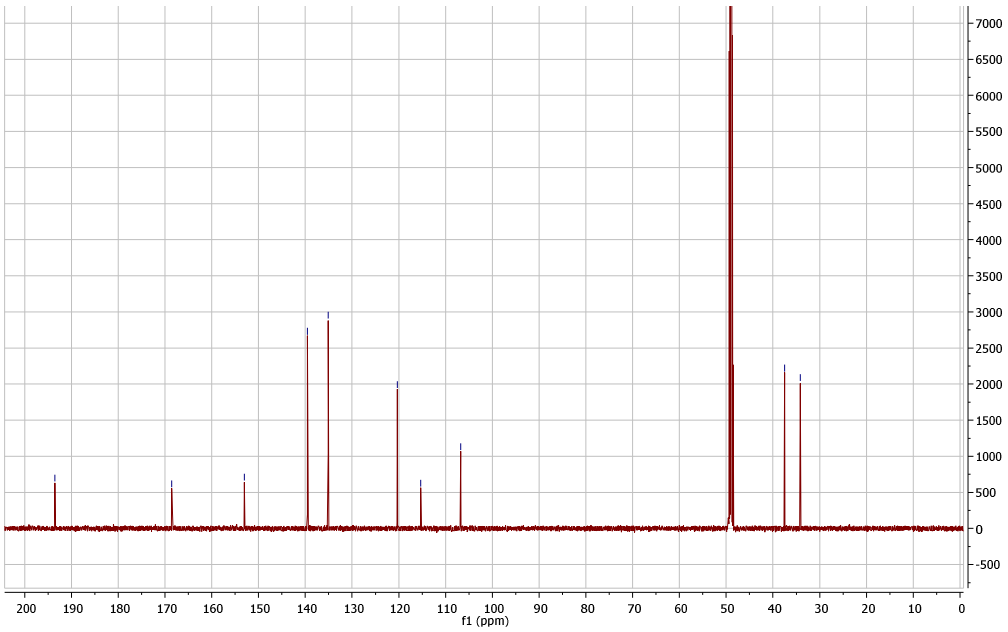
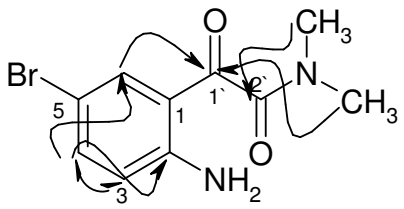


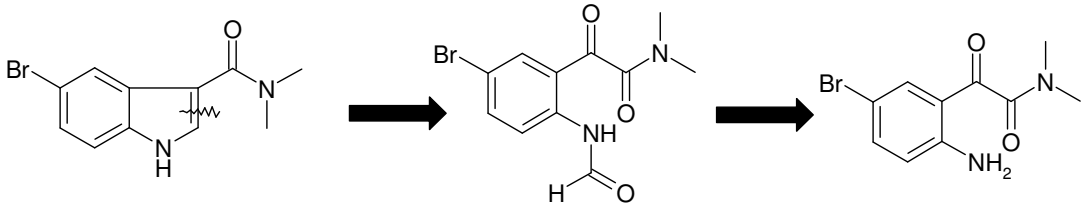
Figure 6: ^{13}C NMR spectrum (CD_3OD, 75 MHz) of 2-(2-amino-5-bromophenyl)-N,N-dimethyl-2-oxoacetamide **(54)**



54

Figure 7: HMBC correlations ( ) of 2-(2-amino-5-bromophenyl)-N,N-dimethyl-2-oxoacetamide (**54**)

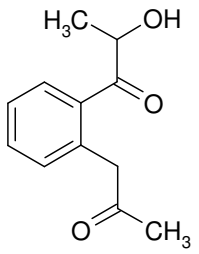
2-Aminophenylglyoxylic acid derivatives have not been reported from bacteria so far. The structure of **54** resembles, however, the kynuramines, and it can be postulated that **54** is formed from 5-bromo-indole-3-carboxylic acid dimethylamide by oxidative ring cleavage: In a first step, the formamide **60** is formed, which is easily hydrolysed to afford **54**.



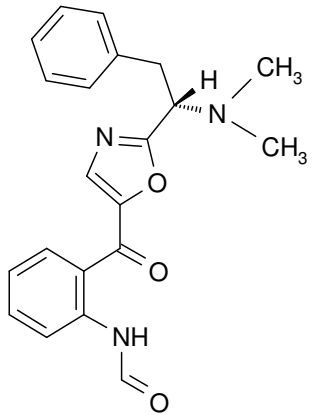
59 **60** **54**

Scheme 1: Oxidative ring opening of 5-bromo-indole-3-carboxylic acid dimethylamide (**58**)

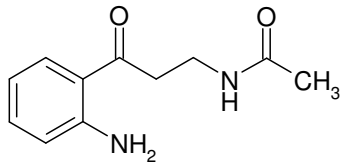
Related natural formamides formed in this way are e.g. 2-(2-hydroxypropionyl)-acetanilide (**61**), almazole A (**62**), and N-acetylkynuramine (**63**). The latter was isolated from the Antarctic shelf ice bacterium ANT V/2-253[78].



61



62



63

Table 1: ^{1}H and ^{13}C NMR data of 2-(2-amino-5-bromophenyl)-N,N-dimethyl-2-oxoacetamide (**54**)

2-(2-Amino-5-bromophenyl)-N,N-dimethyl-2-oxoacetamide (**54**)			
No.	^{1}H (δ_H, mult., *J* Hz)[a]	^{13}C (δ_C) [b]	HMBC
1	-	153.0	-
2	-	135.1	-
3	7.38 (d, *J* = 7.4)	120.3	1, 2, 4, 5
4	7.36 (dd, *J* = 8.7, 2.4)	139.6	2, 3, 5, 6
5	-	106.8	-
6	6.78 (d, *J* = 2.6)	115.3	1, 1', 2, 4, 5
1'	-	193.6	-
2'	-	168.6	-
4'	3.08 (s)	37.5	1', 2', 5'
5'	2.95 (s)	34.2	2', 4'

[a] CD_3OD, 300 MHz. [b] CD_3OD, 125 MHz

3.1.6 5-Bromo-3-hydroxy-3-(2-oxobutyl)indolin-2-one

Compound **56** was obtained as white powder, which was easily soluble in DMSO. ESI MS followed by ESIHRMS showed two peaks of similar intensity confirming the molecular formula $C_{12}H_{12}N_1O_3Br$. The proton NMR spectrum of the compound exhibited an NH signal at δ 10.38 and three further 1H signals of aromatic protons in 1,2,4-position: two doublets at δ 6.76 (*J* = 8.3 Hz) and 7.42 (*J* = 2.2 Hz), and a double doublets at δ 7.35 (*J* = 8.3, 2.0 Hz). In addition, a CH_2 signal was observed in the

aliphatic region at δ 3.03 and 3.38 with a large coupling constant (AB, J = 16.9 Hz, 2H, 8-H) that was attributed to a prochiral methylene group. The presence of a further methylene group was observed at 2.40 (m), and a methyl triplet was found at δ 0.78, indicating an ethyl residue.

The carbon spectrum showed two carbonyl signals δ 207.2 and δ 177.4. The value of the latter is typical for an amide (or an ester), in this case for the amide carbonyl of a 2-oxindole. The position of bromine at position 5 was confirmed by HMBC correlations resulting in a 5-bromo-substituted isatin derivative. The position of an ethyl methyl ketone residue at position 3 was also confirmed by COSY and HMBC correlations between C-8, 9, 10 and 11 as shown in Table 2 and Figure 9

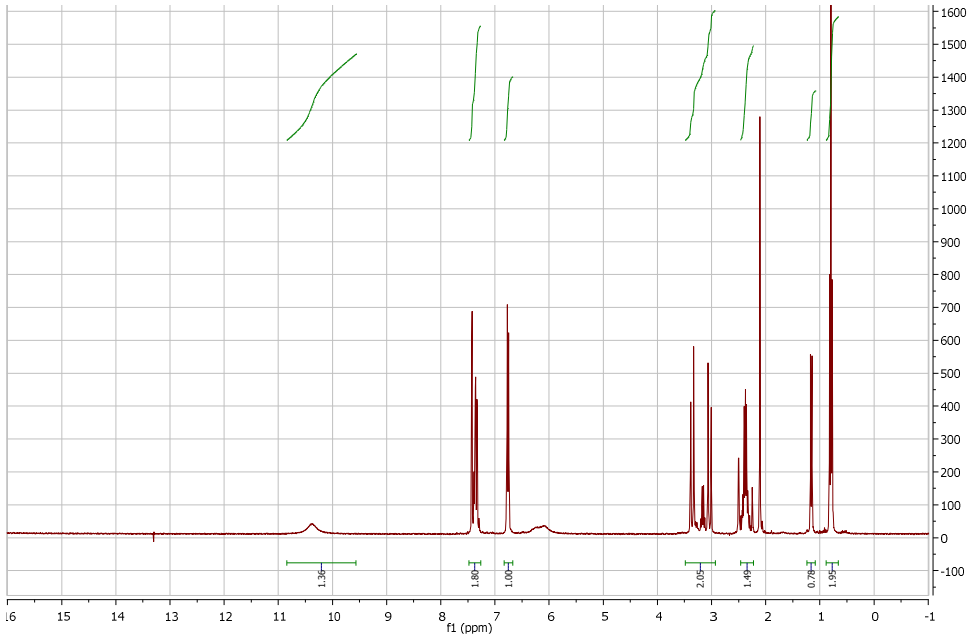


Figure 8: ^{1}H NMR spectrum (DMSO-d_6, 300 MHz) of 5-bromo-3-hydroxy-3-(2-oxobutyl)indolin-2-one (**56**)

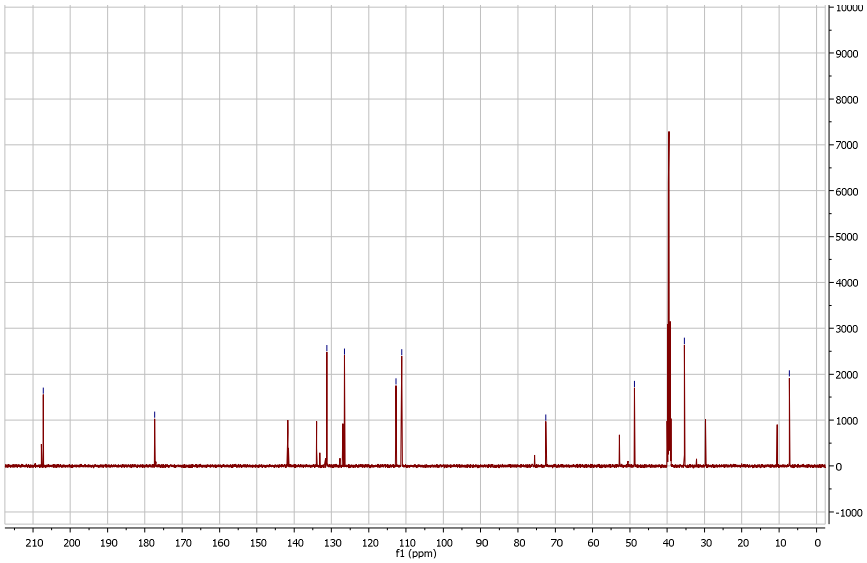
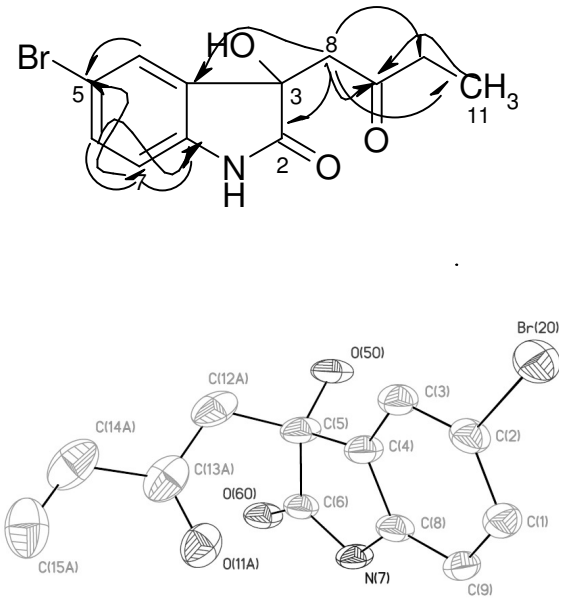


Figure 9: ^{13}C NMR spectrum (DMSO-d_6, 75 MHz) of 5-bromo-3-hydroxy-3-(2-oxobutyl)indolin-2-one (**56**)



56

Figure 10: HMBC correlations (⌒) and X-ray crystallography of 5-bromo-3-hydroxy-3-(2-oxobutyl)indolin-2-one (**56**)

3.1.7 5-Bromo-3-hydroxy-3-(3-oxobutan-2-yl)indolin-2-one

Compound **57** was isolated as colourless solid from the less polar fraction I. During TLC, compound **57** appeared as UV absorbing band, which stained yellow on spraying with anisaldehyde/sulphuric acid. ESI MS showed again a bromo derivative, and ESIHRMS afforded the molecular formula $C_{12}H_{12}N_1O_2Br$.

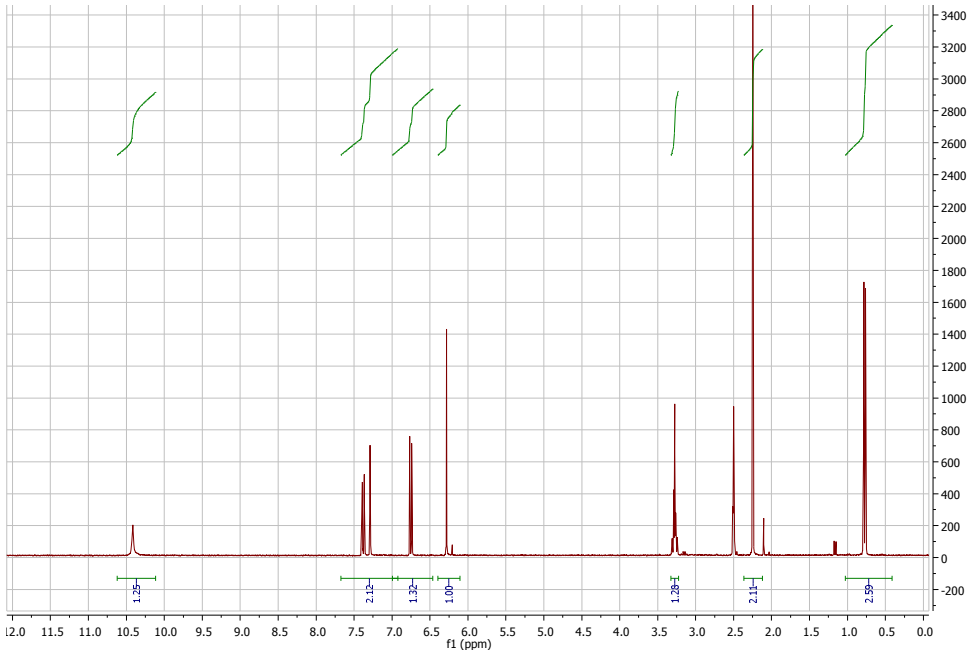


Figure 11: ^{1}H NMR spectrum (DMSO-d_6, 300 MHz) of 5-bromo-3-hydroxy-3-(3-oxobutan-2-yl)indolin-2-one (**57**)

The proton NMR spectrum of compound **57** established again protons in 1,2,4-position as in **54** and **56**. An OH group gave a broadened 1H signal at δ 6.3 with no correlation in the HSQC spectrum. The aliphatic region showed a multiplet at δ 3.22, a methyl singlet at δ 2.25 and a methyl doublet at δ 0.77.

The ^{13}C NMR spectrum showed the expected 12 carbon signals, among them 8 carbon signals in the sp^2 region. The presence of a ketone group was seen by a signal at δ 214.6 and an amide carbon was found at δ 182.4. The signal of an oxygenated carbon appeared at δ 81.6, a methine group was displayed at δ 55.8. An acetyl group was represented by a carbon signal at δ 37.5 and the respective methyl singlet at δ 2.25 in the ^{1}H NMR spectrum. The methyl doublet gave a carbon signal at δ 16.0.

The COSY correlations of the methine group at δ 3.22 with the aliphatic methyl doublet at δ 0.77 and HMBC correlations of the latter to the acetyl group and the oxygenated carbon C-3 at δ 81.6 confirmed the placement of the side chain in the 5-bromo isatin derivative. The HMBC spectra showed also a crucial correlation of the methine group at δ 3.22 with the amide carbon (δ 182.3), which finally confirmed the compound to be 5-bromo-3-hydroxy-3-(3-oxobutan-2-yl)indolin-2-one (**57**).

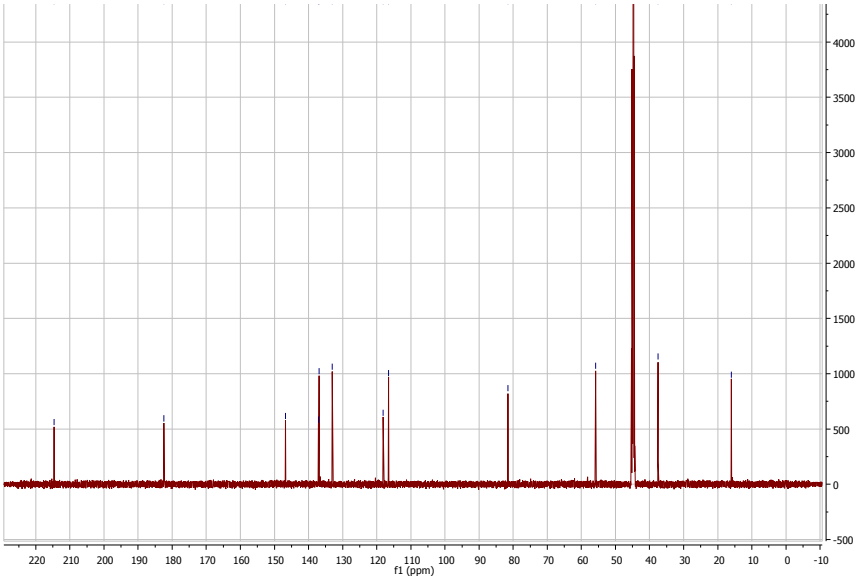
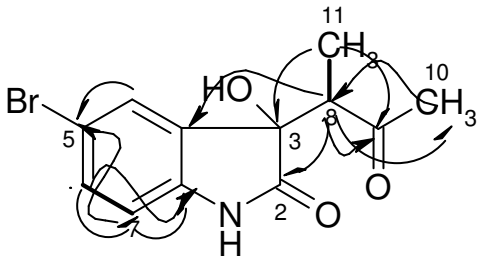


Figure 12: ^{13}C NMR spectrum (DMSO-d_6, 75 MHz) of 5-bromo-3-hydroxy-3-(3-oxobutan-2-yl)indolin-2-one (**57**)



57

Figure 13: ^{1}H-^{1}H COSY (bold bonds) and HMBC correlations ( ) of 5-bromo-3-hydroxy-3-(3-oxobutan-2-yl)indolin-2-one (**57**)

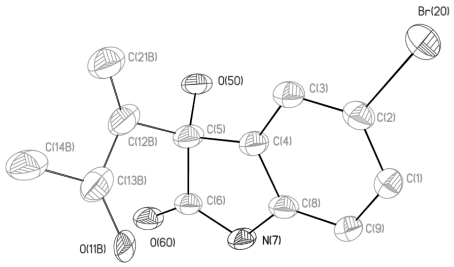


Figure 14: X-ray crystallography of 5-bromo-3-hydroxy-3-(3-oxobutan-2-yl)indolin-2-one (**57**)

Table 2: ^{1}H and ^{13}C NMR of 5-bromo-3-hydroxy-3-(2-oxobutyl)indolin-2-one (**56**) and 5-bromo-3-hydroxy-3-(3-oxobutan-2-yl)indolin-2-one (**57**)

5-Bromo-3-hydroxy-3-(2-oxobutyl)indolin-2-one (**56**)				5-Bromo-3-hydroxy-3-(3-oxobutan-2-yl)indolin-2-one (**57**)			
Position No.	^{1}H (δH, mult., *J* Hz)[a]	^{13}C (δC)[b]	HMBC	Position No	^{1}H (δH, mult., *J* Hz)[a]	^{13}C (δC)[b*]	HMBC
2	-	177.4	-	2	-	182.4	-
3	-	72.5	-	3	-	81.6	-
3a	-	134.0	-	3a	-	133.1	-
4	7.42 (d, *J* = 2.4 Hz)	126.5	3, 3a, 5, 6	4	7.29 (d, *J* = 2.0 Hz)	118.1	3, 3a, 5, 6
5	-	112.8	-	5	-	116.6	-
6	7.35 (dd, *J* = 8.2, 2.0 Hz)	131.3	4, 5, 7, 7a	6	7.39 (dd, *J* = 8.2, 2.1)	137.0	4, 5, 7, 7a
7	6.76 (d, *J* = 8.3, Hz)	111.2	3a, 7a, 6, 5	7	6.75 (d, *J* = 8.3 Hz)	131.1	3a, 7a, 6, 5
7a	-	141.7	-	7a	-	146.8	-
8a	3.03 (d, *J* = 16.8, Hz)	48.8	2, 3, 3a, 9, 10	8	3.22 (m)	55.8	2, 3, 3a, 9, 8 Me, 10
8b	3.38 (d, *J* = 16.8, Hz)						
9	-	207.2	-		-	214.7	-
10	2.40 (m)	35.4	8, 9, 11		2.25 (s)	37.5	9, 10
11	0.78 (t, *J* = 7.3 Hz)	7.2	9, 10		0.77 (d, *J* = 7.0 Hz)	16.0	2, 3, 8, 9, 10
NH	10.38 (s br)				10.42 (s br)		
3-OH		6.1 s br			6.28 s		

[a] DMSO-d_6, 300 MHz. [b] DMSO-d_6, 125 MHz

It is obvious that **56** and **57** are formed similarly as **58** by a reaction of 5-bromoisatin with an aliphatic ketone, in this case with ethyl-methylketone. It is interesting to see that the latter reacted with both CH-acidic sides.

3.1.8 5-Bromo-3-hydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one

From the polar fraction I, compound **64** was isolated as colourless solid from a UV absorbing band, which stained yellow on spraying with anisaldehyde/sulphuric acid. ESI MS and ESIHRMS afforded the molecular formula $C_{14}H_{16}N_1O_3Br$. The proton and carbon data were related to those of **56** and **57** and pointed again to a related 5-bromoisatin derivative. The HMBC correlations of two prochiral methylene protons with the oxygenated quaternary carbon C-3 (δ 72.5), the amide group C-2 (δ 177.4), the aliphatic ketone group (δ 206.6) and a methylene group (δ 51.2) gave two partial structures as shown in Figure 13 and Figure 15. Further correlations (Figure 16) resulted finally in structure **64**

Figure 15: Partial structure of 5-bromo-3-hydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one (**64**)

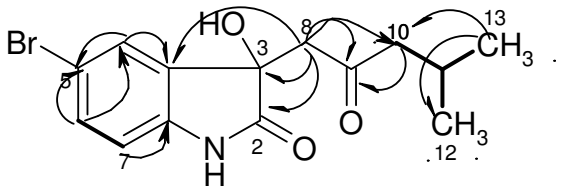


Figure 16: ^{1}H-^{1}H COSY (bold bonds) HMBC correlations (⌒) of 5-bromo-3-hydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one (**64**)

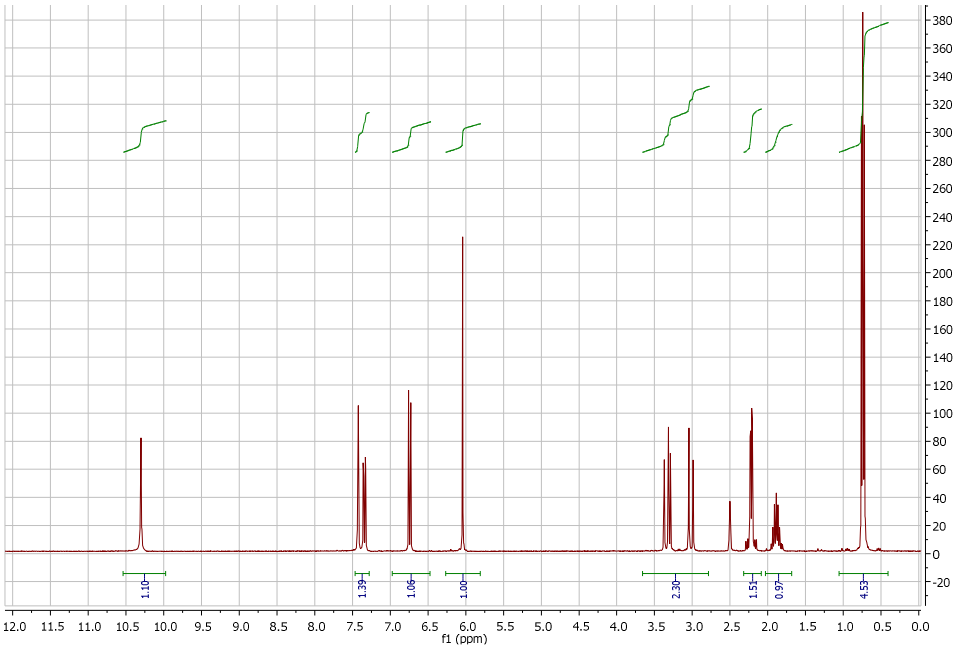


Figure 17: ^{1}H NMR spectrum (DMSO-d_6, 300 MHz) of 5-bromo-3-hydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one (**64**)

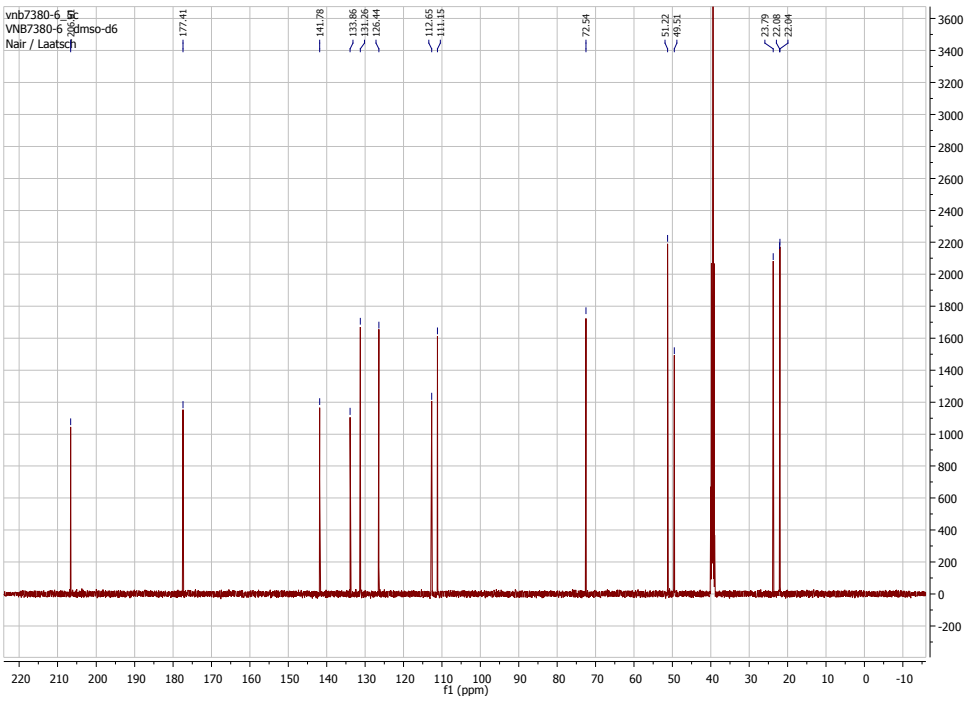


Figure 18: ^{13}C NMR spectrum (DMSO-d_6, 75 MHz) of 5-bromo-3-hydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one (**64**)

3.1.9 3,5-Dihydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one

Compound **65** was isolated from fraction IV as medium polar yellowish-orange UV-absorbing solid. The compound showed a colour change to violet on treatment with anisaldehyde/sulphuric acid spray reagent. The molecular weight of **65** was established as 263 Dalton on the basis of ESI mass spectra. HRESIMS confirmed the molecular formula as $C_{14}H_{17}NO_4$. Also here, the ^{1}H NMR spectrum revealed a 1,2,4-trisubstituted benzene. The placement of a hydroxyl group at C-5 of an indole skeleton was confirmed by NMR spectroscopy. The upfield region of the ^{1}H NMR spectrum showed a signal pattern similar as in **64**, indicating an isobutyl-methyl ketone. The position of this residue at position 3 was confirmed by correlations in the HMBC spectrum as shown in Figure 25 and Table 3. Correspondingly, the derivative was elucidated as 3,5-dihydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one (**65**).

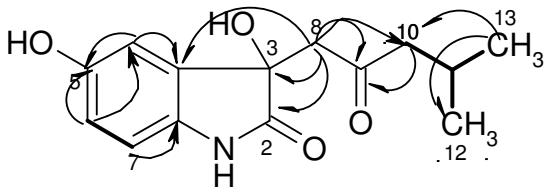


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Figure 19: HMBC correlations (⌒) of 3,5-dihydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one (**65**)

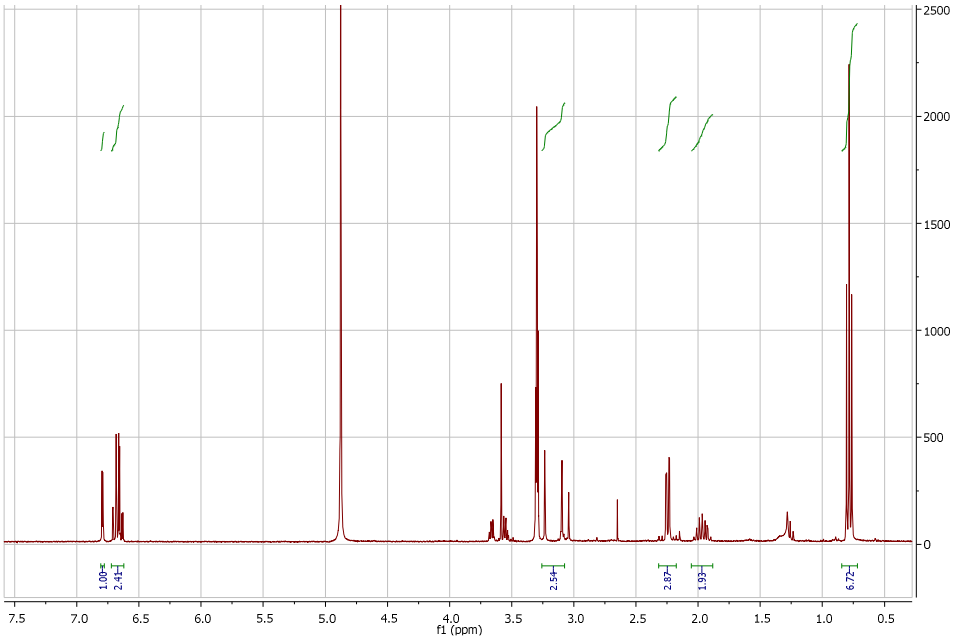


Figure 20: ^{1}H NMR spectrum (CD_3OD, 300 MHz) of 3,5-dihydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one (**65**)

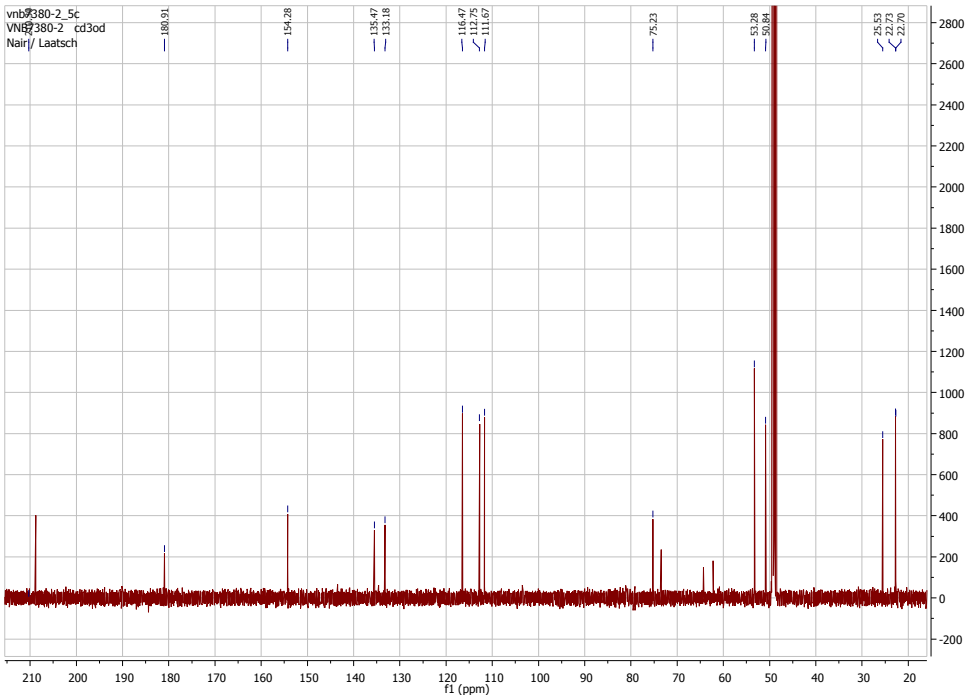


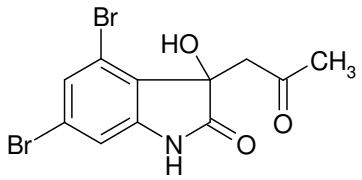
Figure 21: ^{13}C NMR spectrum (CD_3OD, 75 MHz) of 3,5-dihydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one (**65**)

Table 3: ^{1}H and ^{13}C NMR of 3,5-dihydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one (**65**) and 5-bromo-3-hydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one (**64**)

3,5-Dihydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one (**65**)				5-Bromo-3-hydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one (**64**)		
Position.	^{1}H (δ_H, mult., *J* Hz)[a]	^{13}C (δ_C)[b]	HMBC	^{1}H (δ_H, mult, *J* Hz)[c]	^{13}C (δ_C)[d]	HMBC
2	-	180.9	-	-	177.4	-
3	-	75.2	-	-	72.5	-
3a	-	133.2	-	-	131.3	-
4	6.79 (d, *J* = 2.3 Hz)	112.8	3, 3a, 5, 6	7.42 (d, *J* = 2.0 Hz)	126.4	3, 3a, 5, 6
5	-	154.3	-	-	111.2	-
6	6.67 (dd, *J* = 8.3, 2.5 Hz)	116.5	4, 5, 7, 7a	7.35 (dd, *J* = 8.2, 2.0 Hz)	133.9	4, 5, 7, 7a
7	6.69 (d, *J* = 8.3 Hz)	111.7	3a, 7a, 6, 5	6.74 (d, *J* = 8.2 Hz)	112.7	3a, 7a, 6, 5
7a	-	135.5		-	141.8	-
8a	3.17 (d, *J* = 16.4 Hz)	50.8	2, 3, 3a, 9, 10	3.35 (d, *J* = 17.0 Hz)	49.5	2, 3, 3a, 9, 10
8b	3.28 (d, *J* = 16.4 Hz)			3.02 (d, *J* = 17.0 Hz)		2, 3, 3a, 9, 10
9	-	208.8	-	-	206.6	-
10	2.24 (d, *J* = 5.4 Hz)	53.3	8, 9, 11, 12, 13	2.23, 2.20 (ABX, *J* = 2.3 Hz, 5.6 Hz)	51.2	8, 9, 11, 12, 13
11	1.96 (m)	25.5	9, 10, 12, 13	1.90 (m)	23.8	9, 10, 12, 13
12	0.80 (d, *J* = 6.6 Hz)	22.7	9, 10, 11, 13	0.79 (d, *J* = 5.6 Hz)	22.1	9, 10, 11, 13
13	0.78 (d, *J* = 6.6 Hz)	22.7	9, 10, 11, 12	0.78 (d, *J* = 5.6 Hz)	22.0	9, 10, 11, 12
NH				10.30 (s)		
3-OH				6.05 (s br)		

a : CD_3OD, 300 MHz. b : CD_3OD, 125 MHz c : DMSO-d_6, 300 MHz. d : DMSO-d_6, 125 MHz

The brominated isatin derivatives **54, 56, 57, 64** and the new 5-hydroxyisatin derivative **65** are new natural products, which are, however, related with a few other isatin derivatives, e.g. convolutamydine A (**66**) from the marine bryozoan *Amathia convoluta*.[79] According to their rapid formation from isatins and the respective ketones, they may be artifacts, which are formed in a ketone-rich surrounding. In contrast, the samples **56, 57, 64** and **65** showed optical activity. But as the crystal structure analysis of **56** and **57** showed a recemic mixture, a low enantiomeric excess must be assumed. Nevertheless this is of interest, as the involvement of enzymes is indicated.

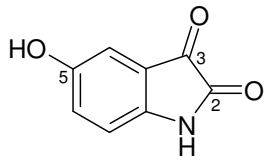


66

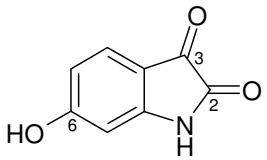
3.1.10 Synthesis of hydroxylated isatins and their acetone adducts

Acetonyloxindole (**58**) is formed easily on standing of isatin acetone solution, and the same can be assumed for the formation of **65** from 5-hydroxyisatin (**72**) or correspondingly, of **54, 56, 57,** and **64** from 5-bromoisatin and the respective ketones. To further confirm structure **65** and to distinguish between the 5-hydroxy and 6-hydroxy isomers, both 5-hydroxy- and 6-hydroxyisatins (**72** and **66**) were synthesized. A further reason for this task was the isolation of 6-hydroxyisatin (**66**) from the marine *Streptomyces* sp. isolate B1848[80] in such a small amount, that it was not possible to distinguish between the 5- and 6-hydroxy isomers just on the basis of NMR data.

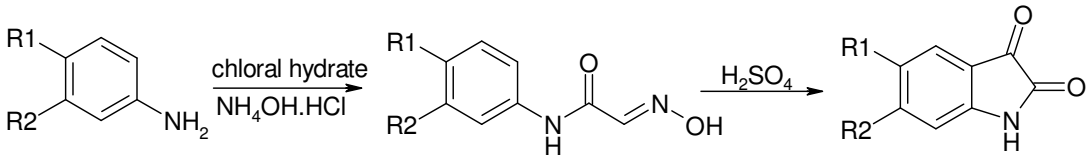
5-Hydroxyisatin (**67**) and 6-hydroxyisatin (**68**) were isolated previously in our group. The structures however could not be unequivocally confirmed due to small amounts. This stimulated the synthesis of **72** and the positional isomer **66** from the **69a** and **72b** (Scheme 2). The synthesis of the latter was achieved by condensation of substituted anilines[81], **70a** and **72b** with chloral hydrate to give the corresponding methoxy substituted isonitrosoacetanilide **71a** and **72b** (Scheme 2). The ^{1}H NMR data of **72a** confirmed the imino proton by a singlet at δ 7.70, along with two singlets of the exchangeable oxime (=N-OH) and amide protons at δ 12.00 and δ 10.00, respectively. The **72a** and **72b** was cyclized by acid catalysis (Scheme 2) to give the corresponding isatins, **72a** and **72b**, respectively. Hydrolysis of 6-methoxy isatin (**72b**) using boron-tribromide gave the hydroxyl analogue[82], whereas the 5-methoxy isatin (**72a**) gave 5-hydroxy-isatin (**72**).



72



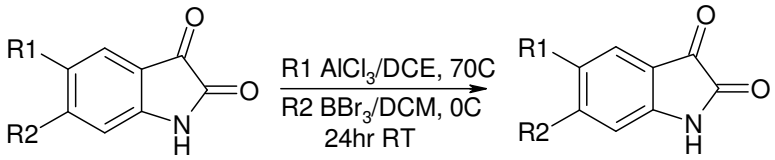
66



72a: $R^1 = OCH_3$
$R^2 = H$
72b: $R^1 = H$
$R^2 = OCH_3$

72a: $R^1 = OCH_3$
$R^2 = H$
72b: $R^1 = H$
$R^2 = OCH_3$

72a: $R^1 = OCH_3$
$R^2 = H$
72b: $R^1 = H$
$R^2 = OCH_3$



72a: $R^1 = OCH_3$
$R^2 = H$

72b: $R^1 = H$
$R^2 = OCH_3$

72: $R^1 = OH$
$R^2 = H$

66: $R^1 = H$
$R^2 = OH$

Scheme 2: Synthesis of the Isatins **66** and **72**

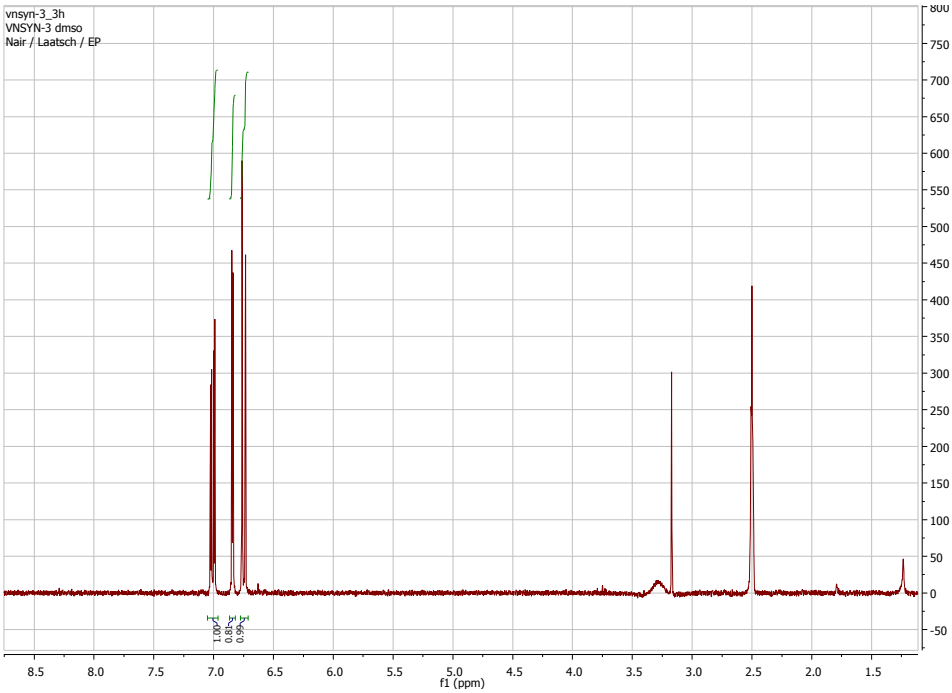


Figure 22: ^{1}H NMR spectrum (DMSO-d_6, 300 MHz) of 5-hydroxyisatin (**72**)

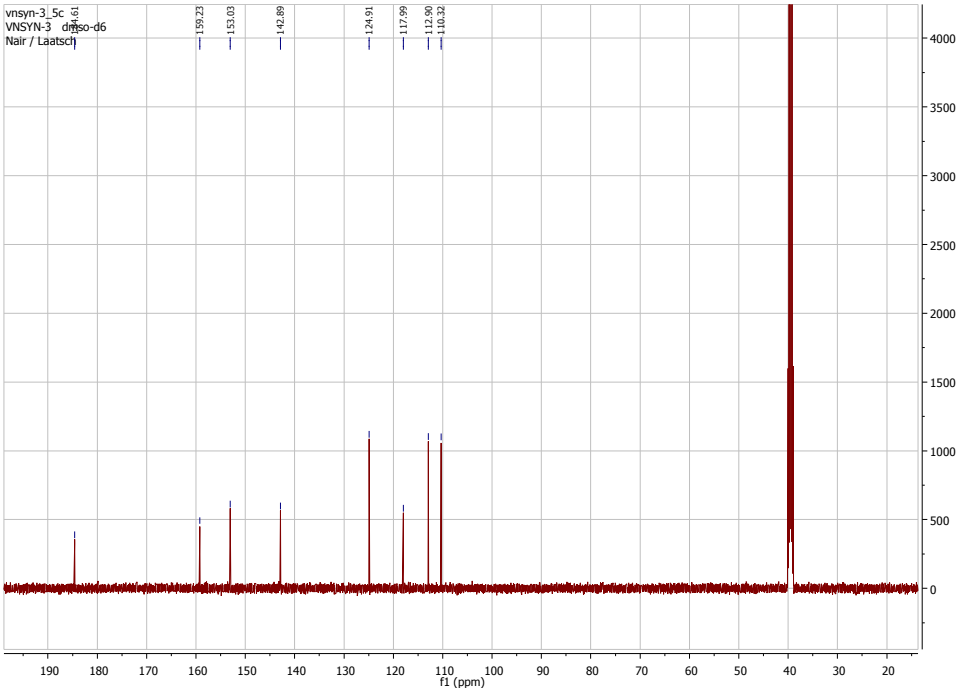


Figure 23: ^{13}C NMR spectrum (DMSO-d_6, 75 MHz) of 5-hydroxyisatin (**72**)

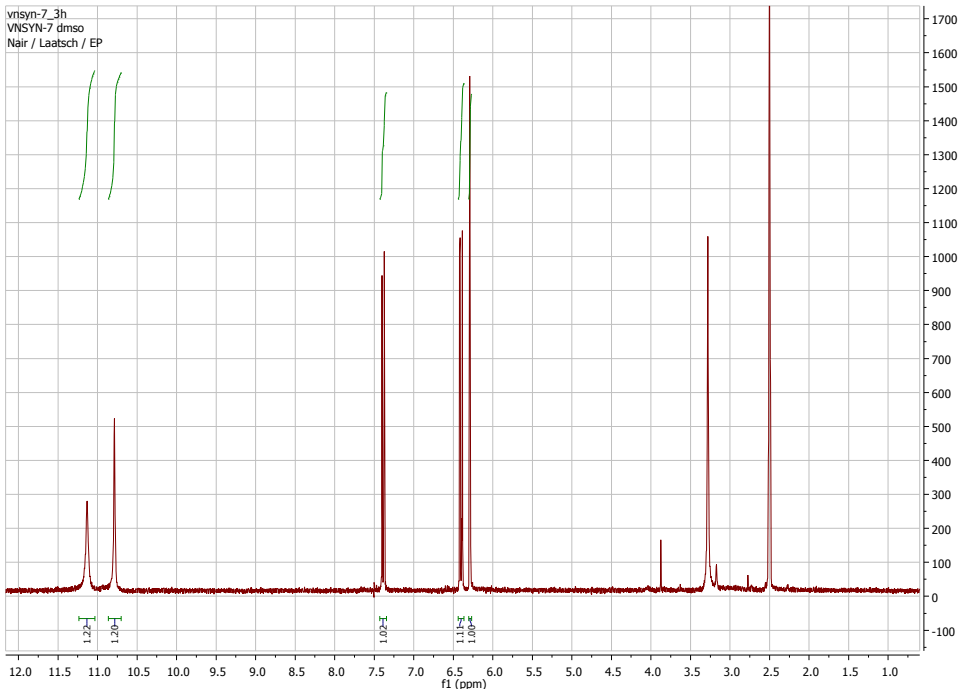


Figure 24: ^{1}H NMR spectrum (DMSO-d_6, 300 MHz) of 6-Hydroxyisatin (**66**)

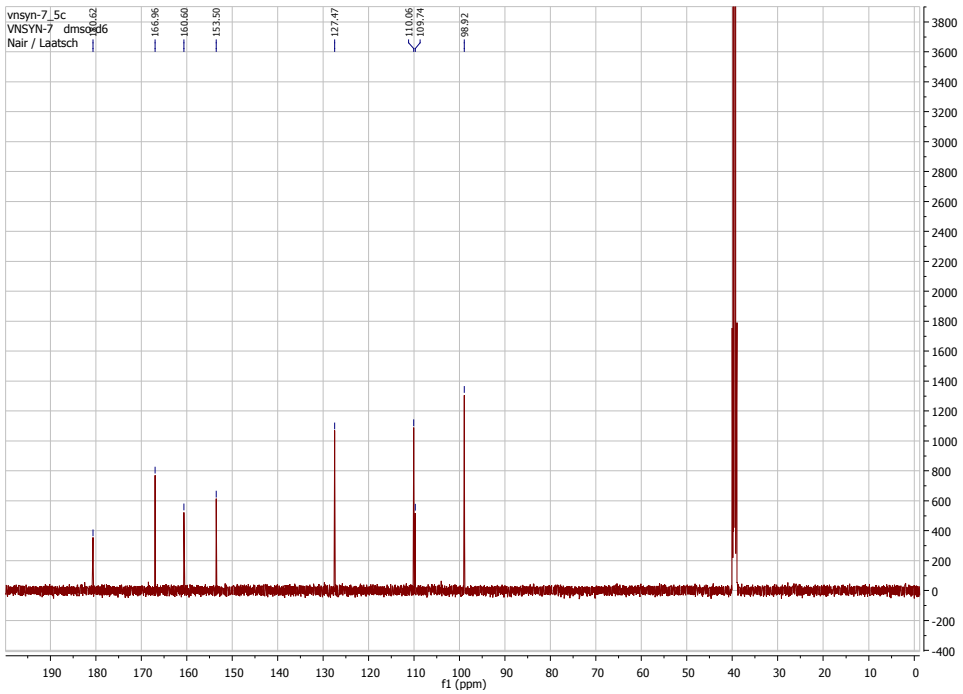
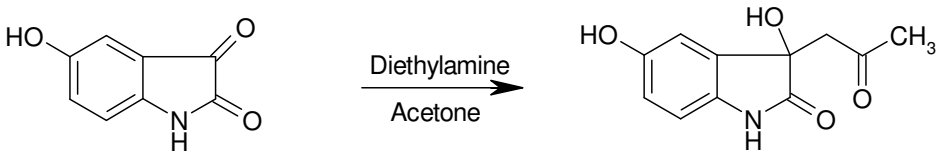


Figure 25: ^{13}C NMR spectrum (DMSO-d_6, 75 MHz) of 6-Hydroxyisatin (**66**)

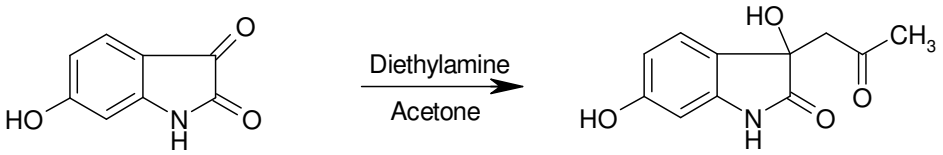
Synthesis of 3,5-dihydroxy-3-acetonylisatin

Synthesis of 3,5-dihydroxy-3-acetonyl-isatin (**73**) was achieved by Knoevenagel condensation of 5-hydroxyisatin with acetone in presence of diethyl amine as base.

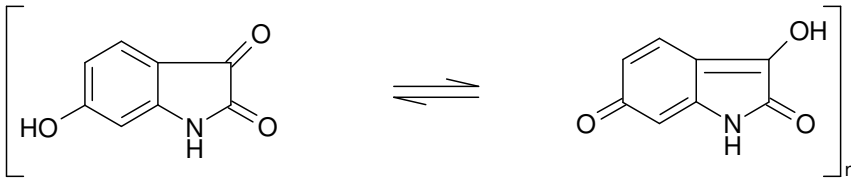


72 **73**

Similarly, the synthesis of 3,6-dihydroxy-3-acetonylisatin (**74**) was also attempted, but the aldol reaction of 6-hydroxyisatin with acetone could not be achieved. As the hydroxyl group is in *para* orientation to the carbonyl at position 3, a prototrop-isomer can be formed, which reduces the carbonyl reactivity.



66 **74**



3.2 Marine *Streptomyces* sp. T262

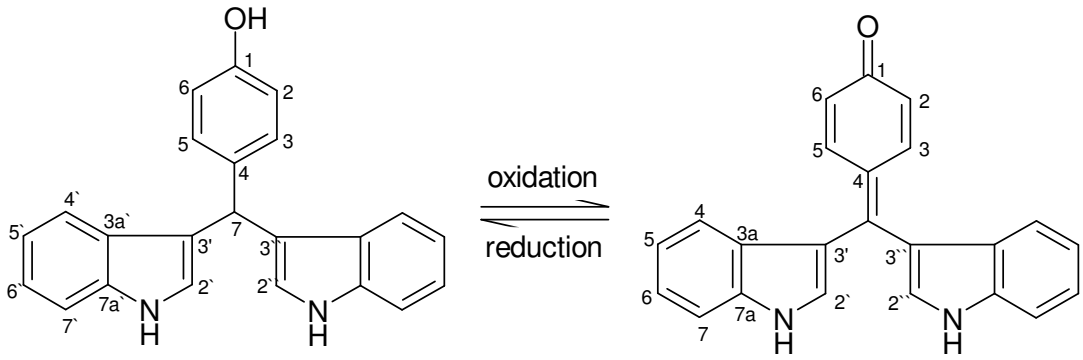
The *Streptomyces* sp. strain T262 was isolated from the Antarctic shelf ice*;* it was chosen because of its high antibiotic activities. The cultivation of the strain was done at the Institute for Biotechnology and Drug Research (Kaiserslautern). The crude extract showed on TLC a series of yellow zones in the polar as well as in the non-polar range, which showed red-brown colour reaction which anisaldehyde/sulphuric acid. Several new indole and tris-indole derivates were isolated, which were different from those isolated from the earlier fermentation, and their structures were established on the basis of NMR, MS, and other spectroscopic methods, and by comparison with known and the previously isolated compounds. Fifteen indole derivatives were isolated, namely 7,7-bis(3-indolyl)-*p*-cresol (**83**), 4-[bis-(1*H*-indol-3-yl)-methyl]-cyclohexa-2,5-dienone (**84**), 3,3'-[(4-butoxy-phenyl)-methylene]-bis-(1*H*-indole) (**85a**), 3,3'-[(4-propoxy-phenyl)-methylene]-bis-(1*H*-indole) (**85b**), 3,3'-[(4-ethoxy-phenyl)-methylene]-bis(1*H*-indole) (**85c**), 4-(1*H*-Indol-3-yl-sulfanyl)-phenol (**86**), turbomycin A (**75**), turbomycin B (**88**), 3,3'-bis-indolylmethane (**76**), trisindoline A (**77**), trisindoline B (**78**), 1,1,1-tris(3-indolyl)-methane (**79**), trisindolal (**80**), trisindonone (**81**), and 3,3'-[(4-methoxy-3,5-dinitrophenyl)methylene]-bis-(1*H*-indole) (**82**).

3.2.1 7, 7-Bis (3-indolyl)-*p*-cresol

Compound **83** has a molecular formula of $C_{23}H_{18}N_2O$, which was determined by high-resolution electron spray ionisation (HRESI) mass spectrometry. ^{1}H and ^{I3}C NMR spectral data summarized in Table 4; ^{1}H-^{1}H COSY data of **62** revealed the presence of a 1,4-disubstituted benzene ring, two 1,2-disubstituted benzene rings, three methines, and two quaternary sp^2 carbons. Assignments of all the protonated carbons were made by HSQC experiments. The HMBC spectrum showed three-bond couplings of H-4' to C-6' and C-7a', H-5' to C-3a' and C-7', H-6' to C-4' and C-7a', and H-7' to C-3a' and C-5'. The proton at δ 6.67 (s, H- 2') showed HMBC correlations to C-3' [δ 119.8 (s)], C-3a' [δ 127.2 (s)], and C-7a' [δ 136.9 (s)]. These findings indicated the presence of a 3-indolyl unit, which was confirmed by the fragment ion peak at *mlz* 222 $[M-C_8H_6N]^+$ on ESIMS of **83**. The integral values (each 2H) of

the protons in the 3-indolyl unit exhibited that **83** has two 3-indolyl units symmetrically arranged in the molecule. The proton signals at δ 6.73 (2H, d, J = 8.3 Hz, H-2 and H-6) and 7.17 (2H, d, J = 8.3 Hz**,** H-3 and H-5) correlated to carbon signals at δ115.0 (d) and 129.8 (d), respectively, arising from aromatic protons of a *p*-hydroxyphenyl unit. This was supported by the presence of a fragment ion peak at *mlz* 245 $[MC_6H_5O]^+$ in the ESIMS**.** The HMBC spectrum showed two- and three-bond couplings for H-7 (δ 5.81, s) to C-3, C-4, C-5, C-2', C-2", C-3', C-3", C-3a', and C-3a", confirming that the two 3-indolyl units and the *p*-hydroxyphenyl unit are attached to C-7.

The antimicrobial activity of compound **83** against several test microorganisms was measured by the paper disc diffusion method. Compound **83** showed activity against *Bacillus subtilis* and *Escherichia coli* at 10 μg/disk but did not affect the growth of *Pseudomonas aeruginosa, Staphylococcus aureus, Saccharomyces viridochromogenes,* and the alga *Chlorella vulgaris.* Compound **84** is a quinonoid oxidation product of **83** and showed a higher cytotoxic potency (mean IC_{50} = 4.2 μg/ml) than the precursor **83** (mean IC_{50} = 7.49 μg/ml).

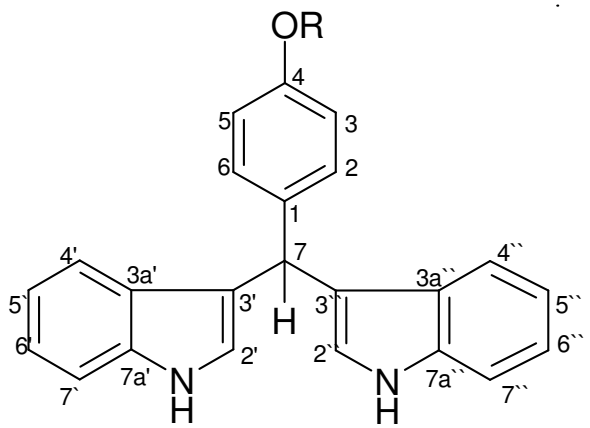


83 **84**

Table 4: ^{1}H NMR and ^{13}C NMR data of 7,7-bis-(3-indolyl)-*p*-cresol (**83**)

No	^{1}H (δ_H, mult, *J* Hz)[a]	^{13}C (δ_C)[b]
1		154.8
2	6.73 (d 8.3)	115.0
3	7.17 (d, 8.3)	129.8
4		135.8
5	7.17 (d, 8.3)	129.8
6	6.73 (d, 8.3)	115.0
7	5.81 (s)	39.5
2', 2"	6.67 (s)	123.7
3', 3"		119.8
3a', 3a"		127.2
4', 4"	7.38 (d, 8.3)	119.9
5', 5"	6.96 (t, 8.3)	118.8
6',6"	7.13 (t, 8.3)	121.6
7', 7"	7.35 (d, 8.3)	111.1
7a', 7a"	6.73 (d 8.3)	136.9

[a] DMSO-d_6, 300 MHz. [b] DMSO-d_6, 125 MHz



85a R = $CH_2CH_2CH_2CH_3$
85b R = $CH_2CH_2CH_3$
85c R = CH_2CH_3

Table 5: ^{1}H and ^{13}C NMR data of 3,3'-[(4-butoxyphenyl)-methylene]-bis(1*H*-indole) (**85a**), 3,3'-[(4-propoxyphenyl)-methylene]-bis(1*H*-indole) (**85b**) and 3,3'-[(4-ethoxyphenyl)-methylene]-bis-1*H*-indole) (**85c**)

	3,3'-[(4-butoxyphenyl]-methylene)-bis(1*H*-indole) (**85a**)			3,3'-[(4-propoxyphenyl]-methylene)-bis(1*H*-indole) (**85b**)			3,3'-[(4-ethoxyphenyl]-methylene)-bis(1*H*-indole) (**85c**)		
Position	^{1}H (δ_H, mult., *J* Hz)[a]	^{13}C (δ_C)[b]	HMBC	^{1}H (δ_H, mult., *J* Hz)[a]	^{13}C (δ_C)[b]	HMBC	^{1}H (δH, mult., *J* Hz)[a]	^{13}C (δ_C)[b]	HMBC
1	-	135.9	-	-	135.9	-	-	135.9	-
2, 6	7.25 (d, *J* = 8.7Hz)	129.5	3, 4, 5, 7	7.25 (d, *J* = 8.7Hz)	129.5	3, 4, 5, 7	7.23 (d, *J* = 8.6Hz)	129.5	3, 4, 5, 7
3, 5	6.80 (d, *J* = 8.7 Hz)	114.1	2, 5, 6	6.80 (d, *J* = 8.7 Hz)	114.1	2, 5, 6	6.90 (d, *J* = 8.7 Hz)	114.0	2, 5, 6
4	--	157.4	-	-	157.4	-	-	157.1	-
7	5.82 (s)	39.4	1, 2, 6, 3', 3", 3a"	5.82 (s)	39.4	1, 2, 6, 3', 3", 3a"	5.83 (s)	39.3	1, 2, 6, 3', 3", 3a"
8	3.92 (t, *J* = 6.5 Hz)	67.6	9, 10, 11	3.89 (t, *J* = 6.5 Hz)	67.6	4, 9, 10,	3.99 (q, *J* = 7.0 Hz)	63.3	4, 9
9	1.75 (tt, *J* = 6.7, 9.7 Hz)	31.5	8, 10, 11	1.82 (tt, *J* = 6.7, 9.7 Hz)	31.5	8, 10	1.28 (t, *J* = 6.7Hz)	14.9	8
10	1.47 (tq, *J* = 7.6, 7.2 Hz)	19.4	8, 9, 11	1.04 (tq, *J* = 7.6, 7.2 Hz)	19.4	3, 4, 5, 7	-	-	-
11	0.96 (t, *J* = 7.33)	13.9	8, 9, 10	-	-	-	-	-	-
1', 1"	7.8 (NH)	-	7a', 7a", 2', 2"	7.72 (NH)	-	7a', 7a", 2', 2"	7.77 (NH)	-	7a', 7a", 2', 2"
2', 2"	6.60 (s)	123.4	1, 1', 3', 3", 7	6.54 (brd, *J* = 1.4)	123.4	1, 1', 3', 3", 7	6.52 (brd, *J* = 1.5)	123.4	1, 1', 3', 3", 7
3', 3"	-	119.1	-	-	119.1	-	-	119.9	-
3a', 3a"	-	126.9	-	-	126.9	-	-	126.9	-
4', 4"	7.36 (d, *J* = 7.94 Hz)	119.9	5', 5" 6', 6", 7a', 7a"	7.32 (d, *J* = 7.94 Hz)	119.9	5', 5" 6', 6", 7a', 7a"	7.31 (d, *J* = 8.1 Hz)	119.8	5', 5" 6', 6", 7a', 7a"
5', 5"	6.99 (ddd, *J* = 8.0, 7.0, 1.0 Hz, 1H)	121.7	3a', 3a", 4', 4", 6', 6", 7', 7"	6.98 (ddd, *J* = 8.0, 7.0, 1.0 Hz, 1H)	121.7	3a', 3a", 4', 4", 6', 6", 7', 7"	6.97 (ddd, *J* = 8.0, 7.0, 1.0 Hz, 1H)	121.7	3a', 3a", 4', 4", 6', 6", 7', 7"
6', 6"	7.15 (ddd, *J* = 8.2, 7.1, 1.1 Hz, 1H)	121.8	4', 4", 5', 5", 6', 6", 7', 7", 7a', 7a"	7.15 (ddd, *J* = 8.2, 7.1, 1.1 Hz, 1H)	121.8	4', 4", 5', 5", 6', 6", 7', 7", 7a', 7a"	7.13 (ddd, *J* = 8.2, 7.1, 1.1 Hz, 1H)	121.8	4', 4", 5', 5", 6', 6", 7', 7", 7a', 7a"
7', 7"	7.38 (d, *J* = 7.94 Hz)	110.9	5', 5", 6', 6, 7a', 7a"'	7.38 (d, *J* = 7.94 Hz)	110.9	5', 5", 6', 6, 7a', 7a"'	7.39 (d, *J* = 7.9Hz)	110.9	5', 5", 6', 6, 7a', 7a"'
7a', 7a"	--	136.6	-	-	136.6		-	136.5	-

[a] $CDCl_3$, 300 MHz. [b] $CDCl_3$, 125 MHz

3.2.2 3,3'-[(4-Butoxyphenyl)methylene]-bis(1*H*-indole)

Compound **85a** was obtained as a pale orange solid, which gave a colourless spot on TLC. Its EI mass spectrum gave a molecular ion at 394 [M^+], ESI-MS showed a *pseudo*molecular ion at 393 $[M+H]^+$, and HRESIMS (393.19615) suggested the molecular formula $C_{27}H_{24}N_2O$, which was due to oxidation. Its ^{1}H NMR spectrum showed two broadened *o*-coupled doublets at δ 7.32 and 7.38 (both J = 7.94 Hz), and two triple doublets at δ 7.15 and 6.99 (each J = 8.0, 7.0, 1.0 Hz) indicating the presence of a 1,2-disubstituted benzene ring. Another proton in the aromatic region at δ 6.60 (s) together with the positive Ehrlich reaction on TLC indicated a 3-substituted indole ring. In addition to these signals, a 1,4-disubstituted benzene ring was observed by signals at δ 6.80 (d, J = 8.7 Hz) and 7.25 (d, J = 8.7 Hz). Furthermore it's ^{1}H NMR spectrum showed signals at δ 3.92 indicating the presence of an oxygenated CH_2 group, and multiplets at δ 1.82, 1.52 and a triplet at δ 0.96 indicated the presence of an alkyl chain, which was identified as an *n*-butyl residue by ^{1}H-^{1}H COSY correlations between H8-H11. An HMBC correlation was observed between H8 and C4, which confirmed the placement of *n*-butyl chain as shown in Figure 26. All the aromatic signals related to the indole system were found to be doubled in their intensity with respect to the alkyl signals. The ^{13}C NMR and HSQC data indicated the presence of only 16 carbon signals of which five were due to quaternary carbon atoms, seven methine carbons, and four alkyl chain groups. The ^{1}H NMR spectrum, the MS fragmentation pattern and high resolution MS confirmed the presence of two indolyl moieties in a symmetrical orientation. From the foregoing spectral data the structure of the compound **85a** was established as 3,3'-[(4-butoxyphenyl)methylene]bis(1*H*-indole).

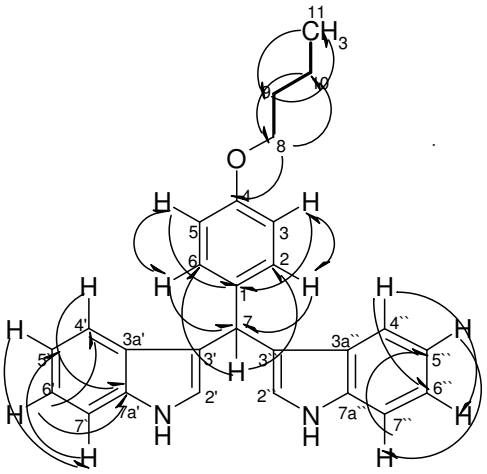


Figure 26: HMBC correlations of 3,3'-[(4-butoxyphenyl)methylene]bis(1*H*-indole) (**85a**)

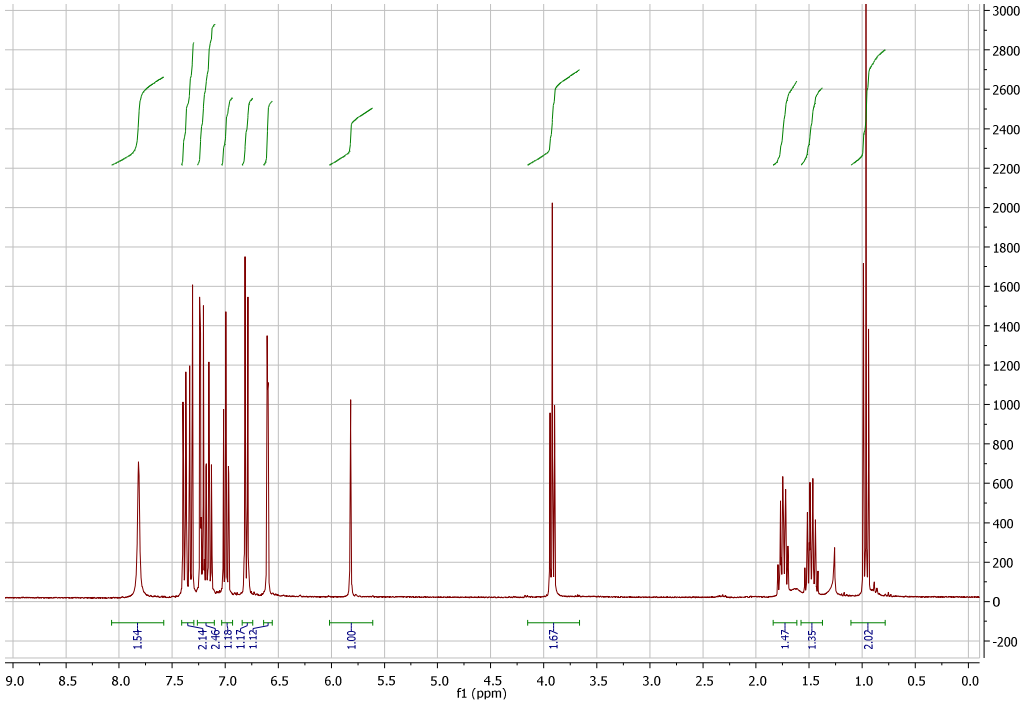


Figure 27: ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of 3,3'-[(4-butoxyphenyl)methylene]bis(1*H*-indole) (**85a**)

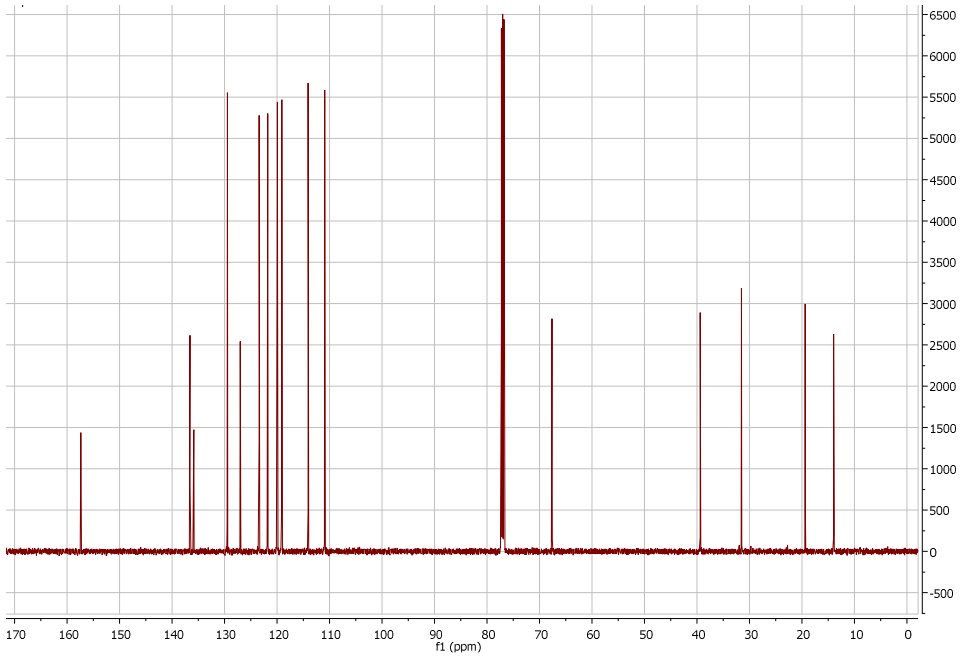


Figure 28: ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of 3,3'-[(4-butoxyphenyl)methylene]-bis(1*H*-indole) (**85a**)

3.2.3 3,3'-[(4-Propoxyphenyl)methylene]-bis(1*H*-indole)

Compound **85b** was isolated as a strongly UV absorbing solid with an EI-MS (380). The HRESIMS (379.15715) data suggested that the molecular formula is $C_{26}H_{22}N_2O$. The doublets at δ 7.38, 7.32 and 6.54 (br d, J = 1.46) and three double doublets at δ 7.15 and 6.98 in the ^1H NMR spectrum revealed the presence of the same 3-substituted indolyl system as found in the previous compound **85a.** Further, its ^1H NMR spectrum displayed a singlet at δ 5.82. All the aromatic signals were found to be doubled in their intensity relative to the higher field signal at δ 5.82. The ^{13}C experiments indicated a total of 15 carbon signals, which included 7 methine and 5 quaternary carbon atoms in the skeleton and 3 methylene carbons. Except for the methine signal at δ 39.4, all the remaining signals were found to be due to the 3-substituted indolyl system. The low number of carbon signals together with the high mass suggested the structure to be highly symmetrical. Signals were observed at δ 6.80 (d, J = 8.7 Hz) and 7.25 (d, J = 8.7 Hz), which were due to 1,4 substitution of an aromatic system. Since the proton H-7 at δ 5.82 was visible and showed correlations with C-3', C-3", C-2', C-2", C-2 and C-6 but the mass suggested 2 protons less than the expected value of 380, which could be due to a redox reaction. According to

the EI mass fragmentation pattern with a signal at δ 117 typical of 3-substituted indoles and an M-118 ion at 243, the structure of the compound was found to be 3,3'-[(4-propoxyphenyl)methylene]-bis(1*H*-indole) (**85b**).

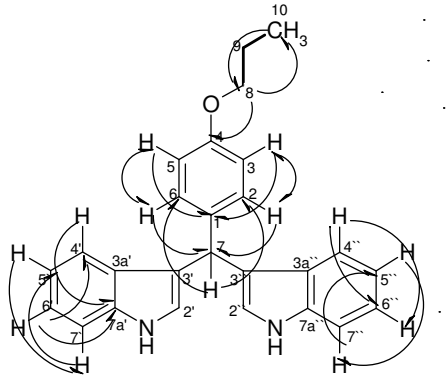


Figure 29: HMBC correlations of 3,3'-[(4-propoxyphenyl)methylene]-bis(1*H*-indole) (**85b**)

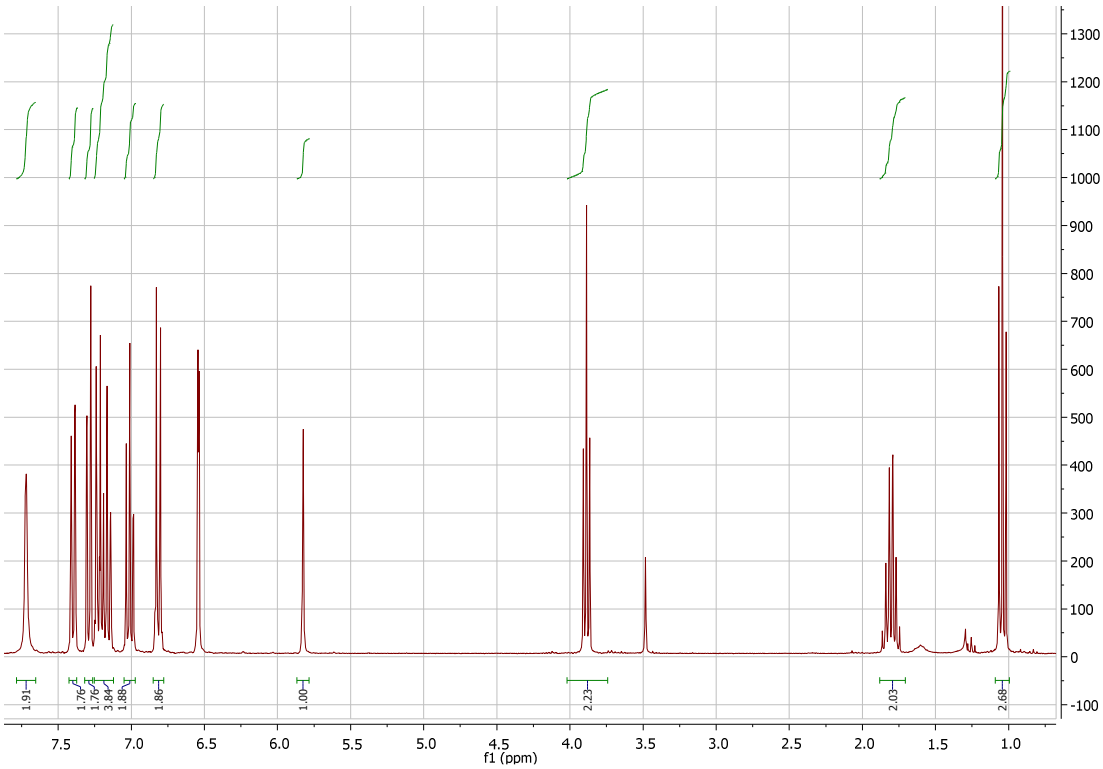


Figure 30: ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of 3,3'-[(4-propoxyphenyl)methylene]-bis(1*H*-indole) (**85b**)

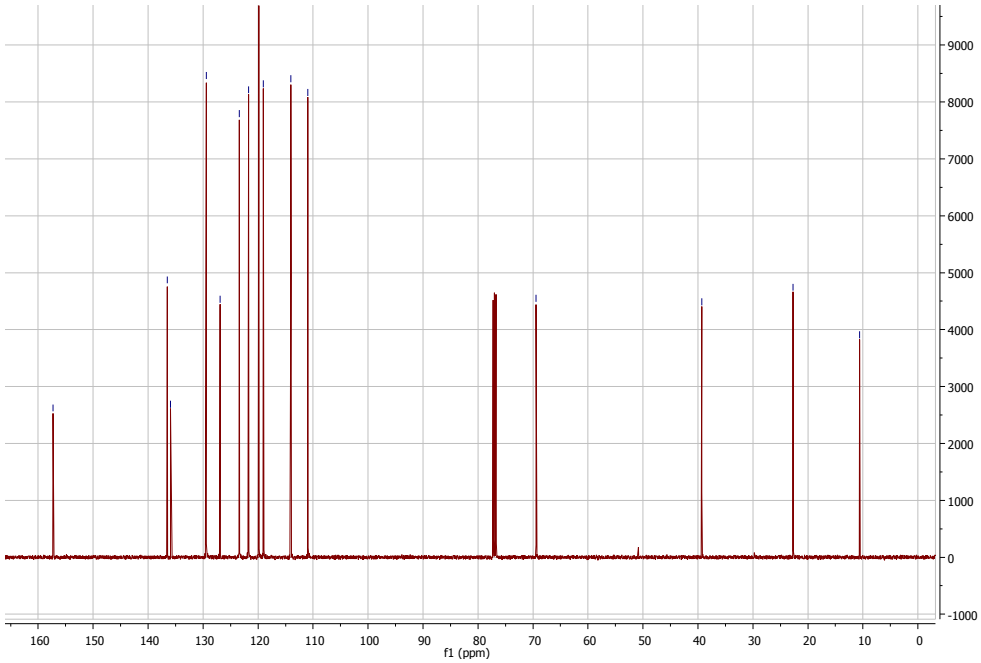


Figure 31: ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of 3,3'-[(4-propoxyphenyl)-methylene]-bis(1*H*-indole) (**85b**)

3.2.4 3,3'-[(4-Ethoxyphenyl)methylene]-bis(1*H*-indole)

Compound **85c** was isolated as a strongly UV absorbing solid by an EI-MS (366). The HRESIMS $[M+H]^+$ (365.16480) suggested that the molecular formula is $C_{25}H_{21}N_2O$, which was obviously due to oxidation. The doublets at δ 7.39, 7.31 and a broad doublet at δ 6.52, and three doublets at δ 7.13 and 6.97 in the ^{1}H NMR spectrum revealed the presence of the same 3-substituted indolyl system as found in the previous compound **85c**. Further, its ^{1}H NMR spectrum displayed a singlet at δ 5.83. All the aromatic signals were found to be doubled in their intensity relative to the higher field signal at δ 5.83. Also signals were observed at δ 6.90 (d, J = 8.7 Hz) and 7.23 (d, J = 8.7Hz) for a 1,4 substituted aromatic sytem as in the previous compounds. This compound was having the same pattern as in the previous compounds but this time having an oxygenated methylene at δ 3.99 and a triplet at δ 1.4 hence making it an oxygenated ethyl group. The dimeric bis indole **85a, 85b** and **85c** has not been reported so far, but a closely related compound, has been reported[83] and also isolated from this strain.

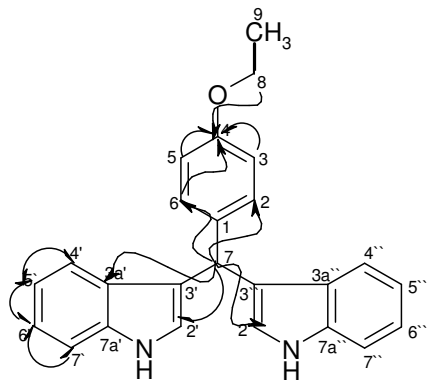


Figure 32: HMBC correlations of 3,3'-[(4-ethoxyphenyl)methylene]-bis(1*H*-indole) (**85c**)

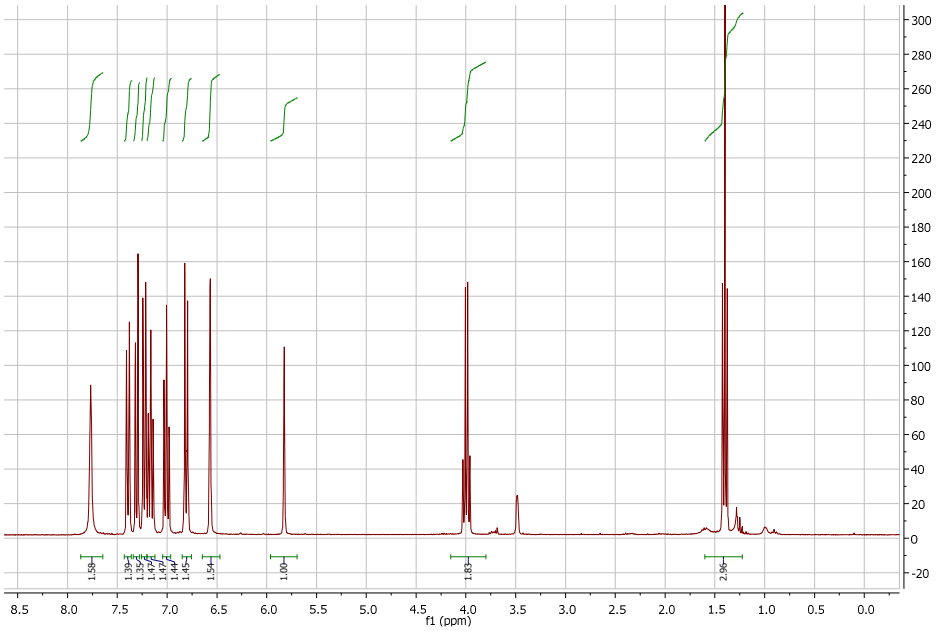


Figure 33: ^{1}H NMR spectrum (300 MHz,$CDCl_3$) of 3,3'-[(4-ethoxy-phenyl)-methylene]-bis-(1*H*-indole) (**85c**)

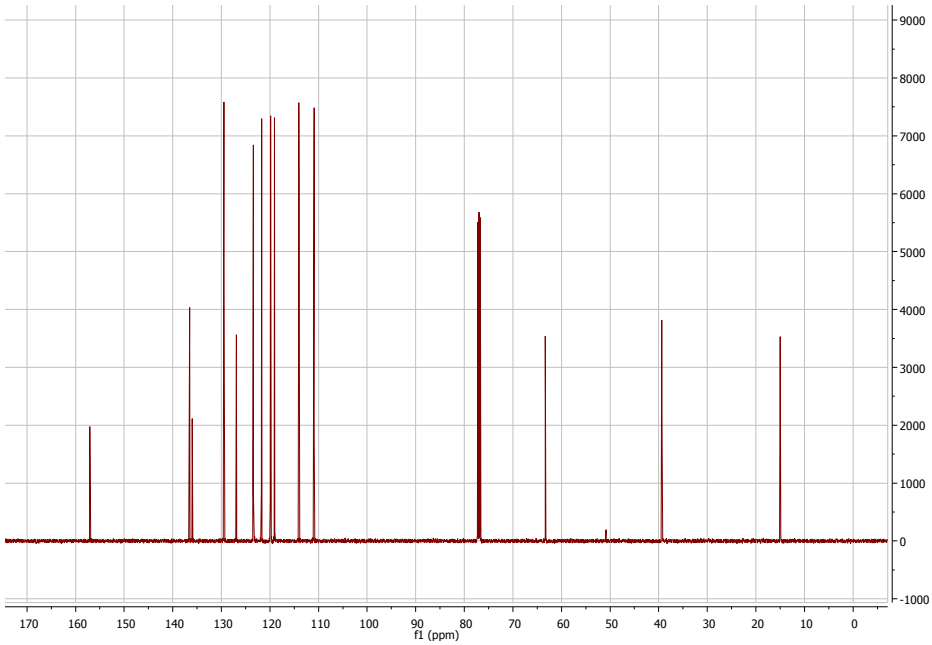
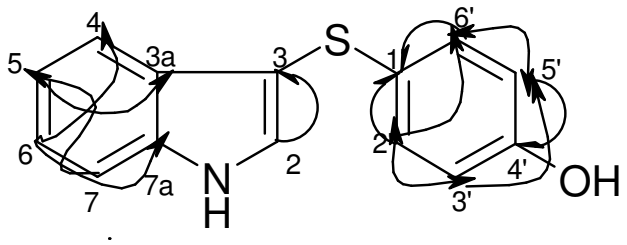


Figure 34: ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of 3,3'-[(4-ethoxy-phenyl)-methylene]-bis1*H*-indole) (**85c**)

3.2.5 4-(1*H*-Indol-3-ylsulfanyl)-phenol

Compound **86** was obtained as a solid, which gave a colourless spot on TLC. Its ESI mass spectrum gave a molecular ion at 240 $[M-H]^-$. The HRESIMS data (240.04896) suggested that the molecular formula is $C_{14}H_{11}N_1S_1O$. Its ^{1}H NMR spectrum showed two overlapped triplets at δ 7.48 and 7.46 and also overlapped signals at δ 7.03 and 7.13 (dd, J = 7.8, 1.4), which is a typical indole pattern. Another proton in the aromatic region at δ 7.49 (s) together with the positive Ehrlich reaction on TLC indicated a 3-substituted indole ring. Doublets at δ 6.58 (d, J = 8.6) and 6.96 (d, J = 8.6) and a large coupling constant indicated a *para* disubstitution. A carbon value of δ 156.4 at C4' and the highfield shift of the *ortho* ^{1}H signals indicated the presence of an OH group. From the foregoing spectral data the structure of compound **86** was established as shown in Figure 35



86

Figure 35: HMBC correlation of 4-(1*H*-Indol-3-ylsulfanyl)-phenol (**86**)

Table 6: ^{1}H and ^{13}C NMR data of 4-(1*H*-indol-3-ylsulfanyl)-phenol (**86**)

4-(*1H*-Indol-3-ylsulfanyl)-phenol (**86**)			
No.	^{1}H ($\delta_{H,}$ mult., *J* Hz)[a]	^{13}C (δ_{C})[b]	HMBC
2	7.49 (s)	131.6	3
3	-	104.4	-
3a	-	130.3	-
4	7.48 (d, *J* = 7.8)	119.8	3a, 5, 6
5	7.03 (dd. *J* = 7.8, 1.4)	120.8	3a, 4, 6, 7
6	7.13 (dd, *J* = 7.8, 1.4)	123.1	4, 5, 7, 7a
7	7.40 (d, *J* = 7.8)	112.7	3a, 7a, 6, 5
7a	-	138.3	1, 2, 5, 10
1’	-	129.7	-
2’, 6’	6.96 (d, *J* = 8.6)	129.6	8, 10,11
3’, 5’	6.58 (d, *J* = 8.6)	116.4	8, 10,11
4’	-	156.4	-

[a]CD_3OD, 600 MHz. [b]CD_3OD, 125 MHz

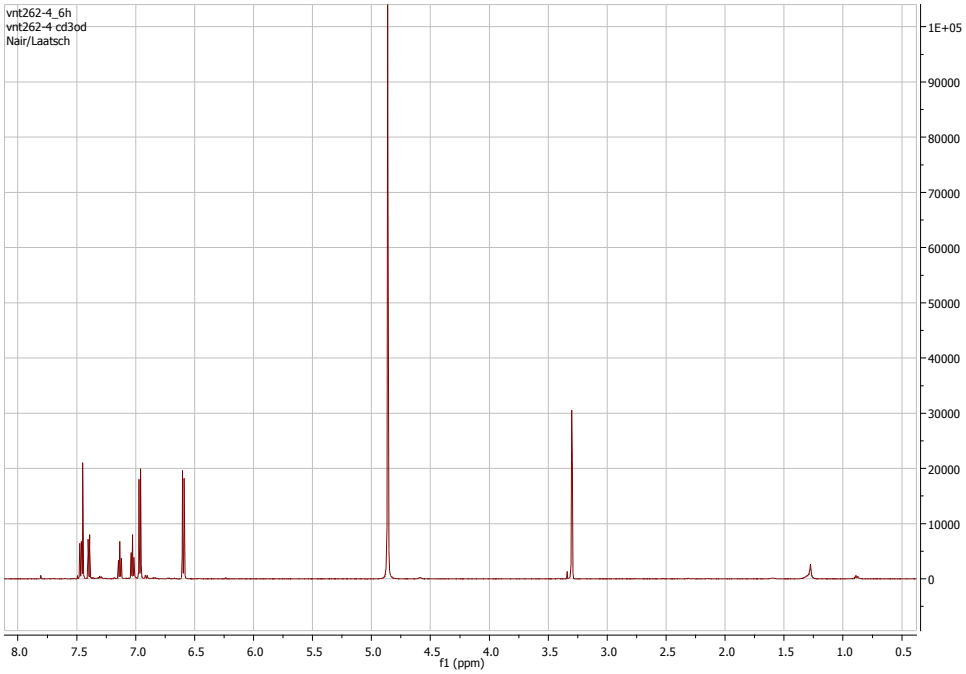


Figure 36: ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of 4-(1*H*-indol-3-ylsulfanyl)-phenol (**86**)

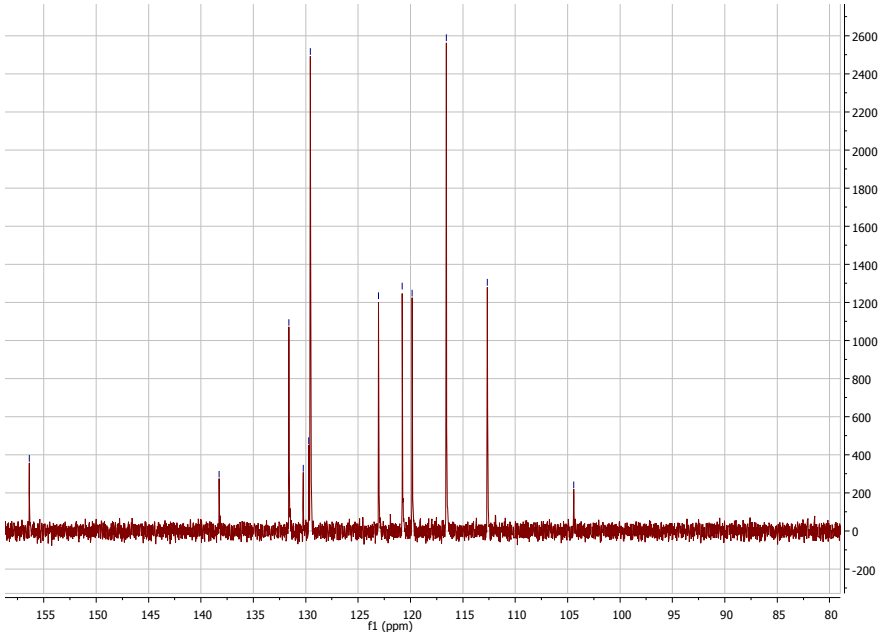
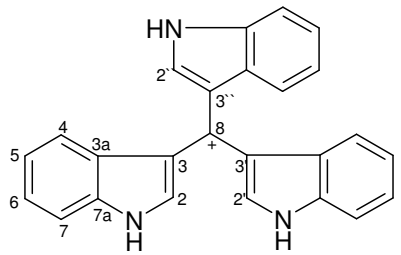


Figure 37: ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of 4-(1*H*-indol-3-ylsulfanyl)-phenol (**86**)

3.2.6 Turbomycin A and B

The ESIHRMS data for turbomycin A indicated a molecular formula of $C_{25}H_{18}N_3$, while ^1H NMR experiments suggested the presence of only five protons and the *ortho*-substituted aromatic ring of the indole was easily identified from the ^1H NMR chemical shifts, and the nitrogen at position 1 was suggested by the deshielding of C-2, C-7a, and H-2. All five protons and nine carbons observed by NMR were present in the C-3 substituted indole partial structure. The structure of turbomycin A (**75**) as the indole trimer was supported by the molecular formula deduced by ESI-MS. In liquid culture, the MIC of turbomycin A (orange) was 6.2 μg/ml for *E. herbicola*, *B. subtilis, S. aureus,* and *S. pyogenes* and 12.5 μg/ml for *Salmonella enterica* serovar. *typhimurium*[84]. For turbomycin B, the presence of the indole was deduced in the same manner as described above for turbomycin A and the phenyl group was apparent from both the ^1H NMR spectrum and ^{13}CNMR experiments. Again, the trimeric structure of turbomycin B (**88**) was suggested by the molecular formula predicted by ESIHRMS, $C_{23}H_{17}N_2$. As with turbomycin A, the proposed structure of turbomycin B was previously confirmed by synthesis[28] from our group; benzaldehyde was heated with indole in 10% acetic acid, and the resulting heterotrimeric methane was then

oxidized *in situ* with tetrachloro-1,4-benzoquinone to yield a red compound that was spectroscopically identical with the compound[85] isolated from the culture media. Turbomycin A has been also previously characterized from a fungal[86] source and was synthesized[85].



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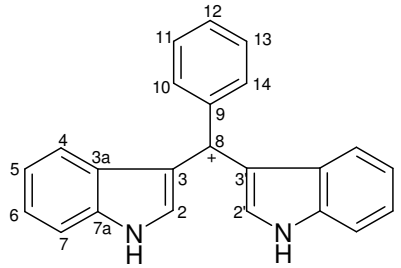
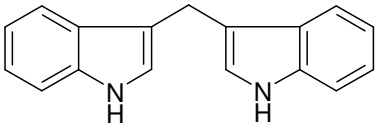


88

3.2.7 3, 3'-Bisindolylmethane

Inspection of the NMR spectra of **76** revealed that the five aromatic signals (δ 6.94, 7.05, 7.14, 7.35 and 7.55) were in the same intensity to the higher field signal of methylene group at δ 4.16 (2H, s). The two doublets of doublets (δ 7.35 and 7.55) and the two doublets of triplets (δ 6.94 and 7.05) were contributed to a 1,2-disubstituted aromatic ring. The other proton in this region (δ 7.14, br s) was coupled through three bonds (J = 2.2 Hz). The latter two fragments fit on an indole system substituted at position 3. The two identical 3-indolyl fragments could finally be constructed with the assistance of AntiBase, which was confirmed by ESI mass spectrometry which showed $[M+Na]^+$ ion peak corresponding to molecular weight 269. Hence the compound was confirmed to be 3,3'-bisindolylmethane (**76**).



76

3.2.8 Trisindoline A and 2,2-Bis-(3-indolyl)-3-indolone

The ^{1}H NMR spectrum of trisindoline showed in total fourteen proton signals, among which five at δ 7.75, 7.69, 7.52, 7.43 and 7.37 were observed each with 2H intensity. Among the twenty-four carbons of **77**, only sixteen showed distinct signals in the ^{13}C NMR spectrum. These signals were assignable to fourteen aromatic carbons, one carbonyl carbon at δ 201.2 and one quaternary carbon at δ 55.7. The ^{1}H, ^{1}H-correlation spectrosopy of **77** revealed the presence of two types of *ortho*-disubstituted benzene moieties, one of which showed two-proton signals at δ 7.75 (d, J = 7.5 Hz), 7.69 (ddd, J = 7.5, 7.5, 1.0 Hz), 7.52 (ddd, 7.5, 7.5, 1.0 Hz) and 7.43 (d, J = 7.5 Hz). The presence of two identical indole moieties in **77** was figured out from the correlations obtained by ^{1}H-^{13}C, COSY and from the HMBC spectrum of **77** and **78**. Based on these findings, it has been presumed that trisindoline posseses a symmetrical structure as shown in

Figure **38**. Trisindoline showed antibiotic activities [16, 17 and 10 mm diameter growth inhibition for *E. coli*, *B. subtilis* and *S. aureus* at 10 μg/disk (diameter of the paper disks = 8 mm)].

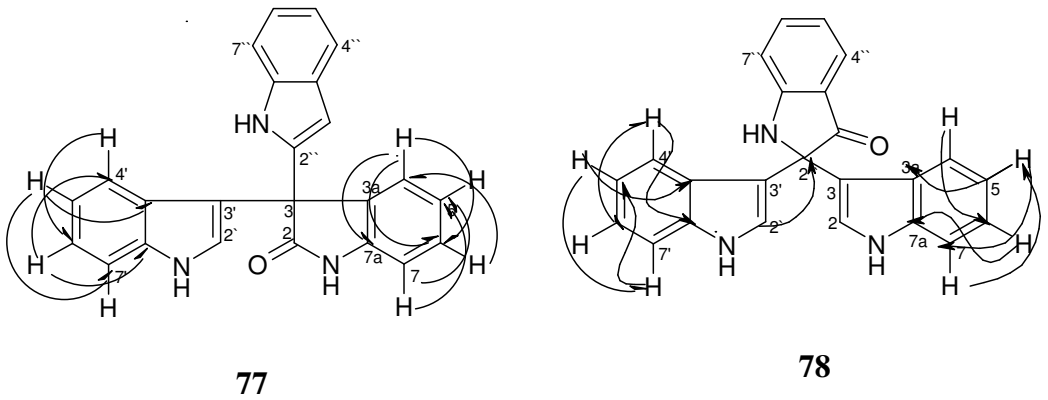


Figure 38: HMBC correlations of trisindoline A (**77**) and 2,2-bis-(3-indolyl)-3-indolone (**78**)

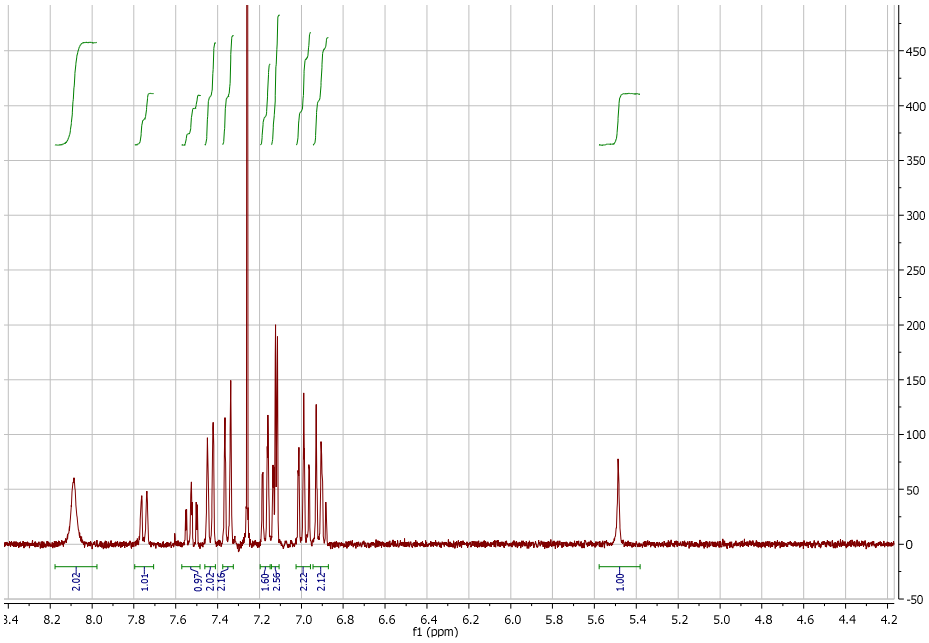


Figure 39: ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of trisindoline A (**77**)

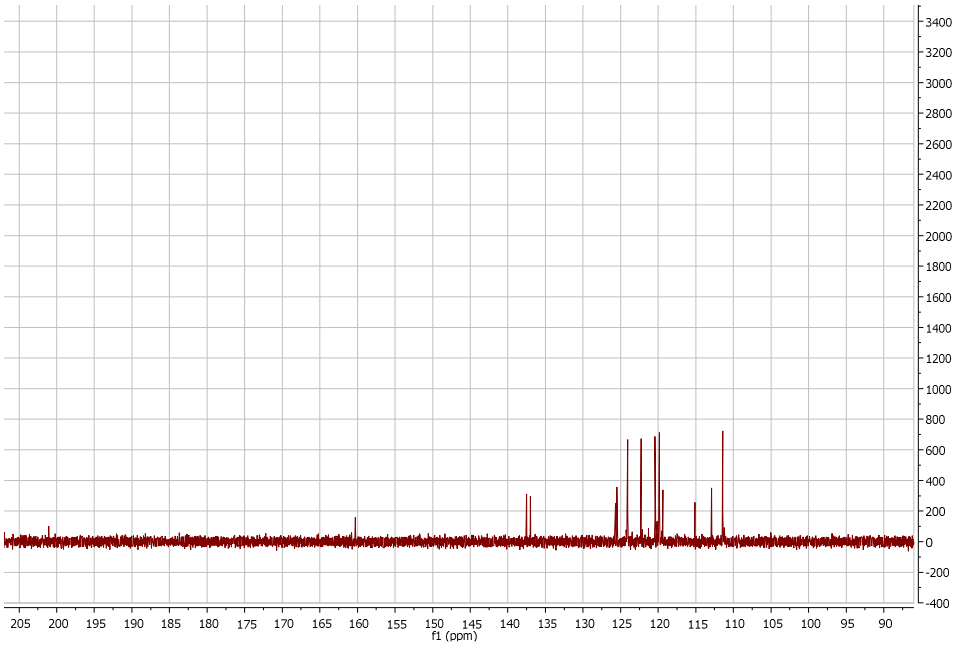


Figure 40: ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of trisindoline A (**77**)

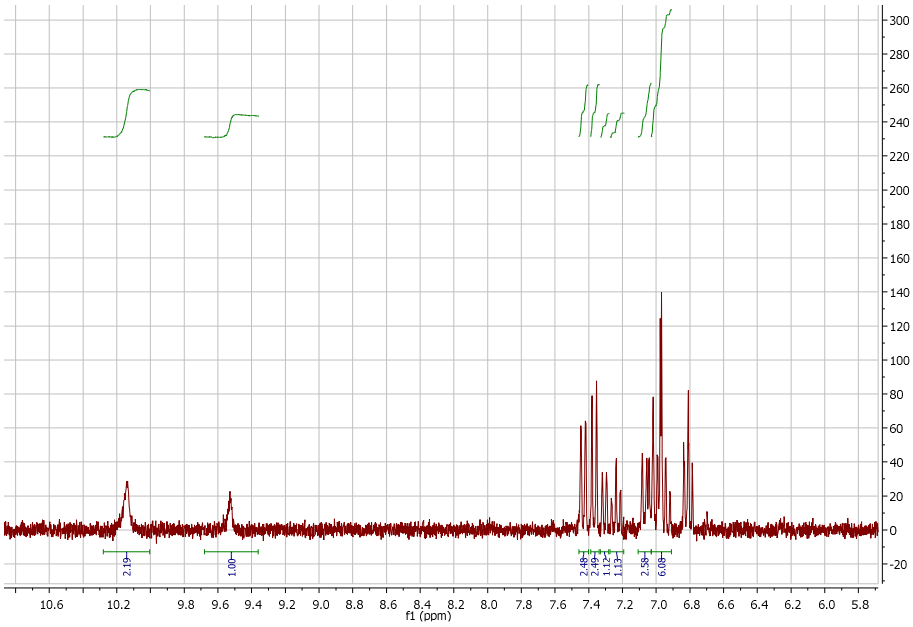


Figure 41: ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of 2,2-bis-3-indolyl-3-indolone (**78**)

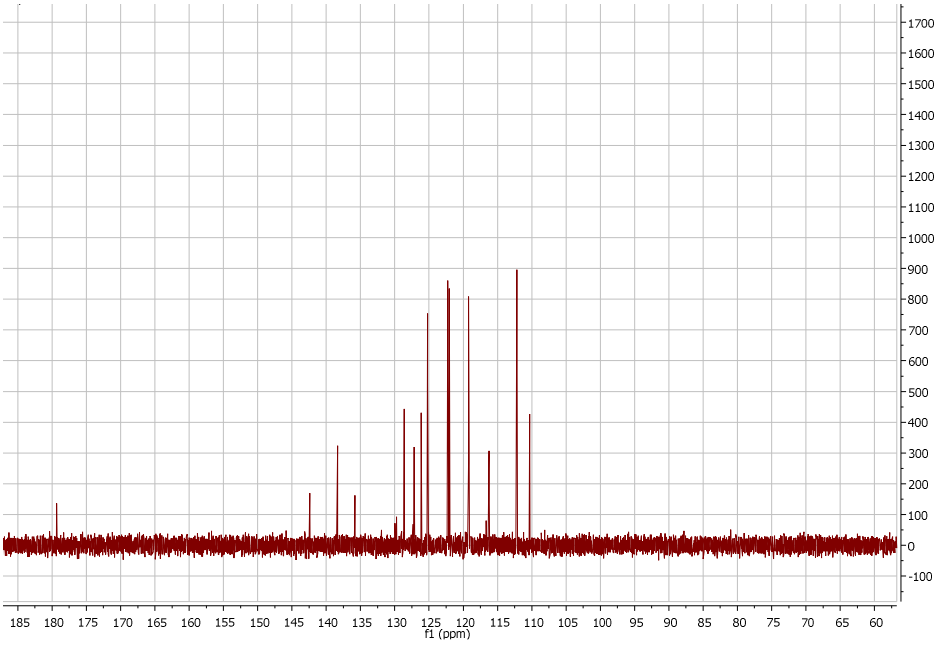
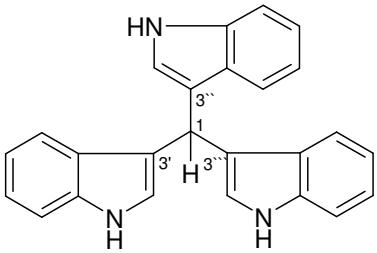


Figure 42: ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of 2,2-bis-3-indolyl-3-indolone (**78**)

3.2.9 1,1,1-Tris(3-indolyl)methane

Compound **79** was isolated as a strongly UV absorbing solid with a high-resolution mass (361.1579) indicating the molecular formula $C_{25}H_{19}N_3$. The doublets at δ 7.37, 7.31 and 6.99, and two triplets at δ 6.91 and 6.82 in the ^{1}H NMR spectrum revealed the presence of the same 3-substituted indolyl system as found in the previous compound **79**. Further, its ^{1}H NMR spectrum displayed a singlet at δ 6.03. All the aromatic signals were found to be tripled in their intensity relatively to the higher field signal at δ 6.03. The ^{13}C NMR indicated a total of only 9 carbon signals, which included 6 methine and 3 quaternary carbon atoms in the skeleton. Except for the methine signal at δ 32.1, all the remaining signals were found to be due to the 3-substituted indolyl system. The low number of carbon signals together with the high mass suggested the structure to be highly symmetrical. The ESI mass spectrometry gave a *pseudo*-molecular ion peak at 361 $[M+H]^+$, the structure of the compound was found to be 1,1,1-tris(3-indolyl)methane (**79**).[87]

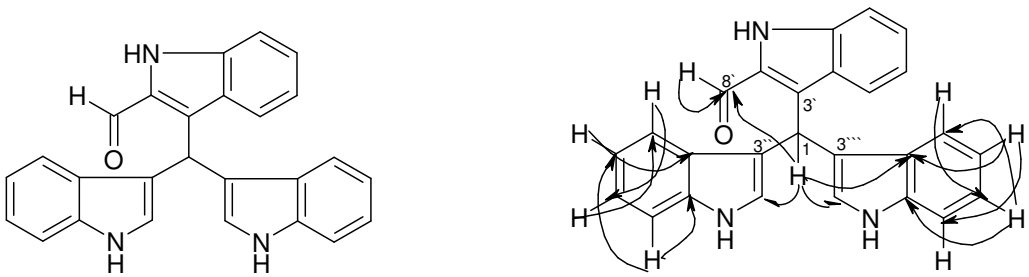


79

3.2.10 Trisindolal

Trisindolal (**80**) was an off-white amorphous solid. The molecular formula $C_{26}H_{19}N_3O$ was established it's the HRESIMS spectrum, which showed a molecular ion peak at *m/z* 389.15281 (calc. for $C_{26}H_{19}N_3O$: 389.44856). The UV spectrum showed absorption maxima at 285, 209, and 208 nm suggesting the presence of an aromatic chromophore. The ^{1}H NMR spectrum was characteristic of 3-substituted indoles which was corroborated by the presence of an *ortho*-coupled protons in the aromatic ring by the ^{1}H NMR spectrum, which showed the expected AA',BB' signals in the aromatic region. In addition, it showed NH signals at δ 10.7 and at δ 10.1 and

additionally an aldehyde signal at δ 9.94 (1H, s). The ^{13}C NMR and HSQC spectrum showed 18 signals, comprising of 16 methine and 10 quaternary carbons. The above spectral data were consistent with a 3-substituted indole type alkaloid with additional aldehyde group at C-2'. Since the intensity of the aromatic signals was tripled with respect to the intensity of a methane group C-1 at δ 6.75, it was established as a tri-substituted indole moiety. The tris-indole moiety was further confirmed by the ^{1}H-^{1}H COSY and HMBC correlations. The proton at δ 6.75 (H-1) showed ^{3}J correlations with C-3a' (δ C 127.0) and C-2' (δ C 127.7). The position of the aldehyde group was identified by HMBC spectra (Figure 43), in which H-1 at δ 6.75 showed a ^{4}J correlation with C-8' (δ C 182.4) and the NH proton at δ 10.7 showed a ^{3}J correlations with C-8' (δ 182.4). The position of the aldehyde group was further confirmed by synthesis (see scheme 2). The assignments of ^{1}H NMR and ^{13}C NMR signals of the synthetic compound were identical to the isolated trisindolal (**80**).



80

Figure 43: HMBC correlations of trisindolal (**80**)

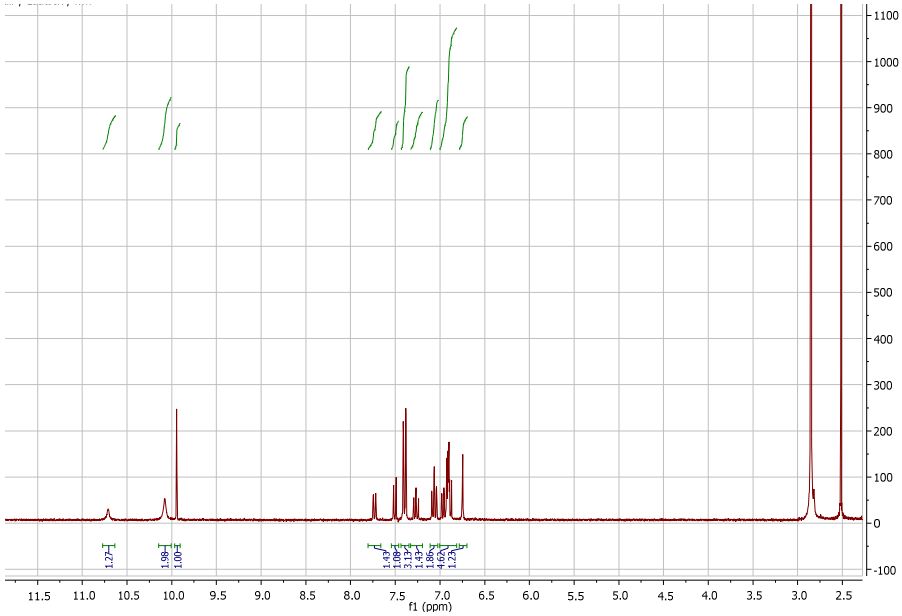


Figure 44: ^{1}H NMR spectrum (300 MHz, acetone-d_6) of trisindolal (**80**)

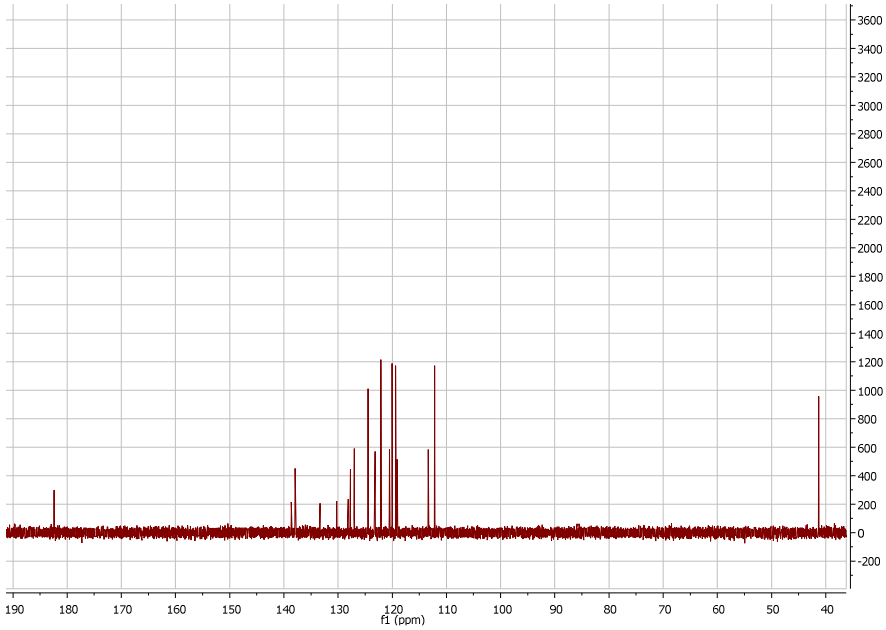
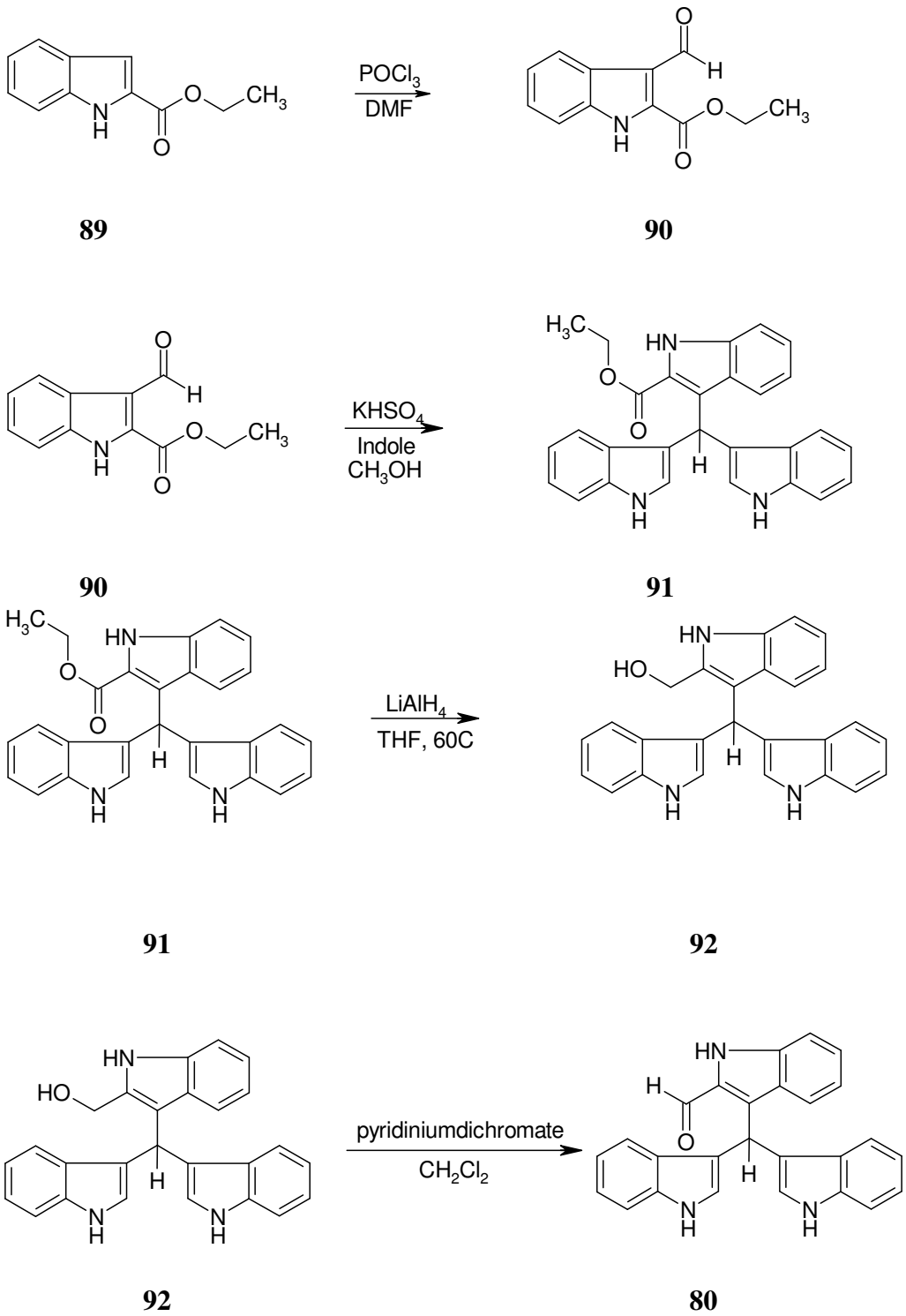


Figure 45: ^{13}C NMR spectrum (125 MHz, acetone -d_6) of trisindolal (**80**)

Table 7: ^{1}H and ^{13}C NMR data of trisindolal (**80**)

Trisindolal (**80**)	(Natural)			(Synthetic)	
	^{1}H (δ_H, mult., *J* Hz)[a]	^{13}C (δ_C)[b]	HMBC	^{1}H (δ_H, mult., *J* Hz)[c]	^{13}C (δ_C)[d]
1	6.75 (s, 1H)	41.3	2', 3', 3a', 4'	6.65	31.4
1'	10.72 (brs, 1 NH)	-	2', 8'	8.89	
2'	-	127.7	-		137.1
3'	-	119.4	-		117.3
3a'	-	127.0	-		129.5
4'	7.73 (dd, *J* = 7.9, 1.5 Hz	120.5	3a', 5', 6'	7.67 d, *J* = 8.2 Hz)	121.2
5'	7.08 (ddd, *J* = 7.9, 7.1, 1.0 Hz)	120.0	3a', 4', 6', 7'		120.4
6'	7.51 (dd, *J* = 8.4, 1.5 Hz)	124.5	4', 5', 7', 7a'		122.2
7'	6.84 (dd, *J* = 8.4, 1.0 Hz)	112.2	5', 6', 7a'		111.2
7a'	-				136.6
8'	9.94 (s, 1H)	182.4	2', 3', 3a'	9.63 (s, 1H)	182.1
1",1"'	10.1 (brs, 2 NH)	-	7a', 7a", 2', 2"	7.97 (brs, 2 NH)	-
2", 2"'	6.84 (d, *J* = 2.0 Hz)	123.2	1, 1', 3', 3"	6.72 (d, *J* = 2.1 Hz)	127.0
3", 3"'	-	119.4	-		119.6
3a", 3a"'	-	135.9	-		127.4
4", 4"'	7.11 (t, *J* = 7.2 Hz)	119.1	5', 5" 6', 6", 7a', 7a"	7.02 (t, *J* = 7.8 Hz)	123.4
5", 5"'	7.40 (d, *J* = 7.9, Hz)	122.1	3a', 3a", 4', 4", 6', 6", 7', 7"	7.36 (m)	122.4
6", 6"'	7.34 (t, *J* = 7.2 Hz)	124.5	4', 4", 5', 5", 6', 6", 7', 7", 7a', 7a"	7.36 (m)	126.6
7", 7"'	7.27 (ddd, *J* = 8.4, 7.1, 1.5 Hz)	113.3	5', 5", 6', 6, 7a', 7a"'	7.04 (t, *J* = 7.8 Hz)	112.1
7a", 7a"'	-	137.9	-		132.4

a: Acetone -d_6, 300 MHz. b: Acetone -d_6, 125 MHz c: $CDCl_3$ -d_6, 300 MHz. d: $CDCl_3$, 125 MHz



Scheme 3: Synthesis of Trisindolal (**80**)

3.2.11 Trisindolone

Trisindolone (**81**) was isolated as a pale yellow amorphous solid with the composition $C_{26}H_{17}N_3O$ (derived from (-)-ESIHRMS). The ^{1}H NMR spectra showed, however, a proton count for 19 protons, and from the ^{13}C NMR spectrum the number of carbon signals was 26; these data agreed better with the 2D data than a structure with 17 protons and one double equivalent less. The ^{1}H- and ^{13}C NMR spectra were similar to those of **80**, except for the replacement of the aldehyde group by a ketone moiety. The ^{13}C NMR and HSQC spectrum showed only 18 signals, comprising of 10 methine and 8 quaternary carbons; some of the signals should therefore have double intensity. The signal at δ 194.6 was assigned to the carbonyl carbon of a ketone. The other signals ranging from δ C 137.4–112.0 were due to aromatic carbons. The ^{1}H NMR spectrum displayed the pattern of AA'XX' type signals and the ^{1}H-^{1}H COSY spectrum of **81** revealed the presence of four adjacent protons in a 1,2-disubstituted benzene. The HMBC spectrum showed three-bond couplings for H-4' to C-6 and C-7a', H-5' to C-3a' and C-7', H-6' to C-4' and C-7a', and H-7' to C-3a' and C-5'. The downfield proton at δ 8.62 (s**,** H-2') showed HMBC correlations to C-3' [δ 117.7], C-3a' [δ 127.4], and C-7a' [δ 137.7]. These findings and the low number of proton signals and their intensity pattern indicated the presence of three indole units, two of them in a symmetrical orientation. From the knowledge of the ^{1}H and ^{13}C NMR values and from the HMBC correlations, the structure of compound was established as trisindolone (**81**) as shown in Figure 46. During the revision of this thesis, structure **81** was found to be patented as 1,2,2-tri(1*H*-indol-3-yl)ethanone and reported as an antitumor compound[88].

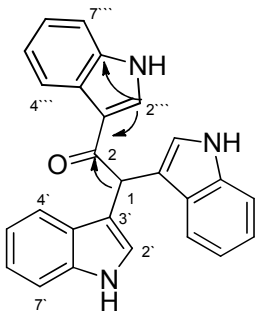


Figure 46: Key HMBC correlations of trisindolone (**81**)

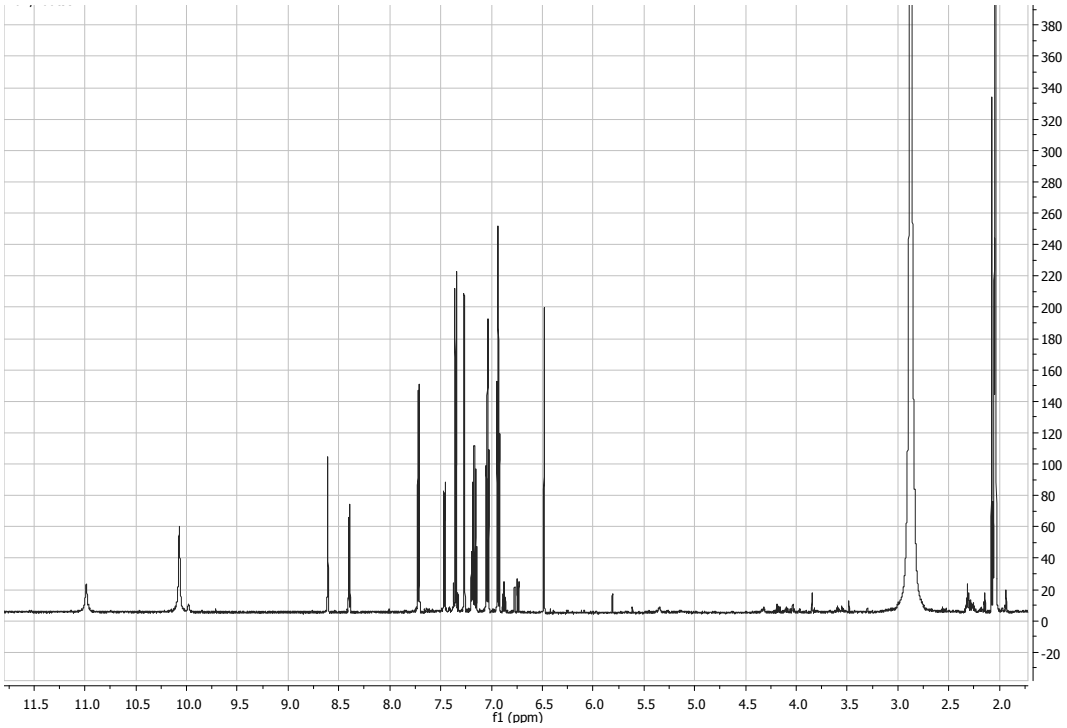


Figure 47: ^{1}H NMR spectrum (300 MHz, Acetone-d_6) of trisindolone (**81**)

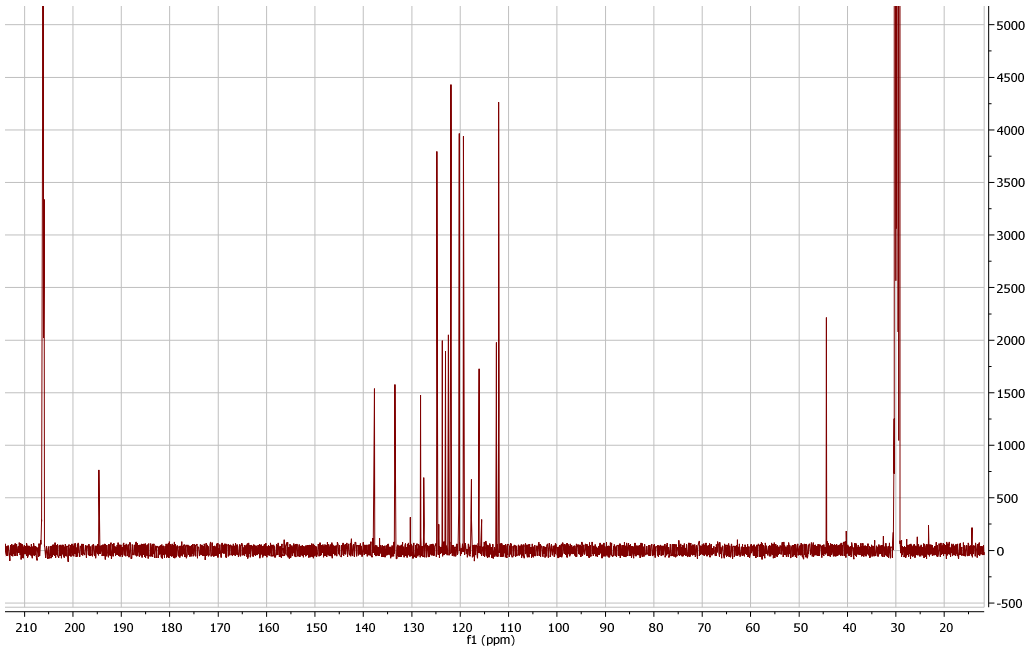


Figure 48: ^{13}C NMR spectrum (125 MHz, acetone-d_6) of trisindolone (**81**)

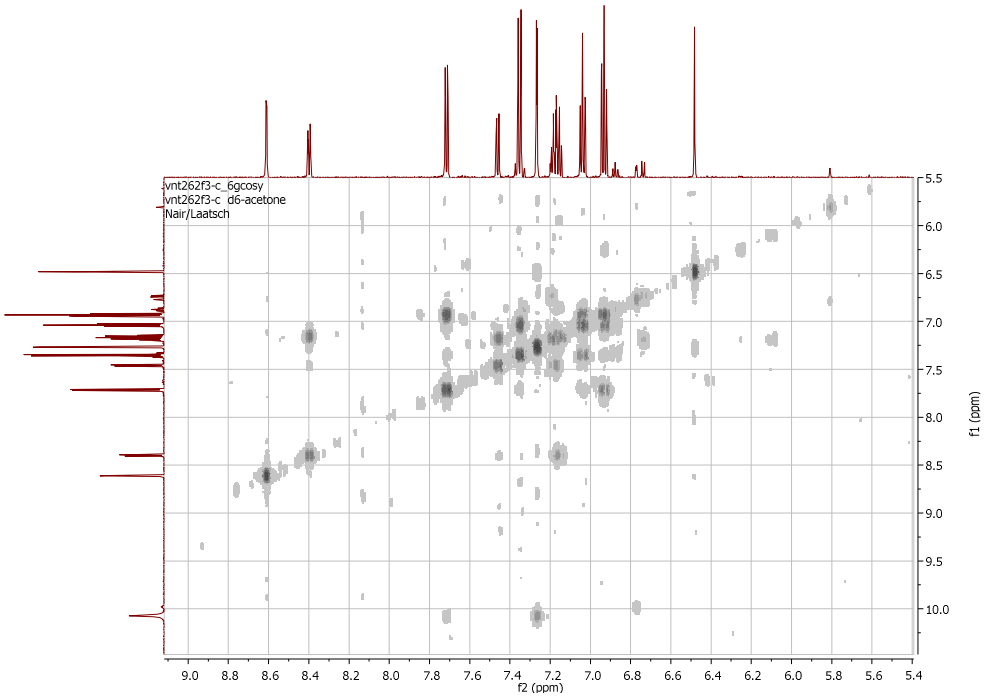


Figure 49: ^{1}H-^{1}H COSY spectrum (300 MHz, acetone-d_6) of trisindolone (**81**)

Table 8: ^{1}H NMR and ^{13}C NMR of trisindolone (**81**)

	^{1}H (δ_H, mult., J Hz)[a]	^{13}C (δ_C)[b]	HMBC
1	6.48	44.4	2’, 3’, 3a’, 4’
2	-	194.6	-
1’	10.99 (1H, s)	-	2’, 8’
2’	8.61 (d, J = 2.1 Hz)	133.5	-
3’	-	117.7	-
3a’	-	127.4	-
4'	7.46 (ddd, J = 7.6, 1.8. 1.0 Hz)	112.6	3a’, 5’, 6’
5’	7.72 (dd, J = 7.9, 1.0 Hz)	120.2	3a’, 4’, 6’, 7’
6’	7.36 (d, J = 2.1)	112.0	4’, 5’, 7’, 7a’
7’	8.41 (ddd, J = 8.4, 1.8, 1.2 Hz)	123.1	5’, 6’, 7a’
7a'	-	137.7	
1”, 1”’	10.08 (1H, s)	-	7a’, 7a”, 2’, 2”
2”, 2”’	7.28 (d, J = 2.1)	124.7	1, 1’, 3’, 3”
3”, 3”’	-	116.1	-
3a”, 3a”’	-	128.3	-
4”, 4”’	6.94 (ddd, J = 7.9, 7.0, 1.0 Hz)	119.3	5’, 5” 6’, 6”, 7a’, 7a”
5”, 5”’	7.19 (t, J = 8.2 Hz)	123.7	3a’, 3a”, 4’, 4”, 6’, 6”, 7’, 7”
6”, 6”’	7.16 dt (dt, J = 7.9, 1.0 Hz) 7.35 (dt, J = 7.9, 1.0 Hz)	122.4	4’, 4”, 5’, 5”, 6’, 6”, 7’, 7”, 7a’, 7a”
7”, 7”’	7.04 (ddd, J = 8.2, 7.1, 1.2 Hz)	121.9	5’, 5”, 6’, 6, 7a’, 7a”’
7a”, 7a”’	-	137.8	-

a: Acetone-d_6, 300 MHz. b: Acetone-d_6, 125 MHz

3.2.12 3,3'-[(4-Methoxy-3,5-dinitrophenyl)methylene]-bis-(1*H*-indole)

Compound **82** was isolated as yellow solid from the fraction II during PTLC purification followed by chromatography on Sephadex LH-20. A reddish brown colour reaction of **82** with anisaldehyde/sulphuric acid reagent and a pink colour with Ehrlich's reagent indicated its indole property. Based on the two *quasi*-molecular ion peaks observed at *m/z* 465 $[M+Na]^+$ and 441 $[M-H]^-$, in ESI MS of positive and negative modes, respectively, the molecular weight of **82** was determined as 442 Dalton. High resolution (+)-ESI MS confirmed the molecular formula to be $C_{24}H_{18}N_4O_5$ (exp. *m/z* 465.1169408, calcd. for $C_{24}H_{18}N_4O_5Na$, 465.11692). The ^1H NMR spectrum showed a broad NH signal with an intensity for 2 protons at δ 8.05 (s, 2H), showed many aromatic signals between δ 7.98~7.00, a singlet at δ 5.95 (s, 1H) and a methoxyl group at δ 4.03. The ^{13}C NMR spectrum showed 14 signals, among them 5 carbon signals accounted for 10 sp^2 methines from two indole rings, one methine at δ 39.4, one downfield signal at δ 128.8 for two methine signals of the substituted benzene ring, six signals located at δ 145.7, 145.1 (2 C-NO_2), 141.7, 136.7, 126.2, 117.1 were indicating quaternary carbons and a signal at δ 64.7 represented a methoxy group. The presence of a bis-indole unit was established by *ortho*-disubstituted benzene moieties, each of which showed two-proton signals at δ 7.39 (dd, J = 8.2, 7.2 Hz, 2H), 7.33 (dd, J = 8.2, 7.2, 2H), 7.24 (t, J = 7.2, 2H) and 7.05 (t, J = 7.2, 2H). Hence the presence of a bis-indolyl-methane moiety in **82** was figured out from the correlations obtained by ^1H-^{13}C, COSY and from the HMBC spectrum of **82**. Based on these findings, the structure of 3,3'-[(4-methoxy-3,5-dinitrophenyl]methylene)-bis-(1*H*-indole) (**82**) has been assumed, a symmetrical molecule as in the case of turbomycins A and B (Figure 52).

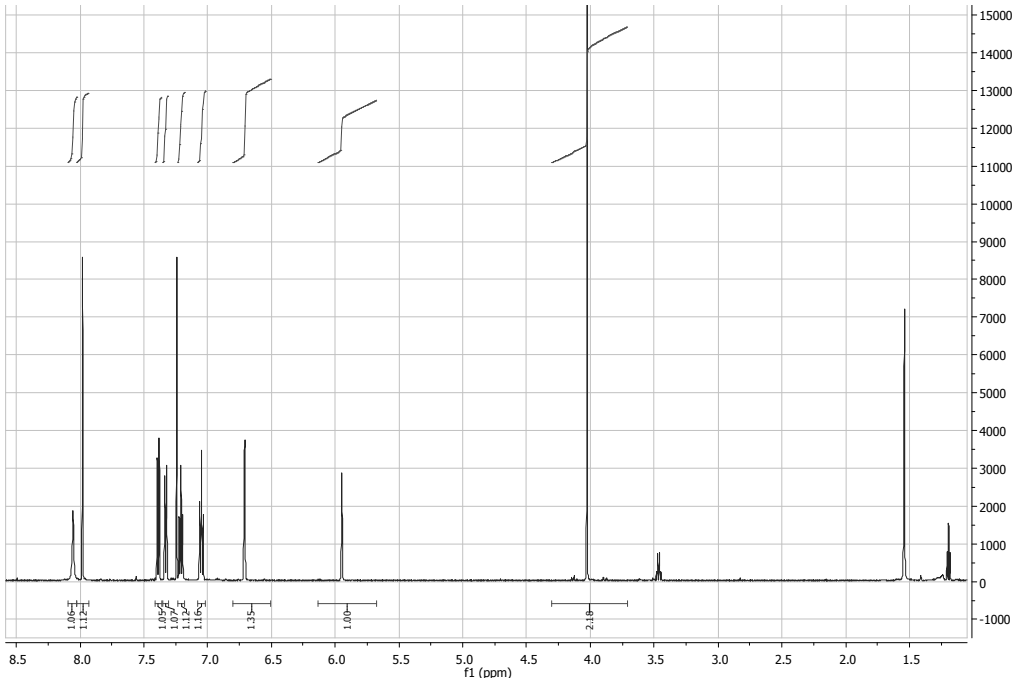


Figure 50: ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of 3,3'-[(4-methoxy-3,5-dinitrophenyl)methylene]-bis-(1*H*-indole) (**82**)

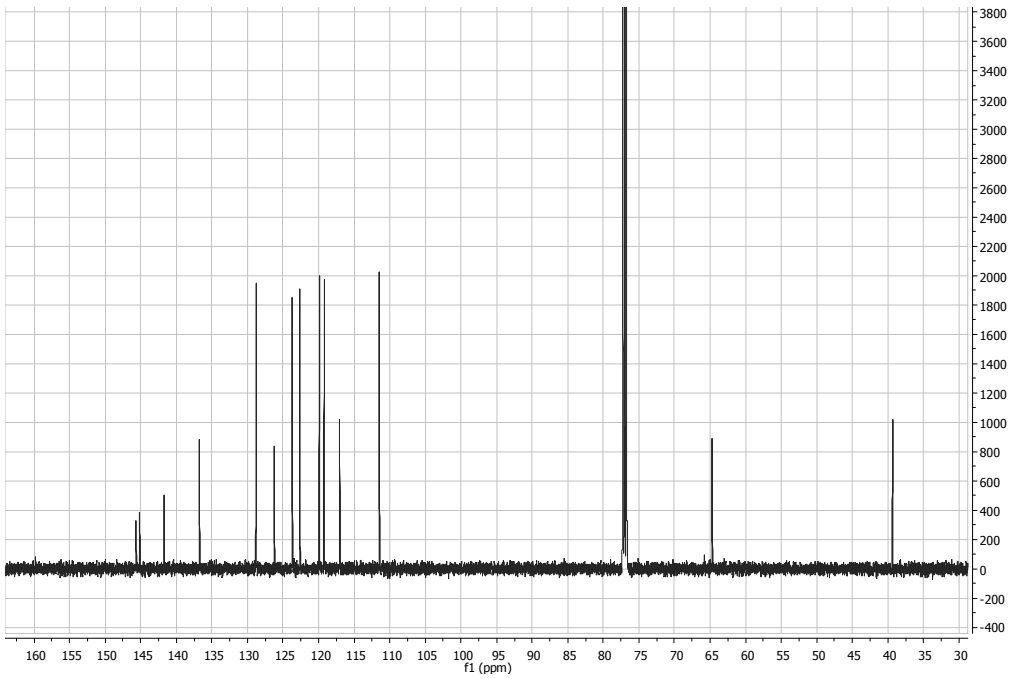


Figure 51: ^{13}C NMR spectrum (300 MHz, $CDCl_3$) of 3,3'-[(4-methoxy-3,5-dinitro-phenyl)methylene]-bis-(1*H*-indole) (**82**)

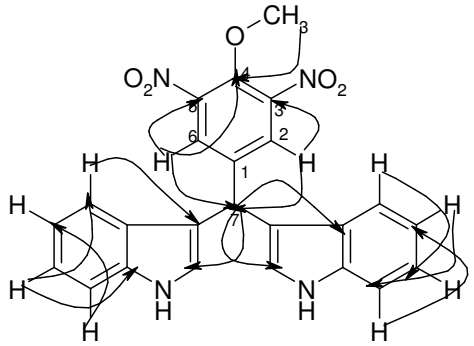


Figure 52: HMBC correlations of 3,3'-[(4-methoxy-3,5-dinitrophenyl)methylene]-bis-(1*H*-indole) (**82**)

Table 9: ^{1}H and ^{13}C NMR data of 3,3'-[(4-methoxy-3,5-dinitrophenyl)methylene]-bis-(1*H*-indole) (**82**)

3,3'-[(4-methoxy-3,5-dinitrophenyl)methylene]-bis-(1*H*-indole) (**82**)			
Position	^{1}H (δH, mult., *J* Hz)[a]	^{13}C (δC)[b]	HMBC
1	-	141.7	-
2, 6	7.98 (s)	128.8	1, 3, 4, 5
3	-	145.1	-
4	-	145.7	-
5	-	145.1	-
7	5.95 (s)	39.4	1, 2, 3, 6, 3', 3", 3a', 3a", 2', 2"
7a', 7a"	-	136.7	-
8	4.02 (s)	64.7	4
1', 1"	8.05 (s)	-	7a', 7a"
2', 2"	6.71 (d, *J* = 2.0)	123.7	3', 3", 7a', 7a"
3', 3"	-	117.1	-
3a', 3a"	-	126.2	--
4', 4"	7.21 (t, *J* = 7.2 Hz)	122.7	3', 3", 3a', 3a", 5', 5", 6', 6", 7a', 7a"
5', 5"	7.33 (dd, *J* = 7.2, 8.1)	119.2	3a', 3a", 4´, 4", 5', 5", 6', 6", 7', 7"
6', 6"	7.39 (dd, *J* = 7.2, 8.1)	111.5	4´, 4", 5', 5", 6', 6", 7', 7"
7', 7"	7.05 (t, *J* = 7.2)	119.9	4´, 4", 5', 5", 6', 6", 7a', 7a"

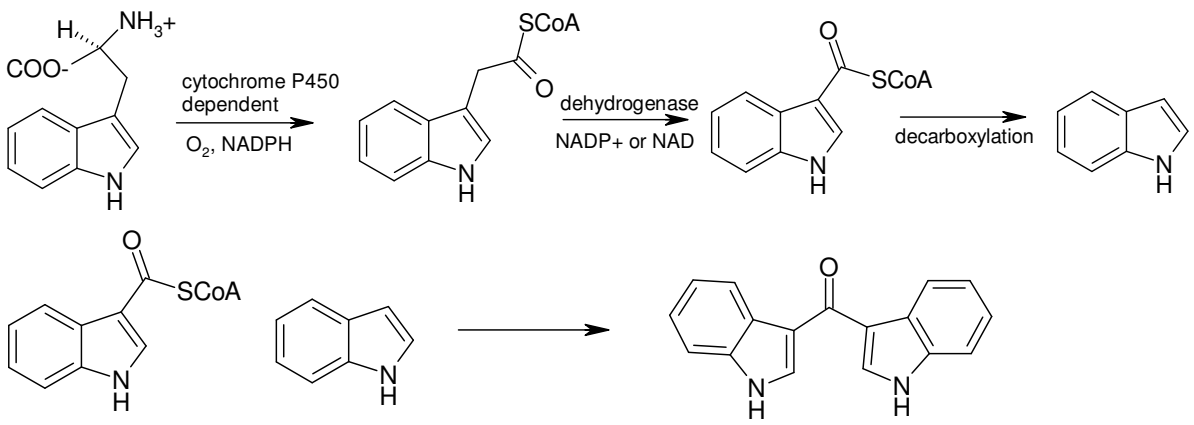
[a] $CDCl_3$, 300 MHz, [b] $CDCl_3$, 125 MHz

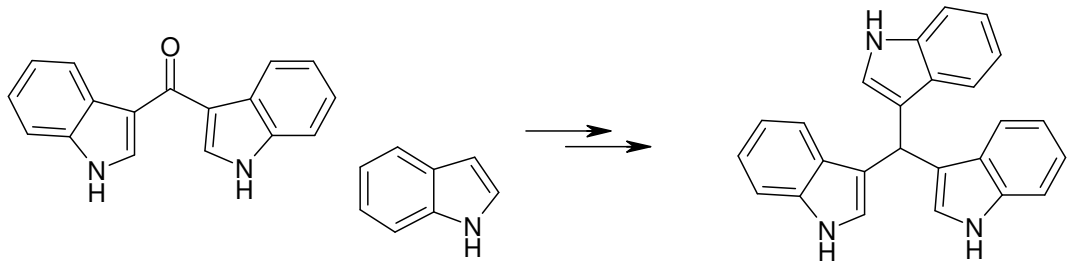
3.2.13 Cytotoxicity of indole derivatives

Cytotoxicity profiling of trisindole derivatives in a panel of up to 11 tumor cell lines indicated promising cytotoxic activity of several derivatives with a pronounced selectivity for cell lines of colourectal cancer, lung cancer, breast cancer, melanoma, ovarian cancer, prostate cancer cells, pleuramesothelioma, renal, gastric and uterus cancer cells. The cytotoxic effect of the indole derivatives trisindolal (**80**), the quinonoid derivative **84**, 7,7-bis(3-indolyl)-*p*-cresol (**83**), turbomycin A (**75**) and turbomycin B (**88**) was determined. Trisindolal (**80**) proved to be active with an overall potency of 0.45 μg/ml (mean IC_{50} value of 11 tumor cell lines tested). It was followed by the quinoid derivative C (**84**, mean IC_{50} = 4.2 μg/ml) and 7,7-bis(3-indolyl)-*p*-cresol (**83**, mean IC_{50} = 7.49 μg/ml) in decreasing order of cytotoxic potency (Table 23). Turbomycin A (**75**) showed concentration-dependent cytotoxicity with a mean IC_{50} value of 3.55 μg/ml. Turbomycin B (**88**) showed pronounced cytotoxic activity with a mean IC_{50} value of 6.096 μg/ml (Table 63). Trisindolal (**80**) displayed significant *in vitro* tumor cell activity towards all tested eleven tumor cell lines with IC_{50} values ranging from 0.316 μg/ml to 0.363 μg/ml (Table 23). Cytotoxic selectivity of the indole alkaloids was observed in 6 of 11 (**80**), 4/11 (**84**), 2/11 (**83**), 2/11 (**75**), and 1 of 11 (**88**) cell lines (Table 23). Thus, antitumor potency of the trisindolal was higher according to its substitution by an aldehyde at C-2 and displayed comparable overall cytotoxicity against all human cancer cell lines. This indicated that the substitution at position C-2 of the indole moiety by an aldehyde group could be crucial for their cytotoxic potency, apart from the trimerisation of the indole moeity of **80,** which obviously both influenced cytotoxic selectivity. In contrast, variation of the quinonoid derivative **84** or C-4 position (**83**) of the quinol as in case of 7,7-bis (3-indolyl)-*p*-cresol of the indole quinol skeleton (**83**), respectively, influenced cytotoxicity (Table 23). Tris-indolal was synthesized and is available for further biological tests. Other derivatives were not tested for cytotoxic activities.

3.2.14 Kinase Assays

Subsequent mining of microbial organisms for anticancer natural products led to the discovery of staurosporine[89] , a kinase inhibitor[90], and later rebeccamycin[91,92], analogs of which are DNA-topoisomerase I inhibitors[93]. Bis-indoles have found clinical application as anticancer drugs; they cause tubulin polymerization with lower toxicity than vinblastine or vincristine[94]. On the basis of the anticancer properties of the indole derivatives isolated from the marine *Streptomyces*. sp. T262, an attempt was made to search for kinase activity, together with Dr. Manfred Konrad, a co-operation partner at the Max Planck Institute for Biophysical Chemistry in Göttingen. The characteristic feature of the compounds isolated from *S*. sp. T262 is their symmetry. Many symmetric compounds are potent inhibitors for choline kinase like hemicholinium (HC-3)[95]. Hence these compounds were tested against choline kinases, which are key enzymes in the synthesis of phospholipids that are essential constituents of cell membranes and are involved in the regulation of cell proliferation. The inhibition by the isolated compounds was, however, not promising in comparison with HC-3. Turbomycin A (**75**) and B (**88**) are charged cation species, which correlates to the charged nitrogen in HC-3. However, turbomycin A and B did not show any inhibition against these kinases. The indole structures invite innovative approaches to explore their possible kinase targets as a potential lead in drug discovery: Another challenging target could be a trial against protein kinases. But as these tests required sophisticated equipments, they were not yet done.

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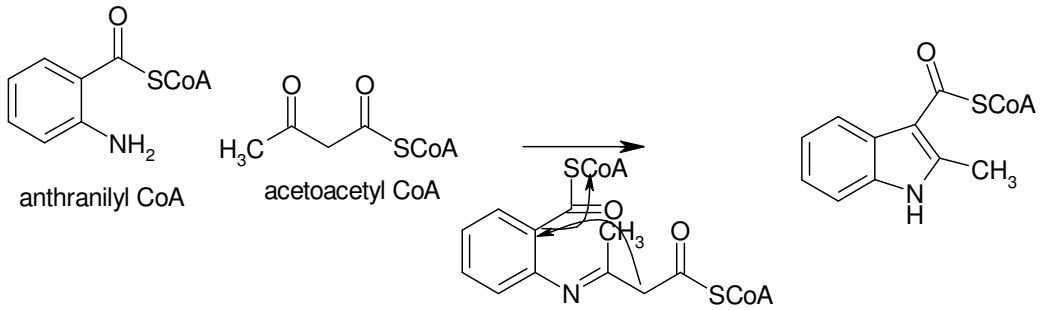
79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O
O
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SCoA
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N
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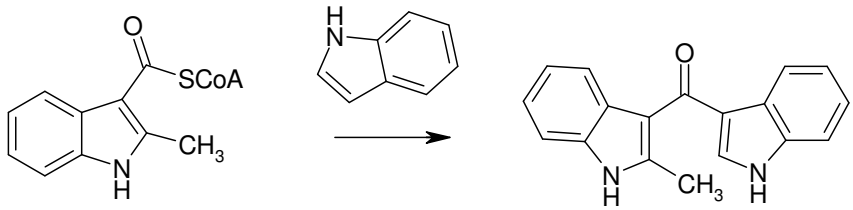
O
SCoA
CH3
N
H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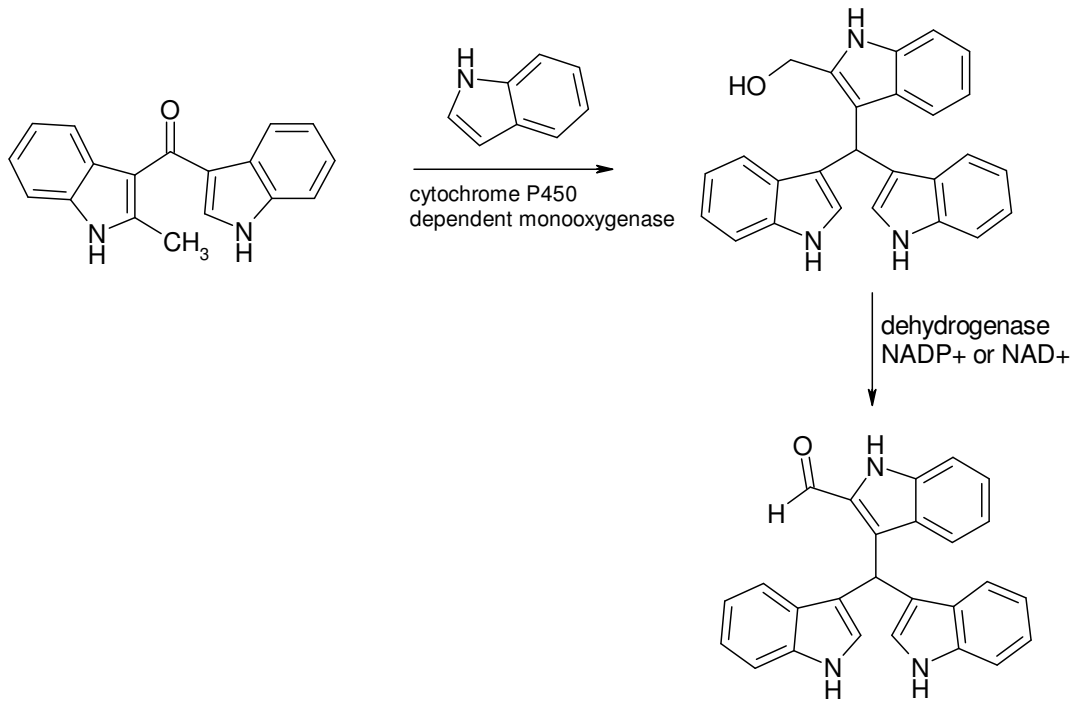


80

Figure 53: Hypothetical biosynthetic pathway to trisindol using L-tryptophan and trisindolal via anthranilyl Co-A

Like other indole alkaloids[96], naturally occurring bis-indoles are generally thought to be derived from the amino acid L-tryptophan[97], and this assumption has indeed proven true for all characterized bisindoles. L-Tryptophan provides an excellent scaffold for dimerization and derivatization, and consequently, production of bis-indole molecules with distinct chemical properties give rise to charecteristic biological properties. In a similar way trisindol could also be derived from L-tryptophan. Anthranilic acid is a precursor of some quinoline and indole alkaloids. Anthranyl Co-A acts as a starter unit for malonate chain extension. Formation of imine occurs from acetoacetyl CoA followed by aromatisation via decarboxylation and cyclisation to give 2-methyl indole acetyl Co-A. This could be a precursor used by nature for the formation of trisindolal (**80**), which could be formed by enzymatic transformation from indole and L-tryptophan as shown in the pathway (Figure 53).

3.3 Marine *Streptomyces* sp. B7354

The crude extract obtained by fermentation of the marine *Streptomyces* sp. isolate B7354 was moderately active against both Gram-positive and Gram-negative bacteria (*Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* and a weakly against *Streptomyces viridochromogenes* Tü 57 as well as against the yeast *Candida albicans*, while it was strongly active against the fungus *Mucor miehei* Tü284 (Table 25). It exhibited also *in vitro* antitumor activity against cancer cells at very low concentrations (Table 45). Chemical screening indicated nonpolar zones, which showed no significant UV absorption, but turned violet to blue with anisaldehyde/sulfuric acid. The brown culture broth was mixed with Celite and filtered through a filter press. The water phase was loaded on a XAD-16 column and the adsorbed metabolites were eluted with methanol. The eluate was evaporated *in vacuo* and the aqueous residue was extracted with ethyl acetate. The biomass was extracted with ethyl acetate and acetone. The dark brown oily crude extract was subjected to column chromatography on silica gel using a CH_2Cl_2/MeOH gradient. Two isomeric daucane sesquiterpenes streptodaucanes A (**99**) and B (**100**) were isolated, along with other known compounds like nonactic acid (**93**), homononactic acid (**94**), bonactin (**95**), dinactin (**96**) and tetralactone (**97**) which were identified by means of AntiBase data[11]. From fraction IV trimethoxy adenosine (**98**) was isolated, which was unknown from nature.

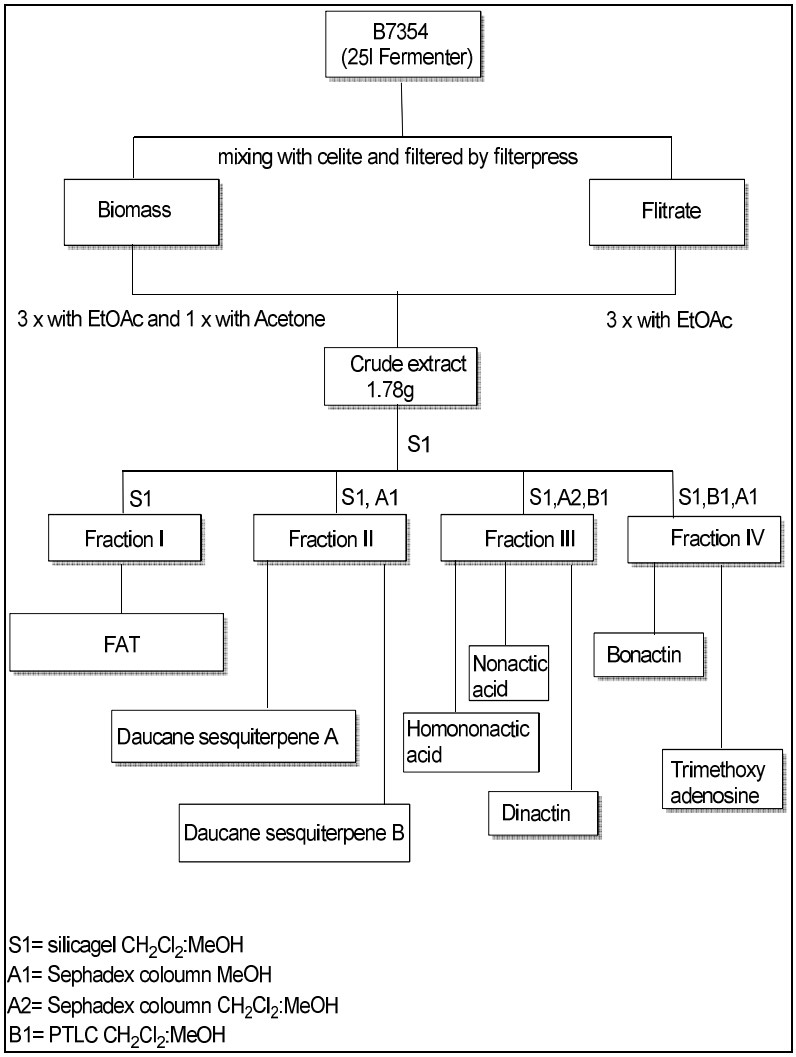


Figure 54: Work up scheme of the strain *Streptomyces* sp. B 7354

3.3.1 Streptodaucane A

Chemical diversity of bacterial metabolites showed that sesquiterpenes are much less abundant as compared to their existence in plants[98], fungi[99-101] and other marine organisms[102]. Only limited number of sesquiterpenes have been isolated from bacteria so far, e.g., africantriol[103], 4*S*,7*R*-germacra-1*E*,5*E*-dien-11-ol,[104] cadin-4-en-1-ol,[105] sesquiterpenol,[106] geosmin,[107] methylisoborneol,[108] and pentalenolactones[109]. Few of them are inhibitors of kinase or show selectivity for human colon tumor cell lines.[110] This is a first report for isolation of sesquiterpenes with the daucane skeleton from bacteria.

Chromatography of the crude extract and isolation of metabolites were performed as usual (see Experimental Section). From fraction F2, two colourless sesquiterpenes **99** and **100** with the molecular formula $C_{15}H_{26}O_2$ (from (+)-HRESIMS) were isolated, which had, according to the HSQC and HMBC NMR data, a daucane skeleton. ^{1}H and ^{13}C NMR spectra of compound **99** (Figure 55) revealed the presence of a 2-hydroxyisopropyl moiety at C-4 and an epoxide ring at C-8 and C-9. The placement of the 2-hydroxyisopropyl substituent at C-4 was derived from HMBC correlations of H-5 (1.70, 1H, td, J = 12.3, 6.2 Hz) and the shielded Me-15 (δ 0.98, s) with C-5 (δ 59.6). Further, the HMBC correlation of Me-12 and 13 with H-5 (1.70, 1H, td, J = 12.3, 6.2 Hz) also confirmed the 2-hydroxyisopropyl moiety. The 1H triplet at δ 2.81 (J = 7.2 Hz) showing HSQC with carbon at δ 63.1 was assigned to the H-9, and the signal at δ 1.31 (3H, s) was due to Me-14, which showed a HMBC correlation with C-8 (δ 62.3) and C-9 (δ 63.1). These correlations indicated the presence of a trisubstituted epoxide at C-8 and C-9. Further HMBC correlations for the methylene protons at C-10 (δ 2.14, δ 1.26) with C-15 (δ 18.8) and C-9 (δ 63.1) confirmed the position of the epoxy substitution in the cycloheptane ring of the daucane skeleton. Finally the HMBC correlations (Figure 55) together with COSY spectrum led to the identification of structure **99** as streptodaucane A (Figure 55). The relative stereochemistry was determined from the NOESY interactions. The NOESY spectrum showed correlations of Me-15 with H-4, Me-14 and H-9, which indicated a *trans* fused ring system with *alpha* orientation of the epoxide oxygen. Further correlations of H-5 with Me-12, Me-13 established the *alpha* orientation for H-5 and the 2-hydroxyisopropyl substituent at C-4 (Fig. **55**).

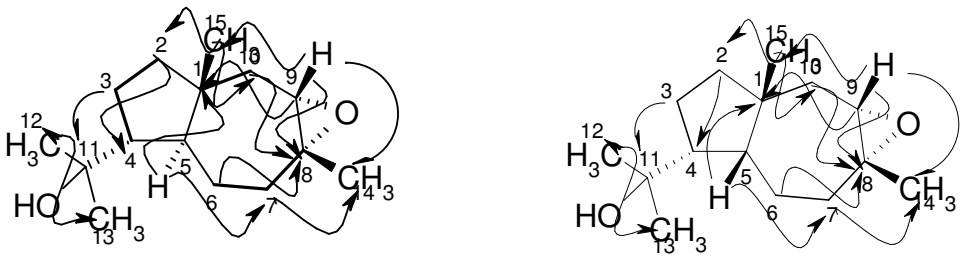
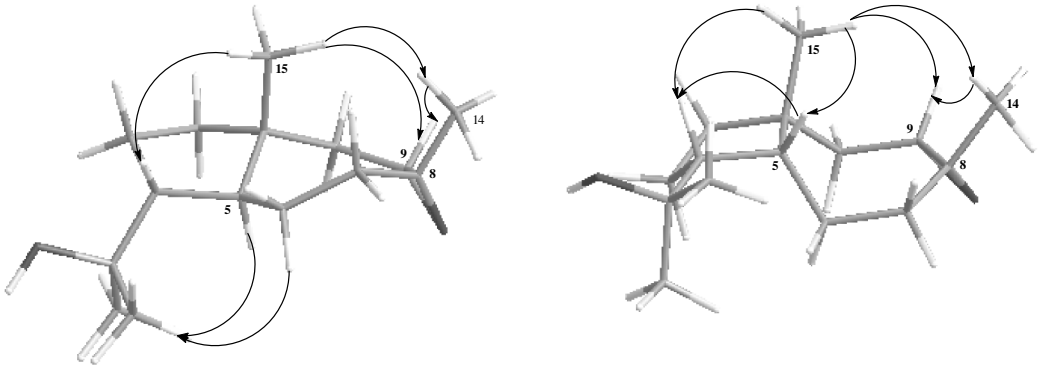


Figure 55: HMBC and COSY correlations for structures **99** and **100**

3.3.2 Streptodaucane B

Compound **100** showed similar spectral properties as **99**; the only difference between the two compounds was the annellation of the two rings: In compound **100** the two rings were *trans* fused whereas in compound **99** the rings were *cis* connected as evident from the NOESY spectrum Figure 56: Me-15 showed correlations with H-5, H-4, Me-14 and H-9, which confirmed the relative structure for **100** as streptodaucane B (Figure 55).



99 **100**

Figure 56: Key NOE correlations for structures **99** and **100**

Table 10: NMR data for **99** and **100** recorded in CD_3OD (125 MHz) at 25 ºC

	streptodaucane A (**99**)				streptodaucane B (**100**)			
No	^1H (δ_H, mult., *J* Hz)[a]	COSY	HMBC	^{13}C (δ_C)[b]	^1H (δ_H, mult., *J* Hz)[a]	COSY	HMBC	^{13}C (δ_C)[b]
1	-	-	-	45.4	-	-	-	44.4
2a, 2b	1.39 (1H, m)* 1.30 (1H, m)	H2ab	C1, C5, C4	41.6	1.40 (1H, m)*, 1.33 (1H,m)	H2ab	C1, C5, C4	40.6
3a, 3b	1.46 (1H, m)* 1.73 (1H, m)	H3a, H2a	C11, C4, C1	28.0	1.49 (1H, m)*, 1.72 (1H, m)	H3a, H2a	C11, C4, C1	27.2
4	2.36 (1H, m)*	H5, H3b	C11	53.8	2.36 (1H, m)*,	H5, H3b	C11	53.9
5	1.70 (1H, td, *J* = 12.3, 6.2 Hz)	H4, H6a, 6b	C6, C15	59.6	1.71 (1H, td, *J* = 13.1 Hz, *J* = 6.9 Hz)	H4, 6a, 6b	C6, C15,	59.9
6a, 6b	2.34, 1.63 (1H, d, *J* = 13.6 Hz)	H6ab, H7a	C8	23.8	2.35, 1.62 (1H, d, 13.9 Hz)	H6ab, H7a	C8	23.5
7a 7b	1.28 (1H, m)* 2.12 (1H, dd, *J* = 13.9, 6.7 Hz)	H6b	C8, C5, C6	39.0	1.26, 2.12 (1H, dd, *J* = 6.6 Hz, *J* = 13.7 Hz)	H6b	C8, C5, C6	39.6
8	-	-	-	62.3	-	-	-	62.7
9	2.80 (1H, t, *J* = 7.2 Hz)	H10ab	C10	63.1	2.78 (1H, t, *J* = 6.9 Hz)	H10ab	C10	63.4
10a, 10b	2.24 (dd, *J* = 7.1 Hz, *J* = 13.6 Hz), 1.14 (1H, m)	H10b H9	C8, C9, C5, C6	43.5	2.24 (dd, *J* = 6.7 Hz, *J* = 12.6 Hz), 1.15 (1H, m)	H10b, H9	C8, C9, C5, C6	42.8
11	-	-	-	74.8	-	-	-	73.9
12	1.18 (3H, s)	-	C11, C5, C13	27.3	1.19 (3H, s)	-	C11, C5, C13	27.6
13	1.14 (3H, s)	-	C11, C12, C5	32.0	1.15 (3H, s)	-	C11, C12, C5	32.4
14	1.31 (3H, s)	-	C7, C8, C9	23.6	1.32 (3H, s)	-	C7, C8, C9	23.9
15	0.98 (3H, s)	-	C1, C2, C5, C10	18.8	0.97 (3H, s)	-	C1, C2, C5, C10	18.8

[a]CD_3OD, 300 MHz. b: CD_3OD, 125 MHz, * = overlapped

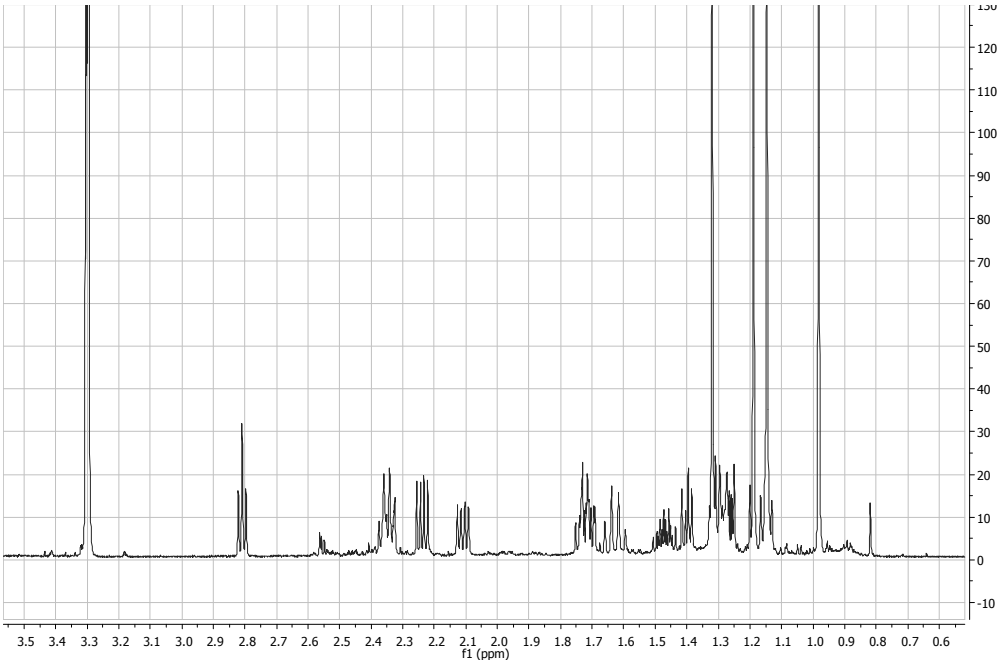


Figure 57: ^{1}H NMR spectrum (300 MHz, CD_3OD) of streptodaucane A (**99**)

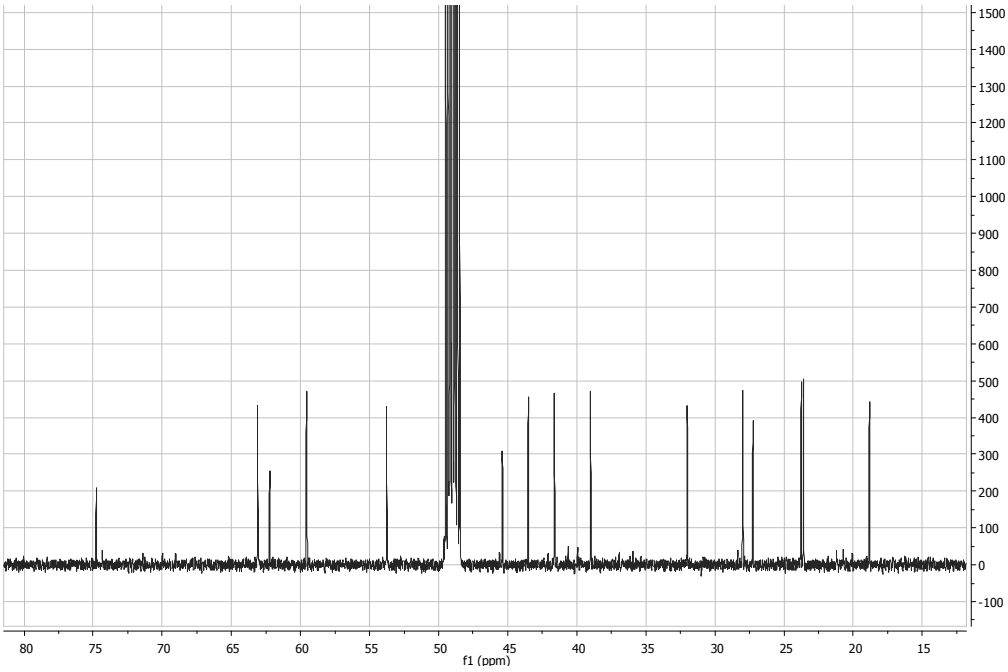


Figure 58: ^{13}C NMR spectrum (125 MHz, CD_3OD) of streptodaucane A (**99**)

Compounds **99** and **100** were tested for their antifungal activites against the oomycetes viz. *Botrytis cineria* (BOTRCI), *Septoria tritici* (SEPTTR), *Pyricularia grisea* (PYRIOR), *Phytophthora infestans* (PHYTIN). The activities for these compounds are listed in Table 11.

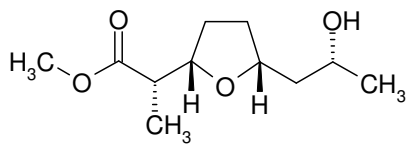
Table 11: Activities against oomycetes

Compound	Doses (ppm)	BOTRCI (%)	SEPTTR (%)	PYRIOR (%)	PHYTIN (%)
99	31	0	58	90.5	8.5
	7.75	93.5	11.5	96.5	100
	2	92	16.5	96.5	100
100	31	0.5	7	89	63
	7.75	76.5	9	97	100
	2	91.5	16	96	100

From the table above it is clear that at 31ppm concentration compound **99** showed complete inhibition of *Phytophthora infestans*. Similarly compound **100** also had a comparative inhibition for *Botatis cineria* and high inhibition activity against *Septoria tritici* at 31ppm concentration. This activity test was performed by BASF.

3.3.3 Nonactic acid

Nonactin, monactin, dinactin, trinactin and tetranactin isolated from a variety of *Streptomyces* species are cyclotetralactones derived from nonactic (**93**) and homononactic acids (**94**) as building units of ionophoretic character. With the exception of nonactin, they exhibit, in addition to antibacterial and antifungal activity, also remarkable acaricidal, insecticidal, coccidiostatic and anthelminthic effects.[111]



93

Compound **93** was isolated as colourless oil from fraction III by subjecting it to Sephadex LH-20 followed by RP18. Fraction III showed a colourless spot on TLC, which turned to dark violet on spraying with anisaldehyde/sulphuric acid and heating. The ^{1}H NMR spectrum indicated signals for 18 protons only in the aliphatic re-

gion of the spectrum. It consisted of two methyl doublet groups at δ 1.10 (H-10) and δ 1.18 (H-9), multiplets at δ 1.58 (4 H), 2.12 (2H), one quartet at δ 2.52 and three signals of oxygenated methines at δ 3.96 (H-3), 4.08 (m, H-8, H-6), respectively. ESIMS of this compound afforded a molecular ion peak at *m/z* 202, and HRESIMS revealed the molecular formula $C_{10}H_{18}O_4$. Searching in AntiBase using the molecular weight, the molecular formula and the ^{1}H NMR data led to nonactic acid (**93**).

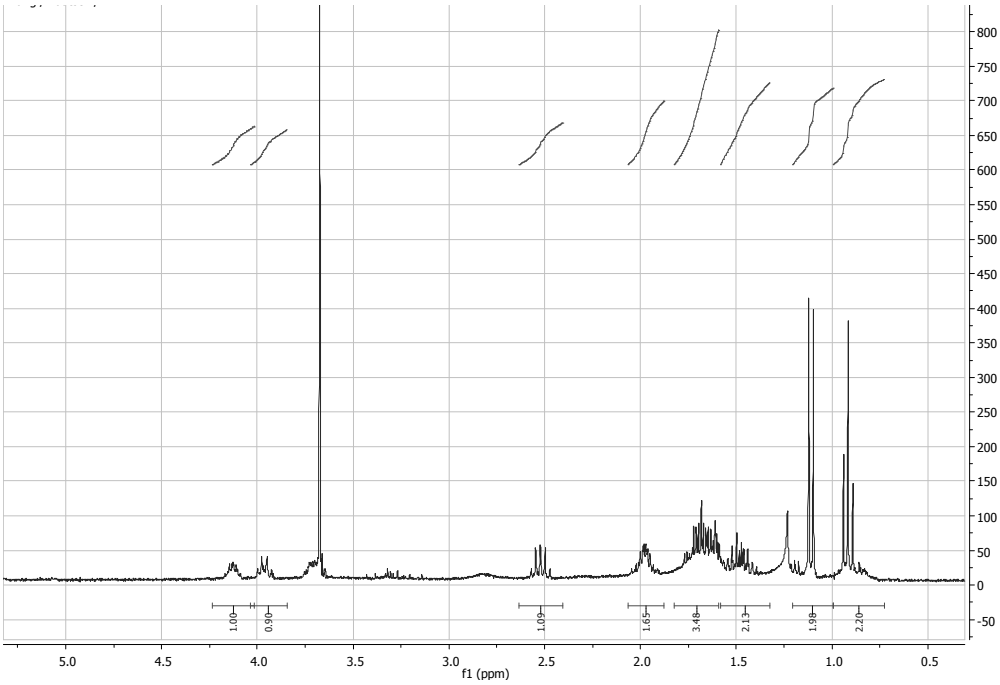
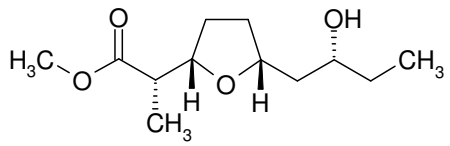


Figure 59: ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of nonactic acid (**93**)

3.3.4 Homononactic acid



94

Fraction III showed a non-polar band, characterized by a violet colour reaction with anisaldehyde/sulphuric acid. Further purification with RP18 followed by Sephadex LH-20 led to colourless oil. The ^{1}H NMR spectrum was nearly identical with that of nonactic acid (**93**). The only difference was that the methyl doublet in nonactic acid (**93**) was replaced by **94**. The structure was confirmed as homononactic acid (**94**) by comparison of the ^{1}H NMR data with literature values; **94** and its triple epimer at the carbon atoms C-2/C-3/C-6 were synthesized by Sharma *et al*.[112]

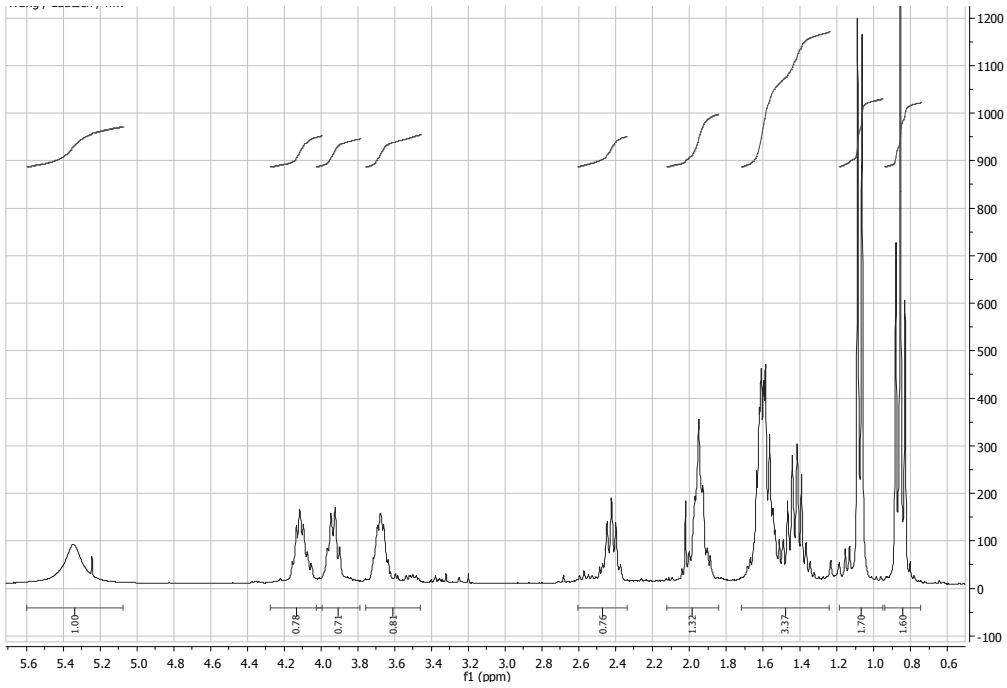
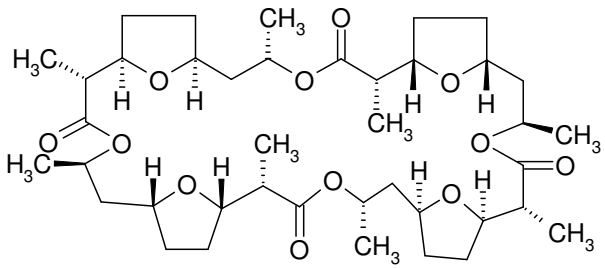


Figure 60: ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of homononactic acid (**94**)

3.3.5 Dinactin

The component of a further fraction IV was recognized as the macrocyclic dinactin (**101**), which was inferred from a set of proton/carbon NMR signals at δ 4.97/68.6. In addition, the ESI-MS spectrum revealed a dominant $[M+Na]^+$ ion peak at *m/z* 787. The molecular formula of $C_{42}H_{68}O_{12}$ was established by HRESI-MS. The aliphatic region of the ^{1}H NMR spectrum of showed three proton signals at δ 4.93 (H-8, 17), 4.02 (H-3, 12) and 3.87 (H-6, 15), which were interpreted as oxygenated methines. Furthermore, one 2H multiplet at δ 2.47 possibly corresponded to a carbon connected to an sp^2 carbon; seven methylene multiplets were displayed in the range of δ 2.01-1.22. The ^{13}C NMR spectrum exhibited signals of 21 carbon atoms, which could be classified as follows: two carbonyls at δ 176.5, 176.1, six oxygenated methine groups in the range of δ 82.6 - 70.2, two methine groups connected to sp^2 carbon at δ 46.9 and 46.7. Seven methylenes groups at δ 42.0 - 28.5, and four methyls at δ 20.9, 12.9 (2 each), 9.1 were present. The ^{1}H NMR and ^{13}C NMR spectra showed half of the number of protons and carbons as expected from the formula, which indicated two identical parts. Searching in AntiBase using the above spectroscopic data led to dinactin (**101**).



101

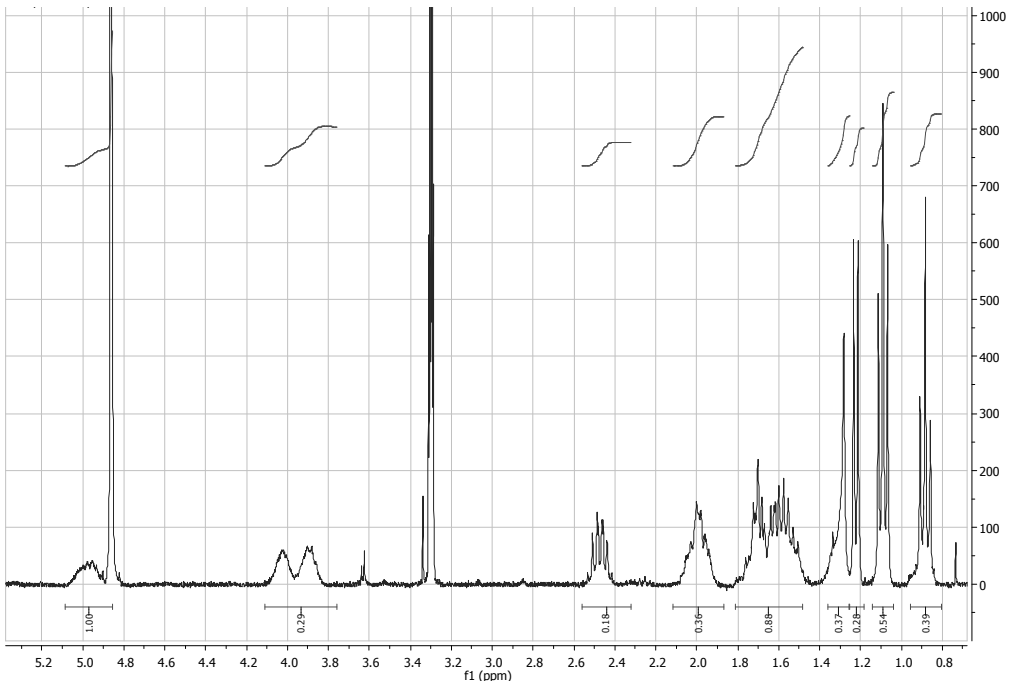
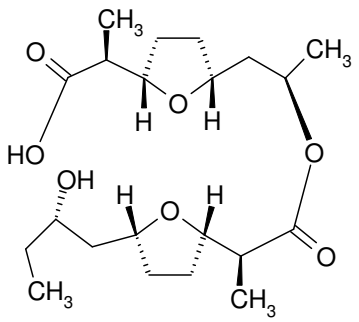


Figure 61: ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of dinactin (**101**)

3.3.6 Bonactin

Bonactin (**102**) was obtained as brown oil from a polar band, which showed a violet colour by anisaldehyde/sulphuric acid. The ^{1}H NMR spectrum of compound **102** showed close structural similarities to dinactin (**101**). It displayed three multiplets for six oxygenated methine at δ 4.18 (H-6'), 4.00 (H-3, 3', 6) and 3.87 (H-8'), as well as a 2H multiplet at δ 2.06 (H-2, 2') of two methine protons linked to sp^2 carbon. Furthermore, a multiplet of two methylene groups was found in the range of δ 2.08-1.93 (H-5, 4). Finally, the ^{1}H NMR spectrum exhibited three doublets and one triplet of methyl groups at 1.25 (CH_3-8), 1.17 (CH_3-2), 1.09 (CH_3-2') and 0.89 (CH_3-10'), of which the last one was assigned to a terminal ethyl group. The ESI mass spectrum showed a molecular ion peak at *m/z* 423 $[M + Na]^+$ affording a molecular weight of 400 Dalton. Searching in AntiBase using the above ^{1}H NMR and the molecular mass resulted in bonactin (**102**), which was further confirmed by direct comparison with the literature.[113]

Bonactin (**102**) showed moderate inhibition of the 3α-hydroxysteroid dehydrogenase, moderate cytotoxicity against L-929, K562 and HeLa cell lines, and exhibited 50% inhibition of Coxackie virus B3 at 25 µg/mL. Bonactin (**102**) was synthesized by Lee and co-workers.[114,115]



102

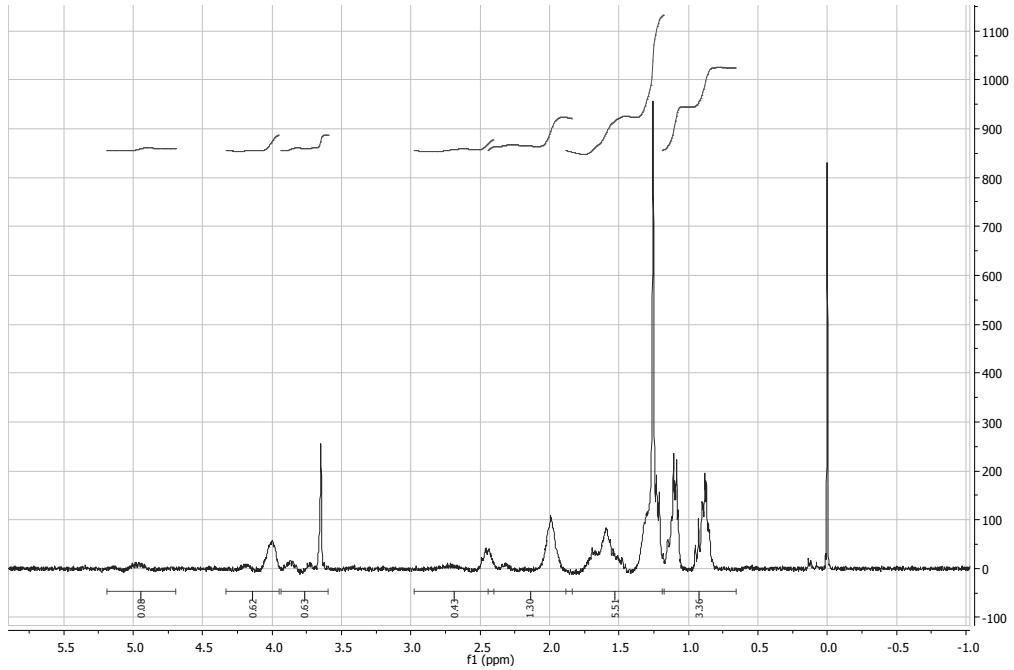
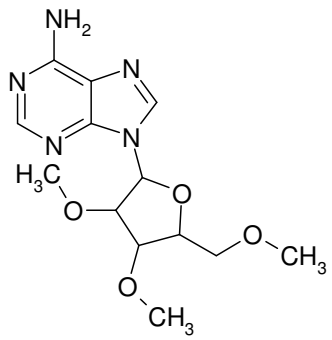


Figure 62: ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of bonactin (**102**)

Literature data on the two linear dimers, nonactin and bonactin, were ambiguous. The former did not exhibit antimicrobial activity[116] up to 2 mg/ml, whereas the latter exhibited excellent activity[117] at a concentration of 1 μg/ml. As compared with previously tested organisms, by using brine shrimp (*Artemia salina*) probably due to a high sodium complex formation.

3.3.7 Trimethoxy adenosine



98

Compound **98** was obtained as a colourless UV absorbing solid from the subfraction IIA. It turned to green with anisaldehyde/sulphuric acid after heating. The molecular weight of **98** was established as 309 Dalton by (+)-ESI mass spectra. (+)-HRESI MS deduced the molecular formula as $C_{13}H_{19}N_5O_4$, containing seven double bond equivalents. The ^{1}H NMR spectrum showed two downfield shifted 1H singlets (δ 8.53, 8.34), similar as in adenosine. In the sugar region, a 1H doublet at δ 6.35, probably of a *hemi*-aminal (N-CH-O) proton was visible, together with three oxymethine signals at δ 4.79, 4.19 and 4.16. Two ABX signals (δ AB 3.60 and 3.44) of an oxymethylene group along with a broad 6H singlet (δ 3.41) for two methyls were visible and an additional broad 3H singlet appeared at δ 3.37. The ^{13}C NMR spectrum of compound **98** showed five aromatic carbon signals, three of which were quaternary (δ 150.6, 141.2, 117.70). The remaining two sp^2 methine carbons exhibited identical chemical shifts (δ 141.2). In the sugar region, a further set of five sp^3 carbon signals was observed; four of them were due to oxygenated methines (δ 86.0~72.3), and the other one to an oxymethylene (δ 71.8). Two methoxy signals appeared at δ

58.9 and 58.8. A search in AntiBase resulted in adenosine analogues, but non of them was fitting with the experimental data.

The trimethoxy derivative **98** was reported as a synthetic compound[118] in the literature and was isolated now for the first time from nature. The structure resembles the primary metabolite adenosin; similar derivatives of thymidine and deoxycytidine were reported in the literature as kinase inhibitors [119]. For **98**, unfortunately no inhibition was found against deoxycytidine kinase, neither at nanomolar nor at micromolar concentration.

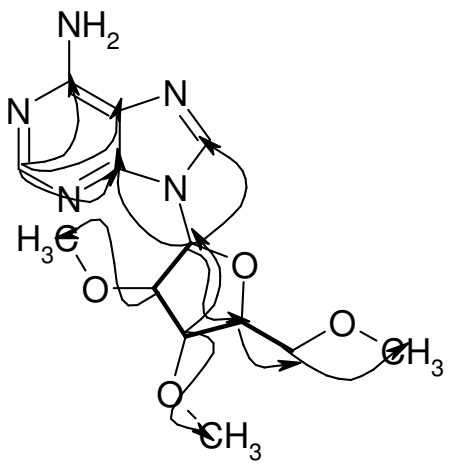


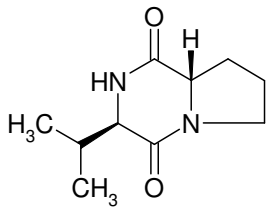
Figure 63: HMBC correlation of trimethoxy adenosine (**98**)

3.4 Marine *Streptomyces* T426A

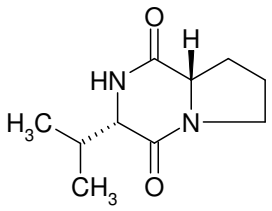
The marine *Streptomyces* sp. T426 A was isolated from the Arctic ice in the Eastern Weddell Sea. Our attention in this strain was evoked by its antibacterial activity and the remarkable metabolic potency: The crude extract showed on TLC a series of UV absorbing zones in polar as well as in the non-polar zones, which showed dark red to violet colour reactions with anisaldehyde. The cultivation of the strain followed by work-up was done at the Institute for Biotechnology and Drug Research (Kaiserslautern). The crude extract was subjected to extensive chromatography and five compounds were isolated: *cis/trans-cyclo*-(Pro-Val) (**103a** and **103b**), *cis/trans-cyclo*-(Tyro-Pro) (**104a** and **104b**) and a cadinane type sesquiterpene **105**.

3.4.1 *Cis-cyclo*-(Pro,Val)

The compound *cis-cyclo*-(Pro,Val) was isolated as colourless crystals and showed a UV absorbing spot, which were visible as with anisaldehyde/sulphuric acid on the pink background. Proton signals were only visible in the aliphatic region: two 1H multiplets were observed at δ 4.19 and 4.03, which indicated electron withdrawing substituents. Two methyl doublets at δ 1.08 and 0.92 and one methine at δ 2.47 formed an isopropyl group. The ^{1}H NMR spectrum revealed further three multiplets at δ 2.31 (1H), 3.52 (2H) and 1.99 (3H). A search in AntiBase resulted in four known structures: two isomers of *cis-cyclo*-(Pro-Val) and two isomers of *trans-cyclo*-(Pro,Val); as the diketopiperazines are characterized by the presence of two chiral centers at the positions 6 and 9, four stereoisomers are possible. Comparison with the authentic ^{1}H NMR spectrum of *cis-cyclo*-(Pro,Val) indicated the identity of **103a**.



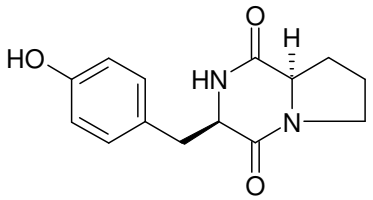
103a



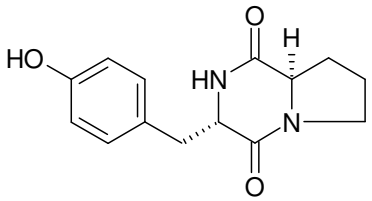
103b

3.4.2 *Cis-cyclo*-(Tyr,Pro)

Cis-cyclo-(Tyr,Pro) was isolated as colourless crystals and showed a UV absorbing spot, which remained white with anisaldehyde/sulphuric acid on the pink background. The ^{1}H NMR spectrum showed a 1,4-disubstituted benzene ring due to the presence of AA´,BB´ signals at δ 7.03 and 6.69 as well as two signals at δ 4.33 and 4.00 for two methines attached to heteroatoms. In addition, the spectrum revealed three multiplets at δ 3.53 (1H), 3.30 (1H) and 3.04 (2H) of an sp^2 bound or hetero atom bearing ABX system of the two methylenes. Three multiplets at δ 2.06 (1H), 1.76 (2H) and 1.22 (1H) for further two methylene groups were also seen in the ^{1}H NMR spectrum. A search in AntiBase led to the structural isomers *trans-cyclo*-(Tyr,Pro) and *cis-cyclo*-(Tyr,Pro). Comparing the discussed data with the literature confirmed the compound as *cis* and *trans* form.



104a

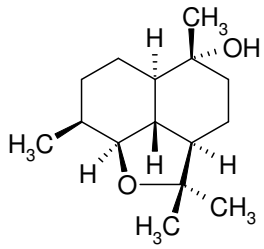


104b

3.4.3 Cadinane type sesquiterpene

Compound **105** was isolated from non-UV absorbing spot, which turned to dark violet with anisaldehyde/sulphuric acid. The molecular formula was established by HR-EIMS ($[M]^+$ *m/z* 238.1928). The ^{1}H and ^{13}C NMR spectra of **105** showed the presence of a methyl doublet [δ 0.90 (3H, d, J = 7.1 Hz)], three methyl singlets [δ 1.05, 1.19, 1.31 (each 3H, s)], and signals of oxygenated carbon atoms at δ 73.0, 81.5, and 81.6. Compound **105** showed correlations between (i) H-4 and C-5, C-6, C-10 and C-15; (ii) H-12/C-6, C-11 and C-13; (iii) H-13/C-6, C-11 and C-12; (iv) H-14 / C-8, C-9 and C-10 in the HMBC spectrum.

The structure of **105** could not be deduced completely, because in the ^{1}H NMR spectrum of **105** several signals were overlapping. The structure was finally established by crystal structure analysis and identified **105** as a cadinane type sesquiterpene, as shown in Figure 65. Toyota *et al.* reported that a solution of (4*S*,7*R*)-germacra-1(10)*E*,5*E*-dien-11-ol (**106**) isolated from the liverwort *Dumoritera hirsuta* in $CHCl_3$ or Et_2O was allowed to stand at RT for a long time to afford compounds **105**, **107, 108** [120]. The NMR data of **105** was identical to that of the literature value. However, the absolute configuration of **105** remains still to be identified.



105

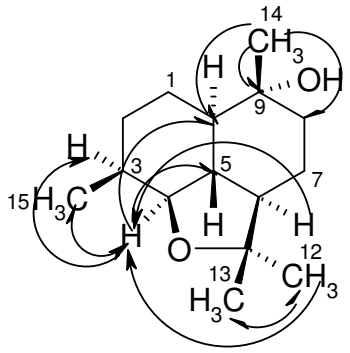


Figure 64: HMBC correlations

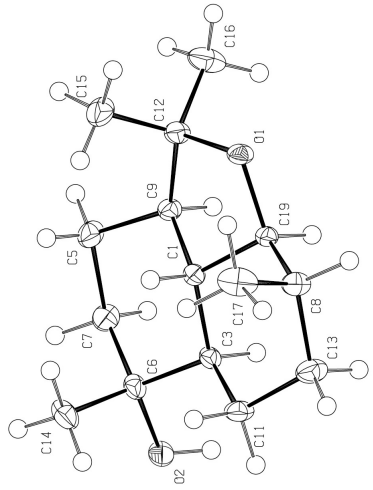
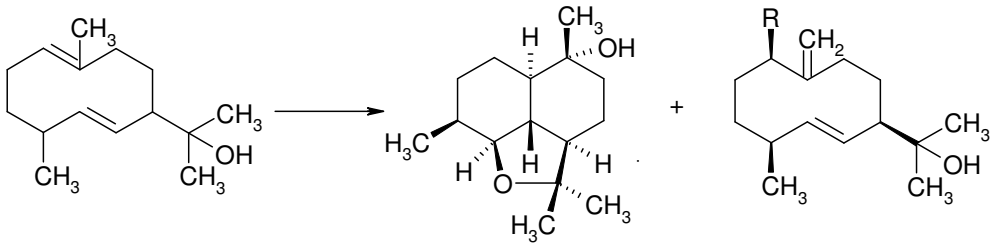


Figure 65: Ortep Diagram of Cadinane sesquiterpene **105**



106 **105** **107** R = OH
108 R = H

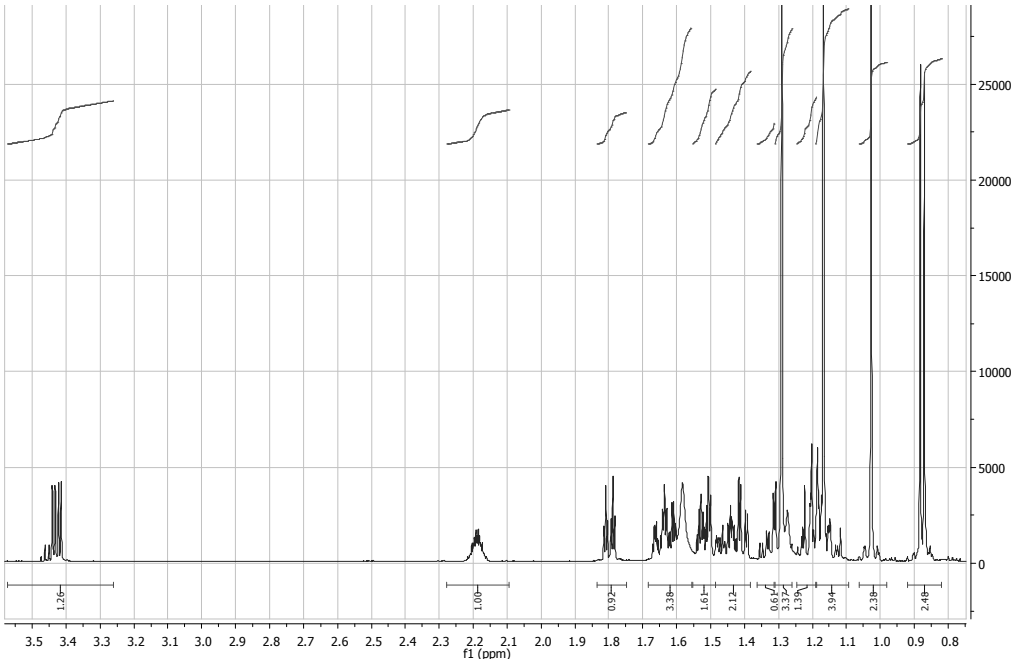


Figure 66: ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of cadinane sesquiterpene **105**

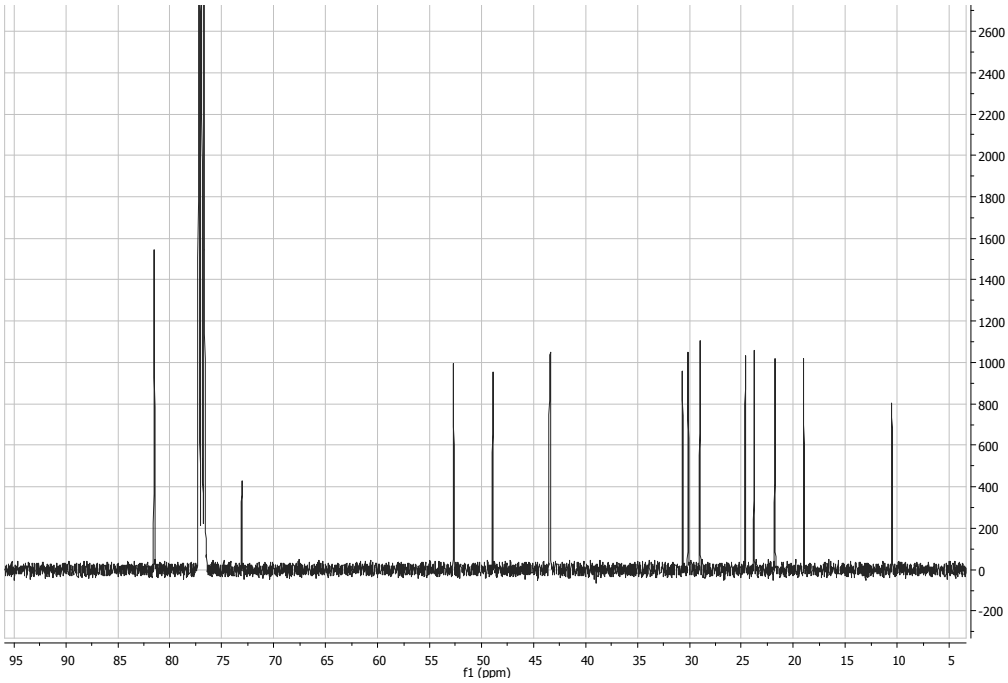
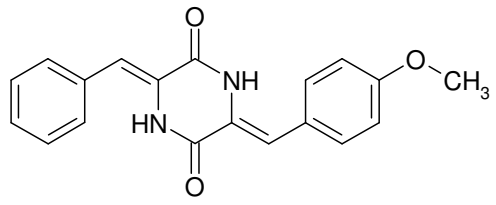


Figure 67: ^{13}C NMR spectrum (300 MHz, $CDCl_3$) of cadinane sesquiterpene **105**

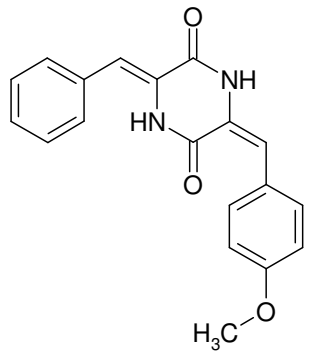
3.5 Marine *Streptomyces* B6003

The extract of the marine streptomycete B6003 has been noticeable in the screening through a selective activity against cancer cell lines. For the further investigation, the strain was twice fermented in a 20-l-fermenter scale. Because the amount of the crude extract was insufficient and its quality in the first fermentation was not satisfactorily, a second trial became necessary. From this strain 4 metabolites were isolated, among them the a new diketopiperazine **115.** Two *E/Z* isomers of *cyclo*-(didehydro-4-methoxyphenyl-alanine,didehydro-phenylalanine)[121] were isolated: the pure isomer **109** and additionally the isomer **110** as a mixture with **109**; (2*S*)-acetamido-3-pentanone (**116**) was also isolated from this strain.

3.5.1 *cyclo*-(Didehydro-4-methoxyphenylalanine,didehydrophenylalanine)



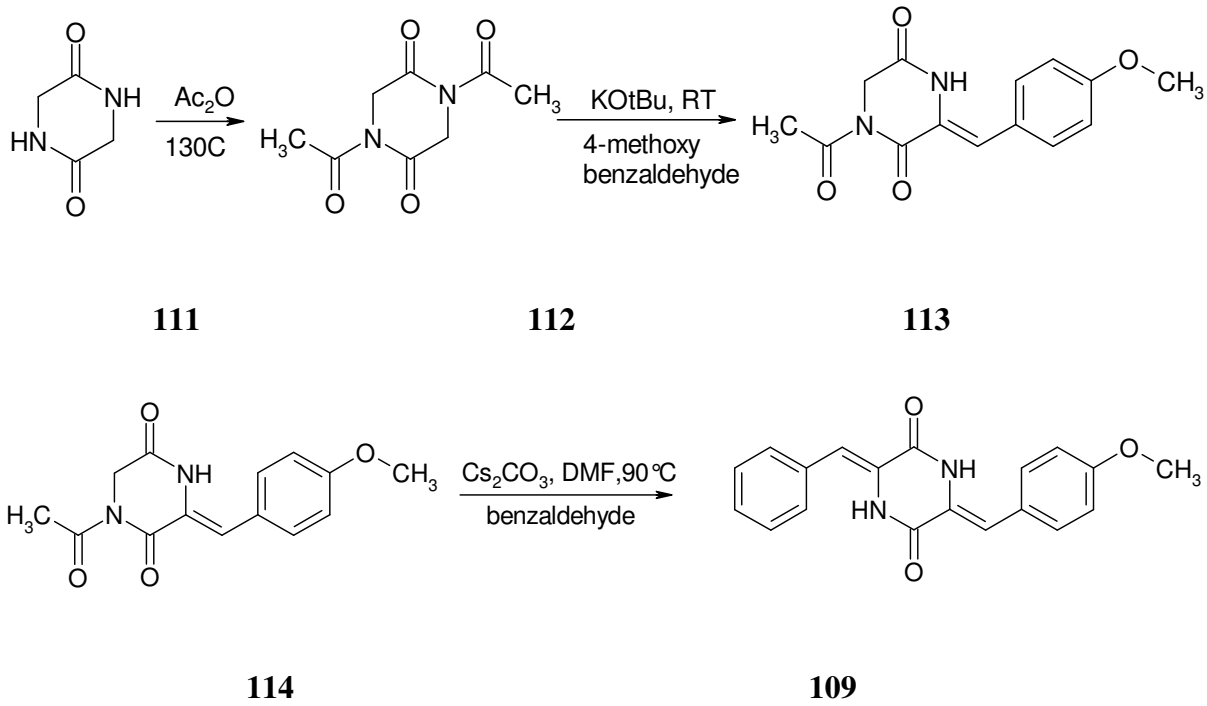
109



110

Compound **109** was isolated as yellow solid having a molecular formula of $C_{19}H_{16}N_2O_3$ as deduced from accurate mass measurements on the molecular ion. The ^{1}H and ^{1}H,^{1}H COSY NMR spectra included one 3H singlet at δ 3.82 corresponding to the presence of a methoxy group, a broad 1H singlet at δ 8.05 and revealed the presence of eleven further aromatic protons corresponding to a substituted and an unsubstituted benzylidene group. The ^{13}C NMR spectrum supported the above conclusions and further indicated the presence of two quaternary carbons resonating at δ 159.6 and 159.9. The structure of compound **109** was elucidated by interpretation of spectral data, including the HSQC and HMBC correlations. The structure of compound **110** was determined in a similar fashion as the *cis*-isomer but was obtained as

a mixture of **109** and **110**. The stereochemistry of **109** found to be 3Z, 6Z based on the identity with the published structure from S. *thioluteus* and biosynthetic consuderations [121]. The proposed structure of **109** was confirmed previously by synthesis[121]. NMR-Verschiebungen diskutieren und mit **109** vergleichen Compound **109** is reported to inhibited plasminogen activator inhibitor-1 (PAI-1) in an *in vitro* tPA-mediated plasmin generation assay (S2251) with IC_{50} values of 51 μM and in an urokinase amidolytic assay (S2444) with an IC_{50} value of 80 μM. Various controls excluded the possibility that the diketopiperazine interfered with the substrate or acted directly on the plasminogen activators.



Scheme4: Synthesis of *cyclo*-(didehydro-4-methoxyphenylalanine,didehydrophenylalanine) (**109**)

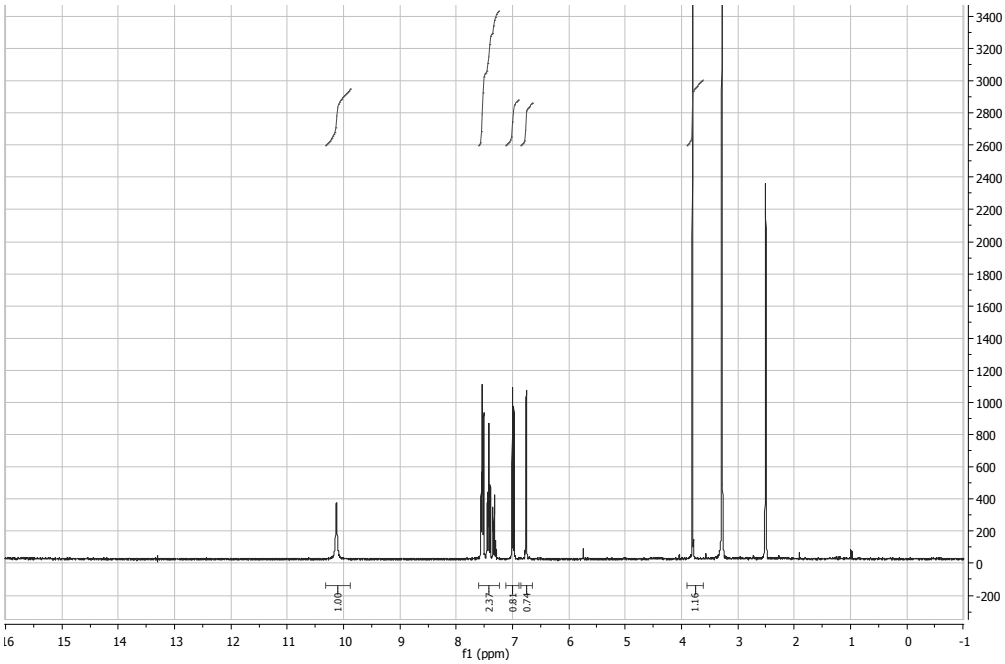


Figure 68: ^{1}H NMR spectrum (300 MHz, DMSO-d_6) of *cyclo*-(didehydro-4-methoxyphenylalanine, didehydrophenylalanine) (**109**)

3.5.2 Albonoursin C

Compound **115** was obtained as pale yellow, crystalline powder having the molecular formula $C_{16}H_{18}N_2O_3$ as determined by HRESIMS and ^{13}C NMR data. Dereplication with the NMR data using AntiBase indicated a new derivative of albonoursin B.[122] The mass information and ^{1}H NMR data gave the inference of an additional methoxyl group in the phenyl group of albonoursin. Although there was a reference that this compound was obtained by synthesis[123,124], analytical data as well as the stereochemistry or any hint about the isolation as natural product were not reported. The ^{1}H NMR spectrum of **115** showed two aromatic proton signals H9/H13 at δ 7.44 (2H, d, J = 8.7) and H10/H12 at δ 6.98 (2H, d, J = 8.76) indicating a *para* substituted aromatic ring system. An aromatic methoxy signal was observed at δ 3.82 and confirmed by an oxygenated carbon at δ 55.1 in the ^{13}C NMR spectrum. The HMBC correlations of the aromatic signals H10/H12 at δ 6.98, the C11 at δ 158.8 and the correlations between H9/H13 at δ 7.44 and C8 at δ 125.3 confirmed the substitution pattern of the aromatic ring system and the partial structure. A signal H7 at δ 6.72 showed further HMBC correlations with C9 at δ 130.7, C13 at δ 130.8 and C5 at δ

157.6. The presence of an isopropyl group was seen at δ 1.01 (6H, d) in the ^{1}H NMR spectrum. COSY correlations between the methine proton H15 at δ 2.96 (1H, m) and δ 1.01 (6H, d) were observed indicating that the isopropyl group is attached adjacent to a methine proton H15. ^{1}H-^{1}H COSY correlations were observed between proton H14 at δ 5.72 (1H, d, J = 10.2) and the methine proton H15 at δ 2.96 (1H, d) and hence these two were adjacent to each other as seen in the structure. Further HMBC correlations of H14 with C2 at δ 157.3, H14 with C16 at δ 22.1 and C17 at δ 22.2 confirmed the structure as methoxyalbonoursin (**115**), which is termed here as albonoursin B. The (*Z*)-configuration was assigned on the basis of the chemical shifts of H-7 and H-14 and was confirmed by a NOESY correlation between H-7 (δ 6.7) and H-14 (δ 5.7) for albonourisin C (**115**).

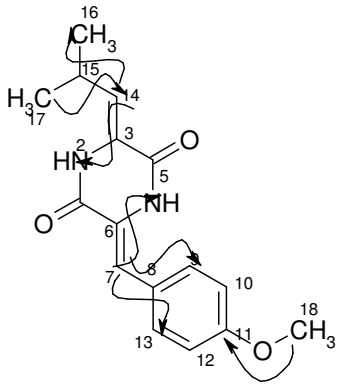


Figure 69: HMBC correlations of albonoursin C (**115**) Albonoursin

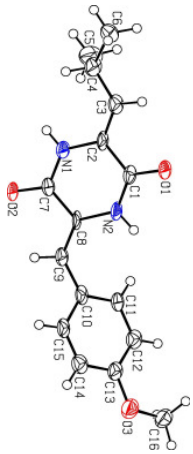


Figure 70: ORTEP diagram of albonoursin C (**115**)

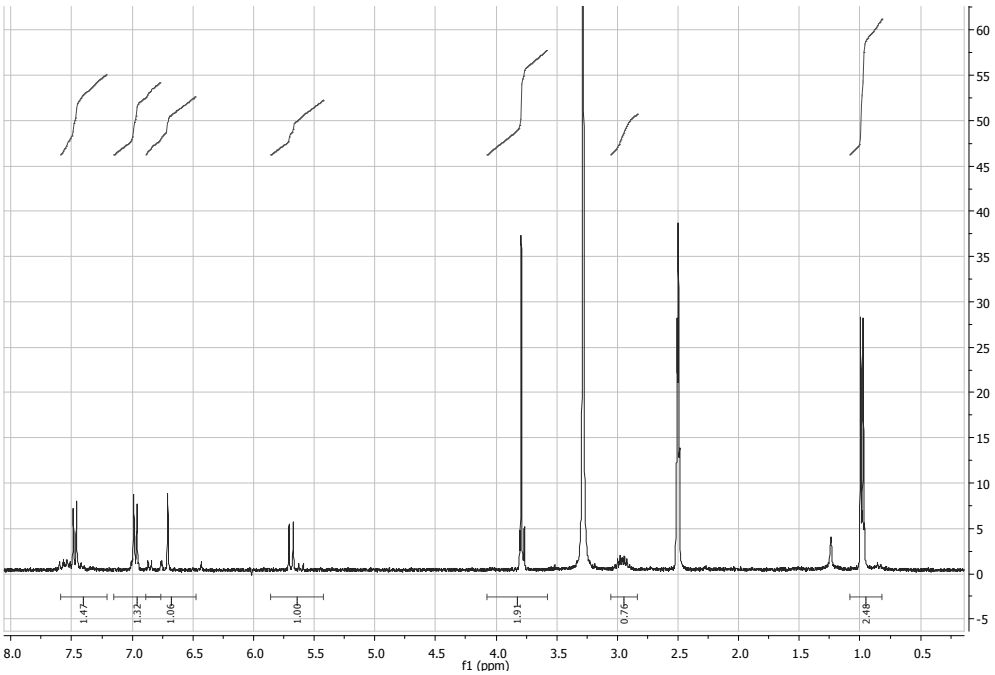


Figure 71: ^{1}H NMR (300 MHz, DMSO-d_6) spectrum of albonoursin C (**115**)

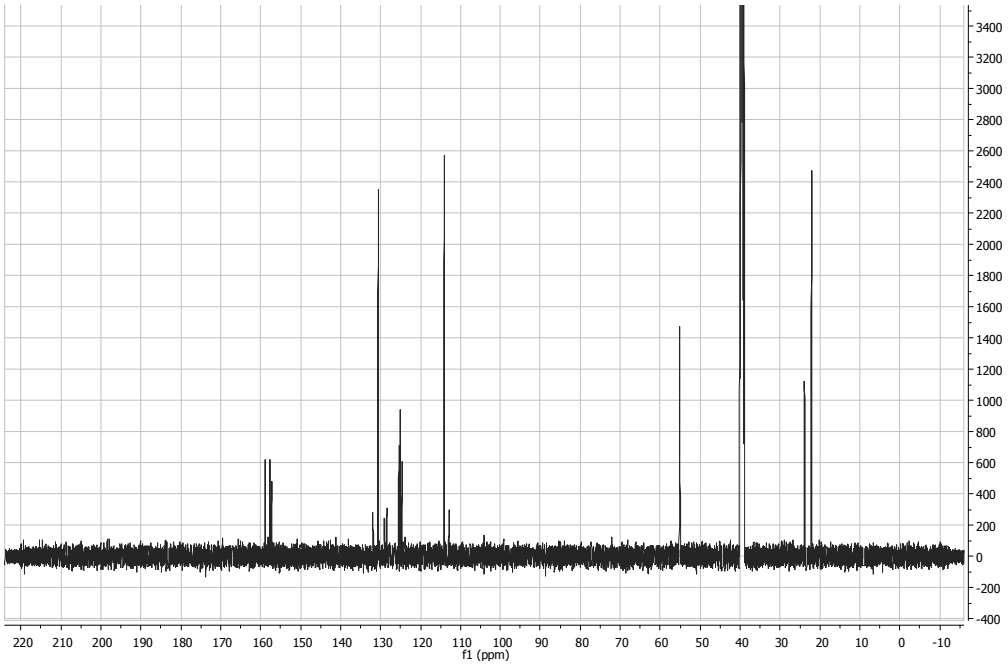


Figure 72: ^{13}C NMR spectrum (125 MHz, DMSO-d_6) of albonoursin C (**115**)

Table 12: NMR data of albonoursin B (**115**) and *cyclo*-(didehydro-4-methoxy-phenylalanine,didehydrophenylalanine) (**109**) in DMSO-d_6

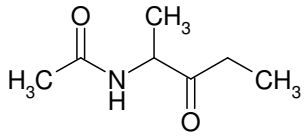
Albonoursin C (**115**)				*cyclo*-(Didehydro-4-methoxy-phenylalanine,didehydrophenylalanine) (**109**)		
Position	δ H[a] (J in Hz)	∂C[b] mult	HMBC	Position	δ H[a] (J in Hz)	∂C[b] mult
2	-	157.3	-	2	159.6	-
3	-	125.1	-	3	125.9	-
5	-	157.6	-	4		8.05 (1H.brs)
6	-	125.1	-	5	159.8	-
7	6.7, s	114.3	5, 7, 13	6	128.9	-
8	-	125.3	-	1’	121.3	7.25 (1H, brs)
9	7.44, d (8.7)	130.7	10, 13	2’	125.9	-
10	6.98, d (8.76)	114.0	9, 10, 12	3’	131.1	7.24 (2H, d, J = 8.8 Hz
11	-	158.8	-	4’	113.8	6.93 (2H, d, J = 8.6)
12	6.98, d (8.76)	114.0	10, 11, 12, 13	5’	159.8	-
13	7.44, d (8.7)	130.8	9, 12	1”	116.9	7.1 (1H, s)
14	5.7, d (10.4)	125.2	2, 16	2”	132.9	-
15	2.96, m	23.8	14, 16, 17	3”	128.4	7.42 (2H, m)
16	1.01, d	22.1	14, 15, 17	4”	129.4	7.42 (2H, m)
17	1.01, d	22.2	14, 15, 16	5”	128.7	7.36 (1H, m)
				OCH_3	55.3	3.80 (3H, s)

[a] DMSO-d_6, 300 MHz. [a] DMSO-d_6, 125 MHz

3.5.3 (2*S*)-Acetamido-3-pentanone

HR-EI-MS measurement of **116** showed a molecular ion at *m/z* 143.0938 (calcd. for $C_7H_{13}NO_2$, 143.0946). In the ^{1}H NMR spectrum of **116**, a broad singlet at δ 6.45 (1H) replaceable with D_2O was attributed to an amide proton. The signals of a triplet (3H) at δ 1.10 and a quartet at δ 2.48 showed the presence of an ethyl group, which was attached to a carbonyl carbon. A singlet (3H) at δ 2.01 was ascribed to acetyl protons and in addition another quartet was assinged to a methine signal at δ 2.60 attached to nitrogen of the amide group. The ^{13}C NMR spectrum showed 2 carbonyl signals and one of them at δ 172.3 indicated an amide carbonyl. In ^{1}H-^{1}H COSY experiments, the broad NH singlet at δ 6.45 correlated with a methine quintet (1H) at δ 4.62, which itself correlated with a quartet (J = 1.1 Hz). The HMBC correlations

confirmed the presence of a -COCH(NHCO-)CH_3 moiety. Thus, the structure was derived as acetamido-3-pentanone (**116**). The (2*S*) isomer was previously isolated[125] from a fungal strain of the genus *Scolicotrichum graminis;* it had no significant activity.



116

3.6 Marine *Streptomyces* sp. ACT7655

The strain was isolated and identified by E. Helmke from the Alfred-Wegener Institute for Polar and Marine Research in Bremerhaven, Germany. The strains formed a white mycelium after incubation on M_2^+ agar medium for 3 days at 28 °C. The antimicrobial assay of crude extracts from the marine *Streptomyces* sp. ACT 7655 showed strong activity against *Bacillus subtilis* and *Staphylococcus aureus* and weak activity against *Streptomyces viridochromogenes* Tü 57 and *Escherichia coli*. The chemical screening showed four UV absorbing bands at 254 nm in the less polar region.

3.6.1 Venturicidin A

Compound **117** was purified by column chromatography and was obtained as a weakly at 254 nm UV absorbing solid. It gave an intensive dark blue colour on spraying with anisaldehyde/sulphuric acid reagent and Ehrlich's reagent. The substance showed a complex signal pattern in the region δ 5.58 – 0.82 of the ^{1}H NMR-spectrum. The existence of 41 carbon atoms in the molecule was inferred from the ^{13}C NMR spectrum. In the ESI spectrum, an ion was found at *m/z* = 772 for $[M+Na]^+$. A search in the database with this information gave a 20-membered lactone ring with a sugar and a substituted C_{11}-alkyl-side chain, the antibiotic venturicidin A (**117**). Comparison of the NMR data with literature values confirmed the identity.[126] It was isolated formerly from *Streptomyces aureofaciens*[127].

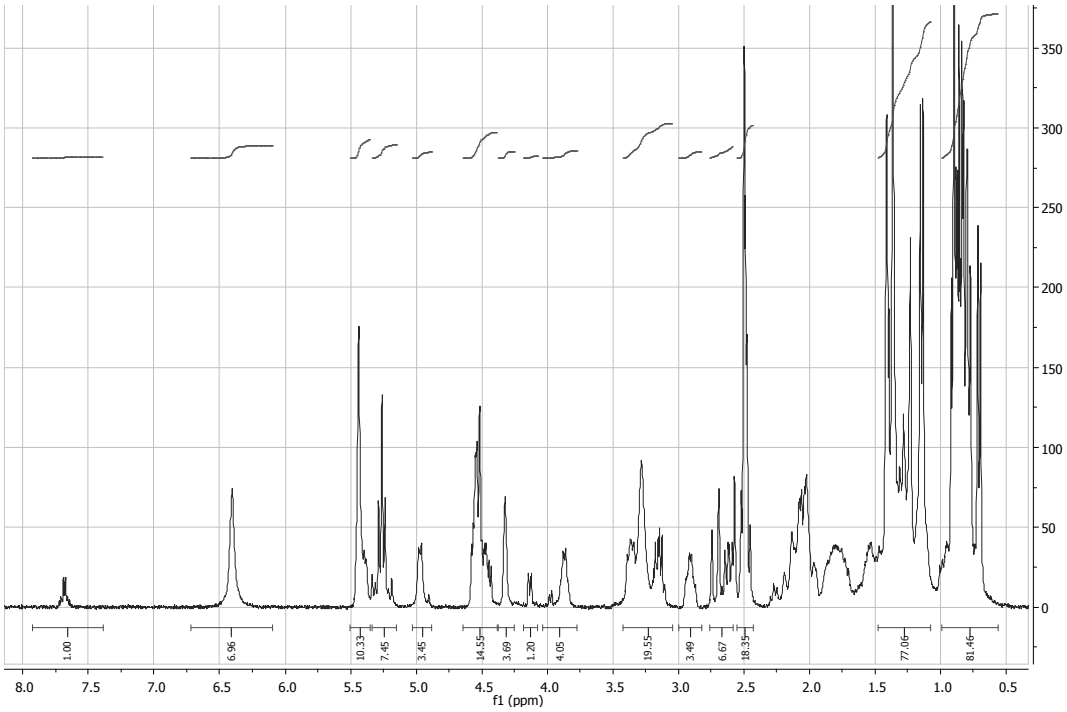
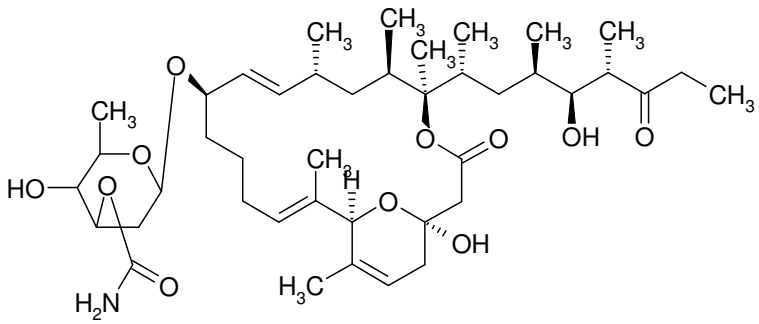


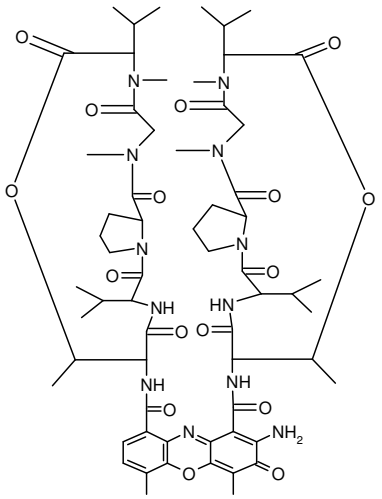
Figure 73: ^{1}H NMR spectrum (300 MHz, DMSO-d_6) of venturicidin A (**117**)



117

3.6.2 Actinomycin D

The sub fraction F3 was purified by preparative thin layer chromatography and purified by silica gel for several times to afford the orange coloured actinomycin D (**118**). It was UV absorbing and showed no colour change with NaOH but turned to red with concentrated sulphuric acid. The actinomycins form a family of chromopeptide antitumor antibiotics isolated from various *Streptomyces* strains. The natural actinomycins all share the same phenoxazinone chromophore, varying only in the amino acid content of their two-depsipentapeptide moieties. ^{1}H NMR spectrum showed two *ortho* protons at δ 7.21 and 7.42 of a 1,2,3,4-tetrasubstituted aromatic ring and two 3H singlets at 2.25 and 2.57 for methyl groups attached to an aromatic system. This and the typical colour are characteristic for the phenoxazinone chromophore in actinomycins. The spectrum showed 4 NH doublets between δ 7.60-8.15. It also exhibited 8 hydrogen signals of oxygenated or α-amino acid protons at δ 6.03 (d), 5.96 (d), 5.25-5.15 (m, 2 H), 4.78 (d), 4.73 (d), 4.61 (dd) and 4.49 (dd). Further 8H signals were exhibited between δ 4.01-3.48 for methylene and methine groups. Six singlets each with intensity of 3H were observed between δ 2.98-2.20, four of them for N-methyl groups. Methylene groups of proline residues appeared between δ 1.80 - 2.24 as multiplets with intensity of 6H. Additionally, the spectrum showed 5 signals between δ 0.75 and 1.41 for five methyl groups.



118

ESI MS revealed the molecular ion peak at *m/z* 1255. A search in AntiBase gave two possible structures, actinomycin D (**118**) and aurantin II. The difference between the two structures is that aurantin II has an ethyl group at C-2' but actinomycin D has no ethyl group. In the ^{1}H NMR spectrum, there is no triplet peak for a methyl group in the highest field. The structure of actinomycin D (**118**) has been further confirmed by comparing with authentic spectra as well as literature data. Actinomycin D (**118**) has found clinical application as anticancer drug, particularly in the therapy of Wilm's tumor[128] and soft tissue sarcoma[129] in children. Actinomycin D (**118**) has been proposed as a therapeutic agent for AIDS, because it is a potent inhibitor of HIV-1 minus-strand transfer.[130]

4 Terrestrial Streptomyces

4.1 Terrestrial *Streptomyces* sp. GW 7/354

The antimicrobial assay of the crude extract of *Streptomyces* sp. GW 7/354 showed moderate activity against *Streptomyces viridochromogenes* (Tü 57) and *Mucor miehei* (Tü 284) but no activity against other bacteria tested. It showed 86% activity against *Artemia salina*. TLC showed various UV active zones by a strong violet colour with anisaldehyde/sulphuric acid. In addition, HPLC/MS showed siderochelin A (**119**) and B (**120**) as main products; other compounds were seen in traces.

4.1.1 Siderochelin A

Siderochelin A was isolated as colourless crystals from the polar fraction I. During TLC, compound **119** appeared as blue fluorescence band under UV. On spraying with anisaldehyde/sulphuric acid, it was stained pink. Its molecular weight was established by ESI as 235 Dalton. ESIHRMS determined the molecular formula as $C_{11}H_{11}N_3O_3$, bearing 7 double bond equivalents. The proton NMR spectrum of compound **119** established the presence of the substituted aromatic moiety giving signals at δ 8.18 (dd, J = 5.58, 2.3 Hz, 1H) and δ 7.70 (m, 2H). The ^{13}C NMR spectrum showed 11 carbon signals; a down field peak among them at δ 175.8 was most likely due to an amide or ester carbonyl group. Three aromatic sp^2 methines appeared at δ 135.2, 128.3 and 126.3. A further methine carbon at δ 71.5 was connected with a proton at δ 4.96 (td, J = 9.2, 2.0 Hz, 1H). The values at δ 181.2 and 140.8 were for quaternary carbon atoms, which were connected with heteroatoms. The methylene

group and a methyl group showed up at δ 43.0 and δ 28.3 respectively in the ^{13}C NMR and their corresponding protons appeared at δ 2.38 (ddd, J = 17.2, 13.5, 6.5 Hz, 1H) and 1.78 (s, 3H) in ^{1}H NMR. HSQC spectrum gave the information and the location of the protonated carbon signals. HMBC spectrum of the aromatic protons at δ 8.18 (dd, J = 5.58, 2.3 Hz, 1H) and δ 7.70 (m, 2H) correlated with quaternary carbon at δ 140.8 and 159.4 and hence 3-hydroxy pyridine moiety was constructed which consumed four double bond equivalence. A downfield value for C-9 at δ 71.5 was unusual for a methine attached to nitrogen. H-9 gave HMBC correlations to C-10, carbonyl amide and C-11 while the singlet methyl at C-12 gave a correlation to C-7 at δ 181.2. Hence from the HMBC, ^{1}H-^{1}H COSY and from the knowledge of remaining double bond equivalence a five membered ring containing nitrogen was constructed. This inference was further supported by X-ray crystallographic analysis where the structure and its absolute configuration were confirmed.

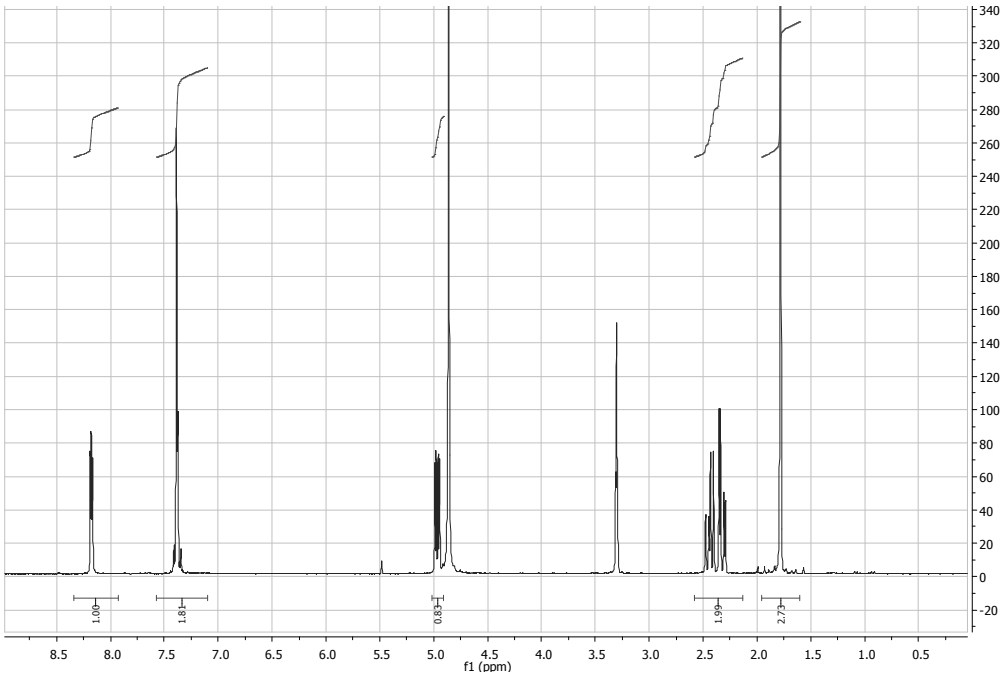
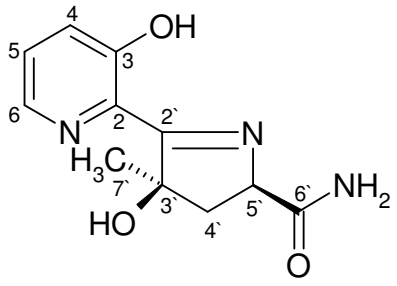
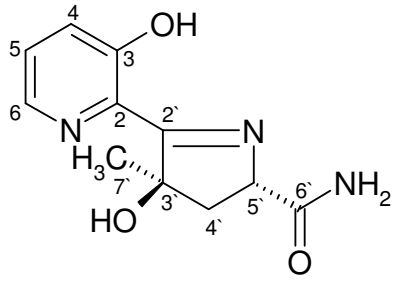


Figure 74: ^{1}H NMR (300 MHz, CD_3OD) spectra of siderochelin A (**119**)



119



120

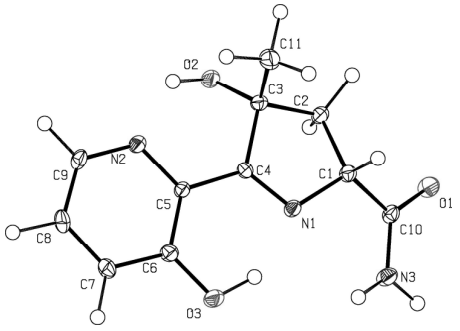


Figure 75: ORTEP diagram of siderochelin A (**119**)

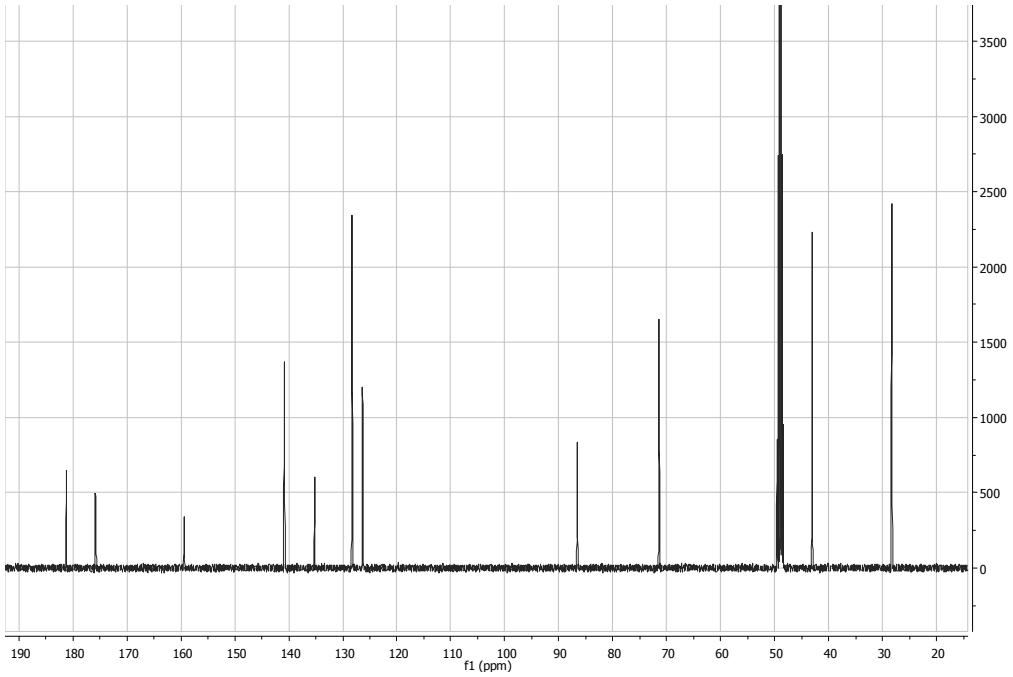


Figure 76: ^{13}C NMR spectrum (125 MHz, CD_3OD) of siderochelin A (**119**)

4.1.2 Siderochelin B

Siderochelin B was isolated as colourless crystals from the same polar fraction II. During TLC, compound **120** appeared as blue fluorescent band under UV. On spraying with anisaldehyde/sulphuric acid, it stained pink. Its molecular weight was established by ESI mass spectrometry as 235 Dalton. ESIHR MS determined the molecular formula as $C_{11}H_{11}N_3O_3$, bearing 7 double bond equivalents. The ^{1}H and ^{13}C NMR spectra were similar to those of siderochelin A (**119**); only the values of the methylene protons at C-4' were different, which appeared in this molecule as AB signals at δ 2.45 (dd, J = 12.7, 7.1 Hz) and at δ 2.21 (td, J = 9.3, 3.3 Hz). Crystal structure analysis determined the isomeric form of siderochelin as siderochelin B. The structure was in agreement with the literature and also determined the sterochemistry.

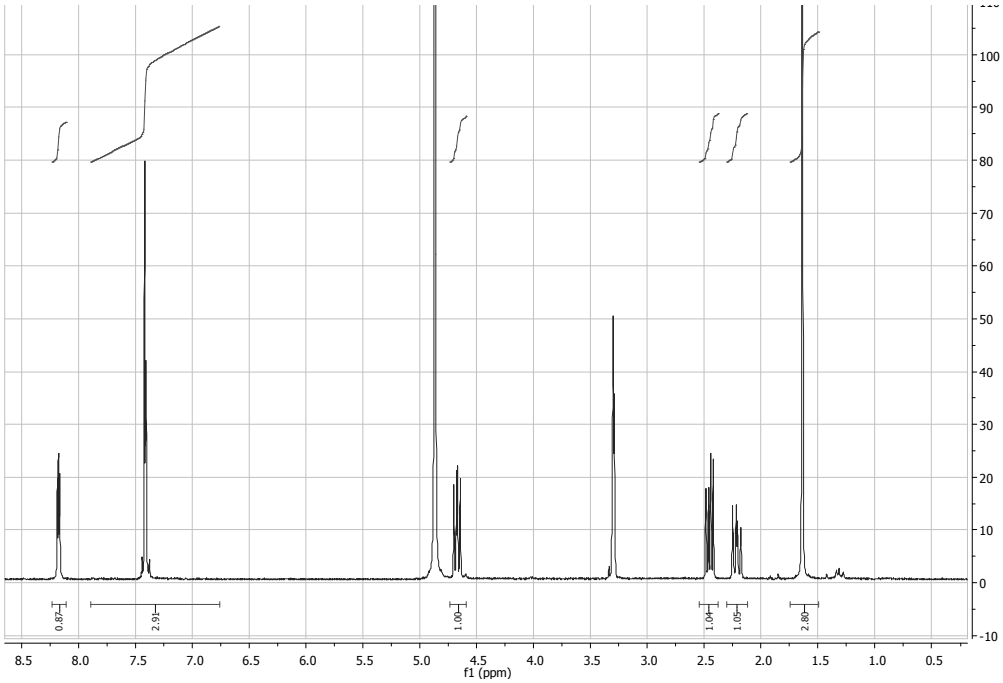


Figure 77: ^{1}H NMR spectrum (300 MHz, CD_3OD) of siderochelin B (**120**)

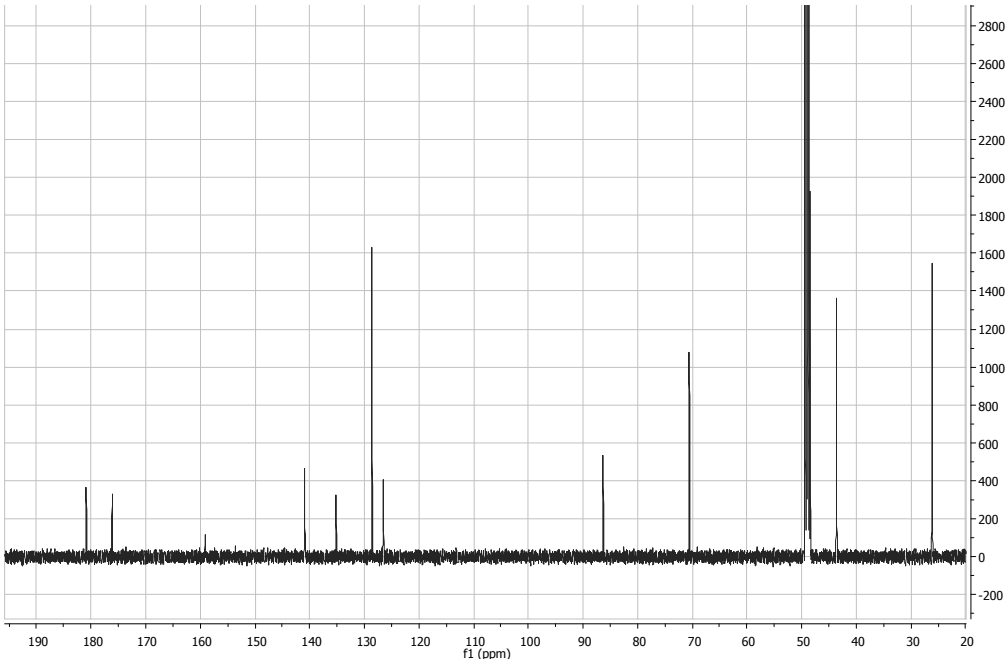


Figure 78: ^{13}C NMR spectrum (125 MHz, CD_3OD) of siderochelin B (**120**)

4.1.3 Siderochelin D

Siderochelin D was isolated as colourless solid from the less polar fraction III. During TLC, compound **121** appeared as blue fluorescent band under UV. On spraying with anisaldehyde/sulphuric acid, it stained pink. Its molecular weight was established by ESI as 218 $[M+H]^+$ and by ESIHRMS, the molecular formula was determined as $C_{11}H_{11}N_3O_2$, having 8 double bond equivalents. The ^1H NMR spectrum showed many broad signals in the aromatic region between δ 8.08-7.10. A singlet at δ 6.72 assigned the presence of an olefinic proton. A 3H singlet at δ 2.35 was due to a methyl group attached to an aromatic/olefinic carbon. The ^{13}C NMR and the HSQC spectrum showed many sp^2 methine signals (δ 133.2~143.2), it showed a down field peak at δ 168.2 for an amide carbonyl group and also showed four quaternary carbons at δ 143.4, 155.6, 180.8 and 125.2. The elucidation of siderochelin D was based on the similarity with the other two known derivatives siderochelins A and B. The mass of Siderochelin D was 217 Dalton and that of siderochelins A and B was 235 Dalton. The difference of 18 Dalton corresponded to a loss of water from the tertiary alcohol. Further 2D correlations (see Figure 81) confirmed the presence of a pyridine moiety and a five-membered ring as in siderochelin A (**119**) and B (**120**) and led to

the discovery of a new deoxygenated derivative of siderochelin named as siderochelin D (**121**).

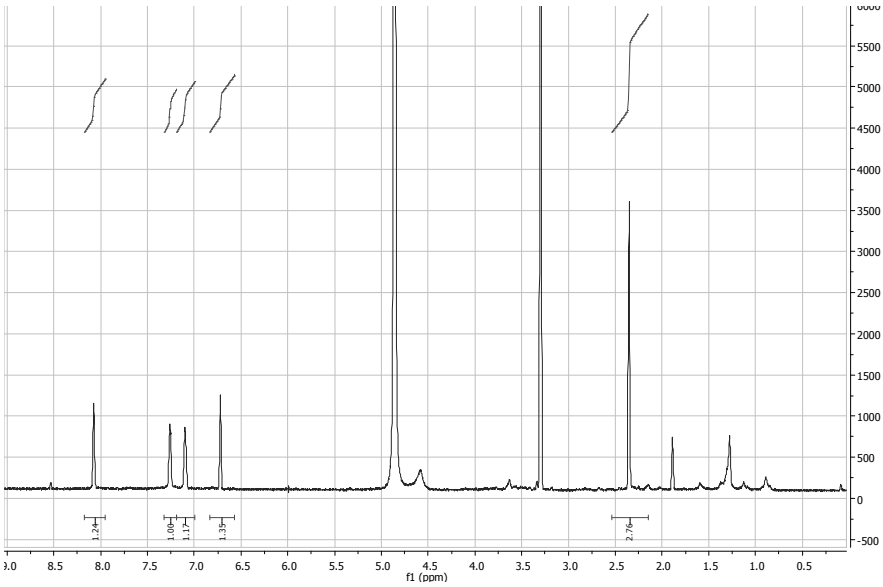


Figure 79: ^{1}H NMR spectrum (300 MHz, CD_3OD) of siderochelin D (**121**)

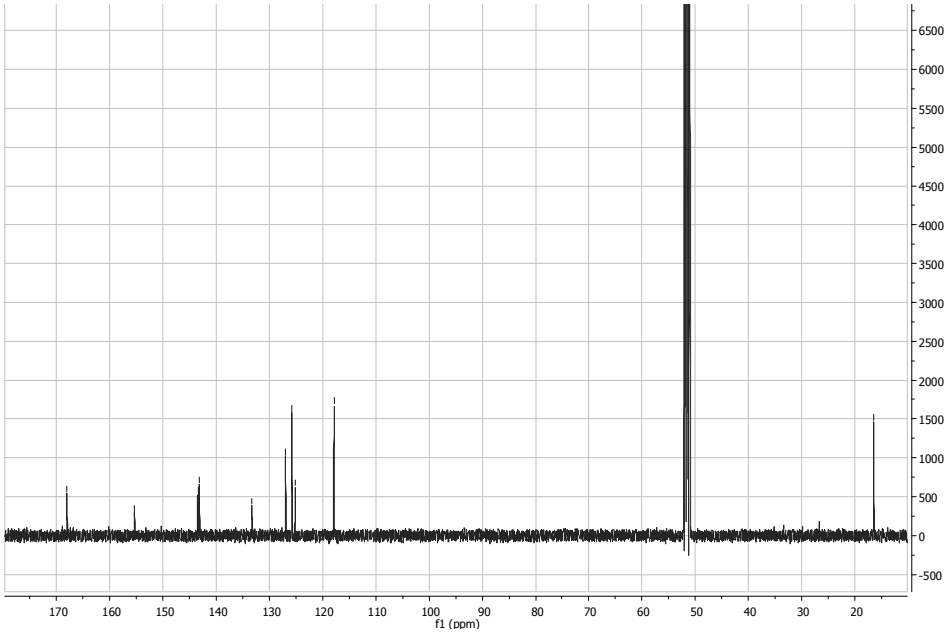


Figure 80: ^{13}C NMR spectrum (125 MHz, CD_3OD) of siderochelin D (**121**)

Table 13: ^{1}H and ^{13}C NMR spectrum of siderochelin A (**119**), B (**120**) and D (**121**)

	Sidersochelin A (**119**)			Sidersochelin B (**120**)			Sidersochelin D (**121**)		
Position	^{1}H (δ_H, mult., *J* Hz)[a]	^{13}C (δ_C)[b]	HMBC	^{1}H (δ_H, mult., *J* Hz)[a]	^{13}C (δ_C)[b]	HMBC	^{1}H (δH, mult., *J* Hz)[a]	^{13}C (δ_C)[b]	HMBC
2		140.8	-		140.9	-		143.4	-
3		159.4	-		159.1	-		155.3	-
4	7.19 (m)	128.3	2, 3, 4, 5	7.6 (m)	128.6	2, 3, 4, 5	8.08 (brs)	133.3	2, 3, 4, 5
5	7.53 (m)	126.3	2, 3, 4,	7.25 (m)	126.6	2, 3, 4,	7.26 (brs)	126.9	2, 3, 4,
6	8.18 (dd, *J* = 5.58, 2.3 Hz, 1H)	135.2	3, 4, 5	8.18 (dd, *J* = 5.58, 2.4 Hz, 1H)	135.2	3, 4, 5	7.10 (brs)	143.2	3, 4, 5
2’	-	181.2	-	-	180.8	-	-	180.8	-
3’	-	71.5	-	-	70.6	-	-	70.6	-
4ab’/4a’	2.38 (dd, *J* = 12.6, 7.0 Hz, 2H)	43.0	2’, 3’, 5’	2.45 (dd, *J* = 12.7, 7.1 Hz)	43.7	2’, 3’, 5’	6.72 (s)	117.8	2’, 3’, 5’
4b’	-	-	-	2.21 (dd, *J* = 12.6, 9.3 Hz)	-	2’, 3’, 5’	-	-	2’, 3’, 5’
5’	4.89 (m)	86.6	3’, 4’, 6’	4.66 (dt, *J* = 22.0, 11.0 Hz,	86.4	3’, 4’, 6’	-	125.2	3’, 4’, 6’
6’	-	175.8	-	-	176.1	-	-	168.0	-
7’	1.78 (s, 3H)	28.3	2’, 3’, 4’, 5’	1.78 (s, 3H)	26.1	2’, 3’, 4’, 5’	2.35 (s, 3H)	16.5	2’, 3’, 4’, 5’

[a] CD_3OD-d_4, 300 MHz. [b] CD_3OD-d_4, 125 MHz

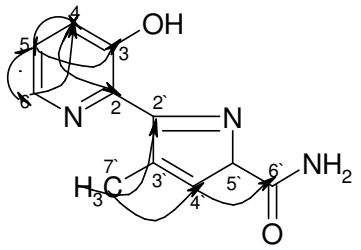
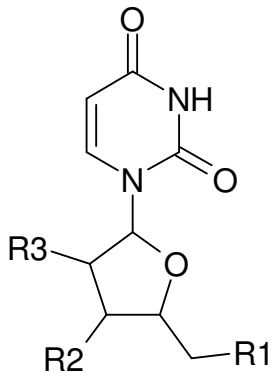


Figure 81: The HMBC correlations of siderochelin D (**121**)

4.1.4 Acetyl uridine A and B

Acetyl uridine A (**122a**) was isolated as colourless solid from fraction II; it showed UV absorption at 254 nm and turned bluish green with anisaldehyde/sulphuric acid reagent. The ^{1}H NMR spectrum showed a signal in the aromatic region at δ 8.0 (d, J = 8.1 Hz) and at δ 6.26 (dd, J = 8.4, 5.9 Hz). It also showed an anomeric proton at δ 5.86 (dt, J = 6.1, 2.0 Hz, 1H) and three oxymethine proton signals between δ 5.3 and δ 3.8; a methylene showed a multiplet at δ 2.52, a methyl singlet at δ 2.08 could be linked to a carbonyl as acetyl group. The ESI mass spectrum of this compound revealed the *pseudo*molecular ion peak at *m/z* 286, which was 32 amu more than the molecular mass of uridine. The HRESI mass spectrum suggested the molecular formula $C_{11}H_{14}N_2O_7$, and the assumption of an acetyl group was confirmed by the occurrence of the fragment ion peak at *mlz* 59 $[M\text{-}COCH_3]^+$ by the ESI-MS of **122a**.

The ^{13}C NMR spectrum showed 11 signals among which two were sp^2 methine carbons (δ 142.1 and 102.9). Two carbonyl signals as for uracil were observed at δ 151.2 and δ 166.1 and the acetyl carbonyl was observed at δ 171.9. In the sugar region, a further set of three sp^3 carbon signals was observed; three of them were oxygenated methines (δ 86.8~76.4) and the other an oxymethylene (δ 62.9). The HMBC correlation of H-5' at δ 3.78 (d, J = 3.1 Hz) to carbonyl of acetyl group at δ 171.9 and the HMBC of the methyl group at δ 2.08 to acetyl carbonyl group confirmed the placement of the acetyl group in acetyl uridine A. Acetyl uridine B (**122b**) was also isolated from the same strain only differing in the position of the acetyl group. The placement of the acetyl group was confirmed by the HMBC correlations of H3'with the acetyl group for **122b**. Other correlations were in accordance with uridine and hence the structure was deduced as shown in the figure 83 and table 14. These metabolites were not known from nature however this molecule has been synthesized[131] for its investigation for pharmacological applications.



	acetyl uridine A	acetyl uridine B
122a R^1	$OCOCH_3$	OH
122b R^2	OH	$OCOCH_3$

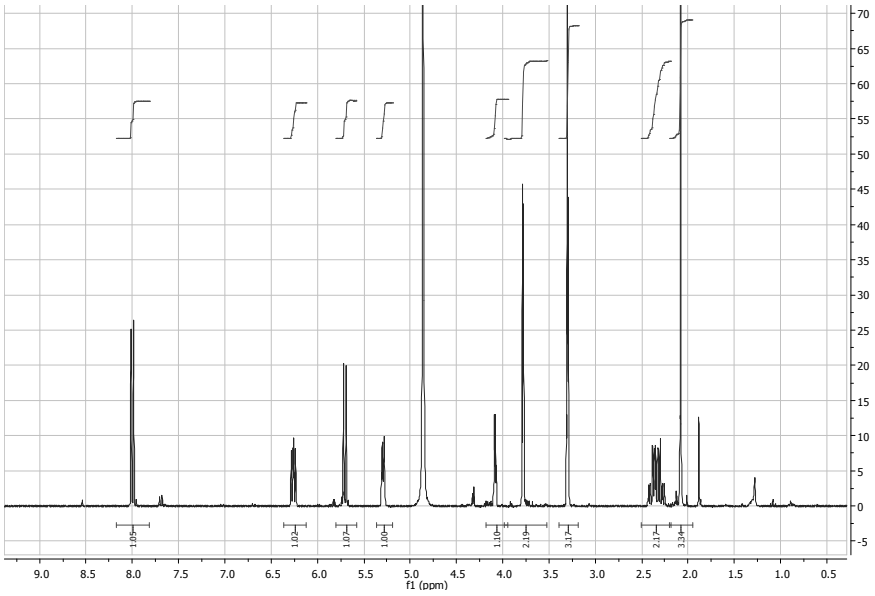


Figure 82: ^{1}H NMR spectrum (300 MHz, CD_3OD) of acetyl uridine A (**122a**)

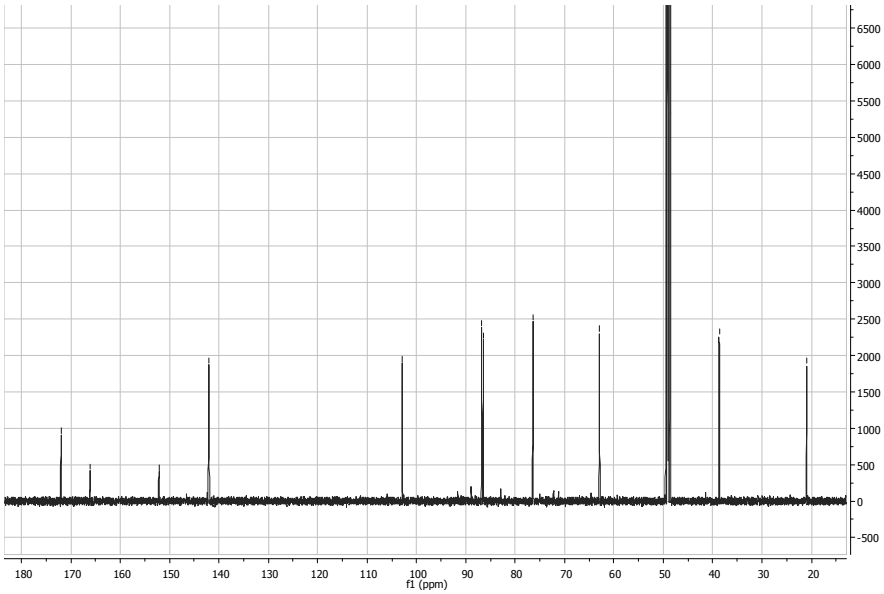


Figure 83: ^{13}C NMR spectrum (300 MHz, CD_3OD) of acetyl uridine A (**122a**)

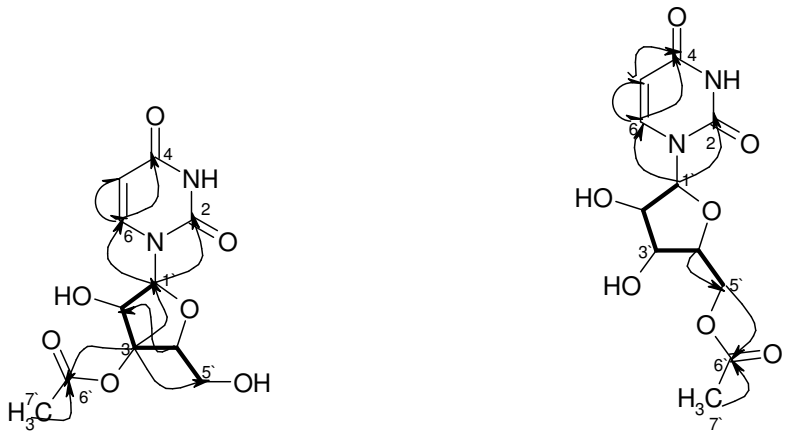


Figure 84: HMBC correlations for acetyl uridine A (**122a**) and B (**122b**)

Table 14: ^{1}H and ^{13}C NMR spectrum of acetyl uridine A (**122a**) and B (**122b**)

Position	^{1}H (δ_H, mult., *J* Hz)[a]	^{13}C (δ_C)[b]	HMBC	^{1}H (δ_H, mult., *J* Hz)[a]	^{13}C (δ_C)[b]	HMBC
2	-	151.2	-		151.9	-
4	-	166.1	-		166.0	-
5	6.26 (dd, *J* = 8.4, 5.9 Hz	142.1	6	7.70 (d, *J* = 8.1 Hz)	141.8	6
6	8.00 (d, *J* = 8.4 Hz	102.9	5	7.97 (d, *J* = 8.1 Hz)	102.8	5
1’	5.29 (dt, *J* = 6.1, 2.0 Hz)	86.8	2, 6	6.24 (m)	86.8	2, 6
2’	4.08 (m)	86.5	1’, 3’, 4’	4.44 (m)	85.5	1’, 3’, 4’
3’	3.88 (m)	76.4	2’, 4’, 5’	3.91 (dd, *J* = 6.8, 3.4 Hz)	72.1	2’, 4’, 6’
4’	2.53 (m)	38.7	2’, 3’, 5’	2.40 (m)	40.8	2’, 3’, 5’
5’	3.78 (d, *J* = 3.1 Hz)	62.9	4’, 6’	3.74 (qd, *J* = 12.0, 3.5 Hz	65.0	4’, 3’
6’	-	171.9	-	-	172.2	-
7’	2.08 (s)	20.9	6’	2.08 (s)	20.7	6’

a CD_3OD-d_4, 300 MHz. b CD_3OD-d_4, 125 MHz

4.2 Terrestrial *Streptomyces* sp. ANK205

The crude extract of the *Streptomyces* sp. ANK205 drew our attention due to its high biological activity against a set of test organisms in the biological screening. The crude extract showed a low and rather unselective cytotoxic activity against a range of human tumor cell lines with a mean IC_{50} of 89 μM (mean IC_{70} =105 μM) and showed antimicrobial activity (Table 28). In the chemical screening, *semi*-polar zones were present which gave unusual yellow, dark green, violet and black colourations with anisaldehyde/sulphuric acid spraying reagent. Additionally, HPLC MS/MS of the crude extract showed many interesting peaks in the positive mode with MS/MS peptide fragmentation patterns. A 25 L shaker culture of *Streptomyces* sp. ANK205 was grown on M_2 medium at 28 °C for 7 days, giving a yellowish-brown culture broth, which was filtered over Celite and adsorbed on Amberlite XAD-16, while the mycelium was extracted with ethyl acetate and acetone. By column chromatography on silica gel, 11 compounds were obtained: tryptamine, 2-hydroxy-1-(1*H*-indol-3yl)-ethanone, tryptophol, methyl 4-hydroxybenzoate, *p*-hydroxy-2-phenylethyl alcohol, 4-hydroxybenzoic acid. The remaining more polar

fraction IV contained 3-(2-hydroxy-4-methoxyphenyl)-propanoic acid, glutamic acid, piperazimycin A (**123**), butanolide A (**124**), and butanolide B (**125**).

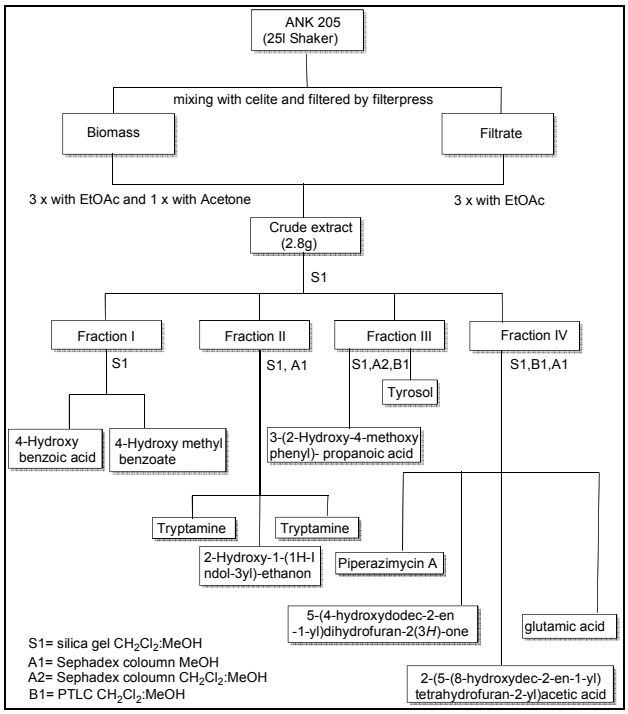


Figure 85: Working up scheme of the terrestrial *Streptomyces* sp. isolate ANK 205

4.2.1 Piperazimycin A

Compound **123** was isolated as white UV absorbing powdery solid from fraction IV during purification by RP-18 silica gel. It gave blue colour with anisaldehyde/sulphuric acid reagent, and based on the two *quasi*-molecular ion peaks observed at *m/z* 749 $[M+Na]^+$ and 725 $[M-H]^-$, in (+)-ESI and (-)-ESI MS, respectively, the molecular weight of **123** was determined as 726 Dalton. Two [M+2] ions in the ratio 3:1 indicated the presence of chlorine, which was confirmed by the corresponding molecular formula $C_{31}H_{47}N_8O_{10}Cl$. The ^1H NMR spectrum displayed four olefinic methine protons between δ 5.98 ~ 5.42, which had coupling constants in the range of $J = 11$ ~ 16 Hz. In addition, a further four δ-methines of amino acids (δ 5.83 ~ 4.96) and their corresponding carbons (~δ 50) were found. Between δ 5.52 ~ 2.0, several signals of ~24 protons were visible, which could be assigned to oxymethine/methylene protons or protons at other electron-withdrawing systems. Finally, one methyl singlet at δ 1.60 along with a doublet at δ 1.14 (6H) of an isopropyl group

was observed. The ^{13}C NMR and HSQC spectra depicted the presence of 31 carbon signals, which were classified into the following categories: six amide and/or ester carbonyl carbons (δ 174.8 - 169.8), four olefinic methine carbons (δ 142.3, 135.4, 125.7 and 124.7), two oxymethylenes (δ 63.2, 63.7), two oxymethines (δ 57.8, 58.6), five δ carbons of amino acids, four of which were methines (δ 53.4- 50.7) and one quaternary carbon (δ 63.0). The remaining signals were of three other methylenes (δ 36.6, 37.2 and 27.5) and three methyls (δ 22.6, 22.7 and 18.9). When this compound was isolated, a substructure search in all databases delivered hits of undetermined structure named sohbumycin, which had the same mass; the published ^{1}H NMR spectrum was very similar to that of **123**, which was later published by Fenical[132].

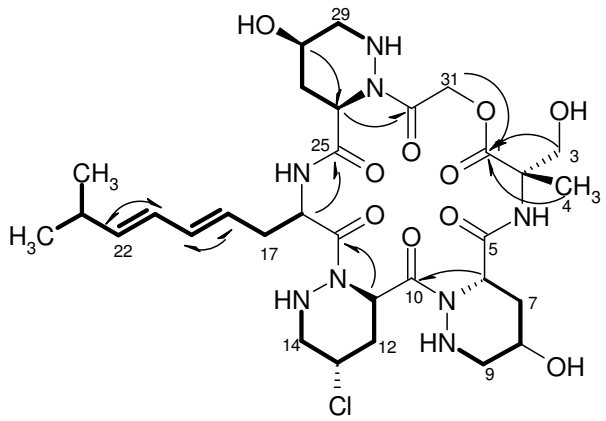


Figure 86: ^{1}H-^{1}H COSY (bold bonds) and selected ( ) HMBC couplings in piperazimycin A (**123**)

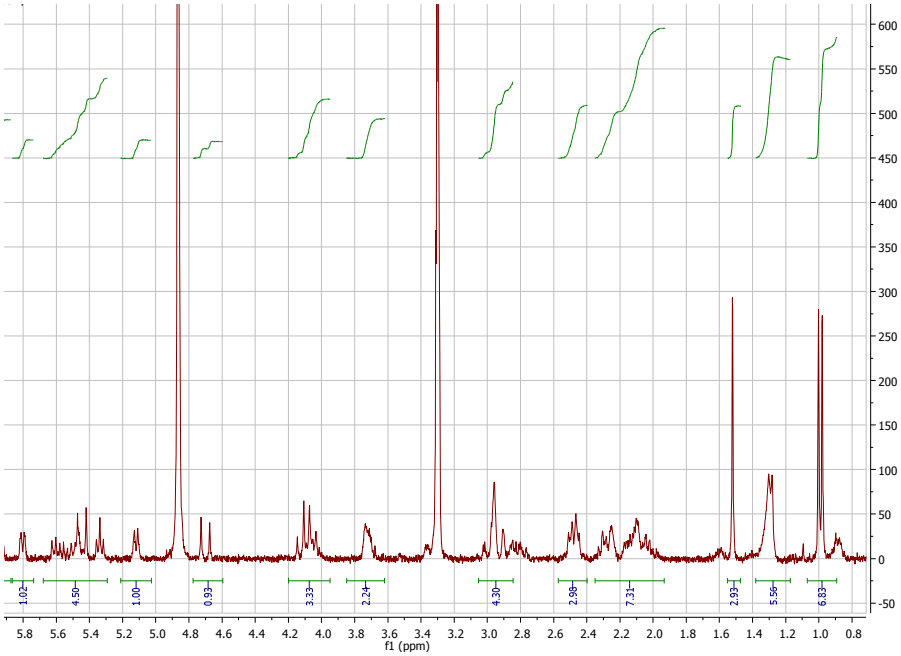


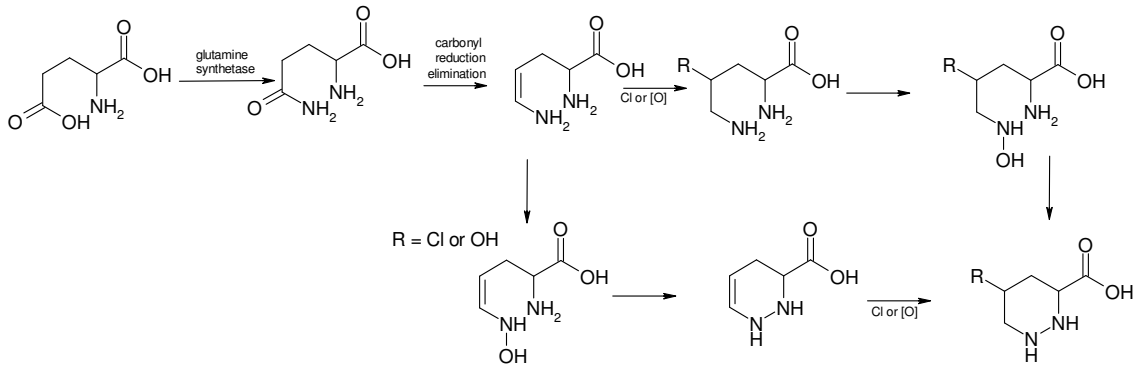
Figure 87: ^{1}H NMR spectrum (CD_3OD, 300 MHz) of piperazimycin A (**123**).

The ^{13}C and ^{1}H NMR data of **123** were in agreement with published values of sohbumycin[133]. As the NMR data were also identical with the recently published structure of piperazimycin A (**123**), it can be concluded that the formerly unidentified antibiotic sohbumycin is identical with piperazimycin A (**123**), a cyclodepsipeptide containing the rare acids γ-hydroxypiperazic acid, γ -chloropiperazic acid, 2-amino-8-methyl-4, 6-nonadienoic acid, 2-amino-8-methyl-4, 6-decadienoic acid, and hydroxyacetic acid. Cyclic peptides, containing piperazic acid, were reported to have high biological activities, e.g. anticancer[134], tuberculostatics[135], anti-inflammatory[136] and anti-HIV agents[137,138]. From this strain glutamine was also isolated. It has been assumed that the formation of this cyclic depsipeptide is *via* glutamic acid pathway followed by reduction of the *δ*- carbonyl and conversion of either the α - or γ -amino group to a hydroxylamine, and intramolecular cyclization (Scheme 5)[139]. Hence this hypothesis can be supported by the fact that glutamine is a precursor for the biosynthetic formation of piperazimycin as shown in the Scheme 5

Table 15: ^{1}H and ^{13}C NMR assignments of piperazimycin A (**123**) in CD_3OD.

Position	**Piperazimycin A** δ_C[a],	δH (*J* [Hz])[b]	Position	**Piperazimycin A** δ_C[a],	δH (*J* [Hz])[b]
1	174.8		16	51.4	5.21 (q, 5.9)
2	63.7		17a	37.2	2.46 (m)
3a	63.2	4.03 (d, 10.7)	17b		2.54 (m)
3b		4.06 (d, 10.7)	18	125.7	5.42 (m)
4	18.9	1.5 (s)	19	142.3	5.98 (t, 10.9)
5	173.5	-	20	135.4	5.95 (t, 11.2)
6	52.1	5.3 (d, 6.8)	21	124.7	5.62 (d, 12.5)
7a	25.6	2.10 (m)	22	31.8	2.32 (m)
7b		2.22 (m)	23	22.6	1.14 (d, 6.7)
8	56.7	3.7 (m)	24	22.7	1.14 (d, 6.7)
9a	53.4	2.87 (m)	25	169.8	-
9b		2.96 (m)	26	51.6	4.89 (d, 7.4)
10	174.3	-	27a	27.5	2.2 (d, 14.5)
11	50.7	5.67 (d, 6.7)	27b		2.34 (d, 14.1)
12a	36.6	2.01 (m)	28	57.8	3.76 (m)
12b		1.98 (m)	29a	54.2	2.95 (m)
13	51.4	3.92 (m)	29b		3.01 (d, 13.2)
14a	52.8	2.68 (dd, 8.5, 1.2)	30	169.6	4.46 (d, 12.8)
14b		3.32 (d, 8.5)	31a	63.5	4.46 (d, 16.4)
15	171.2	-	31b		5.56 (d, 16.4)

[a] CD_3OD-d_4, 300 MHz. [b] CD_3OD-d_4, 125 MHz

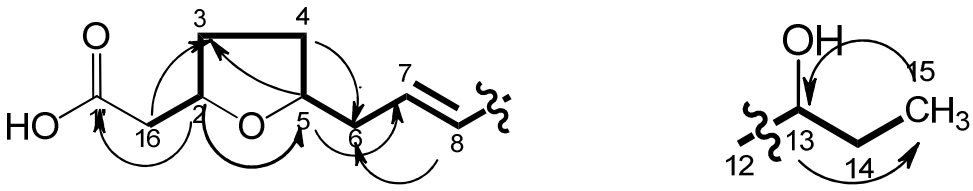


Scheme 5: Hypothetical biosynthetic pathways to γ-substituted piperazic acids.

4.2.2 Butanolide A

Extensive chromatography of *Streptomyces* sp. ANK 205 extracts delivered the compound **124**, a derivative of asperic acid[140], which had not been isolated from nature before. According to the ESI HRMS value, compound **124** analysed for the molecular formula $C_{16}H_{28}O_4$. The compound did not show UV absorption, but a dark green colour reaction with anisaldehyde/sulphuric acid.

The ^{1}H NMR and ^{13}C NMR spectrum of compound **124** corroborated the presence of four oxygenated carbon atoms and a secondary hydroxyl group. The ^{13}C NMR shifts of C-2 at δ 64.8 and C-5 at δ 81.1 confirmed the presence of oxygenated carbon atoms. Since this compound had three double bond equivalents and two were accounted for a carbonyl and a double bond, the third one must be a ring, most likely a tetrahydrofuran ring. The chemical shift at δ 73.2 of C-13 indicated a further oxygenated carbon atom. Additionally, the ^{1}H NMR spectrum showed a clear triplet of a terminal methyl group with an HMBC coupling to an oxymethine and a ^{1}H-^{1}H COSY correlation with a methylene group at δ_H 1.47/1.37, indicating the presence of a terminal ethyl residue adjacent to an oxymethine group. HMBC correlations from the methylene protons at δ_H 2.13/2.20 to C-7 at δ_C 131.9 and C-8 at δ_C 129.6 confirmed the presence of an olefin adjacent to C-6. Also an HMBC correlation was observed from δ_H 2.13/2.20 to C-5 at δ_C 81.1 and C-4 at δ_C 37.2. The protons belonging to the methylene carbon (C-16) at δ_H 3.05/2.80 showed an ABX splitting pattern in the ^{1}H-NMR spectrum with J_{AB} = 13.9, J_{AX} = 0 and J_{BX} = 7.0 Hz. ^{1}H-^{1}H COSY correlations were observed between δ_H 3.05/2.80 and δ_H 4.14. Also ^{1}H-^{1}H COSY correlations were observed between the methine proton of C-2 at δ_H 4.14 and the methylene protons of C-3 at δ_H 1.98/1.78. HMBC correlations from the methine proton at δ_H 4.14 to C-3 at δ_C 35.2, C4 at δ_C 37.2, C5 at δ_C 81.1 confirmed the presence of the furan ring system. An HMBC correlation was observed to the carbonyl group at δ_C 175.9. The presence of free carboxylic acid was inferred based on the fact that a methyl ester was formed on treatment with diazomethane. This sequence resulted in fragments **a** and **b**



124a **124b**

Figure 88: ^{1}H-^{1}H COSY and HMBC ( ) correlations in the partial structures of **124**

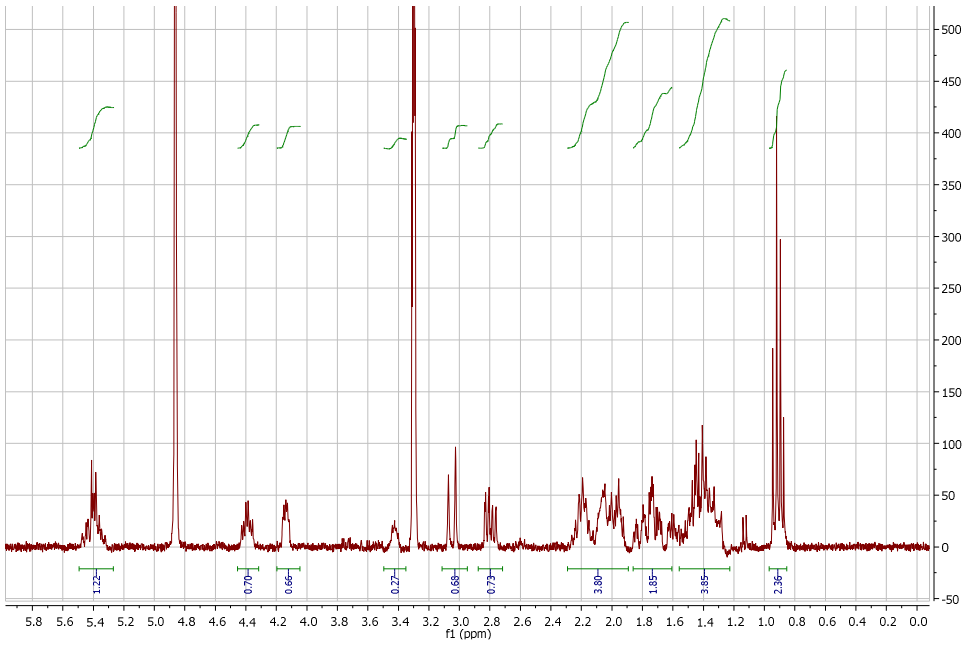


Figure 89: ^{1}H NMR spectrum (CD_3OD, 300 MHz) of butanolide A (**124**)

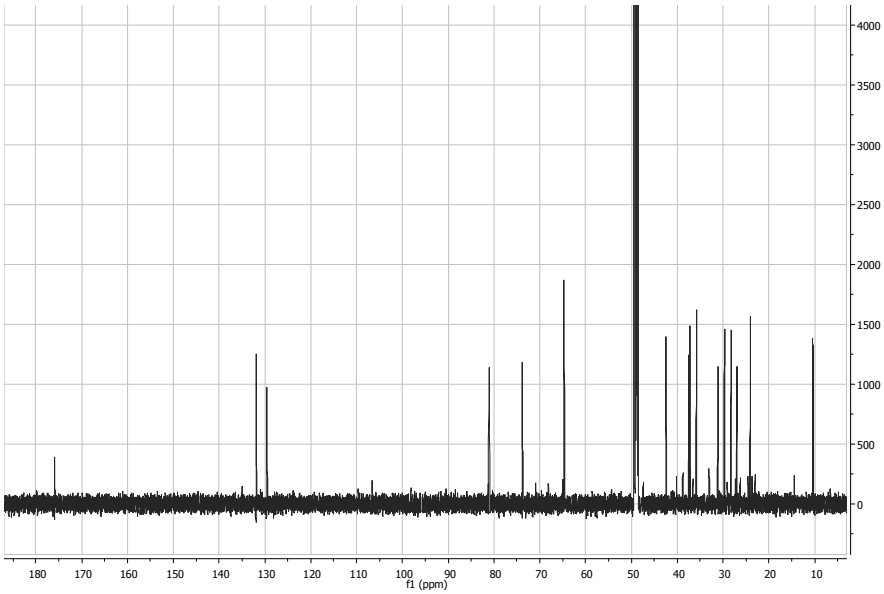
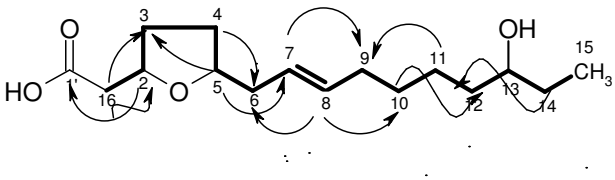


Figure 90: ^{13}C NMR spectrum (125 MHz, CD_3OD) of butanolide A (**124**)

From the information of the molecular formula the remaining C_4H_8 residue should be inserted between carbons C-8 and C-12. It was obviously not easy to verify this from the COSY correlations due to overlapping of the methylene signals, but was supported by HMBC correlations.



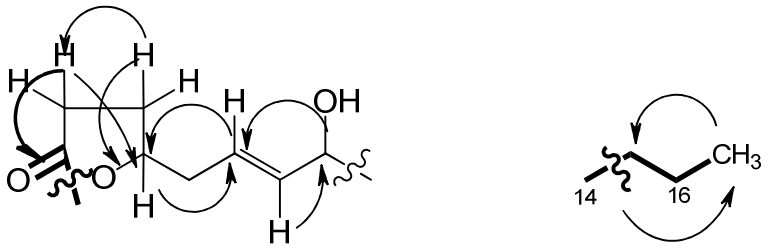
124

Figure 91: ^1H-^1H COSY (bold bonds) and HMBC correlations ( ) of butanolide A (**124**)

4.2.3 Butanolide B

Compound **125** was analysed by ESIHR mass spectromertry for the molecular formula $C_{16}H_{28}O_3$ having one oxygen less than compound **124.** The molecular formula indicated three double bond equivalents for compound **125**. HMBC correlations observed from the methylene protons at δ_H 2.18/2.25 to C-7 at δ_C 130.8 and C-8 at δ_C 137.0 confirmed the presence of an olefin adjacent to C-6. HMBC correlations from δ_H 2.18/2.25 to C-5 at δ_C 81.0 and C-4 at δ_C 37.3 were similar to the previous com-

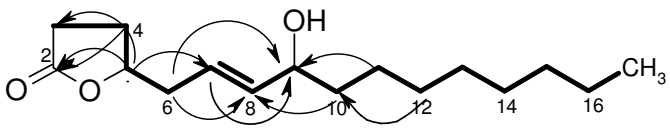
pound **124**. The presence of an allyl alcohol was confirmed by the ^{1}H-^{1}H COSY correlations between δ_H 4.35 and δ_H 5.37 and additionally proven by HMBC correlations of δ_H 4.35 to δ_C 130.8 and δ_C 38.8. The formation of the lactone ring was confirmed from the doublebond equivalents and also by the HMBC correlations of the methine proton at δ_H 4.42 to C-2 at δ_C 179.9, C3 at δ_C 35.6 and C-4 at δ_C 37.3. Hence the partial structure of the molecule **125a** was constructed as shown in the Figure 92. A terminal methyl was inferred by the presence of the triplet at δ_H 0.89; an HMBC correlation was observed from δ_H 0.89 to C-15 at δ_C 32.9 and δ_C 23.8 which gave the partial structure **125b**.



125a **125b**

Figure 92: ^{1}H-^{1}H COSY and HMBC ( ) correlations in the partial structure of **125**

The remaining alkyl residue C_6H_{12} gave overlapping multiplets between 1.91 and 1.29; their individual signals and COSY correlations were difficult to distinguish. However, HMBC correlations were observed from 4.35 to 38.8 and 26.2. Since the partial structure of molecule was confirmed, this alkyl residue should lie in between the two partial structures hence giving a complete structure **125** as seen in Figure 93.



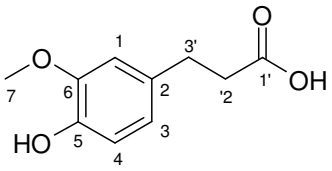
125

Figure 93: The ^{1}H-^{1}H COSY (bold bonds) and HMBC correlations of structure **125**

4.2.4 3-(2-Hydroxy-4-methoxy phenyl)-propanoic acid

The fraction FIII was subjected to Sephadex LH-20 column chromatography with methanol. A crude compound showed a UV absorption at 256 nm and turned to orange-red by spraying with anisaldehyde/sulphuric acid after heating. From the sub fraction F-III further purification was carried out using RP-18 and 3-(2-hydroxy-4-methoxy phenyl)- propanoic acid was obtained. The ^{1}H NMR spectrum of -(2-hydroxy-4-methoxy phenyl)- propanoic acid (**126**) showed two aromatic signals. Signals at δ 6.84 (1H, d, J = 7.2) and δ 6.76 (2H, dd, J = 7.6 Hz, 3.4 Hz) showed the presence an aromatic 1,3,4-substituted system. The aliphatic region showed a singlet δ 3.86 (3H, s), which was due to a methoxy group and two methylene groups were seen at δ 2.8 (2H, t) and 2.78 (2H, t).

EI MS showed the molecular ion peak at *m/z* 196 followed by the loss of a water molecule group (Δ *m/z* 17) to the fragment peak at *m/z* 179. The spectrum revealed the base peak at *m/z* 152 due to the loss of CO (MW 27) from the molecular ion peak. 3-(2-hydroxy-4-methoxy phenyl)-propanoic acid did not show significant biological activity but was weakly active against *Candida albicans.*



126

Many further low molecular weight molecules were isolated from this strain[141]. All other isolated compounds were inactive in the agar diffusion test against *Staphylococcus aureus*, *Bacillus subtilis*, *Streptomyces viridochromogenes* (Tü 57), and *Escherichia coli*, the fungi *Candida albicans* and *Mucor miehei*, and the microalgae *Chlorella vulgaris*, *Chlorella sorokiniana*, and *Scenedesmus subspicatus.* 5-(8-hydroxydec-2-enyl)-tetrahydro-furan-2-yl]-acetic acid (**124**) and 5-(4-hydroxy-dodec-2-enyl)-dihydro-furan-2-one (**125**) exhibited weak inhibition against *Candida albican*s with antifungal activity at 10 μg/ml.

4.3 Terrestrial *Streptomyces* sp. Ank 175

The crude extract of the terrestrial Streptomyces sp. Ank 175 exhibited activity against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Streptomyces viridochromogenes* (Tü 57), *Mucor miehei* and *Candida albicans*. In the chemical screening the extract showed fluorescent (366 nm) and UV absorbing (254 nm) bands, which turned light green on spraying with anisaldehyde/sulphuric acid reagent, while others turned red.

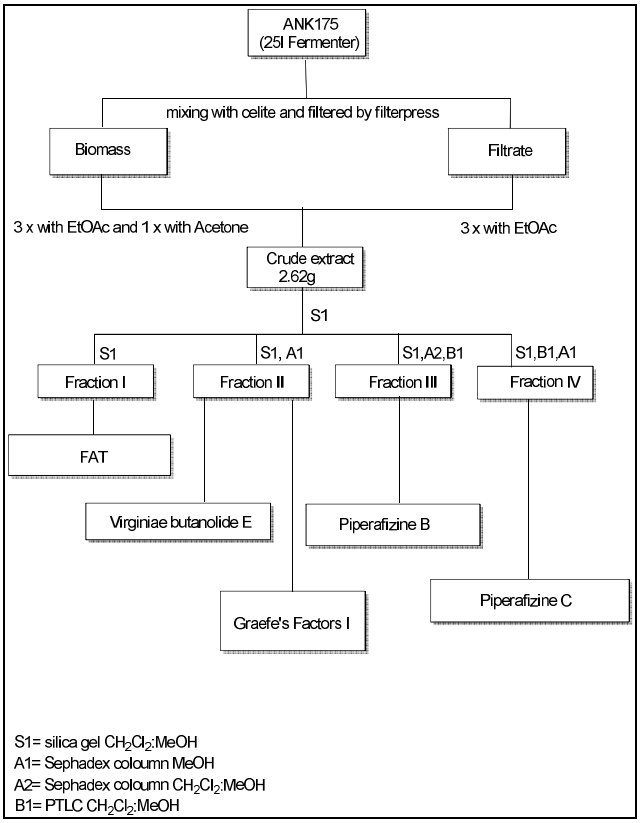
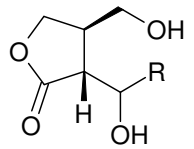


Figure 94: Work-up scheme of the marine *Streptomyces* sp. Ank 175



127a R = $CH_2CH_2CH(CH_3)CH_2CH_3$

127b R = $CH_2CH_2CH_2CH(CH_3)$

4.3.1 Virginiae butanolide B

Compound **127a** was acquired as colourless oil during the purification of fraction II on silica gel. It was a non-UV absorbing compound, which turned greenish-blue by spraying with anisaldehyde/sulphuric acid. The molecular weight was determined by ESI-MS as 230 Dalton by two *quasi*-molecular ion peaks in positive mode at *m/z* 253.0 $[M+Na]^+$ and 455.0 $[2M+Na]^+$. (+)-HRESI-MS delivered the molecular formula as $C_{12}H_{22}O_4$, indicating the presence of two double bond equivalents. The ^1H NMR spectrum of **127a** showed signals for 20 protons, all located in the aliphatic region; a doublet of two equivalent methyls δ 0.91 (d, J = 12.6 Hz, 6H) represented an isopropyl group, two signals at δ 3.70 (2 H) and δ 4.03 were indicative for oxy-methylene and a methine proton, respectively. Furthermore, a methine signal (δ 2.22, m) besides two protons of another oxy-methylene at δ 4.48 (dd, J = 24.8, 18.2 Hz, 1H), 4.10 dd, J = 24.8, 18.1 Hz), were visible. The remaining six protons were positioned in the region of δ 1.33~1.15 as three multiplets from three methylene groups. The ^{13}C NMR spectrum of **127a** displayed all 12 expected carbon signals, among them one quaternary carbon (δ 178.6) for an ester or acid, an oxy-methine (δ 69.6) and two oxy-methylenes (δ 68.5, 63.9). Additionally, three methine signals (δ 52.1, 44.4, 34.7), three methylenes (δ 34.5, 33.4, 32.7) as well as two methyls (δ 28.6, and 22.7) appeared. Based on the spectroscopic data as well as the molecular formula, searching in AntiBase deduced the compound as virginiae butanolide B (**127a**)[142]. Virginiae butanolide B have 2,3-disubstituted butanolide as basic skeleton[143], bearing two hydroxyl groups in the side chains. Virginiae butanolide B is known to induce the production of virginiamycin at low concentrations.

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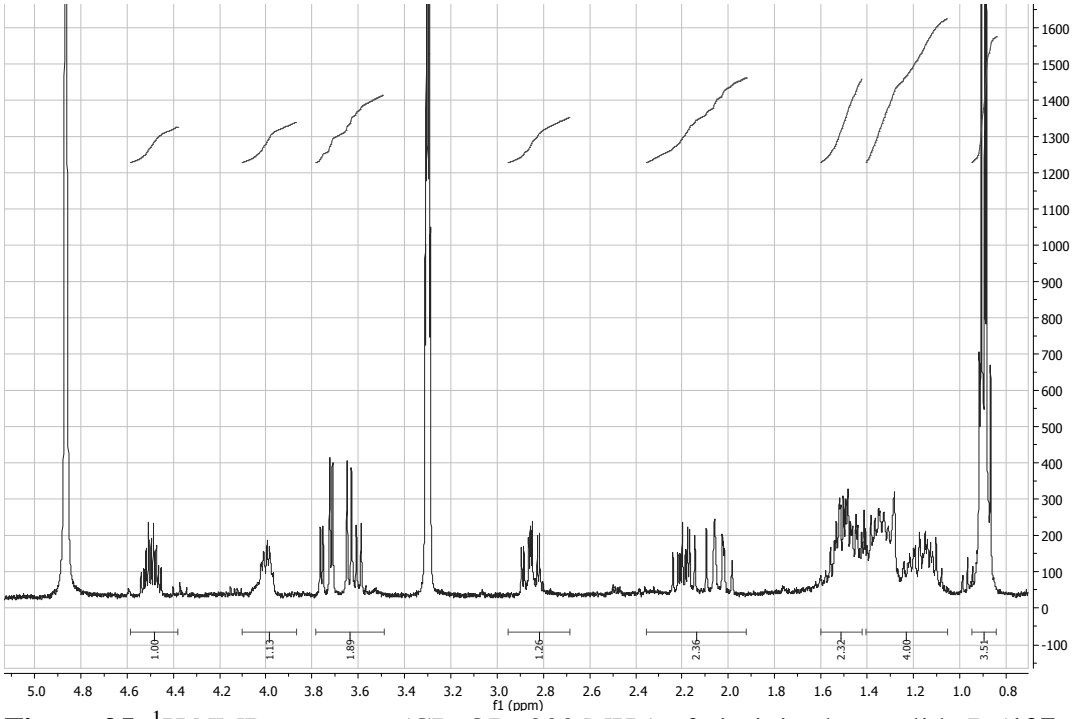


Figure 95: ^{1}H NMR spectrum (CD_3OD, 300 MHz) of virginiae butanolide B (**127a**)

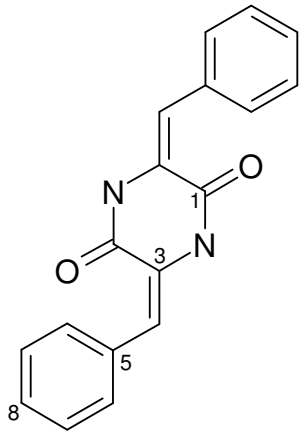
4.3.2 Graefe's Factor I

Compound **127b** was obtained as colourless non-UV absorbing oil, which became blue-violet after spraying with anisaldehyde/sulphuric acid reagent. The (+)-ESI MS spectrometry afforded their molecular weight as 230 Dalton. The ^{1}H NMR pattern of **127b** was closely related to that of virginiae butanolide. Two doublets at δ 0.90 for a methyl group contributed to isopropyl group, which was distinct along with three multiplets of oxygenated methine and methylene protons located at δ 4.52, 4.21 and 3.96. The remaining proton signals with integration of 22 protons located between δ 3.26-1.15 were due to a chain of 11 methylene groups. Based on this description, a search in AntiBase and comparison with the literature[144] identified this compounds as Graefe's Factor I (**127b**).

4.3.3 3,6-Dibenzylidene-diketopiperazine

3,6-Dibenzylidene-diketopiperazine (**128**) was isolated as an UV absorbing pale yellow crystals. On spraying with anisaldehyde/sulphuric acid reagent it stained white on the pink background and ESI MS delivered the molecular weight as 290 Dalton and the ESIHRMS delivered the molecular formula $C_{18}H_{14}N_2O_2$. The ^{1}H NMR showed five aromatic protons signals at δ 7.55 (d, J = 7.5 Hz, 2H), 7.42 (t, J = 7.6 Hz, 2H), 7.33 (t, J = 7.4 Hz, 1H), which could be clearly assigned as a monosubstituted phenyl residue. Another olefinic 1H singlet was seen at δ 6.99.

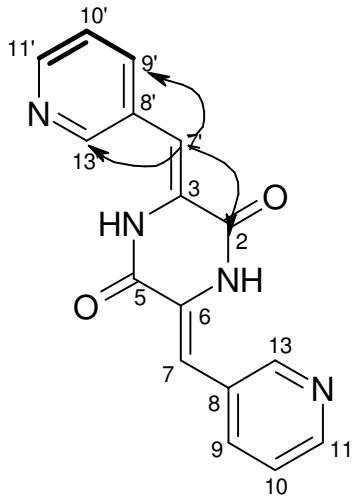
The assignment of the NMR signals on the aromatic ring could be established by the combined COSY and HMBC data. Furthermore, the singlet at δ 6.99 (114.8) showed ^3J correlations with signals at δ 128.5 and 157.7, which was assigned as amide carbonyl. According to the molecular formula, the compound was supposed to be symmetrical, so that two units of the molecule were joined by an amide bond. Piperafizine B (**128**) exhibited cytotoxic activity against mosercells P388 cells and vincristine (VCR)-resistant P388 cells[145]



128

4.3.4 Piperafizine C

The molecular formula of **129** was established as $C_{16}H_{11}N_4O_2$ from high-resolution positive ESI-MS. Dereplication using the high-resolution mass from AntiBase database[11] and Scifinder showed that this molecular formula could not be assigned to any known metabolite. The structure of **129** was further elucidated by extensive 1D and 2D NMR data analysis. Only 8 carbon signals and 4 proton signals (11 protons by integration calculation) were observed in the 1D NMR spectra, indicating the possibility of a symmetric dimer skeleton. The pattern of the aromatic proton signals was showed signals at δ 9.02, δ 8.84, and δ 8.1 signals, and neighbouring aromatic carbons (between δ 8.6). The observation of 8 aromatic carbons (C-8, C-8'-C13, C13') resonances also supported this assumption (see Table 16). Due to low solubility of the compound in DMSO alone, a few drops of TFA (trifluoroacetic acid) was added to measure the sample. The analysis of HMBC correlations further confirmed the presence of an aromatic ring with a nitrogen bridge between C-13 (δ 142.3) and C-11 (δ 146.1) and hence a pyridine moiety, and the proton and carbon signals in the pyridine moiety were assigned unambiguously as shown in Table 16. Further confirmation of the structure **129** was achieved by X-ray crystallography and the stereochemistry was also achieved as (*Z*).



129

Figure 96: HMBC correlations of piperafizine C (**129**)

Table 16: NMR Spectroscopic Data of piperafizine C (**129**) in DMSO-d_6 + TFA

Position	δ_H [a] (J in Hz)	δ_C [b] mult	HMBC
2,5		157.7 qC	
3,6		130.7 qC	
7,7’	6.88, s	107.5 CH	2, 5, 11, 11’, 13,13’
8,8’		133.1 qC	
9,9’	8.84, d (5.05)	140.1 CH	2, 5, 11, 11’, 13,13’
10,10’	8.10, dd (13.8, 5. 76)	126.9 CH	8, 8’, 9, 9’
11,11’	8.62, d (8.16)	146.1 CH	7, 7’, 9, 9’, 11, 11’
13,13’	9.02, s	142.3 CH	7, 7’, 9, 9’, 11, 11’

a : DMSO-d_6, 300 MHz. b: DMSO-d_6, 125 MHz

The UV spectrum of Piperafizine C (**129**) was somewhat complex. However, characteristic absorbances at 170 nm due to the double bond $\pi \rightarrow \pi^*$ transition, 213 nm due to the $\pi \rightarrow \pi^*$ charge transfer, 306 nm due to the n$\rightarrow \pi^*$ charge transfer, and 243 nm due to overlapping of the double bond $n \rightarrow \pi^*$ transition and 352 nm the carbonyl $\pi \rightarrow \pi^*$ transition are critical to the configurational stereoisomer.[146]. The stereochemistry of the pyridyl alanine doublebonds in **129** are in *Z* configuration, as is seen in related molecules such as neihumicin[147] and piperafizines A and B.[148] The conformation was assigned through X-ray crystallographic examination consequently, piperafizine C (**129**) was confirmed to be a (3*Z*, 6*Z*)-diketopiperazine containing a pyridine group.

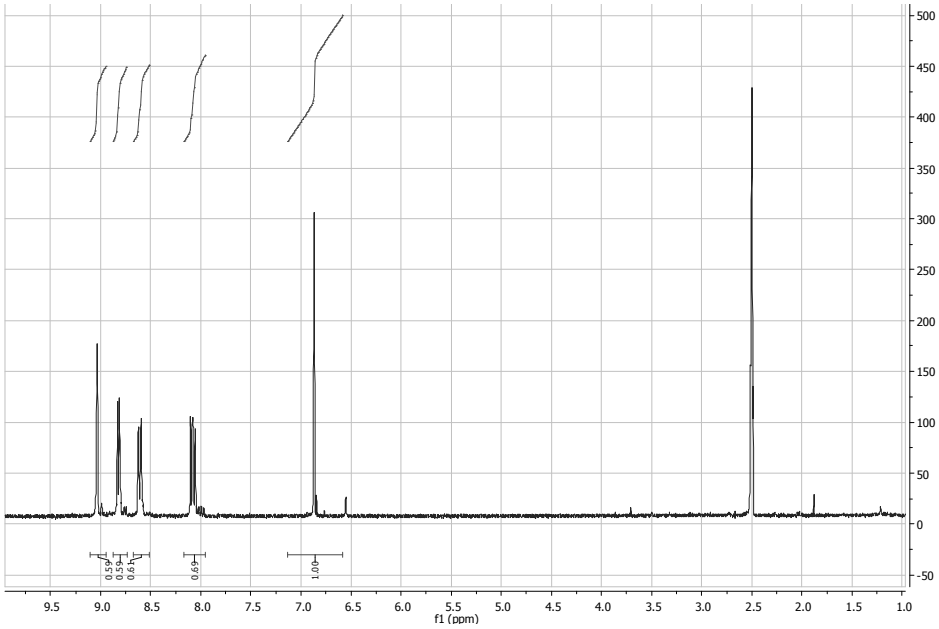


Figure 97: ^{1}H NMR (300 MHz, DMSO-d_6) spectrum of piperafizine C (**129**)

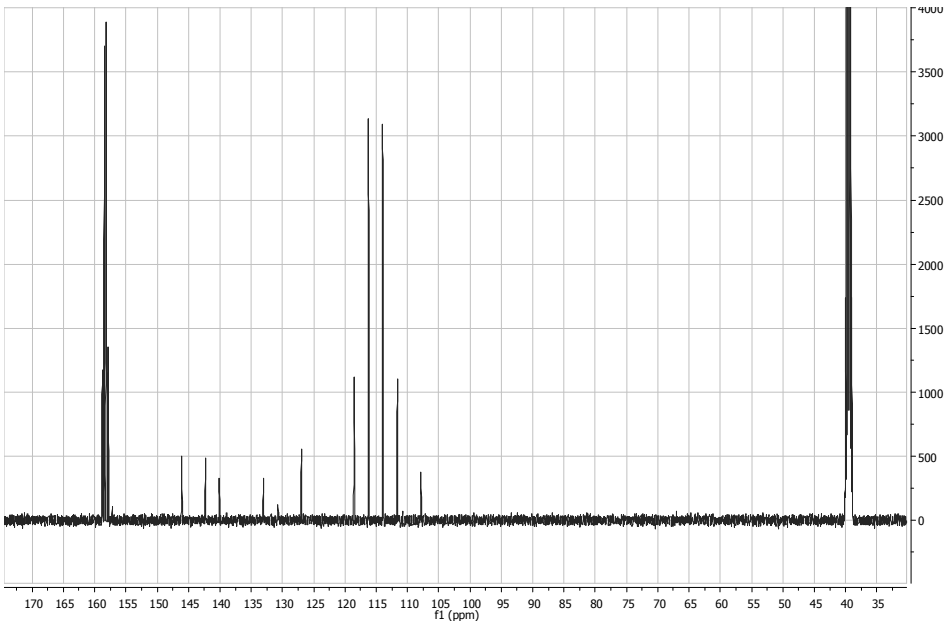


Figure 98: ^{13}C NMR spectrum (300 MHz, DMSO-d_6) of piperafizine C (**129**)

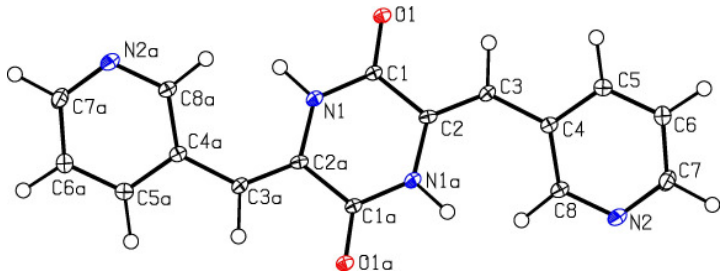


Figure 99: ORTEP diagram of piperafizine C (**129**)

During this work a mixture of three main peaks was observed in the HPLC/MS of the crude extract of the strain. The HPLC/MS chromatogram of the mixture showed two additional peaks whose molecular ion peaks were observed at *m/z* 406, 364 and 322, respectively. The two peaks derived from the conversion of **129** could not be fully purified in this study. However, their HPLC/MS data of the crude suggested that these two peaks could be some derivatives of diketopiperazine formed from pyridyl alanine. The formation of this diketopiperazine takes place when two molecules of di-dehydro-pyridylalanine undergoes Diels-Alder reaction, which can occur non-enzymatically. The amino acid 3-pyridyl alanine undergoes transformation or

post-modification by the bacterium.[149] Other options include the alteration of the bacterial genetic expression of **129** by an unknown bacterial stimulus,[150] induction of novel production by external stressors, or a long-term competitive interaction between the bacterium and the amino acid.

Preceding chemical studies of *Streptomyces* species reported the presence of phenyl alanine,[151] tryptophane, leucine,[152] and phenyl alanine methionin diketopiperazine as in case of haematocin[153]. However L-*ß*-(5-hydroxy-2-pyridyl)-alanine analogues has been reported by Watanabe *et al.*[154]. There were reports on several diketopiperazines,[155] and a multimodular nonribosomal peptide synthetase,[156] although no pyridylalanine metabolites have been isolated from this bacteria. When **129** was examined for *in vitro* cytotoxicity against 11 human cancer cell lines, it showed moderate inhibition at microgram concentration against PXF pleuramesothelioma and UXF1138L uterus body cell lines (IC_{50} 8.7 and 3.8 μg/ml, respectively).

Table 17: Antifungal Bioassay Results for piperafizine C (**129**) and piperafizine A (**128**)

Compound	Doses (ppm)	BOTRCI (%)	SEPTTR (%)	PYRIOR (%)	PHYTIN (%)
128	31	0	58	90.5	8.5
129	31	0.5	7	89	63

From the table above it is clear that at 31-ppm concentration compound **128** showed complete inhibition for BOTRCI (*Botrytis cineria*) and activity against PHYTIN (*Phytophthora infestans*). Similarly compound **129** also had a comparative inhibition for BOTRCI (*Botatis cineria*) and inhibition activity against SEPTTR *(Septoria tritici)* at 31-ppm concentration.

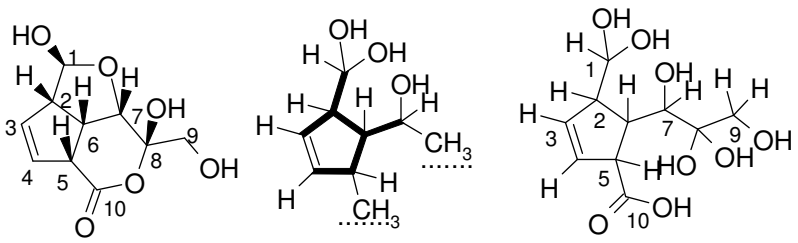
4.4 *Streptomyces* sp. ANK 289

Streptomyces sp. ANK-289 was selected, based on the chemical screening of the crude extract, where it showed violet to deep blue colour spots with anisaldehyde/H_2SO_4 spray reagent, in order to isolate biologically active compounds. Chromatographic purification of the extract led to isolation of two unprecedented tri-

cyclic, highly functionalized ketal-lactone metabolites named as Lucknolide A (**130**) and Lucknolide B (**131**), and one straight chain alcohol **132**.

4.4.1 **Lucknolide A**

Lucknolide A (**130**) was obtained as colourless crystals and gave a deep violet colour reaction with anisaldehyde/H_2SO_4 spray reagent. HRESIMS of the *pseudo*-molecular ion $[M+Na]^+$ confirmed the molecular formula to be $C_{10}H_{12}O_6$. Analysis of ^{13}C NMR data suggested the presence of one double bond, one ester/lactone carbonyl, one acetal methine, one ketal, one oxymethine, one oxygenated methylene along with three methine groups.



130 **130a** **130b**

Figure 100: Structure of lucknolide A (**130**) and; bold bonds indicate COSY correlations.

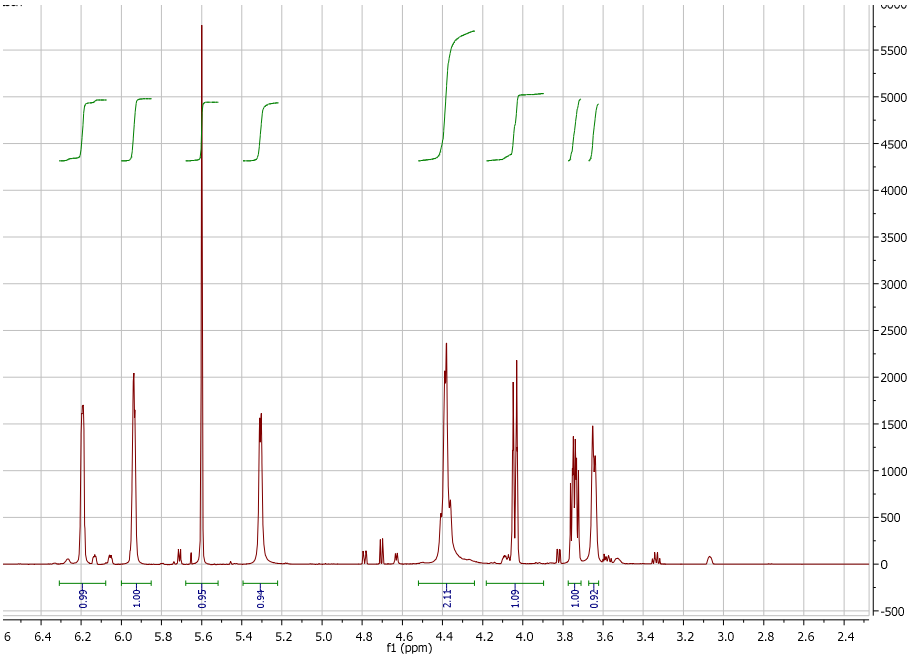


Figure 101: ^{1}H NMR spectrum (300 MHz, pyridine-d_5) of lucknolide A (**130**)

These data accounted for two double bond equivalents out of the five calculated from the formula. Analysis of the HSQC spectrum allowed the assignment of proton to carbon signals as well as (indirectly) their multiplicities (Table 18). The ^{1}H-^{1}H COSY couplings of H-1 - H-7 (see Figure 100) clearly indicated a fused cyclopentene system. A 2H AB signal at δ 4.36/4.34 suggested isolated diastereotopic oxymethylene protons (H_2-9), which showed HMBC correlations with the quaternary ketal carbon C-8 at δ 102.81 and with C-7 at δ 74.3, resulting in fragment **130b** (Figure 100). Protons H-5 and H-6 correlated with C-10 at δ 171.9, indicating the presence of a carbonyl group attached to C-5. Further HMBC correlations (Figure 102) of H-1 with C-7 suggested an oxygen bridge between C-1 and C-7. Moreover, the quaternary ketal carbon C-8 had to be adjacent to the oxymethylene C-9, and the oxymethine C-7 had to be in a lactone form to the presence of a ketal and to satisfy the remaining three double bond equivalents, resulting in a tricyclic structure as depicted in structure **130.** The conclusion was further reaffirmed by the results of a COCON simulation on the basis of the experimental 1D and 2D NMR data: We received again only structure **130** as the only possible result.[157]

The NOESY correlations (Figure 102) of proton H-6 with H-2, H-5 and H-7 indicated their *syn* orientation. An unambiguous proof of the assignment of the absolute configuration was achieved by a single-crystal X-ray diffraction (XRD) experiment (Figure 4).[158] Structure determination was possible from the signal mainly of the oxygen atoms by full-matrix least-squares refinement of the Flack parameter[159] after invariom refinement.[160] While in an independent atom model (IAM) refinement the value and standard uncertainty of the Flack parameter was -0.06(12), it could be significantly reduced to -0.08(8) using an aspherical scattering model. Hence absolute stereochemistry of all the chiral centres was determined to be (1*S*,2*S*,5*R*,6*S*,7*S*,8*S*) (Figure 103).

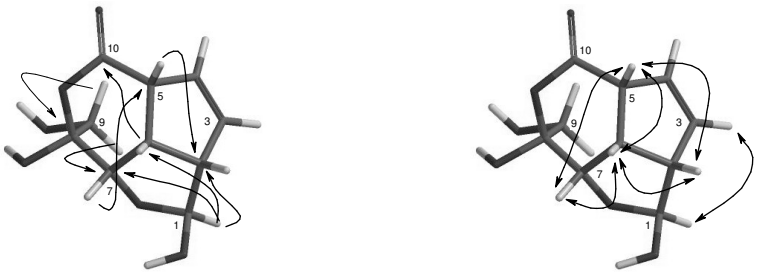


Figure 102: HMBC (left) and NOESY (right) correlations of lucknolide A (**130**)

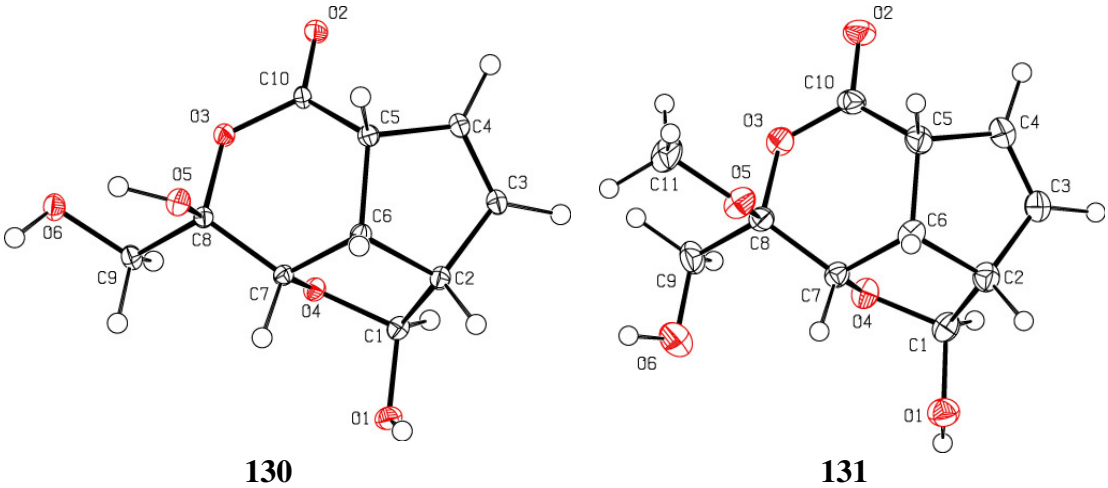


Figure 103: ORTEP representation of lucknolide A (**130**) and lucknolide B (**131**) Ellipsoids with 50% probability

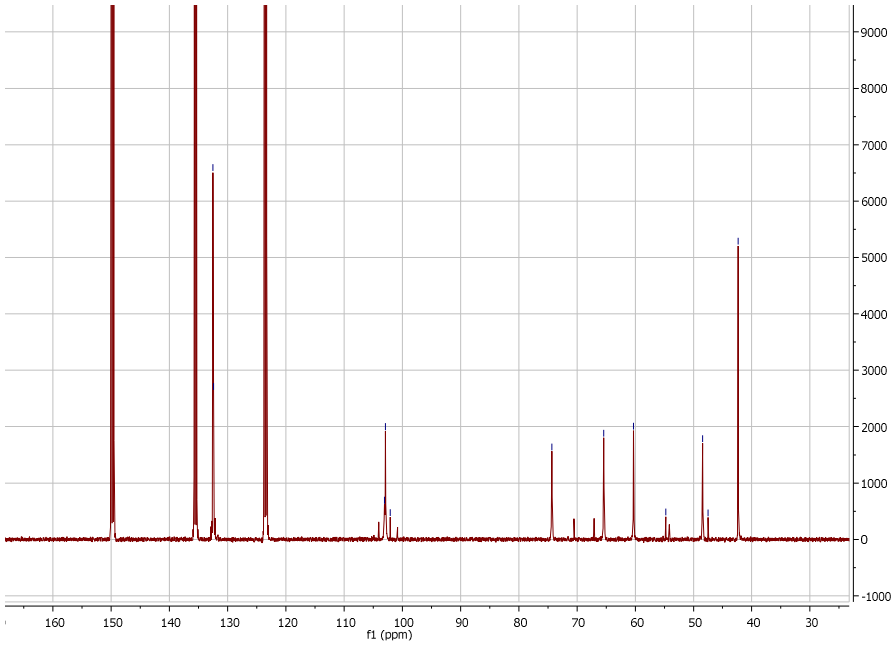


Figure 104. ^{13}C NMR spectrum (125 MHz, pyridine-d_5) of lucknolide A (**130**)

4.4.2 Lucknolide B

Lucknolide B (**131**) was obtained as white solid and gave a deep violet colour reaction with anisaldehyde/H_2SO_4 spray reagent as well. The molecular formula of $C_{11}H_{14}O_6$ was derived from the HRESIMS. ^1H and ^{13}C NMR data (Table 18) of **131** were similar to those of **130,** but indicated the presence of an additional methoxy group, in agreement with the empirical formula. Spectroscopic comparison of **131** with **130** revealed that both shared the same tricyclic structural framework with the same substitution pattern. The methoxyl group was placed at C-8 based of its HMBC correlations (Figure 100 and Figure 101) with C-8 at δ 105.4. Further HMBC correlations (Figure 100) led to structure **131** (Figure 106 and Figure 107). The same stereostructure for both compounds was revealed from the similar NOESY correlations (Figure 103 and Figure 107) and from the X-ray diffraction experiment (Figure 4): The molecular connectivity of lucknolide B (**131)** could also be confirmed by XRD.[161] However, here values of the Flack parameter and its standard deviation did not allow an unambiguous sub-structure search in AntiBase for the carbon framework of **130b** gave echinosporin and deoxyechinosporin as related compounds,[162,163] which differ, however, in functionalities and the mode of cyclisation.

Literature reports showed that the echinosporin biosynthesis follows the shikimate pathway *via* chorismate as possible late intermediate. A similar biosynthetic origin of lucknolide A and B may be speculated.

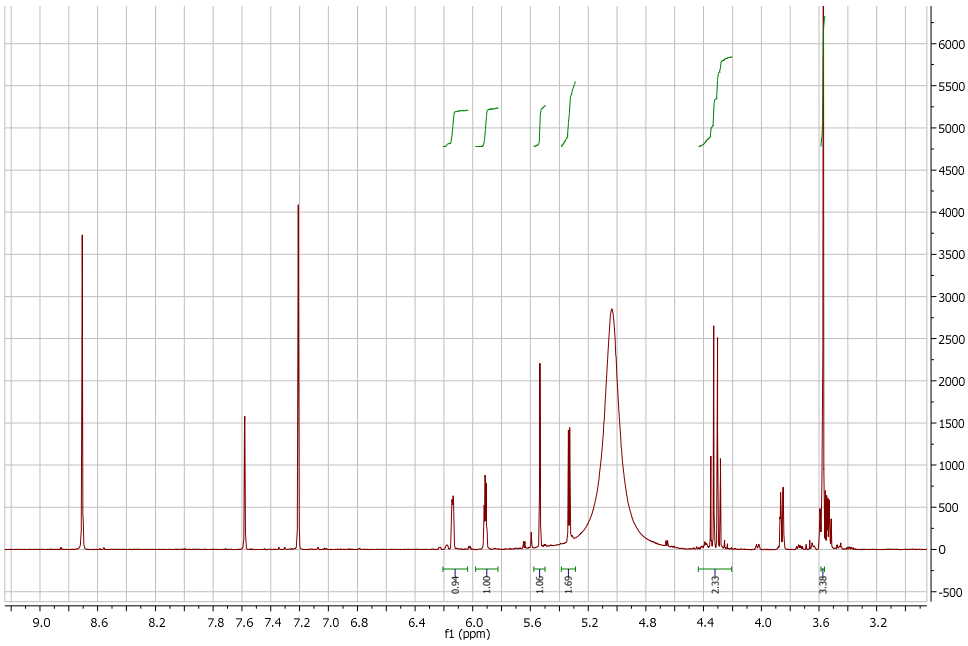
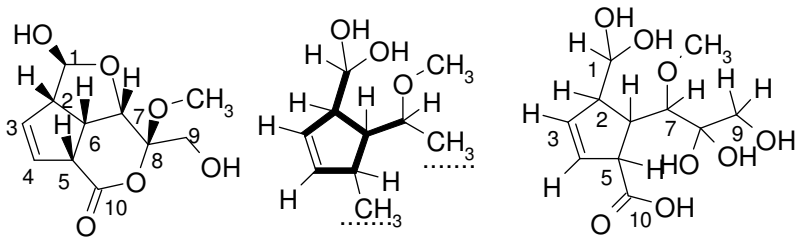


Figure 105: ^{1}H NMR spectrum (300 MHz, pyridine-d_5) of lucknolide B (**131**)



131 **131a** **131b**

Figure 106: Structure of lucknolide B (**131**) and; bold bonds indicate COSY correlations.

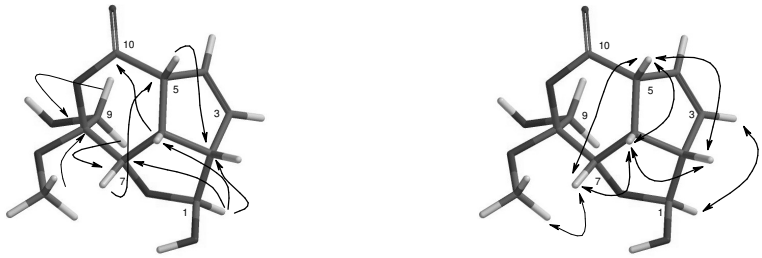


Figure 107: HMBC and NOESY correlations of lucknolide B (**131**)

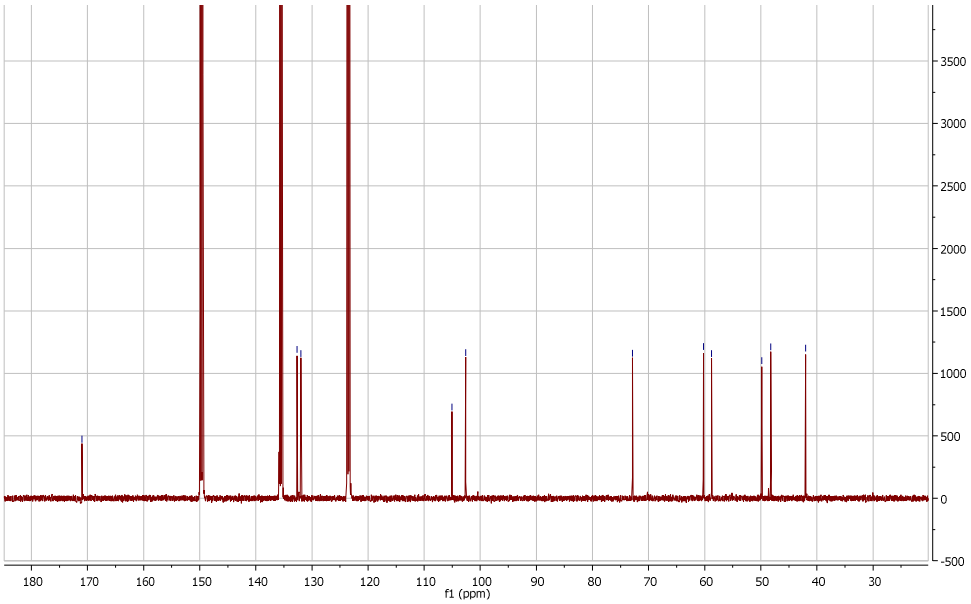


Figure 108: ^{13}C NMR spectrum (125 MHz, pyridine-d_5) of lucknolide B **(131)**

Compounds **130** and **131** were found to be inactive in the agar diffusion test against *Escherichia coli*, *Bacillus subtilis*, *Streptomyces viridochromogenes* and *Staphylococcus aureus*; antifungal activities against the oomycetes *Botrytis cinerea*, *Septoria tritici*, *Pyricularia oryzae*, and *Phytophthora infestans* were also not found. Compound **132** could not be tested due to insufficient amounts. The unusual structural framework with a highly functionalized fused tricyclic ring system reported here for **130** and **131** has no counterpart in the literature. Their structures invite innovative approaches for synthesis and to explore their possible biological targets.

Table 18: NMR data of compounds **130, 131** and **132**

	Lucknolide A (**130**)			Lucknolide B (**131**)			2,7-dimethyl-nonane-1,3,4,8-tetraol (**132**)		
No.	^{1}H (δ_H, mult., *J* Hz)[a]	^{13}C (δ_C)[b]	HMBC	^{1}H (δ_H, mult., *J* Hz)[a]	^{13}C (δ_C)[b]	HMBC	^{1}H (δ_H, mult., *J* Hz)[c]	^{13}C (δ_C)[d]	HMBC
1	5.58 (br s)	102.82	2, 3, 6, 7	5.58 (br s)	103.0	2, 3, 6, 7	3.51(dd, 10.7, 5.6) 3.69 (dd, 6.4, 5.6)	64.8	2, 3, 11
2	3.63 (br d, 7.8)	60.3	1, 3, 4, 5, 6	3.61 (br d, 7.8)	60.6	3	1.92 (d, 5.4)	38.2	1, 3, 4, 11
3	6.16 (m)	132.46	1, 2, 4, 5, 6	5.94 (m)	133.0	2, 5, 6	3.36 (m)	78.7	1, 2, 4, 5, 11
4	5.91 (m)	132.41	2, 3, 5, 6	6.17 (m)	132.4	2, 5, 6	3.50 (m)	74.1	2, 3, 5, 6
5	4.01 (dt, 10.1, 2.4)	48.4	3, 4, 6, 10	3.89 (dt, 9.9, 2.2)	48.7	4, 10	1.72 (m) 1.32 (m)	31.4	3, 4, 6, 7
6	3.72 (m)	42.3	1, 2, 5, 10	3.57 (ddd, 9.9, 8.0, 5.4)	42.4	1, 3, 4, 10	1.73 (m) 1.17 (m)	30.3	4, 5, 7, 8
7	5.30 (d, 5.2)	74.3	5, 9	5.36 (d, 5.4)	73.3	5	1.41 (m)	41.4	5, 6, 8, 9
8	-	102.81	-	-	105.4	-	3.64 (m)	71.7	6, 7, 9
9	4.36 (d, 11) 4.34 (d, 11)	65.3	7, 8	4.34 (d, 12.2) 4.28 (d, 12.2)	59.2	7, 8	1.13 (d, 6.4)	20.4	6, 7, 8
10	-	171.9	-	-	171.4	-	0.92 (d, 6.8)	15.1	6, 7, 8, 9
OMe	-	-	-	3.60 (s)	50.2	8	-	-	-
11							0.97 (d, 7.1)	14.9	1, 2, 3, 4

a Pyridine-d_5, 600 MHz. b Pyridine-d_5, 125 MHz , c Methanol-d_4, 600 MHz, d Methanol-d_4, 125 MHz

4.4.3 2,7-Dimethyl-nonane-1,3,4,8-tetraol

For a third compound **132** (2,7-dimethyl-nonane-1,3,4,8-tetraol), HRESI MS of the *pseudo*molecular ion at *m/z* 243.15702 ($[M+Na]^+$, confirmed the molecular formula as $C_{11}H_{24}O_4$. Analysis of ^{13}C NMR and HSQC data indicated the presence of three oxygenated methines and one oxygenated methylene group, but no quaternary carbon atom. Analysis of the HSQC spectrum delivered the positions of protonated carbons and their hydrogen assignments as shown in Table 18. The $^1H,^1H$ COSY couplings between the protons H-1~H-8 clearly indicated a straight carbon chain, and further COSY and HMBC correlations confirmed the positions of the methyl groups. From 1D and 2D NMR analysis, compound **132** resulted as depicted in Figure 109

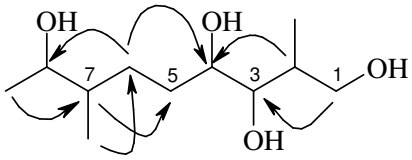


Figure 109: Structure and HMBC correlations of compound (**132**)

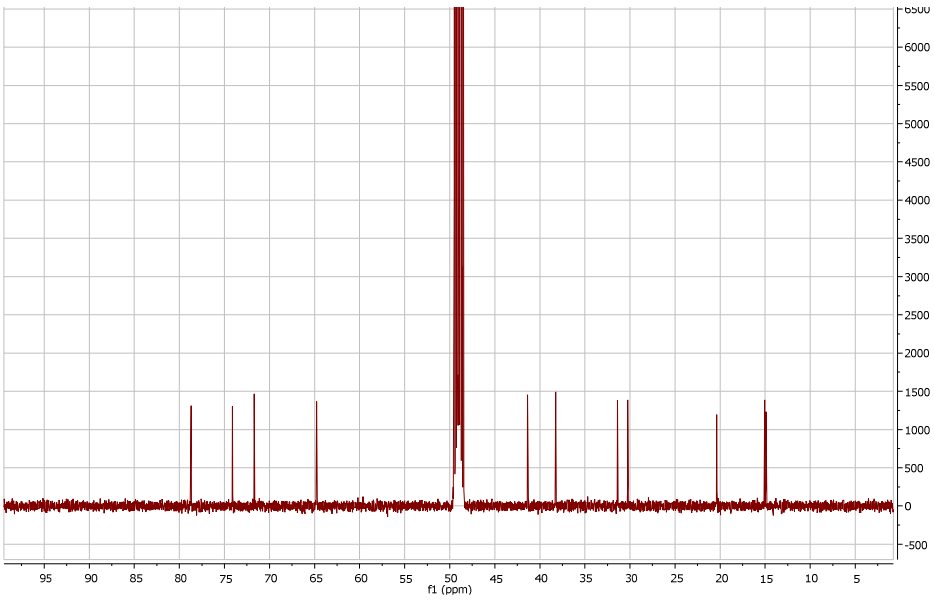


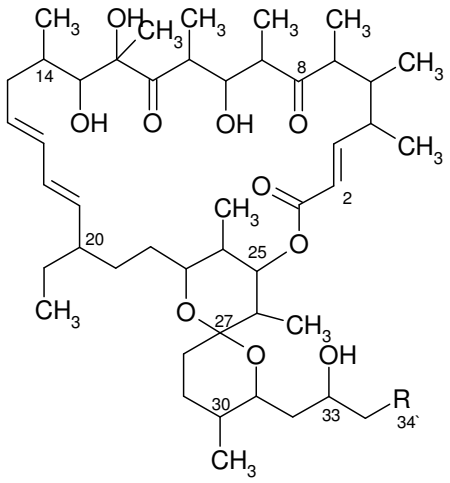
Figure 110: ^{13}C NMR spectrum (125 MHz, methanol-d_4) of 2,7-dimethyl-nonane-1,3,4,8-tetraol (**132**)

4.5 Terrestrial *Streptomyces* sp. ANK 316

The terrestrial *Streptomyces* sp. ANK 316 was selected to isolate metabolites with biological activities. TLC of the crude extract obtained from a 20 L shaker culture using M_2 medium exhibited a number of yellow, UV absorbing bands. Three further UV absorbing bands turned blue or reddish-brown on spraying with anisaldehyde/sulphuric acid. In the antimicrobial assay, the extract showed activity against *Bacillus subtilis*, *Escherichia coli, Streptomyces viridochromogenes* (Tü 57) and *Candida albicans,* moderate activity against either *Mucor miehei* (Tü 284) or *Staphylococcus aureus*. Purification of the extract was carried out by different chromatographic techniques resulted isolation of Oligomycin F (**132**), 2-methyl-4-(1-glyceral) furan (**135**) and N-acetyltyramine (**136**).

4.5.1 Oligomycin F

Compound **132** was isolated from the middle polar fraction by RP-18 column chromatography. It was obtained as a non-UV absorbing white solid which turned green to dark brown colour reaction on spraying with anisaldehyde/sulphuric acid reagent. (+)-ESI MS of compound **132**showed signals at *m/z* 828 and 1633, representing $[M+Na]^+$ and $[2M+Na]^+$ ions, respectively. (-)-ESI MS showed a signal at *m/z* 804 of $[M-H]^-$. Accordingly, the molecular weight was deduced as 805 Dalton. Comparing the information from the mass spectrometry and NMR data with Anti-Base database led to the assumption that is a homologue of oligomycin A (**133**).The ^1H and ^{13}C NMR data of the compound **132** were very similar to oligomycin A **133**, but differed by an additional methylene group, as indicated by the molecular weight. Hence the compound was confirmed as oligomycin F (**132**). Oligomycin F (**132**) was found to be highly active against plant pathogenic fungi[164]. Both oligomycin A (**133** and F (**132**) showed extremely potent suppressive agent[164] for various immunological systems.



133 R = H

134 R = CH_3

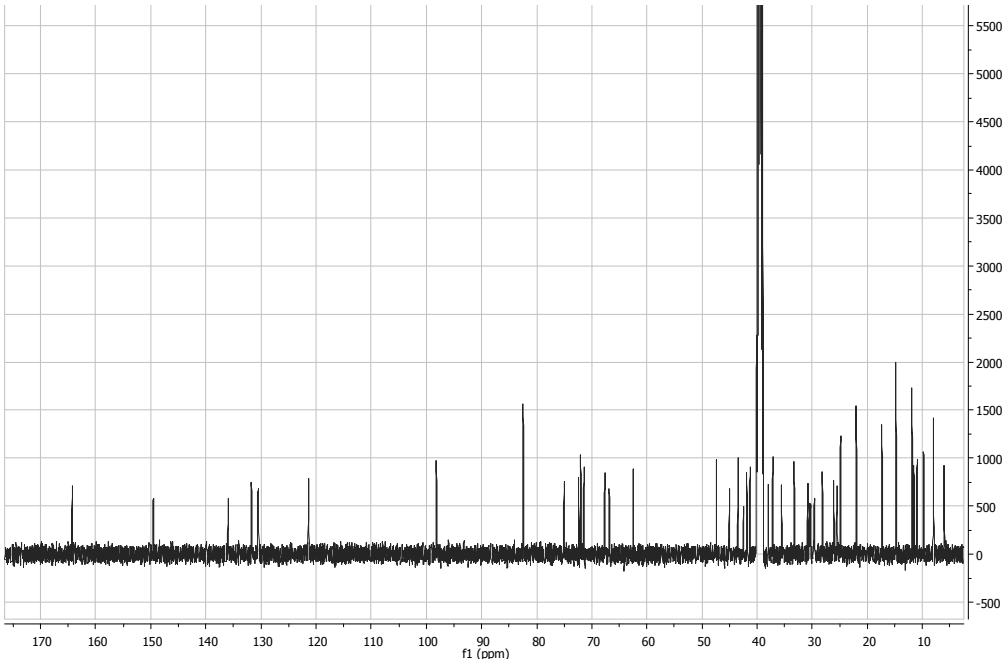


Figure 111: ^{13}C NMR spectrum (125 MHz, DMSO-d_6) of oligomycin F (**132**)

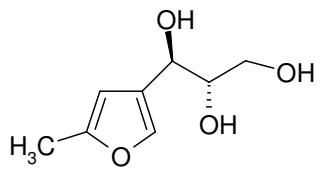
Table 19: ^{1}H and ^{13}C NMR of oligomycin F (**132**)

No.	^{13}C (δ_C)[a]	^{1}H (δ_H, mult., *J* Hz)[b]	No.	^{13}C (δ_C)[a]	^{1}H (δ_H, mult., *J* Hz)[b]
1	165.2	-	24	35.9	2.2 (m)
2	122.8	5.90 (dd, *J* = 15.5, 0.7)	25	76.3	4.96 (dd, *J* = 11.5, 15.0)
3	148.4	6.75 (dd, *J* = 15.5, 10.0)	26	37.7	1.83 (dq, *J* = 11.5, 6.3)
4	40.2	2.4 (dd, *J* = 5.5, 6.5)	27	99.2	
5	73.0	3.79 (d, *J* = 5.5, 6.5)	28	26.1	1.96, 1.25 (m)
6	46.6	2.73 (dq, *J* = 7.3, 1.3)	29	26.6	2.12, 1.42 (m)
7	220.4		30	30.7	1.59 (m)
8	41.9	3.62 (dq, *J* = 7.3, 1.3)	31	67.3	4.04 (m)
9	72.7	3.97 (m)	32	40.3	1.66, 1.25 (dd, *J* = 10.5, 2.5)
10	45.7	2.78 (m)	33	69.8	3.75 (m)
11	220.0	-	34	31.2	1.49, 1.41 (m)
12	83.1	-	34’	9.7	0.97 (t, *J* = 7.0)
13	72.3	3.97 (m)	35	17.9	1.19 (d, *J* = 6.6)
14	33.5	1.92 (dt, *J* = 9.5, 4.5)	36	8.3	1.1 (d, *J* = 7.3)
15	38.5	2.21, 1.96 (m)	37	14.1	1.12 (d, *J* = 6.8)
16	129.4	5.46 (ddd, *J* = 15.0, 10.5, 3.5)	38	9.3	1.05 (d, *J* = 6.9)
17	132.5	6.04 (ddd, *J* = 15.0, 10.5, 1.8)	39	21.0	1.14 (s)
18	130.3	5.94 (dd, *J* = 15.0, 10.5)	40	14.5	0.83 (t, *J* = 7.5)
19	137.9	5.36 (dd, *J* = 15.0, 9.7)	41	28.6	0.85 (t, *J* = 6.9)
20	46.1	1.86 (m)	42	12.1	0.98 (d, *J* = 6.3)
21	31.5	1.25, 1.41 (m)	43	6.1	0.85 (d, *J* = 6.7)
22	31.0	1.55, 1.49 (m)	44	11.9	0.92 (d, *J* = 7.0)
23	69.1	3.78 (dt, *J* = 11.5, 2.5)	45	11.3	0.89 (d, *J* = 6.7)

a : DMSO-d_6, 300 MHz. b : DMSO-d_6, 125 MHz

4.5.2 2-Methyl-4-(1-glyceral)furan

Compound **135** was obtained as a non-UV absorbing colourless oil giving a deep blue colour reaction with anisaldehyde/sulphuric acid. The molecular formula was determined to be $C_8H_{12}O_4$ by ESI-MS. The ^{1}H and ^{13}C NMR spectra showed the presence of two oxymethine signals at δ 4.54/76.4 and δ 3.53/68.7. It also showed one oxygenated methylene group at δ 3.48 and 3.38 and their corresponding carbon signal at δ 64.5. The presence of a methyl singlet at δ 2.25 suggested its attachment to a double bond. Comparison of the available information with AntiBase suggested it to be 2-methyl-4-(1-glyceryl) furan.



135

4.5.3 N-Acetyl-tyramine

Compound **136** was found in fraction II as an UV absorbing zone and stained to violet with anisaldehyde/sulphuric acid. Compound **136** was a colourless solid, the ^{1}H NMR spectrum showed two acidic protons as broad singlets at δ 9.11, and 7.81, which could be due to phenolic hydroxyls or amide groups. A 1,4-disubstituted aromatic system was established due to the existence of two 2H doublets at δ 6.68 and 6.73. In the aliphatic region, two signals were observed at δ 3.18 (t) and 2.58 (t) as two methylene groups. A singlet of a methyl group was observed at δ 1.72 (s, 3H), which could be assigned as an acetyl group. A search in AntiBase resulted in *p*-hydroxyphenethyl acetamide (**136**) [78]. Compound **136** and its derivatives are used as antitumor agents[165]. Compound **136** was isolated recently as a secondary metabolite from the marine fungus *Fusarium* sp.[166], *S. griseus*[167], as well as from plants[168]. It was found also as metabolite from pathogenic fungi, *Mycobacteria*, *Enterobacteria* and from *Bombyx mori* at the chrysalis stage[169].

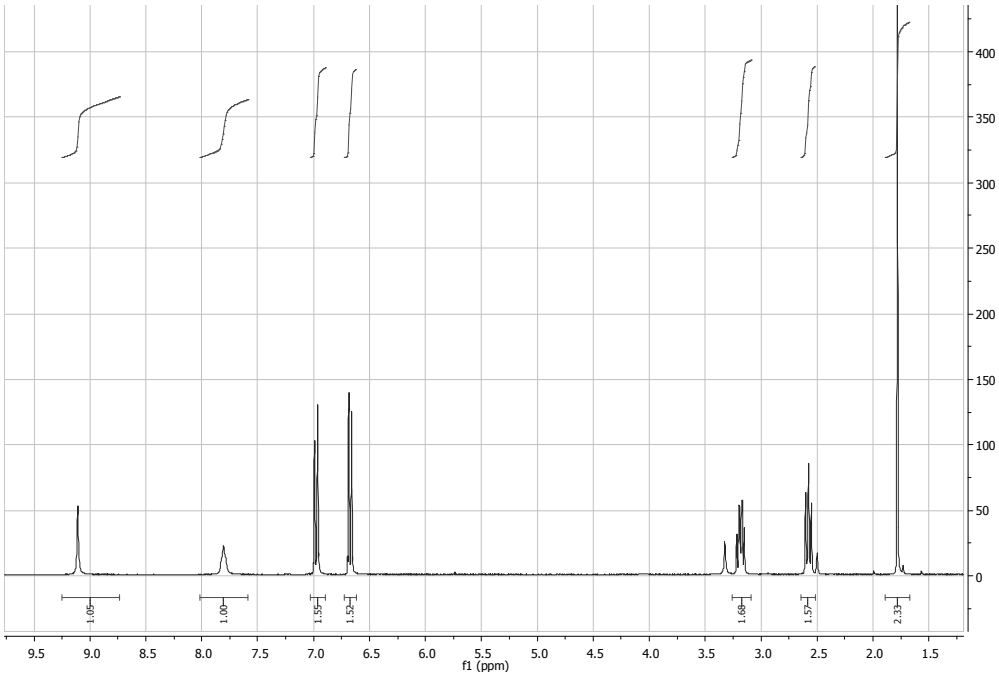
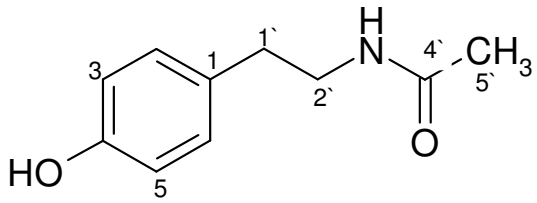


Figure 112: ^{1}H NMR spectrum (300 MHz, DMSO-d_6) of N-Acetyl-tyramine (**136**)



136

5 Summary

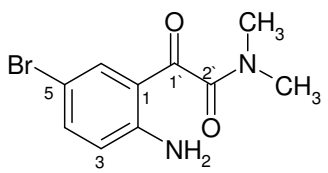
Microorganisms take a large influence on ecological and symbiotic interactions in long-term relationships that are moderated by secondary metabolites with often-unique structure and biological activity. Different habitats influence the development of the secondary metabolites produced by bacteria. Marine microorganisms, particularly bacteria, have provided new incentives for investigating marine natural products over the few years, and also continue to be the subject of vigorous chemical investigation. This highlights their importance as a source of natural products. So, the search for new biologically active natural metabolites in bacteria may enable us to cure unusual drug resistances and infectious diseases.

In the present study, five terrestrial Streptomycetes and six marine-derived bacterial strains were selected on the basis of a chemical and biological screening. The isolates were scaled up under standard conditions, and the culture broths was usually extracted with ethyl acetate or passed through Amberlite XAD-column, while the mycelial cake was extracted with ethyl acetate, followed by acetone. The crude extracts were purified using various chromatographic methods, including silica gel columns, Sephadex LH-20 and PTLC. The isolated compounds were dereplicated by means of AntiBase, the Dictionary of Natural Products (DNP) and the Chemical Abstracts. Structure elucidation occurred with the aid of spectroscopic techniques (MS, MS/MS, 2D NMR, etc.). Some structures were confirmed by synthesis or x-ray crystallography. Some of the isolated compounds were investigated for various biological activities (e.g. as antifungal, anticancer agents, etc.).

5.1 Secondary Metabolites from marine *Streptomyces* sp.

The marine *Streptomyces* sp. B7380 formed white mycelial colonies after incubation on M_2^+ agar medium. The antimicrobial assay of this strain showed a high activity against *Staphylococcus aureus, Escherichia coli*, and *Bacillus subtilis*. It also showed high activity against the fungus *Mucor miehei* and moderate cytotoxic activity against *Artemia salina*. The chemical screening showed highly blue fluorescent regions, which stained to yellow and brown with anisaldehyde/sulphuric acid. Investigation of this strain gave four novel bromine-containing isatine derivatives **54**, **56, 57** and **64**, which are related to convolutamydine A (**66**) from the marine bryozoan

Amathia convoluta. The related 3-hydroxy-3-acetonyloxindole (**58**) was also isolated from this strain, together with the new 5-hydroxyisatin derivative **65**. The position of the hydroxy group in the isolated compound **65** was confirmed by synthesis of 5-hydroxy- and 6-hydroxyisatins. The ketone adducts **56**, **57**, and **64** showed optical activity and were therefore not racemic, although they are formed very easily in a solution of the respective isatin and the ketone. The absolute configuration of **56**, **5764** could be achieved by X-ray crystallographic methods and was determined as (*R*). Compounds **54, 56, 57**, **64** and **65** showed weak activities against gram positive and gram-negative bacteria at 10 μg/disk (see Table 21). Additionally, several known butanolides were isolated.



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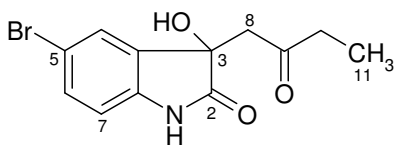

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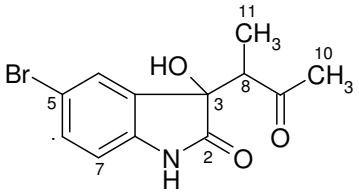

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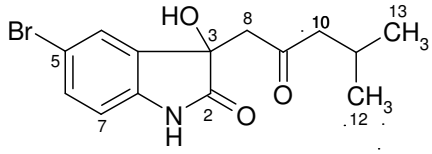
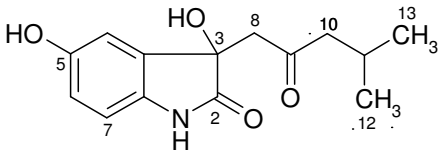


64



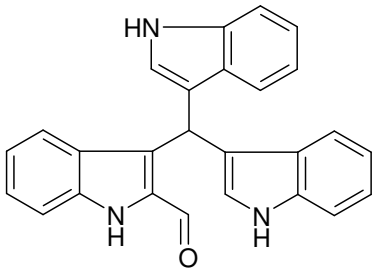
65

From extracts of the marine bacterium T262, a series of 15 bis- and tris-indole derivatives was isolated, among them seven new compounds and additionally 3,3'-bisindolylmethane (**76**), 3,3',3" trisindolyl-methane (**79**), turbomycin A and B (**75** and **88**), trisindoline (**77**), 2,2-di-(3-indolyl)-3-indolone (**78**), and 7,7'-bis(3-indolyl)-*p*-cresol (**83**), which showed cytotoxic activity of a mean IC_{50} 7.1 μg/ml. One of the compounds was a simple quinonoid oxidation product of 7,7'-bis (3-indolyl)-*p*-cresol (**84**), which showed cytotoxic activity with a mean IC_{50} of 4.2 μg/ml; this oxidised form of compound **83** which was a quinonoid derivative was not reported in the lit-

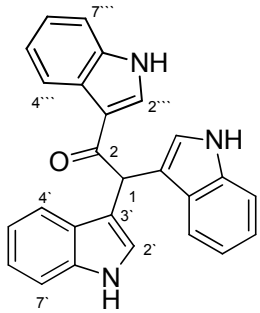
erature. The indoles were tested against choline kinases, which are key enzymes in the synthesis of phospholipids that are essential constituents of cell membranes and are involved in the regulation of cell proliferation. The inhibition by these compounds was, however, not promising in comparison with hemicholinium (HC-3).

The new compound trisindolal (**80**) showed selective toxicity against six cell lines with IC_{50} values of 0.45 μg/ml, IC_{70} 0.77 μg/ml and IC_{90} 1.41 μg/ml (Table 23). This compound was also synthesized to confirme the position of the aldehyde in the structure and is being tested now against 40 cell lines. Turbomycin A (**87**) and B (**88**) showed antifungal activity against zoospores of *Botrytis cinerea* and *Phytophthora infestans* at 2.5 μg/ml. The new trisindolal (**80**) also showed antifungal activity against *Botrytis cinerea* and *Phytophthora infestans* at 31 μg/ml. It also showed moderate antibacterial activity against *Staphylococcus aureu*s and *Streptomyces viridochromogenes* Tü 57 by causing inhibition zones of 15 and 16 mm at 10 µg/disk, and had a strong and selective antitumor activity at 20 μg/ ml. The three 7,7'-bis(3-indolyl)-*p*-cresol ethers **85a**, **85b**, **85c** showed activity against both Gram positive and Gram negative bacteria at 10 μg/disk. The new indolyl-3-thiophenyl derivative **86** showed activity against *Bacillus subtilis*, *Staphylococcus aureu*s, *Streptomyces viridochromogenes* (Tü 57), *Escherichia coli* and *Candida albicans* at 10 μg/disk. A ketone derivative of trisindole, trisindolone (**81**) was isolated from the same strain as a new tris-indole, however, due to the small available quantity, the biological activity was not investigated but during the revision of the thesis the same compound was patented as 1,2,2-tri(1*H*-indol-3-yl) ethanone[88]. A dinitro derivative **82** of the same family was isolated as a new indole alkaloid, which showed antimicrobial activity and 82% mortality rate against brine shrimps (*Artemia salina*).

From the above investigations and the potent biological activities against a range of bacteria, fungi and brine shrimps it can be concluded that indole alkaloids could be a potential lead in drug discovery.



80



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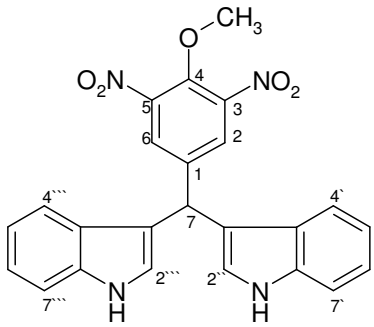
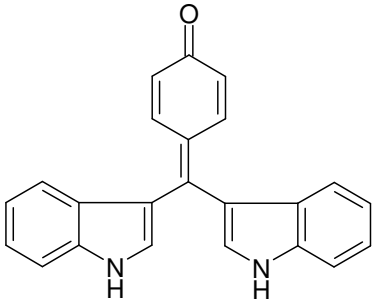
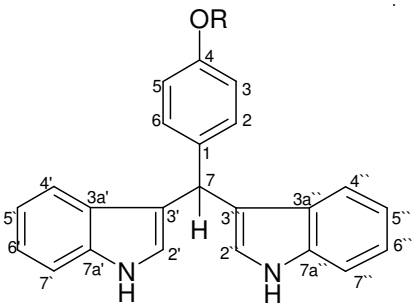


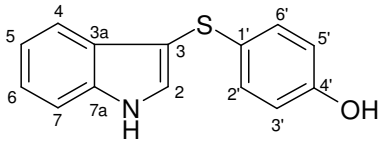
82



84



85a R= $CH_2CH_2CH_2CH_3$
85b R= $CH_2CH_2CH_3$
85c R= CH_2CH_3

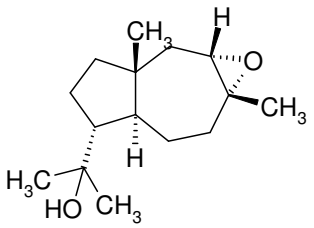


86

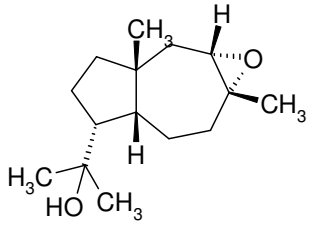
The crude extract of the marine *Streptomyces* sp. B7354 exhibited activity against *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* and showed a weak inhibition of *Streptomyces viridochromogenes* (Tü 57) in the biological screening. The crude extract also showed some cytotoxic activity. Chemical screening indicated nonpolar zones, which showed no significant UV absorption, but turned violet to blue with anisaldehyde/sulphuric acid. Separation of the crude extract by extensive chromatography led to the isolation of two new isomeric daucane sesquiterpenes. They were tested for antifungal activities against different oomycetes, *Botrytis cinerea*, *Septoria tritici*, *Pyricularia oryzae*, and *Phytophthora infestans.* Daucane-

sesquiterpene isomer A (**99**) showed antifungal activity against *Botrytis cinerea* and the isomeric daucane-sesquiterpene B (**100**) against *Phytophthora infestans* at 31 μg/ml. The cytotoxic activity of this strain came from nonactin and nonactin trilactone.

Among further compounds isolated from this strain was a trimethoxy sugar derivative **98** of adenosine, which was reported as a synthetic compound[118] in the literature, but was isolated now for the first time from nature. Similar derivatives were reported in the literature to inhibit thymidine and deoxycytidine kinases [170]. An attempt was made to test this compound against deoxycytidine kinase, which showed, however, no significant activity.



99



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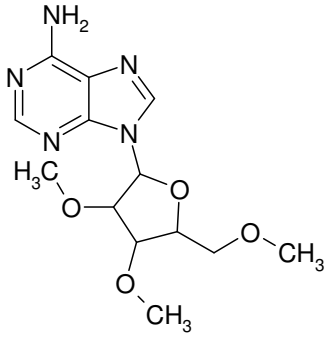
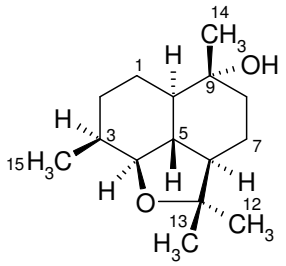


98

The strain T426A was isolated from Antartic sea ice by Dr. Helmke at the Alfred-Wegener-Institute for Polar and Marine research in Bremerhaven. The extract showed no significant biological activity, but chemical screening indicated some interesting dark brown spots with anisaldehyde/sulphuric acid. From this extract, four compounds were isolated: the diketopiperazines *cis*-(3S,7aS)-*cyclo*(Pro,Val) (**103**), trans-(3R,7aS)-*cyclo*(Pro,Val), *cis*-*cyclo*(Tyr,Pro) (**104**) and a tricyclic cadinane-type sesquiterpene **105**, which was known from Liverwort species[171], but was isolated for the first time now from bacteria. The crystal structure analysis of this compound revealed also the absolute configuration.



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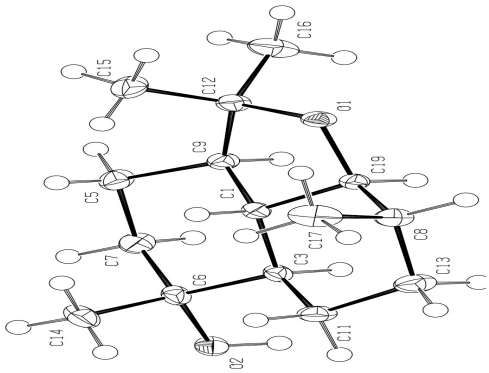
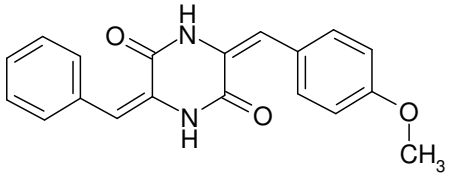


ORTEP Diagram of Cadinane sesquiterpene **105**

The extract of the marine streptomycete B6003 has been noticeable in the screening through a selective activity against cancer cell lines. From this strain, four metabolites were isolated, among them a diketopiperazine **115**, which is an albonoursin derivative[172], but had been unknown from nature. The configuration of **115** was (*Z*) according to X-ray crystallography. Another diketopiperazine, *cyclo*-(di-dehydro-4-methoxyphenylalanyl,di-dehydrophenylalanyl) (**109**) and a mixture of *cis/trans* isomers of *cyclo*-(didehydro-4-methoxyphenylalanyl,didehydrophenylalanyl) (**110**) was also obtained. Albonoursin C (**115**) showed antifungal activity against *Phytophthora infestans.* (2S)-Acetamido-3-pentanone (**116**) was isolated from this isolated from a fungal strain of *Scolicotrichum graminis*. strain and previously



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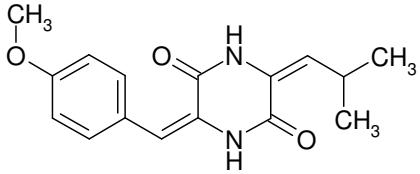
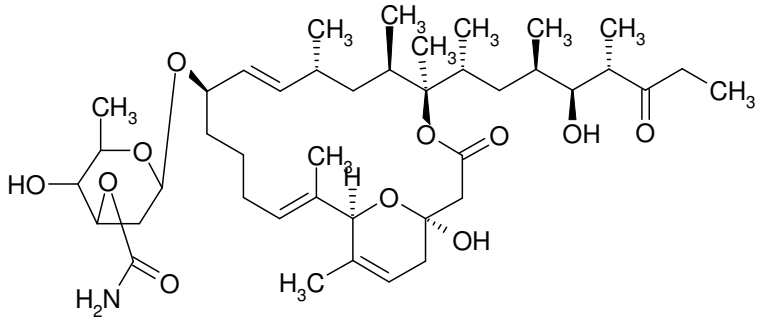


110

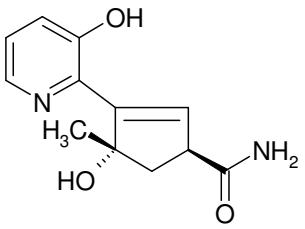
From the strain ACT 7655, the antifungal venturicidin A (**117**) and actinomycin D (**118**) were isolated.



117

5.2 Secondary Metabolites from Terrestrial *Streptomyces* sp.

The extract of the terrestrial *Streptomyces* GW 7/354 showed high cytotoxic activity against nematodes and also high activity against *Escherichia coli*, *Staphylococcus aureus* and *Streptomyces viridochromogenes* (Tü 57). It also inhibited the growth of microalgae. Chromatography of extracts of this strain gave five zones, which on spraying with anisaldehyde/sulphuric acid gave a red brown colouration. Extensive chromatographic purification yielded the two isomeric siderochelins A (**119**) and B (**120**) along with the deoxy derivative **121**, which is new as natural product. The mycelial extract gave further two isomeric acetyl uridine derivatives **122a-122b**, that were for the first time isolated from nature; the synthesis of **122a** and **122b** was reported in the literature[173]. Siderochelin A and B[174] (**119** and **120**) are having high antibiotic activity. The new deoxygenated siderochelin D (**121**) showed activity against both Gram-positive and Gram-negative bacteria and it also showed moderate antifungal activity.



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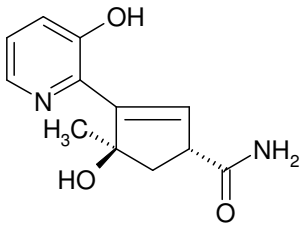

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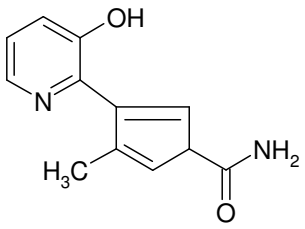

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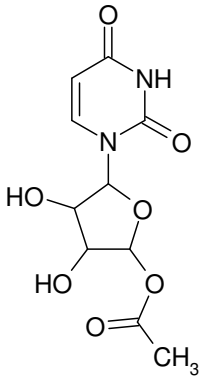
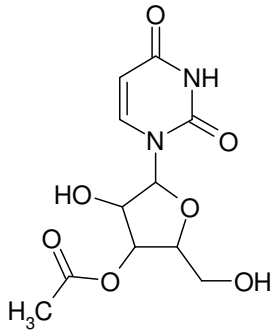
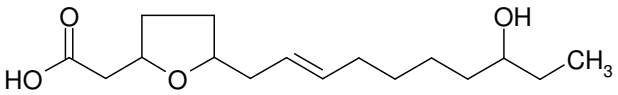


122a



122b

The crude extract of the *Streptomyces* sp. ANK205 drew our attention due to its high biological activity against a set of test organisms in the biological screening. The crude extract showed a low and rather unselective microbial activity against *Streptomyces viridochromogenes* (Tü 57) and *Escherichia coli*; by column chromatography on silica gel, 11 compounds were obtained: tryptamine, 2-hydroxy-1-(1*H*-indol-3yl)-ethanone, tryptophol, 4-hydroxybenzoic acid methyl ester, tyrosol, and 4-hydroxybenzoic acid. The remaining more polar fraction IV contained 3-(2-hydroxy-4-methoxyphenyl)-propanoic acid, glutamic acid, new butanolides A and B (**124** and **125**) and piperazimycin A (**123**). Piperazimycin A (**123**) showed high but unselective cytotoxic activity against a range of human tumor cell lines with an IC_{50} of 0.130 μg/ml and IC_{70} of 0.210 μg/ml. Butanaloides A and B (**124** and **125**) showed moderate activity against *Streptomyces viridochromogenes* (Tü 57) and *Escherichia coli.*



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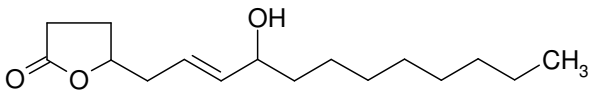

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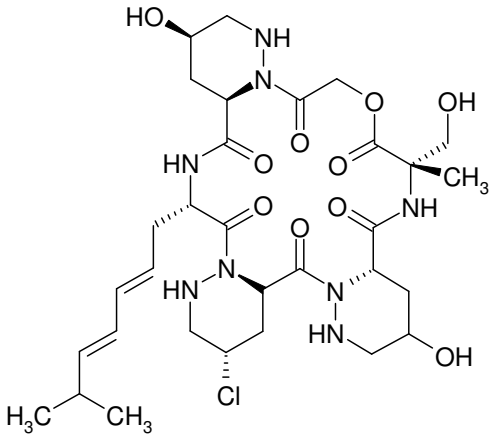
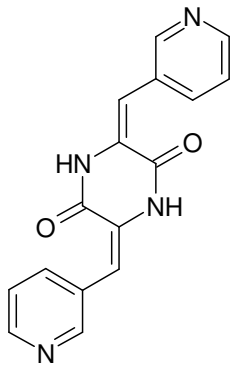


123

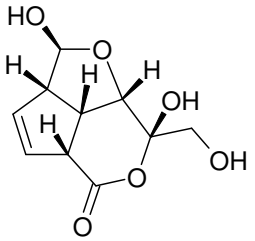
The crude extract of strain Ank 175 showed a strong cytotoxic activity with 100% lethality of *Artemia salina*. Chromatographic separation delivered the diketopiperazines 3,6-bis-[1-phenyl-methylidene]-piperazine-2,5-dione (**128**), 3,6-bis-[1-pyrid-3-yl-methylidene]-piperazine-2,5-dione (**129**), Virginae butanolide B (**127a**), and Graefes Factor I (**127**)**b**. Unsaturated diketopiperazines are known to possess cytotoxic activities, and so the high activity of the strain was not surprising. Piperafizine C (**129**) is formed from pyridylalanine, which has never been isolated from nature before. This compound was inactive against *B. subtilis* and *S. aureus* but it showed activity against *Artemia salina* and moderate activity against *Botrytis cinerea.* When **129** was examined for *in vitro* cytotoxicity against 11 human cancer cell lines, it showed moderate inhibition at microgram concentration against PXF Pleuramesothelioma and UXF1138L Uterus Body cell lines (IC_{50} 8.7 and 3.8 μg/ml, respectively). It also showed antifungal activity against *Botrytis cinerea.*



129

The investigation of *Streptomyces* sp. ANK-289 was based on the chemical screening of the crude extract, where it showed spots developing violet/deep blue colour on TLC with anisaldehyde/sulphuric acid. Chromatographic purification of the extract led to the isolation of two novel unprecedented tricyclic highly functionalized ketal-lactones, lucknolides A and B (**130** and **131**), in rather large amount. The crystal structure analysis of these compounds revealed the absolute configuration of one of the isolated compounds lucknolide A. Additionally a new straight chain secondary metabolite **132** was isolated from the same strain. The ketal-lactone metabolites were inactive in our test systems. The other compounds were not available in sufficient amounts to be tested. The unusual structural framework of these ketal-lactones had no counterpart in the literature and shows a highly functionalized fused

tricyclic ring system, which invites innovative approaches for synthesis of these molecules and exploring their biological targets.



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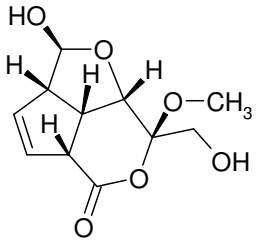

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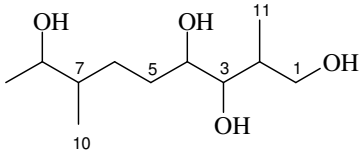
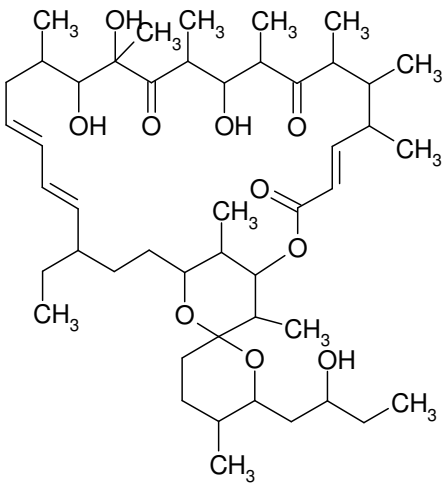
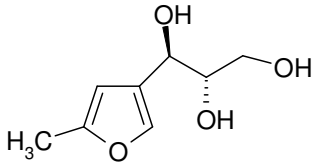


132

The terrestrial *Streptomyces* sp. ANK 316 was selected to isolate several metabolites with biological activities. TLC of the crude extract obtained from a 20 L shaker culture using M_2 medium exhibited a number of yellow, UV absorbing bands. Three further UV absorbing bands turned blue or reddish-brown on spraying with anisaldehyde/sulphuric acid. In the antimicrobial assay, the extract showed activity against *Bacillus subtilis*, *Escherichia coli, Streptomyces viridochromogenes* (Tü 57) and *Candida albicans,* moderate activity against *Mucor miehei* (Tü 284) and *Staphylococcus aureus*. Purification of the extract was carried out by different chromatographic techniques and resulted in the isolation of oligomycin F (**132**), 2-methyl-4-(1-glyceryl)furan (**135**) and N-acetyltyramine (**136**). Oligomycin F (**132**) was found to be highly active against plant pathogenic fungi. Oligomycin F (**132**) was extremely potent immunosuppressive agents[164] in various immunological test systems.



132



136

5.3 Conclusion

Eleven bacterial strains were selected and subjected to culture optimisation, working up and isolation of their metabolites. From the extracts of these strains, 67 compounds were isolated, among them 26 were new. Of the latter, 14 were isolated from marine *Streptomyces* spp., six new compounds were isolated from terrestrial *Streptomyces*. spp. The remaining six compounds were known from synthesis but were isolated here for the first time from nature.

Table 20: Total number of isolated compounds from the bacterial strains in this thesis

Strains	No. of strains	No. of compounds	No. of new compounds
Marine streptomycetes	6	40	16
Terrestrial streptomycetes	5	27	10

6 Materials and Methods

6.1 General

IR spectra: Perkin-Elmer 1600 Series FT–IR; Perkin-Elmer 297 infrared spectro-photometer; Beckman DU-640; Shimadzu FT-IR; (KBr tablet and film). - **UV/VIS spectra:** Perkin-Elmer Lambda 15 UV/VIS spectrometer. - **Optical rotations:** Polarimeter (Perkin-Elmer, model 243), the concentration were given in [mg/ml]. – **^{1}H NMR spectra:** Varian Unity 300 (300.145 MHz), Bruker AMX 300 (300.135 MHz), Varian Inova 500 (499.8 MHz), Varian Inova 600 (600 MHz). Coupling constants (*J*) in Hz. Abbreviations: s = singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, m = multiplet, br = broad. – **^{13}C NMR spectra:** Varian Unity 300 (75.5 MHz), Varian Inova 500 (125.7 MHz), Varian Inova 600 (150.7 MHz). Chemical shifts were measured relatively to tetramethylsilane as internal standard. Abbreviations: APT (Attached Proton Test): CH/CH_3 up and C_q/CH_2 down. - **2D NMR spectra:** H,H COSY (^{1}H,^{1}H-Correlated Spectroscopy), HMBC (Heteronuclear Multiple Bond Connectivity), HMQC (Heteronuclear Multiple Quantum Coherence) and NOSY (Nuclear Overhauser Effect Spectroscopy). - **Mass spectra**: EI MS at 70 eV with Varian MAT 731, Varian 311A, AMD-402, high resolution with perflurokerosene as standard. DCI-MS: Finnigan MAT 95 A, 200 eV, Reactant gas NH_3. ESI MS was recorded on a Finnigan LCQ with quaternary pump Rheos 4000 (Flux Instrument). ESI HRMS were measured on Micromass LCT mass spectrometer coupled with a HP1100 HPLC with a diode array detector. Reserpine (MW = 608) and leucine-enkephalin (MW = 555) were used as standards in positive and negative mode. High-resolution mass spectra (HRMS) were recorded by ESI MS on an Apex IV 7 Tesla Fourier-Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA). ESI MS/MS was performed with normalized collision energy of 35% samples were infused with a flow rate of 2 μL/min. **CD-Spectra:** Circular dichroism (CD)-Spectra were measured on a Jasco J 500 Spectrometer. The molar ellipticity θ is given in [10^{-1} grad cm^2 mol^{-1}]. – **High performance liquid chromatography (HPLC): Instrument I**: **Analytical**: Jasco multiwavelength detector MD-910, two pumps type Jasco Intelligent Prep. Pump PU-987 with mixing chamber, injection valve (type Rheodyne) with sample loop 20 μl, Borwin HPLC-software. **Preparative**: sample loop 500 µl. **Analytical column:** 1)

Eurochrom 4.6 × 125 mm without pre-column: stationary phase: Hypersil, ODS 120 × 5 µm; 2) Vertex 4.6 × 250 mm, stationary phase: Nucleosil NP 100-C-18, particle size 5 µm; **Preparative column**: 1) Vertex 16 × 250 mm with 16 × 30 mm pre-column, stationary phase: Eurospher C-18 RP 100 × 5 µm; 2) Vertex 16 × 250 mm with 16 × 30 mm pre-column, stationary phase: Nucleosil NP 100-C-18, particle size 5 µm, pore diameter 100 Å (Macherey–Nagel & Co.). **Instrument I**: sample loop 20 µl. **HPLC solvents:** Acetonitrile/water azeotrop (83.7% acetonitrile, bp. 78.5 °C). The azeotrop was redistilled, filtered through a membrane filter (pore Ø: 0.45 µm, regenerated cellulose, Sartorius, Göttingen) and then degassed for 15 min by ultrasonic. – **Filter press:** Schenk Niro 212 B40. - **Photo reactor for algal growth:** Cylindrical photo reactor (Ø: 45 cm) with ten vertical neon tubes Philips TLD 15 W/25.

6.2 Materials

Thin layer chromatography (TLC): DC-Folien Polygram SIL G/UV_{254} (Macherey-Nagel & Co.). – **Glass plates for chemical screening:** Merck silica gel 60 F254, (10 × 20 cm). - **Preparative thin layer chromatography (PTLC):** 55 g Silica gel P/UV_{254} (Macherey-Nagel & Co.) is added to 120 ml of demineralised water with continuous stirring for 15 minutes. 60 ml of the homogenous suspension is poured on a horizontal held (20 × 20 cm) glass plate and the unfilled spaces are covered by distributing the suspension. The plates are air dried for 24 hours and activated by heating for 3 hours at 130 °C. - **Column chromatography (CC):** MN silica gel 60: 0.05-0.2 mm, 70-270 mesh (Macherey-Nagel & Co); silica gel (230-400 mesh) for flash chromatography: 30-60 µm (J. T. Baker); size exclusion chromatography was done on Sephadex LH-20 (Lipophilic Sephadex, Amersham Biosciences Ltd; purchased from Sigma-Aldrich Chemie, Steinheim, Germany). Amberlite XAD-16 resin was obtained from Rohm and Haas, France.

6.3 Spray Reagents

Anisaldehyde/sulphuric acid: 1 ml anisaldehyde was added to 100 ml of a stock solution containing 85 ml methanol, 14 ml acetic acid and 1 ml sulphuric acid. **Ehrlich's reagent:** 1 g 4-dimethylaminobenzaldehyde was dissolved in a mixture of 25 ml hydrochloric acid (37%) and 75 ml methanol; it gives a red or violet colour-

ation with indoles and yellow with other N-heterocycles. **Ninhydrin:** 0.3 g ninhydrin (2,2-dihydroxyindan-1,3-dione) was dissolved in 95 ml *iso*-propanol. The mixture was added to 5 ml collidin (2,4,6-trimethylpyridin) and 5 ml acetic acid (96%). This reagent gave a blue to a violet colouration with amino acids, peptides and polypeptides with free amino groups. Ninhydrin in ethanol (0.1 %) was also directly used. **Palladium(II)-chloride reagent:** Palladium(II)-dichloride (0.5 g) was dissolved in water (100 mL) with some drops hydrochloride acid (25 %) as reagent for sulphur containing compounds. **Chlorine/*o*-dianisidin reaction**: The reagent was prepared from 100 ml *o*-dianisidin solution (0.032% in 1 N acetic acid), 1.5 g $Na_2WO_4 \cdot 2\ H_2O$ in 10 ml water, 115 ml acetone and 450 mg KI. The moistened TLC plate was kept ca. 30 min in a chlorine atmosphere (from 0.5 g $KClO_3$ + 2 ml conc. HCl) and then dried for ca. 1 h, till the excess of chlorine was evaporated and then dipped into the reagent. **NaOH or KOH:** 2 N NaOH or KOH solutions are used to identify *peri*-hydroxyquinones by deepening of the colour from orange to violet or blue.

6.4 Microbiological Materials

Fermentor: The 50 L fermentor type U20 (Biostat) consisted of a 70 L metallic container (50 L working volume), propeller stirrer, and culture container covered with thermostat for autoclaving, cooling and thermostating (Braun Melsungen, Germany). – **Storage of strains:** Deep-freeze storage in a Dewar vessel, 1'Air liquid type BT 37 A. - **Capillaries for deep-freeze storage:** diameter 1.75 mm, length 80 mm, Hirschmann Laborgeräte Eberstadt. – Soil for soil culture**:** Luvos Heilerde LUVOS JUST GmbH & Co. Friedrichshof (from the health shop). - **Ultraturrax:** Janke & Munkel KG. – **Shaker:** Infors AG (CH 4103 Einbach) type ITE. - **Laboratory shaker:** IKA-shaker type S50 (max. 6000 Upm). - **Autoclave:** Albert Dargatz Autoclave, volume 119 l, working temperature 121 °C, working pressure 1.2 kg/cm^2. - **Antibiotic assay discs:** 9 mm diameter, Schleicher & Schüll No. 321 261. - **Culture media:** glucose, bacto peptone, bacto agar, dextrose, soybean, mannit, yeast extract and malt extract were purchased from Merck, Darmstadt. - **Antifoam solution:** Niax PPG 2025; Union Carbide Belgium N. V. (Zwiijndrecht). – **Petri dishes:** 94 mm diameter, 16 mm height, Fa. Greiner Labortechnik, Nürtingen. – **Celite:** Celite France S. A., Rueil-Malmaison Cedex. - **Sterile filters:** Midisart 2000, 0.2 µm, PTFE-Filter, Sartorius, Göttingen. - **Laminar-Flow-Box:** Kojar KR-125, Reinraum-

technik GmbH, Rielasingen-Worblingen 1. - **Brine shrimp eggs (*Artemia salina*):** SERA Artemia Salinenkrebseier, SERA Heinsberg. - Salinenkrebsfutter: micro cell DOHSE Aquaristik KG Bonn (brine shrimp eggs and food can be obtained from aquaristic shops).

6.5 Recipes

All cultures were autoclaved at 1.2 bar and 120 °C. Sterilisation time for 1 L shaker culture: 33 min, 2 l concentrated medium for fermentor: 50 min and fermentor containing 16 l water: 80 min.

Artificial Seawater

Iron citrate	2 g (powder)
NaCl	389 g
$MgCl_2 \cdot 6H_2O$	176 g
Na_2SO_4	68.8 g
$CaCl_2$	36.0 g
Na_2HPO_4	0.16 g
SiO_2	0.30 g
trace element stock soln.	20 ml
stock soln.	200 ml
tap water	ad 20 l

Trace element stock solution

H_3BO_3	0.611 g
$MnCl_2$	0.389 g
$CuSO_4$	0.056 g
$ZnSO_4 \cdot 7\ H_2O$	0.056 g
$Al_2(SO_4)_3 \cdot 18\ H_2O$	0.056 g
$NiSO_4 \cdot 6\ H_2O$	0.056 g
$CO(NO_3)_3 \cdot 6\ H_2O$	0.056 g
TiO_2	0.056 g
$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	0.056 g
LiCl	0.028 g
$SnCl_2$	0.028 g
KI	0.028 g
tap water	ad 1 l

Stock solution

KCl	110 g
$NaHCO_3$	32 g
KBr	16 g
$SrCl_2 \cdot 6H_2O$	6.8 g (dissolved separately)
H_3BO_3	4.4 g
NaF	0.48 g
NH_4NO_3	0.32 g
tap water	ad 2 l

6.5.1 Nutrients

M_2 medium (without seawater)

malt extract	10 g
glucose	4 g
yeast extract	4 g
tap water	ad 1 l

The pH was adjusted to 7.8 using 2N NaOH. Solid medium was prepared by adding 18 g of bacto agar

M_2^+ medium (M_2 medium with seawater)

malt extract	10 g
glucose	4 g
yeast extract	4 g
artificial sea water	500 ml
tap water	500 ml

The pH was adjusted to 7.8 using 2N NaOH. Solid medium was prepared by adding 18 g of bacto agar.

M_2 100% Seawater + $CaCO_3$

malt extract	10 g
glucose	4 g
yeast extract	4 g
$CaCO_3$	0.5 g
artificial sea water	1000 ml

The pH was adjusted to 7.3 using 2N NaOH. Solid medium was prepared by adding 18 g of bacto agar.

$CaCl_2$ Medium

malt extract	40 g
glucose	5 g
$CaCl_2$	45 g
tap water	1000 ml

The pH was adjusted to 7.8 using g 2N NaOH. Solid medium was prepared by adding 18 g of bacto agar.

Luria-Bertani Medium (LB)

trypton	10 g
yeast extract	5 g
NaCl	10 g
tap water	1000 ml

The pH was adjusted to 7.8 using 2N NaOH. Solid medium was prepared by adding 18 g of bacto agar.

Soja-Mannitol Medium

soybean meal (defatted)	20 g
D(-)-mannitol	20 g
tap water	1000 ml

The pH was adjusted to 7.8 using 2N NaOH. Solid medium was prepared by adding 18 g of bacto agar.

M Test Agar (for test organisms *Escherichia coli*, *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus, Mucor miehei* (Tü 284):

malt extract	10 g
yeast extract	4 g
glucose	4 g
bacto agar	20 g
demineralised water	1000 ml

The pH was adjusted to 7.8 using 2N NaOH.

Sabouraud-Agar

(for test organism *Candida albicans*)

glucose	40 g
bacto peptone	10 g
agar	20 g
demineralised water	1000 ml

The pH was adjusted to 7.8 using 2N NaOH.

Nutritional solution A

soybean meal (defatted)	30 g
glycerol	30 g
$CaCO_3$	2 g
artificial sea water	750 ml
demineralised water	250 ml

Nutritional solution B

starch	10 g
NZ-amine	5 g
soybean meal	2g
yeast extract	5 g
KNO_3	3 g
algal extract	2.5 ml
artificial sea water	750 ml
demineralised water	250 ml

6.6 Stock Solutions and Media for Cultivation of Algae

Fe-EDTA

0.7 g of $FeSO_4 \cdot 7\ H_2O$ and 0.93 g EDTA (Titriplex III) are dissolved in 80 ml of demineralised water at 60 °C and then diluted to 100 ml.

Trace element Solution II:

Solution A:

$MnSO_4 \cdot H_2O$	16.9 mg
$Na_2MoO_4 \cdot 2H_2O$	13.0 mg
$Co(NO_3)_2 \cdot 6H_2O$	10.0 mg

Salts are dissolved in 10 ml of demineralised water.

Solution B:

$CuSO_4 \cdot 5H_2O$	5.0 mg
H_3BO_3	10.0 mg
$ZnSO_4 \cdot 7H_2O$	10.0 mg

Salts are dissolved each in 10 ml of demineralised water. Solutions A is added to B and diluted to 100 ml with demineralised water.

Bold's Basal medium (BBM): (for algae *Chlorella vulgaris, Chlorella sorokiniana* and *Scenedesmus subspicatus*.

$NaNO_3$	0.250 g
KH_2PO_4	0.175 g
K_2HPO_4	0.075 g
$MgSO_4 \cdot 7H_2O$	0.075 g
NaCl	0.025 g
$CaCl_2 \cdot 2H_2O$	0.025 g
Fe-EDTA	1.0 mL
trace element solution II	0.1 mL

Salts are dissolved in 10 ml of demineralised water and added to Fe-EDTA and trace element solution II. The mixture made to one litre with demineralised water. Solid medium was prepared by adding 18 g of bacto agar.

6.7 Microbiological and Analytical Methods

6.7.1 Storage of Strains

All bacteria strains were stored in liquid nitrogen for long time. The strains were used to inoculate agar plates with the suitable media at room temperature.

6.7.2 Pre-Screening

The microbial isolates (obtained from culture collections) were cultured in a 1 L scale in 1 L-Erlenmeyer flasks each containing 200~250 ml of M_2 or (for marine strains) M_2^+ medium. The flasks were shaken for 3-5 days at 28 °C after, which the entire fermentation broth was freeze-dried and the residue extracted with ethyl acetate. The extracts were evaporated to dryness and used for the antimicrobial tests in a concentration of 50 mg/ml.

6.7.3 Biological Screening

The crude extract was dissolved in $CHCl_3$/10% MeOH (at concentration of ~100 μg/platelet), in which the paper disks were dipped, dried under sterile conditions (flow box) and put on an agar plates inoculated with the Gram-positive bacteria; *Bacillus subtilis* (ATCC6051), *Staphylococcus aureus* and *Streptomyces viridochromogenes* (Tü 57), the Gram-negative *Escherichia coli,* the yeast *Candida albicans* and the fungi *Mucor miehei* (Tü 284) along with the three microalgae *Chlorella vulgaris*, *Chlorella sorokiniana*, and *Scenedesmus subspicatus*.

The plates were incubated at 37 °C for bacteria (12 hours), 27 °C for fungi (24 hours), and 24-26 °C under daylight for micro-algae (96 hours). The diameters of the inhibition zones were measured by ruler.

6.7.4 Chemical and Pharmacological Screening

Samples of the extracts were separated on silica gel glass plates (10 × 20 cm) with $CHCl_3$/5% MeOH and $CHCl_3$/10% MeOH. After drying, the plates were photographed under UV light at 254 nm and marked at 366 nm, and subsequently stained by anisaldehyde and Ehrlich's reagent. Finally, the plates were scanned for documentation. For the pharmacological investigations, approximately 25 mg of the crude extract was sent to industrial partners.

6.7.5 Brine shrimp Microwell Cytotoxicity Assay

To a 500 ml separating funnel, filled with 400 ml of artificial sea water, 1 g of dried eggs of *Artemia salina L.* and 1 g food were added. The suspension was aerated by bubbling air into the funnel and kept for 24 to 48 hours at room temperature. After aeration had been removed, the suspension was kept for 1 h undisturbed, whereby the remaining unhatched eggs dropped. In order to get a higher density of larvae, one side of the separating funnel was covered with aluminium foil and the other illuminated with a lamp, whereby the phototropic larvae were gathering at the illuminated side and could be collected by pipette. 30 to 40 shrimp larvae were transferred to a deep-well microtiter plate (wells diameter 1.8 cm, depth 2 cm) filled with 0.2 ml of salt water and the dead larvae counted (number N). A solution of 20 μg of the crude extract in 5 to 10 μl DMSO was added and the plate kept at r.t. in the dark. After 24

h, the dead larvae were counted in each well under the microscope (number A). The still living larvae were killed by addition of *ca*. 0.5 ml methanol so that subsequently the total number of the animals could be determined (number G). The mortality rate M was calculated in%. Each test row was accompanied by a blind sample with pure DMSO (number B) and a control sample with 1 µg/test actinomycin D. The mortality rate M was calculated using the following formula:

$$M = \left[\frac{(A - B - N)}{(G - N)} \right] \cdot 100 \qquad \text{With}$$

M = percent of the dead larvae after 24 h.
A = number of the dead larvae after 24 h.
B = average number of the dead larvae in the blind samples after 24 h
N = number of the dead larvae before starting of the test.
G = total number of brine shrimps

The mortality rate with actinomycin must be 100%.

6.7.6 Antitumor Test

A modified propidium iodide assay was used to examine the antiproliferative activity of the compounds against human tumor cell lines. The test procedure was described elsewhere[175]. Cell lines tested were derived from patient tumors engrafted as a subcutaneously growing tumor in NMRI nu/nu mice, or obtained from American Type Culture Collection, Rockville, MD, USA, National Cancer Institute, Bethesda, MD, USA, or Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

6.8 Origin of the Investigated Strains

All streptomycetes with names starting with the signature "GW" are of terrestrial origin and were obtained from the collection of the "Labor für Bodenkunde" (Dr. Grün-Wollny, Lohra-Kirchvers. The marine *Streptomyces* spp. (numbers Bxxxx and Actxxxx) were obtained from the collection of E. Helmke, Alfred-Wegener Institute for Polar and Marine Research, Bremerhaven. The North Sea bacteria strains (Meixxx) are from the Collection of Marine Streptomycetes (Prof. Meiners, Fachhochschule Emden). The origin of a few further strains is mentioned at the corresponding place.

6.9 Primary Screening Results

Bases of evaluation:

Antibiotic screening (disk diffusion test): The test is performed using paper discs with a diameter of 8 mm under standardized conditions (see above). If the inhibition zone is ranging from 11 to 20 mm, the compound is considered to be weakly active (+), from 21 to 30 mm designated as active (++) and over 30 mm is highly active (+++). - **Chemical screening:** evaluation of the separated bands by the number, intensity and colour reactions with different staining reagents on TLC. – **Toxicity test:** By counting survivors after 24 hrs, the mortality of the extracts was calculated (see above). The extracts, fractions or isolated compounds were considered inactive when the mortality rate was lower than 10% (-), from 10 to 59% as weakly active (+), from 60 to 95% as active (++) and over 95% as strongly active (+++).

6.9.1 Fermentation in 20 L Fermentor

The 20 L fermentor was filled with 16 L of water and closed with the metal lid. The aeration, acid/base and antifoam systems were connected to the fermentor and the inlet and outlet openings and tubes were closed with aluminium-foil and clamps. The pH electrode port was closed with a glass stopper. The fermentor was autoclaved for 90 minutes at 120 °C, after that it was taken out of the autoclave and the air supply, stirring motor and water circulation pumps were switched on. The acid (2N HCl), base (2N NaOH) and antifoam flasks (1% Niax/70% EtOH) were connected and filled. The pH electrode was sterilised for 30 minutes with 70% EtOH and then connected with the lid. Parallel to the preparation of the fermentor itself, two litres of medium containing suitable nutrients were prepared and autoclaved for 50 minutes at 120 °C. After cooling, the medium was added to the fermentor and the pre-culture was used to inoculate the fermentor.

7 Metabolites from Selected Strains

7.1 Marine *Streptomyces* sp. B7380

The strain Streptomyces sp. B7380 was isolated and identified by E. Helmke from the Alfred-Wegener Institute for Polar and Marine Research in Bremerhaven, Ger-

many. The strains formed a white mycelium after incubation on $M2^+$ agar medium for 6 days at 28° C.

7.1.1 Pre-screening

The biological screening of the crude extract showed activity against *Staphylococcus aureus, Mucor miehei* and *Streptomyces viridochromogenes* (Tü57). The TLC showed UV absorbing bands, which gave green, and orange colour after spraying with anisaldehyde/sulphuric acid.

Tested Microorganisms	Inhibition zone ϕ (mm)
Staphylococcus aureus	16
Streptomyces viridochromogenes (Tü57)	25
Mucor miehei (Tu 284)	21

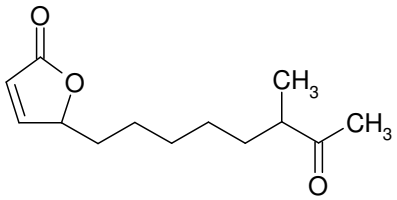
7.1.2 Fermentation, Extraction and Isolation

The marine *Streptomyces* sp. isolate B7380 was pre-cultivated on $M2^+$ agar plates (with 50% sea water) at 28 °C for 7 days. Pieces of a well-grown agar subculture of the marine isolate B7380 were used for the inoculation of a 25 l shaker culture on $M2^+$ medium. After 7 days of cultivation at 28 °C, a brown-yellow culture broth was obtained and filtered over Celite with the aid of the filter press. The filtrate was extracted using Amberlite XAD-16, then washed with methanol, while the mycelium was extracted with ethyl acetate followed by acetone. The biomass was extracted three times with ethyl acetate followed by acetone. Thin layer chromatography of the crude extracts from the water phase and the biomass showed identity, so they were combined. The crude extract (4.67 g), obtained after usual work-up from the 25 L fermentation, was separated over silica gel with a CH_2Cl_2/MeOH gradient to afford four fractions. 5-(6-Methyl-7-oxooctyl)furan-2(5*H*)-one (**52**) was obtained from fraction I by applying to Sephadex LH-20 (MeOH). Fraction II was purified using silica gel column chromatography followed by PTLC and Sephadex LH-20 to give 5-(6-hydroxy-6-methyloctyl)furan-2(5*H*)-one (**53**), 3-hydroxy-3-(2-oxopropyl)indolin-2-one (**58**) and 2-(2-amino-5-bromophenyl)-N,N-dimethyl-2-oxoacetamide (**54**). Fraction III was purified in the same way to afford 5-(6,7-dihydroxy-6-methyloctyl)furan-2(5*H*)-one (**55**), 5-bromo-3-hydroxy-3-(2-oxobutyl)indolin-2-one (**56**) and 5-bromo-3-hydroxy-3-(3-oxobutan-2-yl)indolin-2-one (**57**) . Fraction IV gave 5-

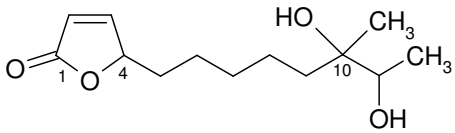
bromo-3-hydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one (**64**) and 3,5-dihydroxy-3-(4-methyl-2-oxopentyl)indolin-2-on (**65**).

Table 21: Antimicrobial activity of the compounds

	Conc./paper disk µg/ paper disc	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus subtilis*	*Streptomyces viridochromogenes*	*Mucor miehei*	*Chlorella vulgaris*
52	10	-	10	12	-	-	-
53	10	-	12	14	-	-	-
55	10	-	10	12	-	-	
54	10	12	16	10	18	-	-
56	10	14	15	10	12	-	-
57	10	15	12	17	11	-	-
64	10	12	17	13	16	-	-
65	10	12	16	10	18	-	-
58	10	15	-	-	18	-	-



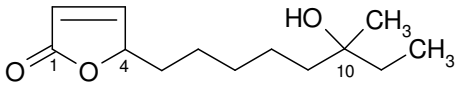
5-(6-Methyl-7-oxooctyl)furan-2(5*H*)-one (**52**): Colourless oil, 7.5 mg, R_f = 0.55 (CH_2Cl_2/5%MeOH), no UV absorption at both 254 and 366 nm, violet with anisaldehyde/sulphuric acid. - **^{1}H NMR** (300 MHz, CD_3OD): δ = 7.71 (dd, *J* = 5.7, 1.5 Hz, 1H, H-3), 6.11 (dd, *J* = 5.7, 1.9, 1H, H-4), 5.13 (m, 1H, H-4), 2.56 (m, H-6), 2.22 (s, 3H, CH_3-13), 2.13 (s, 3H) 1.85-1.77 (m, 4 H), 1.70-1.50 (m, 2H), 1.50-1.22 (m, 9H, CH_2-6, 7, 8, 9), 1.06 (d, 3H, *J* = 1.4 Hz). - **^{13}C NMR** (125 MHz, CD_3OD): δ 215.7 (C-2), 175.8 (C-11), 159.8 (C-3), 121.5 (C-4), 85.7 (C-5), 33.4 (C-6), 30.7 (C-7), 33.3 (C-8), 28.2 (C-8), 25.9 (C-9), 17.03 (C-13). – (+)-**ESIMS** *m/z* 225 ([M + H]$^+$, 3), 247 ([M + Na]$^+$, 100), 471 ([2M + Na]$^+$, 92).



5-(6-Hydroxy-6-methyloctyl)furan-2(5H)-one (**53**) Colourless oil, 2.1 mg, R_f = 0.55 (CH_2Cl_2/5%MeOH), no UV absorption at both 254 and 366 nm, violet with anisaldehyde/sulphuric acid. – **^{1}H NMR** (300 MHz, $CDCl_3$): δ = 7.45 (dd, *J* = 5.6, 1.5 Hz, 1H, H-3), 6.11 (dd, *J* = 5.4, 1.9, 1H, H-2), 5.05 (m, 1H, H-4), 3.48 (q, 1 H, H-11), 2.03-1.54

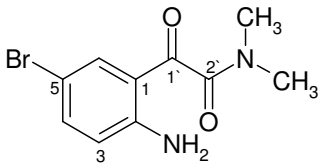
(m, 9H, CH_2-5, 6, 7, 8, 9), 1.42 (m, H, x-H), 1.17 (d, J = 5.2, 3H, H-12), 1.09 (s, 3H, CH_3-13). – (+)-**ESIMS** *m/z* 225 ([M + H]$^+$, 3), 247 ([M + Na]$^+$, 100), 471 ([2M + Na]$^+$, 92).

5-(6,7-Dihydroxy-6-methyloctyl)furan-2(5H)-one (55):

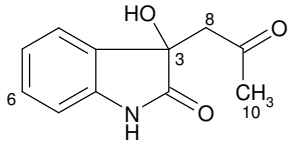


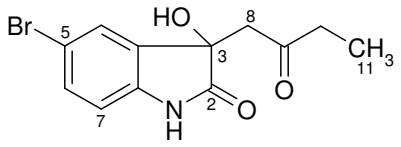
Colourless oil, 3.5 mg, R_f = 0.55 (CH_2Cl_2/5%MeOH), no UV absorption at both 254 and 366 nm, violet with anisaldehyde/sulphuric acid. – **^{1}H NMR** (300 MHz, $CDCl_3$): δ = 7.45 (dd, J = 5.7, 1.5 Hz, 1H, H-3), 6.10 (dd, J = 5.7, 1.9 Hz, 1H, H-2), 5.05 (m, 1H, H-4), 3.64 (m, 1H, H-10), 1.79 (m, 2H, CH_2-5), 1.42 (m, 2H, CH_2-9), 1.15 (s, 3H, CH_3-10), 1.11 (t, J = 7.4 Hz, 3H, CH_3-12). – (+)-**ESIMS** *m/z* 243 ([M + H]$^+$, 9), 266 ([M + Na]$^+$, 100), 509 ([2M + Na]$^+$, 56).

.**2-(2-Amino-5-bromophenyl)-N,N-dimethyl-2-oxoacetamide (54):** yellow UV-absorbing solid at 254 nm, colour change to yellow on treatment with anisaldehyde/sulphuric acid spray reagent. – R_f = 0.5 (CH_2Cl_2/5% MeOH). **UV/VIS** (MeOH): λ_{max} (log □) 390 (2.58), 229 (3.18); (MeOH/HCl): 390 (2.65), 229 (3.19); (MeOH/NaOH): 390 (2.62), 229 (3.22) nm. – **^{1}H** and **^{13}C NMR**, see Table 1. – **(+)-ESI MS:** *m/z*: 293 ([M+Na]$^+$, 86). **(–)-ESI MS:** *m/z*: 269 ([M-H]$^-$, 84), 561 ([2M-2H+Na]$^-$, 37). – **(-)**-ESI HRMS 270.99122 [M-H]$^-$ (calcd. for $C_{10}H_{11}N_2O_2Br$, 270.9931138). – **^{1}H-^{1}H COSY and HMBC:** see Figure 7.

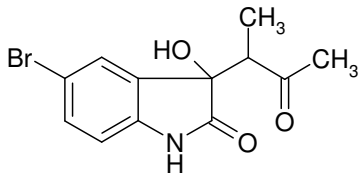


3-Hydroxy-3-(2-oxopropyl)indolin-2-one (58): white solid, UV-active (254 nm). – R_f = 0.23 (CH_2Cl_2/MeOH 95:5). – Colour reaction: with anisaldehyde/sulphuric acid violet, Ehrlich's reagent (green) – **^{1}H NMR** (CD_3OD, 300 MHz): δ =7.37 (d, J = 8.2 Hz, 1H, 4-H), 7.28 (t, J = 7.9 Hz, 1H, 6-H), 7.06 (t, J = 8.1 Hz, 1H, 5-H), 6.89(d, J = 8.0 Hz, 1H, 7-H), 3.21, 2.99 (AB, J = 15.2 Hz, 2H, 8-H), 2.21 (s, 3H, 10-H). – **ESI MS:** *m/z*: (228[M+Na]$^+$)

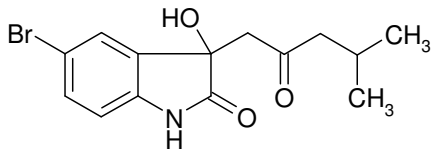




5-Bromo-3-hydroxy-3-(2-oxobutyl)indolin-2-one (56): $C_{12}H_{12}N_1O_3Br$ (298), white UV-absorbing solid at 254 nm, colour change to yellow on treatment with anisaldehyde/sulphuric acid spray reagent. – R_f = 0.5 (CH_2Cl_2/5% MeOH). $[\alpha]_D^{20}$ = -1.93 (*c* 0.10, MeOH) **UV/VIS** (MeOH): λ_{max} (log ε) 260 (3.69), 213 (3.22); (MeOH/HCl): 390 (2.62), 226 (3.22) ; (MeOH/NaOH): 259 (2.67), 226 (3.26) nm. – **IR** (KBr): ν = 3489, 2928, 1732, 1645, 1578, 1483, 1449, 1373, 1299, 1051, 959, 875, cm^{-1}– **^{1}H** and **^{13}C NMR**, see Table 2. – **(+)-ESI MS:** *m/z*: (619 $[2M+Na]^+$, 86). **(-)-ESI MS:** *m/z*: (297 $[M-H]^-$, 84), (617 $[2M-2H+Na]^-$, 37). **(-)-**ESI HRMS 297.99083 $[M-H]^-$ (calcd. for $C_{12}H_{12}N_1O_3Br$, 297.99278) – **^{1}H-^{1}H COSY** and **HMBC:** see Figure 12.

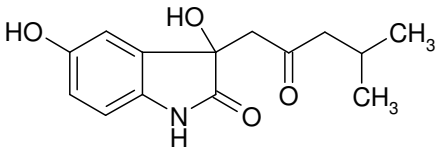


5-Bromo-3-hydroxy-3-(3-oxobutan-2-yl)indolin-2-one (57): white UV-absorbing solid at 254 nm, colour change to yellow on treatment with anisaldehyde/sulphuric acid spray reagent. – R_f = 0.52 (CH_2Cl_2/5% MeOH). $[\alpha]_D^{20}$ = +22.5 (*c* 0.1, MeOH) **UV/VIS** (MeOH): λ_{max} (log ε) =: 289 (3.72), 327 (3.77); (MeOH/HCl): 288 (3.72), 336 (3.72); (MeOH/NaOH): 290 (3.67), 331 (3.77) nm. – **IR** (KBr): ν = 3478, 3425, 1742, 1663, 1580, 1456, 1432, 1371, 1295, 1233, 1133, 1090, 1051, 936, 868, cm^{-1} – **^{1}H and ^{13}C NMR**, see Table 2. – **(+)-ESI MS:** *m/z*: (619$[2M+Na]^+$, 86). **(–)-ESI MS:** *m/z*: (297 $[M-H]^-$, 84), (617 $[2M-2H+Na]^-$, 37). **(-)-**ESI HRMS 297.99075 $[M-H]^-$ (calcd. for $C_{12}H_{12}N_1O_3Br$, 297.9927794) confirmed the molecular formula to be $C_{12}H_{12}N_1O_3Br$). **^{1}H-^{1}H COSY** and **HMBC**: see Figure 13.



5-Bromo-3-hydroxy-3-(4-methyl-2oxopentyl)indolin-2-one (64): UV-absorbing white solid at 254 nm, colour change to yellow on treatment with anisaldehyde/sulphuric acid spray reagent. – R_f = 0.46 (CH_2Cl_2/5% MeOH). $[\alpha]_D^{20}$ = +22.5 (*c* 0.1, MeOH) **UV/VIS** (MeOH): λ_{max} (log ε) =: 289 (3.72), 327 (3.77); (MeOH/HCl): 288 (3.72), 336 (3.72); (MeOH/NaOH): 290 (3.67), 331

(3.77) nm. – **IR** (KBr): ν = 3470, 3424, 2941, 1758, 1677, 1586, 1491, 1458, 1375, 1299, 1233, 1133, 1090, 1046, 920, 866 cm^{-1}– **^{1}H** and **^{13}C NMR**, see Table 3. – **(+)-ESI MS:** *m/z*: (675[2M+Na]$^+$, 86). **(–)-ESI MS:** *m/z*: (324[M-H]$^-$, 89), (651[2M-2H+Na]$^-$, 37). **(-)**-ESI HRMS 326.03872 [M-H]$^-$ (calcd. for $C_{14}H_{16}N_1O_3Br$, 326.0386324) **^{1}H-^{1}H COSY** and **HMBC:** see Figure 13 and Figure 15



3,5-Dihydroxy-3-(4-methyl-2-oxopentyl)indolin-2-one (65): white UV-absorbing solid at 254nm, colour change to yellow on treatment with anisaldehyde/sulphuric acid spray reagent. – R_f = 0.35 (CH_2Cl_2/7% MeOH). **$[\alpha]_D^{20}$** = +22.5 (*c* 0.1, MeOH) **UV/VIS**: λ_{max} (log ε) = 325 (1.97), 260 (2.54), 208 (2.96); (MeOH/HCl): 260 (2.46), 208 (2.91). – **IR** (KBr): ν = 3466, 3420, 1760, 1650, 1578, 1483, 1478, 1379, 1289, 1233, 1233, 1060, 1053, 945, 860 cm^{-1} – **^{1}H** and **^{13}C NMR**, see Table 3 and Figure 16. – **(+)-ESI MS:** *m/z* = 286 ([M+Na]$^+$, 86), 549 ([2M+Na]$^+$, 65). **(–)-ESI MS:** *m/z*: (262[M-H]$^-$, 83), 525([2M-H]$^-$, 26; (-)-ESI HRMS**:** *m/z* = 286.10497 [M-H]$^-$ (calcd. for $C_{11}H_{24}O_4Na$, 286.104971).

7.2 Marine sp. T262

Marine sp. T262 was fermented in M11 media with different marine salts concentrations and at different temperatures to select the optimum condition for the bacterial growth and the production of antimicrobial compounds. The optimum temperature was 22-27 °C with 33.5–75 g/l of marine salts mixture in M11. Five fermentations (100 litres) were carried out with marine sp. in M11 at 25 °C with 150 rpm, by pH 4 and aeration of 3-4 l/min. The duration of fermentation (Biostat U20) varied between 46-72 hours. The fermentation process was ended when the oxygen partial pressure started to increase again. 3.62 g of an oily crude extract were obtained and exhibited UV absorbing spots on TLC, which developed a characteristic yellow to orange colour reaction upon spraying with anisaldehyde/sulphuric acid. The strain was fermented in a small scale (5x 2L with M1 medium). The chromatogram of the crude extract of T262 showed in the non-polar range a large number of peaks that

had indole-like UV spectra in the analytical HPLC. After various methods of separation such as silica gel column chromatography, PTLC, Sephadex (MeOH) LH-20 series of bis-tris indole derivates were isolated.

Compounds **83, 85a, 85b, 85c**, **75**, **88, 76, 77, 78, 79, 80, 82**, **91** and **92** were tested against fungi, gram positive and gram negative bacteria using the agar diffusion test Table 22), and cytotoxic test (Table 23).

Table 22: Antimicrobial activities of compounds from marine sp. T262 in agar diffusion test

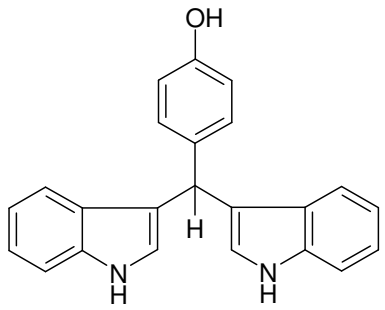
	(μg/disk)	BS	SA	SV	EC	CA	MM	CV	CS	SS	RS	PU
83	10	25	0	0	20	0	0	0	0	0	0	0
85a	10	20	0	12	16	0	0	0	0	0	0	0
85b	10	18	0	12	16	0	0	0	0	0	0	0
85c	10	18	0	12	16	0	0	0	0	0	0	0
86	10	20	14	12	14	12	10	0	0	0	0	0
75	10	12	13	0	14	12	14	0	0	0	0	0
88	10	12	14	0	14	14	15	0	0	0	0	0
76	10	0	0	0	0	0	0	0	0	0	0	0
77	10	17	10	0	16	0	0	0	0	0	0	0
78	10	17	10	0	12	0	0	0	0	0	0	0
79	10	0	0	0	0	0	0	0	0	0	0	0
80	10	18	15	16	12	16	16	0	0	0	0	++
91	10	10	0	0	16	0	0	0	0	0	0	0
92	10	12	0	0	14	0	0	0	0	0	0	0
81	10	-	-	-	-	-	-	-	-	-	-	
82	10	10	14	0	15	12	10	0	0	0	0	++

[a]*Bacillus subtilis,* [b]*Staphylococcus aureus,* [c]*Streptomyces viridochromogenes (Tü 57),* [d]*Escherichia coli,* [e]*Candida albicans,* [f]*Mucor miehei,* [g]*Chlorella vulgaris,* [h]*Chlorella sorokiniana,* [i]*Scenedesmus subspicatus,* [j]*Rhizoctonia solani,* [k]*Pythium ultimum;* ++ = active, +++ = highly active; - = not tested

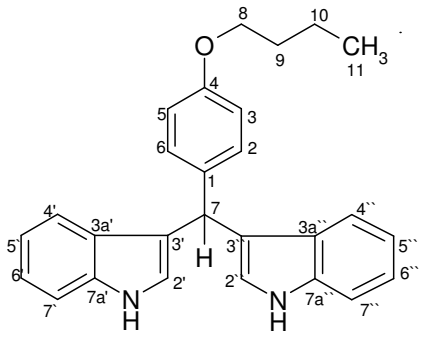
Table 23: Cytotoxic activity of indole derivatives against tumour cell lines

Tumour type	Cell line	IC_{50} μg/ml				
		80	**84**	**83**	**75**	**88**
Colourectal	CXF HT29	0.316	3.336	16.926	4.545	8.540
Gastric	GXF 251L	0.316	2.382	3.807	3.106	6.309
Lung	LXF 529L	0.340	2.848	3.106	3.519	6.012
	LXF 629L	0.234	3.034	2.669	3.206	5.584
Breast	MAXF 401NL	0.264	3.644	4.328	3.603	5.792
Melanoma	MEXF 462NL	0.196	3.706	25.560	3.448	5.658
Ovarian	OVXF 899L	2.869	18.077	16.451	3.162	5.216
Pleuramesothelima	PAXF 1657L	0.362	6.906	9.521	4.071	7.139
Prostrate	PRXF 22RV1	0.356	2.551	5.541	3.487	5.692
Renal	RXF486L	3.044	> 10.0	>10.0	3.480	5.623
Uterus	UXF1138L	0.363	2.743	4.061	2.968	4.923
Mean		0.454	4.247	7.058	3.549	6.096

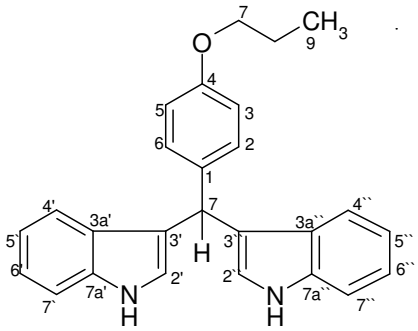
7,7-Bis(3-indolyl)-*p*-cresol (**83**): A yellow-orange solid stained to dark red-brown by treatment with anisaldehyde/sulphuric acid reagent. – R_f = 0.44 (hexane/EtOAc 20%). – **^{1}H** and **^{13}C NMR**, see Table 4. – **(+)-ESI-MS**: *m/z* = 338 $[M+H]^+$. – **HRESIMS**: *m/z* = 337.13359 $[M+H]^+$; oxidation product, (calcd. 337.133592 for $C_{23}H_{17}N_2O$).



3,3'-((4-Butoxyphenyl)methylene)bis(1*H*-indole) (**85a**): Yellow-orange solid, which stained to dark red-brown by treatment with anisaldehyde/sulphuric acid reagent. – R_f = 0.54 (hexane/EtOAc 10%). **UV/VIS** (MeOH): λ_{max} (log ε) = 282 (2.27); (MeOH/HCl): 279 (3.71); (MeOH/NaOH): 281 (3.71). – **^{1}H** and **^{13}C NMR**, see Table 5. – **EI MS** (70 eV): *m/z* (%) = 394 ($[M]^{\cdot+}$, 100), 335 (40), 293 (55), 237 (34), 144 (78). – **(+)-ESI-MS**: *m/z* = 393 $[M+H]^+$; oxidation product. – **(+)-HRESIMS**: *m/z* = 393.19615 $[M+H]^+$ (calcd. 393.19615 for $C_{27}H_{25}N_2O$).– **^{1}H-^{1}H COSY**, **HMBC:** see Figure 26.

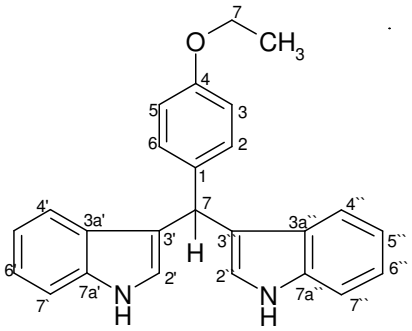


3,3'-((4-Propoxyphenyl)methylene)bis(1*H*-indole) (**85b**) a yellow-orange solid, an UV blue fluorescent (254, 366 nm) stained to dark red-brown by treatment with anisaldehyde/sulphuric acid reagent.– R_f = 0.52 (hexane/EtOAc 10%). **UV/VIS** (MeOH): λ_{max} (log ε) =: 443 (2.12), 278 (4.14), 218 (4.58) sh; (MeOH/HCl): 486 (3.43), 423 (3.37), 280 (4.17); (MeOH/NaOH): 279 (4.07) sh nm. – **^{1}H** and **^{13}C**



NMR, see Table 5. **– EI-MS** (70 eV): *m/z* (%) = 380 ([M]$^{\cdot+}$, 100), 338 (40), 322 (20), 245 (60), 114 (46). **– HRESI-MS**: *m/z* = 379.18046 [M+H]$^+$, (calcd. 379.18050 for $C_{26}H_{23}N_2O$, oxidation product). **– ^{1}H,^{1}H COSY**, **HMBC:** see Figure 29.

3,3'-((4-Ethoxyphenyl)methylene)bis1*H*-indole) (**85c**):



a yellow-orange solid, an UV blue fluorescent (254, 366 nm) stained to dark red-brown by treatment with anisaldehyde/sulphuric acid reagent.– R_f = 0.52 (hexane/EtOAc 10%). **UV/VIS**: λ_{max} (log ε) = (MeOH): 443 (0.823), 212 (1.869), 208 (1.878), 205 (1.874), 202 (1.854) sh;(MeOH/HCl): 428 (0.998), 268 (1.404), 208 (1.920), 206 (1.927), 202 (1.809) sh; (MeOH/NaOH): 442 (0.888), 216 (1.812), 215 (1.810), 213 (1.804), 202 (1.432), sh, nm. – **^{1}H** and **^{13}C NMR**, see Table 5. **– EI MS** (70 eV): *m/z* (%) = 366 ([M]$^{\cdot+}$, 100), 365 (10). **– (+)-ESI-MS:** *m/z* = 365 (100, oxidation product), 389 [M+Na]$^+$, 56), 755 [2M+Na]$^+$, 60). **– (-)-ESI-MS:** *m/z* = 365 [M-H]$^-$. **HRESI-MS**: *m/z* = 365.16480 [M+H]$^+$, (calcd. 365.16485 for $C_{25}H_{21}N_2O$); obviously oxidation product. **– ^{1}H-^{1}H COSY**, **HMBC:** see Figure 32.

4-(1*H*-Indol-3-ylsulfanyl)-phenol (**86**): a red-orange solid, an UV blue fluorescent (254, 366 nm) stained to dark red-brown by treatment with anisaldehyde/sulphuric acid reagent. – R_f = 0.52 (hexane/EtOAc 10%). **UV/VIS**: λ_{max} (log ε) = (MeOH): 465 (1.74), 349 (1.59), 283 (1.83),sh; (MeOH/HCl): 479 (2.13), 283 (2.17) sh; (MeOH/NaOH): 465 (2.66), 349 (2.54), 283 (2.84), sh, nm. – **^{1}H** and **^{13}C NMR**, see Table 6. **– ^{1}H-^{1}H COSY**, **HMBC:** see

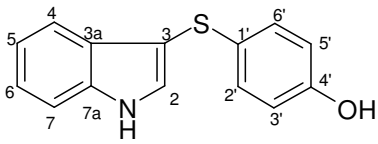
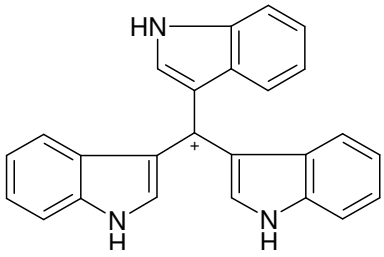
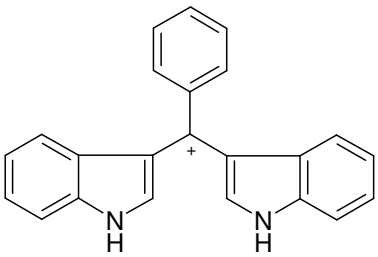


Figure **35**. **– EI MS:** (70 eV): *m/z* (%) = 241 ([M]$^{\cdot+}$, 20), 58 (25), 43 (100). **– (-)-ESI MS**: *m/z* (%) = 240 ([M-H]$^-$, 100), 481([2M-H]$^-$, 30). **– (-)-HRESI MS**: *m/z* 240.04896 (calcd. 241.048852 for $C_{14}H_{11}N_1OS_1$).

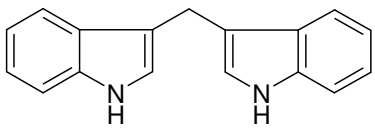


Turbomycin A (**75**): Yellowish solid an UV blue fluorescent (254, 366 nm) stained to dark reddish brown by treatment with anisaldehyde/sulphuric acid reagent; R_f = 0.57 (CH_2Cl_2/MeOH 9:1). **^{1}H NMR** (CD_3OD, 300 MHz) 7.47 (d, *J* = 8.0 Hz, 3H- 4, 4', 4''), 7.38 (d, *J* = 8.0 Hz, 3 H, H-7,7',7''), 7.20 (td, *J* = 7.6, *J'* = 1.0 Hz, H-6,6',6''), 6.96 (td, *J* = 7.6, *J'* = 0.8 Hz, H-5,5',5'')-, 6.74 (d, *J* = 2.2 Hz, 3 H, H-2,2',2''). – **(+)-ESI-MS:** *m/z* **=** 360 $[M+H]^+$

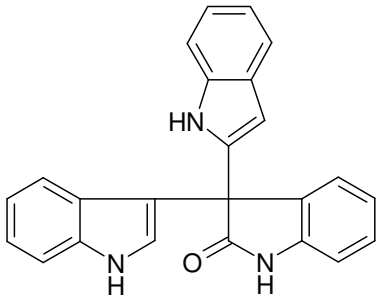
Turbomycin B (**88**): UV absorbing red solid. - R_f = 0.57 (CH_2Cl_2/MeOH 9:1). – **^{1}H NMR** ($CDCl_3$, 300 MHz): δ = 7.82 (br, 2H, 2-NH), 7.34 (d, *J* = 7.1 Hz, 2H), 7.33 (t, *J* = 7.2 Hz, 2H), 7.26 (d, *J* = 7.1 Hz, 2H), 7.25 (d, *J* = 7.6 Hz, 2H), 7.18 (t, *J* = 7.2 Hz, 1H), 7.06 (t, *J* = 8.1 Hz, 2H), 6.98 (t, *J* = 8.0 Hz, 2H), 6.62 (d, *J* = 2.5 Hz, 2H). - (-)-ESI MS *m/z* (%) = 321 ($[M+H]^+$, 100), 367 ($[M+HCOO^-]$, 10), 643 $([2M+H]^+$



3,3'-Bisindolylmethane (**76**): Colourless oil. - R_f = 0.40 (hexane/EtOAc 9:1). – **^{1}H NMR** (DMSO-d_6, 300 MHz): δ = 10.71 (br, 2H, 2 NH), 7.48 (d, *J* = 8.0 Hz, 2H, 4,4'-H), 7.35 (d, *J* = 8.0 Hz, 2H, 7,7'-H), 7.12 (d, *J* = 1.2 Hz, 2H, 2,2'-H), 7.02 (t, *J* = 8.0 Hz, 2H), 6.85 (t, *J* = 8.0 Hz, 2H), 4.20 (s, 2H). – **ESI MS** *m/z* 269 ($[M+Na]^+$ 45), 515 ($[2M+Na]^+$, 60).

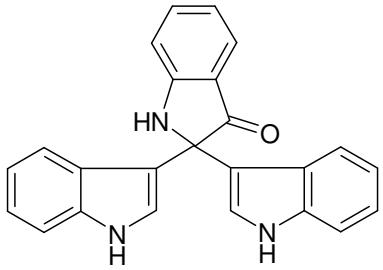


Trisindoline (**77**): UV absorbing amorphous solid; **^{1}H NMR** (300 MHz, acetone-d_6): δ 8.01 (1H, NH), 7.75 (2H, br s, H-l'), 7.69 (1H, dd, *J* = 0.9 and 7.1 Hz, H4), 7.52 (lH, dt, *J* = 0.9 and 7.1 Hz, H-6), 7.43 (2H, d, *J* = 7.9 Hz, H-4'), 7.37 (2H, d, *J* = 7.9 Hz, H-7'), 7.16 (2H, d, *J* = 2.0 Hz, H-2'), 7.12 (2H, dt, *J* = 0.8 and 7.9 Hz, H-6'), 6.94 (1H, d, *J* = 7.1 H-7), 6.93 (2H, dt, *J* = 0.8, 7.9 H-5'), 5.48 (lH, br s, H-1); **^{13}C NMR** ($CDCl_3$, 125 MHz) δ 201.2 (s, C-3), 160.2 (s, C-7a), 137.4 (C-6), 137.0 (C-7a'), 125.5 (C-3a'), 124.2 (d, C-4), 122.4 (C-2'), 120.4 (C-6'), 119.8

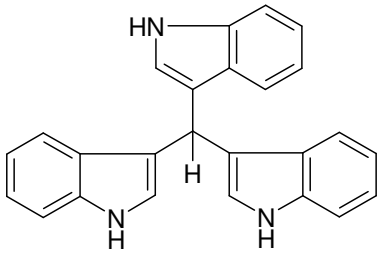


(C-4'), 119.4 (C-3a), 115.1 (C-5,5'), 112.9 (C-3'), 111.4 (C-7), 111.2 (C-7'); – **^{1}H-^{1}H COSY**, **HMBC:** see Figure 38. **– HREIMS** (70 eV) *m/z* 363.1404 ($M^{\cdot+}$, 17, calcd 363.14036 for $C_{24}H_{17}N_3O$).

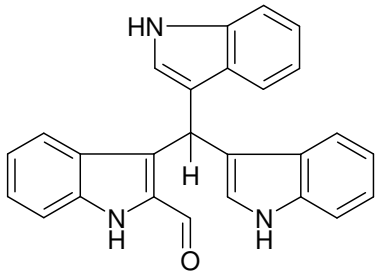
2,2-Bis-(3-indolyl)-3-indolone (**78**): UV absorbing blue fluorescent (254, 366 nm) colourless solid; stained to dark red-brown by treatment with anisaldehyde/sulphuric acid reagent. **^{1}H NMR** (300 MHz, CD_3OD) δ: 7.43 (2H, ddd, J = 7.5, 7.5, 1.0 Hz, 5', 5"-H), 7.31 (2H, s, 2',2"-H), 7.07 (1H, td, J = 7.5, 1.0 Hz, 5-H), 7.06 (2H, td, J = 7.5, 1.0 Hz, 6', 6"-H), 7.03 (1H, d, J = 7.5Hz, 7-H), 6.97 (1H, ddd, J = 7.5, 7.5, 1.0 Hz, 6-H), 6.81 (3H, d, J = 7.5 Hz, 4, 4', 4"-H). – **^{13}C NMR** (125 MHz, CD_3OD) 179.3 (C-2), 143.1 (C-7a), 142.3 (2C, C-7'a, 7a"), 138.2 (C-3a), 128.6 (C-6), 127.2 (2C, C-4',4"), 126.1 (2C, C-6', 6"), 125.3 (2C, C-5, 5"), 122.5 (2C, C-3',3"), 112.1 (2C, C-7',7"), 110.2 (C-7), 55.7 (C-3). – **^{1}H-^{1}H COSY**, **HMBC:** see Figure 38 . – ESI MS *m/z* 364 $[M+H]^+$ – HRESIMS *m/z* 364.13567 (calcd. 364.140891 for $C_{24}H_{17}N_3O$).



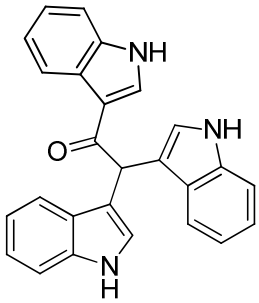
1,1,1-Tris(3-indolyl)methane (**79**): Yellowish solid an UV blue fluorescent (254, 366 nm) stained to dark reddish brown by treatment with anisaldehyde/sulphuric acid reagent; **^{1}H NMR** (DMSO-d_6, 300 MHz) δ 10.68 (s br, 3 NH), 7.37 (d, J = 8.0 Hz, 3H- 4, 4', 4''), 7.31 (d, J = 8.0 Hz, 3 H, H-7,7',7''), 6.99 (td, J = 7.6, J' = 1.0 Hz, H-6,6',6''), 6.91 (td, J = 7.6, J' = 0.8 Hz, H-5,5',5''), 6.82 (d, J = 2.2 Hz, 3 H, H-2, 2',2''), 6.03 (1 H, H-1); **^{13}C NMR** (DMSO-d_6, 75.5 MHz) δ 136.3 (C-7'a), 126.5 (s, C-3'a), 122.9 (d, C-2'), 120.3 (C-6'), 120.6 (C-4'), 118.9 (C-3'), 118.0 (C-5'), 117.6 (d, C-7'), 111.1 (C-3), 32.1 (d, C-1); all dashed atoms are of threefold intensities; **– ESIMS** *m/z* 361 $[M+H]^+$.



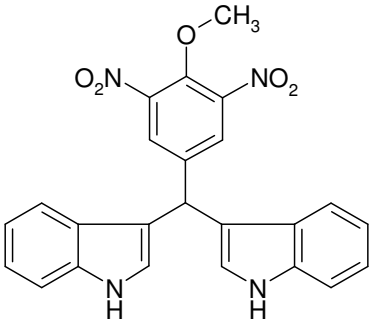
Trisindolal (**80**): Yellow-orange solid, UV blue fluorescent on TLC (254, 366 nm), stained to dark red-brown by treatment with anisaldehyde/sulphuric acid reagent. **–** R_f = 0.58 (CH_2Cl_2/MeOH 9:1). **UV/VIS**: (MeOH) λ_{max} (log ε)



=: 285 (2.58), 209 (3.12), sh;(MeOH/HCl): 349 (2.24), 301 (2.72), 288 (2.76), sh; (MeOH/NaOH): 282 (2.75), 215 (3.21), sh, nm. – **^{1}H** and **^{13}C NMR**, see Table 7. – **^{1}H-^{1}H COSY**, **HMBC:** see Figure 43. – **(-)-ESI MS**: *m/z* (%) = 388 ([M-H]$^{-}$, 100), 799 (35) ([2M-2H+Na]$^{-}$). – **(+)-ESI MS**: *m/z* (%) = 179 (100), 412 (27) [M+Na]$^{+}$, 801 (24) [2M+Na]$^{+}$. **(+)-HRESI MS**: *m/z* = 412.1411 (calcd. for 412.14204 $C_{26}H_{19}N_3ONa$).



Trisindolone (**81**): a yellow-orange solid, an UV blue fluorescent (254, 366 nm) stained to dark red-brown by treatment with anisaldehyde/sulphuric acid reagent. – R_f = 0.45 (CH_2Cl_2/MeOH 9:1). **UV/VIS**: λ_{max} (log ε) = (MeOH): 216 (3.22); (MeOH/HCl): 290 sh (2.42), 214 (3.31); (MeOH/NaOH): 290 sh (2.36) nm. – **^{1}H** and **^{13}C NMR**, see Table 8. – (+)-ESI MS: *m/z* (%) = 410 (45) [M+Na]$^{+}$, 797 (40) [2M+Na]$^{+}$. (-)-ESI MS: *m/z* (%) = 386 ([M-H]$^{-}$, 100), 795 (60) [2M-2H+Na]$^{-}$. – **(-)-HRESI MS**: *m/z* 386.47659 (calcd. 386.47659 for $C_{26}H_{16}N_3O$ [M-H]$^{-}$). – **^{1}H-^{1}H COSY**, **HMBC:** see Figure 46.



3,3'-[(4-Methoxy-3,5-dinitrophenyl]methylene)-bis-(1*H*-indole) (**82**): a yellow-orange solid, an UV blue fluorescent (254, 366 nm) stained to dark red-brown by treatment with anisaldehyde/sulphuric acid reagent. – R_f = 0.52 (hexane/EtOAC 9:1). **UV/VIS**: λ_{max} (log ε) = (MeOH): 240 sh (3.24), 280 sh (2.74); (MeOH/HCl): 235 sh (3.64), 280 sh (2.42); (MeOH/NaOH): 210 (4.12), 245 (3.17), 350 sh (2.46) nm. – **^{1}H** and **^{13}C NMR**, see Table 9. – (+)-ESI MS: *m/z* (%) = 465 (10) [M+Na]$^{+}$, 908 (4) [2M+Na]$^{+}$. (-)-ESI MS: *m/z* (%) = 441 ([M-H]$^{-}$, 100), 883 (16) [2M-H]$^{-}$. – **(+)-HRESI MS**: *m/z* 465.11692 (calcd. for 465.11694 $C_{24}H_{18}N_4O_5Na$) – **^{1}H-^{1}H COSY**, **HMBC:** see Figure 52.

7.3 Marine-derived *Streptomyces* sp. B7354

The marine-derived *Streptomyces* sp. B7354 was pre-cultivated on agar plates using $M2^+$. The incubation was performed at 28 °C for 3 days, exhibiting white mycelium.

7.3.1 Prescreening

The above subcultures were served to inoculate 1 L on a rotary shaker at 28 °C for 7 days. After extraction of the culture broth with ethyl acetate, a yellow oil crude extract was afforded. The extract was applied to antimicrobial assay, displaying an activity against the listed microorganisms (Table 24). The chemical screening showed UV absorbing spots during TLC in addition to several non-UV absorbing spots, which were mostly stained as violet-blue after spraying with anisaldehyde/sulphuric acid reagent.

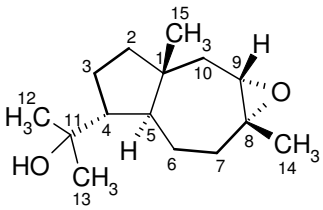
Table 24: Biological activity of the crude extract of the strain B7354

Microorganisms	Inhibition zone ∅ (mm)
Bacillus subtilis	10
Staphylococcus aureus	7
Streptomyces viridochromogenes (Tü 57)	15
Escherichia coli	14
Candida albicans	25
Mucor miehei (Tü 284)	36
Chlorella vulgaris	21
Chlorella sorokiniana	13
Scenedesmus subspicatus	16

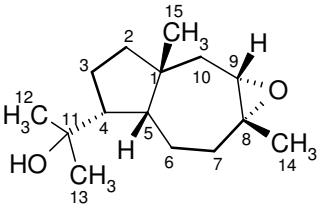
7.3.2 Isolation and Workup

With pieces of well-grown agar subculture of the strain, 30 L Shaker culture were cultivated at 28 °C with 250 rpm for 9 days. The well-grown culture broth was mixed with Celite and filtered in vacuo. The mycelium and filtrate were separately extracted with ethyl acetate and through XAD- 16, respectively; the organic phases showed a similar composition and were combined and evaporated under vacuum to dryness. The oily brownish crude extract (1.78 g) was chromatographed on silica gel using a stepwise CH_2Cl_2/MeOH gradient to yield four fractions. Fraction II gave isolated two isomeric daucane class of sesquiterpenes streptodaucane A (**99**) and B (**100**). Fraction III gave known compounds like nonactic acid (**142**), homononactic acid (**143**), bonactin (**102**). Fraction IV gave other derivatives of nonactic acid like dinactin

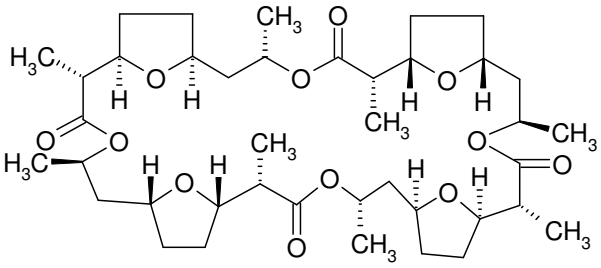
(**101**) from the same fraction also trimethoxy adenosine (**144**) was isolated which was unknown from nature.



Streptodaucane A (**99**): Colourless solid, R_f = 0.46 (CH_2Cl_2/5% MeOH); $[\alpha]_D^{25}$ +22.4 (*c* 0.18, MeOH); IR (KBr) ν_{max} 3406, 2967, 2932, 2361, 2338, 1457, 1376, 1299, 1163, 1021 932, 906 cm^{-1}; **NMR data**, see Table 10; (+)-ESIMS *m/z* (%) 256 $[M + NH_4]^+$ (43), 238 $[M - H_2O + NH_4]^+$ (100), 221 $[M - H_2O + H]^+$ (20), 163 (74); – (+)-**HRESIMS** *m/z* 256.22711 (calcd for $[M + NH_4]^+$, $C_{15}H_{26}O_2(NH_4)$, 256.22710). – **^{1}H-^{1}H COSY**, **HMBC:** see Figure 55.

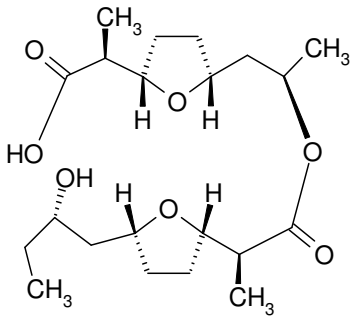


Streptodaucane B (100): Colourless solid, R_f 0.46 (CH_2Cl_2/5% MeOH); $[\alpha]_D^{25}$ -16.8 (*c* 0.18, MeOH); IR (KBr) ν_{max} 3426, 2897, 2969, 2667, 2627, 1469, 1284, 1319, 1072, 1045, 946, 908 cm^{-1}; **NMR data**, see Table 10; (+)-ESIMS *m/z* (%) 256 $[M + NH_4]^+$ (43), 238 $[M - H_2O + NH_4]^+$ (100), 221 $[M - H_2O + H]^+$ (20), 163 (74); **–** (+)-**HRESIMS** *m/z* 256.227110 (calcd. for $[M + NH_4]^+$, $C_{15}H_{26}O_2$+NH_4, 256.22710).–**^{1}H-^{1}H COSY**, **HMBC:** see Figure 55.

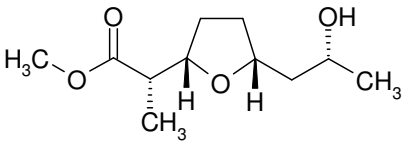


Dinactin (**101**): Non UV absorbing, colourless oil, R_f = 0.27 (CD_3OD/10% MeOH), which gave a violet band on spraying with anisaldehyde/sulphuric acid. – **^{1}H NMR** (CD_3OD, 300 MHz): δ = 4.97 (m, 2 H, H-8, 17), 4.02-3.89 (m, H-3, H-6, 12, 15), 2.47 (m, 2H, H-2, 11), 2.01 (m, 2 H, CH_2-5, 14), 1.69 (m, 2H, CH_2-7), 1.56 (m, 4H, CH_2b-4, 13, CH_2-18), 1.28 (m, 2H, CH_2-5, 14), 1.22 (d, ^3J = 6.2 Hz, 3H, CH_3-8), 1.09 (d, ^3J = 7.0 Hz, 3H, CH_3-11), 1.08 (d, ^3J = 7.0 Hz, 3H, CH_3-2), 0.88 (t, ^3J = 7.4 Hz, 3 H, CH_3-19). – **^{13}C NMR** (CD_3OD, 125 MHz) δ = 176.5 (Cq-1), 176.1 (Cq-10), 82.6 (CH-12), 82.2 (CH-3), 77.1 (CH-6), 77.1 (CH-15), 72.9 (CH-17), 70.2 (CH-8), 46.9 (CH-11), 46.7

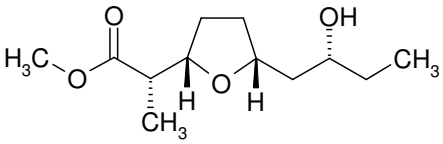
(CH-2), 42.0 (CH_2-7), 39.7 (CH_2-16), 32.5 (CH_2-5), 30.8 (CH_2-14), 29.4 (2 CH_2-4, 13), 28.5 (CH_2-18), 20.9 (CH_3, 8-CH_3), 12.9 (CH_3, 2- CH_3), 9.1 (CH_3-19). –(+)-ESI MS *m/z* 787 ([M + Na]$^+$, 100).



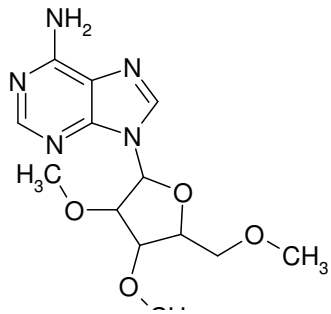
Bonactin (**102**): Non UV absorbing, colourless oil (13 mg), R_f = 0.27 (CH_2Cl_2 /10% MeOH) coloured to violet by anisaldehyde/sulphuric acid after heating. –**^{1}H NMR** (CD_3OD, 300 MHz) δ= 5.12-4.97 (m, 1H, H-8), 4.18 (m, 1H, H-6'), 4.00 (m, 3H, H-3, 3',6), 3.87 (m, 1H, H-8'), 2.08-1.93 (m, 4H, CH_2-5,4), 1.86-1.42 (m, 10H, CH_2-5',4',7,7',9'), 1.25 (br s, 3H, CH_3-8), 1.17 (d, ^3J = 7.2 Hz, 3H, CH_3-2), 1.09 (d, ^3J = 7.2 Hz, 3H, CH_3-2'), 0.89 (t, ^3J = 6.2 Hz, H, CH_3-10').



Nonactic acid (**93**): Non UV absorbing, colourless oil, R_f = 0.42 (CH_2Cl_2/10% MeOH), stained violet by anisaldehyde/sulphuric acid and heating. – **^{1}H NMR** ($CDCl_3$, 300.132 MHz) δ 4.08 (m, 1H, H-6), 3.97-3.96 (m, 2H, H-8, H-3), 3.62 (s, 3H, OCH_3), 2.52 (dq, ^3J = 8.3, 7.2 Hz, 1H, H-2), 2.02 (m, 2H, Ha-4, Ha-5), 1.58 (m, 4 H, Hb-4, Hb-5, 7-CH_2), 1.18 (d, ^3J = 6.4 Hz, 3H, H-9), 1.10 (d, ^3J = 7.2 Hz, 3H, H-10).



Homononactic acid (**94**): Non UV absorbing, colourless oil, R_f = 0.40 (CH_2Cl_2/ 5% MeOH), turns to violet with anisaldehyde/sulphuric acid. – **^{1}H NMR** ($CDCl_3$, 300 MHz) δ 4.04 (m, 1H, H-6), 3.97-3.96 (m, H-8, H-3), 3.76 (m, 1H, H-8), 2.48 (dq, ^3J = 8.3, 7.0 Hz, 1H, H-2), 2.03 (m, 1H, CH_2-4), 2.01 (m, 1H, CH_2-5), 1.70 (m, 2H, CH_2-7), 1.68- 1.45 (m, 4H, CH_2-4, CH_2-5, CH_2-9), 1.14 (d, ^3J = 7.1 Hz, 3H, CH_3-11), 0.92 (t, ^3J = 7.6 Hz, 3H, CH_3-10).



Trimethoxyladenosine (98): white solid, UV absorbing, turned greenish-blue with anisaldehyde/sulphuric acid. **-** R_f = 0.40 (CH_2Cl_2/7% MeOH). **- ^{1}H NMR** (DMSO-d_6, 300 MHz): δ = 8.26 (s, 1H, 2-H), 5.93 (s, 1H, 6-H), 4.79 (t, ^3J = 5.4 Hz, 1H, 2'-H), 4.53 (dd, 5.0, 3.3 Hz, 1H, 3'-H), 4.15 (q, 5.8 Hz, 1H, 4'-H), 4.03 (m), 3.56 (*ABX*, ^3J = 12.3, 2.8 Hz, 1H, 5'a-H, 5'b-H), 3.41 (s, 3H, CH_3), 3.30 (brs, 6H, CH_3). **- ^{13}C NMR** (CD_3OD, 150 MHz): δ = 154.3 (Cq-6), 151.7, 150.2, 137.9 (CH-2, CH-8, Cq-4), 119.7 (Cq-5), 87.2 (CH-1'), (CH-1'), 82.8 (CH-4'), 72.3 (CH-2'), 70.6 (CH-3'), 71.9 (CH_2-5'), 58.9 (OMe), 58.8 (OMe) **- (+)-ESI MS:** *m/z* (%) = 613 ([2M+Na]$^+$, 40), 318 ([M+Na]$^+$, 100). –(+)-**HRESI MS**: *m/z* = 309.1353480 [M+H]$^+$ (calcd. 309.135325 for $C_{13}H_{19}N_5O_4$). –**^{1}H-^{1}H COSY**, **HMBC:** see Figure 63.

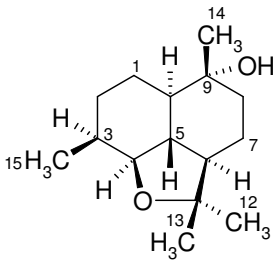
7.4 Strain T426A

The fermentation was carried out by IBWF (Kaiserslautern). The optimum temperature for growth was between 21-27 °C in M1 medium without marine salt. The growth culture was filtrated and the mycelium was extracted with ethyl acetate. The crude extract (400mg) was separated by silica gel with mobile phase (CH_2Cl_2/ MeOH), which gave two fractions A and B. Both fractions were separated by Sephadex LH-20 (MeOH) following by silica gel chromatography and gave **103a**, **103b, 104a**, **104 b** and **105.**

Table 25: **Biological activity**

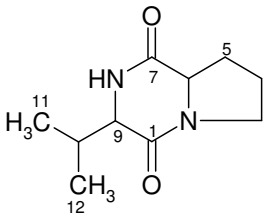
Microorganism	Inhibition zone
Bacillus brevis	12
Bacillus subtilis	13
Nematospora coryli	12
Micrococcus luteus	12
*Mucor miehei (*Tü 284)	16
*Mucor miehei (*Tü 284)	10

Cadinane Sequiterpene 105: colourless prisms R_f 0.56 ($CHCl_3$: 10% MeOH), non UV absorbing band at 254 nm, violet colour with anisaldehyde-sulphuric acid; $[\alpha]_D^{20}$ + 23.7 (c1.0, $CHCl_3$); **^{1}H NMR** ($CDCl_3$): 0.90 (3H, d, J = 7.1 Hz, H-15), 1.05 (3H, s, H-12), 1.19 (3H, s, H-14),

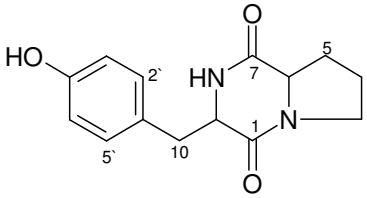


1.20 (1H, m, H-1β), 1.23 (1H, m, 7 β), 1.23 (1H, ddd, *J* = 10.7, 11.8, 12.1 Hz, H-5), 1.31 (3H, s, H-13), 1.33 (1H, ddd, *J* = 3.8, 11.8, 11.8 Hz, H-10), 1.43 (1H, ddd, *J* = 3.6, 12.1, 12.1 Hz, H-6), 1.47 (1H, m, H-2α), 1.51 (1H, ddd, *J* = 3.3, 12.9, H-8α), 1.54 (1H, m, H-1α), 1.64 (1H, m, H-7), 1.67 (1H, m, H-2β), 1.82 (1H, ddd, *J* = 3.3, 12.9, H-8β), 2.10 (1H, m, H-3), 3.45 (1H, dd, *J* = 4.9, 10.7 Hz, H-4); **^{13}C NMR** ($CDCl_3$: 10.4 (C-15), 18.9 (C-1), 21.7 (C-14), 23.7 (C-7), 24.5 (q, C-13), 30.0 (C-2), 30.6 (C-3), 43.3 (C-8), 43.4 (d, C-5), 48.8 (C-10), 52.6 (C-6), 73.0 (C-9), 81.5 (C-4), 81.6 (C-11). **– EIMS**: *m/z* 238 $[M]^+$, 233, 205, 162, 122 (100%), 95; **– HR-EIMS**: $[M]^+$ m/z 238.1928; $C_{15}H_{26}O_2$ calc for 238.1933). – **^{1}H-^{1}H COSY**, **HMBC:** see Figure 64.

Crystal data for sesquiterpinoid: Orthorhombic, space group $P2_1P2_1P2_1$ a = 8.390 (3) A^0, b = 11.306 (5) A^0, C = 14.988 A^0, v = 1422 (11) A^{03}, α = 90.00^0, β = 90.00^0, γ = 90.00^0, z = 2, D_x = 114 Mg m^{-3}, μ (Mo, K_α) = 0.071 for 1416 reflections.



***Cis-cyclo*-(Pro-Val)** (**103**): Colourless crystals R_f = 0.56 ($CHCl_3$: 10% MeOH), UV absorbing band at 254 nm, white colour with anisaldehyde-sulphuric acid. **– ^{1}H NMR** ($CDCl_3$, 300 MHz): 6.18 (brs, 1NH), 4.09 (t, *J* = 8.1 Hz, 1H, H-3), 4.1-3.96 (dd, *J* = 3.56, 9.5 Hz, H-6), 3.53 (m, 2H, CH_2-5), 2.31(m, 2H, H-4), 1.92 (m, H-3), 1.5 (m), 0.97(d, *J* = 6.5 Hz, 3H, H-11), 0.92 (d, *J* = 6.5 Hz, 3H, H-12).

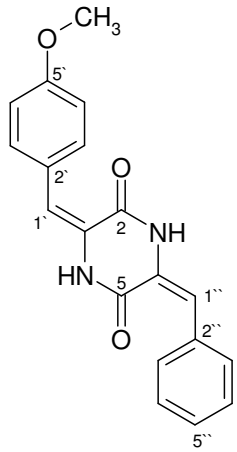


***Cis-cyclo*-(Tyr,Pro)** (**104**): Colourless crystals, 7 mg, R_f = 0.56 ($CHCl_3$/10% MeOH), white colour with anisaldehyde-sulphuric acid under pink background. **– ^{1}H NMR** (DMSO-d_6, 300 MHz): δ = 9.28 (brs, 1H, OH), 8.06 (d, 1H, NH), 6.96 (d, *J* = 8.6 Hz, 2H, 3',5'-H), 6.70 (d, *J* = 8.6 Hz, 2H, 2',6'-H), 3.93 (dd, *J* = 7.8, *J* = 2.1 Hz, 1H, 3-H), 3.43 (t, *J* = 1H, 10-Ha), 3.18 (dd, *J* = 9.2 Hz, *J* = 5.1 Hz, 1H, 10-Hb), 2.89(m), 2.79 (dd, *J* = 0.5 Hz, *J* = 0.6 Hz), 2.00 -1.52 (2m, 3H, 7-, 8-CH_2).

7.5 Marine *Streptomyces* sp B6003

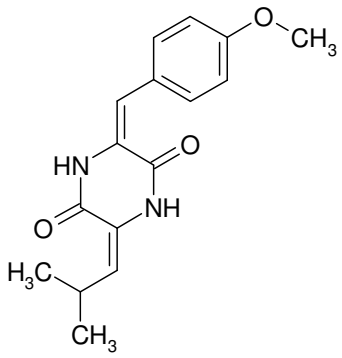
7.5.1 Fermentation procedure and work-up

The marine *Streptomyces* sp B6003 was cultivated using M_2^+ medium for five days on a linear shaker culture. For the further investigation, the strain was twice fermented in a 20-l-Fermenter scale. Because the amount of the crude extract was insufficient and its quality in the first fermentation was not satisfactorily, a second trial became necessary. The brown culture broth was filtrated under filter press followed by the extraction of the mycelium cake 4 times with ethyl acetate and finally with acetone. The water phase was chromatographed on XAD-16 and eluted with methanol. The extract of the marine streptomycete B6003 has been noticeable in the screening through a selective activity against cancer cell lines. The methanolic phase was evaporated under dryness. The mycelium and water extracts was mixed together on the view of their TLC comparison and delivered 8g of crude extract. The 8g was first chromatographed on Sephadex LH-20 using (CH_2Cl_2/50%MeOH) as eluent and delivered four fractions. The oily fraction I was discarded. The chromatography of fraction 3 on silica gel, followed by PTLC delivered *cis/trans* isomers of *cyclo*-(-didehydro-4-methoxy-phenylalanine,-di-dehydro-phenylalanine) (**109**) and (**110**) . Triturating of fraction 4 with methanol delivered *cyclo*-(didehydro-4-methoxy-phenylalanine,didehydrophenylalanine) (**109**). The filtrate was chromatographed on Sephadex LH-20 (CH_2Cl_2/50% MeOH) and delivered sub-fractions A and B. Sub-fraction A was chromatographed on sephadex LH-20 using MeOH as eluent followed by PTLC to give uracil and albonoursin C (**115**, 8 mg). Silica gel chromatography of fraction B delivered in addition 2(S)-2-acetamido-3-pentanone (**116**, 15 mg).

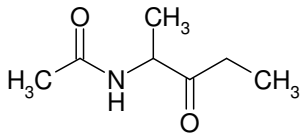


***cyclo*-(Didehydro-4-methoxyphenylalanine,didehydrophenylalanine)** (**109**): UV absorbing, colourless solid, R_f = 0.75 (CH_2Cl_2/1%MeOH), yellow colouration with anisaldehyde/sulphuric acid. – **^{1}H NMR** (300 MHz, CD_3OD) and – **^{13}C NMR** (125 MHz, CD_3OD) see Table 12. – (-)-ESIMS *m/z* 321

$[M+H]^+$, 36); 160(100). 663 ($[2\ M+Na]^+$, 15) – ESIHRMS *m/z* 343.08665 $[M + Na]^+$ (calcd for $C_{19}H_{16}N_2O_3Na$, 343.06872)



Albonoursin C (115): UV absorbing, colourless solid, R_f = 0.58 (CH_2Cl_2/3% MeOH), yellow colouration with anisaldehyde/sulphuric acid. UV (MeOH): max = 263 (1.80) nm. - – **^{1}H NMR, ^{13}C NMR** see Table 12. – (+)-ESI MS *m/z* (%) = 309 ($[M+Na]^+$, 76), 595 ($[2M+2Na]^+$, 100). – (-)-ESI MS *m/z* (%) = 285.0 ($[M-H]^-$, 100), 593 ($[2M-2H+Na]^-$, 50). – (+)-**HREIMS**: 287.02229 $[M+H]^+$ (calcd. 287.052621 for $C_{16}H_{18}N_2O_3$).



2(S)-2-Acetamido-3-pentanone (116). Colourless oil, R_f = 0.45 (CH_2Cl_2/5% MeOH), yellow colouration with anisaldehyde/sulphuric acid. – **^{1}H NMR** (CD_3OD): δ 4.20 (1H, m, 1H), 3.45 (m, 1H), 3.13(1H, q, J = 7.1 Hz, 2-H), 2.5 (1H, dq, J = 18.0, 7.3Hz, 4-H), 2.12 (3H,s, CH_3), 1.91(3H, d, J = 7.1 Hz, 1-H), 1.71 (3H, t, J = 7.2Hz, 5-H). CI-MS *m/z* (%): 144 $[M+H]^+$, 161 $[M+NH4]^+$, 287 $[2M+H]^+$, 304 $[M+NH4]^+$.

7.6 Marine *Streptomyces* sp. ACT7655

The strain was isolated and identified by E. Helmke from the Alfred-Wegener Institute for Polar and Marine Research in Bremerhaven, Germany. The marine *Streptomyces* sp. ACT7655 was growing with a white aerial mycelium on agar plates using M_2^+ medium at 28 °C for 3 days.

7.6.1 Pre-screening

TLC of the strain showed four UV absorption bands, which changed on spraying with anisaldehyde/ sulphuric acid to a pink, violet, and red colour.

Table 26: Antimicrobial activity of the crude extract from strain ACT7655 on M_2^+ medium (40μL (100 mg/ml), diameter of inhibition zones in mm).

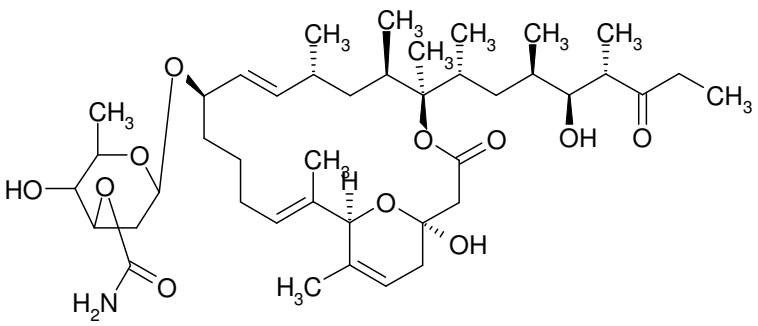
Tested microorganism	Inhibition zone ∅ [mm]
Bacillus subtilis	23
Staphylococcus aureus	18
Streptomyces viridochromogenes (Tü 57)	12
Escherichia coli	11
Candida albicans	0
Mucor miehei (Tü 284)	0

7.6.2 Fermentation and Workup

Well grown agar cultures of the strain were cut into small pieces to inoculate 100 of 1-L Erlenmeyer flasks each containing 250 mL of M_2^+ medium (with 50% artificial sea water) of pH 7.8 as shaker culture (at 28 °C for 7 days). The resulting culture broth was filtered with the aid of the filter press. The water phase and the biomass were obtained. The water phase was subjected to Amberlite XAD-2 column with methanol/water and then the eluent was extracted with ethyl acetate. The biomass was extracted with ethyl acetate and acetone for three times. Thin layer chromatography of the crude extracts from the water phase and the biomass showed identity. They were combined therefore and worked up together.

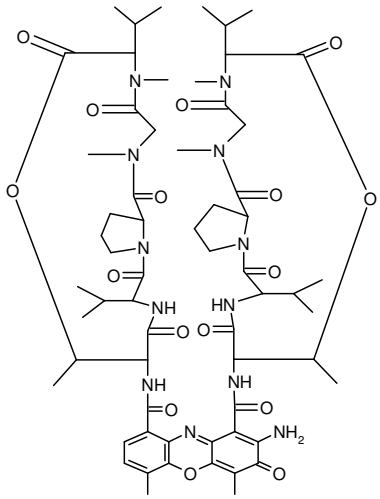
7.6.3 Workup and Isolation

The crude extract was subjected to silica gel column chromatography by using a dichloromethane/methanol gradient to afford three fractions. They were further chromatographed in different ways to get their constituents in pure form (see work up scheme, Figure 4). Fraction III showed four UV absorbing bands at 254 nm, which gave a red, violet, blue colour by spraying with anisaldehyde/sulphuric acid after heating. These compounds were purified by Sephadex LH-20 column with methanol to get venturicidin A (**117**, 10.5mg), anthranalic acid (16mg) and actinomycin D (**118,** 21mg).



Venturicidin A (117): $C_{41}H_{67}NO_{11}$ (750), colourless crystal, weakly UV absorbing (254 nm). – R_f = 0.28 (CH_2Cl_2/ MeOH 9:1). – Colour reaction: anisaldehyde/sulphuric acid (dark

hyde/sulphuric acid (dark blue), Ehrlich (dark blue). **^{1}H NMR** (DMSO-d_6, 300 MHz): δ = 6.41(1H, s, NH), 5.44 (m, 1H, 14-H), 5.25 (m, 1H, 5-H), 4.97(s br, 1H, 3-OH), 5.38 (m, 1H, 9-H), 5.27 (m, 1H, 15-H), 4.82 (br, 2H, $CONH_2$), 4.64 (m, 2H, 19-CH, 3´-H), 4.54 (m, 1H, 1´-H), 4.32 (s, 1H, 7-H), 3.83 (m, 1H, 13-H), 3.51 (m, 1H, 23-H), 3.25 (m, 1H, 5´-H), 3.22 (m, 1H, 4´-H), 3.14 (br, 1H, 4´-OH), 2.92 (m, 1H, 24-H), 2.59 (m, 2H, 2-H), 2.57 (m, 1H, 26-HA), 2.53 (s br, 1H, 23-OH), 2.30 (m, 1H, 26-HB), 2.24 (m, 1H, 2´-HA), 2.14 (m, 3H, 4-H, 16-H), 2.08 (m, 1H, 10-HA), 1.90 (m, 1H, 10-HB), 1.83 (m, 1H, 18-H), 1.77 (m, 1H, 20-H), 1.67 (m, 1H, 2´-HB), 1.60 (m, 1H, 22-H), 1.47 (s, 3H, 6-CH_3), 1.44 (m, 2H, 12-H), 1.44 (m, 1H, 11-HA), 1.39 (s, 3H, 8-CH_3), 1.31 (d, 3H, 5´-CH_3), 1.20 (m, 1H, 17-HA), 1.17 (m, 1H, 11-HB), 1.15 (m, 1H, 21-HA), 1.08 (m, 3H, 24-CH_3), 1.03 (m, 1H, 17-HB), 1.03 (m, 3H, 27-H), 0.98 (m, 1H, 21-HB), 0.92 (d, 3H, 16-CH_3), 0.85 (m, 3H, 22-CH_3), 0.82 (m, 3H, 20-CH_3), 0.82 (m, 3H, 18-CH_3). – **(+)-ESI-MS**: *m/z* (%) = 772 $[M+Na]^+$ (100). – **(-)-ESI-MS**: *m/z* (%) = 748 $[M-H]^-$ (60).



Actinomycin D (**118**): isolated as reddish-orange powder (21 mg) from sub fraction III-2 was subjected to PTLC ($CHCl_3$/5%MeOH) and Sephadex LH-20 (two times MeOH). On TLC, it turned to red by anisaldehyde/ sulphuric acid but no colour change happened on treatment with 2 N NaOH. – R_f = 0.37 ($CHCl_3$/MeOH 95:5). – **^{1}H NMR** ($CDCl_3$, 300 MHz): δ = 8.23 (d br, ^3J = 6.4 Hz, 1 H, NH-Val$_\alpha$), 7.97 (d br, ^3J = 6.4 Hz, 1 H, NH-Val$_\beta$), 7.81 (d br, ^3J = 6.4 Hz, 1 H, NH-Thr$_\beta$), 7.67 (d, ^3J = 7.9 Hz, 1 H, 8-H), 7.35 (d, ^3J = 7.9 Hz, 1 H, 7-H), 6.88 (d br, ^3J = 6.4 Hz, 1 H, NH-Thr$_\alpha$), 5.99 (d, ^3J = 9.1 Hz, 1 H, 2-H Pro$_\alpha$), 5.96 (d, ^3J = 9.1 Hz, 1 H, 2-H Pro$_\beta$), 5.20 - 5.15 (m, 2 H, 3-H, Thr$_{\alpha,\beta}$), 4.81- 4.03 (m, 9H, 2-H$_a$, Sar$_\alpha$ 1 H, 2-H$_a$, Sar$_\beta$, 2-H Thr$_\alpha$, 2-Thr$_\beta$), 4.02-3.40 (m, 8 H, 2-H$_b$ Sar$_{\alpha,\beta}$, 2-H, Val$_{\alpha,\beta}$, 5-H$_2$, Pro$_{\alpha,\beta}$), 2.93, 2.90, 2.88 (3 s, 3 H + 3 H + 6 H, NCH_3 Me- Val$_{\alpha,\beta}$, NCH_3, Sar$_{\alpha,\beta}$), 2.67, 2.57 (m + s, 6 + 3, 3-Ha Pro$_{\alpha,\beta}$, 2,3-H, MeVal$_{\alpha,\beta}$, 6-CH_3), 2.38-1.76 + 2.23 (m + s, 8 + 3 H, 3-H$_b$-, 4-H2 Pro$_{\alpha,\beta}$, 3-H Val$_{\alpha,\beta}$, 4-CH_3), 1.28 (m, 6 H, CH_3, Thr$_{\alpha,\beta}$), 1.17-1.07 (m, 6 H, CH_3 Val$_{\alpha,\beta}$), 0.99-0.82 (m, 12 H, CH_3 Val$_{\alpha,\beta}$, CH_3 Me Val$_{\alpha,\beta}$), 0.75 (d, ^3J = 4.9 Hz, 6

H, CH_3 $MeVal_{\alpha,\beta}$). **ESI MS**: *m/z* (%) = 1277 ([M + Na]$^+$, 100), 1255 ([M + H]$^+$, 6). – (-)-**ESI MS**: *m/z* (%) = 1254 ([M]$^-$).

7.7 Terrestrial *Streptomyces* sp. GW7/354

7.7.1 Fermentation, Workup and Isolation

Fully developed agar subcultures of terrestrial *Streptomyces* sp. GW7/354 were used to inoculate 80 1-l Erlenmeyer flaks containing each 250 ml of M2 medium at standard conditions. The flasks were placed on a linear shaker at 28°C for 4-5 days. The culture broth was worked up and the obtained crude extract was subjected to flash chromatography on silica gel using CH_2Cl_2/MeOH gradient and resulted in four fractions. The TLC of the crude extract obtained from the extraction of the water phase exhibited strong UV fluorescent bands from the ethyl acetate extract, which became pinkish-brown on spraying with anisaldehyde/sulphuric acid. The purification of this crude extract finally on preparative TLC (CH_2Cl_2/MeOH5%) and Sephadex-LH20 (CH_2Cl_2/50%MeOH) delivered siderochelin A (**119,** 40 mg) and B (**120,** 15 mg). Fraction III was subjected to Sephadex-LH20 (CH_2Cl_2/50%MeOH) followed by silica gel chromatography and sidercochelin D (**121**, 2.5mg) was isolated. The mycelial cake was extracted with ethyl acetate, the water phase was passed through XAD-16 and latter eluted with methanol. The ethyl acetate of the mycelium and methanol extracts were evaporated to dryness and worked separately on the view of their TLC. The ethyl acetate fraction was subjected to Sephadex LH-20 using (CH_2Cl_2/50% MeOH) and four fractions were obtained. The PTLC (CH_2Cl_2/5% MeOH) of the fraction IV delivered acetyl uridine A (7 mg) and acetyl uridine B (4mg)

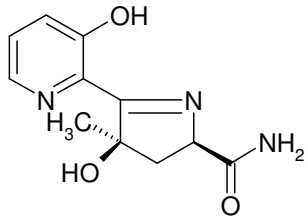
Siderochelin A (**119**) and B (**120**) with 20 μg per paper disc showed high activity against, *Bacillus subtilis*, *Escherichia coli, Staphylococcus aureus, Streptomyces viridochromogenes* (Tü 57), *Mucor miehei*, *Chlorella vulgaris.* Siderochelin D (**121**) also showed moderate activity against both gram positive and gram-negative bacteria. Antibacterial and antifungal activities were determined using the agar diffusion

method with 10 mm paper disc with 40 μg for acetyl uridine A (**122a**) and B (**122b**)/disk.

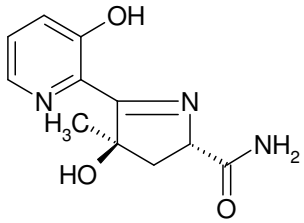
Table 27: **Biological activities of the compounds**

	Conc./paper disk μg/ paper disc	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus subtilis*	*Streptomyces viridochromogenes*	*Mucor miehei*	*Chlorella vulgaris*
119	20	20	35	40	35	45	45
120	20	20	22	40	35	25	30
121	20	10	25	20	20	18	12
122a	40	10	10	15	17	-	-
122b	40	20	10	15	17	-	-

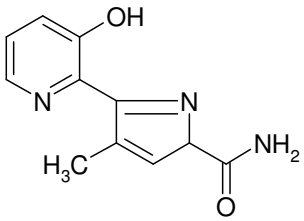
Siderochelin A (**119**): colourless crystals. - R_f (CH_2Cl_2/MeOH10%): 0.33, showed pinkish-brown colour reaction with anisaldehyde/ sulphuric acid NMR data see Table 13. – (+)-**ESI MS** *m/z* (%) 258 $[M+Na]^+$, 100), 493 ($[2M+Na]^+$, 40). - (+)- **HRESIMS** *m/z* 258.088205 $[M+Na]^+$ (calcd. 258.084910 for $C_{11}H_{13}N_3O_3Na$).

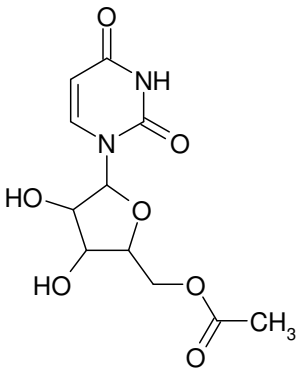


Siderochelin B (**120**): – colourless crystals. - R_f (CH_2Cl_2/ MeOH10%): 0.36, showed pinkish-brown colour reaction with anisaldehyde/ sulphuric acid NMR data see Table 13. – (+)-**ESI MS** *m/z* (%) 258 $[M+Na]^+$, 100), 493 ($[2M+Na]^+$, 40). – (+)– **HRESIMS** *m/z* 258.088205 $[M+Na]^+$ (calcd. 258.084910 for $C_{11}H_{13}N_3O_3Na$).

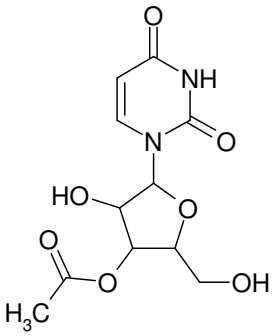


Siderochelin D (**121**): colourless crystals. - R_f (CH_2Cl_2/ MeOH10%): 0.34, showed pink colour reaction with anisaldehyde/ sulphuric acid NMR data see Table 13. – (+)-**ESI MS** *m/z* (%) 218 $[M+H]^+$, 100), – (+)- **HRESIMS** *m/z* 218.09250 $[M+H]^+$ (calcd. 218.0924031 for $C_{11}H_{11}N_3O_2$). – **^{1}H-^{1}H COSY**, **HMBC:** see Figure 81.





Acetyl uridine A (**122a**): UV absorbing colourless solid. - R_f (CH_2Cl_2/MeOH10%): 0.26, showed blue-green colour reaction with anisaldehyde/ sulphuric acid. **NMR data** see Table 14. – (-)-**ESI MS**: *m/z* (%) = 285 ([M-H]-, 100), 593 ([2M-2H+Na]-, 30); **EI MS**: *m/z* (%) = 286 $[M+H]^+$, 30), 227 (12), 211 (52), 112 (100); – (+)-**ESI MS** *m/z* (%) 309 $[M+Na]^+$, 100), 595 ($[2M+Na]^+$, 40). – (+)-**HRESIMS** *m/z* 309.088205 $[M+Na]^+$ (calcd. 309.084910 for $C_{11}H_{14}N_2O_7$) – **^{1}H-^{1}H COSY**, **HMBC:** see Figure 84.



Acetyl uridine B (**122b**): UV absorbing colourless solid. - R_f (CH_2Cl_2/MeOH10%): 0.22, showed blue-green colour reaction with anisaldehyde/sulphuric acid **NMR data** see Table 14. – (-)-**ESI MS**: *m/z* %) = 285 ($[M-H]^-$, 100), 593 ($[2M-2H+Na]^-$, 30); **EI MS**: *m/z* (%) = 286 ($M^{\cdot+}$, 30), 227 (12), 211 (52), 112 (100); – (+)-ESI MS *m/z* (%) 309 $[M+Na]^+$, 100), 595 ($[2M+Na]^+$, 40). – (+)-**HRESIMS** *m/z* 309.088205 $[M+Na]^+$ (calcd. 309.084910 for $C_{11}H_{14}N_2O_7$).). – **^{1}H-^{1}H COSY**, **HMBC:** see Figure 84.

7.8 Terrestrial -derived *Streptomyces* sp. ANK205

7.8.1 Prescreening

The terrestrial-derived *Streptomyces* sp. ANK205 showed a white aerial mycelium. Five 1L Erlenmeyer flasks, containing 250 ml of $M2^+$ medium, were incubated at 28 °C on a linear shaker, to afford a dark brown broth. The broth was lyophilized and the residue was extracted by ethyl acetate followed by concentration *in vacuo*. The obtained yellowish-brown oily extract was applied to biological and chemical screening The chemical screening of the extract during TLC showed appeared several UV absorbing spots and non-UV absorbing spots, which stained with anisaldehyde/sulphuric to yellowish-brown and violet-blue. Biologically, the extract exhibited antimicrobial activities against *Staphylococcus aureus* and *Candida albicans* using agar disc diffusion method (Table 28). The extract was examined against a set of cancer cells, and it showed a potent *in vitro* antitumor activity against all selected cells.

7.8.2 Fermentation and Isolation

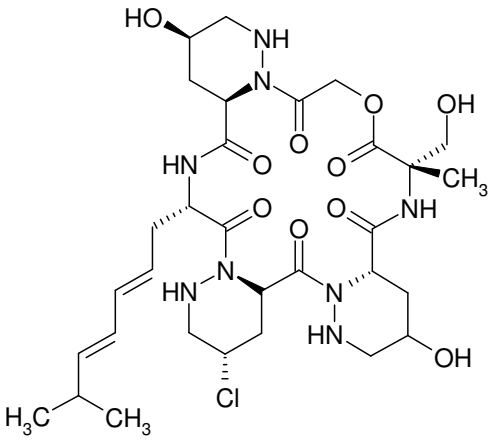
The strain was cultured as 20 L, using 80 of 1 L Erlenmeyer flasks each containing 250 ml of M2 + medium at 28 °C for five days using a linear shaker. The dark brown culture broth was harvested and filtered to separate the mycelium. The mycelial cake was extracted with ethyl acetate, while the filtrate was passed through a column of XAD-2. The column was eluted with 25 L demineralised to exclude the unwanted water-soluble materials, followed by elution with 15 L methanol. The methanolic extract was concentrated under reduced pressure and the water residue was extracted by ethyl acetate. Both ethyl acetate extracts were combined based on TLC. The yielded crude extract (2.8 g) was chromatographed on silica gel column and eluted with CH_2Cl_2/MeOH-gradient (CH_2Cl_2, 1 L; 3% MeOH, 1.5 L; 6% MeOH, 0.5 L; 10% MeOH, 0.3 L; 20 % MeOH, 0.5 L; 50% MeOH, 0.4 L; 100% MeOH, 0.2 L). After TLC, four fractions (I-IV) were collected. From them fraction I: 4-Hydroxy benzoic acid and 4-Hydroxy methyl benzoate was isolated. Purification of fraction II using silica gel column (CH_2Cl_2) followed by Sephadex LH-20 (CH_2Cl_2/40% MeOH) delivered tryptamine, 2-Hydroxy-1-(1*H*-Indol-3yl)-ethanone and tryptophol . Application of fraction III to Sephadex LH-20 (MeOH) lead to the sub-fractions

which on further purification using different chromatographic methods, they delivered two compounds: 3-(2-Hydroxy-4-methoxy phenyl)- propanoic acid (**126**, 3mg, whites solid) and Tyrosol (6 mg, colourless solid). Fraction IV on treatment with sephadex followed by RP 18 gave piperazimycin A (**123** 4.5 mg, white solid), Butanolide A (**124**, 3.6 mg, colourless oil) and butanolide B (**125** 2.5 mg, colourless oil).

Table 28: Antimicrobial activity of the crude extract from the marine-derived *Streptomyces sp*. ANK 205 (1 mg/ml).

Tested microorganisms	**Inhibition zone ∅ [mm]**
Bacillus subtilis	0
Staphylococcus aureus	-
Streptomyces viridochromogenes (Tü 57)	12
Escherichia coli	14
Candida albicans	-
Mucor miehei (Tü 284)	-
Chlorella vulgaris	-
Chlorella sorokiniana	-
Scenedesmus subspicatus	-

Antibacterial, antifungal and antialgal activities were *semi* quantitatively determined using the agar diffusion method with 9 mm paper disks and 40 μg of the pure compounds. Butanolide A (**124**), butanolide B (**125**), piperazimycin A (**123**) and 3-(2-Hydroxy-4-methoxy phenyl)-propanoic acid (**126**) were tested. Compounds **124** and **125** were moderately active against all test microbes except three algae *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus subspicatus* for **123**.



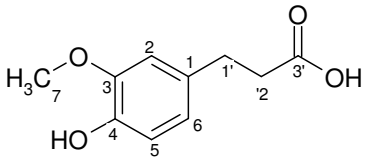
Piperazimycin A (123): white crystals, UV absorbing, stained to blue by exposing to anisaldehyde/sulphuric acid reagent. **–** R_f = 0.26 (CH_2Cl_2 / 5% MeOH). **– ^{1}H NMR** (300 MHz, CD_3OD): **– ^{13}C NMR** (125 MHz, CD_3OD): see Table 16. **– (+)-ESI-MS**: *m/z* (%) = 1475 ([2M+Na]$^+$, 30), 749 ([M+Na]$^+$, 100). **– (-)-ESI-MS**: *m/z* = 725 [M-H]$^-$. **– (+)–**

HRESI-MS: *m/z* 727.3176330 $[M+H]^+$, (calcd 727.3176353 for $C_{31}H_{48}N_8O_{10}Cl$), 749.2995570 $[M+Na]^+$ (calcd 749.299575 for $C_{31}H_{47}N_8O_{10}Cl_1Na$).

5-(8-Hydroxy-dec-2-enyl)-tetrahydro-furan-2-yl]-acetic acid (124): colourless oil, R_f = 0.3 ($CHCl_3$/MeOH 95:5); $[\alpha]^{20}_D$ +9 (c 0.43, MeOH); IR (KBr) ν_{max} 3430, 2925, 2855, 1717, 1633, 1281, 1157, 1023, 578 cm^{-1}; **^{1}H NMR** (300 MHz, CD_3OD) δ 5.45 (m, 1H, H-7), 5.40 (m, 1H, H-8), 4.39 (m, 1 H H-5), 4.14 (m, 1 H, H-2), 3.42 (q, J = 6.0 Hz, 3H, H-13), 3.05, 2.80 (ABX, J_{AB} = 13.9, J_{AX} = 0, J_{BX} = 7.0 Hz; 2 H, H-16a,b), 2.20, 2.13 (m, 2H, H_2-6), 2.09, 1.97 (m, 2H, H_2-9), 1.98, 1.78 (m, 2H, H_2-3), 1.97, 1.73 (m, 2H, H_2-10), 1.76, 1.60 (m, 2H, H_2-4), 1.49, 1.39 (m, 2H, H_2-12) 1.48, 1.36 (m, 2H, H_2-10), 1.48, 1.36 (m, 2H, H_2-11), 1.47, 1.37 (m, 2H_2, H-14), 0.89 (t, J = 6.0 Hz, 3H, H_3-15); **^{13}C NMR** (125 MHz, CD_3OD), δ 175.9 (C-1'), 131.9 (CH-7), 129.6 (CH-8), 81.1 (CH-5), 73.7 (CH-13), 64.7 (CH-2), 42.5 (CH_2-16), 37.5 (CH_2-4), 37.2 (CH_2-10), 35.8 (CH_2-3), 31.1 (CH_2-12), 30.1 (CH_2-13), 29.7 (CH_2-10), 28.2 (CH_2-9), 24.1 (CH_2-14), 10.4 (CH_3-15). **(+)-ESIMS** *m/z* (%) 307 $[M + Na]^+$ (100), 591 $[2M+Na]^+$ (6); – (+)-**HRESIMS** *m/z* 307.18793 (calcd for $[M + Na]^+$, $C_{16}H_{28}O_4Na$, 307.18798). – **^{1}H-^{1}H COSY**, **HMBC:** see Figure 91, Figure 88, Figure 93.

5-(4-Hydroxy-dodec-2-enyl)-dihydro-furan-2-one (125): colourless oil, R_f = 0.34 ($CHCl_3$/MeOH 95:5); $[\alpha]^{20}_D$ +3 (c 0.38, MeOH); IR (KBr) ν_{max} 3426, 2859, 1720, 1450, 1284, 1256, 1176, 1015, 562 cm^{-1}; ^{1}H NMR (300 MHz, CD_3OD) δ 5.45 (m, 1H, CH-7), 5.37 (ddt, J = 10.7, 9.0, 1.4 Hz, 1H, CH-8), 4.42 (td, J = 9.1, 3.9 Hz, 1H), 4.35 (td, J = 8.2, 4.4 Hz, 1H, CH-9), 2.78a, 2.54b (m, 2H, CH_2-3), 1.73a, 1.55b (m, 2H, CH_2-4), 2.25a, 2.18b (m, 2H, CH_2-6), 1.69a, 1.38b (q, 1H, J = 6.4 Hz, CH-10), 1.29 (m, 1H, CH_2-11, 15, 16), 1.91 (m, 1H, CH_2-11, 13), 0.89 (t, J = 6.2 Hz, 3H CH_3-17); **^{13}C NMR** (125 MHz, CD_3OD) δ 178.9(C-1), 137.0 (CH-8), 130.8 (CH-7), 81.0 (CH-5), 68.2 (CH-9), 35.6 (CH_2-3), 37.3 (CH_2-4), 24.7 (CH_2-6), 38.8 (CH_2-10), 26.2(CH_2-11), 29.1 (CH_2-12), 24.1 (CH_2-13), 23.7 (CH_2-15), 32.9 (CH_2-16), 14.1 (CH_3-17); –

(+)ESIMS *m/z* (%) 291[M + Na]$^+$(28); – (+)-**HRESIMS** *m/z* 291.4068499 (calcd for [M + Na]$^+$, $C_{16}H_{28}O_3Na$, 291.40685). – **^{1}H-^{1}H COSY**, **HMBC:** see Figure 91.



3-(2-Hydroxy-4-methoxy phenyl)-propanoic acid (**126**): A crude compound showed a UV absorption at 256 nm and turned to orange-red by spraying with anisaldehyde/sulphuric acid after heating. From the sub fraction F-3 further purification was carried out by RP 18 and 3-(2-hydroxy-4-methoxy phenyl)- propanoic acid was obtained R_f = 0.56 ($CHCl_3$ / 5 % MeOH). **^{1}H NMR** (CD_3OD, 300 MHz): 6.78(1H, s), δ6.66 (2H,dd, *J* = 4.6,3.2 *Hz*), 2.8 (3H, s), 2.53 (2H, t, *J* = 13.6 Hz.). **EI MS (70 eV)**: *m/z* (%) 196 ([M]$^+$, 27), 152 ([CO]$^+$, 100), 103 (16), 77 (28).

7.9 Terrestrial *Streptomyces* sp. ANK 175

7.9.1 Prescreening

The terrestrial *Streptomyces* sp. ANK 175 grew on M_2 agar with white aerial mycelium and brown pigmentation in the agar after five days of incubation at 28 °C. The strain was cultured in 4 of 1 L Erlenmeyer flasks each containing 250 ml M2 medium on a round shaker with 95 rpm for 72 h at 28 °C, which yielded 200 mg of dark reddish-brown crude extract for the biological and chemical pre-screening. The biological screening of this crude extract using agar diffusion method, revealed antibacterial activities, which were summarized in

Table **29**.

Table 29: Antimicrobial activity of the crude extract of Terrestrial Streptomyces sp. ANK175

Tested microorganisms	Inhibition zone ∅∅ [mm]
Streptomyces viridochromogenes (Tü57)	12
Mucor miehei (Tü 284)	18

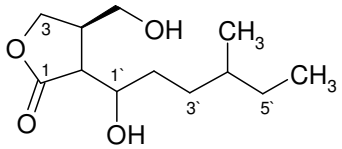
The crude extract showed 92% activity against *Artemia salina*.

7.10 Fermentation and Work-up

For scaling up, the strain was cultivated on a 30 L scale at 28 °C for 7 days on a linear shaker. The brown culture broth was mixed with 1 kg diatomaceous earth and then filtered using a filter press. The water phase was adsorbed on XAD-16, the resin washed with demineralised water (10 L) followed by methanol (15 L). The methanolic phase was evaporated in vacuo to the remaining water phase, which was extracted with ethyl acetate (3 ×1 L). The solid phase was extracted with ethyl acetate (3 ×1 L) followed by acetone (3×1L). The filtrate was passed through the XAD-16 column and mycelium was separately extracted four times with ethyl acetate (2 L). During the extraction of the mycelial cake, it was treated with ultrasonic radiation for 15 min each time. The yellow organic solutions were combined and the solvent was removed under vacuum resulting in 2.62 g of dark yellowish brown extract.

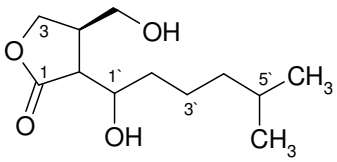
7.10.1 Isolation

The crude extract of the terrestrial *Streptomyces* sp. ANK 175, was fractionated over a silica gel column (CH_2Cl_2/CH_3OH gradient 0-50% MeOH) to give four fractions. Fraction II was subjected to Sephadex LH-20 (MeOH) to give three subfractions, which were re-chromatographed over a silica gel column, followed by RP 18 to give Virginiae butanolide B (**127a**, 2.4 mg) and Graefe's Factor I (**127b** 1.6 mg). Fraction III on purification with silicagel column followed by Sephadex LH-20 (MeOH) gave piperafizine B (**128**, 7mg) and fraction IV was purified using number of chromatographic techniques to give Piperafizine C (**129**, 5.1 mg).

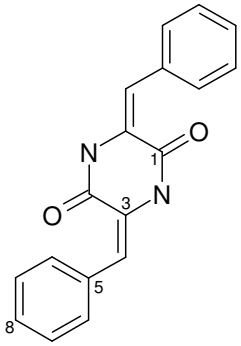


Virginiae butanolide B; (127a): colourless oil, UV nonabsorbing, turn-ed greenish-blue by anisaldehyde/sulphuric acid, R_f = 0.33 (CH_2Cl_2/5% MeOH). – **^{1}H NMR** (300 MHz, CD_3OD): δ = 4.37 (dd, J = 24.8, 18.2 Hz, 1H), 4.14 (dd, J = 24.8, 18.1 Hz, 1H), 3.56 (dd, J = 24.8, 14.2 Hz, 1H), 3.29 (dd, J = 24.8, 14.2 Hz, 1H), 2.78 (dt, J = 17.8, 12.5 Hz, 1H), 2.49 (m, 2H), 1.15-1.47 (dd, J = 22.8, 17.5 Hz, 1H), 1.35 – 1.18 (m, 5H), 0.90 (d, J = 12.6 Hz, 6H). – **(+)-ESIMS**: *m/z* (%) =

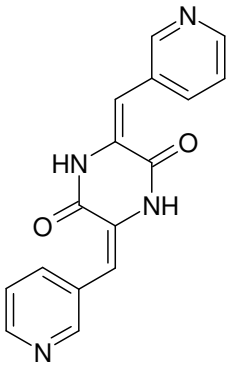
455 ([2M+Na]$^+$, 22), 239 ([M+Na]$^+$, 100), 217 ([M+H]$^+$, 8). – **(-)- ESI-MS**: *m/z* = 261 ([M+HCOO]$^-$.



Graefe's Factor I (127b) colourless oil, UV nonabsorbing, turned blue-violet by anisaldehyde/sulphuric acid. – R_f = 0.54 (CH_2Cl_2/5% MeOH). – **^{1}H NMR** (300 MHz, CD_3OD): δ =4.49 (m, 1H), 4.17 – 3.96 (m, 1H), 3.65 (qdd, *J* = 26.0, 12.5, 10.2 Hz, 2H), 2.86 (m, 1H), 2.15 – 1.99 (m, 2H), 1.15 – 1.28 (m, 3H), 1.22 – 1.05 (m, 4H), 0.88 (d, *J* = 12.5 Hz, 6H). **–(+)-ESI-MS**: *m/z* (%) = 483 ([2M+Na]$^+$, 40), 253 ([M+Na]$^+$, 70).



Piperafizine B (**128**): Yellow solid R_f = 0.62 (CH_2Cl_2/MeOH 5%), UV absorbing band at 254 nm, white with anisaldehyde/sulphuric acid. – **^{1}H NMR** (DMSO-d_6, 300 MHz) δ 7.55 (d, *J* = 7.5 Hz, 2H, H-6), 7.42 (t, *J* = 7.6 Hz, 2H, H-7), 7.34 (t, *J* = 7.4Hz, 1H, H-8), 6.79 (s, 1H, H-4), **^{13}C NMR** (DMSO-d_6, 125 MHz) 157.6 (cq, C-1), 129.0 (Cq, C-5), 128.4 (CH, C-7), 127.8 (CH, C-8), 126.0 (Cq, C-3), 114.8 (CH, C-4). – (-)-**ESIMS** *m/z* 289 ([M-H]$^-$, 100).



Piperafizine C (**129**): Yellow solid, 5.1 mg, R_f = 0.62 (CH_2Cl_2/MeOH 5%), UV absorbing band at 254nm, white with anisaldehyde/sulphuric acid. – **^{1}H NMR** (DMSO-d_6, 300 MHz) & **^{13}C NMR** (DMSO-d_6, 125 MHz) see Table 16. – (-)-**ESIMS** m/z 291.088791 ([M-H]$^-$, calcd. 291.0900 for $C_{16}H_{11}N_4O_2$). – **^{1}H-^{1}H COSY**, **HMBC:** see Figure 96.

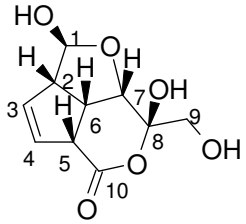
7.11 Terrestrial *Streptomyces* strain ANK289

The *Streptomyces* strain ANK289 has been derived from a soil sample and was isolated on YMG agar at room temperature (YMG agar: 2 g/L yeast extract, 5 g/L malt extract, 5 g/L glucose, 15 g/L agar, 30 mg/L cycloheximide). Its almost complete 16 S rRNA gene sequence (GenBank Accession Nr. HM367877) shows high similarities to *Streptomyces hawaiiensis* NBRC 12784 (GenBank Accession Nr. AB184143). The strain is deposited in the culture collection at the Institute of Organic and Biomolecular Chemistry, Göttingen, Germany. During the primary screening of the strain cultivated on M2 medium (7-days at 28 °C), three middle polar non-UV-absorbing bands were detected during TLC, which turned dark blue, by anisaldehyde/sulphuric acid. HPLC MS/MS of the extract showed two peak signals with masses of 251 and 265, corresponding to a moderate activity against Grampositive and Gram-negative bacteria. The extract displayed cytotoxic activity with mortality of 13.49% against the brine schrimp *Artemia salina*, as test organism.

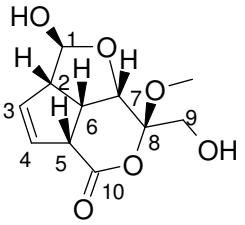
Tested microorganisms	**Inhibition zone ∅ [mm]**
Bacillus subtilis	17
Staphylococcus aureus	16
Streptomyces viridochromogenes (Tü 57)	12
Escherichia coli	14

7.11.1 Fermentation of *Streptomyces* sp. Isolate ANK289 and Workup.

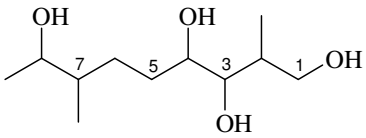
The *Streptomyces* sp. ANK289 was cultivated in 100 of 1L Erlenmeyer flasks each containing 250 mL of M_2 medium at 28 °C, while shaking for 5 days on a linear shaker. The culture broth was mixed with ca. 1.5 kg of Celite and filtered under pressure. The water phase was extracted with Amberlite XAD-16, the resin was washed with distilled water and eluted with methanol, while the mycelium was extracted first with ethyl acetate and then acetone. All extracts were combined and separated on silica gel (column 50 × 3 cm, CH_2Cl_2/MeOH gradient), yielding four fractions, A-D. Purification of fraction B on Sephadex LH-20 (MeOH) gave four subfractions, F2A - F2D. Fraction F2B yielded on silica gel followed by RP-18 column chromatography with H_2O/MeOH (85:15) lucknolides A (**130**) (30 mg) and B (**131**) (4 mg). Chromatography of fraction C on RP-18 silica gel with H_2O/MeOH (90:10) gave compound **132** (1.5 mg).



Lucknolide A (**130**): Colourless crystals, R_f = 0.43 ($CHCl_3$/MeOH 90:10) $[\alpha]_D^{25}$+12.5 (*c* 0.04, MeOH); IR ν_{max}^{KBr} cm^{-1}: 3368, 2911, 1708, 1414, 1320, 1218, 1016, 959, 604; CD (*c* 2.6 × 10^{-4} M, MeOH, 22 °C) $[\theta]_{213}$ -14500; **^{1}H NMR** (600 MHz, pyridine-d_5) and **^{13}C NMR** (125 MHz, pyridine-d_5) data see Table 18; (+)-ESIMS: *m/z* 251 $[M+Na]^+$, 229 $[M+H]^+$; **–** (-)-ESIMS: *m/z* 227 $[M-H]^-$; **–** (+)-HRESIMS *m/z* 251.05270 $[M + Na]^+$ (calcd. for $[C_{10}H_{12}O_6Na]^+$, 251.05263).). – **^{1}H-^{1}H COSY**, **HMBC:** see Figure 100.



Lucknolide B (**131**): White solids, R_f = 0.52 ($CHCl_3$/MeOH 90:10) $[\alpha]_D^{25}$+40.0 (*c* 0.04, MeOH); **IR** $\nu_{max}$$cm^{-1}$: 3451, 2951, 1714, 1324, 1204, 1061, 1013, 967, 603; CD (*c* 2.4 x 10^{-4} M, MeOH, 22 °C) $[\theta]_{222}$ -21000; **^{1}H NMR** (600 MHz, pyridine-d_5) and **^{13}C NMR** (125 MHz, pyridine-d_5) data see Table 18; **–** (+)-**ESIMS**: *m/z* 265 $[M+Na]^+$; **–** (+)-**HRESIMS** *m/z* 265.06838 $[M+Na]^+$ (calcd. for $C_{11}H_{14}O_6Na$, 265.06827).). –**^{1}H-^{1}H COSY**, **HMBC:** Figure 107.



2,7-Dimethyl-nonane-1,3,4,8-tetrol (**132**); colourless oil, R_f = 0.34 ($CHCl_3$/MeOH 9:1), $[\alpha]_D^{25}$ +8.8 (*c* 0.01, MeOH); ^{1}H **NMR** (600 MHz, CD_3OD) and **^{13}C NMR** (125 MHz, CD_3OD) data see Table 18; (+)-ESIMS: *m/z* 243 $[M+Na]^+$; (+)-HRESIMS *m/z* 243.15702 $[M+Na]^+$ (calcd. for $C_{11}H_{24}O_4Na$, 243.15669).

7.12 Terrestrial *Streptomyces* sp. ANK 316

The terrestrial *Streptomyces* strain ANK 316 was inoculated on agar plates with M2 medium. After 4 days of incubation at 28 °C, faint yellowish-brown mycelous colonies had grown, and the surrounding agar showed a dark yellow colour.

7.12.1 Pre-screening

The strain was cultivated as 1 liter-shaker culture at 28 °C for 5 days with 95 rpm. After 5 days, the mycelium and nutritional medium were orange-yellow coloured. After extraction with ethyl acetate and evaporation *in vacuo*, 200 mg of orange residue were obtained. TLC analysis of the extract exhibited numerous unpolar yellow bands, which turned to red by treatment with NaOH, and brown by sulphuric acid. They showed orange UV fluorescence.

Table 30: Antimicrobial activity of the crude extract produced by the strain ANK 316

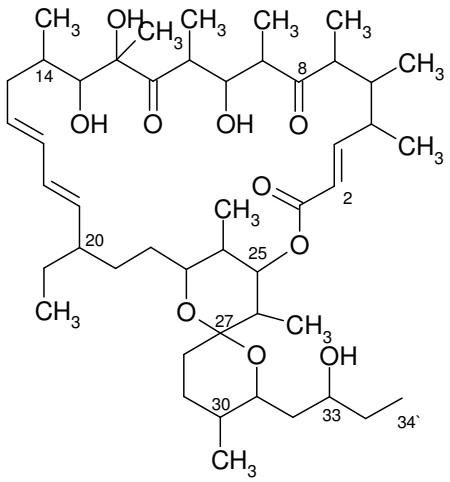
Tested Microorganism	Inhibition zone
Bacillus subtilis	16
Staphylococcus aureus	20
Streptomyces viridochromogenes (Tü57)	30
Mucor miehei (Tü284)	25
Candida albicans	20
Chlorella vulgaris	11
Chlorella sorokiniana	11
Scenedesmus subspicatus	11

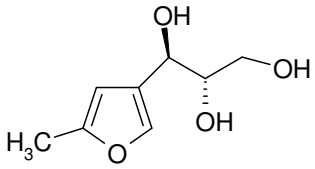
7.12.2 Fermentation workup and Isolation

The strain was cultured at 28 °C for 4 days on a rotary shaker (95 rpm) in 12 of 1 liter- rlenmeyer flasks, each containing 250 ml of M_2 medium. The pre-culture was used to inseed a 25 liter fermenter, charged with 22 liters of M_2 medium, which was kept at 28 °C for 96 hours. The fermentation broth (pale yellow, 25 liters) was filtered with the aid of celite, and the resulting mycelial cake was extracted with acetone. After removal of the acetone, the aqueous residue was extracted with ethyl acetate. The filtered broth was extracted repeatedly using ethyl acetate. The combined organic phases were evaporated to dryness under vacuum at 40 °C, giving 3.2 g of brownish-yellow crude extract. The organic extract was applied to a flash column of silica gel (30 × 600 mm), and eluted with $CHCl_3$/MeOH-gradients. As a result, four

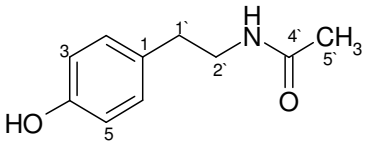
fractions were obtained by TLC monitoring. TLC analysis of fraction III (1.12 g) exhibited a middle polar, blue UV fluorescence band. By purification of the fraction III by PTLC ($CHCl_3$/7%MeOH) and Sephadex LH-20 (CH_2Cl_2/MeOH 6:4), a pale yellow solid (10 mg) of N-Acetyl-tyramine (**136**) was obtained. Pieces of agar (*ca.* 1 cm^2) from the ANK 316 culture were used to inoculate 96 of 1 liter-Erlenmeyer flasks, each containing 250 ml of M2 medium. The flasks were kept at 28 ºC for 6 days and 95 rpm. After harvesting, celite (~1 kg) was added to the culture broth (25-liter), and the mixture was homogenized by mechanical stirring for 1~3 min. By filtration using a filter press, the liquid phase was separated and extracted with ethyl acetate. The mycelial cake was extracted three times with acetone (5 liters), and the acetone was removed in *vacuo*, and the aqueous layer was extracted with ethyl acetate. For the isolation of metabolites, the combined organic layers were evaporated in *vacuo* at 40 °C to give 3.6 g of brown crude extract. The crude residue was chromatographed on Sephadex LH 20 (30 × 1000 mm, MeOH) yielding four fractions. The main fraction II containing all the yellow components was subjected to further fractionation, using PTLC (CH_2Cl_2/ 2% MeOH), to give 3 yellow components. These were further purified with Sephadex LH-20 (CH_2Cl_2/MeOH 6:4), and identified as oligomycin F (**132**) and 2-Methyl-4- (1-glyceral) furan (**135**).

Oligomycin F (**132**): white solid (22.0 mg); turned violet on spraying with anisaldehyde/sulphuric acid reagent. R_f = 0.62 (CH_2Cl_2/5 % MeOH) **– (+)-ESI MS:** *m/z* (%) = 828 $[M+Na]^+$, 40, 1679 $[2M+Na]^+$, 100 **–** HRESIMS (*m/z*) 827.527983 $[M+Na]^+$, (calcd for $C_{46}H_{76}O_{11}Na$ *m/z* 827.2433), NMR data, **^{1}H** and **^{13}C NMR**: see Table 19.





2-Methyl-4-(1-glyceral)furan (**135**): Colourless oil (14 mg); turned deep violet with anisaldehyde/sulphuric acid reagent – **^{1}H NMR** (CD_3OD, 300 MHz): δ = 7.34 (s, 1 H), 6.10 (s, 1 H), 4.54 (d, ^3J = 8.8 Hz, 1 H, 2, 6-H), 3.53 (dd ^3J = 7.6 Hz, 3.4 Hz, 1 H, 7-H), 3.48 (dd, J = 17.2 Hz, 12.9, 8-H_A), 3.38 (dd J = 15.2 Hz, 10.6, 8-H_B). – **^{13}C NMR** (CD_3OD, 125 MHz): δ = 138.9 (CH-1), 128.6 (Cq-3), 106.3 (CH-4), 153.8 (CH-5), 13.5 (CH_3-6), 76.0 (CH-7), 68.2 (CH-8), 64.3(CH_2-9). – **(+)-ESI MS:** *m/z* (%) = 195 $[M+Na]^+$, 40), 367 $[2M+Na]^+$, 100). **EI MS** (70 eV): *m/z* (%) = 172 (20), 142 (42), 82 (16) – **(+)-HRESI MS:** *m/z* 195.3112430 $[M+Na]^+$, (calcd. 195.31122 $[M+Na]^+$ for $C_8H_{12}O_4Na$).



N-Acetyl-tyramine (**136**): $C_{10}H_{13}NO_2$ (179.21), R_f = 0.38 (CH_2Cl_2/MeOH 7%) middle polar UV absorbing colourless solid (60.5 mg), which turned to violet by anisaldehyde/sulphuric acid, and pink with Ehrlich's reagent. – **^{1}H NMR** (DMSO-d_6, 300 MHz): δ = 9.11(brs, 1NH), 7.81 (s br, 1 H, OH), 6.98 (d, J = 7.8 Hz), 6.67 (d, J = 7.8 Hz), 3.18 (q, ^3J = 6.2 Hz, 2 H, 2'-CH_2), 2.58 (t, ^3J = 6.1 Hz, 2 H, 1'-CH_2), 1.72 (s, 3 H, 5'-CH_3).

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