Investigation of Secondary Metabolites of North Sea Bacteria: Fermentation, Isolation, Structure Elucidation and Bioactivity

Dissertation

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Herrn Prof. Dr. Axel Zeeck danke ich für sein stetes Interesse und viele wertvolle Diskussionen, die den Weg zu der vorliegenden Arbeit begleitet haben.

For my parent, my husband and my son

A. Theoretical Part

I. Introduction

1. Development of natural product research

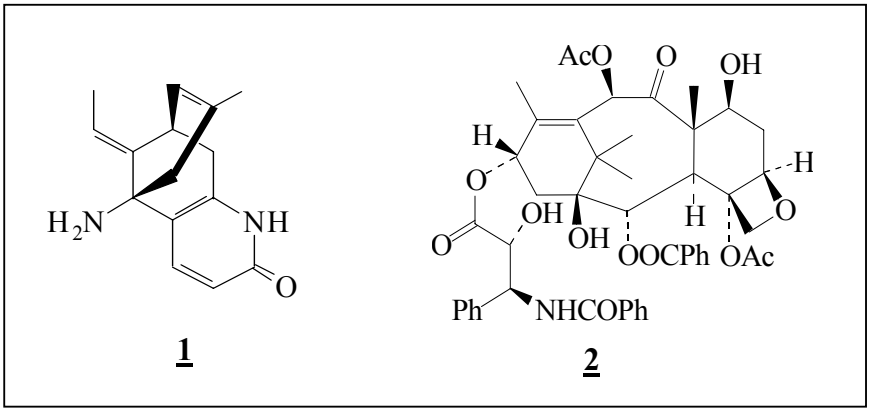
Natural products (NP) chemistry has been continuously developed during the past 4 decades. In pharmaceutical research, natural products have been playing an important role due to their chemical diversity and various bioactivities against diseases. Moreover, due to the low cost and availability, crude natural products have been an important source of medicine especially in poor countries all over the world. It has been estimated by the World Health Organization that 80% of the people on earth mainly depend on the traditional medicines for their health care.[1]

Traditional medicine based on plant sources has its roots in the thirtieth century BC when "Nei Ching", one of the earliest health anthologies, was written by the Chinese Yellow Emperor.[2] For thousands of years especially Asians, Egyptians and the Greek used parts of plants and animals, several minerals or self-cooked crude extracts, derived from mixtures of many different natural materials.

In 1806 the first pure commercial natural product morphine was isolated from plant opium, and the manufacturing of the analgetic drug was introduced in 1826 by E. Merck in Darmstadt, Germany. Till today, no alternative drug has been found to treat patients suffering from severe pain in the late stage of cancer.[3] In the last century, chemists discovered numerous bioactive compounds from traditional medicines. In China, over 10,000 compounds were identified from 11,146 kinds of plants belonging to traditional Chinese medicines.[4]

An example that demonstrates the impact of traditional Chinese medicine on modern drugs is huperzin A (**1**). This alkaloid was isolated from the plant *Huperzia serrata* by a Chinese research group in 1986.[5] It has been a traditional Chinese medicine against fever, Alzheimer and inflammation for hundreds of years. Medical studies showed that **1** possesses very strong anticholinesterase activity and markedly increases efficiency for learning and memory. Therefore it is a clinical candidate for the treatment of Alzheimer.[6] Due to the fact, that this plant is hardly available and need 8 – 10 years to develop fully (and the low production (0.01%) of **1**), scientists have synthesized more than 100 derivatives. Furthermore their structure-activity-relationship (SAR) has been studied. Bioassay data indicated that the natural (–)-**1** enantiomer has the anticholinesterase activity with an IC_{50} of 44.5 nM but synthetic (+)-**1** is only 1/33 as potent as (–)-**1**.[7] The X-ray crystal structures and kinetic study

of them separately with Torpedo californica acetylcholinesterase (TcAChE) showed us that every part of (−)-**1** bound closely with TcAChE and had a smaller dissociation constant.[8] Now a synthetic approach was developed and scaled up to provide sufficient quantities for both preclinical toxicology studies and clinical trials.



1 **2**

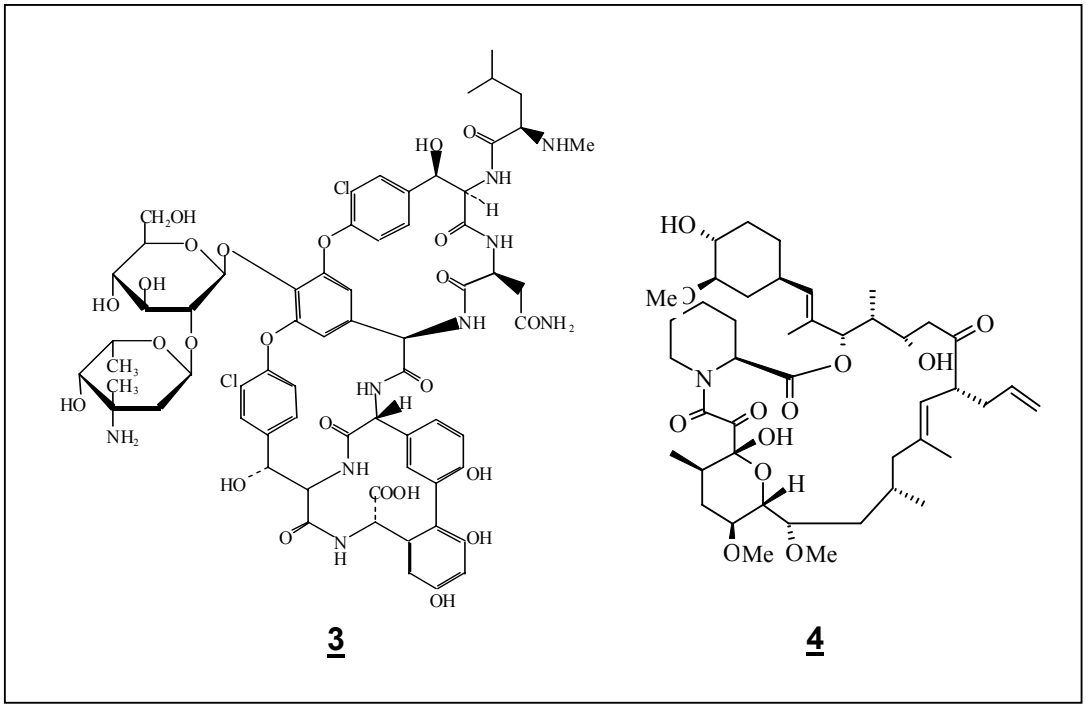
Probably the most famous example of a natural product from a plant is paclitaxel (**2**), which was isolated first in 1967 from the stem bark of the western yew[9] and used as a cytostatic in the clinical practice since 1982. **2** is a diterpene ester with unique structural features and many asymmetric centers. Unlike other antimicrotubule agents in clinical use (e.g., vincristine, colchicine) that inhibit mitotic spindle formation, **2** promotes assembly of microtubules, stabilizes them against depolymerization and inhibits cell replication. It displays considerable antileukemic and tumor inhibitory properties.[10]

The discovery of penicillin in 1928 by Alexander Fleming had heralded the era of antibiotics which ultimately showed that microorganisms are a rich source of clinically useful natural products.[11] Since then many antibiotics have been isolated from various microorganisms like actinomycetes, other bacteria, fungi, mushrooms, etc. A lot of antibiotics, such as antibacterial vancomycin (**3**), daptomycin, cephalosporin, streptomycin, antifungous amphotericin B, griseofulvin, antiviral aciclovir, antitumor actinomycin D, doxorubicin and also antidiabetic acarbose, play a very important role in medicine.[12]

Various studies have shown that the metabolites obtained from the microorganisms are structurally more diverse, and exhibit more interesting bioactivities compared to those of plant origin. Instead of monotonous alkaloids from plants, microorganisms produce many different classes of chemical structures, such as polyketides (e.g. FK506 (**4**)), terpenes, polysaccharides (e.g. acarbose), polyether, and nitrogen-containing compounds, such as indoles, peptides, pyrrols, glycopeptides (e.g. **3**), etc.

Vancomycin (**3**) is an aminoglycopeptide and has a high affinity to the cell wall building enzymes of bacteria. It is a very important antibiotic because at the present time it is a "last resort" that is used to fight against multi-drug resistant (MDR) pathogenic bacteria.

In 1983 immunosuppressant cyclosporin A facilitated the transplantation of organs.[13] After four years, the Japanese company Fujisawa isolated the novel immunosuppressant agent FK506 (**4**), which was discovered in a screening for inhibitors of interleukin-2 release in T-cells as the active principle of the culture broth of *Streptomyces tsukubaensis*.[14] Being 100-fold more potent **4** was expected to replace cyclosporin A.



3 **4**

Not all natural products serve as antibiotics. Some of them function as signal substances between microorganisms. One of the most intensively studied bacterial intercellular signal substance is the A-factor (γ-butylrolactone), which stimulates the production of streptomycin by *Streptomyces griseus* and it is also responsible for the formation of mycelium and pigments.[15]

There is no doubt that the discovery of antibiotics has revolutionized the world of medicine and saved the lives of countless patients. However, 65 years after the discovery of penicillin, humans are far from winning the battle against infectious diseases. Out of the 150 presently commercially available antibiotics there are only six different mechanisms by which antibiotics attack bacterial cells.[16] The increased frequency in clinically observed cases of antibiotic resistance and the appearance of vancomycin-resistant *Enterococci* (VRE) in 1988

cannot be ignored. In addition, many wide spread diseases such as diabetes, Alzheimer and cardiovascular diseases can not be cured till now and show an upwards tendency. New pathogens like HIV expand quickly and extend all over the world.

In the meantime the rate of rediscovery of antibiotics approaches 99.9%.[17] Searching for new bioactive natural products with new skeletons is becoming an urgent task for chemists, microbiologists, biochemists and pharmacists.

Researchers follow different approaches to get new natural products.

New compounds can be isolated from known strains. Changing culture conditions [OSMAC method (One Strain MAny Compounds)], for example, nutrients, aeration, pH, light, duration or temperature, one strain can produce different secondary metabolites.[18] For example, strain Tü 3634 (*Streptomyces griseoviridis*) produces various acyl and phenyl α-L-rhamnopyranosides in the presence of different supplements.[19] Minor components from known organisms were often overlooked because of difficulties with the isolation and structure elucidation. Nowadays they can be isolated by advanced preparative HPLC technology, and the increased sensibility of NMR and the improved technology of mass spectrometry make structure elucidation easier.

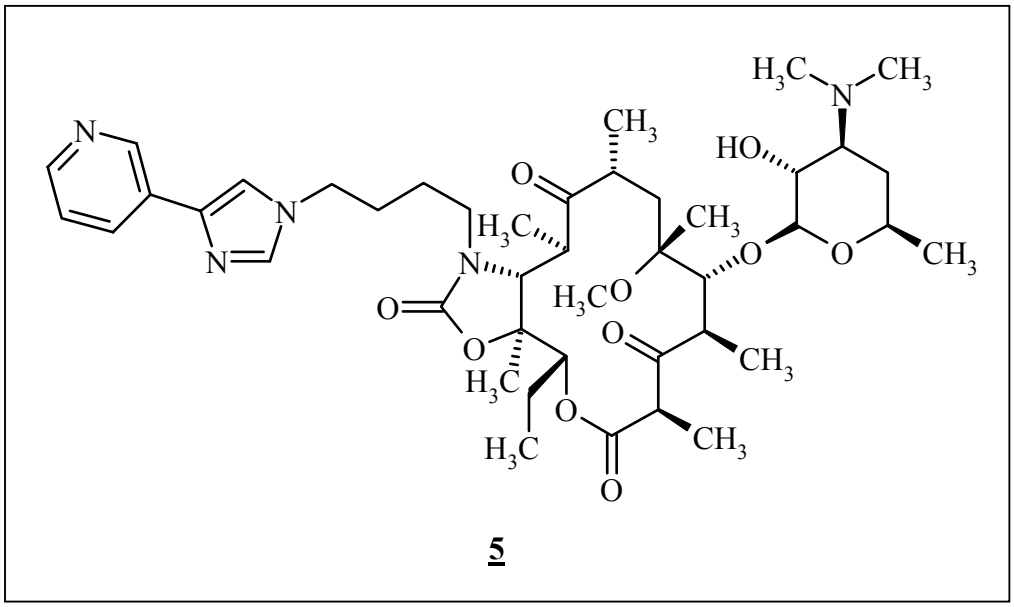
Known compounds might reveal new bioactivities when tested against new targets. Advances in instrumental analysis such as X-ray diffraction, Maldi-Tof mass spectrometry and heteronuclear three-dimensional NMR techniques facilitate the determination of the structure and provide dynamic information of many proteins associated with diseases.[20] With the development of some new drug targets based on these proteins, known compounds might be possibly found as new drugs.[21] A newly discovered metabolite, whose mode of action is specific, even if its antibiotic activities are not significant enough to take it as a therapeutic compound, will be useful as a new lead compound for chemical synthesis.

Organisms from new sources should be exploited, e.g., marine, coast, desert, the Antarctic or high saline content environments. They will open up more chances to find new metabolites because of their special survival conditions. Detailed description about marine natural products is given in the third part of this introduction.

Until now unculturable microorganisms can be studied. The number of culturable microorganisms in soil represents 1% or fewer of the total microbial community.[22] The unculturable microorganisms are gaining access to their metabolites by a direct cloning of soil DNA into a bacterial artificial chromosome (BAC) vector, without relying on traditional culturing methods.[23]

New artifical "natural products" can be produced by combinatorial biosyntheses. In recent years, genes of diverse enzymes were located and isolated. With this knowledge manufactured microorganisms can be used to generate new metabolites by realigning the extensive synthetic capacities of different species. Novel natural products will be optimized on the basis of their biological activities to yield effective chemotherapeutic and other bioactive agents.[24]

New derivatives of natural products can be obtained by biotransformation techniques. This method enables derivatisation of known compounds by esterification, reduction, oxidation, demethylation or glycosylation utilizing enzymes of living microorganisms, while the chemical route is time-consuming, requires many steps, needs chemicals and produces by-products. For example, because some desoxysugar-containing natural metabolites were found as good antibiotics (e.g. bleomycin[25]), the microbial glycosylation have become an important bioprocess in the past few years.



5

Traditional chemical synthesis or semi-synthesis can produce diverse molecules by combinatorial chemistry and rational drug design due to the advance of the studies of proteins or receptors. In order to optimize the pharmacological properties of some antibiotics, it is necessary to transform the structure of known natural products. Erythromycin is an antibacterial macrolide isolated in 1952.[26] Its semi-synthetic ketolide derivative telithromycin (**5**), which contains a 3-keto substituent in place of the cladinose sugar, is superior to the parent antibiotic with MIC values 10 – 40 times lower than the macrolides.[27] The ketolides are a new class of antibiotics possessing excellent inhibitory abilities against Gram-positive

or Gram-negative cocci like Staphylococcus, Streptococcus, and Entercoccus, which are resistant against macrolides and other antibiotics.[28] **5** is on the German market since 2001 as a new antibiotic.

The study of natural products is time-consuming and expensive. But both the profit of pharmaceutical companies and people's benefit are impressive. A study by Cragg et al. shows that over 60% of the new antibiotics and anticancer drugs approved between 1983 and 1994 were derived originally from natural products.[29] Nine of the 30 top-selling drugs are natural products.[30] It is estimated that the market of antibiotics alone yields currently US $25 billion of benefit and grows at a rate of approximately 5% per annum.[1]

2. Different screening methods for new natural products

The discovery of penicillin and the actinomycins led to the new research field "antibiotic screening". In order to discover new bioactive compounds crude extracts can be evaluated by chemical screening or by various biological or pharmacological screening approaches. The latter can focus on looking for a bioactive substance and often provides the advantages of greater sensitivity and high sample throughput like industrial High-Throughput-Screening (HTS).[31] But novel compounds, which may be active against other targets, are overlooked.

In order to overcome this problem, Zähner, Zeeck and other researchers began to investigate systematically chemical screening methods in the 1980s.[32] The chromatographic characteristics of metabolites on thin layer chromatography (TLC) plates, as well as their chemical reactivity towards staining reagents under defined reaction conditions, allows visualizing an almost complete fingerprint of a secondary metabolite pattern.[33] Using this method all metabolites are first isolated and unknown compounds are biologically tested. Of course known compounds were often isolated repeadly.

Along with the increase of the sensitivity of MS and NMR instruments and the availability of diverse natural products databases the screening approaches using HPLC-DAD, HPLC-CD, HPLC-MS HPLC-NMR-MS or GC-MS systems have been developed. This allows researchers to distinguish between known compounds and new molecules directly from crude extracts. Thus, the tedious isolation of known compounds can be avoided, and a selected isolation of constituents presenting novel or unusual spectroscopic features can be undertaken.

In recent years considerable attention has been turned on the marine organisms for natural products. But the marine bacteria show a low production rate under normal incubation conditions like terrestrial bacteria. To increase the screening efficiency for secondary

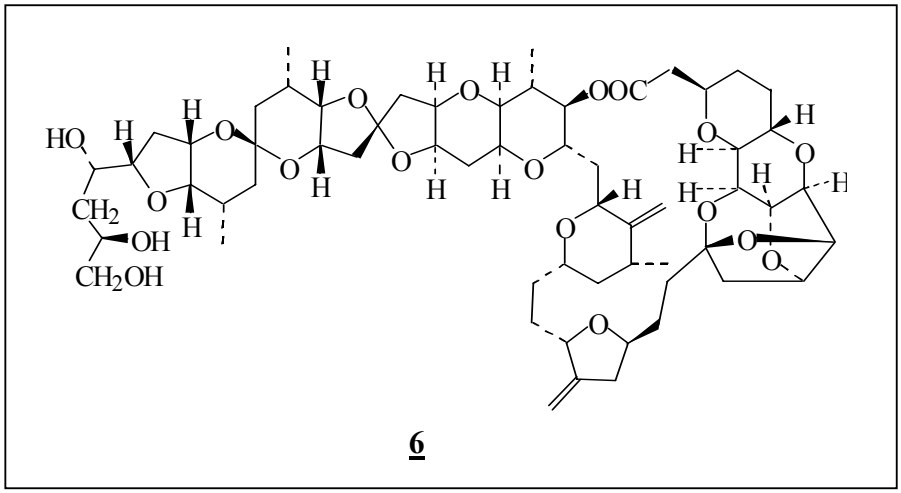
metabolites of marine bacteria microbiologists developed a PCR-based (Polymerase Chain Reaction) screening assay for genes e.g. polyketide synthases, non-ribosomal polypeptide synthetases (NRPSs), dNDP-glucose dehydratases or halogenases.[34] This method can limit the amount of strains for the next chemical screening approach; predict the efficiency of strains and the types of their metabolites. Furthermore the predicted metabolites can be produced by applying biotechnology to activate the strains.

In summery every screening method has itself advantages and disadvantages. To date there are over 159,000 natural products described, which were isolated from microorganisms or plants by biological or chemical screening.[35] This shows that the "natural products screening" is very successful.

3. New natural products from the marine source

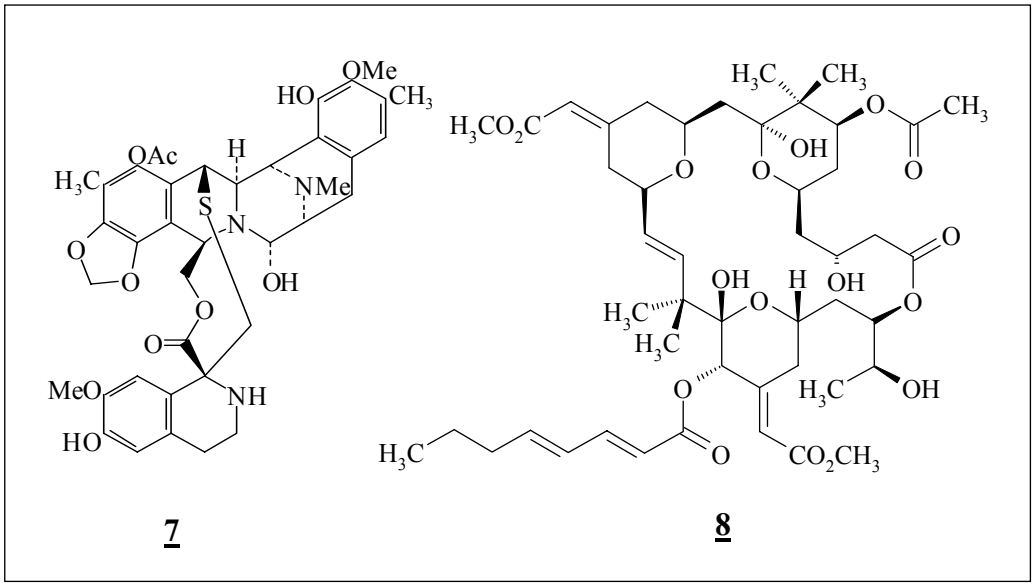
About 70% of our globe is covered by oceans. The oceans are full of living organisms and contain more flora and fauna compared to the land.[36] Marine natural products are mainly derived from marine sponges, also from corals, snails, mussels, tunicates, fungi, marine algae, bacteria etc. In the course of evolution, marine organisms have adapted excellently to the marine environment, such as high salt concentration, low temperature, high pressure and low nutrient availability. These extreme conditions require unique adaptation strategies leading to new natural products, which differ from known structures of terrestrial organisms. For example, the carbonimidic dichloride functionality (-N=CCl_2) and the sulfamate group (-OSO_2NHR) have only been found in marine natural products.[37] Moreover marine natural products comprise all chemical classes of natural products. Even though marine organisms are difficult to cultivate and normally have a low productivity, the novel compounds are very attractive as lead structures or with their potent biological activity. Furthermore these difficulties will be resolved by an increasing knowledge about them or by the application of biotechnology, combinatorial chemistry or combinatorial biosynthesis.

To resolve the problem of low concentration and limited natural sources, many research groups use different ways to get a sufficient amount of a needed natural product. Halichondrin B (**6**), a polyether macrolide, was first isolated from the sponge *Halichondria Okadai* as the most potent member of a series of related antitumor compounds.[38] But because of its low concentration in the ranges of 10^{-5} – 10^{-6} % in sponges, it is impossible to collect enough biomass. A total synthesis involves many steps with low yields.[39] Therefore the mass cultivation of the macroorganism, i.e. the sponge *Lissodendoryx* sp., has been first established for pharmaceutical purposes.[40]



6

Ecteinascidin 743 (**7**)[41] was discovered in a marine animal tunicate and contains a novel skeleton of 9 cycles based on tetrahydroisoquinoline. Its activities are orders of magnitude more potent than any other tetrahydroquinolines against B16 melanoma. The exciting aspect of **7** is its unique mode of action, constituting a new subclass of antitumor agents that could be active against resistant cell lines. Currently it is in phase II clinical trials in the United States.[42]

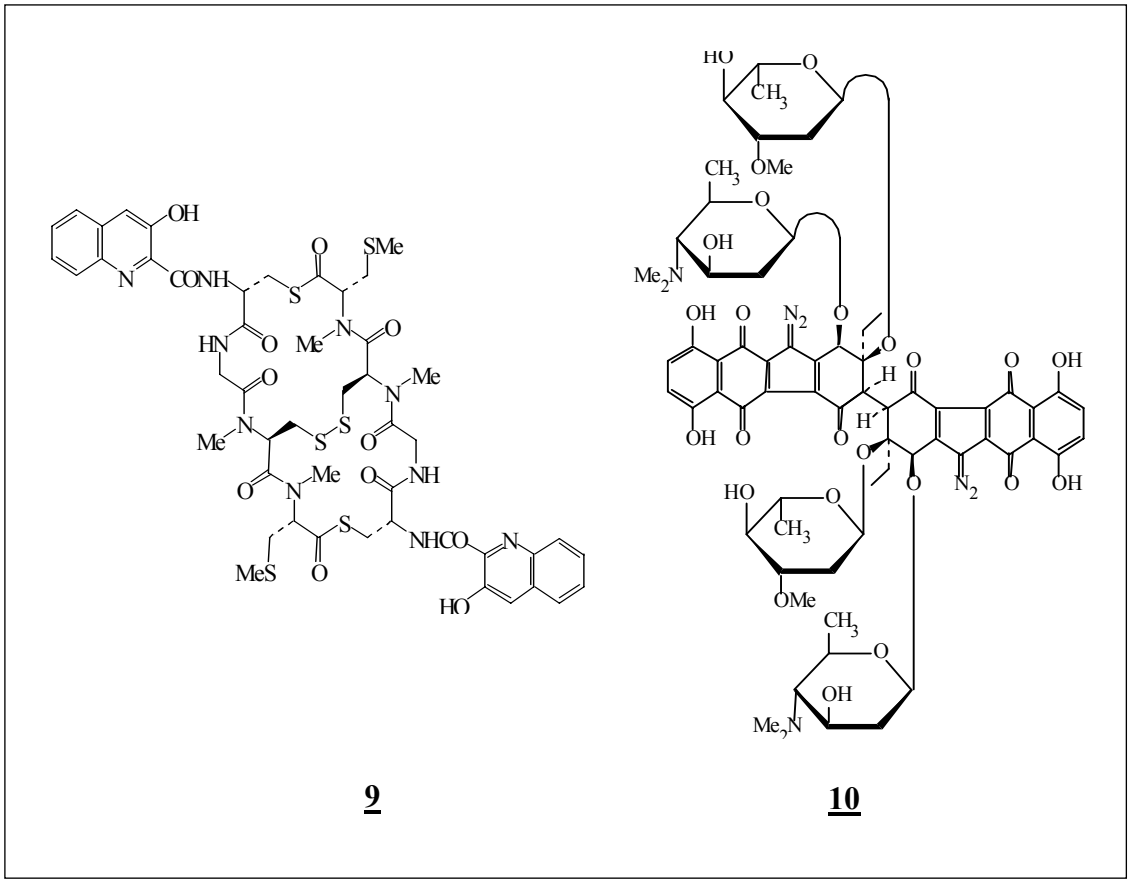


7 **8**

Symbiotic and endophytic microbes associated with terrestrial and marine macroorganisms were supposed to be responsible for metabolites in their hosts. A mounting body of evidence suggests that many marine natural product chemicals are not produced by the source invertebrate, but rather attributable to symbiotic microorganisms living within the tissues of

the invertebrate. The studies on their collection have a considerable meaning, because it is easier to get mass production from cultures of microorganisms than from macroorganisms.

Bryostatin 1 (**8**) is the first potent anticancer compound of marine origin. It is a member of the large polyketide family, which are of biomedical importance. **8** was isolated in very small amount (0.00014%) from the sponge bryozoan *Bugula neritina* in the 1970s[43] and its structure was determined by X-ray crystallography in 1982.[44] It is currently in phase II clinical trials. Supply of material is always the problem by marine natural products. Whether aquaculture could supply enough material for commercial exploitation of **8** is not known. But there is increasing evidence for an important role of the bacterial endosymbionts in the bryostatin-producing *B. neritina*.[45] This opens a way for biotechnological manipulation of the biosynthetic genes.[46] Wender et al. have prepared a simplified analog of **8** that retains the biological activity.[47] From a commercial viewpoint, the synthetic analog may represent a strong challenge to **8**.



9 **10**

Due to advances in molecular genetics, scientists fed encouraged to study marine microorganisms regardless of their low productivity. Only in 2000, 31 new natural products derived from marine bacteria.[48]

Thiocoraline (**9**), a new sulfur-containing depsipeptide with antitumor activity produced by the marine actinomycete *Micromonospora marina*, isolated from a marine soft coral collected in the Indian Ocean near the coast of Mozambique. It inhibits RNA synthesis and shows a potent antimicrobial activity against Gram-positive microorganisms and cytotoxic activities with an IC_{50} value of nM level. This antibiotic strongly inhibits the synthesis of RNA.[49] **9** is almost exclusively an exception, which enables industrial production with about 10 mg/L yield among marine drug candidates. It is now in preclinical development at PharmaMar S. A. in Spain.

Many living organisms, especially sea creatures, produce a wide range of poisons, and these are constantly being screened for possible uses as anticancer agents. Sea squirts - a group of marine invertebrates - produce a range of chemicals, some of which have pharmaceutical potential. When the researchers isolated actinomycetes from sea squirts, and grew them in fermentation vats, they discovered a previously unidentified strain that generates a substance highly active against tumor cells. They defined the bacterium as *Micromonospora lomaivitiensis* and named its active components as lomaiviticin A (**10**) and B. These compounds also act as antibiotics and show activity against *Staphylococcus aureus*, one of the virulent, antibiotic-resistant bugs that cause infection and death in surgical treatments. Its MICs of tumor cells are lower than 10 pg/mL.[50]

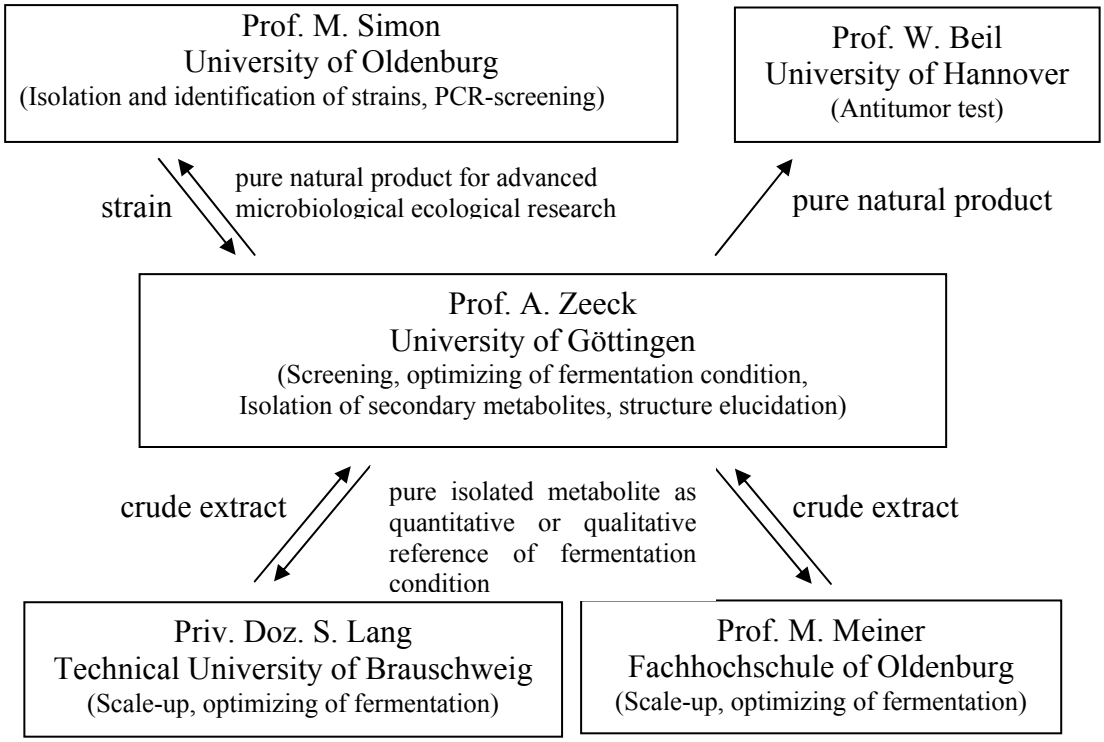
Although the research on marine natural products has going on for only about 40 years, over 5,000 compounds have been published.[51] Many of them possess unique functional groups or skeletons and also potent biological activities. It is noteworthy that presently published new compounds are almost exclusively found in marine organisms. Some of them got already into preclinical phase and will lead to pharmaceutical products.[52] Exploring marine organisms will be one of the main focuses in the next years and should be highly successful.

4. Project description

As the initiator of exploiting for bacteria of the German North Sea in Germany, Prof. Dr. A. Zeeck has coordinated the project "Marine biotechnology" in Lower Saxony, which was supported by the VW-foundation and focused on microorganisms and their secondary metabolites of the Wadden Sea as part of the German North Sea. The North Sea is an unexplored marine and special ecological area due to the tide. Therefore its microbiological and chemical features should differ from other marine environments. Based on this idea, 19 research groups (from universities of Lower Saxony) set up an interdisciplinary network. It was aimed at optimizing the efficiency of research by cooperation between natural product chemists, synthetic chemists, microbiologists, biologists, pharmacists.

The present work is one part of this project. The relevant theme is named "Secondary metabolites in new microorganisms from the North Sea". The internal cooperation connected with this present work is described in scheme 1.

All strains investigated in the present work were isolated from the North Sea by Dr. T. Brinkhoff, Dr. HP. Grossart, Dipl.-Biol. H. Steven and Dipl.-Biol. T. Martens (group of Prof. M. Simon). The fermentations were finished by Dipl.-Biol. T. Heidorn (group of Prof. M. Meine), Priv.-Doz. S. Lang and in the group of Prof. A. Zeeck. The antitumor activity was tested by Prof. W. Beil. The activities against Microalgae, Nematodes, and brine shrimps were carried out by Prof. H. Laatsch.



Scheme 1 Cooperation of the relevant groups in this work

5. Scope and aims of the present study

The main goal of the present investigation was the isolation and identification of the new and preferably biologically active secondary metabolites from marine bacteria. This work can be divided into five main parts.

a) Biological and chemical screening of bacterial metabolites:

The biological or genetically interesting strains have to be screened by biological and chemical screening methods. The biological screening is mainly based on agar diffusion assays for antibacterial, antifungal and antialgal activity. The chemical screening mainly uses TLC stained with different staining reagents or HPLC in order to estimate qualitative and semi-quantitative control of metabolites.

b) Isolation of metabolites:

In order to get a sufficient amount of pure metabolites selected strains should be cultured on a large-scale after optimizing culture conditions. The metabolites extracted from the fermentation broth have to be separated using different chromatographic methods. To identify bioactive compounds, bioautography and bio-assay guided isolation had to be attempted.

c) Elucidation of the molecular structures of metabolites:

The molecular structures of isolated metabolites have to be elucidated by spectroscopic methods such as NMR, MS, UV, IR and X-ray crystal analysis if necessary.

d) Biological evaluation of isolated pure compounds:

The isolated new metabolites should be evaluated in different bio-assays. This includes the same assays as used for the crude extracts and further specific assays, such as antinematodic, antisalinecrabs, antitumor, ion channel and herbicide activities, performed in cooperation with other groups and industries.

e) Activating of NRPS in bacteria and identification of peptides in crude extract:

Bacteria express their genes only under suitable conditions. NRPS strains produce peptides only under special conditions. This work should try to activate the NRPS by changing the culture medium or fermentation conditions, and to identify peptides in crude extracts. Through this work the feasibility of PCR-based screening should be verified and a suitable method for identifying peptides in crude extracts should be set up.

II Chemical and biological screening of the North Sea strains

1. Origin of the strains

This work focused on the screening of strains of the North Sea bacteria possessing antimicroalgal, antibacterial and antifungal activities or containing novel metabolites in the crude extracts. In total 38 bacterial strains were studied, which were isolated from different samples collected by Dr. T. Brinkhoff (T-series), Dr. H. P. Grossart (HP-series) and Dipl.-Biol. H. Steven (RK-series and H-series) from water sample derived from the German North Sea, where the average temperature of water is 9.6 °C, the pH value is 7.9, the saturation of oxygen is 94%, and the salinity is 3.4%. The bacteria were isolated from a sample of water (free swimming bacteria) and the surface of algae (aggregate-associated bacteria). The MPN technique[53] was used for the isolation of all strains. The 16S rDNA of part of the strains were sequenced and compared to the database *Blast*. They mainly consist of α –, β –, γ – proteobacteria, Gram-positive bacteria and actinomycetes. A part of the strains were not characterized.

2. Cultivation of the strains

In contrast to terrestrial strains, the information regarding cultivation and isolation of metabolites from marine bacteria are very poor and most of marine bacteria are known to yield only few metabolites. Normally terrestrial streptomycetes need three days to be cultivated at 28 °C and its biomass is much higher than that of marine bacteria. Marine bacteria were cultivated under different conditions. For example, marine bacterium *Flavobacterium*, the producer of flavocristamide A and B, was grown statically in Zobell broth [Pepton 0.1%, yeast extract 0.02% in 90% sea water, pH 7.6] for 10 days at 25 °C.[54] One marine streptomycete, the producer of anthranilamides, was isolated on casein peptone medium containing 50% natural sea water with incubation at 18 °C, but its optimum of growing is near 30 °C for three days.[55] The marine strain *Streptomyces platensis* TP-A0598 produces the antibiotics TPU-0037-A, B, C, D at 32 °C for 10 days with the medium consisting of glycerol 4%, NZ-case 0.5%, pharmamedia 2%, CaCO3 0.5% and HP20 1%.[56] Thus the studied strains should not be limited by using unified culture conditions. The focus was set on the variation of culture temperature and duration. According to the observation of strain-growth on the agar plates, all strains were separated into four groups, which were cultivated separately under different conditions. Among them, T1, T4, HP18, HP20a, HP21, HP34, HP38 were cultivated at 20 °C for six days; HP9w, HP35, HP37, HP42, HP47 were

cultivated at 28 °C for four days; RK2207, RK1099, RK342, HP4, HP5, HP8, HP16, HP17, HP22, HP23, HP27, HP30, HP44 were cultivated at 28 °C for six days; HP11, HP12, HP29, HP42 were cultivated at 15 °C for 14 days. MB2216 is the special cultivation medium for marine microorganisms, which is offered commercially by DIFCO. It is composed of different amino acids and inorganic salts which imitates the composition of sea water. Thus it could be the common cultivation medium of overall balance. In this work all strains were cultivated using unified MB cultivation medium.

Microorganisms communicate by releasing compounds. Some observations were made by co-cultivation of two strains. In the Ph. D. thesis of Dr. R. Höfs[57] several mixed cultivations were attempted, but no new metabolites were found although the interaction of the two strains was observed on agar plates. It can be supposed that the yield of signal compounds was so low that they were undetectable in crude extracts.

In order to understand the interaction between the strains living in the same environment, an interaction screening was carried out in cooperation with Dr. H. P. Grossart. Fig. 1 illustrates this method. One pre-cultivated strain was first spread out on an agar plate with MB medium and other pre-cultivated strains were dropped on it. After cultivation it was observed that some of them showed inhibitory activity of other bacterial growth and some of them showed the induced ability of other strains. All HP-strains were reciprocally tested against other HP-strains.

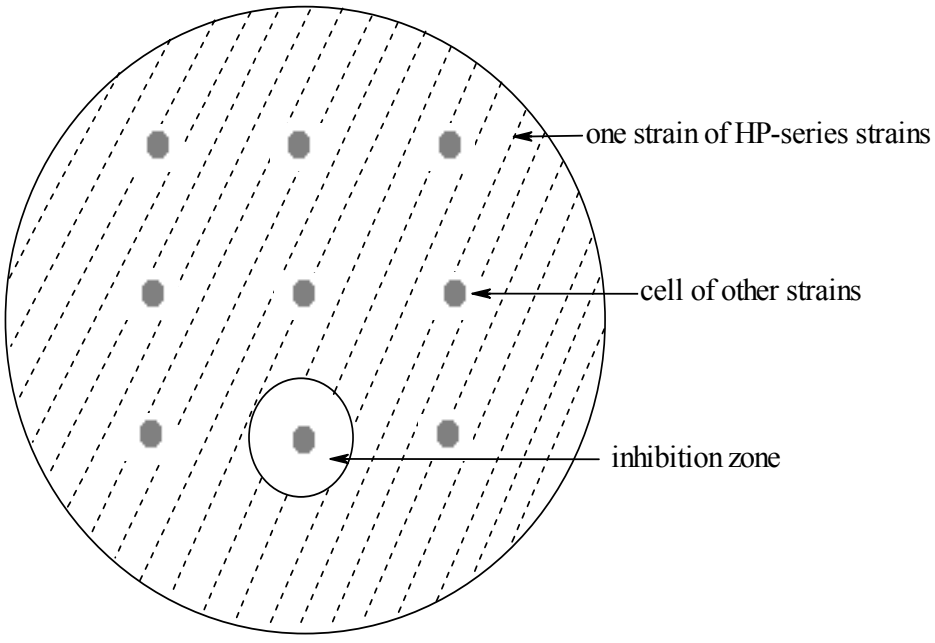


Fig. 1 Interaction screening

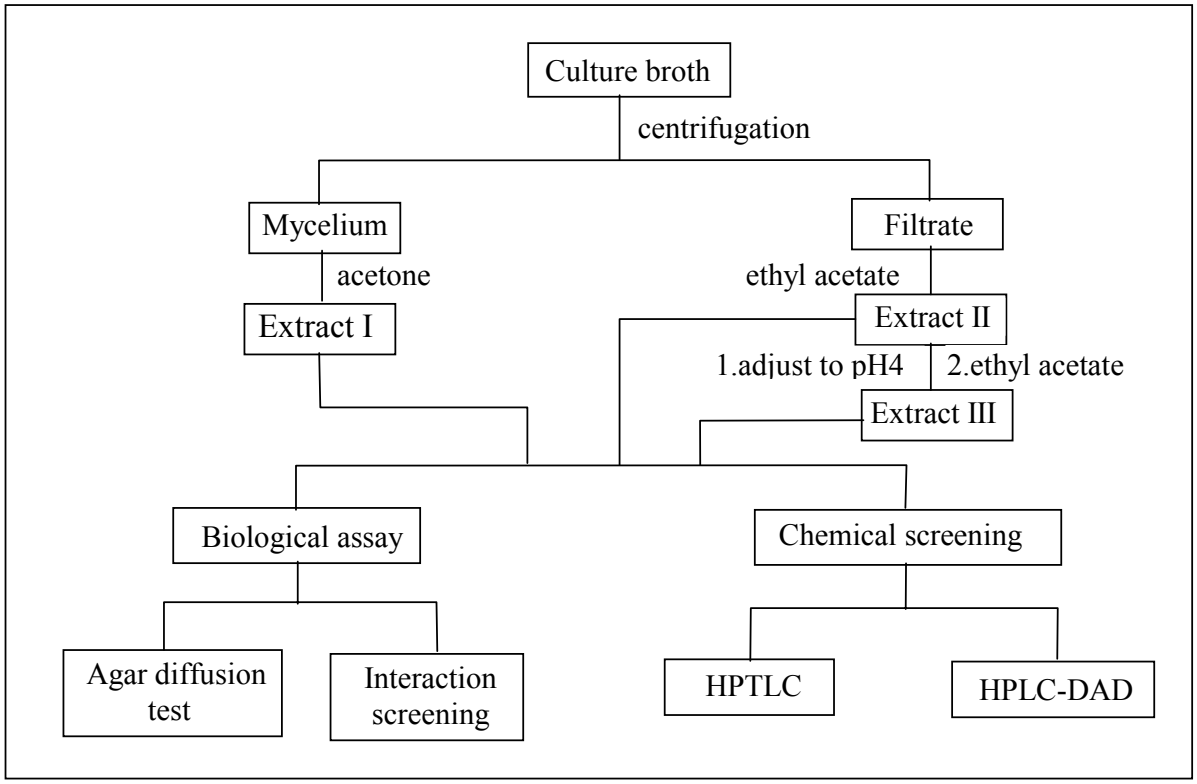
Based on results of this interaction screening, an attempt to obtain new antibiotics by co-cultivation of several strain pairs was carried out. Six strains were selected for three mixed cultivations, i.e. HP12 and HP29 were cultivated together at 15 °C for 14 days; HP14 and HP15 were cultivated together at 28 °C for eight days; HP26 and HP33 were cultivated together at 15 °C for 28 days. It was expected that one strain would produce new biologically active metabolites in the presence of its hostile strain. However, no special interesting spots on TLC and peaks in HPLC chromatogram could be detected. This is probably due to the common reason that marine bacteria produce only few metabolites in low yields.

In order to increase the productivity of these organisms various techniques have been carried out. One of the methods was the addition of resin like XAD into the culture broth, which adsorbs metabolites and minimizes the toxic effects of the produced metabolites. If there is a feedback regulation, the microorganisms will produce more metabolites to keep their concentration in the culture broth.[58] In the screening process the broths of two strains were added XAD-2 into MB medium.

3 Work-up of the culture broth

According to literature and our own experience it can be stated that many metabolites possess weak acidic properties. Therefore they are present in the culture filtrate as salts, which are soluble in water and not soluble in organic solvents. Hence a modified work-up procedure was used, i.e. extraction of culture filtrate at acidic conditions (pH 3 – 4), where the salt is converted into the acid and thus can be extracted by organic solvents. A flow diagram of the screening procedure is shown in Scheme 2.

In order to study their metabolite pattern in detail all strains were screened by the chemical screening approach with thin-layer-chromatography (TLC) and the advanced physicochemical screening approach (HPLC-DAD). An additional biological screening procedure was carried out on the strains of RK- and T-series using agar diffusion assay with Gram-positive (*B. subtilis*, *S. aureus*), Gram-negative bacteria (*E. coli*) and fungi (*C. albicans*). The interaction screening was carried out among the strains of HP-series. This technique has been described in an earlier part of this section.



Scheme 2 Screening procedure of North Sea bacteria

4. Results of the chemical and biological screening

Most of the 38 strains showed good growth in MB at 15 °C, 20 °C, 23 °C and 28 °C except for the strain RK342. The higher the temperature was, the more quickly the bacteria grew between 15 °C and 28 °C. But their cell densities were generally lower than those of streptomycetes. Furthermore TLC and HPLC analyses showed that the number and yield of metabolites is very low.

In the Kromasystem (HPLC-DAD program) an UV spectra database, obtained from identified metabolites and single peaks of chromatography of crude extracts, was set up and several indole derivatives, phenylacetic acid and anthranilic acid in crude extracts were identified by comparing their UV spectra with the self-set UV spectra library. For example, 3-hydroxyacetyl indole was found in crude extracts of RK2207 and HP9w via this method. Fig. 2 shows the result. HPLC-DAD screening of HP44 revealed one UV spectrum, which was identical with that of one component of RK377 (see Fig. 3). After the metabolites of RK377 were isolated and characterized in detail, it was clear that the HP44 also produced the new natural product 3-(4´-hydroxyphenyl)-4-phenylpyrrole-2,5-dicarboxylic acid (**35**) without further investigation.

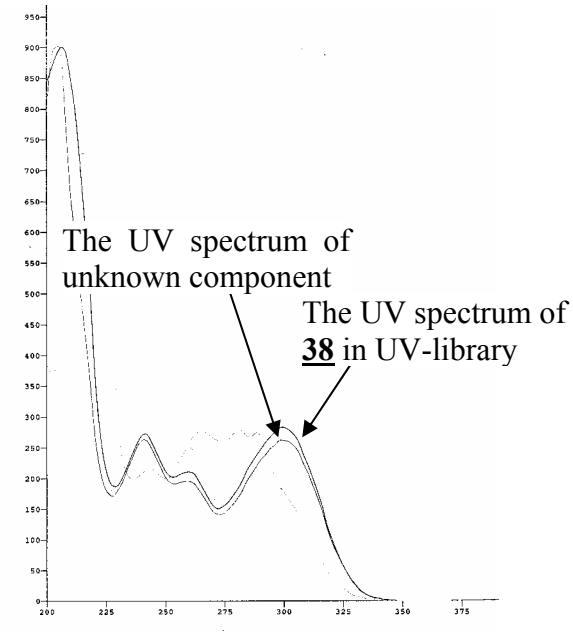


Fig. 2 The UV spectrum of one component in the filtrate extract (pH 8.5) from strain RK2207 was compared with an UV library. It was identical with that of 3-hydroxyacetylindole (**38**). The other two spectra drawn by light-line belong to the next best hits.

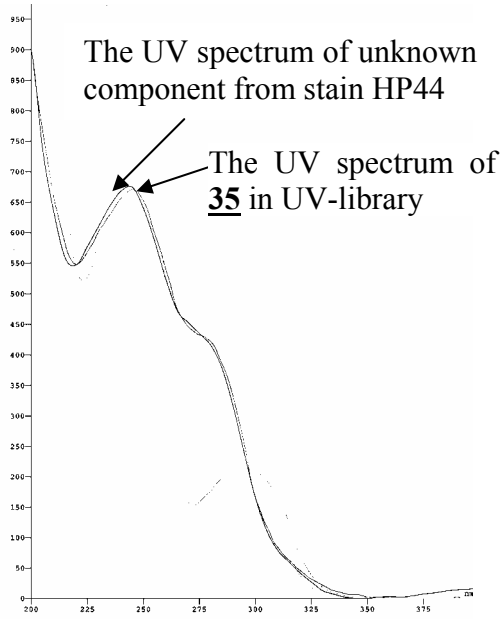


Fig. 3 The UV spectrum of one component in the filtrate extract (pH 8.5) from strain HP44 was compared with an UV library. It was identical with that of the elucidated compound (**35**) produced by strain RK377. The other two spectra drawn by light-line belong to the next best hits.

Because there were only a limited number of UV spectra to compare, many HPLC peaks could not be assigned to corresponding substances via their UV spectra. Therefore a complete UV spectra library would be very useful in the screening of natural products. It is known, that in some pharmaceutical companies HPLC-DAD screening was performed by comparison of retention times and UV spectra of screened components with that of known natural products in libraries. This used to be an efficient way of finding new natural products.

Additionally most of the compounds are polar according to HPLC chromatograms (peaks emerged at the range of 100% - 70% water) and TLC (R_f = 0 - 0.5 with $CHCl_3$/methanol = 9 : 1). They are easily to oversee because normally most nutrients are in this region. Thus biological screening is very necessary for marine bacteria.

In the biological screening the extracts of T5 and RK377 were active against *B. subtilis*. The strains of HP-series 5, 8, 9w, 14, 18, 20a, 21, 26, 29, 34, 38, and 42 had different inhibitory activities against other strains, which were concluded from interaction screening.

The pH, color and foam of all culture broths were observed or measured after cultivation. The pH value is an important parameter as an indicator for biosynthetic activities. Most of the

culture pH values changed from 7.6 (at the beginning) to about 8.6 (at the end of cultivation) in cases of good growth.

The two strains HP4 and HP30, supplemented with XAD-2, showed the production of more metabolites in TLC than other strains. This method could be used to enhance the amount of metabolites and particularly in order to get more polar metabolites. Other XAD types like XAD-8 or XAD-16, which adsorb more polar compounds, should be tried out.

III. Biosynthesis efficiency and metabolites of strain T5

1. Isolation and identification of the strain T5

Strain T5 was isolated from a water sample collected from the surface near Neuharlingersiel in the German North Sea. It was identified as *Ruegeria* sp. by 16S-rRNA analysis and comparison with the library *BLAST*. It is 98% relevant to *Rossbacter gallaeciensis* and belongs to the alpha-subgroup of proteobacteria.

2. Optimization of culture conditions

From the screening it was known that marine bacteria grow under very different conditions. Thus it is necessary to optimize the cultivating condition of each strain individually. T5 was first cultivated in Erlenmeyer flasks on a rotary shaker at 20 °C, 23 °C, and 28 °C for 6, 12 hours and 1 – 7 days, respectively. The inhibition diameters of the culture broth against *B. subtilis* were used to monitor the production of bioactive substances. At 20 °C and 23 °C the production reached a maximum within three days and after that the inhibition was lost; maybe the active substance was changed to an inactive form. At 28 °C the production reached a maximum after six hours.

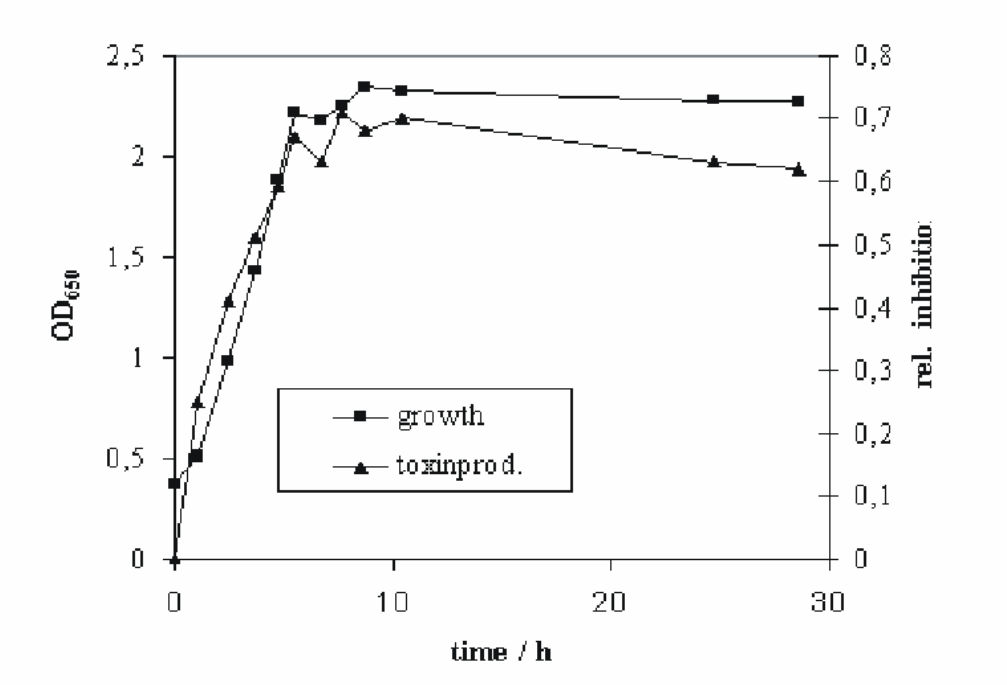


Fig. 4 Relationship of T5's growth at 28 °C and production of bioactive compounds

A detailed study of the culture condition was carried out by Dipl.-Biol. T. Heidorn in Willheimshaven. The temperature was varied between 25 °C – 35 °C and the pH value was varied between 6.0 – 8.5. Since the organism was isolated from a marine environment, the influence of the salinity was studied in the range of 0 – 65%. The growth of T5 was verified by measuring the optical density of the culture broth at 650 nm (OD_{650}). It could be determined that the optimal temperature was about 30 °C, the optimal pH value was about pH 7.5 and the optimal salinity was about 32 g/L. The pH-optimum was nearby the naturally occurring pH of 7.6 and also the optimal salinity corresponded to the naturally occurring values. However, the optimal growth temperature of about 30 °C is not usual in the Wadden Sea at the coastline of North Germany.

In order to determine the optimal duration of the fermentation, the correlation between cell growth and the production of the active substance was observed. The production of the active substance was measured by the relative inhibition, which is the diameter ratio of the inhibition zones of the culture broth and penicillin G on agar plates inoculated with *B. subtilis*. Fig. 4 shows that the production rate of the active substance has nearly the same slope as the growth of the bacterium in the log-phase, but decreases in the stationary phase. This differs from the usual behaviour of actinomycetes, where metabolites are produced at the end of the log phase. In the case of T5 it could be shown the pure bioactive compound inhibits its growth, pointing to a feedback effect between the metabolite and the producer.

3. Assignment of the bioactive compound

As a first step in the process of isolation it was necessary to identify the bioactive substance via TLC and HPLC. To achieve this, an efficient technique was used, named “bioautography”. The developed TLC-plates (carrier material: aluminium) were cut into stripes and put on the test agar inoculated with *B. subtilis*. The agar plate was stored at 35 °C. After 24 hours the inhibition zone was measured. The corresponding R_f value was compared to a parallel developed TLC plate, which was marked under UV light or stained by different staining reagents. Fig. 5 shows the method of bioautography. It is very useful for the assignment of bioactive components in crude extracts.

In order to identify the bioactive substance by HPLC the spot based on bioautography was cut out of the TLC plate and dissolved in methanol. After centrifugation the sample was examined by HPLC. The peak representing the bioactive compound together with its characteristic UV spectrum was used to simplify the process of isolation (see Fig. 6).

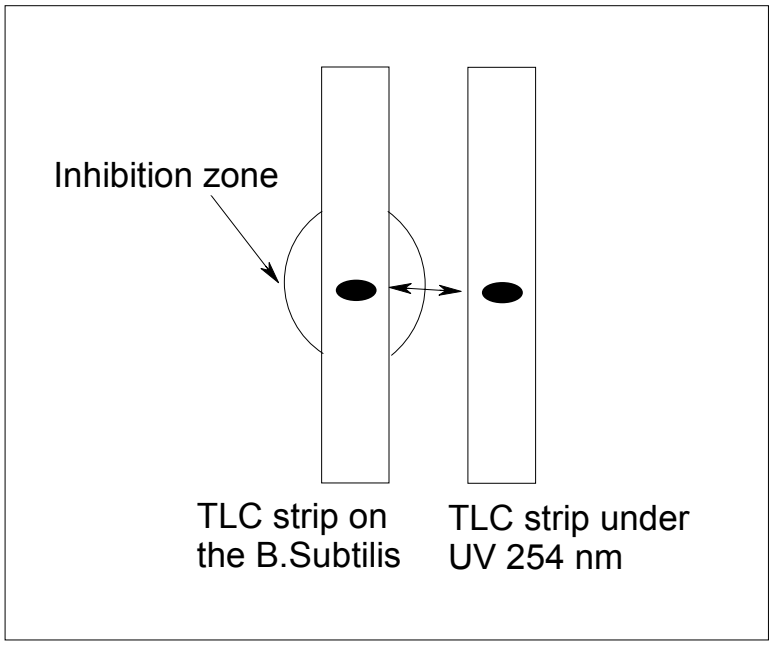


Fig. 5 Method of bioautography

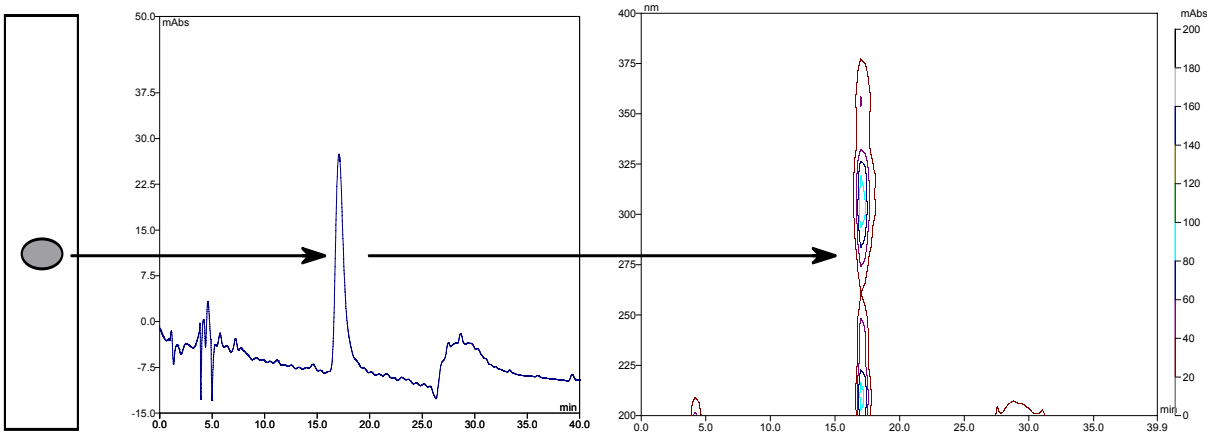


Fig. 6 The spot on TLC was assigned to the peak in HPLC and its UV spectrum

Fig. 7 shows the HPLC chromatogram of crude extract of an acidified T5 culture filtrate, where almost no peak of the bioactive substance could be observed because of its low concentration. But after the first isolation step the peak in the concentrated fraction emerges clearly (Fig. 8).

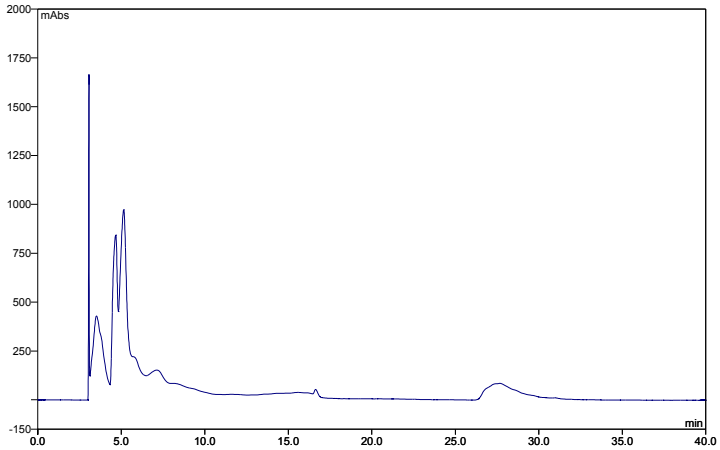


Fig. 7 HPLC-chromatogram of the crude extract of T5 culture broth at pH = 4.0

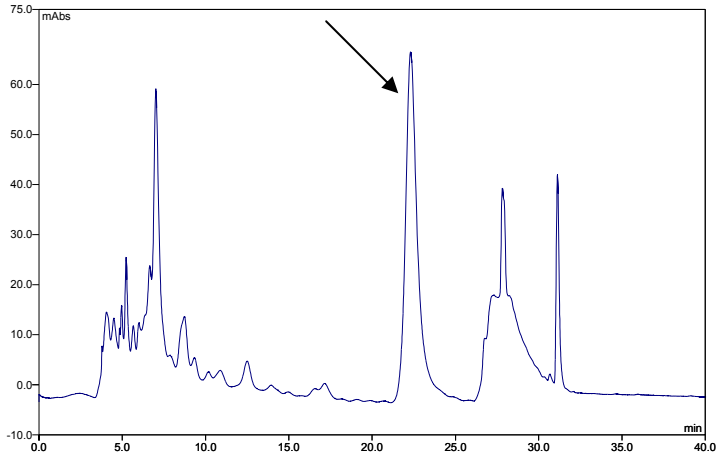
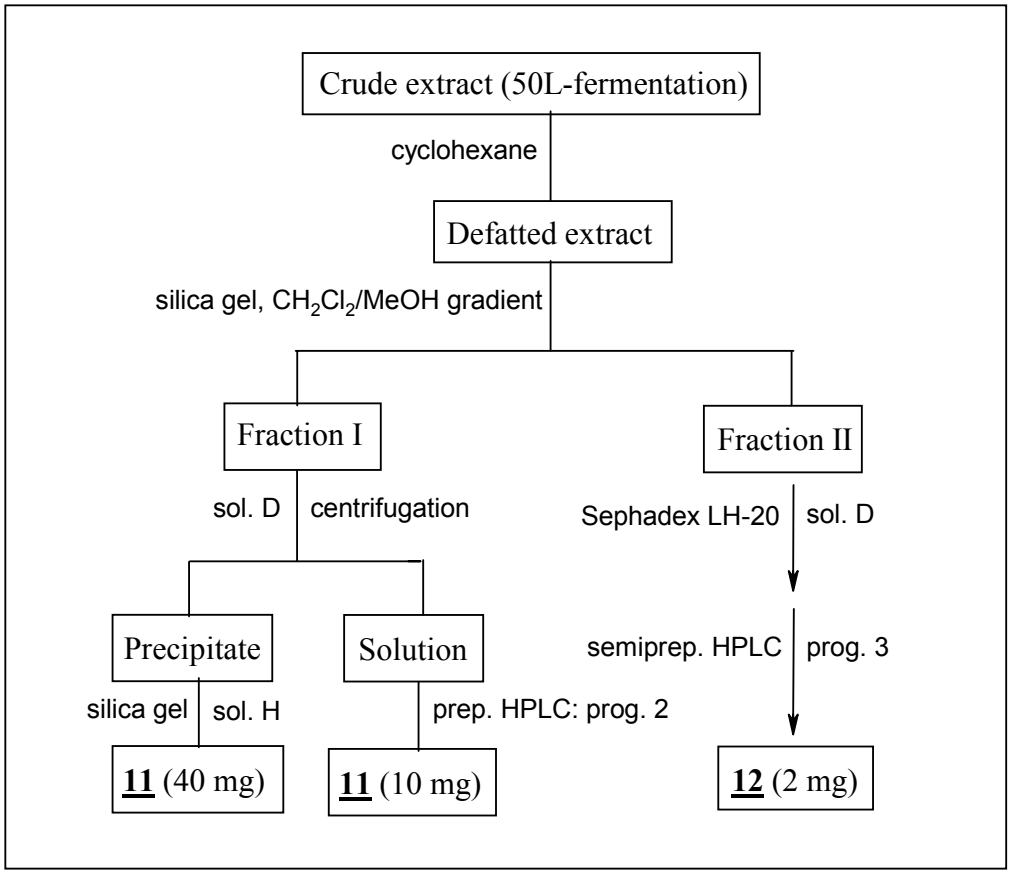


Fig. 8 HPLC-chromatogram of a concentrated fraction of T5 culture extract

4. Isolation of metabolites

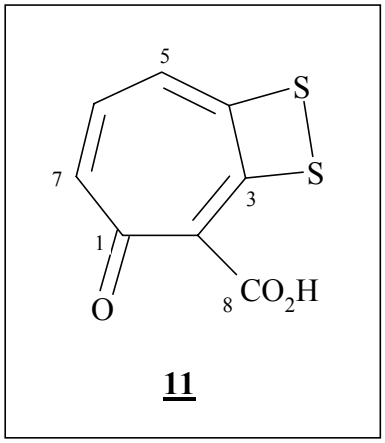
A 50 L-fermentation of T5 was carried out with MB medium for 12.5 hours. The filtrate was adjusted to pH 3 with HCl and extracted with ethyl acetate. This acidification process is very important because the bioactive compound exists as anion in basic or neutral medium and thus cannot be extracted with organic solvents. From the crude extract **11** as main component and **12** as minor component were obtained via column chromatography on silica gel and Sephadex LH-20 according to Scheme 3. Because of **11** being difficult to dissolve in normal solvents when concentrated, further purification could not easily be carried out. Thus two portions were obtained by adding 5 mL of methanol to fraction I and centrifugation. The solution was purified by preparative HPLC. The precipitate containing mostly **11** was applied to a silica gel column and eluted with 5 % formic acid-containing chloroform. The total yield

of **11** from the two portions was 1 mg/L. If the entire concentrated fraction was subjected to a silica gel column, it was difficult to get pure **11** due to the disturbance by other components and when isolation was done by preparative HPLC, it was difficult to get a completely soluble sample.



Scheme 3 Isolation of **11** and **12**

5. Structure elucidation of tropodithietic acid (11)



11

Compound **11**, named tropodithietic acid, was obtained as orange solid that decomposed at 222 ~ 225 °C but didn't show a distinct melting point until 300 °C. **11** was soluble in dimethylsulfoxide, tetrahydrofuran and chloroform; scarcely soluble in acetone, ethyl acetate and methanol. It was insoluble in ethyl ether and water. It changed to red-brown color when stained with $PdCl_2$, which indicated the presence of sulfur. It revealed a yellow color when stained with anisaldehyde reagent but no color-reaction with Ehrlich and orcin reagents could be observed.

The molecular formula $C_8H_4O_3S_2$ was established by high-resolution EI-MS. In the EI-MS spectrum the fragment at m/z = 168 $[M\text{-}CO_2]^+$ was determined as base peak, which suggested that the molecule contains the carboxylic group. The IR spectrum (see Fig. 9) showed the absorption of a conjugated carbonyl group at 1632 cm^{-1} and that of hydroxyl group at 3443 cm^{-1}.

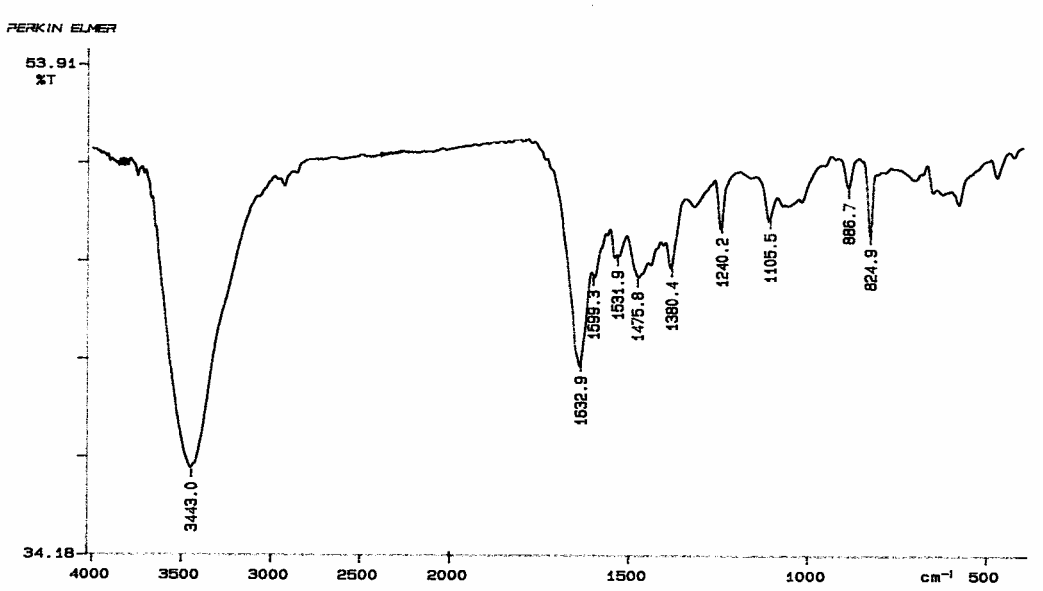


Fig. 9 IR spectrum of **11**

Its UV spectrum (Fig. 10) exhibited broad strong UV absorption bands at 303 nm, a shoulder at 356 nm and a weak broad absorption around 450 nm.

Its ^{1}H-NMR spectrum showed one proton present as an intramolecular hydrogen bridge at downfield (δ= 16.55 ppm) and three aromatic protons with an ABC-spin pattern. Their assignments and coupling constants could be revealed with the help of a computer program. Fig. 11 shows the consistency of calculated and experimental spectrum of this ABC-spin system.

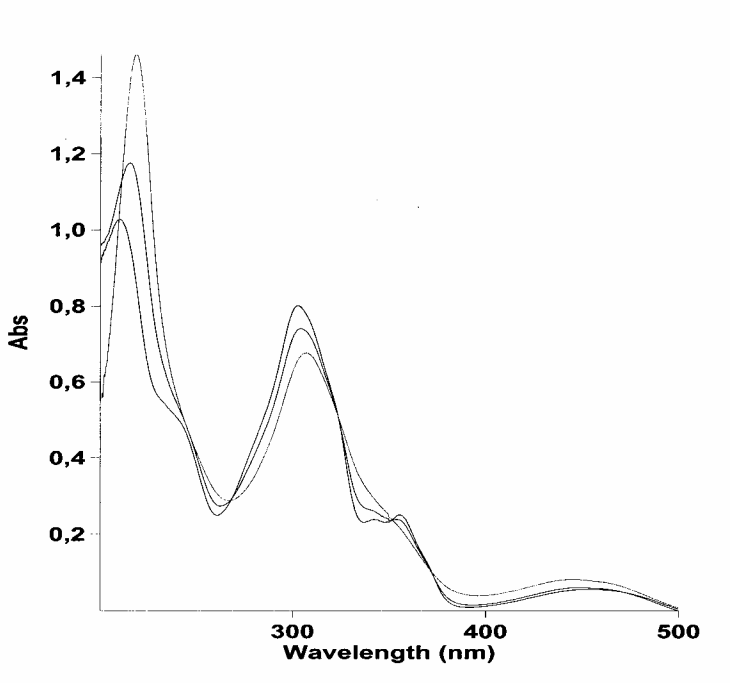


Fig. 10 UV spectra of **11** in different mediums

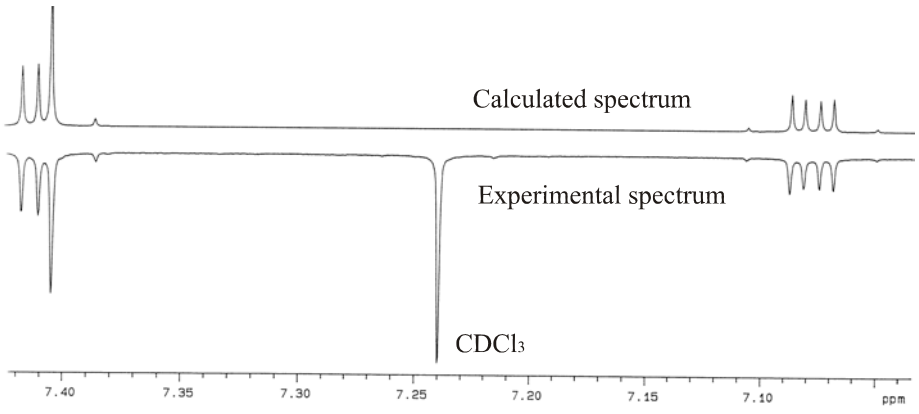


Fig. 11 Calculated and experimental ^{1}H-NMR spectrum of ABC-spin pattern of **11**

The ^{13}C-NMR spectra of **11** in dimethylsulfoxid and chloroform displayed eight carbon signals with one α, β – unsaturated enolic carbon, one carboxylic carbon, six aromatic carbons including two carbons connected with heteroatoms.

Due to the overlapping of two aromatic protons it was difficult to differentiate the correlation of protons and carbons in the 2D-spectra (HSQC and HMBC). To get a better resolution of signals ^{1}H-NMR experiments were carried out in different deuterated solvents such as DMSO, THF, methanol and benzene. In benzene, compound **11** displayed three clearly resolved proton signals (see Fig. 12).

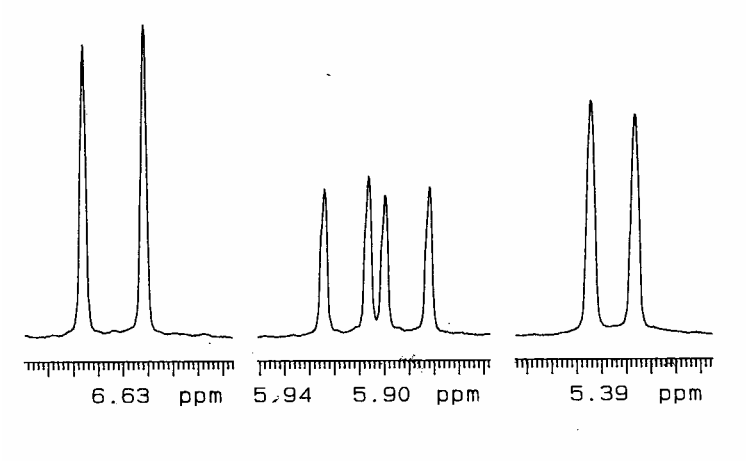


Fig. 12 ^{1}H-NMR of **11** (500 MHz) in d_6-benzene

Fig. 13 shows the spectrum of the $^{1}H - ^{13}C$ coupling projection of **11** in d_6-benzene, where almost all couplings (^{1}J, ^{2}J, ^{3}J, ^{4}J) could be observed. From this an entire structural correlation could be revealed. Fig. 14 shows the long-range coupling in the structure of **11**.

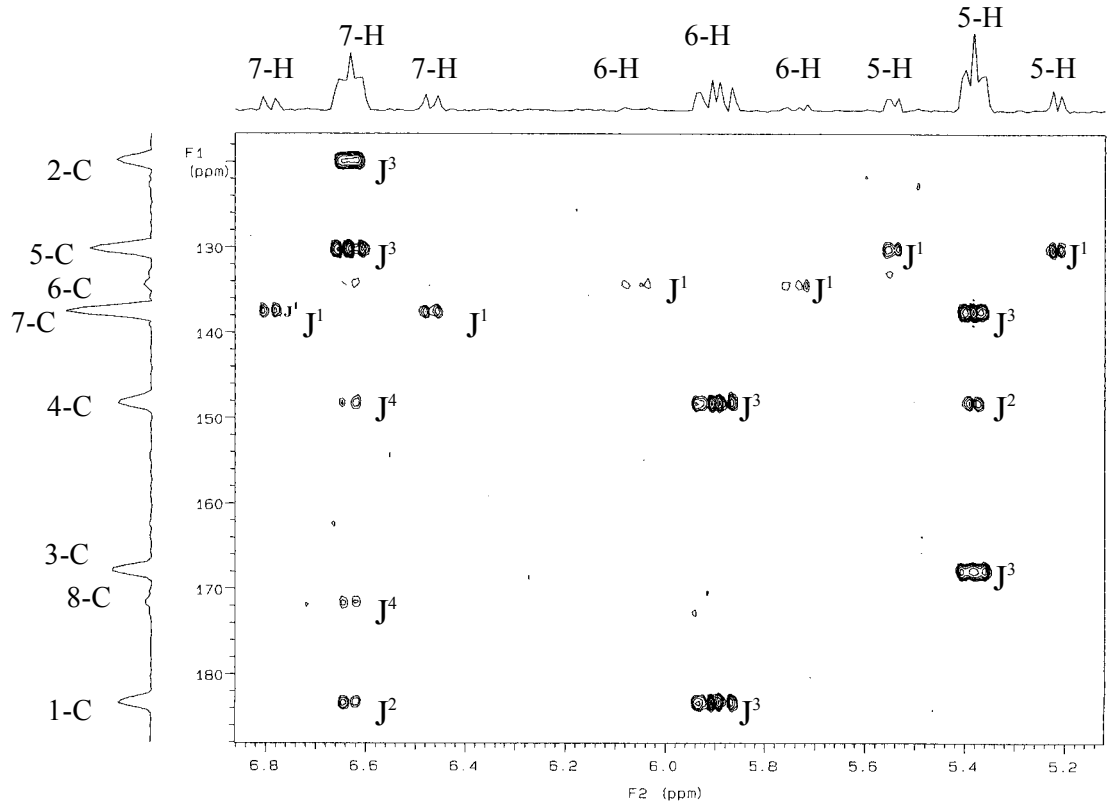


Fig. 13 Projection of C-H coupling of **11** in d_6-benzene

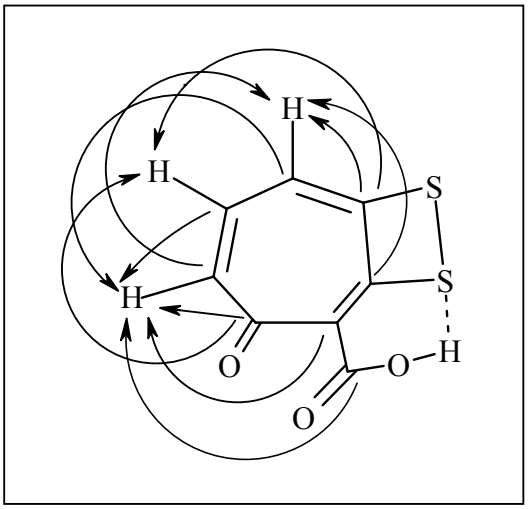
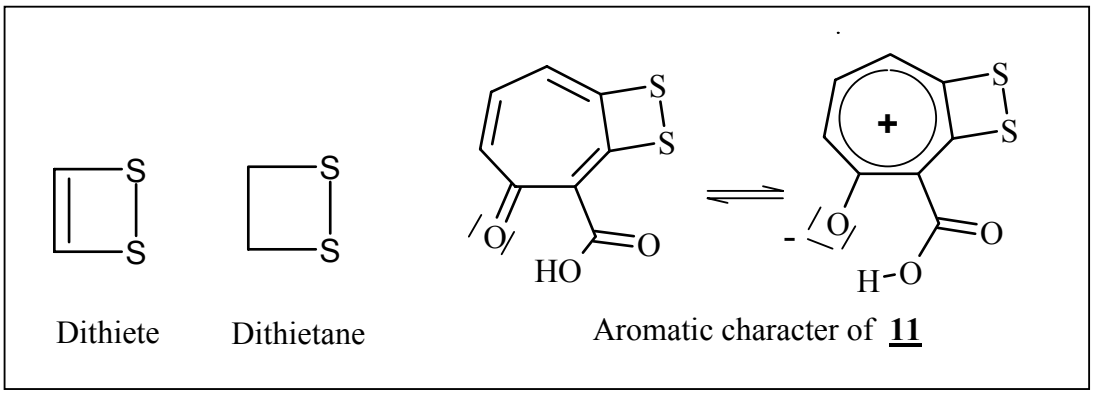


Fig. 14 C-H long range correlation of **11**

Compound **11** contains a carboxylic tropone skeleton connected with a four-membered disulfide ring system. Since the carbons in the four-membered ring show sp^2 hybridization the ring has to be considered as a dithiete moiety rather than as saturated and quite unstable dithietan. Furthermore the aromatic character of tropones via a mesomeric zwitterion (Scheme 4) should also support properties of a dithiete. Neither a dithiete nor a dithietan moiety has been found in natural products so far.



Scheme 4 Structure of **11**

6. The interaction of 11 with DMSO

Table 1 lists the NMR data of **11** in different solvents. Normally the ^{13}C chemical shift is not very much influenced by different solvents, but in d_6-DMSO the ^{13}C signals of C-5 and C-6 have more than 2 ppm distance compared with that in d_6-benzene and $CDCl_3$ (bold types). In addition, **11** showed darker color in DMSO than in other solvents. When the dark-brown

solution was extracted with ethyl acetate **11** got into the ethyl acetate phase and showed an orange color again.

Tab. 1 NMR spectroscopic data of **11**

C	δ_c (δ_H, m, J = Hz) in C_6D_6 125.7 MHz (500 MHz)	δ_c (δ_H, m, J = Hz) in $CDCl_3$ 125.7 MHz (500 MHz)	δ_c in d_6-DMSO 125.7 MHz
1	183.5	183.3	182.4
2	119.9	120.2	120.0
3	168.1	168.6	168.1
4	148.4	149.3	150.2
5	**130.6** (1H, 5.38, d, J = 8.9)	**131.8** (1H, 7.08, d, J = 9.16)	**133.4**
6	**134.6** (1H, 5.90, dd, J = 12.2, 8.9)	**135.8** (1H, 7.42, dd, J = 12.20, 9.16)	**137.3**
7	137.6 (1H, 6.63, d, J = 12.2)	138.5 (1H, 7.41, d, J =12.20)	137.5
8	171.6 (1 H, 16.80, s)	171.6 (1H, 15.55, s)	170.6

In order to investigate the interaction of **11** with DMSO, the UV spectra of **11** in DMSO were measured from the beginning of dissolution to four days. The red-shift from 303 nm (in methanol) to 319 nm (in DMSO) could be observed at the beginning (see Fig. 15). After eight hours the absorption shifted to 307 nm and a new peak emerged at 566 nm (see Fig. 16). Now the DMSO was removed, the solid was dissolved in methanol and its UV spectrum was measured again. As shown in Fig. 17, the peak at 566 nm still exists. The above experiments proved that **11** and DMSO can bind via a weak bond, which can be cleaved by extraction with ethyl acetate. However, the bound DMSO cannot be removed under high vacuum.

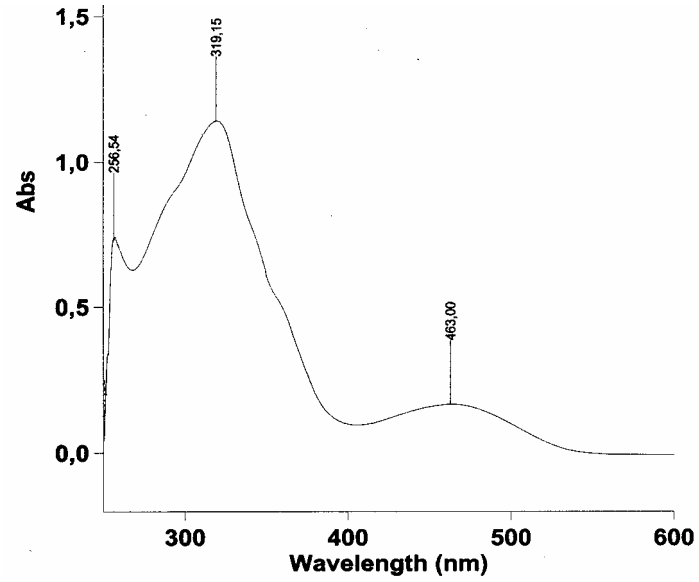


Fig. 15 UV spectrum of **11** in DMSO at the beginning

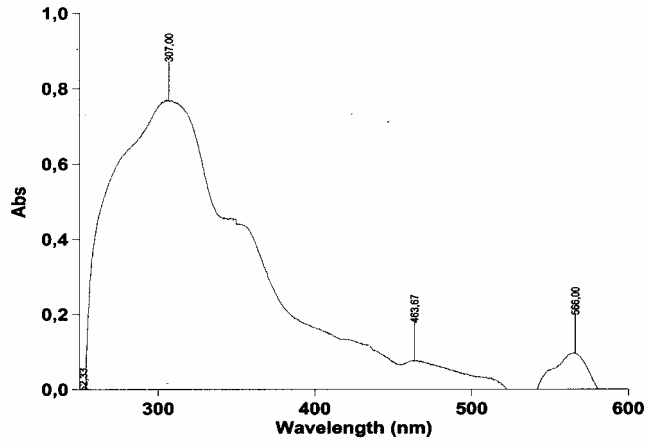


Fig.16 UV spectrum of **11** in DMSO after 8 hours

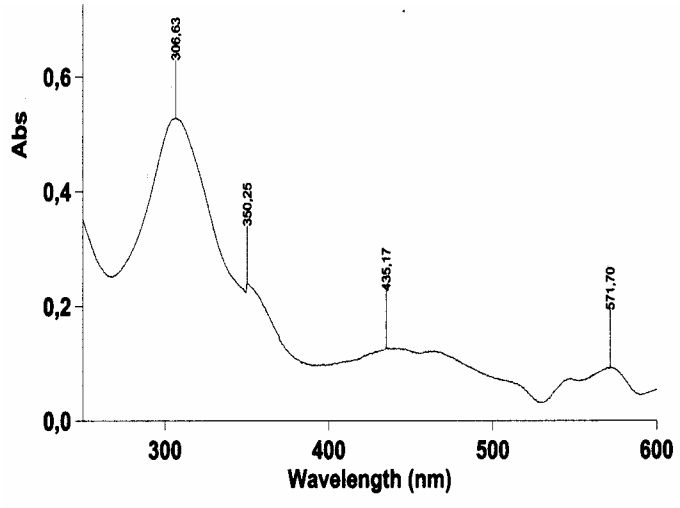
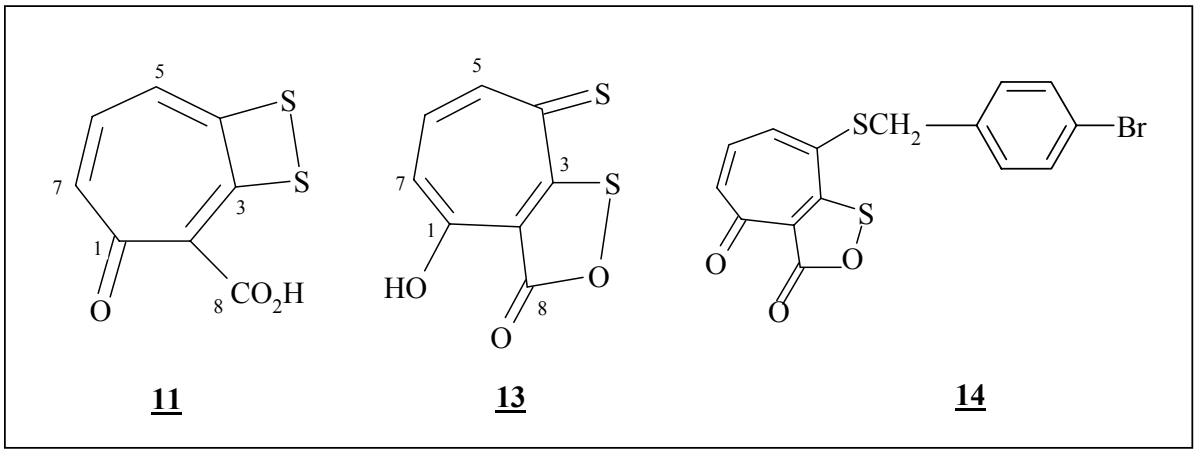


Fig. 17 UV spectrum of **11** in methanol after evaporation of DMSO

7. Comparison of 11 with thiotropocin (13) by experimental and calculating methods

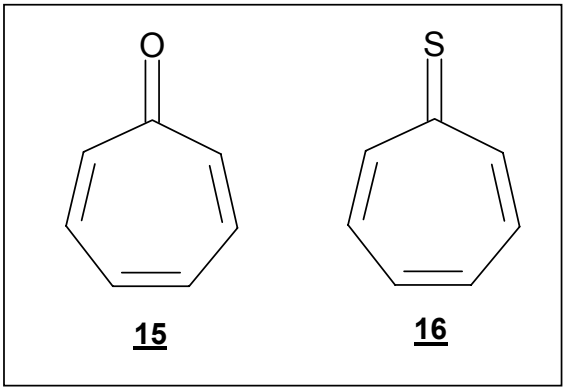


A database search of the formula of **11** in the database "*Antibase*" provided only **13**. **11** and **13** have almost the same physical, chemical and biological properties. **13** was isolated from *Pseudomonas* sp. CB-104 reported by Harada et al. in 1984[59] and also from marine bacterium *Caulobacter* sp. *PK654* by Kawano et al. in 1997.[60] In order to elucidate the structure of **13**, its derivative **14** was synthesized and crystallized.[59] Regarding the high similarity of the physico-chemical data of **11** and **13**,we had to take into consideration that both substances are identical with the consequence that one of the given structures was wrong. In order to make a decision, a more detailed investigation was carried out. First the NMR data of both structures were calculated by the ACDLabs program.

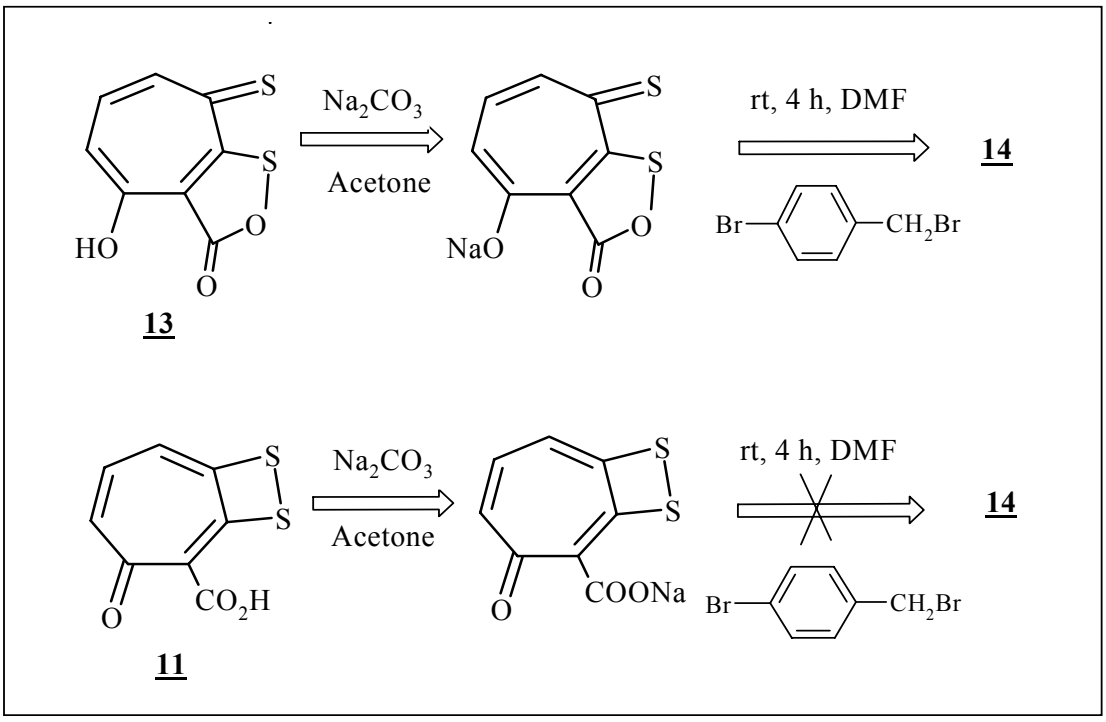
Tab. 2 Experimental and calculated ^{13}C-NMR data of **11** and **13**

Position	Cal. Data of **11** δ_c (ppm)	Exp. data of **11** δ_c (ppm) in d_6-DMSO	Cal. data of **13** δ_c (ppm)	Exp. data of **13** δ_c (ppm) in d_6-DMSO
1	177.6	182.4	155.9	150.1
2	118.6	120.0	**134.9**	**120.0**
3	**178.2**	**168.1**	165.8	167.7
4	151.3	150.2	**223.4**	**182.6**
5	133.4	133.4	133.7	137.7
6	**128.0**	**137.3**	131.5	137.6
7	141.6	137.5	**105.0**	**133.8**
8	166.0	170.6	169.0	170.6

Table 2 lists their experimental and calculated ^{13}C-NMR data. In **13** the chemical shifts of C-2, C-4, C-7 showed differences of 15 – 40 ppm between experimental and calculated ^{13}C-NMR data, while the chemical shifts of **11** show maximal 10 ppm difference. All data differing more than 10 ppm are expressed with bold types in Tab. 2. Therefore the structure proposal of **11** was more convincing than that of **13**.



In addition, NMR data of the prototypes tropone **15** and thiotropone **16** were previously compared. It was proved that the ^{13}C-NMR shift of the carbonyl group appears at 188 ppm, whereas the signal of the thiocarbonyl carbon appears at 213 ppm.[61] Therefore the most intriguing chemical shift (δ_C = 182 ppm) of both compounds **11** and **13** fits to a tropone C=O but it does not fit to C=S.[62]



Scheme 5 Derivatisation of **13** and **11**

Although the NMR data indicated the structure of **11**, complete confirmation was not possible. In order to compare their properties, **11** was treated with 4-bromobenzylbromide according to the procedure shown in Scheme 5.

The EI-MS analysis showed that no product was formed, while **13** was transferred to the derivative of *p*-bromobenzyl thioether **14**.[59] The negative result indicated again that **11** didn't contain a thiocarbonyl group reacting with 4-bromobenzylbromide.

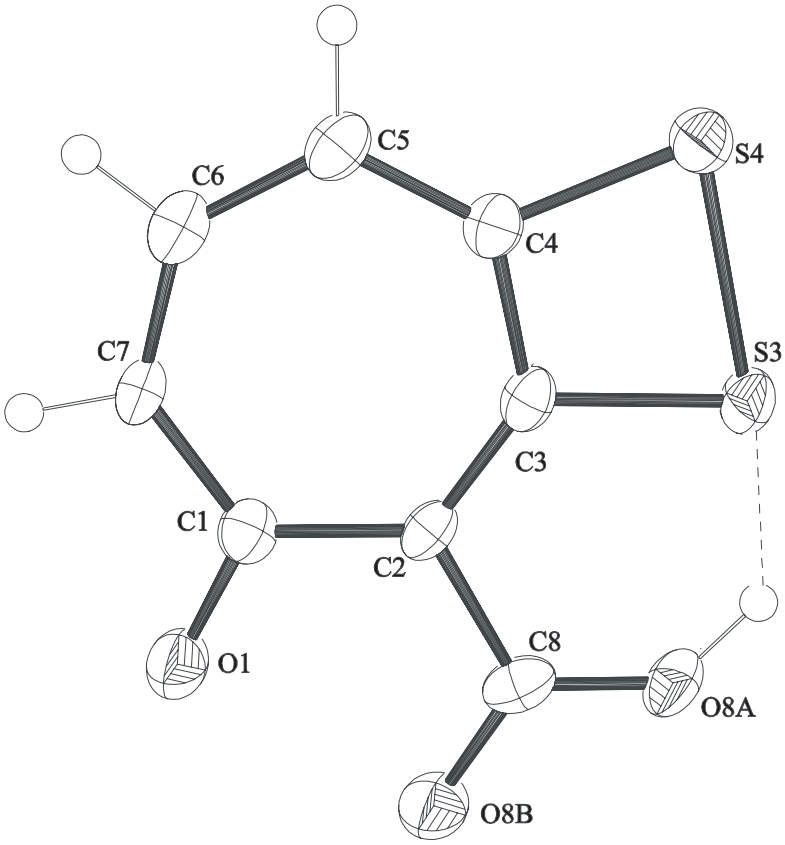
8. Crystal structure of 11

In order to confirm the structure of **11**, many efforts to get single crystals were made. Single crystals can be obtained by various methods. In all cases it is important that the solution is saturated and kept without moving. **11** were dissolved in different solvents such as chloroform, methanol, pyridine, tetrahydrofuran or mixtures of them and kept at different temperatures (room, refrigerator, and freezer). Single crystals of **11** suitable for X-ray structural analysis were obtained from the solvent system of chloroform/formic acid by slow evaporation of the solvent at room temperature for two weeks. Fig. 18a and 18b show two different prospective views of the molecular structure of **11**.[63] Thus X-ray analysis of **11** proves the structure as the same structure previously derived from spectroscopic analysis.

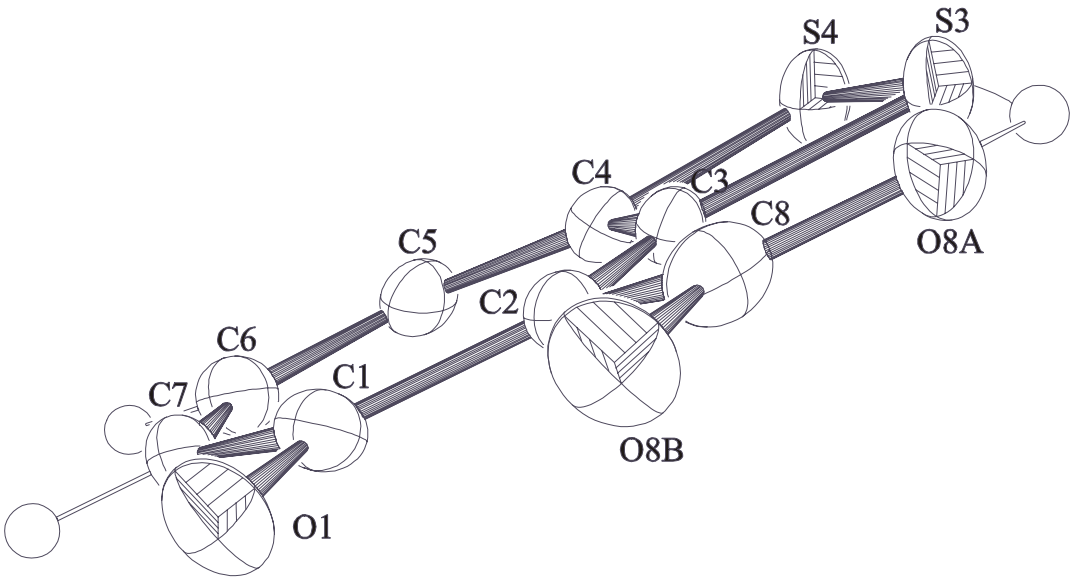
Compound **11** crystallizes in the monoclinic space group *P*2(1) and contains a perfect planar seven-membered ring involving an intramolecular O–H···S hydrogen bond. The other C (C-8), O (O-1, O-8A and O-8B) and S (S-3 and S-4) atoms are almost on the same plane of the seven-membered ring. Table 3 selectively lists some bond lengths (Å) and angles (°) of **11**.

The hydrogen-bonded donor-acceptor separation (S(3)···O(8A) 2.461 Å; O(8A)–H 0.962 Å; S(3)···H 1.745 Å; O(8A)–H–S(3) 128.51°) is greatly shorter than that of the intermolecular interaction found in other similar compounds (O···S 3.455(2) Å; O–H–S 136(4)°).[64] This proton has 16.55 ppm of the chemical shift in ^{1}H-NMR spectrum.

The C(2)–C(3) (1.362(6) Å), C(4)–C(5) (1.366(5) Å) and C(6)–C(7) (1.383(6) Å) formal double bond and the C(1)–C(2) (1.460(5) Å), C(3)–C(4) (1.438(6) Å), C(5)–C(6) (1.412(6) Å) and C(1)–C(7) (1.444(5) Å) formal single bond lengths are comparable to those found in tropolone (scheme 5; C=C 1.373(4) Å, 1.341(4) Å and 1.379(4) Å; C–C 1.454(4) Å, 1.393(4) Å, 1.410(4) Å and 1.410(3) Å)[65] and in 3-azidotropone (scheme 6; C=C 1.327 Å, 1.342 Å and 1.348 Å; C–C 1.445 Å, 1.425 Å, 1.430 Å and 1.448 Å),[66] respectively, which are typical for the substituted aromatic compounds.



(a)

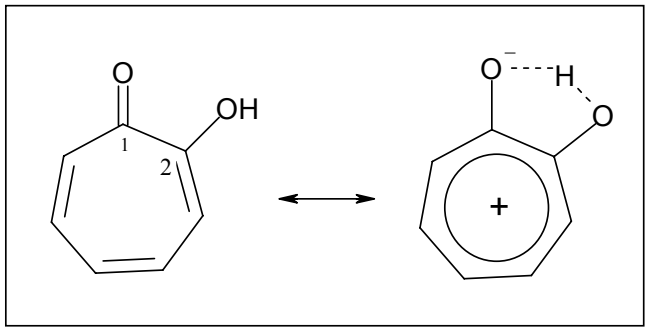


(b)

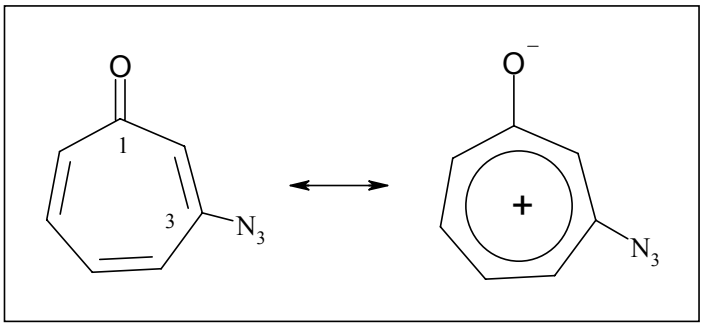
Fig. 18 (a) and (b): Two different views of the molecular structure of **11** (50 % probability ellipsoids).

Tab. 3 Selected bond lengths (Å) and angles (°) of **11**

C(1)–C(2)	1.460(5)	C(2)–C(3)	1.362(6)
C(3)–C(4)	1.438(6)	C(4)–C(5)	1.366(5)
C(5)–C(6)	1.412(6)	C(6)–C(7)	1.383(6)
C(1)–C(7)	1.444(5)	C(1)–O(1)	1.288(5)
C(2)–C(8)	1.503(5)	C(3)–S(3)	1.736(3)
C(4)–S(4)	1.752(4)	C(8)–O(8A)	1.275(5)
C(8)–O(8B)	1.253(5)	S(3)–S(4)	2.137(1)
S(3)···O(8B)	2.461	C(2)–C(1)–C(7)	124.4(3)
C(1)–C(2)–C(3)	126.3(3)	C(2)–C(3)–C(4)	133.1(3)
C(3)–C(4)–C(5)	129.0(4)	C(4)–C(5)–C(6)	125.0(4)
C(5)–C(6)–C(7)	131.3(3)	C(1)–C(7)–C(6)	130.8(3)
C(2)–C(1)–O(1)	118.3(3)	C(3)–S(3)–S(4)	79.1(1)
C(4)–S(4)–S(3)	77.74(13)	O(8A)–C(8)–O(8B)	124.2(4)

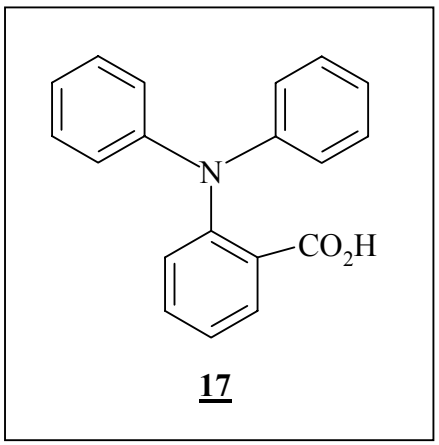


Scheme 6 Dipolar ionic character of the C(1)–O(1) bond and intramolecular hydrogen bond of tropolone

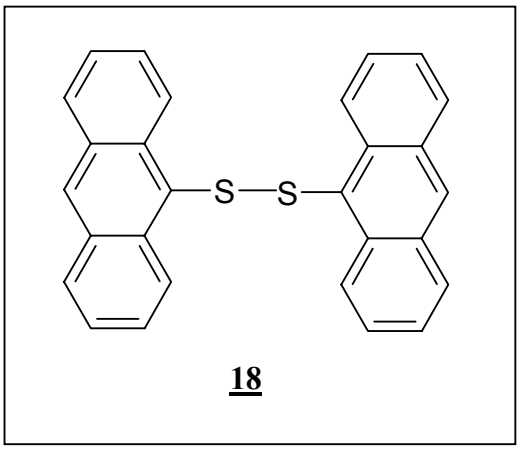


Scheme 7 Dipolar ionic character of the C(1)–O(1) bond of 3-azidotropone

The C(1)–O(1) (1.288(5) Å) bond of **11** is slightly longer than that of tropolone (1.261(3) Å, Scheme 6), and significantly longer than the C=O bond found in 3-azidotropone (1.234(3) Å, Scheme 7).[67] This indicates that **11** has a more distinct dipolar ionic character of the C(1)–O(1) bond. In addition, the C(2)–C(1)–C(7) angle (124.4(3)°) is larger than those in tropolone (124.2(2)°, Scheme 6), which also suggests the significant carbonyl polarization of C(1)–O(1) bond in **11**.[68] This leads to an aromatic character (6π-electrons delocalize in the seven-membered ring) of **11** in agreement with the chemical shifts in ^{13}C- and ^{1}H-NMR of **11**, whereas the bond length difference of 0.022 Å between C(8)–O(8A) (1.275(5) Å) and C(8)–O(8B) (1.253(5) Å) is less pronounced than that in 2-(N, N'-diphenylamino)benzoic acid (0.072 Å) (**17**), in which the carboxylic groups are linked through two intermolecular hydrogen bonds.[69]



17



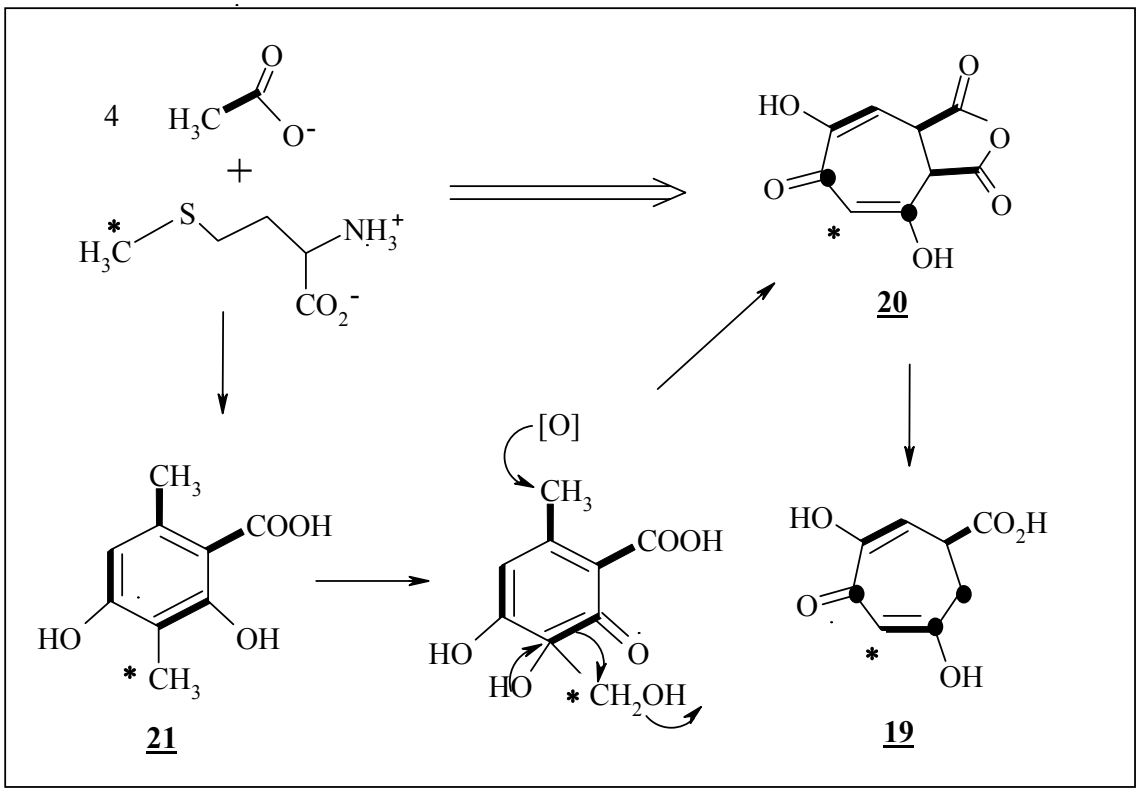
18

The C(3)–S(3) (1.736(3) Å), C(4)–S(4) (1.752(4) Å) and S(3)–S(4) 2.137(1) Å) bond distances are comparable to those exhibited by the compounds di-9-anthryl disulfide (**18**; C–S 1.776(3) Å and 1.770(2) Å; S–S 2.1089(12)Å).[70] Obviously, due to the formation of the

intramolecular hydrogen bond, the C(3)–S(3) bond length in **11** becomes shorter than that of the C(4)–S(4) bond.

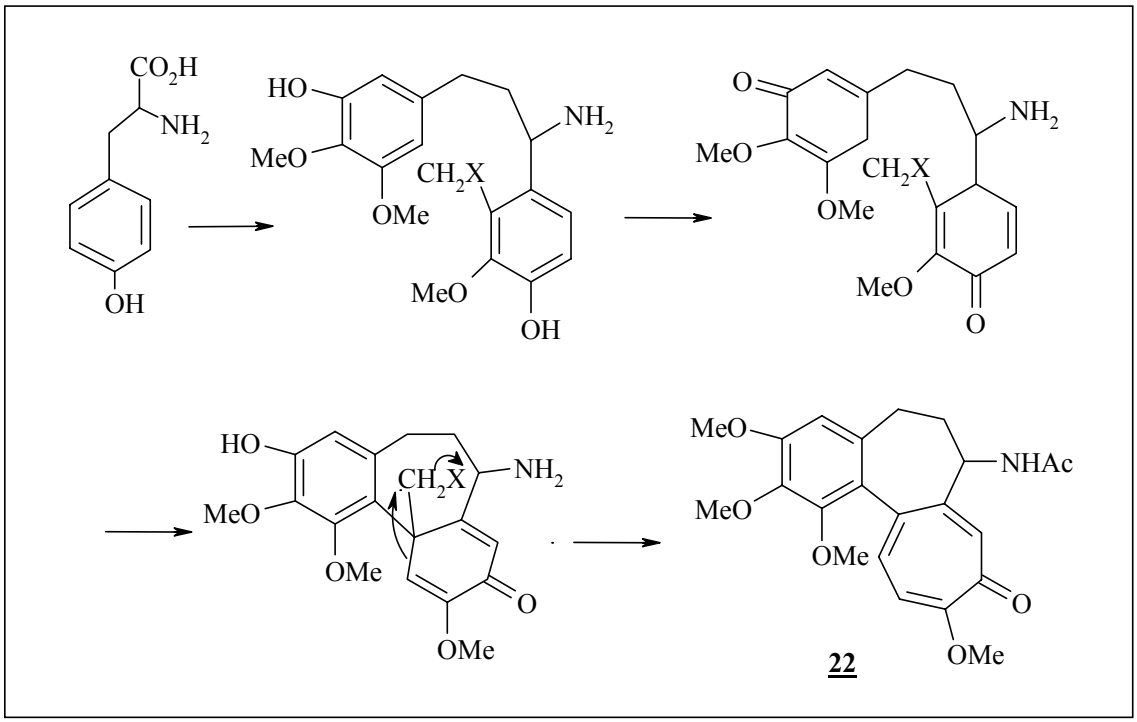
9. Biosynthesis of 11

Among the previously known naturally occurring aromatic tropolone derivatives are some fungal metabolites, including stipitatic acid (**19**) and stipitatonic acid (**20**) from *Penicillium stipitatum*.[71] Labelling studies have established the biogenetic origin of these latter compounds from acetate and methionine and led to the proposal that the common seven-membered tropolone ring could be formed by oxidative ring expansion of a methylated aromatic tetraketide (**21**) as illustrated in Scheme 8.[72]



Scheme 8 Biosynthesis of **19** and **20**

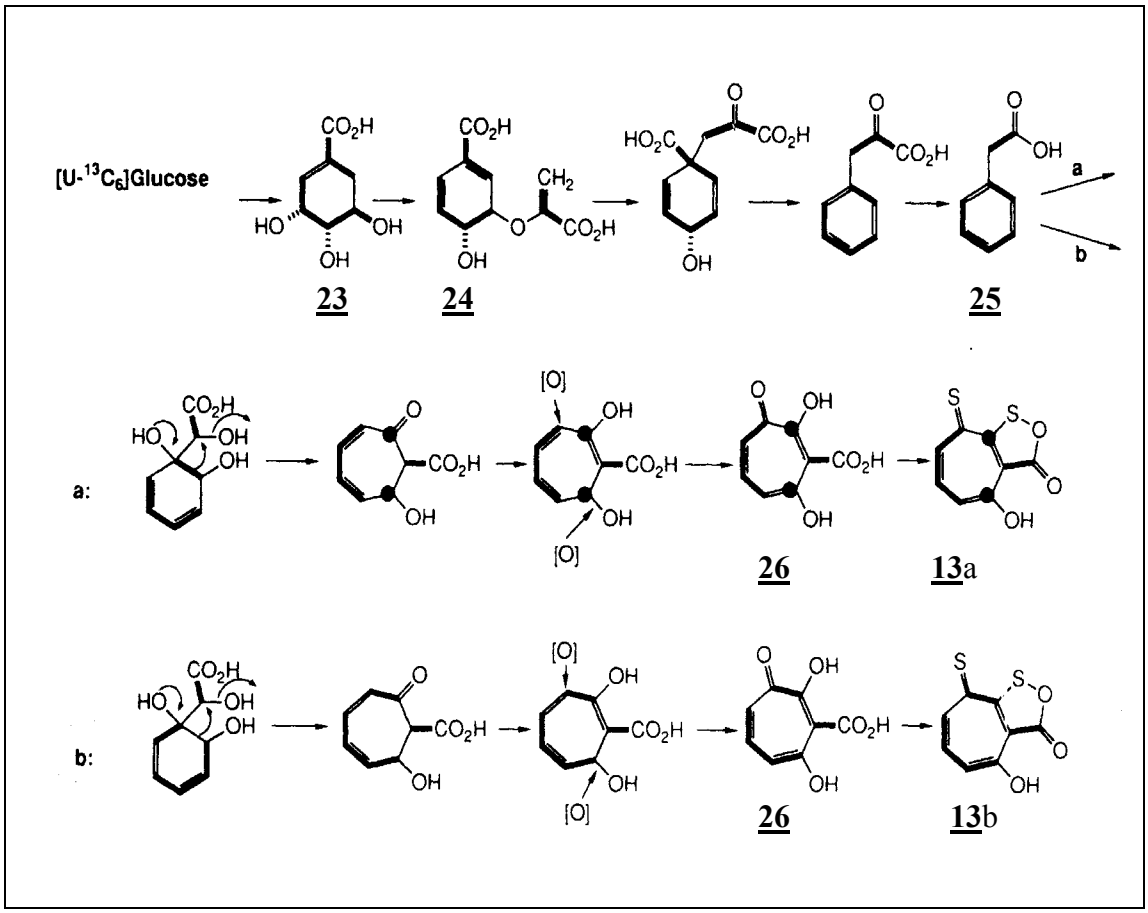
In contrast, the alkaloidal tropolone derivative colchicine (**22**) has been shown to be biosynthesized from tyrosine (see Scheme 9).[73]



Scheme 9 Biosynthesis of **22**

Several alkyltropolones, such as noonkatinol,[74] have been found in the heartwood of various *Cupressales* and are of possible terpenoid biogenetic origin.[75]

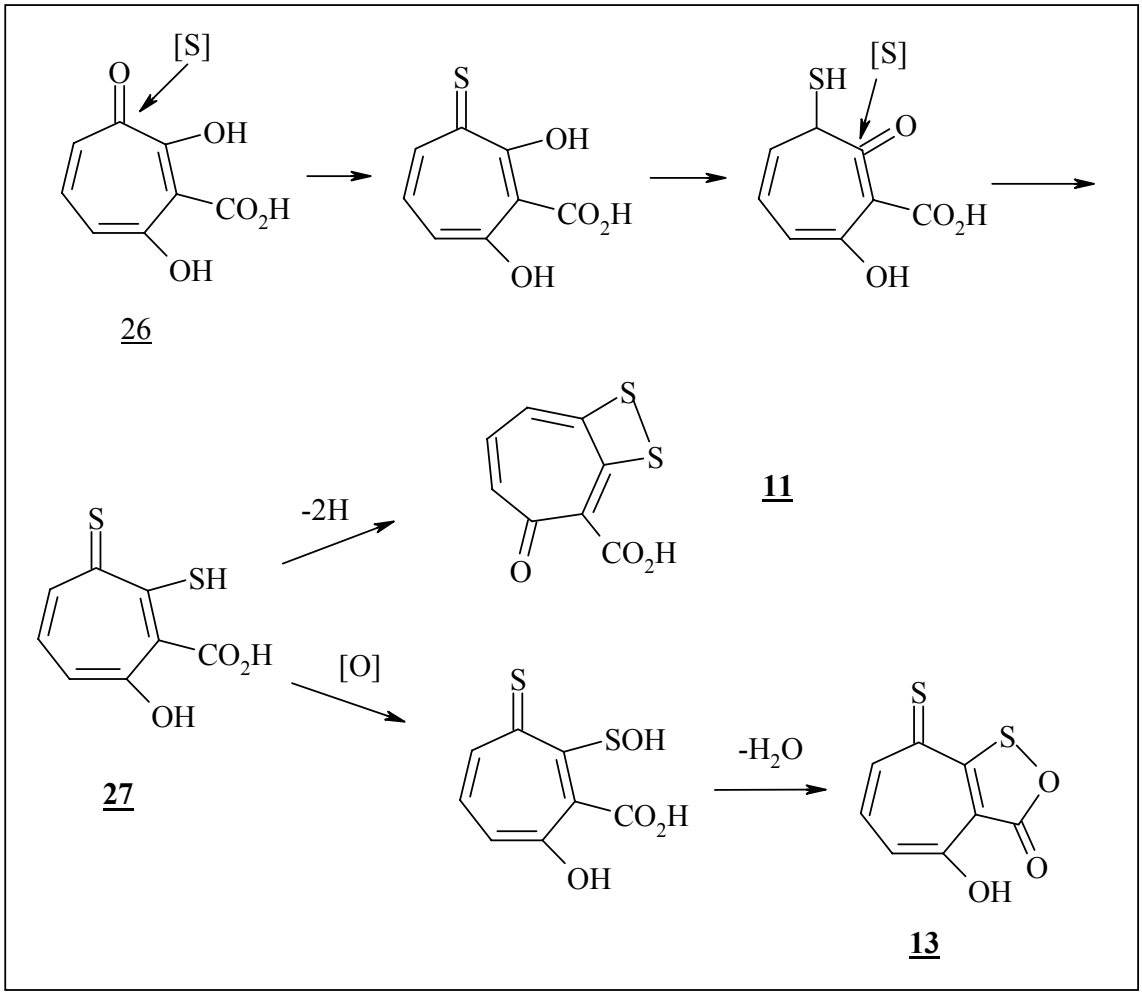
The biosynthesis of thiotropocin (**13**) was investigated in detail by Cane et al. by feeding of labelled [U-$^{13}C_6$] glucose.[76] It was confirmed that **13** was synthesized by an oxidative ring expansion of phenylacetic acid (**25**), itself biosynthesized by the shikimate (**23**)/chorismate (**24**) pathway,[77] and further oxidation to generate a 4-hydroxytropolone carboxylic acid (**26**) (Scheme 10).



Scheme 10 Biosynthesis of **13**

Compound **11** is an isomer of **13** and they have very similar structures. The difference in their biosynthesis could be located in the last step.

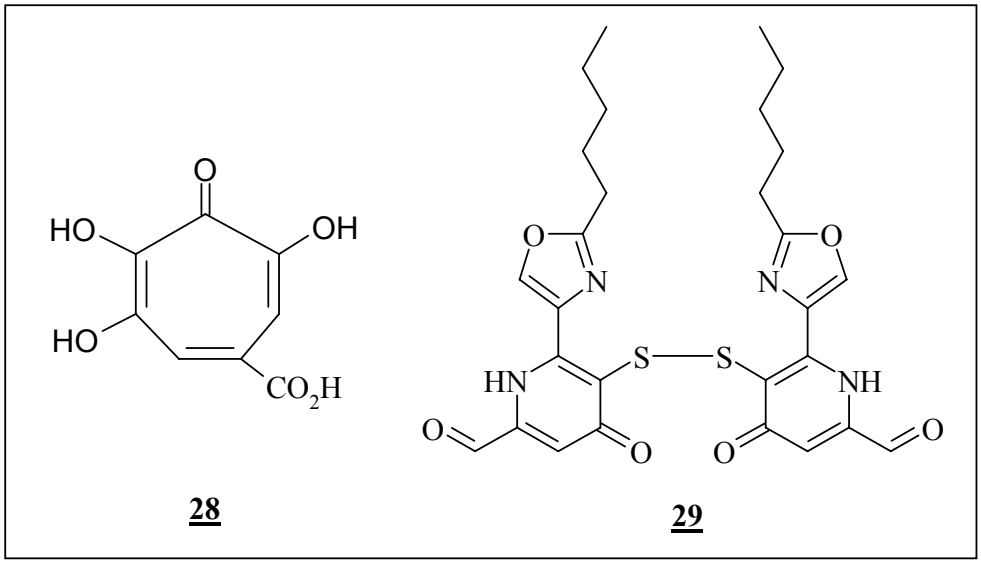
Scheme 11 shows a proposed biosynthesis pathway from **26** to **11** and **13**. The procedures include the attack of nucleophilic sulfur species to the tropolone carbonyl group followed by tautomerization and analogous introduction of the second sulfur atom (**27**), which could proceed via two ways, one leads to **11** and another one leads to **13**. All reactions are carried out by the action of enzymes.



Scheme 11 Proposed biosynthesis pathway of **11** and **13** from **26**

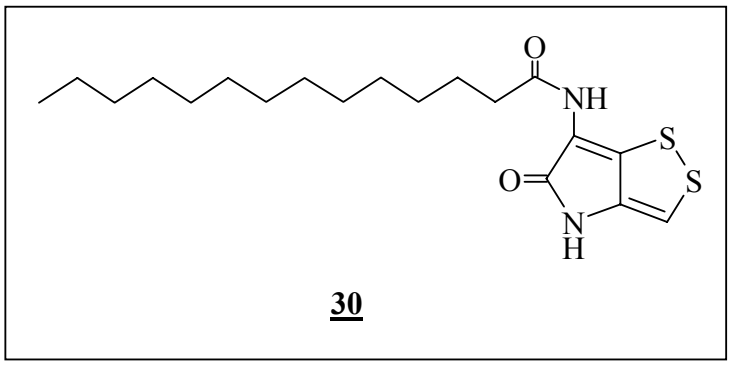
10. Biological activities of 11

The occurrence of tropolone and polysulfide derivatives in nature is now well established.[78] Most of them have biological activities. For example, **19** and puberulic acid (**28**), produced by *Penicillium puberulum,*[79] contain both a tropolone moiety and a carboxylic group like **11**. They showed activities against bacteria, fungi and microalgae.



28 **29**

Sulfur-containing compounds have often been isolated from marine organisms. For example, Antibiotic B-90063 (**29**), isolated from a marine *Blastobacter* sp., is a potent endothelin-converting enzyme inhibitor (ECE).[80]



30

But not all sulfur-containing compounds exhibit bioactivities. One example is tetradecanoyl holothine (**30**), produced by a marine *Alteromonas* sp., for which no biological activities have been reported.[81]

Tropodithietic acid (**11**) showed a broad but moderate antimicrobial activitiy against Gram-positive and Gram-negative bacteria, fungi and microalgae (Tab. 4). It has an especially strong activity against *C. candida* (43 mm inhibition zone diameter at 0.7 mg/mL in agar diffusion assay).

Tab. 4 Agar diffusion assay of **11** against microorganisms

Test organisms	Inhibition zones (mm)
Bacillus subtilis DSM10 (ATCC 6051)	15
Staphylococcus aureus DSM 20231 (ATCC 12600)	27
Escherichia coli K12	25
Candida. albicans Tü164	43
Chlorella vulgaris	17
Chlorella sorokiniana	23
Scenedesmus subspicatus	24

50 μL substance on ∅ = 9 mm filter-paper disk, c = 0.7 mg/mL.

Tab. 5 Activities of **11** against higher organisms

Nematodes (*Caenorhabditis elegans*)	IC_{50} 25 μg/mL
Arthropodes (*Artemia salina*)	100 % mortality with 1 mg/mL

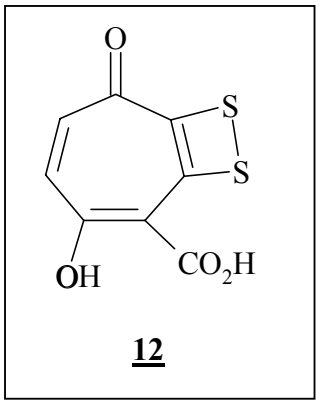
Tab. 6 Antitumor activities of **11**

Cell lines	TGI [μg/mL]	GI_{50} [μg/mL]	LC_{50} [μg/mL]
Stomach carcinoma HM02	> 10	6.7	> 10
Mamma carcinoma MCF 7	8.3	6.2	> 10
Liver carcinoma HEP G2	> 10	5.0	> 10

TGI: Concentration which causes complete inhibition of cell growth.
GI_{50}: Concentration which causes 50% inhibition of cell growth.
LC_{50}: Concentration which causes 50% reduction of the original cell number.

The isolation of a biologically active compound with a previously unknown skeleton from a marine bacterium has reaffirmed the marine environment as a source for interesting metabolites.

11. Structure elucidation of hydroxytropodithietic acid (12)



12

Compound **12** as a minor component had only a yield of 0.004 mg/L, i.e. 2 mg were obtained from 50 L culture. It is a pale yellow amorphous solid with UV adsorption at 237, 268, 355, 399 nm (Fig. 19). The high resolution EI-MS spectrum showed the molecular ion peak at m/z = 228 with the molecular formula $C_8H_4O_4S_2$. Deduced from the structure of major component **11** and the mass difference of m/z = 16, it was easy to suppose that **12** had an additional hydroxy group compared with **11**.

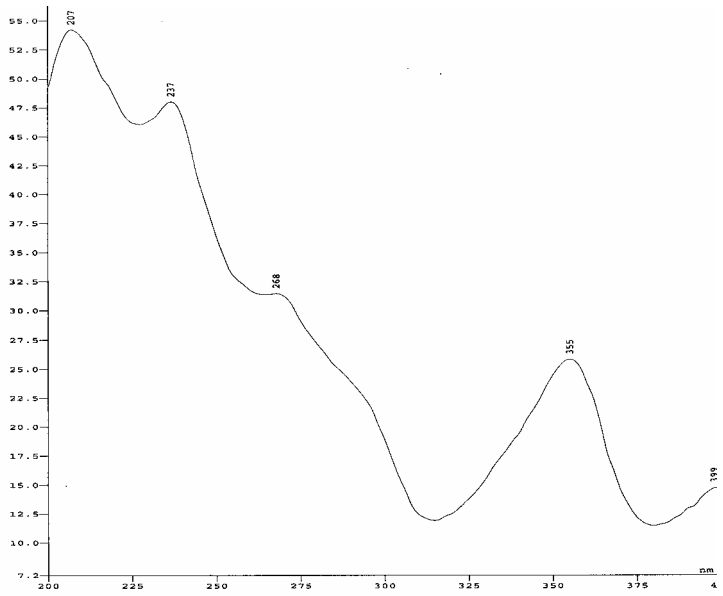


Fig. 19 UV spectrum of **12** derived from HPLC-DAD ($ACN/H_2O/H_3PO_4$)

The ^{1}H-NMR spectrum of **12** in d_4-methanol showed two doublets at δ = 7.31 and 7.04 ppm, with a coupling constant of J = 8 Hz. Since **12** was hardly soluble in other solvents, the

measurement of NMR spectra in solvents without exchangeable protons was impossible. It is obvious that the two aromatic protons are vicinal in **12** and the hydroxyl group should be located at the position of C-5 or C-7. Because of the quite small amount of **12**, the ^{13}C-NMR spectrum revealed only three signals, one at δ = 176.0 ppm, and two signals at 137.6 ppm and 136.2 ppm, indicating two aromatic methine carbons. Four additional carbon signals were observed as projection via their cross signals in the HMBC spectrum. Fig. 20 shows the C-H long-range correlation. The structural elucidation could not be confirmed due to incomplete data with spectroscopy methods alone.

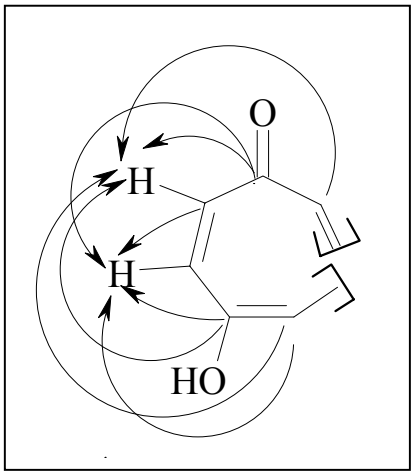
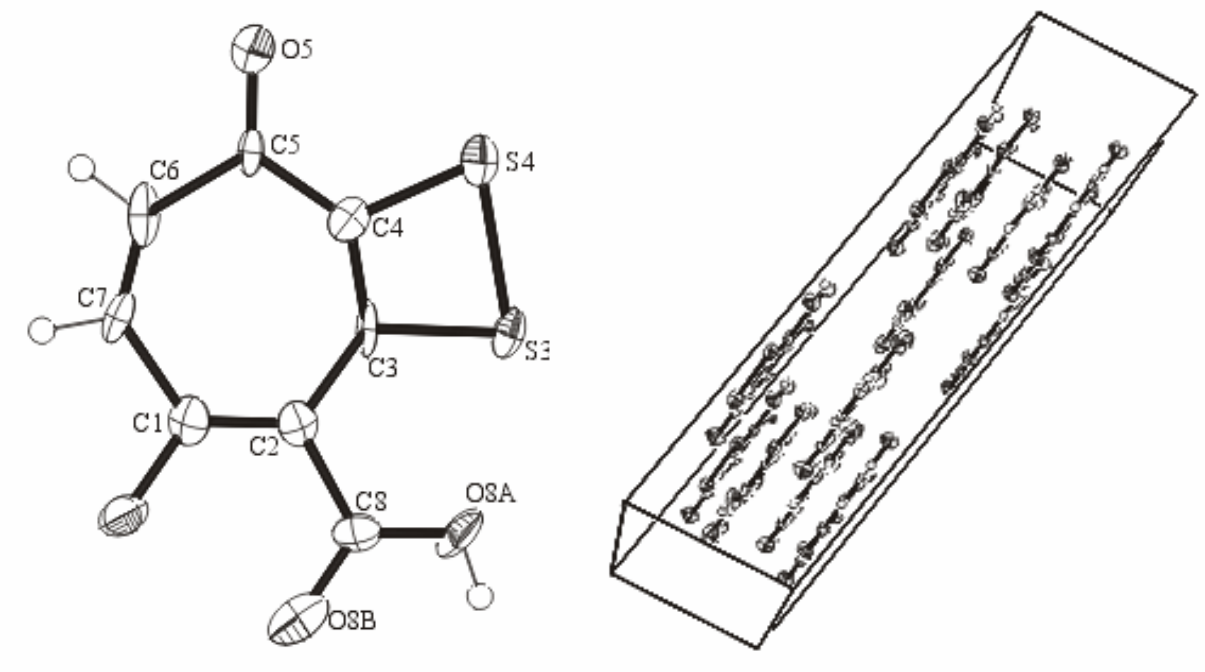


Fig. 20 HMBC correlation of **12**

12. Crystal structure of 12

In order to confirm the structure of **12**, a single crystal was grown. It was obtained from methanol by maintaining the solution at room temperature for two weeks. **12** crystallizes in the monoclinic space group *P*2(1). As shown in Fig. 21a, it contains a planar seven-member ring, and the remaining S, C and O atoms are located almost in the ring plane. The molecular arrangement in the unit cell (Fig. 21b) shows that the planes of all molecules are exactly paralleled in the solid state.



(a) (b)

Fig. 21. (a) The perspective view of **12** (50 % probability ellipsoids and the protons bonded to O atoms are omitted). (b) A packing diagram of **12**.

Tab. 7 Selected bond lengths (Å) and angles (°) of **12**

C(1)–C(2)	1.32(3)	C(2)–C(3)	1.43(2)
C(3)–C(4)	1.33(3)	C(4)–C(5)	1.45(2)
C(5)–C(6)	1.49(2)	C(6)–C(7)	1.25(3)
C(1)–C(7)	1.51(3)	C(1)–O(1)	1.38(2)
C(2)–C(8)	1.51(3)	C(3)–S(3)	1.752(16)
C(4)–S(4)	1.757(18)	C(8)–O(8A)	1.26(3)
C(8)–O(8B)	1.26(3)	S(3)–S(4)	2.128(7)
C(2)–C(1)–C(7)	126.1(18)	C(1)–C(2)–C(3)	124.5(17)
C(2)–C(3)–C(4)	133.7(16)	C(3)–C(4)–C(5)	133.8(17)
C(4)–C(5)–C(6)	115.4(15)	C(5)–C(6)–C(7)	134.7(17)
C(1)–C(7)–C(6)	131.6(17)	C(2)–C(1)–O(1)	123.2(17)
C(3)–S(3)–S(4)	77.8(6)	C(4)–S(4)–S(3)	76.0(6)
O(8A)–C(8)–O(8B)	123.5(17)		

Table 7 lists some selected bond lengths and angles for **12**. All the formal single bonds in the seven-membered ring are considerably shorter than normal single bonds and the formal double bonds are longer than usual double bonds. This indicates that the seven-membered ring of **12** consists of a conjugated system of bonds similar to that found in **11**.

13. The variation of culture medium

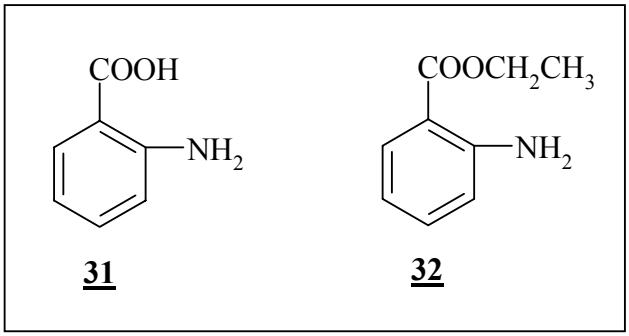
Strain T5 is of particular interest among the screened strains due to its antibiotic production and its ecological meaning seen by interaction with the other coexistent strains (ex. T1, T4). Therefore the biosynthetic potential of strain T5 was investigated in detail.

An investigation of the relationship between cell growth and available amino acids (performed by Dipl.-Biol. T. Heidorn) indicated that the existence of phenylalanine in the culture broth is essential for the growth of strain T5. In order to increase the yield of **11**, phenylalanine (0.8 g/L) and histidin (0.08 g/L) were fed during the cultivation of strain T5. The HPLC analysis showed that the yield of **11** was dramatically increased. A 7 L-fermentation was accomplished and the amount of isolated **11** (according to Scheme 3) was enhanced from usual 1 mg/L to 6 mg/L.

14. Metabolites from new culture medium

When strain T5 was cultivated in a tryptophan-containing medium, no production of **11** could be detected via HPLC. But instead two other major metabolites could be found in the chromatogram. The corresponding UV spectra were completely different from those of **11** and **12**. The fermentation was carried out in a 1 L-fermenter and afforded 76 mg of crude extract. The two compounds could be isolated by silica gel column chromatography (chloroform/methanol 9 : 1) in yields of about 30 mg/L, either.

15. Structure elucidation of 2-aminobenzoic acid (31) and its ethyl ester (32)



31 **32**

Both compounds showed an intense orange color when stained with Ehrlich's reagent. The EI-MS spectra showed the masses of 137 and 165. The corresponding fragments patterns [119 (phenylcarbamate), 92 (alkylbenzole), 65 (5-member aromatic cation)] were in complete accordance with 2-aminobenzoic acid as shown in Fig. 22 and its ethyl ester (omitted). ^{1}H-NMR showed the signals of four vicinal aromatic protons and thus the vicinal substitution pattern could be confirmed.

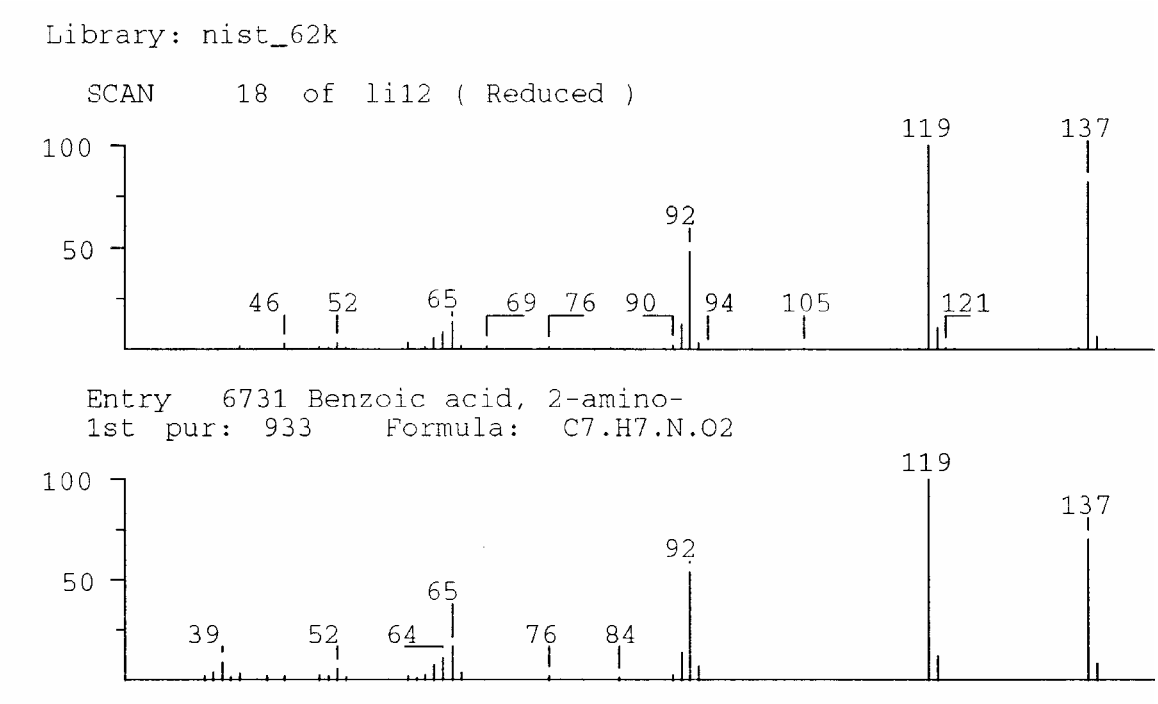


Fig. 22 The mass spectrum of **31** is compared with the database

The two compounds are metabolites of many bacteria, fungi and especially marine bacteria.[82] However, this strain produces **31** in high yields, while most marine bacteria produce their metabolites with low yields and a lot of minor components.

IV. Biosynthetic efficiency and metabolites of strain RK377

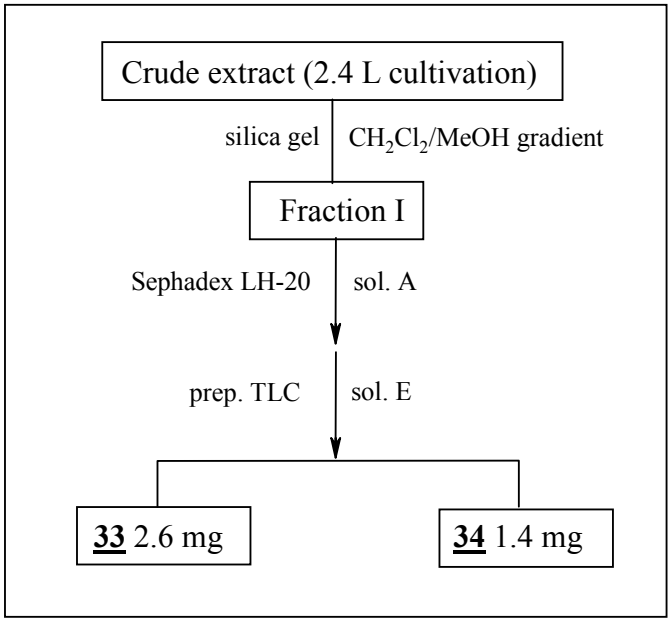
1. Description of strain RK377

Strain RK377 was isolated from a water-sample collected in Norddeich-Mole. It was identified as *Halomonas* sp. (γ–proteobacteria).

2. Cultivation in shaking flasks and structure elucidation of secondary metabolites

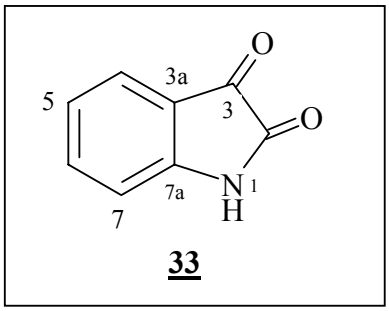
2.1. Cultivation of strain and isolation of secondary metabolites

In the previous screening work, the crude extract of strain RK377 showed noticeable bioactivity and a striking pattern of metabolites. A 2.4 L cultivation of strain in MB medium was at first accomplished at 28 °C for six days in 24 Erlenmeyer flasks. Two compounds were isolated from the crude extract of the culture filtrate (adjusted to pH 4 prior to extraction) according to Scheme 12.



Scheme 12 Isolation of **33** and **34**

2.2. Structure elucidation of isatin (**33**)



33

Compound **33** was isolated as an orange solid, which was readily soluble in acetone, chloroform or methanol. It had an R_f-value of 0.5 in $CHCl_3$/MeOH 9:1; it changed to a yellow color stained with anisaldehyde spray reagent. The UV spectrum showed absorption maxima at 242 and 301 nm. The EI-MS spectrum gave a molecular ion peak at m/z = 147. Comparison with the EI-MS database revealed that this compound had the same molecular weight and fragment pattern as isatin (**33**). Its ^{1}H-NMR spectrum is also in agreement with isatin. It displayed a pattern of four vicinal aromatic protons at δ = 7 – 8 ppm and one exchangeable proton at δ = 10 ppm in d_6-acetone. The X-ray crystallography analysis confirmed the structure.

Single crystals of **33** suitable for X-ray structural analysis were obtained from chloroform by maintaining the solution at room temperature for two weeks. As shown in Fig. 23 and Tab. 8, the X-ray diffraction analysis revealed that **33** is a planar molecule (the sum of the angles at C(2) and C(3) is 360°). The O(1)-C(2) (1.216(3) Å) and O(2)-C(3) (1.206(3) Å) bond lengths are slightly shorter than those found in compound **11** (1.288(5) Å) and tropolone (1.261(3) Å), and comparable with other similar compounds.

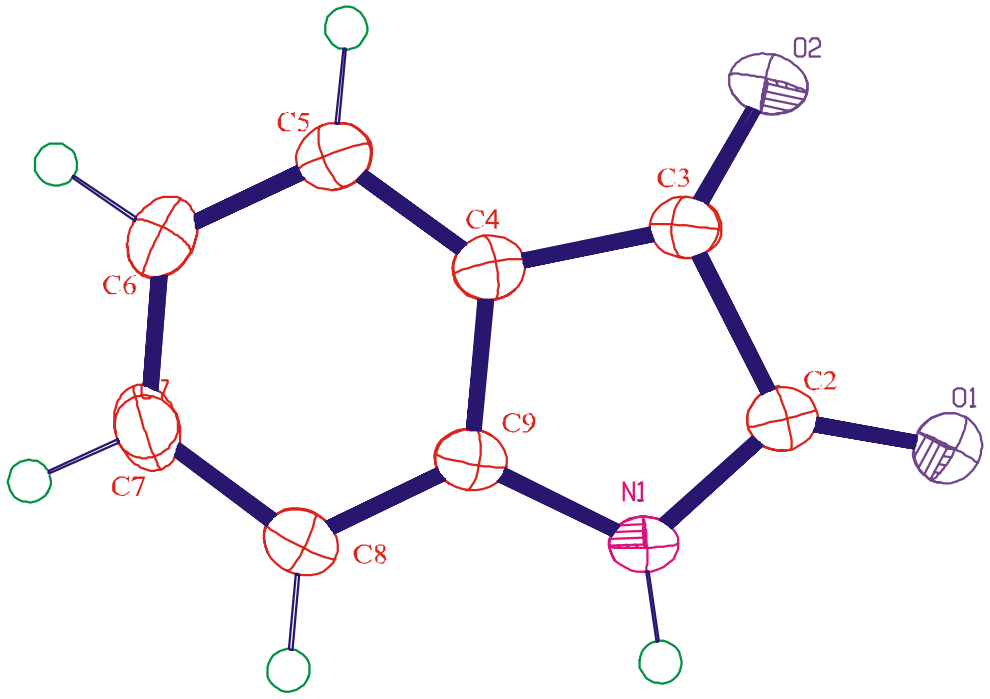


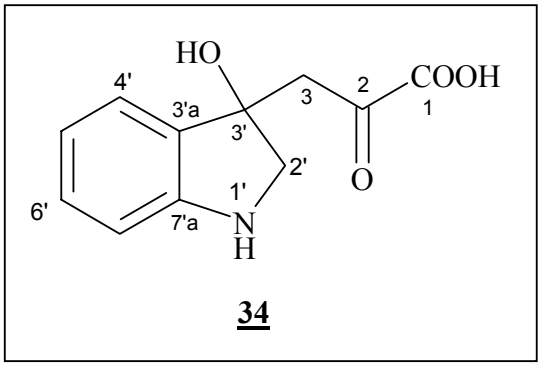
Fig. 23 Perspective view of **33** (50 % probability ellipsoids)

Tab. 8 Selected Bond Lengths (Å) and Angles (°) of **33**

O(1)-C(2)	1.216(3)	O(2)-C(3)	1.206(3)
N(1)-C(2)	1.359(3)	N(1)-C(9)	1.402(3)
C(2)-C(3)	1.563(3)	C(3)-C(4)	1.460(3)
O(1)-C(2)-C(3)	126.7(2)	O(1)-C(2)-N(1)	127.8(2)
N(1)-C(2)-C(3)	105.5(2)	O(2)-C(3)-C(4)	131.5(2)
O(2)-C(3)-C(2)	123.5(2)	C(4)-C(3)-C(2)	105.0(2)
C(5)-C(4)-C(3)	132.4(2)	C(9)-C(4)-C(3)	107.1(2)
N(1)-C(9)-C(4)	110.6(2)	C(8)-C(9)-N(1)	128.1(2)

Isatin (2,3-dioxoindole, **33**) is not only an important raw material for the industry, but also a widespread natural product, additionally it plays a role in human physiology. It was isolated before as a fungicide from *Alteromonas* sp., a symbiont of shrimp larves,[83] and from the pathogenic basidiomycete fungus *Schizophyllum commun*.[84] In animal experiments, **33** showed the stimulation of an anxiety-like action where its level is increased during stress and anxiety.[85] **33** was also isolated from the extract of dried fruits of the cannon ball tree *C. quianensis Aubl.* as precursor of indirubin.[86]

2.3. Structure elucidation of 3-(3-hydroxy-2,3-dihydro-1*H*-indol-3-yl)-2-oxo-propionic acid (34)



34

Compound **34** was isolated as a colorless amorph in a yield of 0.8 mg/L. It was soluble in acetone, chloroform and methanol. It had an R_f-value of 0.32 in $CHCl_3$/MeOH 9:1; Stained with anisaldehyde it changed to a blue color. The UV spectrum showed absorption maxima at 255 and 292 nm. The EI-MS spectrum gave the molecular ion peak at m/z = 205 and the HREI-MS established the molecular formula $C_{11}H_{11}NO_3$. Fig. 24 shows the EI-MS spectrum of **34**.

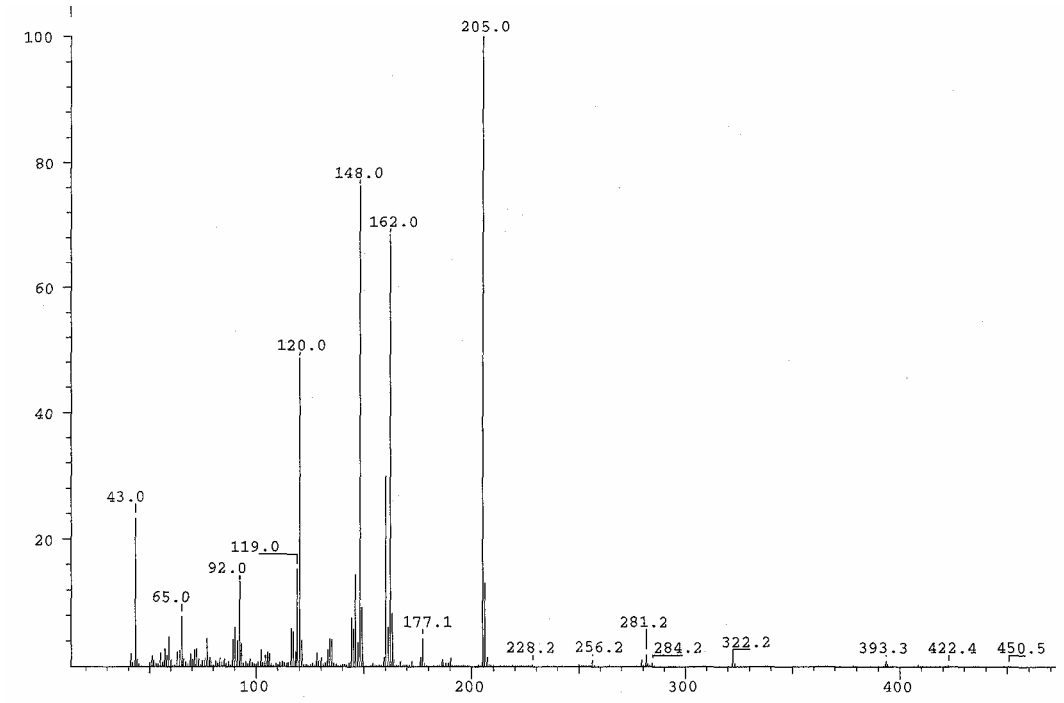


Fig. 24 EI-MS spectrum of **34**

The ^{1}H-NMR spectrum indicated four aromatic vicinal protons, two methylene groups at δ = 3.39 ppm (singlet), δ = 3.32 and 3.10 ppm (two doublets with J = 16.5 Hz) and two

exchangeable protons at δ = 9.28 ppm and δ = 5.02 ppm. The ^{13}C-NMR spectrum showed 11 signals. Among them, the peak at δ = 206.7 ppm indicates a ketone group; the peak at δ = 173.0 ppm is attributed to a carboxylic group; three signals at aliphatic lower field δ = 73.0, 71.8 and 51.4 ppm revealed that their position are near to O or N atom; other six carbon signals between δ = 111 – 147 ppm should build up a benzene ring. A HMBC correlation is drawn by analysis of the HMBC spectrum (Fig. 25). This is only a proposal structure. Further structure elucidation needs more amount of substance. In databases no similar structures were found and thus a comparison of NMR data was not possible.

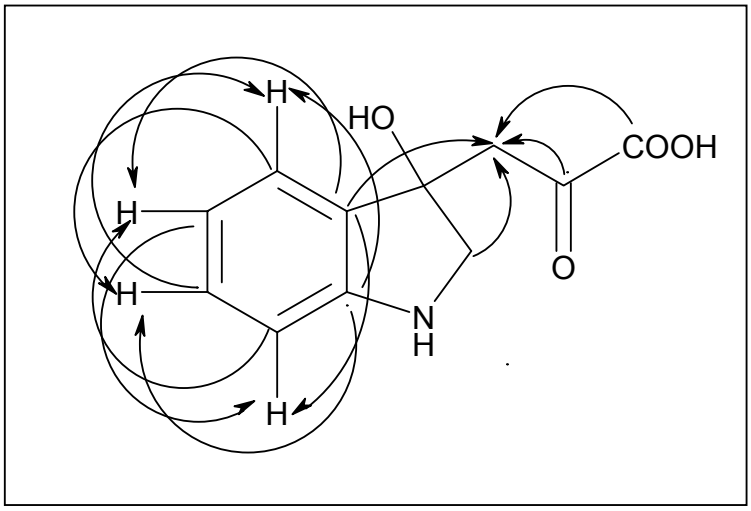
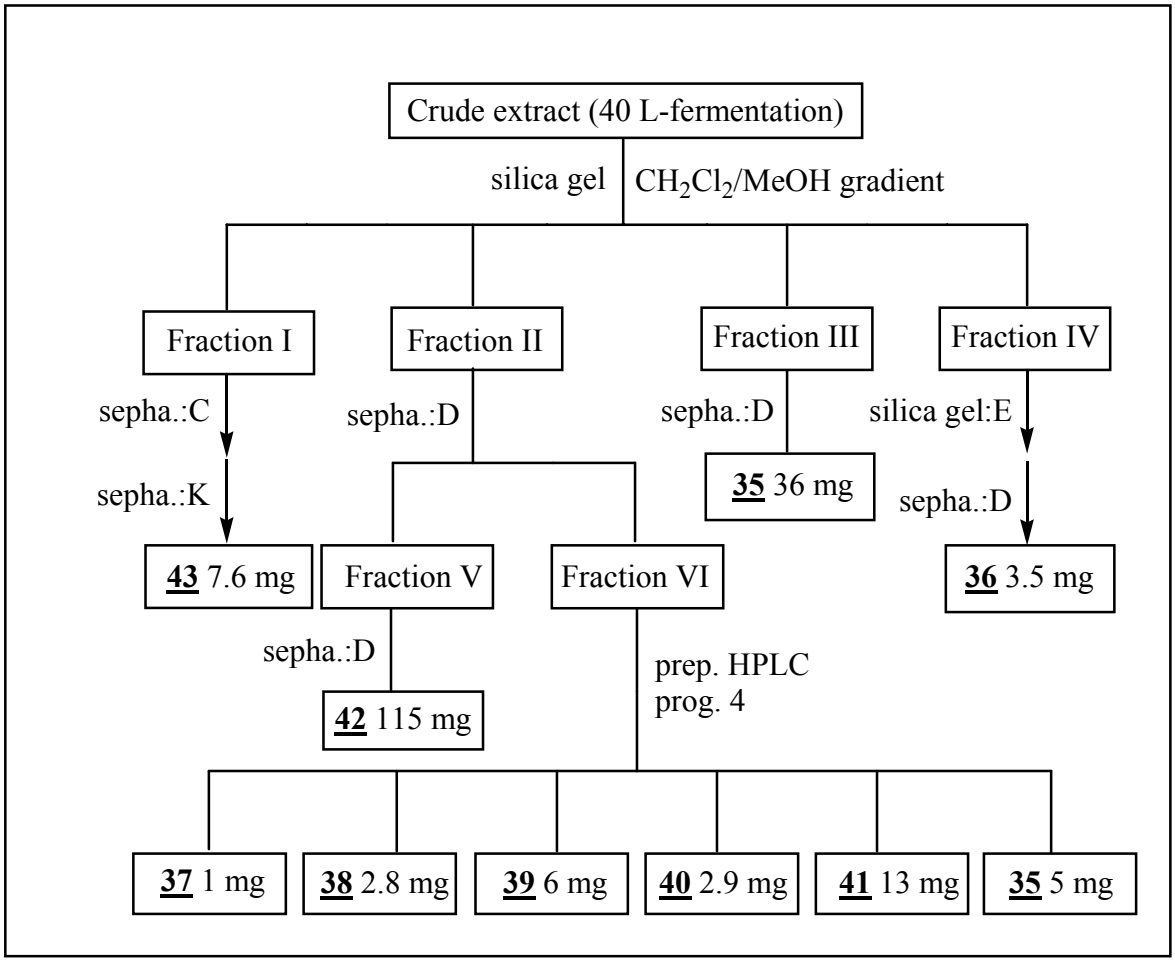


Fig. 25 Selected HMBC correlation of **34**

3. Scale-up fermentation of strain with MB medium for generating further secondary metabolites

3.1. Fermentation of strain and isolation of secondary metabolites

A 40 L-fermentation was carried out with the medium MB and artificial seawater at 27 °C for three days. 3.7 g crude extract of the culture filtrate (adjusted to pH 4 prior to extraction) was separated according to Scheme 13 by column chromatography and preparative HPLC.



* sepha: Sephadex LH-20; C, D, E, K: solvent systems

Scheme 13 Isolation procedure for the compounds **35** to **45**

Fraction VI (92 mg) contained several minor components, which exhibited quite similar R_f values on TLC [R_f ~ 0.4 $CHCl_3$/MeOH (9 : 1, 1% formic acid)]. The whole fraction was separated and purified by preparative HPLC (prog. 4). After optimization of the separation conditions the six components showed a good resolution in the chromatogram (Fig. 26).

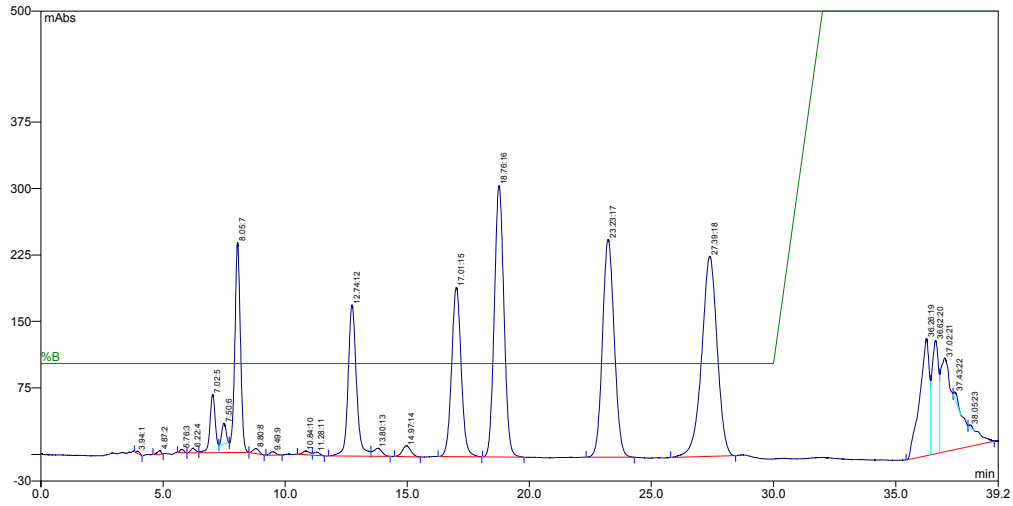
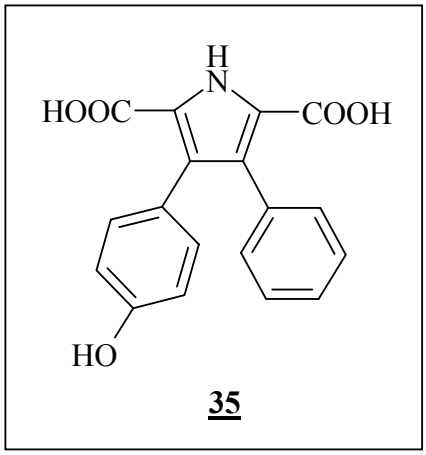


Fig. 26 HPLC chromatogram of Fraction VI for the preparative HPLC

3.2. Structure elucidation of the isolated secondary metabolites

3.2.1. Structure elucidation of 3-(4′-hydroxyphenyl)-4-phenylpyrrole-2,5-dicarboxylic acid (35)



35

Compound **35** is a colorless amorphous solid isolated as a second major component (1 mg/L). It is well soluble in methanol and DMSO, scarcely in chloroform and other nonpolar solvents. It showed a R_f-value of 0.28 in $CHCl_3$/MeOH 9 : 1. Staining with anisaldehyde led to strong blue color. The EI-MS spectrum exhibited the highest peak at m/z = 235. The formula $C_{16}H_{13}NO$ was established by the HREI-MS. The IR spectrum indicated the presence of O-H or N-H groups (3407 cm^{-1}), a benzene ring (1593 cm^{-1}) and a substituted benzene ring (strong bands between 700 and 900 cm^{-1}). The UV spectrum showed an absorption maximum at 245 nm, which indicates also the presence of a benzene ring.

The ^{1}H-NMR spectrum exhibited four multiple peaks of aromatic protons with a ratio of 3 : 2 : 2 : 2 and one exchangeable proton signal. The ^{1}H-^{1}H COSY spectrum showed coupling of the signals at $\delta = 6.55$ and 6.88, 7.08 and 7.15 ppm. The ^{13}C-NMR and APT spectra provided 14 carbon signals, two of them located at about δ = 163 ppm and 12 of them located between δ = 115 – 157 ppm, which revealed the presence of two α, β–unsaturated carboxylic carbons, five unsubstituted aromatic carbons and seven substituted aromatic carbons (Fig. 27).

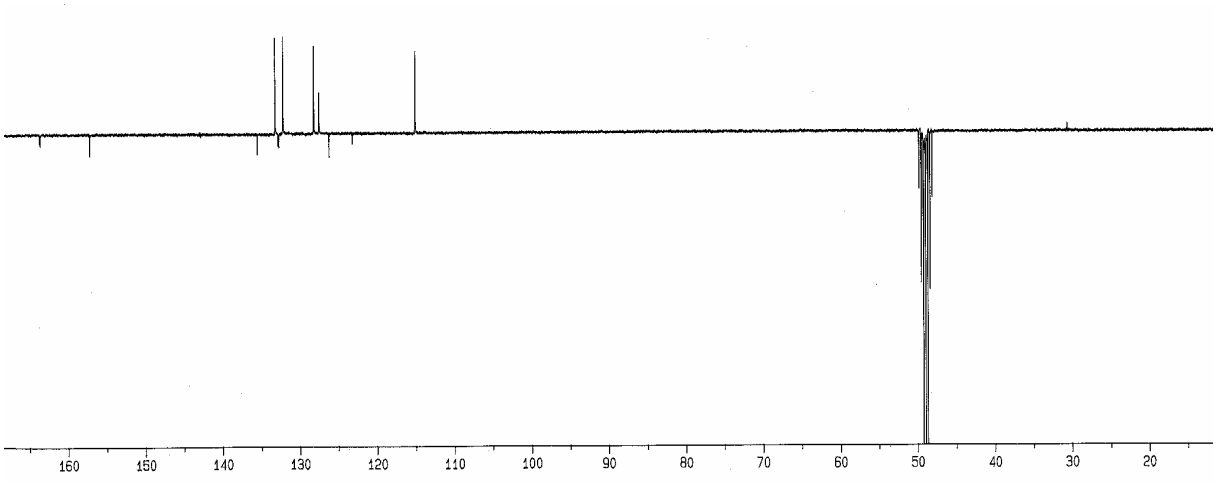


Fig. 27 APT-NMR spectrum (75.5 MHz, CD_3OD) of **35**

From the HMBC spectrum two moieties could be deduced (Fig. 28).

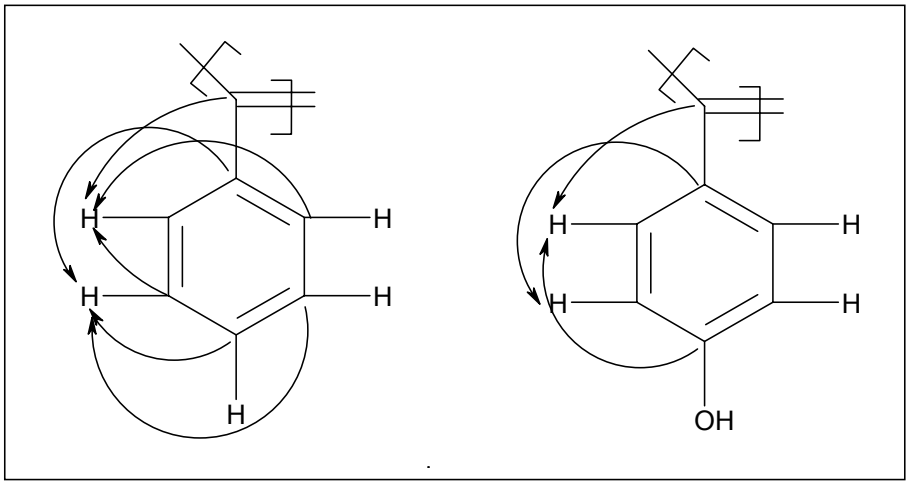


Fig. 28 Selected C-H long range correlation of **35**

A database search (*Chapman & Hall* and *Antibase*) by various strategies delivered no structure which fits the above NMR data. In order to get single crystals many solvent systems were tried for X-ray analysis. Crystals grew successfully from saturated DMSO solution by

keeping it at 0 °C for two weeks. EI-MS analysis of the crystal provided the molecular ion peak m/z = 323 and the formula $C_{18}H_{13}NO_5$ was afforded by HREI-MS. The peak m/z = 235 indicated the fragment $[M\text{-}2CO_2]^+$, which indicated the presence of two carboxylic acid groups.

Compound **35** crystallizes in the monoclinic space group *C*2/*c*. As shown in Fig 29 (b) and Fig. 30, **35** cocrystallizes with three DMSO solvent molecules involving an extensive hydrogen bond between carboxylic acid, the protons of amine and the oxygen atoms in DMSO. The hydrogen-bonded donor-acceptor separations (2.624 Å, O–H 1.022 Å, O···H 1.626 Å, O–H···O 164.11° and O···O 2.567 Å, O–H 1.022 Å, O···H 1.570 Å and O–H···O 163.81°) are within the range of other similar interactions exhibited by compound 3,4-diphenylpyrrole-2,5-dicarboxylic acid acetic acid disolvate (O···O 2.630(8) Å and 2.686(7) Å).[87] The N–H···O hydrogen-bond distance (N···O 2.910 Å, N–H 1.061 Å, H···O 1.902 Å, N–H···O 170.57°) is comparable to that in 3,4-diphenylpyrrole-2,5-dicarboxylic acid/acetic acid disolvate (N···O 2.969(7) Å). The C(51)–O(51) (1.199(6) Å) and C(21)–O(21) (1.206(6) Å) double bond and the C(21)–O(22) (1.317(6) Å), C(51)–O(52) (1.322(6) Å) as well as C(44)–O(4) (1.370(6) Å) single bond lengths are similar with those found in 3,4-diphenylpyrrole-2,5-dicarboxylic acid acetic acid disolvate (C=O 1.208(8) Å and 1.229(7) Å; C–O 1.312(8) Å and 1.324(8) Å).

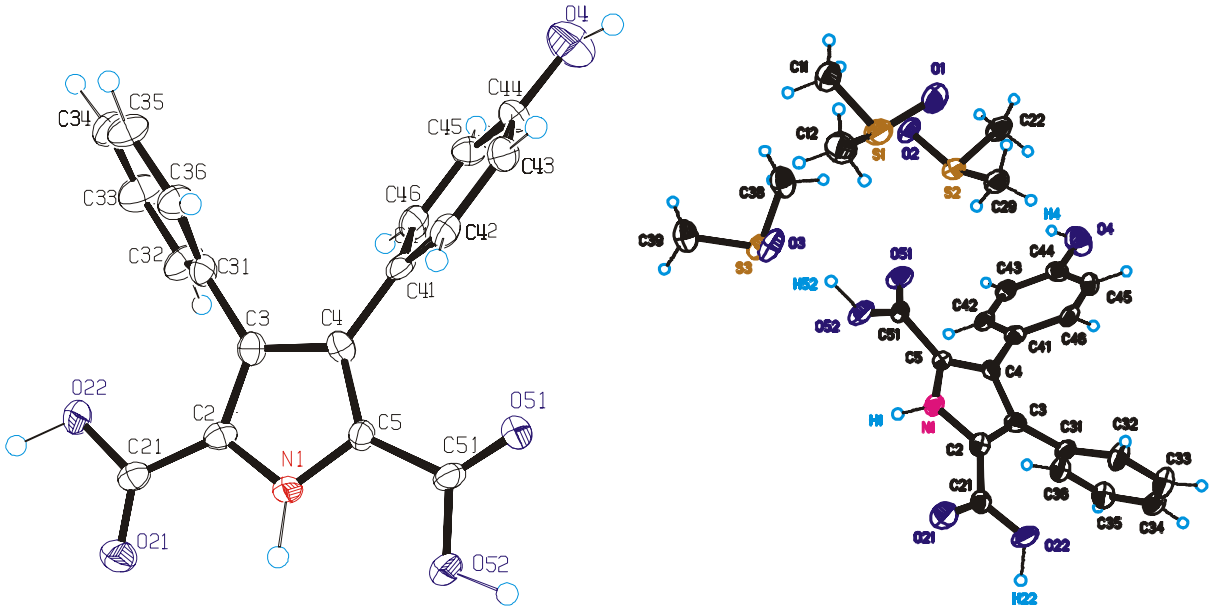


Fig. 29 (a) Prospective view of **35** (b) Molecular structure of **35** with three DMSO molecules (50 % probability ellipsoids)

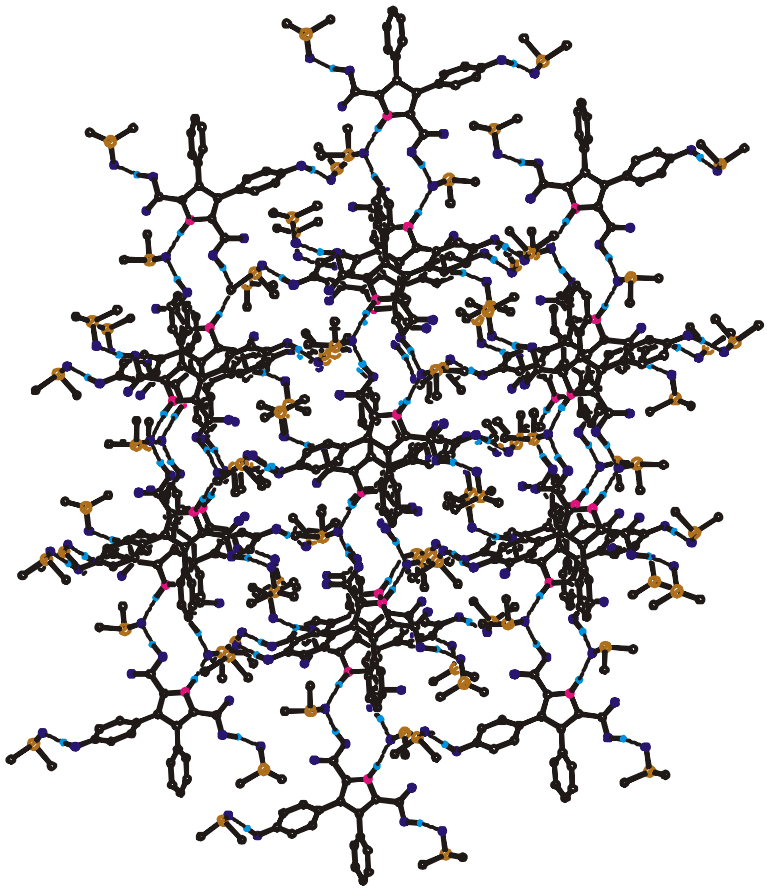
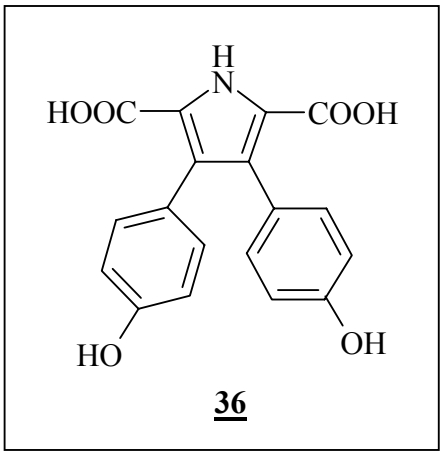


Fig. 30 A packing diagram of **35**

Tab. 9 Selected Bond Lengths (Å) and Angles (°) of **35**

N(1)–C(2)	1.377(6)	N(2)–C(5)	1.364(6)
C(2)–C(3)	1.383(7)	C(3)–C(4)	1.416(7)
C(4)–C(5)	1.382(7)	C(2)–C(21)	1.471(7)
C(3)–C(31)	1.480(7)	C(4)–C(41)	1.483(7)
C(5)–C(51)	1.487(7)	C(21)–O(21)	1.206(6)
C(21)–O(22)	1.317(6)	C(51)–O(51)	1.199(6)
C(51)–O(52)	1.322(6)	C(44)–O(4)	1.370(6)
C(2)–N(1)–O(5)	108.1(4)	N(1)–C(2)–C(3)	108.7(4)
C(2)–C(3)–C(4)	107.1(5)	C(3)–C(4)–C(5)	106.7(4)
N(1)–C(5)–C(4)	109.4(4)	N(1)–C(2)–C(21)	118.9(4)
C(4)–C(3)–C(31)	124.9(5)	C(5)–C(4)–C(41)	128.3(4)
N(1)–C(5)–C(51)	119.9(4)	C(2)–C(21)–O(21)	123.2(5)
C(5)–C(51)–O(51)	124.5(5)		

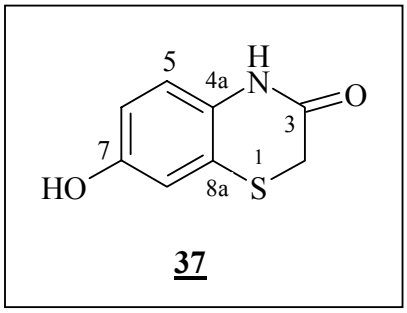
3.2.2. Structure elucidation of 3,4-di(4´-hydroxyphenyl)pyrrole-2,5-dicarboxylic acid (**36**)



36

Compound **36** is a colorless amorphous solid isolated as a minor component. It had the R_f-value 0.1 in the solvent system of $CHCl_3$/MeOH 9 : 1 (1% formic acid) and changed to a strong blue color with anisaldehyde spray. It is well soluble in methanol but not in other nonpolar solvent. EI-MS and DCI-MS have the molecular weight 251 and high resolution EI-MS established its formula $C_{16}H_{13}NO_2$, but the ESI-MS spectrum provided the molecular weight 339. The IR spectrum showed the broad band of O-H or N-H and a strong band of $\alpha, \beta-$ unsaturated carbonyl group at 1655 cm^{-1}. The UV spectrum showed the maximum at 250 nm, which revealed the presence of a benzene ring. The ^{1}H-NMR spectrum showed only two multiples of aromatic protons and one broad peak of exchangeable proton. The ^{13}C-NMR showed seven signals, one located at $\delta = 167$ ppm, six located at $\delta = 115 - 157$ ppm, which revealed the presence of a carboxylic group and a phenol group. Considering the structure of **35** and its EI-MS result, it was easy to deduce that **36** has the molecular weight 339, not 251. Based on the m/z = 16 difference of their molecular weight between **35** and **36**, the structure of **36** was established and all data were in agreement with it.

3.2.3. Structure elucidation of 7-hydroxy-2H-benzo[1,4]thiazin-3-one (37)



37

Compound **37** was obtained by preparative HPLC (1.1 mg). It is a colourless solid and soluble in methanol, acetone and chloroform. **37** showed an R_f-value of 0.44 in the solvent system $CHCl_3$/MeOH 9:1 (1% formic acid) and showed a light-brown color stained with anisaldehyde spray. The UV spectrum showed absorption maxima at 196 and 245 nm. The EI-MS spectrum provided the molecular weight m/z = 181 and high resolution EI-MS gave the molecular formula of $C_8H_7NO_2S$ indicating six double bond equivalents.

The ^{1}H-NMR spectrum indicated three aromatic protons, two of them in vicinal position (J = 8.6 Hz), and another proton in *meta*-position with one of the vicinal protons (J = 2.6 Hz). In addition, the resonance for a methylene group at δ = 3.36 ppm was observed. Two broad exchangeable proton signals at δ = 9.34, 3.00 ppm emerged in d_6-acetone, which should be attributed to one N-H proton and one O-H proton. The ^{13}C-NMR spectrum showed eight carbon signals, six of them are aromatic carbons with one phenolic carbon (δ = 154.9 ppm), one of them is a carbonyl carbon (δ = 167.8 ppm) and one is a methylene carbon (δ = 30.7 ppm). The numbers of carbon atoms and protons were in agreement with the formula supplied by HREI-MS.

According to the NMR data the molecule contained one phenyl group and one amide moiety, which included five double bond equivalents. Another double bond equivalent should result from a ring. In the databases *Antibase* and *Chapman & Hall* no similar molecules were found. But in the database *Scifinder* two possible structures were found, which are shown in Fig. 31. The HMBC spectrum of **37** supported structure a. Structure b (6-OH) could be ruled out due to the lack of corresponding ^3J coupling. All physicochemical data of **37** were consistent with the literature data of structure a, 7-hydroxy-2H-benzo[1, 4]thiazin-3-one.[88]

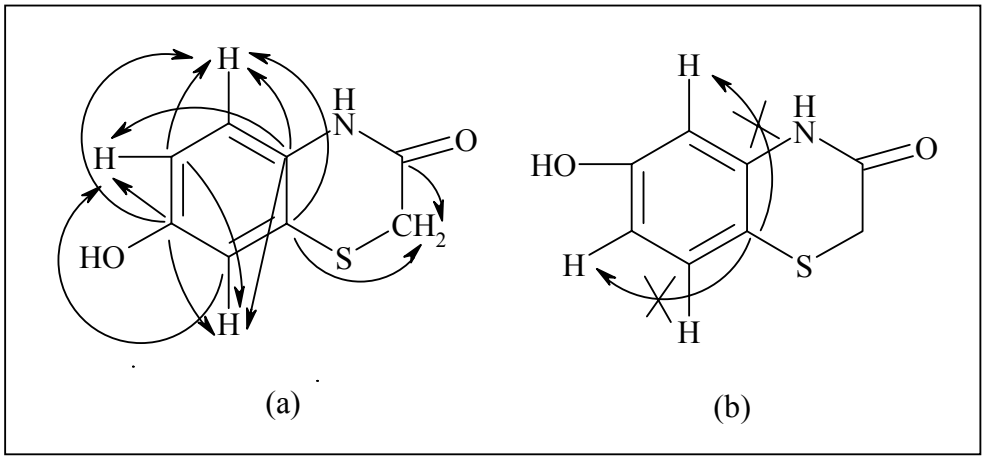
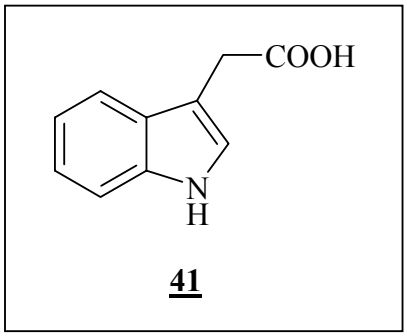


Fig. 31 C-H long range correlation of **37**

Compound **37** has been reported only as a synthetic intermediate.[88] Thus it is a new natural product.

3.2.4. Structure elucidation of indole-3-acetic acid (41)



41

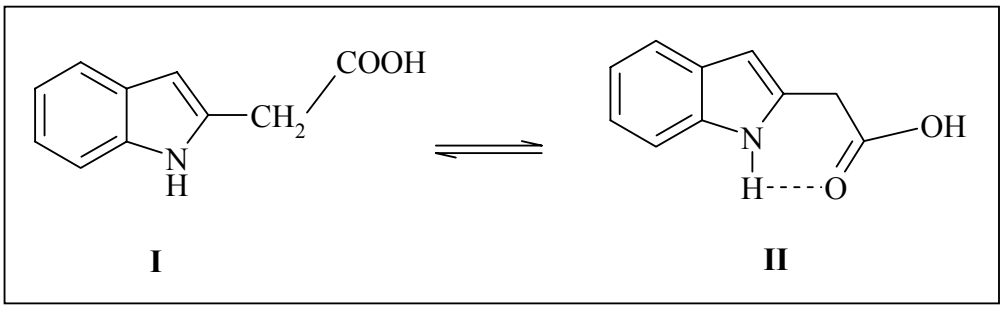
Compound **41** was isolated as a colourless solid by column chromatography and preparative HPLC from the culture filtrate of RK377. It was soluble in methanol, acetone or chloroform. It showed an R_f-value of 0.47 in the solvent system $CHCl_3$/MeOH 9:1 (1% formic acid) and had a brown color stained with anisaldehyde spray. The UV spectrum showed absorption maxima at 218 and 279 nm. The EI-MS spectrum gave the molecular ion peak of m/z = 175. The base peak m/z = 130 indicated the molecule fragment which had lost a carboxylic group. The peak m/z = 116 indicated the skeleton of indole. The mass database *Nist* afforded the possibility of indole-1-acetic acid. The ^{13}C-NMR spectrum showed 10 carbon signals, which indicated one carbonyl carbon (δ = 176.5 ppm), seven aromatic carbons (δ = 108 – 138 ppm), and one aliphatic carbon (δ = 32.0 ppm). The ^{1}H-NMR spectrum of **41** revealed four vicinal coupled aromatic protons (δ = 6.90 – 7.70 ppm), one single aromatic proton (δ = 7.14 ppm),

one methylene group (2H, δ = 3.74 ppm), and one exchangeable N-H proton (δ = 10.1 ppm), respectively. The presence of the N-H proton repudiated a substitution at N atom. The single peak at δ = 7.14 ppm indicated the substitution at C-3 rather than at C-2, because the chemical shift of the proton in 3-position should be at higher field due to the influence of delocalized lone electron pair of nitrogen. The ^{1}H-NMR data of three of indolylacetic acid isomers are listed in Tab. 10.[89] Values which have been useful for the differentiation between the isomers are expressed with bold types.

Tab. 10 Comparison of selected NMR data of **41** and three different indole acetic acid isomers

Compounds	δ CH_2 (ppm)	δ 3-H (ppm)	δ 4-H, 5-H, 6-H, 7-H (and 2-H) (ppm)	δ NH (ppm)
41	3.74 (2-H)		6.90-7.70 (5-H)	**10.1** (1-H)
Indole-3-acetic acid	3.80 (2-H)		6.90-7.75 (5-H)	**9.05** (1-H)
Indole-2-acetic acid	3.75 (2-H)	**6.25** (1-H)	6.75-7.50 (4-H)	**5.65** (1-H)
Indole-1-acetic acid	**4.85** (2-H)	**6.40** (1-H)	6.80-7.60 (5-H)	

In comparison, indole-2-acetic acid exhibits the chemical shift of the N-H proton at higher field at δ = 5.65 ppm due to the formation of an intramolecular hydrogen bond illustrated by Scheme 14. Meanwhile, the resonance of methylene protons of indole-1-acetic acid occurs at lower field (δ = 4.85 ppm) due to the electron withdrawing effect of the nitrogen atom. All data of **41** are in agreement with the data of indole-3-acetic acid as reported in the literature.[89]

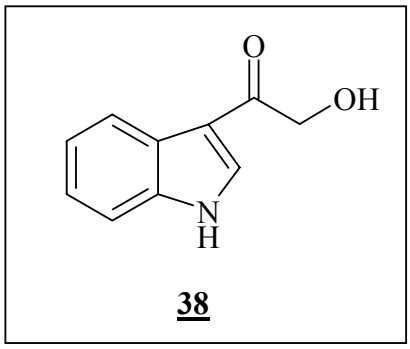


Scheme 14 The intramolecular hydrogen bond of indole-2-acetic acid

Indole-3-acetic acid (**41**) is widely distributed in higher plants and was also isolated from marine algae. It is the major plant growth hormone (auxin) and it is involved in the regulation

of almost every step in plant development.[90] Indole 3-acetic acid was also isolated from *Rhizobium sp*, a simbiotic bacterium isolated from healthy and mature root nodules of a leguminous tree, *Dalbergia lanceolaria*.[91] It was also supposed that indole-3-acetic acid is produced by symbiotic bacteria in algae.[92]

3.2.5. Structure elucidation of 3-hydroxyacetylindole (38)

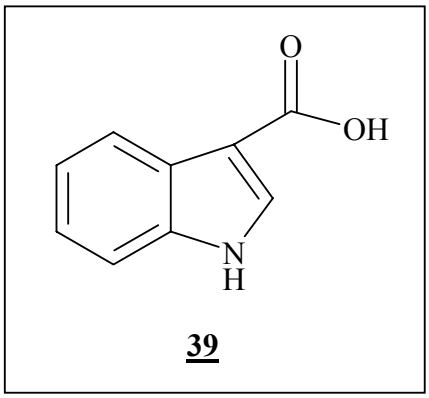


38

Compound **38** was isolated together with **41**. It showed a R_f-value of 0.38 in the solvent system $CHCl_3$/MeOH 9 : 1 (1% formic acid) and had a brown color stained with anisaldehyde. The EI-MS spectrum gave the molecular ion peak m/z = 175. The base peak m/z = 144 indicated the fragment $[M\text{-}CH_2OH]^+$ and the peak m/z = 116 revealed the skeleton of indole. The ^{13}C-NMR spectrum showed 10 carbon signals, which indicated one α, β – unsaturated ketone carbon (δ = 196.0 ppm), eight aromatic carbons (δ = 122-138 ppm), and one aliphatic carbon attached to oxygen (δ = 66.3 ppm). The ^{1}H-NMR spectrum of **38** displayed four vicinal coupled aromatic protons (δ = 7.18 – 8.22 ppm), one single aromatic proton (δ = 8.19 ppm), one methylene group (δ = 4.70 ppm), one exchangeable N-H proton (δ = 11.18 ppm), and one exchangeable O-H proton (δ = 3.88 ppm), respectively. Therefore the structure of **38** was established as 3-hydroxyacetyl-indole. All data of **38** are in agreement with those of the literature.[93]

38 was previously isolated from red algae,[94] from the sponge *T. ignis*, [93] and also from *Micrococcus sp*., growing in association with *T. ignis*.[95] It was proved that **38** is a tryptophan metabolite in myxobacteria.[96]

3.2.6. Structure elucidation of indole-3-carboxylic acid (39)

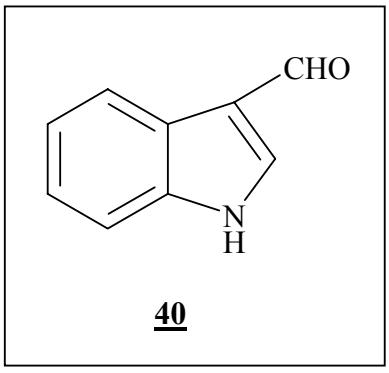


39

Compound **39** was isolated together with **41**. The EI-MS spectrum gave the molecular ion peak of m/z = 161. The peak m/z = 144 indicated the fragment $[M\text{-}OH]^+$ and the peak m/z = 116 represents the skeleton of indole. The ^{13}C-NMR spectrum showed nine carbon signals, which indicated one carbonyl carbon (δ = 169.3 ppm) except for the eight aromatic carbons of indole (δ = 108 – 138.2 ppm). In addition, two broad signals of exchangeable protons were observed at δ = 11.0 ppm (N-H proton) and δ = 4.7 ppm (COOH proton) in the ^{1}H-NMR spectrum of **39**. Combining these data **39** was elucidated as indole-3-carboxylic acid and confirmed by the data in the literature.[97]

Indole-3-carboxylic acid was isolated previously from plants and marine algae.[98]

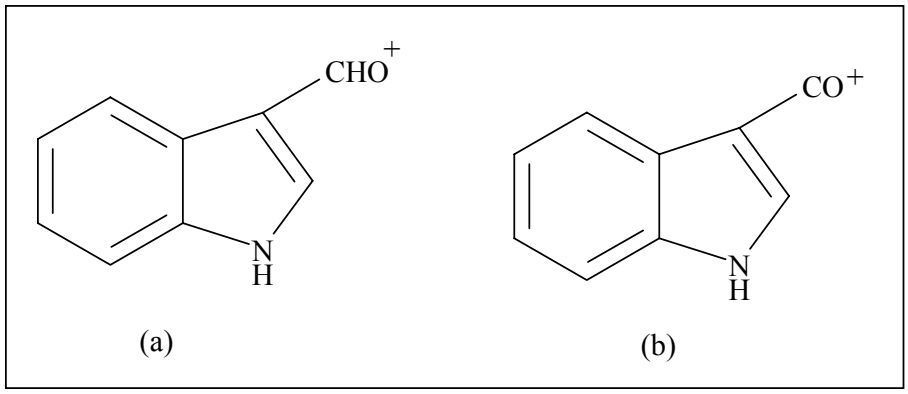
3.2.7. Structure elucidation of indole-3-carboxaldehyde (40)



40

Compound **40** was isolated together with **41**. The EI-MS spectrum gave the molecular ion peak of m/z = 145. The peak m/z = 116 indicated the skeleton of indole and the fragment [M-

CHO]$^+$. The ^{13}C-NMR spectrum showed one additional $\alpha,\beta-$ unsaturated aldehyde carbon ($\delta = 187.4$ ppm). The ^{1}H-NMR spectrum of **40** displayed also the proton of aldehyde ($\delta = 9.88$ ppm). Besides the molecular ion peak m/z = 145 (see Scheme 15, structure **a**), the EI-MS spectrum showed the base peak at m/z = 144 [M-1]$^+$, which could be explained by structure **b** in Scheme 15.

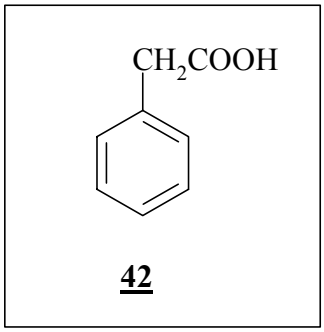


Scheme 15 Characteristic fragments in the EI-MS spectrum of **40**

From these data compound **40** can be identified as indole-3-carboxaldehyde, which was confirmed by comparison with reference data.[99]

Indole-3-carboxaldehyde (**40**) is widely distributed in plants and also in red algae.[100]

3.2.8. Structure elucidation of phenylacetic acid (42)



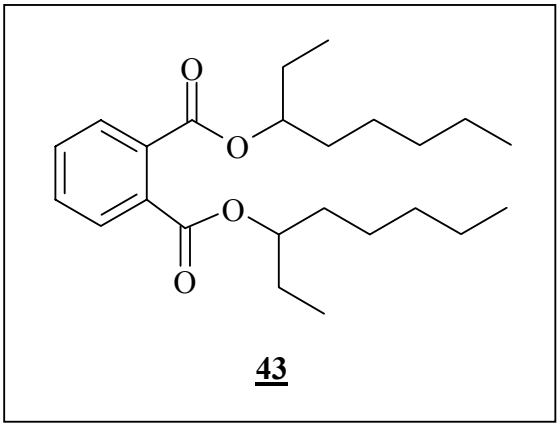
42

Compound **42** was produced as major product by strain RK377 (3 mg/L). It gave no colour-reaction with usual staining reagents. The EI-MS spectrum showed the molecular ion peak m/z = 136 and basic peak m/z = 91. The difference m/z = 45 between the two peaks m/z =136 and m/z = 91 indicated the presence of a carboxylic group. The ^{1}H-NMR spectrum showed a

multiple with five protons between δ = 7.2 – 7.4 ppm and a single signal at δ = 3.6 ppm with two protons. Combining these data **42** was easily deduced as phenylacetic acid.

Phenylacetic acid occurred in a fungus as phytotoxin[101] and was also isolated from a marine streptomyces.[102]

3.2.9. Structure elucidation of bis(2-ethylhexyl)phthalate (43)



43

The colorless, oily compound **43** is well soluble in acetone and chloroform, poorly in methanol, and insoluble in water. It showed a R_f value of 0.58 in the solvent system cyclohexane/ethyl acetate = 6 : 1. It absorbed UV-light of 254 nm and changed to a brown color stained with anisaldehyde.

The EI-MS spectrum gave the molecular ion peak m/z = 390, which was confirmed by ESI-MS. The ^{1}H-NMR spectrum indicated two vicinal aromatic protons at δ = 7.4 – 7.8 ppm, one oxygen-linked methylene group (2H, δ = 4.2 ppm), one methine proton (δ = 1.65 ppm), a multiple peak of methylene protons in the range of δ = 1.2 – 1.5 ppm, and two methyl groups (δ = 0.85 – 0.95 ppm).

The ^{13}C-NMR spectrum showed 12 carbon signals and an APT experiment (Fig. 32) indicated two methyl carbons (δ = 10.9, 14.0 ppm), one methine carbon (δ = 38.7 ppm), five methylene carbons (δ = 23.0, 23.7, 28.9, 30.4, 68.1 ppm), two protons bearing aromatic carbons (δ = 128.8, 130.9 ppm), one quaternary aromatic carbon (δ = 132.4 ppm) and one carbonyl carbon (δ = 167.7 ppm). It was deduced from the analysis of the molecular weight and the NMR data that the compound had a symmetric phthalate structure. Phthalates include several derivatives, which are different at the side chain of the ester. The ^{1}H-^{1}H-COSY spectrum of **43** revealed that the methine group was located between the oxygen-bonded methylene group and the

other methylene groups. Therefore **43** was assigned as bis(2-ethylhexyl)phthalate. All data are identical with the literature data.[103]

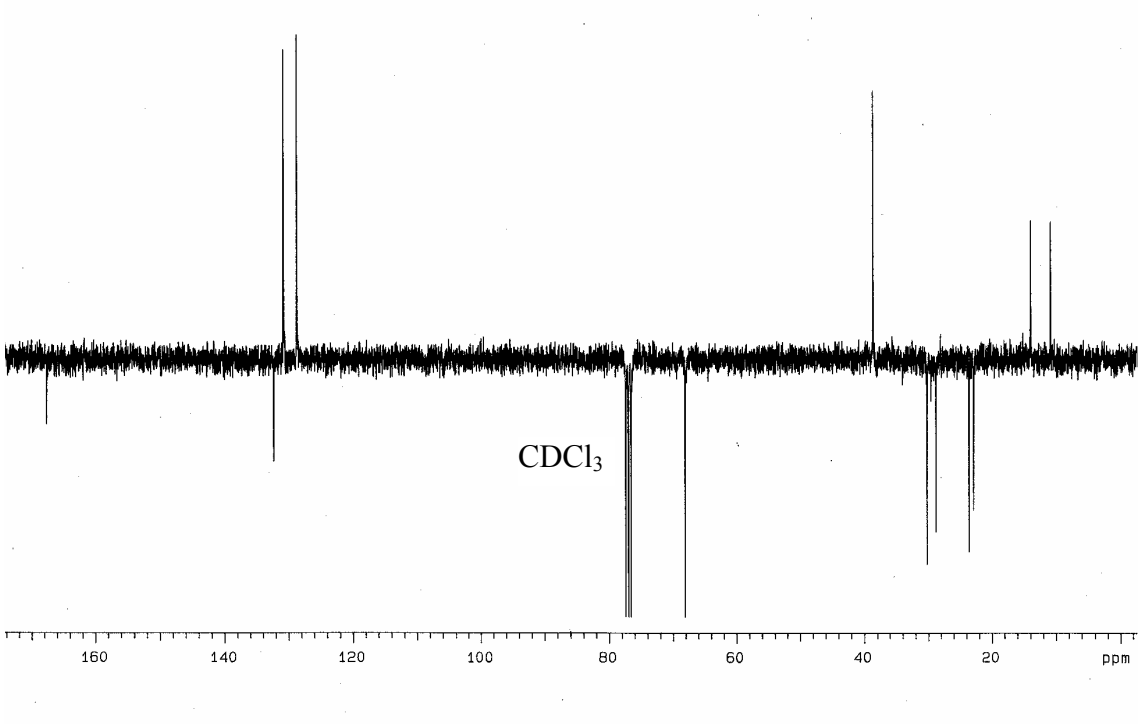


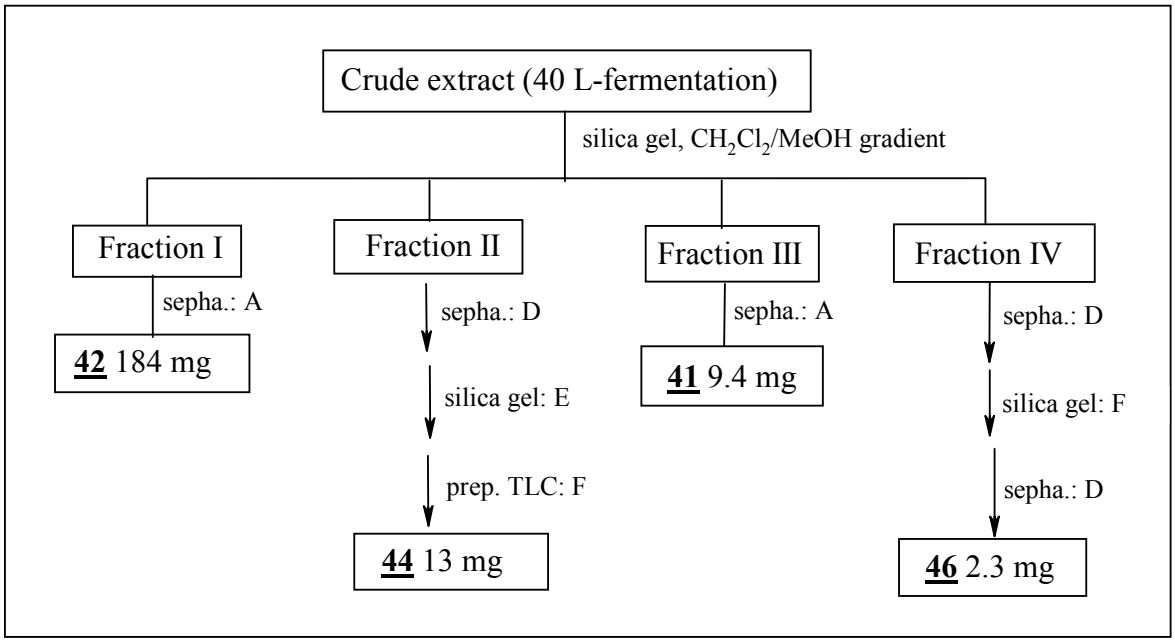
Fig. 32 APT-NMR spectrum (75.5 MHz, CD_3Cl_3) of **43**

Compound **43** was reported as natural product isolated from *Streptomyces* sp.,[104] and from *Penicillium olsonii*.[103] But it is also often used as additive in plastic products. In this situation we do not rule out that **43** is a contaminant.

4. Scale-up fermentation of RK377 with SJ medium for generating further secondary metabolites

4.1. Fermentation and isolation

In order to investigate the biosynthetic potential of RK377, cultivation of RK377 in SJ medium was attempted. New spots were found on TLC plate stained with $PdCl_2$, which were supposed to belong to a sulphur-containing compound. A 40 L-fermentation was carried out and the culture filtrate was extracted with ethyl acetate after adjustment to pH 3 – 4 with HCl. The isolation was accomplished according to Scheme 16.



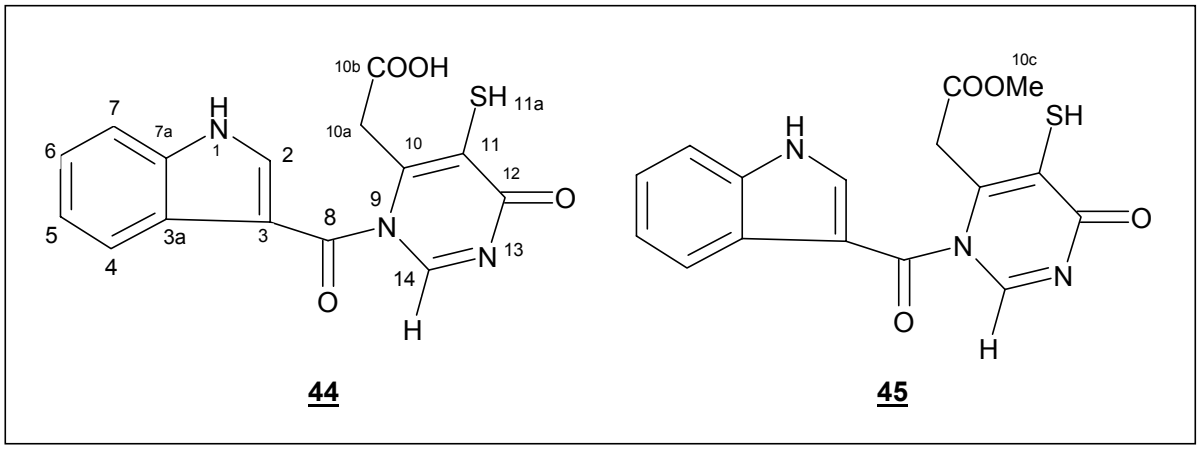
*sepha: Sephadex LH-20; A, D, E, and F: solvent system.

Scheme 16 Isolation procedure of substances **41**, **42**, **44** and **46**

The already described phenylacetic acid (**42**) and indole-3-acetic acid (**41**) were isolated as main components (4.6 mg/L and 0.23 mg/L, respectively). But the two new pyrrole derivatives **35** and **36** were not produced in the new medium.

4.2. Structure elucidation of isolated secondary metabolites

4.2.1 Structure elucidation of Glusun I (44)



44 **45**

Compound **44** was isolated as a yellow solid and was soluble in methanol and scarcely in other nonpolar solvents. Stained with $PdCl_2$ it showed an orange color. The R_f-value of **44**

was determined as 0.43 in the solvent system $CHCl_3$/MeOH 9 : 1 (1% formic acid). Its UV spectrum showed absorption maxima at 272, 278 and 357 nm. The ESI-MS spectrum afforded the molecular weight M = 329 g/mol. The ^{1}H-NMR spectrum of **44** revealed four vicinal aromatic protons, two single aromatic protons at δ = 9.30, 8.42 ppm and one single methylene peak at δ = 4.12 ppm. The ^{13}C-NMR spectrum showed 14 carbon resonances, three of them located at δ = 178.6, 171.6, 163.1 ppm, 10 of them located at δ = 111 – 152 ppm, one was at δ = 43.5 ppm.

In order to get its EI-MS spectrum methylation of **44** was carried out by the reaction with an excess of diazomethane at room temperature. **44** was converted almost completely into **45**, which was monitored by TLC. **45** afforded the EI-MS spectrum (M^+: m/z = 343), HREI-MS led to the molecular formula $C_{16}H_{13}SN_3O_4$.

From the molecular formula of **45**, the molecular formula $C_{15}H_{11}SN_3O_4$ was deduced for **44** and it was consistent with the result of the ESI-MS spectrum (m/z = 329). Fig. 33 shows the EI-MS spectrum of **45**, where the fragments m/z = 144, 116, 89 indicates the presence of the indole skeleton described in Scheme 17. [105]

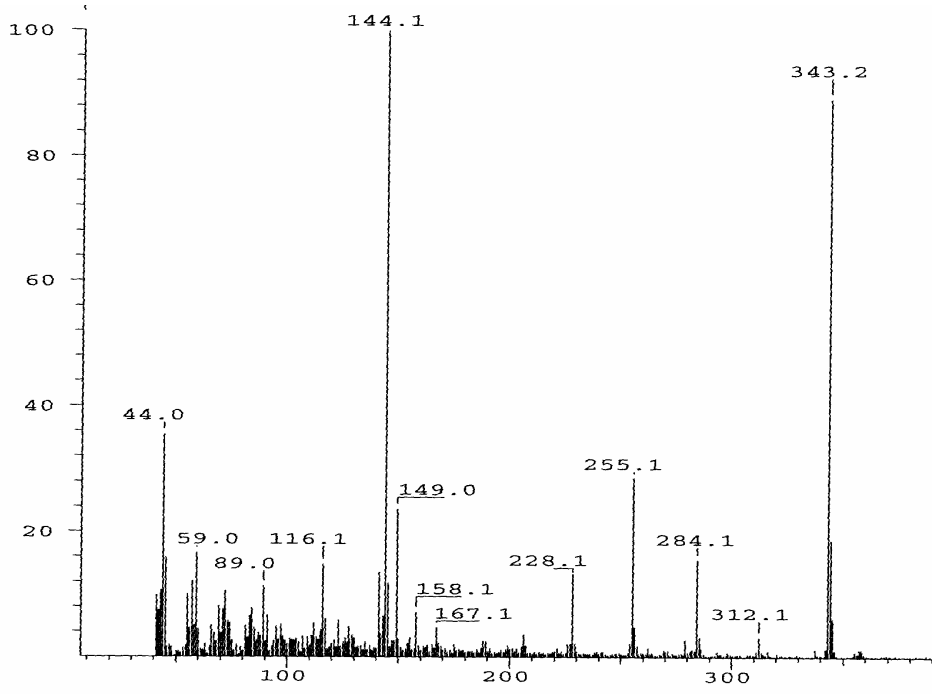
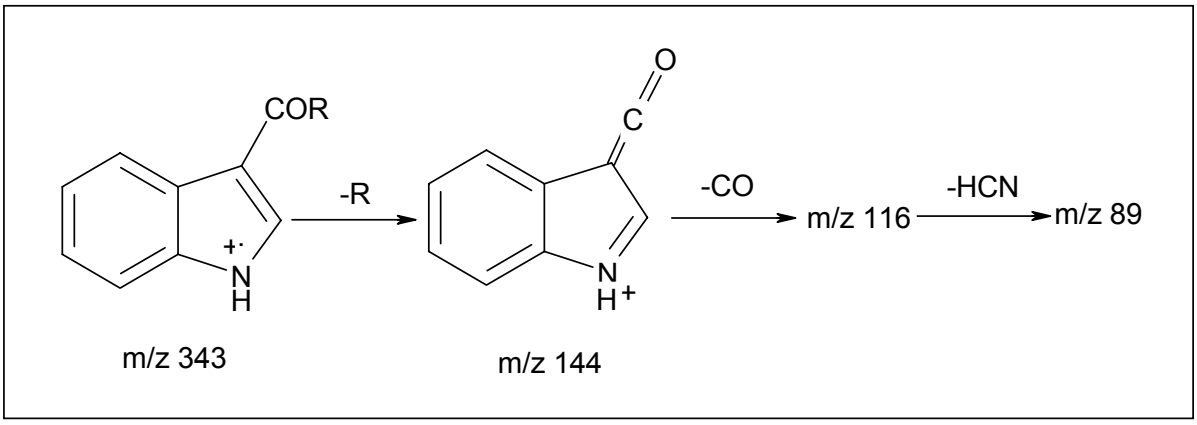


Fig. 33 EI-MS spectrum of **45**



Scheme 17 Fragments of **45** indicated the presence of indole

The ^{1}H-NMR spectrum of **45** additionally revealed the presence of two exchangeable protons at δ = 9.14, 7.69 ppm and one signal of a methyl group. The ^{13}C-NMR spectrum of **45** revealed two additional carbons, one carbon at δ = 177.6 ppm, which was not observed in **44**, and one carbon at δ = 52.7 ppm, which obviously belongs to the methyl carbon. All NMR data of **44** and **45** are listed in Tab. 11

Apart from an indole moiety and a carbonyl group the remaining elements must consist of an aromatic ring containing three carbons, one of them attached to a proton. Fig. 34 shows part of the HMBC spectrum. Combining all COSY, HSQC and HMBC data of **44** and **45**, a proposal for the moieties of **44** has been drawn in Fig. 35.

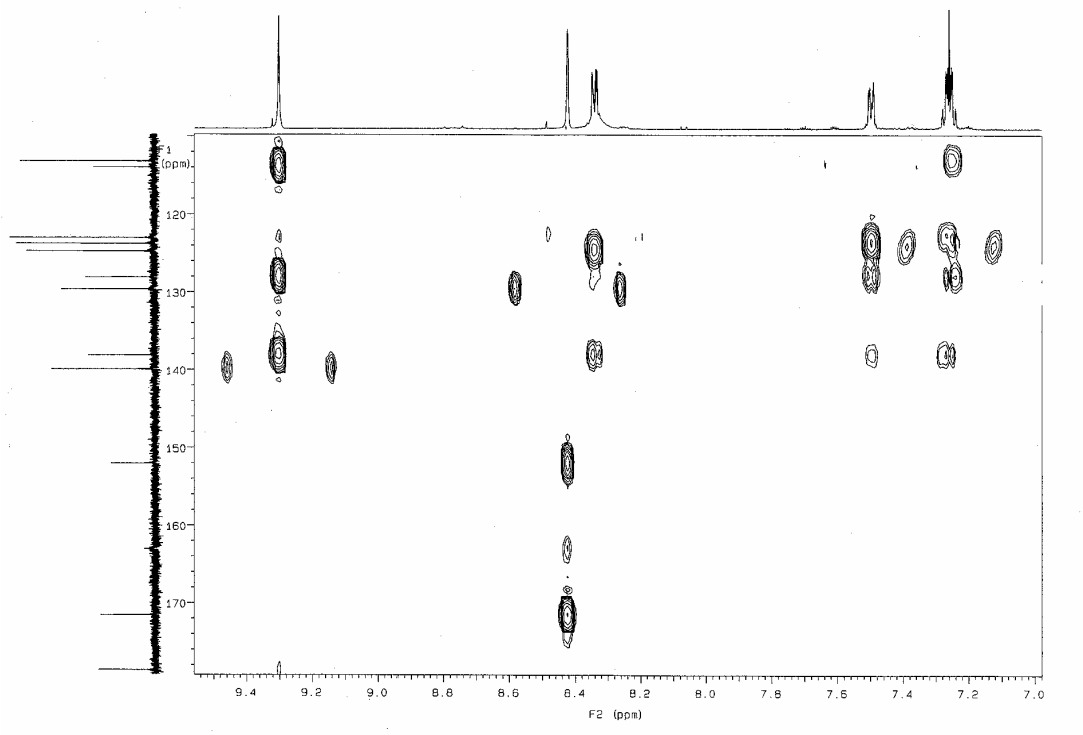


Fig. 34 Part of HMBC spectrum (600 MHz, CD_3OD) of **44**

Tab. 11 NMR data of **44** and **45**

Assign.	^{13}C-NMR of **44** (ppm) in d_4-methanol 150.8 MHz	^{1}H-NMR of **44** (ppm) in d_4-methanol 600MHz	^{13}C-NMR of **45** (ppm) in CD_2Cl_2 150.8 MHz	^{1}H-NMR of **45** (ppm) in CD_2Cl_2 600MHz
1	N			9.14 (1H, s)
2	140.0	9.30 (1H, s)	136.6	9.35 (1H, s)
3	114.0		113.9	
3a	128.2		126.8	
4	123.1	8.34 (1H, dd, *J*=6.5, 2 Hz)	122.6	8.44 (1H, m)
5	124.8	7.26 (1H, m)	124.5	7.34 (1H, m)
6	123.8	7.26 (1H, m)	123.5	7.34 (1H, m)
7	113.2	7.50 (1H, dd, *J*=6.5, 2 Hz)	112.0	7.52 (1H, m)
7a	138.2		136.3	
8	171.6		170.6	
9	N			
10	152.1		150.8	
10a	43.5	4.18 (2H, s)	41.4	4.25 (2H, d, *J* = 5.5 Hz)
10b	178.6		170.4	
10c			52.7	3.79 (3H, s)
11	absence		177.6	
11a	S			7.69 (1H, br. dd, *J* = 5.5, 5.5 Hz)
12	163.1		161.0	
13	N			
14	129.7	8.42 (1H, s)	129.0	8.38 (1H, s)

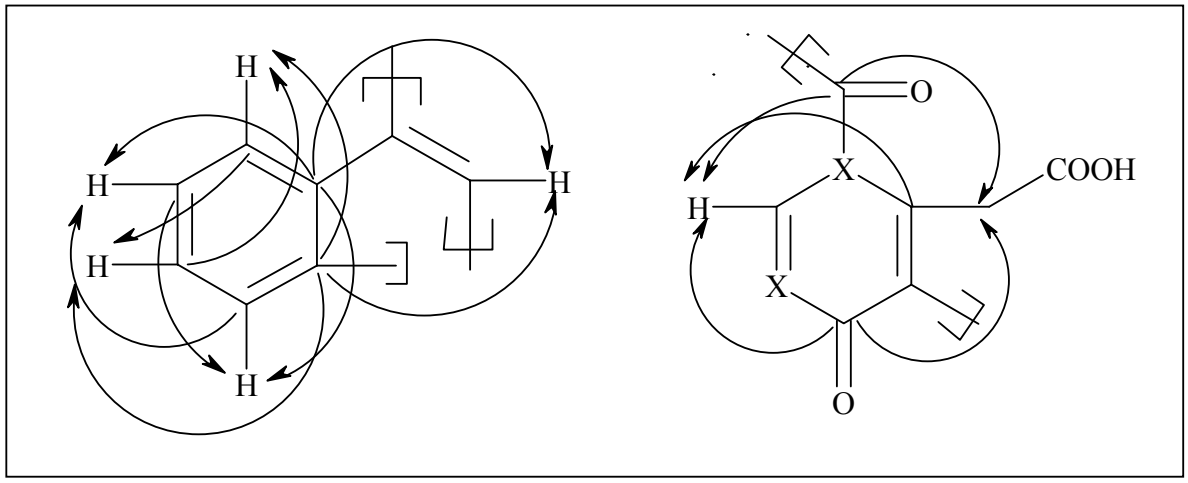
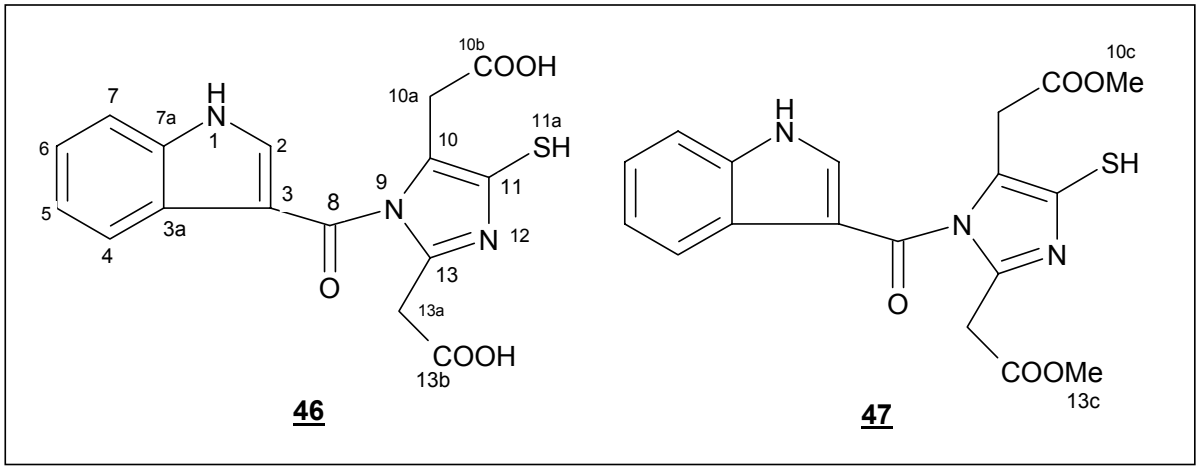


Fig. 35 Substructures of **44** derived from HMBC spectrum

Considering all data the two nitrogen elements should be located in the aromatic ring and the sulfur atom should be attached as a side chain. Thus structure **44** was supposed. The carbonyl polarization of C(12)–O(12) bond should lead to an aromatic character of **44** and **45** in agreement with the NMR data. Between the data of **44** and **45,** only one significant difference was observed at C-11. The signal for C-11 in **45** is located at δ=177.6 ppm, whereas there was no signal in this region for **44**. Due to the electron withdrawing effect of S and C-12 (carbonyl polarization gives rise to bear the positive charge for C-12), it is possible that C-11 shifted to downfield. Additionally it was supposed that due to the sp^2 hybrid, C-8 joined the two aromatic rings (indole and 12-oxo-9,12-dihydropyrimidin) and forms a conjugated π–system. Thus the color of **44** could be explained. Since the structure of **44** could not be elucidated directly, this should be seen as a proposal.

4.2.2. Structure elucidation of Glusun II (46)



Compound **46** was isolated as an orange solid and showed a red color stained with $PdCl_2$. It was soluble in methanol and scarcely in other nonpolar solvents. It had an R_f-value of 0.32 in the solvent system of $CHCl_3$/MeOH 5 : 1 (5% formic acid). Without the addition of acid the spot stayed at the start point. Its UV spectrum showed absorption maxima at 272 and 365 nm. The ESI-MS spectrum afforded its molecular weight as 359 and EI-MS had no defined result. The IR spectrum indicated the presence of N-H or O-H at 3441 cm^{-1} and carbonyl group at 1650 cm^{-1}. The ^{1}H-NMR spectrum in d_4-methanol showed five aromatic protons. Four of them are coupled with each other; one gave a singlet at δ = 7.70 ppm. Two singlets at δ = 4.10 and 3.62 ppm, which indicated two methylene groups attached to electron-withdrawing groups. The ^{13}C-NMR spectrum showed 16 carbons, which included three carbonyl carbons (δ = 165.2, 168.9, 173.9 ppm), 11 aromatic carbons (between δ=113 and 139 ppm) and two aliphatic carbons (δ = 42.8, 24.8 ppm).

In order to get an EI-MS spectrum methylation of **46** was carried out with an excess of diazomethane at room temperature. The reaction led almost completely to a single product, which was monitored by TLC. Since only 1 mg of **46** was used in this reaction, the product was analyzed without further purification. The EI-MS spectrum afforded the molecular ion peak at m/z = 387. The fragments of **47** indicated the presence of an indole. The HREI-MS of **47** led to the molecular formula $C_{18}H_{17}SN_3O_5$ and thus the molecular formula $C_{16}H_{13}SN_3O_5$ of **46** was deduced. Its molecular weight was consistent with the result of ESI-MS.

The ^{1}H-NMR spectrum of the methylated sample in CD_2Cl_2 indicated only the dimethyl derivative, namely two carboxylic groups (δ = 170.3, 165.3 ppm) were methylated. In addition, two exchangeable protons at δ = 8.80 and 8.03 ppm were observed. The former one was coupled with the proton at δ = 7.61 ppm, which indicated the N-H proton of the indole ring; the latter was coupled with a methylene group at δ = 4.16 ppm, which indicated their vicinal position. Table 12 lists all NMR data of **46** and **47**.

In addition to the indole moiety and a carbonyl group the remaining part of the molecule should consist of an aromatic ring containing three carbons. Fig. 36 shows the HMBC spectrum of **47**, from which the moieties of **46** have been deduced (see Fig. 37).

Tab. 12 NMR data of **46** and **47**

Assign.	^{13}C-NMR of **46** (ppm) in d_4-methanol 125.7 MHz	^{1}H-NMR of **46** (ppm) in d_4-methanol 500 MHz	^{13}C-NMR of **47** (ppm) CD_2Cl_2 150.8 MHz	^{1}H-NMR of **47** (ppm) CD_2Cl_2 600 MHz
1	N			8.80 (1H, d, *J* = 2 Hz)
2	132.7	7.70 (1H, s)	130.7	7.61 (1H, d, *J* = 2 Hz)
3	113.2		113.2	
3a	126.5		125.4	
4	121.1	7.56 (1H, d, *J* = 8 Hz)	120.0	7.52 (1H, dd, *J* = 8, 1.1 Hz)
5	121.7	7.16 (1H, dd, *J* = 8, 8 Hz))	121.6	7.19 (1H, ddd, *J* = 8, 8, 1.1 Hz)
6	123.7	7.21 (1H, dd, *J* = 8, 8 Hz)	123.5	7.25 (1H, ddd, *J* = 8, 8, 1.1 Hz)
7	113.1	7.44 (1H, d, *J* = 8 Hz)	112.2	7.45 (1H, *J* = 8, 1.1 Hz)
7a	138.7		136.6	
8	165.2		163.0	
9	N			
10	132.7		131.6	
10a	42.8	4.10 (2H, s)	41.6	4.16 (2H, d, *J* = 6 Hz)
10b	173.9		170.4	
10c			52.5	3.75 (3H, s)
11	138.3		136.9	
11a	S			8.03 (1H, dd, *J* = 6 Hz)
12	N			
13	138.9		137.8	
13a	24.8	3.62 (2H, s)	23.7	3.62 (2H, s)
13b	168.9		165.3	
13c			52.2	3.52 (3H, s)

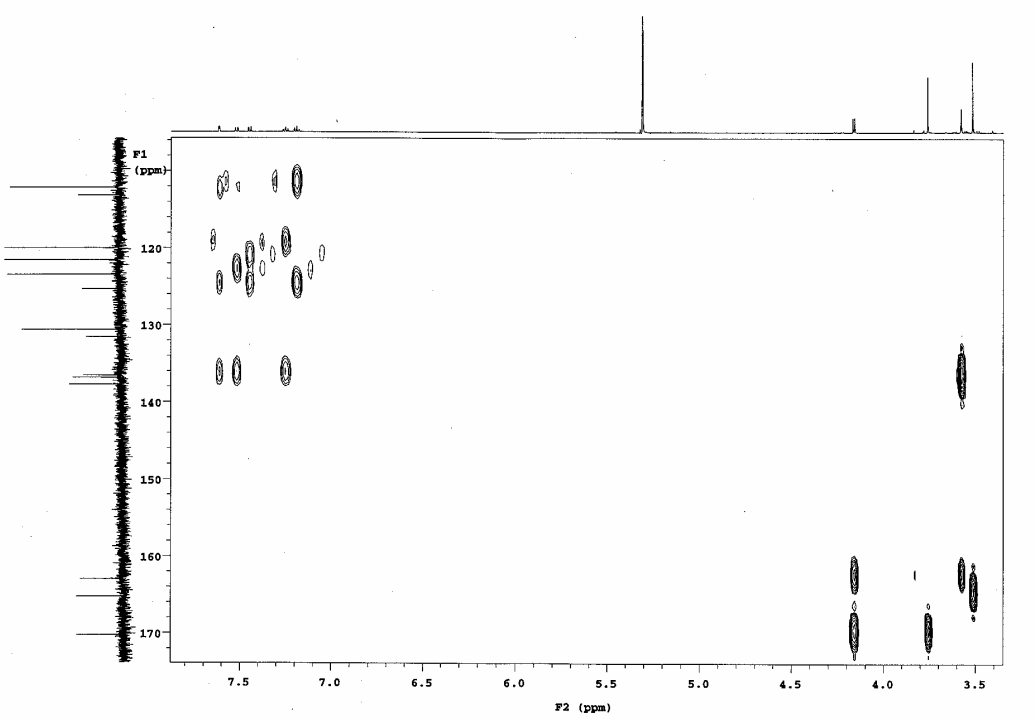


Fig. 36 HMBC spectrum (600 MHz, CD_2Cl_2) of **46**

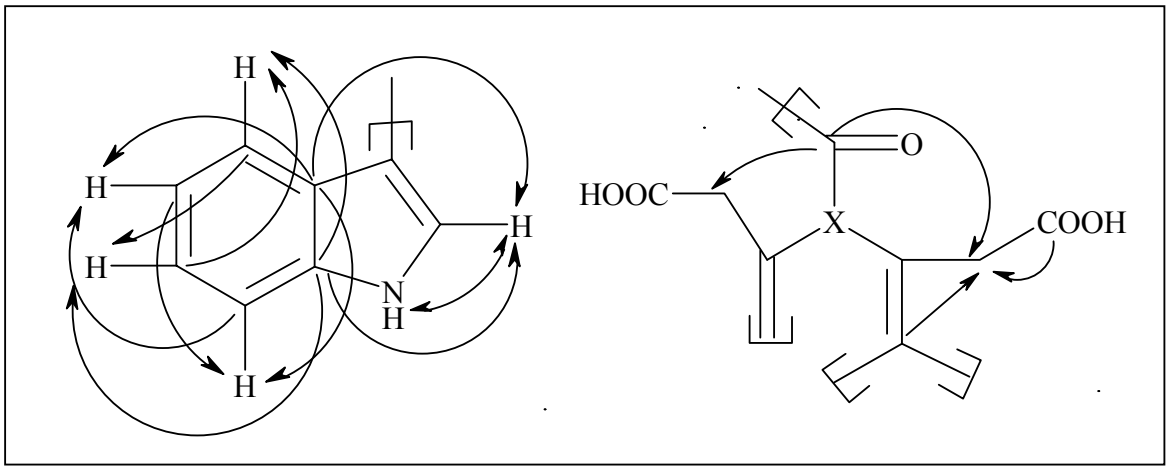
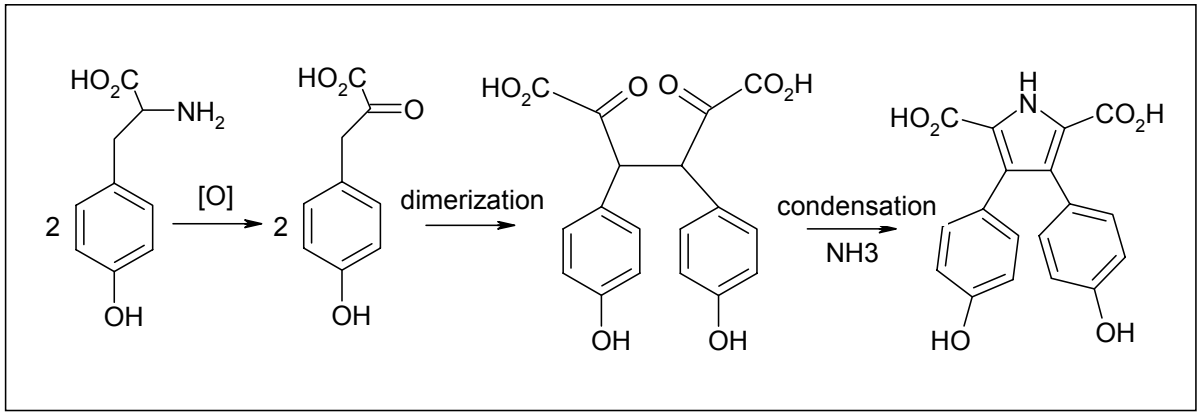


Fig. 37 C-H long range correlation of **46**

Considering all data the two nitrogen elements should be located in an aromatic ring and the sulfur atom should be attached as a side chain. Thus the structure of **46** was inferred. As previously discussed for **44** and **45**, it was supposed that C-8 joined the two aromatic rings of indole and imidazole and thus formed a conjugated π–system causing the orange color of **46**. The structure of **46** is a proposal and needs to be confirmed by further data.

5. Putative biosynthethic pathways of 35 and 36

Alkaloides with a 3,4-diarylpyrrole unit occur in a variety of marine organisms. Many of these compounds are of special interest due to their important biological activities.[106] They suggest a common biosynthesis commencing with the oxidative dimerization of two molecules of aryl (or heteroaryl)-pyruvic acid as already proposed by W. Steglich.[107] Reaction of the resulting 1, 4–diketones with ammonia or primary amines lead to 3,4–diarylpyrrole-2, 5-dicarboxylic acids. Further modifications in various ways generate alkaloids of increasing complexity. Scheme 18 depicts a putative biosynthetic pathway of **36**. The biosynthesis of **35** should follow the same path starting from tyrosine and phenylalanine, respectively. Since **35** and **36** represent prototypes of the pure dimerization products without further modification, their isolation as natural products described in the present work strongly supports the proposed biosynthetic pathway of 3,4.diarylpyrroles.[107]

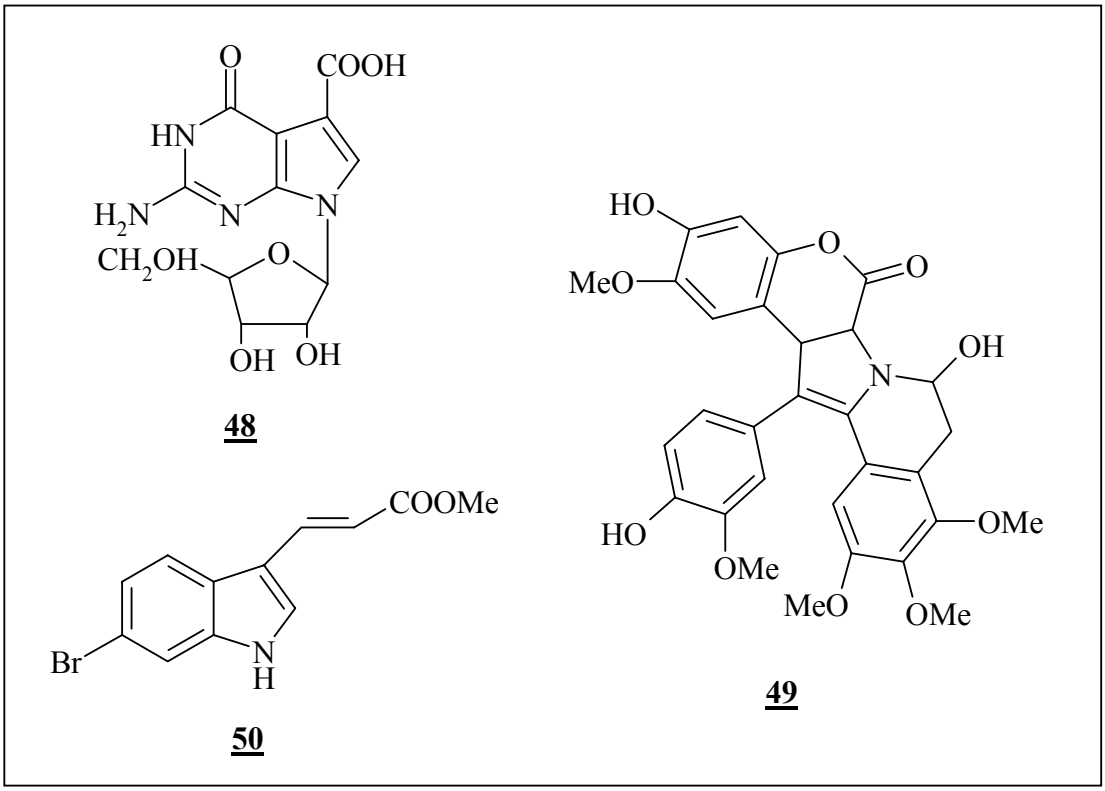


Scheme 18 Putative biosynthetic pathway of **36**

6. Results and discussion

Marine organisms yield a series of alkaloids such as indole, pyrrole, imidazole, pyrimidine or their combined systems. Many of them are of considerable interest because of the novelty of their structures and due to their striking biological activities.[108]

Most of these natural products exhibit various bioactivities. Most pyrimidin derivatives are combined with pyrrole, namely pyrrolepyrimidine. For example, Cadeguomycin (**48**), produced by *Streptomyces hygroscopicus*., inhibited tumor growth and metastasis in association with a modulation of the immune system.[109]



48

49

50

Lamellarin A-P series have been isolated from prosobranch mollusks[110] and ascidians[106] and lamellarin A-N (see Lamellarine A (**49**)) inhibited equally the growth of several tumor cell lines and reversed the P-glycoprotein mediated multidrug resistance (MDR) of tumor cells at very low concentration.[111] A great variety of simple and more complex functionalized indole derivatives were isolated and characterized from several marine organisms, most of them probably biogenetically derived from the amino acid tryptophan. For example, the methyl (E)-3-(6-bromo-3-indolyl)-3-propenoate (**50**) isolated from the sponge *Lotrochota* sp.[112] represents a structurally simple but interesting natural product of the vinyl indole series. Its chemistry and biology are widely documented.[113]

Phenylacetic acid and simple indole derivatives occur in many plants and also as metabolites of microorganisms. Most of them are able to promote the plant growth in plants.[114]

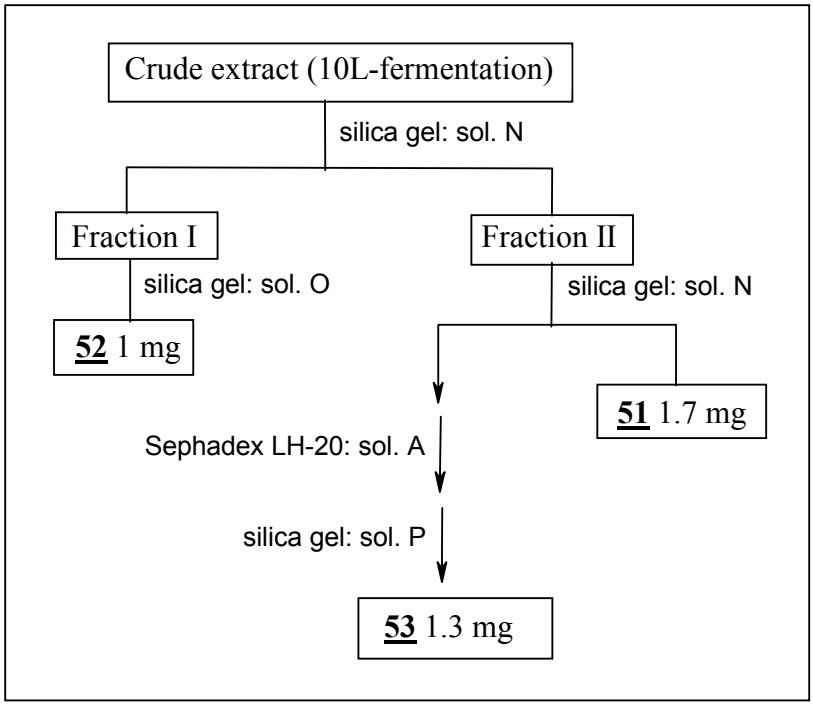
The isolation of two new pyrrole derivatives is of significance for the study of the biosynthesis of many complex bioactive pyrrole derivatives because they could be the intermediates of the latter.

In total strain RK377 produced 13 metabolites and gave indole into the profile of metabolites, which could be detected in North Sea bacteria. From this many metabolites in other screened strains were identified by HPLC-DAD screening, comparing their UV spectra.

V. Investigation of strain RK2207

1. Fermentation of strain and isolation of secondary metabolites

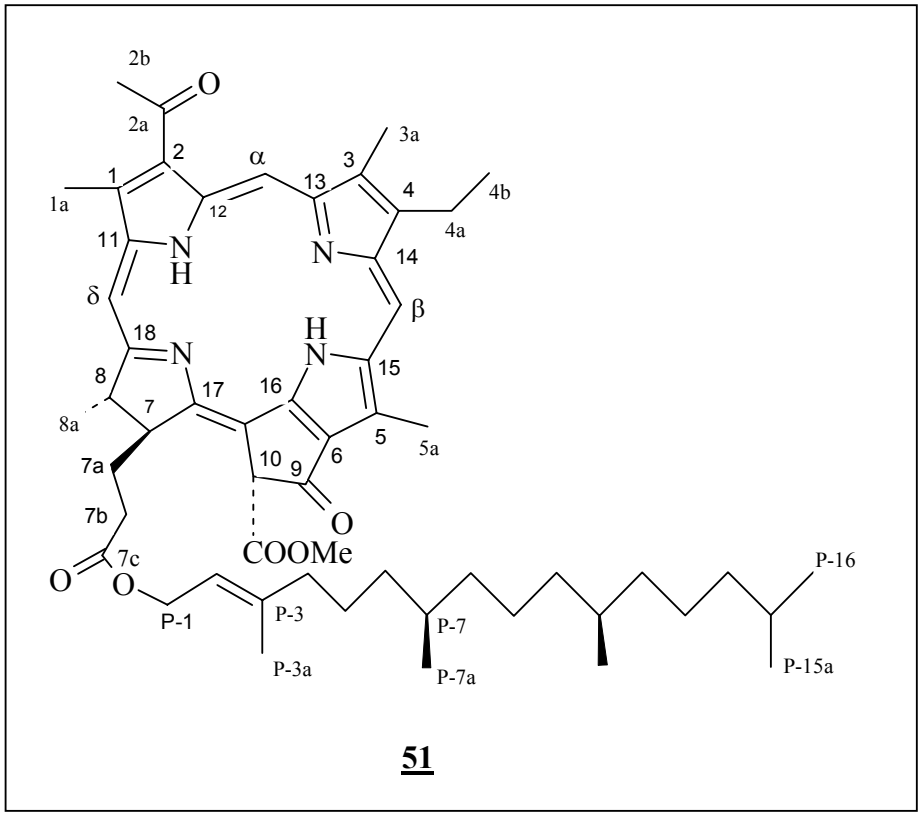
Strain RK2207 was isolated from a water sample of the North Sea. The bacterium is 96% related to *Mesorhizobium loti* (α-proteobacterium). Because of the intriguing deep red colour colonies and also due to several interesting spots observed on TLC in the chemical screening, strain RK2207 was chosen for further investigation. A 10 L-fermentation was carried out in SK medium at 30 °C for 3 days. HPLC-DAD analysis of a crude extract of the culture filtrate was carried out. Comparison of UV spectra indicated the presence of known indole derivatives; hence the isolation of the metabolites was not promising. The cell crude extract showed two red, several green and two violet spots on TLC without staining. Most of the pigments were unstable. Therefore all chromatography was carried out at 4 °C. The crude extract (327 mg) was separated according to Scheme 19.



Scheme 19 Isolation procedure of cell crude extract of strain RK2207

2. Structure elucidation of the isolated secondary metabolites

2.1. Structure elucidation of bacteriopheophytin a_L (51)



51

The dark-green pigment **51** showed UV absorption maxima at 681, 624, 544, 510, 408 and 381 nm, which indicated the chromophore of phorphynoids. In addition, split absorption is found in the Soret region (408 and 381 nm), which is characteristic of porphynoids containing a cyclopentanone ring.[115]

The ESI-MS spectrum gave the molecular ion peak m/z = 888 $[M+1]^+$. The HRESI-MS afforded the probable formula $C_{55}H_{74}N_4O_6$ with 25 double bond equivalences.

The ^{1}H-NMR spectrum showed in total 74 proton signals. It indicated three singlet protons at δ = 9.98, 9.62, 8.76 ppm, one olefinic proton at δ = 5.12 ppm, six methine protons, 13 methylene groups, 12 methyl groups, and two striking protons at δ = 0.73 and –1.98 ppm.

The signal pattern of the ^{13}C-NMR spectrum can be roughly divided into two regions: the sp^3-hybridized or aliphatic carbon region with δ values in the range 10 – 70 ppm and the sp^2-hybridized or aromatic – olefin carbon region with δ values from 90 to 200 ppm.

A wealth of structure information could be obtained by the ^{1}H-NMR spectrum based on the large magnetic anisotropy of the aromatic macrocycle (ring-current). Remarkable shifts occurred at δ = –1.96 ppm (broad) and at δ = 9.98, 9.62, 8.76 ppm. The latter signals are correlated to carbon signals at δ = 100.7, 104.1 and 94.3 ppm.

Fig. 38 shows the ^{1}H-NMR spectrum of **51**, which is characteristic for pheophyrin.[116]

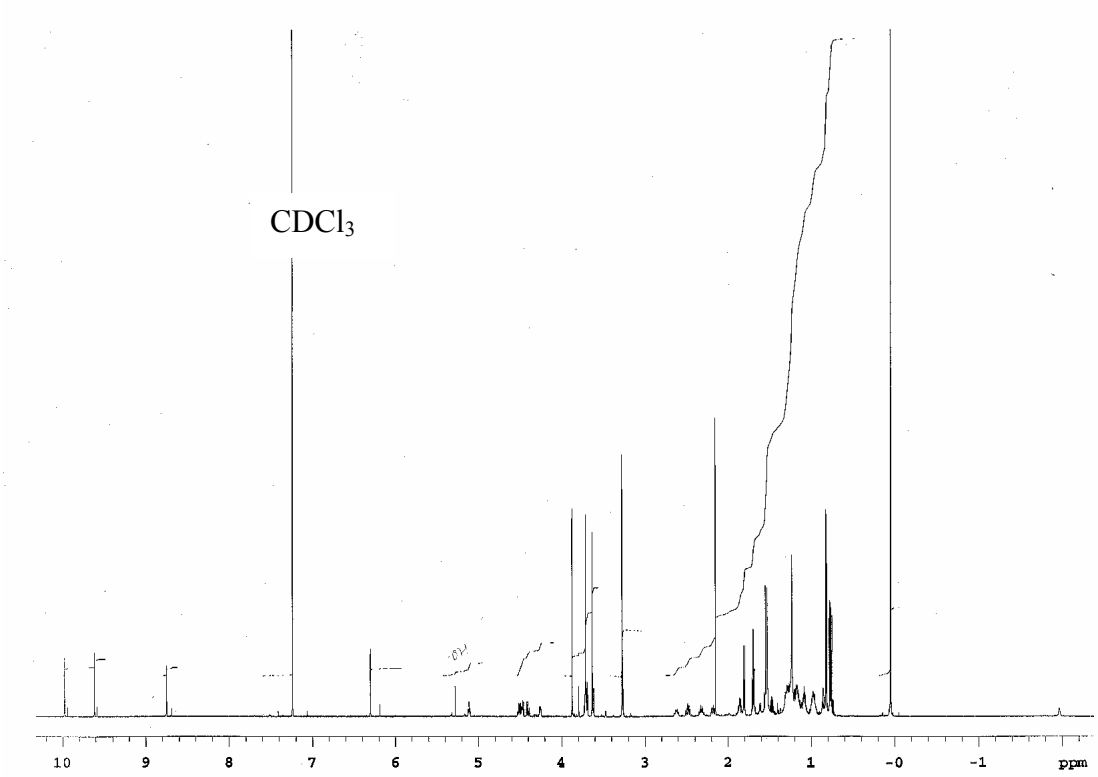


Fig. 38 ^{1}H-NMR (600 MHz, $CDCl_3$) of **51**

The three peaks at significant downfield were assigned to the aromatic methine protons, which suffered a deep field shift of 2.7 ppm compared to the analogous peaks in benzene, while the broad peaks at δ = –1.98 and 0.73 ppm were attributed to the N-H protons, which showed a high field shift of 9 - 11 ppm compared to the N-H proton resonance in pyrrole. In contrast, the strong, sharp signal at δ = 3.88 ppm, which corresponds to the methyl group of the ester chain, was found exactly at the field range expected for it due to comparable data derived from other molecules (e. g., methyl acetate δ (CH_3O) = 3.65 ppm). The methyl groups attached directly to the ring were assigned to the signals at around δ = 3.5 ppm, which were significantly shifted from the position expected for methyl groups attached directly to unsaturated carbon atoms (e.g., CH_2=C(CH_3)CH=CH, δ = 1.84). The methylene quartet at δ = 3.70 (^{13}C at δ = 19.50 ppm) and methyl triplet at

δ = 1.70 ppm, which are coupled to each other, indicated -CH_2CH_3 attached to the porphyrin ring. The signal at δ = 5.15 ppm can be assigned to an olefin proton due to the chemical shift of its attached carbon at δ = 117.6 ppm. The remaining aliphatic groups belong to the substituents at the ester of C-7c.

Comparing the NMR data of **51** with known pheophytins, a striking similarity was found with the data of pheophytin a ($C_{55}H_{74}N_4O_5$).[116] However, two olefin carbons and three olefin protons are absent in **51** and one oxygen atom is absent in pheophytin a. Comparing the data with bacteriopheophytin $a_{p,}$ the latter ($C_{55}H_{76}N_4O_6$) processes two more protons than **51**. Therefore it was supposed that in **51** one acetyl group replaced the ethylene group at C-2.

Due to the high overlapping of signals in the ^{1}H- and ^{13}C-NMR spectra it was difficult to assign all C-H correlations visible in the HMBC spectrum. Fortunately the key correlation could be observed (Fig. 39).

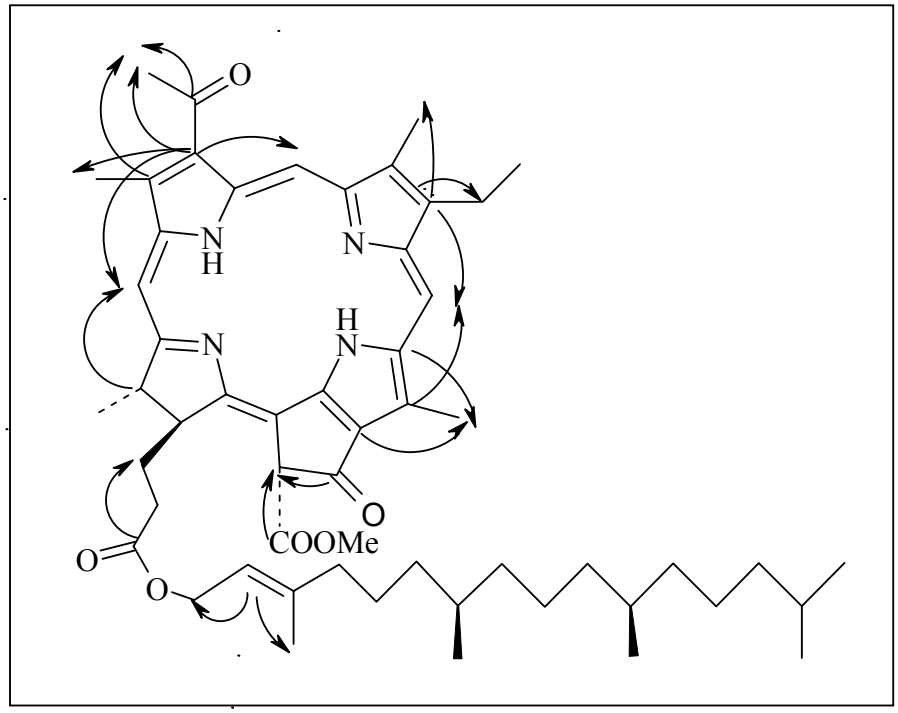


Fig. 39 Part of C-H long range correlations of **51**

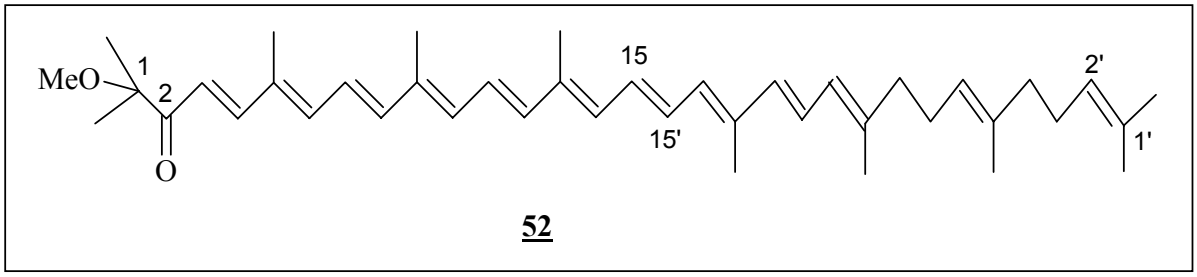
Tab. 11 lists the NMR data of **51**. Combining all data of **51** and the two known compounds, **51** was identified as a new bacteriopheophytin, which contains a C-3/C-4 double bond within the central porphyrin ring in contrast to bacteriopheophytin $a_{p.}$[115] Furthermore **51** differs from pheophytin a in the substitution of C-2.[116] It was termed bacteriopheophytin $a_{L.}$

Tab. 11 NMR data of **51**

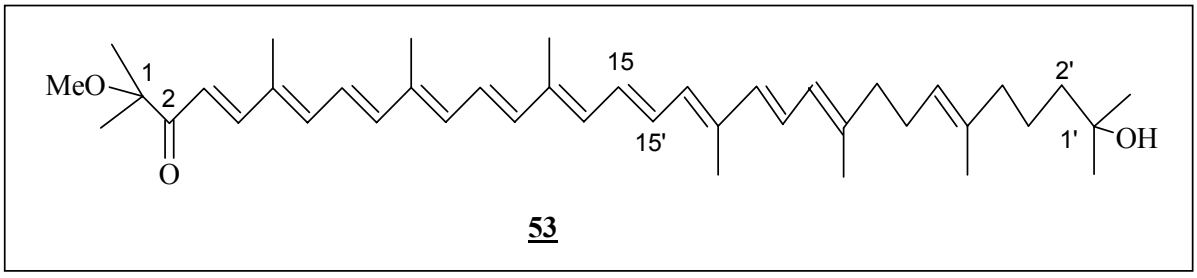
Assign.	^{13}C-NMR data of **51** (ppm) in $CDCl_3$ 150.5 MHz,	^{1}H-NMR data of **51** (ppm) in $CDCl_3$ 600 MHz	Assign.	^{13}C-NMR data of **51** (ppm) in $CDCl_3$ 150.5 MHz	^{1}H-NMR data of **51** (ppm) in $CDCl_3$ 600 MHz
1	132.4		5a	12.3	3.72 (3H, s)
2	136.0		7a	31.9	2.63, 2.34 (2H, m)
3	136.0		7b	29.8	2.49, 2.19 (2H, m)
4	145.1		7c	172.8	
5	128.8		8a	23.3	1.81 (3H, d, *J* = 7.0 Hz)
6	130.9		10a	169.4	
7	51.4	4.28 (1H, m)	10b	52.9	3.88 (3H, s)
8	49.9	4.45 (1H, m)	P1	61.5	4.48, 4.46 (2H, m)
9	189.5		P2	117.6	5.15 (1H, t, *J* = 7.0, 7.0 Hz)
10	64.8	6.32 (1H, s)	P3	142.9	
11	142.9		P3a	16.3	1.55 (3H, s)
12	145.1		P4	39.3	1.85 (2H, m)
13	152.0		P5	25.0	1.0-1.3 (2H, m)
14	150.0		P6	37.2	1.0-1.3 (2H, m)
15	139.5		P7	33.7	1.48 (1H, m)
16	149.9		P7a	19.6	0.82 (3H, d, *J* = 7.0 Hz)
17	167.7		P8	37.3	1.0-1.3 (2H, m)
18	171.8		P9	24.4	1.0-1.3 (2H, m)
α	100.7	9.98 (1H, s)	P10	37.3	1.0-1.3 (2H, m)
β	104.1	9.62 (1H, s)	P11	33.5	
γ	105.8		P11a	19.7	0.82 (3H, d, *J* = 7.0 Hz)
δ	94.3	8.76 (1H, s)	P12	37.4	1.0-1.3 (2H, m)
1a	13.5	3.64 (3H, s)	P13	24.8	1.0-1.3 (2H, m)
2a	187.2		P14	39.8	1.0-1.3 (2H, m)
2b	32.6	3.29 (1H, s)	P15	28.0	0.85 (1H, m)
3a	11.3	3.29 (1H, s)	P15a	22.6	0.78 (3H, d, *J* = 7.0 Hz)
4a	19.5	3.70 (2H, m)	P16	22.8	0.76 (3H, d, *J* = 7.0 Hz)
4b	17.4	1.70 (3H, t, J =7.0, 7.0 Hz	N-H		0.73 (1H, br) -1.98 (1H, br)

The isolation of bacteriopheophytin a_L (**51**) could be of some importance for the study of reaction centres within bacterial photosynthesis complexes.

2.2. Structure elucidation of spheroidenone (52) and hydroxyspheroidenone (53)



52



53

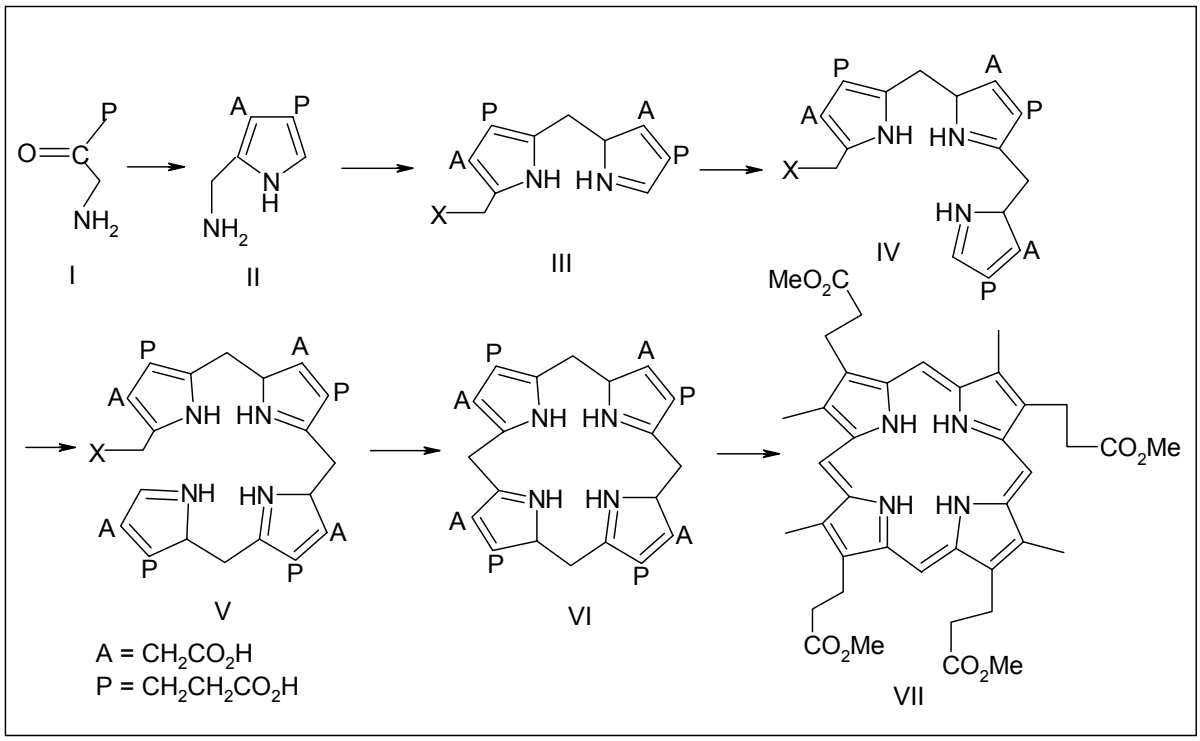
Compounds **52** and **53** were isolated as red amorphous solids from the cell extract of strain RK2207. Their UV spectrum showed absorption peaks at 457, 333, 284 nm and 476, 368, 296 nm, respectively. These (also the red color) indicated the presence of a conjugated double bond system. The EI-MS spectrum gave the molecular weight as 582 and 600 for **52** and **53**, respectively. HREI-MS established the molecular formulas $C_{41}H_{58}O_2$ and $C_{41}H_{60}O_3$ with 13 and 12 double bond equivalences, respectively. This indicated the hydratisation of one double bond in **53**. The ^{1}H-NMR spectrum showed a group of olefinic protons (δ = 5 – 7.5 ppm), one methoxy group (δ = 3.2 ppm) and a group of methyl or methylene protons (δ = 1 – 2.2 ppm). This data indicated that **52** and **53** belong to the carotenoid family. A database search in "*Chapman and Hall*" resulted in four different carotenoids with the same formula $C_{41}H_{58}O_2$, which differ in the two ends of the long chain of conjugated double bonds. The EI-MS spectra showed diagnostically important fragment ions. The fragment of m/z = 73 and 69 in **52** indicated a terminal tertiary methoxy group [-C(OCH_3)(CH_3)$_2$] and a terminal isopentene group [-C_5H_9], respectively. **53** revealed also the fragment of m/z = 73, which indicated the same terminal [-C(OCH_3)(CH_3)$_2$]. Hence **52** and **53** were deduced as spheroidenone and hydroxyspheroidenone. Synonyms are 3,4-didehydro-1,2,7′,8′-tetrahydro-1-methoxy-caroten-

2-one and 3,4-didehydro-1,2,7′,8′-tetrahydro-1-methoxy-1′,2′-dihydro-1′-hydroxy-caroten-2-one, respectively. They belong to the bacterial carotenoids and are produced by photosynthetic purple nonsulphur bacteria.[117]

3. Biosynthesis of the natural porphyrin

Because of low yield of natural porphyrin the assignments of NMR data is difficult. Many studies have been carried out via feeding experiments with ^{13}C-labelled precursors during the last decades.[118] Scheme 20 shows a general biosynthesis pathway.

By the catalyzation of the enzyme system deaminase-cosynthetase labelled δ–aminolevulinic acid (ALA) (I) is converted in four molecules of porphobilinogen (II). These are connected head-to-tail to generate the unrearranged bilane (V); successively the urogen-III (VI) is rapidly produced and undergoes chemically dehydrogenation, decarboxylation, and esterification to coproporphyrin-III-tetramethyl ester (VII).



Scheme 20 Biosynthesis of porphyrins

4. Results and discussion

The purple strain RK2207 belongs to the family of *Rhizobiaceae*, which were often isolated from plants as symbionts and are responsible for the fixing of atmospheric nitrogen within root nodules.[119] Pheophytin has been isolated for the first time as natural product from a

marine bacterium. Its isolation could be of some importance for the understanding of the life-function of this class of bacteria.

Porphyrins represent a large family with a large number of different derivatives. The principle chlorophylls are chlorophyll a and b found e.g. in green plants and in algae, bacteriochlorophyll (produced by purple photosynthetic bacteria), and chlorobium chlorophyll (green photosynthetic bacteria). Due to the 18-π-aromatic system this class of compounds plays a very important role in biology as active participants in the respiratory chain, storage of energy, electron transport, and enzyme oxidation. Pheophytins are demetallated descendants ($M = H_2$) of chlorophylls and consist of various derivatives due to different substituents at the positions of C-2, C-3, C-10 and C-7c. Bacteriopheophytins act as the primary electron acceptor in the bacterial photosynthesis process and play a complementary role for bacteriochlorophylls.

Finally the results provide some evidence that pigments of algae might be produced by symbiontic bacteria.

VI. Using the OSMAC approach to activate the NRPS of strain H260

1. Description of the strain

Strain H260 was identified as *Kokuria sp*. It was isolated from a water sample collected in Neuharlingersiel.

2. Peptide antibiotics

Metabolites derived from amino acids, like penicillin, gramicidin S, and bacitracin, represent one type of antibiotics, namely peptide antibiotics. As a new source for natural products marine organisms have been intensively explored in recent years. A number of peptides with unusual structures and significant biological activities have been isolated from sponges, marine microbes, bryozoans, tunicates, and algae. For example, didemnin B, a cyclic depsipeptide from a tunicate, is now in advanced clinical trials with prospective applications as antiviral, antitumor, and immunosupressive drug.[120]

Ribosomally biosynthesized peptides, often consisting of more than 100 amino acids, are referred to as protein antibiotics. In nonribosomal peptide antibiotic synthesis, amino acids are activated and peptide bonds are formed employing a multienzyme system without ribosomal involvement.[121]

A PCR based approach to investigate marine strains concerning their genetic potential to produce nonribosomal peptides (NRP) was carried out by the group of Prof. Simon within this project. The strain H260 was selected in the course of the PCR screening. Thus it was further investigated for its secondary metabolite production, especially for peptides biosynthesized by a nonribosomal peptide synthetase (NRPS).

3. Using the OSMAC approach to activate the NRPS of strain

It is widely recognized that growth conditions of strain influence the production of secondary metabolites. Therefore the OSMAC approach was used to activate a potentially existant NRPS of strain H260 to produce the corresponding peptide.

In order to find out the growth conditions of strain H260, three mediums MB, SM and SM supplemented with 20 g/L NaCl were used for the cultivation of H260 during 3, 4 and 5 days, respectively. In biological tests the extract from the cultivation on the SM medium showed strong activities after 3 and 4 days, but no activity after five days. Therefore three days were taken for the other cultivations. MB medium with demineralised water, and seawater (with and without XAD) were also used but no good results were produced.

The mediums consisting of peptone and yeast with and without glucose, which worked well for the strains T5 and RK377, were also used for the cultivation of strain H260 and resulted in strong biological activities.

In recent years, microbiological studies have disclosed a common regulation system for secondary metabolism and cell differentiation in streptomycetes.[122] Extracellular molecules can influence intracellular processes. For example, in streptomycetes low molecular-weight substances containing a butyrolactone serve as chemical signal molecules or microbial hormones for secondary metabolism and/or cellular differentiation.[123] In this work N-octanoyl-DL-homoserine lactone, N-hexanoyl-DL-homoserine lactone, and hormaomycin were supplemented to 24 h-old culture broths. The crude extracts of the former two experiments showed only weak activity against *S. aureus*. The cell crude extract of the last experiment showed very strong activity against *E. coli* and its crude extract of acidified filtrate showed strong activity against three microalgae.

There are several pathways to promote the activity of enzymes and influence the peptide yield. One of them is the method of directed biosynthesis, where precursors are fed to the fermentation broth.[124] In this work, several amino acids such as phenyl alanine, proline, asparagine, and lysin were fed to the culture broth of strain H260. But no enhanced activities and no new spots on TLC were observed. When the producing strains are cultured in nutritionally rich media, high levels of secondary metabolites are usually produced when the cell growth is finished. A nutrient limitation can result in a slow growth rate and the expression of genes for the biosynthesis of secondary metabolites. Many micronutrients are necessary for the growth of strains, but excess quantities may be toxic. Therefore in several cultures micronutrients were halved. The nutrient level can directly influence the antibiotic biosynthesis. Glucose, usually an excellent carbon source, is known to interfere with the biosynthesis of many antibiotics; but for strain H260 no significant impacts were observed.

4. Detection methods of peptides

One aim of this work was optimising the detection method for peptides. Two methods were used to detect peptides, namely the chemical screening approach and the HPLC-ESI-MS-MS method.

4.1. Identification of peptides by the chemical screening approach

The crude extracts were first developed on TLC with two different polar solvent systems ($CHCl_3$/methanol 9 : 1 and BuOH/AcOH/H_2O 4 : 5 : 1), and then stained by Ehrlich, ninhydrin and dianisidine, which are special reagents for the amino group. In the chemical

screening approach one big spot in almost all acidic extracts changed to a blue color with Ehrlich, dianisidin, to an orange color with ninhydrin, which was identified later as indole-3-acetic acid. The spot above indole-3-acetic acid showed orange color stained with Ehrlich. It was isolated with small amount and could not be identified. Another region, found to contain a bioactive component, changed to an orange color with ninhydrin, blue with dianisidin, no color with Ehrlich. This compound was later identified as a peptide. Generally the chemical screening approach for peptides is not effective due to the low concentration and unspecific color.

4.2. Identification of peptides by HPLC-ESIMS-MS

The selected crude extracts were further investigated by HPLC-ESI-MS-MS experiments. The separation of the crude extracts was first optimized by varying the HPLC program. Then the samples were measured by HPLC-ESI-MS-MS with this optimized program. Additionally hormaomycin was added as a reference peptide. Its molecular ion peak at m/z = 1129 $[M+H]^+$ was observed but no further fragmentation took place due to the lower collision energy under unified experimental conditions. In another word, it is difficult to set an appropriate unified energy for all samples.

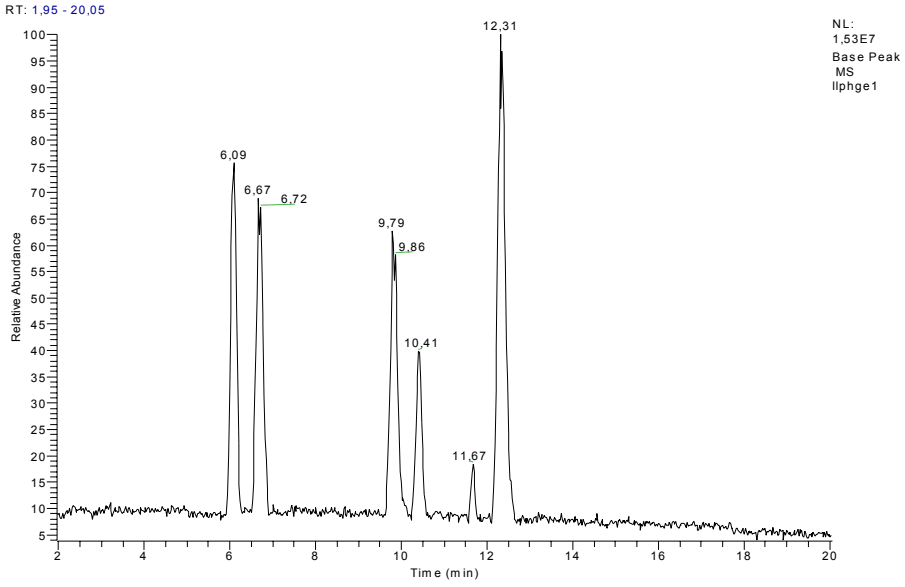


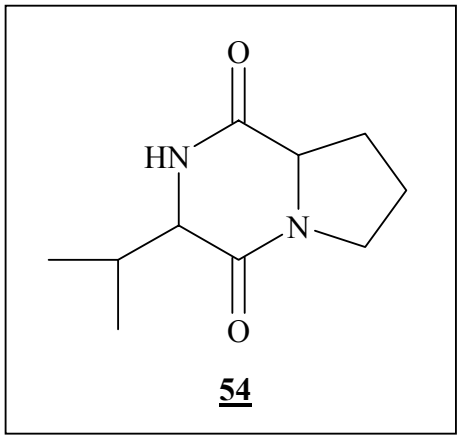
Fig. 40 HPLC chromatogram of one sample in HPLC-ESI-MS-MS

Fig. 40 shows the chromatogram of one sample with six peaks at m/z = 261, 197, 211, 211, 245 and 245, respectively. The fragments of each peak in the HPLC chromatogram were analyzed by comparing them with known masses for the usual amino acids and the in-house

ESI-MS-MS database, which was developed by the group of Prof. Laatsch and the department of mass spectroscopy at Göttingen University. In the crude extracts of peptone supplemented with or without glucose and with hormaomycin, six diketopiperazines were found.

5. Structure elucidation of the peaks in HPLC-ESI-MS chromatogram

5.1. Identification of cyclo-(Val-Pro) (54)



54

The peak at R_t = 6.67 min (HPLC chromatogram) showed the molecule ion peak at m/z = 197 $[M+H]^+$ (ESI-MS spectrum). Its fragment at m/z = 70 indicated the related ions $[M-CO+H]^+$ of proline. Its fragment at m/z = 72 indicated the related ion $[M-CO+H]^+$of valine. Compared with the MS-MS database, the fragment pattern is identical with cyclo-(Pro-Val) (see Fig. 41).

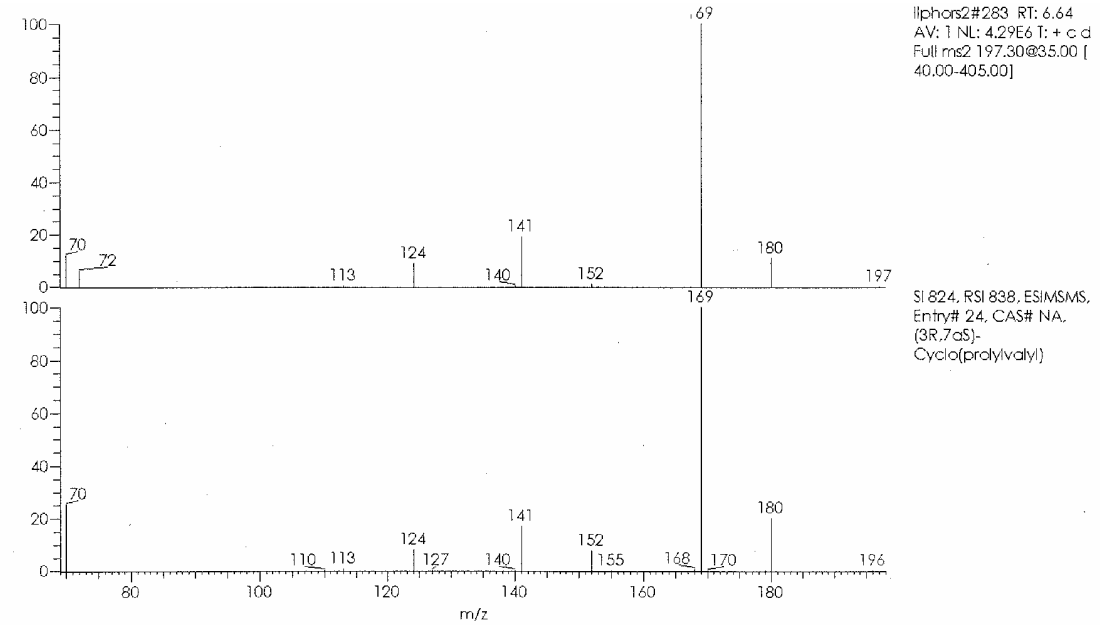
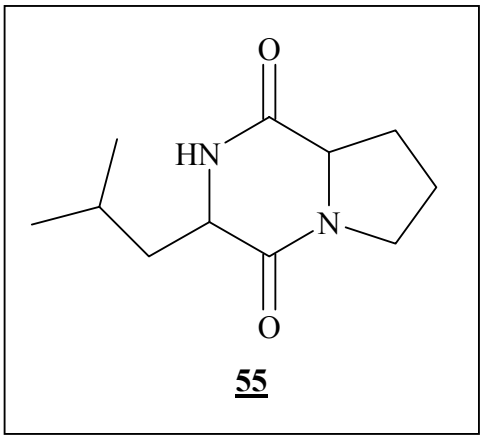


Fig. 41 ESI-MS spectrum of **54** and the corresponding spectrum of database

5.5. Identification of cyclo-(Leu-Pro) (55)



55

The two peaks at R_t = 9.79 and 10.41 min (HPLC chromatogram) showed the molecule ion peak at m/z = 211 $[M+H]^+$ (ESI-MS spectrum). Its fragment m/z = 70 indicated the related ion $[M-CO+H]^+$of proline. Its fragment at m/z = 86 indicated the related ion $[M-CO+H]^+$ of leucin (or isoleucin). Compared with the MS-MS database, the fragment pattern was identical with cyclo-(Leu-Pro) (see Fig. 42). The two peaks in the HPLC chromatogram can be easily considered as two stereoisomers.

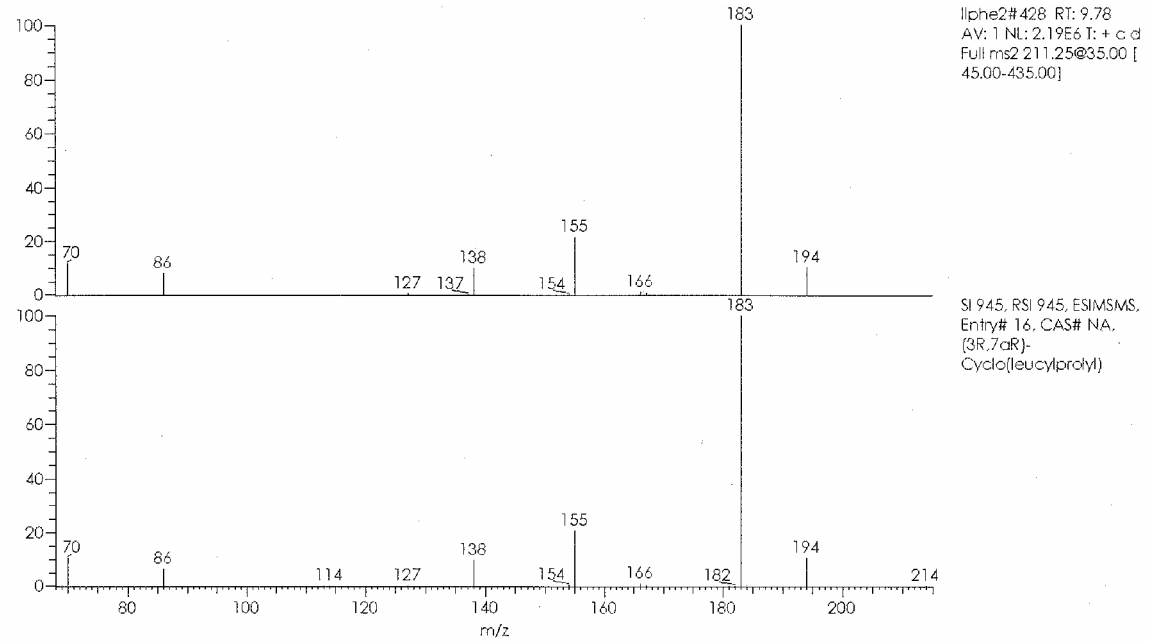
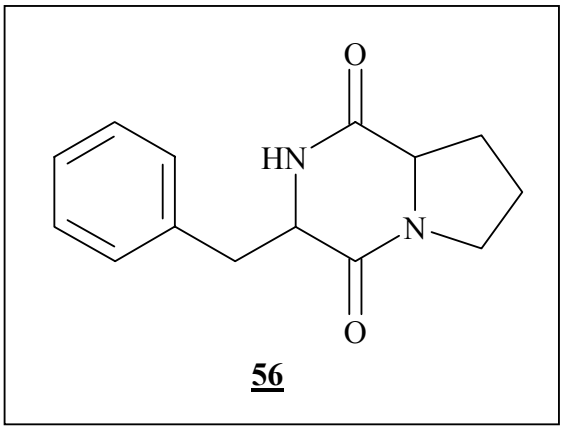


Fig. 42 ESIMS spectrum of **55** and the corresponding spectrum of the database

5.3. Identification of cyclo-(Phe-Pro) (56)



56

The two peaks at R_t = 11.67 and 12.31 min (HPLC chromatogram) showed the molecule ion peak at m/z = 245 $[M+H]^+$. The fragments at m/z = 70 and 120 were observed in the MS-MS spectrum. Because the database did not include the molecules with molecule ion peak at m/z = 245, the comparison of the fragments was not possible. But the peak at m/z = 70 could be assigned to the related fragment $[M-CO+H]^+$ of proline and the peak at m/z = 120 could be assigned to related fragment $[M-CO+H]^+$ of phenylalanine. This compound was thus assigned as cyclo-(Phe-Pro). The two peaks in the HPLC chromatogram could be easily considered as two stereoisomers. Fig. 43 shows the ESI-MS spectrum and the corresponding MS-MS spectrum.

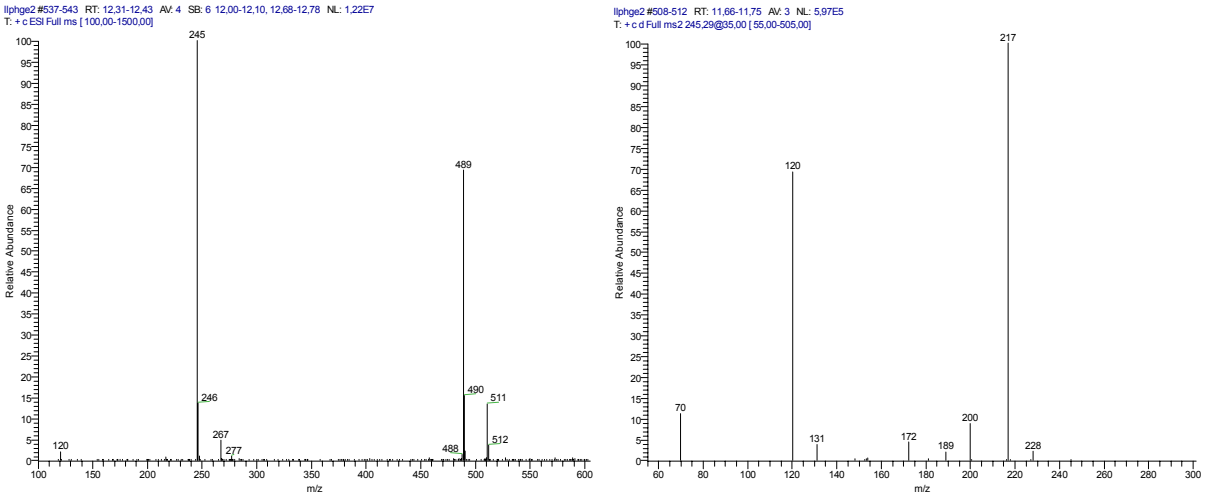
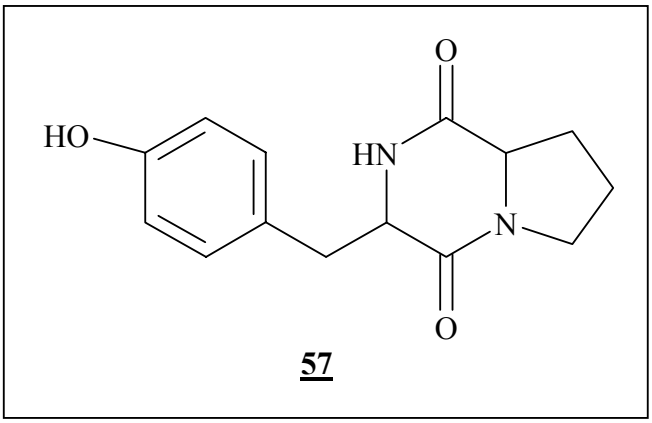


Fig. 43 ESI-MS and MS-MS spectra of **56**

5.4. Identification of cyclo-(Tyr-Pro) (57)



57

The peak at R_t = 6.09 min (HPLC chromatogram) showed the molecule ion peak at m/z = 261 $[M+H]^+$. Because the database did not contain the molecules with the molecule ion peak at 261, the comparison of the fragments was not possible. In *Antibase* two diketopiperazines have molecular weight 260: one was cyclo(Leu-Phe), another one was cyclo-(Tyr-Pro). The former should have the fragments at m/z = 72 and 120, the latter should have the fragments at m/z = 70 and 136. In MS-MS spectrum the fragment at m/z = 136 indicated the related ion $[M-CO+H]^+$ of tyrosine. The difference of m/z = 97 between m/z = 260 and 163 (the molecular weight of tyrosine) indicated the presence of proline, although its related ion fragment at m/z = 70 was not observed. Therefore this component was deduced as cyclo-(Tyr-Pro). Fig. 44 shows the ESI-MS spectrum of **57**.

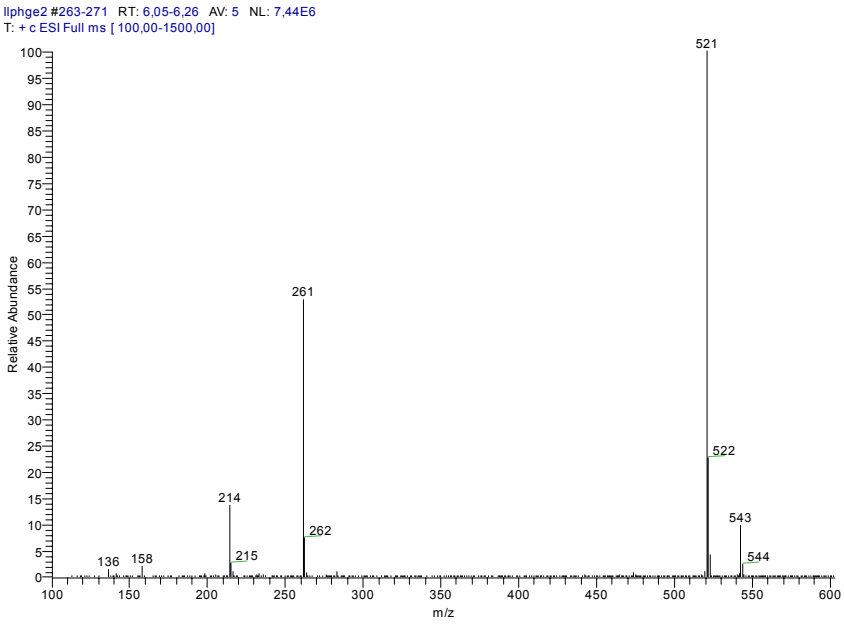


Fig. 44 ESI-MS of **57**

6. Scale-up fermentation as well as isolation and structure elucidation of the bioactive component

6.1. 50 L-fermentation of strain and work-up of the culture broth

From the peak of analytical HPLC it was known that the bioactive component was produced in a low yield. In order to get enough of the pure compound a 50 L-fermentation of strain H260 was carried out. Since the bioactive compound is very polar, the extraction of biomass was done by several steps, first with acetone, then with methanol, followed by methanol/water 1 : 1 and 2 : 8. 100 mL of filtrate were extracted at pH 8.5 and pH 4, respectively. Another 100 mL of filtrate were dried directly under freezing vacuum. All samples were dissolved in 1 : 1 methanol/water with a concentration of 50 mg/mL and their biological activities were tested against *E. coli* (see Tab. 14). The biggest inhibition zone was located in the last extraction step using the most polar solvent system but not in the first extraction, and also in direct filtrate but not in its ethyl acetate extract. When the crude extract was partitioned between water and ethyl acetate, the bioactive component was found in the water phase. These indicated that the compound was very polar.

Tab. 14 Bioactivity against *E. coli* of different extracts of culture broth of strain H260

Sample	Inhibition zone (mm)
1. Cell extract with acetone	23
2. Cell extract with methanol	28
3. Cell extract with MeOH/H_2O = 1 : 1	31
4. Cell extract with MeOH/H_2O = 2 : 8	34
Filtrate extract (at pH 8.5) with ethyl acetate	0
Acidified filtrate extract (at pH 4.0) with ethyl acetate	0
Dried filtrate	34

6.2. Isolation of the bioactive compound

The bioactive compound is difficult to identify on the TLC due to its low content and the disturbance of other components. Also in HPLC there is no single peak although many HPLC programs and different columns were tried. Fig. 45 shows the HPLC chromatogramm of concentrated fraction. The component at R_t = 10.32 min proved to be active against *E. coli*.

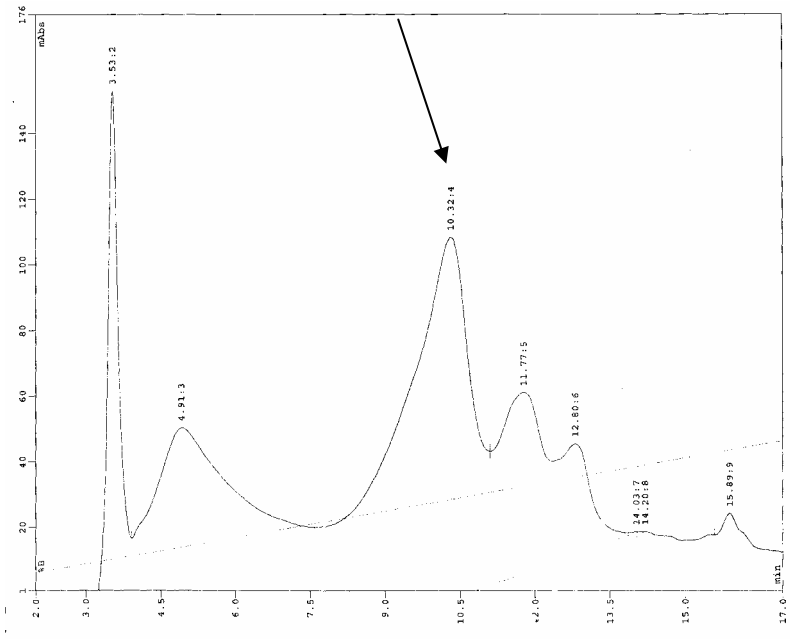


Fig. 45 HPLC chromatogram of the crude extract (concentrated fraction)

Therefore the first isolation was carried out by middle pressure reverse phase C-18 column (MPLC) and the fractions were tested against *E. coli*. Only the fraction 12 showed bioactivity. This fraction was repeated chromatographed by preparative HPLC and 3.9 mg of the compound were obtained.

6.3. Characterization of the bioactive compound

The bioactive compound showed a weak violet color with a retention value of R_f 0.26 (developed by 1 : 3 mixture of chloroform/methanol on the TLC) and absorbed the UV light under 254 nm. It was well soluble in water and DMSO, methanol, scarcely in chloroform and other nonpolar solvents. The NMR spectra indicated nine amide carbonyl signals between δ= 164 – 178 ppm. The 11 carbon signals between δ= 44 – 60 ppm represented the α–carbons of peptides. Additionally about 20 carbon signals between δ= 14 and 40 ppm indicated the presence of aliphatic carbons. The ^{1}H-NMR spectrum indicated protons both in the range of aromatic protons and aliphatic protons. Due to the impurity of the sample and the limitation of the used solvent, accurate integration of protons was not possible. The Mass spectrum could not be interpreted due to the impurity of the sample. But the result has already proved that the bioactive component is a peptide. Further purification will be necessary by repeated preparative HPLC to get more amount of compound.

7. Result and discussion

The identification of a bioactive peptide proved the result from the NRPS-screening that strain H260 contains NRPS. Additionally it has indicated that the NRPS-screening can be used for the pre-screening of strains. But the diketopiperazines (**54** – **57**) are not produced by a NRPS. According to Sammes,[125] diketopiperazines (DKP) are ubiquitous throughout nature and are most commonly isolated from terrestrial yeast, lichen and fungi culture filtrates and also observed in the culture broths of marine bacteria,[126] marine actinomycetes,[127] sponges[128] and starfish.[129] Most of the diketopiperazines were reported to have biological activities such as hormones, antibiotics, antitoxins, or ion carriers. For example, (3R, 7S)-cyclo(Pro-Val) possesses specific inhibitory activity of glucosidase,[130] while its stereo-isomer (3S-7S)-cyclo(Pro-Val) has antibiotic and phytotoxic activities.[131] (3S, 7S)-cyclo(Leu-Pro) possesses antibiotic activities,[132] while (3R, 7S)-cyclo(Leu-Pro) is a phytotoxin.[133] Cyclo(L-Tyr-D-Pro) possesses cytotoxic activity [134]and is a plant growth regulator,[135] but cyclo(D-Tyr-D-Pro) is a phytotoxin.[126] As antagonists of N-acylhomoserine lactone (AHL) DKPs are also capable of activating an AHL biosensor. Similarly, DKPs were found to be capable of activating or antagonizing other LuxR-based quorum-sensing systems, such as the N-butanoylhomoserine lactone-dependent swarming motility of *Serratia liquefaciens*. Although the physiological role of these DKPs has yet to be established, their activity suggests the existence of "cross" talk among bacterial signalling systems.[136]

The identification of peptides in crude extracts by chemical screening is difficult due to disturbance by other components and low specificity of the staining reagents. The HPLC-ESI-MS-MS method is more effective, but the optimization of the HPLC program and the analysis of ESI-MS-MS data are time-consuming. Some peptides like hormaomycin can not be fragmented under the chosen collision energy. Besides, a database containing the data of all known peptides measured under the same conditions, should be prepared.

The isolated bioactive compound is very unstable. At room temperature it changed to an inactive form, which quite likely is its stereoisomer due to their identical UV spectrum and unseparable peak in HPLC chromatogram. Additionally the bioactive compound is so polar that it could not be extracted by organic solvents.

VII. Conclusion

In this thesis 39 strains of North Sea bacteria (*Ruegeria sp*., *Halomonas sp*., *Mesorhizobium sp*., *Kokuria sp*., *actinomycetes,* etc.) from the project of marine biotechnology were studied. Of these 38 strains were screened by biological and chemical methods. Four strains were studied in detail under defined conditions. One strain was studied with regard to its biosynthetic ability to produce peptides using a nonribosomal peptide synthetase (NRPS).

1. Chemical and biological screening of the North Sea bacteria

1) Through systematic and broad variation of the fermentation conditions (temperature, duration and cultivation medium) it was understood that marine bacteria can grow at normal temperature (15 °C – 32 °C) and duration (12 h – 6 days), contrary to the hypothesis that marine bacteria grow more slowly than streptomycetes. But the yield of secondary metabolites is lower (0.01 – 1 mg/L) and can not be raised by changing temperature and duration.

2) The yield of secondary metabolites can be raised by directed supplementing of precursors or addition of adsorption resins (XAD).

3) The culture broth was extracted under different conditions to obtain three crude extracts. The extraction of acidified filtrate proved to be an important step for marine bacteria.

4) All extracts were screened by biological (agar test against *E. coli, B. Subtilis, S. aureus, C. albicans*, three microalgae or interaction screening), chemical (TLC developed with different solvent systems and stained with different staining reagents), physicochemical (HPLC-DAD) approaches. Due to the low productivity of marine bacteria, the HPLC-DAD screening proved to be helpful because of the comparison of the UV-spectra with the database.

5) The biosynthetic potential of the North Sea bacteria was appraised. Most of them produce small aromatic metabolites in low yield.

6) According to the screening results four strains were chosen for further investigation.

2. Metabolites of strain T5

1) The cultivation condition of the strain T5 was optimized for the scale-up fermentation and thereby led to a good yield and reproducible production of tropodithietic acid (**11**).
2) The 6-fold increase of yield of tropodithietic acid (**11**) was carried out by the supplementation of phenylalanin and histidine to the medium of strain T5 (from 1 mg/L to 6 mg/L).

3) The isolation and purification procedure of tropodithietic acid (**11**) has been simplified. Thereby the strong adsorption of this compound on silica gel and Sephadex LH-20 has been avoided.
4) The structure of tropodithietic acid (**11**) was elucidated by X-ray analysis and spectroscopic data. In this case it was avoided to arrive at a wrong conclusion (thiotropocin **13**) due to their similar physicochemical data.
5) The solvent complex effect of DMSO with tropoditietic acid (**11**) was observed and documented.
6) As a minor component hydroxytropodithietic acid (0.04 mg/L) was isolated. Its structure was elucidated by X-ray analysis.
7) Tropodithietic acid (**11**) was biologically tested against bacteria, fungi, different tumor cell lines, saline shrimps, nematode and ion channel activities. It showed activities in all assays except for the ion channel.
8) The biosynthetic pathway for tropodithietic acid (**11**) is supposed.
9) The biosynthetic potential of strain T5 was studied by variation of nutrients. Anthranilic acid and its ethyl ester were isolated as major components from peptone medium supplemented with tryptophan and histidine.

3. Metabolites of strain RK377

1) From the shaking culture on the MB medium, isatin (**33**) and 3-(3-hydroxy-2,3-dihydro-1*H*-indol-3-yl)-2-oxo-propionic acid (**34**) were isolated and characterized by spectroscopic methods and X-ray analysis, respectively.
2) From 40 L-fermentation of strain RK377 in the MB medium in the artificial seawater, three new natural products, namely 3-(4'-hydroxyphenyl)-4-phenylpyrrole-2,5-dicarboxylic acid (**35**), 3,4-di(4'-hydroxyphenyl)pyrrole-2,5-dicarboxylic acid (**36**), 7-hydroxy-2H-benzo[1,4]thiazin-3-one (**37**), were identified. Additional four indole derivatives, namely indole-3-acetic acid (**41**), indole-3-carboxylic acid (**39**), indole-3-carboxaldehyde (**40**), 3-hydroxyacetylindole (**38**), and also phenylacetic acid (**42**), bis (2-ethylhexyl)phthalate (**43**) were isolated and characterized by spectroscopic methods and X-ray analysis.
3) From 40 L-fermentation of strain RK377 on SJ medium, two new imidazol and pyrimidin derivatives, namely glusun I (**44**) and gluson II (**46**) were isolated and characterized by spectroscopic methods. Because of the low yields the structures are proposals only.

4) All isolated compounds of strain RK377 were tested against *E. coli, B. subtilis, S. aureus, and C. albicans*. *N*o activities have been observed so far.
5) The putative biosynthesis approaches of 3-(4'-hydroxyphenyl)-4-phenylpyrrole-2,5-dicarboxylic acid (**35**) and 3,4-di(4'-hydroxyphenyl)pyrrole-2,5-dicarboxylic acid (**36**) were discussed.
6) The biosynthetic potential of strain RK377 was proved by the isolation of different metabolites from the culture broth using different mediums.

4. Investigation of strain RK2207

From a 10 L-fermentation of strain RK2207 the new bacteriopheophytin a_L (**51**) and two bacteriocarotines (**52** and **53**) were isolated and characterized by spectroscopic methods.

5. Investigation of strain H260, which was selected by genetic screening for NRPS

1) Using the OSMAC approach strain H260 was cultivated in different mediums and variation of the cultivation duration. The extracts of their mycelium, culture filtrate and acidified culture filtrate were screened by biological assays against *E. coli* and three different microalgae, and chemical screening and HPLC-ESI-MS-MS experiments.
2) The cell extract from a peptone medium supplemented with or without glucose showed the strongest activities against *E.coli* and microalgae.
3) The detection of polar peptides using a chemical screening approach was not very effective, because the detection was disturbed by the constituents of the cultivation mediums.
4) The HPLC-ESI-MS-MS method was used to prove the presence of peptides. Six diketopiperazines were characterized, namely cyclo-(Pro-Val) (**54**), cyclo-(Pro-Leu) (**55**, two stereoisomers), cyclo-(Phe-Pro) (**56**, two stereoisomers), cyclo-(Tyr-Pro) (**57**).
5) A bioactive component was assigned to the zone on the TLC by bioautography and to the peak of the HPLC chromatogram by the "peak survey" method. It is known that this bioactive component is very polar and unstable.
6) From 50 L-fermentation of this strain one peptide was isolated and characterized by spectroscopic methods. Additionally the strain produces indoleacetic acid as the major component.
7) The production of the peptide has proved the presence of NRPS in strain H260. This result shows that the NRPS-screening is a valuable tool to find producers of NRPS.

B. Experimental Methods

I. General Remarks

1. Instrument analysis

Melting point: Reichert hot-stage microscope (not corrected).

Mass spectra: EI-MS: Varian MAT 731, 70ev, Finnigan MAT 311 70eV, high resolution with perfluorokerosine as standard; **DCI-MS:** Finnigan MAT 95A, 200eV (NH_3); **ESI-MS:** VG Quattro (Micromass), Finnigan LC-Q; **HRESI:** MAT-95 (Thermoquest).

Infrared spectrum: Perkin-Elmer Model 1600 (KBr disc).

Electron spectra: Kontron Uvikon 860 and Varian Cary 3E. One drop of HCl (1 M) or NaOH (1 M) was added to a methanol solution (2 mL) to prepare the MeOH/HCl or MeOH/NaOH sample.

^{1}H-NMR spectra: Bruker AMX 300 (300 MHz), Varian Unity 300 (300 MHz), Varian Inova 500 (500 MHz), and Varian Inova 600 (600 MHz). Chemical shifts are expressed in δ-values (ppm) using the solvent as internal reference. Coupling constants (J) are in [Hz]. Abbreviations of multiplicity: s = singlet, d = doublet, dd = doublet of doublet, ddd = doublet of doublet of doublet, t = triplet, q = quartet, dq = doublet of quartet, m = multiple, br = broad.

^{13}C-NMR spectra: Bruker AMX 300 (75.5 MHz), Varian Unity 300 (75.5 MHz), Varian Inova 500 (125.7 MHz), and Varian Inova 600 (150.8 MHz). Chemical shifts are expressed in δ-values (ppm) using the solvent as internal reference. APT represents Attached Proton Test (CH/CH_3 up and C/CH_2 down), ^{13}C-^{1}H-multiplicity was deduced from correlation of HMQC data and APT data. Abbreviations: s = singlet, d = doublet, t = triplet, q = quartet.

2D-NMR spectra: ^{1}H,^{1}H-COSY (^{1}H,^{1}H-**Co**rrelated **S**pectroscop**y**), **HMBC** (**H**eteronuclear **M**ultiple **B**ond **C**onnectivity), **HMQC** (**H**eteronuclear **M**ultiple **Q**uantum **C**oherence),

HSQC (**H**eteronuclear **S**ingle **Q**uantum **C**oherence), and **NOESY** (**N**uclear **O**verhauser **E**ffect **S**pectroscop**y**)

X-ray analysis: STOE-AED2 four-circle and STOE IPDS II diffractometers. The structures were solved by direct methods using either SHELXS-90[137] or SHELXS-97[138] programs and refined against F^2 on all data by full-matrix least-squares with SHELXL-97.[139]

2. Chromatography methods

Solvent: All solvents for the chromatography were distilled. Analytical pure solvents were used for other uses.

Abbreviation of used mix-solvent for the TLC and column chromatography

A: acetone B: ethyl acetate C: chloroform D: methanol

E: dichloromethane/methanol	9 : 1 + 1 % formic acid
F: dichloromethane/methanol	5 : 1 + 5 % formic acid
G: dichloromethane/methanol	1 : 1
H: chloroform/formic acid	95 : 5
I: chloroform/methanol	9 : 1 + 1 % formic acid
J: chloroform/methanol	9 : 1
K: cyclohexane/ethyl acetate	16 : 1
L: dichloromethane/methanol	7 : 1 + 5 % formic acid
M: chloroform/methanol	5 : 1 + 5 % formic acid
N: dichloromethane/methanol	99 : 1
O: ethyl acetate/cyclohexane	1 : 6
P: ethyl acetate/cyclohexane	1 : 3

Thin Layer Chromatography (TLC): Pre-coated silica gel plates: silica gel 60 F_{254} on aluminum foil or glass, 0.25 mm layer thick (Merck); **HPTLC**: Pre-coated nano plates (10 x 10 cm): silica gel 60/$UV_{254/366}$ on glass, 0.25 mm layer thick (Macherey & Nagel); **RP-18**: Pre-coated plates: silica gel F_{254} on aluminum foil, 0.25 mm layer thick (Merck); **Preparative thin layer chromatography (PTLC**): Pre-coated plates (10 x 20 cm, 2 mm layer thick): silica gel 60 F_{254} with concentrating zone (Merck).

Staining reagents: According to directions of "Reagenzen, Merck, Anfärbereagenzien für die Dünnschicht Chromatographie",[140] all TLC plates were heated to about 100 °C after spraying with staining reagents.

Anisaldehyde (No. 21): Anisaldehyde (1.0 mL) was dissolved in methanol (85 mL), acetic acid (10 mL) and concentrated sulfuric acid (5 mL).

Vanillin/H_2SO_4 (No. 322): Vanillin (1.0 g) was dissolved in concentrated sulfuric acid (100 mL).

Ehrlichs Reagent (No. 91): 4-dimethylaminobenzaldehyde (1.0 g) was dissolved in hydrochloride acid (36 %, 25 mL) and methanol (75 mL).

Orcin (No. 120-122): Iron (III)-chloride (1.0 g) was dissolved in concentrated sulfuric acid (100 mL) and successively mixed with the same volume of orcin solution (6 % in ethanol).

Palladium(II) Chloride (No. 251): Palladium(II) dichloride (0.5 g) was dissolved in water (100 mL) with some drops hydrochloride acid (25 %).

Ninhydrin (No. 232): Ninhydrin in ethanol (0.1 %) was directly used.

Dianisidine: O- or p-dianisdine (300 mg) was dissolved in acetic acid (0.6 g) and acetone (115 mL). TLC plate must be first put in a closed chamber with a bottle containing potassium perchlorate (0.4 g) and concentrated HCl (1.0 mL). It was taken out, dried up and then sprayed with dianisdine solution.

Column Chromatography: Silica gel 60, 0.032-0.063 mm, (ICN Biomedicals GmbH), Sephadex LH-20 (Pharmacia).

Analytical HPLC: Pump: Kontron 322 System; Autosampler: Kontron 360; Detector: Kontron Diode Array Detector 440; Data system: Kontron Kroma-System 2000 Version 1.60; Columns: Knauer Nucleosil 100 C18 (5 µm, 3 x 250 mm), Kromasil 100 C18 (5 µm, 4 x 250 mm); Solvent system: A = redistilled water with phosphoric acid (0.1%), B = Acetonitrile with redistilled water (1.0 %). Flow rate: 0.5 mL/min.

Preparative HPLC: Pump: Jasco PU-1587 System; Detector: Jasco UV-1575 (Dioden Array Detector); Data system: Jasco-Borwin 1.50; Columns: Jasco Kromasil 100 C18 (7 µm, 20 x 250 mm). Solvents system: A = redistilled water with formatic acid (0.1%), B = Acetonitrile. Flow rate: 20 mL/min.

HPLC-ESIMS: Finnigan LCQ with HPLC-pump Rheos 4000 (Flux) and UV-detector UVIS-205 (Linear); Column: Grom Superspher 100 RP-18 endc. (4 μm, 2 x 100 mm); Solvents system: A = redistilled water with formatic acid (0.1%), B = methanol. Flow rate: 0.3 mL/min.

Program of HPLC: A = redistilled water with different acid (0.1%), B = acetonitrile

Prog 1: gradient 0 – 100 % B in 30 min.

Prog 2: isocratic 28 % B in 20 min and then 28 % – 100 % B in 10 min.

Prog 3: gradient 15 – 100 % B in 30 min.

Prog 4: isocratic 25 % B in 30 min and then 25 % – 100 % B in 10 min.

Prog 5: gradient 10 % - 60 % B in 20 min and then 60 % – 100 % B in 10 min.

Prog 6: isocratic 100 % A in 4 min and then 0 % – 60 % in 16 min.

Redistilled water and HPLC-pure solvent were degassed for 10 min in ultrasonic wave bath and by helium gas.

3. Microbiological methods

Cultivation mediums: The nutrients were produced by following Corporation.

MB2216	Difco	MB2216 agar	Difco
Glucose	Merck	Soybean meal	Henselwerk GmbH
Yeast	Gibco BRL	Casein peptone	Brunnengräber
D - Mannit	Riedel de Haen	Bacton peptone	Difco
Bacton tryptone	Difco		

Redistilled water was used and the entire mediums were sterilized for 30 min at 121 °C before fermentation for all of the following media unless otherwise specified.

Constitute of nutrients

MB medium (g/L): Sojapeptone (5), yeast (1), NaCl (19.45), $MgCl_2$ (5.9), Na_2SO_4 (3.24) $CaCl_2$, (1.8), KCl (0.55), $NaHCO_3$ (0.16), Fe(III)-citrat (0.1), KBr (0.08), $SrCl_2$ (0.034), H_3BO_3 (0.022), Na_2HPO_4 (0.008), Na_2SiO_3 (0.004), NaF (0.0024), NH_4NO_3 (0.0016), pH = 7.6.

SC medium (g/L): Peptone (5), yeast (1), 3.3 % XAD, ASW : deion. H_2O = 1 : 1.

SD medium (g/L): Peptone (5), yeast (1), glucose (1), 3.3% XAD, ASW : deion. H_2O = 1 : 1.

SE medium (mg/L): Phenylalanine (825), histidine (7.8), 3.3% XAD, ASW : deion. H_2O = 1 : 1.

SF medium (g/L): Peptone (5), yeast (1), NaCl (21.8), $MgCl_2 \cdot 6H_2O$ (10), Na_2SO_4 (3.6), $CaCl_2 \cdot 2H_2O$ (1.8), $NaHCO_3$ (0.3), KCl (0.6).

SG medium (g/L): L-phenylalanine (0.8), histidine (0.08), NaCl (21.8), KCl (0.6), $MgCl_2 \cdot 6H_2O$ (10), Na_2SO_4 (3.6), $CaCl_2 \cdot 2H_2O$ (1.36), $NaHCO_3$ (0.3).

SH medium (g/L): Tryptophane (0.5), histidine (0.008), NaCl (21.8), KCl (0.6), $MgCl_2 \cdot 6H_2O$ (10), Na_2SO_4 (3.6), $CaCl_2 \cdot 2H_2O$ (1.36), $NaHCO_3$ (0.3), glucose (5), NH_4Cl (0.3), K_2HPO_4 (0.035), KH_2PO_4 (0.025), $SrCl_2 \cdot 6H_2O$ (0.057), trace element stock soln. (1 mL), nicotine acid (0.05), thiamine (0.05), pantothenate (0.05), pyridoxal-HCl (0.05), cyanocobalamine (0.05), riboflavine (0.05), biotine (0.05), folacid (0.05), inositole (0.05), PABA (0.05) in 1 L deion. water.

SJ: medium (g/L): Tryptone (10), yeast (5), glucose (25), ASW, pH = 7.0.

SK medium (g/L): Tryptone (10), yeast (5), ASW, pH = 7.0.

SL medium (g/L): Peptone (5), yeast (1), glucose (1), ASW : deion. H_2O = 1 : 1.

SM medium (g/L): D-mannitol (20), soybean meal (20), pH = 7.0.

The trace element stock solution for SH medium (mg/L): KBr (80), H_3BO_3 (22), Na_2SiO_3 (4), NaF (2.4), NH_4NO_3 (1.6).

ASW (Artificial Sea Water) (g/L): Iron citrate (0.1, powder), NaCl (19.45), $MgCl_2 \cdot 6H_2O$ (8.8), Na_2SO_4 (3.24), $CaCl_2$ (1.8), Na_2HPO_4 (0.008), SiO_2 (0.015), trace element stock soln. (1 mL), stock soln. (10 mL).

Trace elements stock solution for ASW (g/L): H_3BO_3 (0.611), $MnCl_2$ (0.389), $CuSO_4$ (0.056), $ZnSO_4 \cdot 7H_2O$ (0.056), $Al_2(SO_4)_3 \cdot 18H_2O$ (0.056), $NiSO_4 \cdot 6H_2O$ (0.056), $Co(NO_3)_3 \cdot 6H_2O$ (0.056), TiO_2 (0.056), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.056), LiCl (0.028), $SnCl_2$ (0.028), KI (0.028).

Stock solution for ASW (g/L): KCl (55), $NaHCO_3$ (16), KBr (8), $SrCl_2 \cdot 6H_2O$ (3.4), H_3BO_3 (2.2), NaF (0.24), NH_4NO_3 (0.16).

Centrifuge: Sigma 2K1, Sigma 4K15, Sigma 210 M.

Separation of cell from 50 L culture broth: Janke & Kunkel KG Ultra-Turrax.

Rotary shaker: Braun Inkubationsschüttelschrank BS4; Braun Certomat® RM; Braun Certomat® BS-1; Long-shaker Göttingen University (105 spm, 28 °C).

Fermenter with air-regulator: Braun Biostat M 1 L (1.6 L/min, 700 upm), Braun Biostat U 50 L (33 L/min, 200 upm), The Analytical Development Co Ltd. Carbon dioxygen-Analysator, Ingold oxygen-electrode.

4. Biological tests

Paper discs (ϕ 9 mm, thickness 0.5 mm) were soaked with 50 μL of a solution of the pure compound in a suitable solvent (c = 1 mg/mL or 0.7 mg/mL) or crude extract of methanol and distilled water (1 : 1, c = 50 mg/mL), dried under sterile flow box and put on agar plates inoculated with the test organisms (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Mucor hiemalis*, *Candida albicans*, *Chlorella sorokiniana*, *Chlorella vulgaris*, *Scenedesmus subspicatus*). The plates were incubated at 37 °C (bacteria) or 27 °C (fungus) for 1 day or at 24 - 26 °C under day-light for 4 days (microalgae). Afterwards then the diameters of the inhibition zone were measured.

Media for microorganisms

Medium for *S. aureus, E. coli*: Bacto nutrient broth 8 g/L, NaCl 5 g/L, agar 15 g/L.

Medium for *B. subtilis*: Glucose 5 g/L, Na-citrat·$2H_2O$ 0.5 g/L, KH_2PO_4 3 g/L, K_2HPO_4 7 g/L, $MgSO_4$ 7 H_2O 0.1 g/L, $(NH_4)_2SO_4$ 1 g/L, agar 15 g/L.

Medium for *C. albicans*: Hefe extract 4 g/L, malz extract 10 g/L, glucose 4 g/L, agar 15 g/L, pH = 5.5.

Medium for microalgae: NaCl 0.25 g/L, $MgSO_4$ $7H_2O$ 0.75 g/L, $NaNO_3$ 2.5 g/L, $CaCl_2 \cdot 2H_2O$ 0.25 g/L, K_2HPO_4 0.75 g/L, KH_2PO_4 1.75 g/L, Fe-EDTA stock solution 1 mL, trace element stock solution. 1 mL.

Fe-EDTA stock solution: $FeSO_4 \cdot 7H_2O$ (0.7 g) and EDTA (0.93 g) are dissolved in H_2O (80 mL) at about 50 °C, and then diluted to 100 mL.

Trace element stock solution for algae: $MnSO_4 \cdot H_2O$ (169 mg), $Na_2MoO_4 \cdot 2H_2O$ (130 mg), $Co(NO_3)_2 \cdot 6H_2O$ (100 mg), $CuSO_4 \cdot 5H_2O$ (50 mg), H_3BO_3 (100 mg), $ZnSO_4 \cdot 7H_2O$ (100 mg) are dissolved in distilled water (100) mL to obtain trace elements stock solution, respectively.

II. Chemical and biological screening of the North Sea strains

1. Description of sampling site and sampling

Samples were taken monthly between 21 April 1999 and 29 June 2000 in the Wadden Sea (North Sea, Germany) near Norddeich Mole and Neuharlinger Siel. Sediment cores (for MPN and enrichment culture) were taken with Plexiglas tubes (ϕ 36 mm) at low tide, water samples at high tide. For DNA extraction sediment cores were sliced (2 mm from the oxygenated layer and the adjacent zone) and water samples were taken in sea water flushed canisters transported immediately (on ice packs) to the laboratory and filtered on 0.2 μm Nucleopore filters (pore sizes: free water fraction: 0.2 μm; aggregate fraction: first filtration on 0.2 μm followed by filtration on 5.0 μm). The samples were stored at -80 °C. For bacteria phytoplanctons the samples from the canisters were taken immediately at the sampling site, fixed with formol (2 – 4 %) and stored at 4 °C.

2. Storage of strains

All strains were stored in liquid nitrogen for a long time. The conserved strains were used to inoculate an agar plate (MB medium) at room temperature (3 – 10 days) and kept in the refrigerator at 4 °C for maximally three months.

3. Method for cultivation of screened strains

A 1 cm^2 piece of agar from 7 days-old grown culture on the MB2216 agar was used to inoculate 100 mL of the medium contained in a 300 mL-Erlenmeyer flask. The flasks were stored on a rotary shaker under different temperatures for different duration (see Tab. 15).

4. Method for work-up of culture broth

After addition of celite into the culture broth, the mixture was centrifuged for 30 min and the aqueous layer was discarded. The liquid phase was extracted twice with the equal volume of ethyl acetate at the final pH condition of culture. Then the aqueous phase was adjusted to pH 4 with HCl (2 M) and extracted twice with equal volume of ethyl acetate. The mycelium was extracted two times with acetone (2 × 200 mL). All organic phases were separately evaporated under reduced pressure and freeze-dried.

5. Method for chemical screening and OSMAC of H260

The obtained crude extracts were analyzed by HPTLC and HPLC-DAD (UV detector). The solution of the crude extract (methanol/distilled water = 9 : 1) was dripped on the HPTLC and developed separately in both solvent systems of chloroform/methanol (9 : 1) and the above layer of mixture of butanol/acetic acid/water (4 : 1 : 5). After drying the plates were marked under 254 nm and 366 nm UV-light and subsequently stained by different staining reagents under heating.

6. Evaluation of the biological and chemical screening

Tab. 15 gives the culture conditions and screening-results of all strains.

Tab.15: Results of the chemical -biological screening of North Sea bacteria

Strain	Source	Species	Condition of Incubation	Evaluation of biol.screening	Eva.chem. screening	Identified metabolites
T1	Dr. T. Brinkhoff	n.c.	20 °C, 6 d	-	-	-
T4	Dr. T. Brinkhoff	n.c.	20 °C, 6 d	-	-	-
T5	Dr. T. Brinkhoff	*Ruegeria* sp., α–proteobacteria	28 °C, 3 - 5 d 23 °C, 3 - 7 d 15 °C, 3 -10 d	++, i. most organisms, but its growth induced by HP4	-	Tropodithietic acid; Hydroxytropodithietic acid; Anthranilic acid and its ethyl ester
RK377	Dipl. Biol. H.Stevens	*Halomonas* sp., γ–proteobacteria	28 °C, 6 d	i. *B. subtilis*	+	Pyrrol derivate Indol derivate Thioazo derivative Imidazolindole Pyrimidinindole
RK2207	Dipl. Biol. H.Stevens	*Mesorhizobium* sp.	28 °C, 6 d	-	+	Carotines, Bacteriopheo-phytin, Indole derivative
RK1099	Dipl. Biol. H.Stevens		28 °C, 6 d		+	Anthranilic acid
RK342	Dipl. Biol. H.Stevens		28 °C, 6 d, no cultureable.	-	-	-
H260	Dipl. Biol. H.Stevens	*Kokuria* sp.	28 °C, 3 d	i. *E. coli*, ++, microalgae +	+	Peptides, Indole-3-acetic acid
HP4	Dr. H. P. Grossart	93% *Oleiphilus messinensis*	28 °C, 6 d, XAD		+	-
HP5	Dr. H. P. Grossart	98% *Frigobacterium* actinomyceten)	28 °C, 6 d	i. HP19	-	-
HP8	Dr. H. P. Grossart	n.c.	28 °C, 6 d	i. HP16,17,20a	-	-
HP9w	Dr. H. P. Grossart		28 °C, 4 d	i. HP1		Indole derivative, Phenylacetic acid
HP11	Dr. H. P. Grossart	γ-proteobacteria	15 °C, 14 d	-	-	-
HP12	Dr. H. P. Grossart	n.c.	*HP29,15 °C, 14 d	-	+	
HP14	Dr. H. P. Grossart	γ-proteobacteria	*HP15, 28 °C, 8 d	i.HP10,15,20a	-	-
HP15	Dr. H. P. Grossart	98% *Marinobacter*	*HP14,28 °C, 8 d	-	-	-

Tab.15: Results of the chemical -biological screening of North Sea bacteria (continued)

Strain	Source	Species	Condition of Incubation	Evaluation of biol.screening	Eva.chem. screening	Identified metabolites
HP16	Dr. H. P. Grossart	n.c.	28 °C, 6 d	-	+	-
HP17	Dr. H. P. Grossart	n.c.	28 °C, 6 d	-	+	Anthranilic acid
HP18	Dr. H. P. Grossart	α-proteobacteria	20 °C, 6 d	i. HP7,20a,35,42	-	Phenylacetic acid
	Dr. H. P. Grossart	actinomyceten	20 °C, 6 d	-	+	
HP21	Dr. H. P. Grossart	Gram-positive	20 °C, 6 d	i. HP20a,	+	
HP22	Dr. H. P. Grossart	α-proteobacteria	28 °C, 6 d	-	-	-
HP23	Dr. H. P. Grossart	γ-proteobacteria	28 °C, 6 d	-	-	-
HP26	Dr. H. P. Grossart	n.c.	*HP33, 15 °C, 28 d	i. HP33	+	Anthranilic acid
HP27	Dr. H. P. Grossart	n.c.	28 °C, 6 d	-	+	-
HP29	Dr. H. P. Grossart	Gram-positive	*HP12,15 °C, 14 d	i. HP12	+	-
HP30	Dr. H. P. Grossart	n.c.	28 °C, 6 d, XAD	-	+	-
HP33	Dr. H. P. Grossart	99% *Rhizobium*	*HP26, 15 °C, 28 d		+	-
HP34	Dr. H. P. Grossart	γ-proteobacteria	20 °C, 6d	i. HP20a	+	
HP35	Dr. H. P. Grossart		28 °C, 4 d, 16 d			-
HP37	Dr. H. P. Grossart		28 °C, 4 d			-
HP38	Dr. H. P. Grossart		20 °C, 6 d	i. HP20a	+	-
HP42	Dr. H. P. Grossart	actinomyceten	28 °C, 4 d, 15 °C, 14 d	i. HP35		-
HP44	Dr. H. P. Grossart		28 °C, 6 d			Indole derivative (**35**)
HP46	Dr. H. P. Grossart		28 °C, 4 d			-
HP47	Dr. H. P. Grossart		28 °C, 4 d			-

++: very active, +: interesting or active, -: not striking;

d: day; n.c.: not confirmed; i.: inhibition; *:co-cultivating.

III Biosynthesis efficiency and metabolites of strain T5

1. The studies of optimal growing parameters

1.1. Determination of optimal pH value and temperature

In each experiment the fermenter was inoculated with a 24 h-old pre-culture (10 %). Growth was determined by measuring the optical density at 650 nm offline in a photometer (LKB, England).

The optimum pH value and the temperature for growth were determined by growth experiments in a temperature- and pH-controlled 1.5 L-batch fermenter system (FairMenTec, Germany). MB, sterile filtrated and supplemented with ferric citrate (0.1 g/L), was used as growth medium. Oxygen and pH value were monitored by an oxygen electrode (Mettler Toledo, Germany) and a pH-electrode (Mettler Toledo, Germany), respectively. The pH value was adjusted by automatic titration with NaOH (1 M) or H_2SO_4 (1 M). The temperature was controlled by a circulation thermostat (Lauda, Germany).

For determining the temperature-optimum, the pH value was kept at pH 7.0 and the temperature was varied from 25 °C to 35 °C in steps of 5 °C. For determining best pH value, the temperature was kept at 30 °C and the pH value was adjusted to 6.0, 7.0, 7.5, 8.0 and 8.5, respectively.

1.2. Determination of optimal salinity and ferric citrate concentration

The experiments were done in the 250 mL-shaking flasks (each containing 50 mL of SF medium) at 25 °C and 180 rpm. For determining the optimal salinity no ferric citrate was supplemented and the salinity was varied from 0 (only about 0.4 g/L out of the preculture) to 65 g/L. A concentrated salt-stock solution of 106 g/L with following components (g/L): NaCl (72), KCl (2.1), $MgCl_2$ (15.5), $CaCl_2$ (3.4), Na_2SO_4 (12), $NaHCO_3$ (0.9), was used.

1.3. Determination of correlation between growth of strain and production of bioactive compound

The coherence between growth and production of bioactive compound was investigated in a temperature- and pH-controlled 1.5 L-batch fermenter system (FairMenTec, Germany). The experiment was carried out at 30 °C and pH 7.5. The MB medium, sterile filtrated and supplemented with 0.1 g/L ferric citrate, was used as growth medium.

Growth of cell was determined by measuring the optical density at 650 nm offline in a photometer (LKB, England). The production of bioactive compound was determined by using an agar diffusion test with *B. subtilis* (DSM No. 7) as test organism. For this purpose 10 μL of

the culture broth were dropped on a ϕ6 mm filter-paper disc and placed on a test plate. On each plate one disc with 5 μL penicillin G (0.1 g/L) served as reference. After incubation overnight at 30 °C the diameters of the inhibition zones were measured. The relative inhibition was calculated as the ratio of the sample to the reference.

2. 50 L-fermentation of strain with MB medium

The strain T5 was incubated at 28 °C for 12.5 h with the MB medium and 46 L of deionised water, which was inoculated with 4 L of seed culture cultivated under the same condition as the main culture for 40 h. The filtrate of the culture broth was at first separated using a presser and then adjusted to pH 3 with HCl (2 M), and then extracted with ethyl acetate. The crude extract was further purified by column chromatography and preparative HPLC.

3. Bioautography method

In order to identify the responsible zone for biological activity on the TLC, the crude extract was dripped on TLC and developed in a suitable mixture of solvents under sterile flow box. Then the plate was taken out, dried and cut into narrow, flat and long strips under sterile box. One of the strips was put on the test agar inoculated with corresponding organisms. The other strips were marked under UV light or stained with different staining reagents. The active zone was confirmed after the incubation of test organisms and compared with the other strips.

4. Derivatization of 11

Acetone (2 mL) was added to the mixture of **11** (4 mg, 0.02 mmol) and sodium carbonate (2 mg, 0.02 mmol) at room temperature. Stirring was continued for 3 h. After removal of the solvent p-bromobenzyl bromide (4.9 mg, 0.02 mmol solution in tetrahydrofuran) was added to the residue. The resulting mixture was stirred for 4 h at room temperature. The solvent was removed.

5. 7 L-fermentation of strain with SG medium

Seed preparation was a sequence of two steps. 50 mL of SF medium in 250-mL-Erlenmeyer flask inoculated with glycerin culture of strain T5 was incubated at 25 °C and 180 rpm for 7 h. Four 1 L-Erlenmeyer flasks (each containing 200 mL of SF medium) were inoculated with the seed (10 mL), respectively, and then incubated at the same condition overnight. Then the preculture was centrifuged and the cell was diluted to 200 mL. 8 L-fermentation with SG medium was carried out in 10 L-bioreactor (Biostat C) for 38 h at 30 °C and 300 rpm.

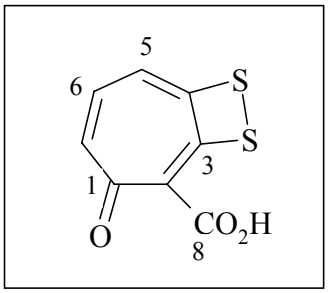
The culture filtrate was adjusted to pH 3 and subjected to a column of XAD-16. The methanol eluate was concentrated *in vacuo* and the crude extract (1.8 g) was obtained.

6. 1 L-fermentation of strain with SH medium

The glycerin culture of strain was inoculated into three 250 mL-Erlenmeyer flasks (each containing 50 mL SH of medium). The resulting medium was incubated at 25 °C and 180 rpm overnight. Fermentation was carried out in 1.5 L fermenter containing 1 L of SH medium at 30 °C and 300 rpm for 23 h. The culture broth was centrifuged and the filtrate was adjusted to pH 3 with HCl (2 M). After extraction with ethyl acetate the metabolites were isolated by a silica gel column eluted with the mixture of dichloromethane and methanol (9 : 1).

7. Characterization of isolated metabolites

Tropodithietic acid (11)



$C_8H_4O_3S_2$ (212.25)

Rf-value: sol. H: 0.27.

Color-reaction: orange color (nature), red-brown ($PdCl_2$)

Mp.: 220-229 °C (dec.)

EI-MS (70 eV): m/z (%) = 212 (100) $[M]^+$, 168 (66) $[M\text{-}CO_2]^+$, 140 (29), 96 (26).

HREI-MS: for $C_8H_4O_3S_2$, calculated: 211.9601, found: 211.9601 ± 2 ppm

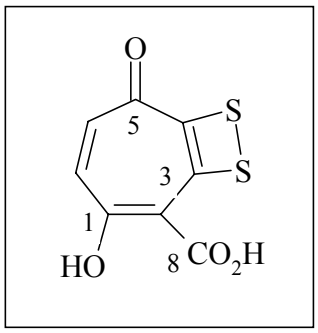
DCI-MS: m/z = 230 $[M+NH_4]^+$, 442 $[2M+NH_4]^+$

IR (KBr) (cm^{-1}): $\tilde{\nu}$ = 3443, 1633, 1599, 1531, 1475, 1380, 1240, 1105, 886, 825

UV [$\lambda_{max,}$ nm (lg ε)] **:** MeOH: 216 (4.36), 303 (4.16), 354 (3.67), 448 (3.08)
MeOH/HCl: 210 (4.30), 303 (4.19), 355 (3.69), 452 (3.04)
MeOH/NaOH: 219 (4.45), 307 (4.11), 445 (3.16)

^{1}H-NMR and ^{13}C-NMR: see Tab. 2.

Hydroxytropodithietic acid (12)



$C_8H_4O_4S_2$ (228.25)

Rf-value: sol. I: 0.5

Color-reaction: yellow color (nature), red-brown ($PdCl_2$).

EI-MS (70 eV): m/z (%) = 228 (3) $[M]^+$, 184 (100) $[M\text{-}CO_2]^+$, 156 (16), 128 (9).

HREI-MS: for $C_8H_4O_4S_2$, calculated: 227.9551, found: 227.9551 ± 2 ppm.

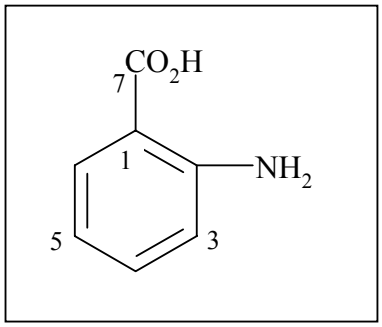
ESI-MS (negative ion): m/z = 227 $[M\text{-}H]^-$.

UV [$\lambda_{max,}$ nm]: ACN/H_2O/H_3PO_4 (from HPLC-DAD): 237, 268, 355, 399.

^{1}H-NMR (600 MHz, CD_3OD): δ = 7.04 (1H, d, J = 8 Hz, 6-H), 7.31 (1H, d, J = 8 Hz, 7-H).

^{13}C-NMR (150.8 MHz, CD_3OD): δ = 176.0 (s, C-5), 169.0 (s, C-1), 137.6 (d, C-7), 136.2 (d, C-6), 131.0 (s, C-4), 111.0 (s, C-2).

2-Aminobenzoic acid (31)



$C_7H_7NO_2$ (137.14)

Rf-value: sol. H: 0.31.

Color-reaction: orange color (Ehrlich).

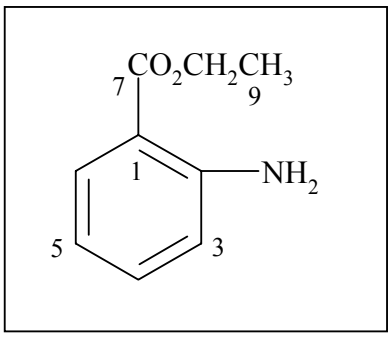
EI-MS (70 eV): m/z (%) = 137 (84) $[M]^+$, 119 (100), 92 (48), 65 (14).

UV [$\lambda_{max,}$ nm]: neutral: 218, 248, 335.

^{1}H-NMR (300 MHz, d_6-acetone): δ = 7.84 (1H, dd, J = 8, 1 Hz, 6-H), 7.25 (1H, td, J = 8, 8, 1 Hz, 4-H), 6.68 (1H, dd, J = 8, 1 Hz, 3-H), 6.56 (1H, td, J = 8, 8, 1 Hz, 5-H), 3.98 (br, -NH_2, -OH).

^{13}C-NMR (75.5 MHz, d_6-acetone): δ = 170.3 (s, C-8), 152.6 (s, C-2), 134.7 (s, C-6), 132.3 (s, C-4), 117.2 (s, C-3), 115.8 (s, C-5), 110.8 (s, C-1).

2-Aminobenzoic acid ethyl ester (<u>32</u>)



$C_9H_{11}NO_2$ (165.19)

Rf-value: sol. H: 0.23.

Color-reaction: orange color (Ehrlich).

EI-MS (70 eV): m/z (%) = 165 (38) $[M]^+$, 137 (60), 119 (100), 92 (48).

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.87 (1H, d, J = 8 Hz, 6-H), 7.23 (1H, m, 4-H), 6.63 (2H, m, 3/5-H), 4.32 (2H, q, 8-H), 1.37 (3H, t, 9-H).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δ = 168.2 (s, C-7), 150.6 (s, C-2), 134.0 (s, C-4), 131.2 (s, C-6), 116.7 (s, C-5), 116.2 (s, C-3), 111.0 (s, C-1), 60.3 (t, C-8), 14.4 (q, C-9).

IV. Biosynthesis efficiency and metabolites of strain RK377

1. 2.4 L cultivation of strain with MB medium in shaking flasks

The agar culture of strain was inoculated into 24 1 L-Erlenmeyer flasks (each containing 100 mL of MB medium), respectively. The resulting medium was incubated at 28 °C and 180 rpm for 6 d. After harvesting the culture broth was centrifuged, and the filtrate was adjusted to pH 4 with HCl (2 M). After extraction with ethyl acetate, the crude extract (298 mg) was obtained.

2. 40 L-fermentation of strain with MB medium

Seed preparation was a sequence of two steps. The MB medium (100 mL) was first inoculated with the slant culture of RK377, and then incubated at 27 °C and 100 rpm for 3 d. Eight 500 mL of MB medium were inoculated with 10 mL of seed obtained at the first step, respectively, and then incubated at the same condition with the first step. 40 L-fermentation

with MB medium was carried out in a 40 L-bioreactor (B. Braun P50) at 27 °C and 200 rpm for 42.5 h.

The culture broth was adjusted to pH 3 with HCl (5 M) and centrifuged. After extraction with ethyl acetate, the crude extract (3.74 g) was obtained.

3. 40 L-fermentation of strain with SJ medium

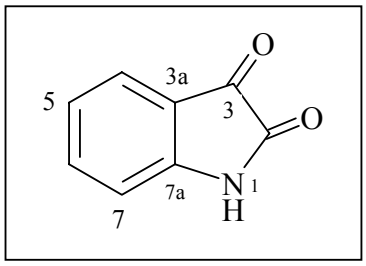
Instead of MB medium SJ medium was used. Other conditions and work-up procedures were the same as 40 L-fermentation with MB medium (see **2.**)

4. Methylation of 44 and 45

44 (about 1 mg) was dissolved in methanol (1 mL), and diazomethane (1 mL cold solution in ether) was added to the solution. The mixture was stirred at room temperature for 6 h (the reaction was monitored by TLC to about 100 %). Diethyl ether and surplus diazomethane were evaporated at 40 °C *in vacuo*.

5. Characterization of isolated metabolites

Isatin (33)



$C_8H_5NO_2$ (147.13)

Rf-value: sol. J: 0.5.

Color-reaction: orange natural color, yellow (anisaldehyde).

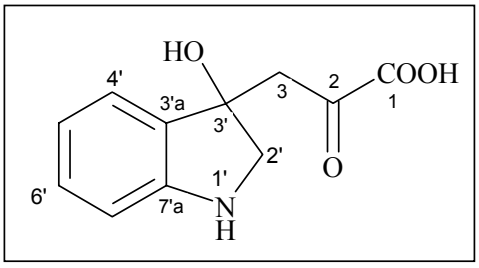
EI-MS: m/z (%) = 147 (98) $[M]^+$, 119 (100), 92 (48), 64 (16).

UV [$\lambda_{max,}$ nm] : ACN/H_2O/H_3PO_4 (from HPLC-DAD): 242, 301.

Mp.: 203.5 °C (dec.).

^{1}H-NMR (500 MHz, d_6-acetone): δ = 10.0 (1H, br, N-H), 7.62 (1H, ddd, J = 8, 8, 1 Hz, 6-H), 7.53 (1H, dm, J = 8 Hz, 4 H), 7.12 (1H, ddd, J = 8, 8, 1 Hz, 5-H), 7.02 (1H, dm, J = 8 Hz, 7-H).

3-(3-hydroxy-2,3-dihydro-1*H*-indol-3-yl)-2-oxo-propionic acid (34)



$C_{11}H_{11}NO_3$ (205.21)

Rf-value: sol. J: 0.32.

Color-reaction: violet (anisaldehyde).

EI-MS (70 eV): m/z (%) = 205 (100) $[M]^+$, 162 (68), 148 (76), 120 (48), 119 (15), 92 (12).

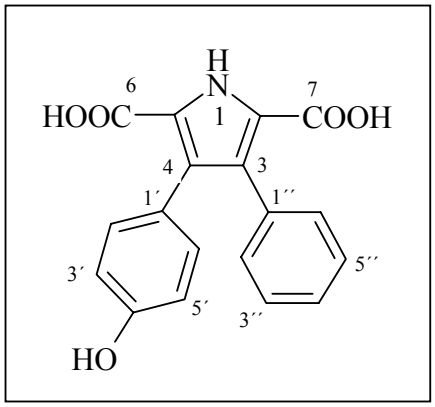
HREI-MS: for $C_{11}H_{11}NO_3$, calculated: 205.0738, found: 205.0738 ± 2 ppm.

UV [λ_{max}, nm]: ACN/H_2O/H_3PO_4 (from HPLC-DAD): 255, 291.

^{1}H-NMR (500 MHz, d_6-acetone): δ = 9.30 (1H, br, N-H), 7.31 (1H, d, J = 7.5 Hz, 4′-H), 7.19 (1H, ddd, J = 7.5, 7.5, 1 Hz, 6′-H), 6.94 (1H, ddd, J = 7.5, 7.5, 1 Hz, 5′-H), 6.86 (1H, d, J = 7.5 Hz, 7′-H), 5.00 (1H, br, O-H), 3.39 (2H, s, 2′-H), 3.32 (1H, d, J = 16.5 Hz, 3-H), 3.10 (1H, d, J = 16.5 Hz, 3-H).

^{13}C-NMR (150.8 MHz, d_6-acetone): δ = 206.7 (s, C-2) 179.0 (s, C-1), 143.0 (s, C-7′a), 132.2 (s, C-3′a), 130.7 (d, C-6′), 125.4 (d, C-4′), 123.2 (d, C-5′), 111.1 (d, C-7′), 73.0 (s, C-3′), 71.8 (t, C-3), 51.4 (t, C-2′).

3-(4′-Hydroxyphenyl)-4-phenylpyrrole-2,5-dicarboxylic acid (35)



$C_{18}H_{13}NO_5$ (323.30)

Rf-value: sol. I: 0.28.

Color-reaction: blue (anisaldehyde).

Mp.: 213 °C (dec.).

EI-MS (70 eV): m/z (%) = 323 (92) $[M]^+$, 305 (28) $[M-H_2O]^+$, 279 (40) $[M-CO_2]^+$, 261(66) $[M-CO_2-H_2O]^+$, 235 (100) $[M-2CO_2]^+$.

DCI-MS: m/z = 236 $[M-2CO_2+H]^+$, 253 $[M-2CO_2+NH_4]^+$, 270 $[M-2CO_2+NH_3+NH_4]^+$, 471 $[2M-4CO_2+H]^+$.

HREI-MS: for $C_{18}H_{13}NO_5$, calculated: 323.0794, found: 323.0794 ± 2 ppm.

ESI-MS: positive ions: m/z = 346.3 $[M+Na]^+$, 669.0 $[2M+Na]^+$;

negative ion: m/z = 322.2 $[M-H]^-$.

IR (KBr) (cm^{-1}): $\tilde{\nu}$ = 3407, 1593, 1355, 1239, 815, 795, 700.

UV [$\lambda_{max,}$ nm (lg ε)] **:** MeOH: 245 (4.26);

MeOH/HCl: 243 (4.26);

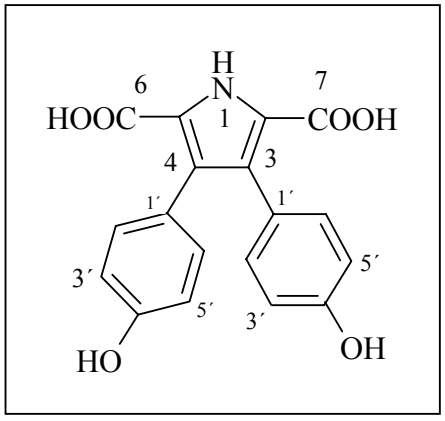
MeOH/NaOH: 249 (4.27).

^{1}H-NMR (300 MHz, CD_3OD): δ = 7.15 (3H, m, 3´´/4´´/5´´-H), 7.08 (2H, m, 2´´/6´´-H), 6.88 (2H, m, 2´/6´-H), 6.55 (2H, m, 3´/5´-H).

^{1}H-NMR (300 MHz, d_5-pyridine): δ = 12.9 (1H, br, N-H), other data are omitted.

^{13}C-NMR (75.5 MHz, CD_3OD): δ = 163.6 (s, C-6/7), 157.2 (s, C-4´), 135.4 (s, C-1´´), 133.2 (d, C-2´/6´), 132.8 (s, C-4), 132.7 (s, C-3), 132.1 (d, C-2´´/6´´), 128.1 (d, C-3´´/5´´), 127.5 (d, C-4´´), 126.2 (s, C-1´), 123.1 (s, C-2/5), 115.0 (d, C-3´/5´).

3,4-Di(4´-hydroxyphenyl)pyrrole-2,5-dicarboxylic acid (<u>36</u>)



$C_{18}H_{13}NO_6$ (339.30)

Rf-value: sol. I: 0.1.

Color-reaction: blue (anisaldehyde).

Mp.: 213 °C (dec.).

EI-MS (70 eV): m/z (%) = 251 (100) $[M-2CO_2]^+$.

HREI-MS: for $C_{16}H_{13}NO_{2:}$ calculated: 251.0946, found: 251.0946 ± 2 ppm.

DCI-MS: m/z = 252.0 (100) $[M-2CO_2+H]^+$.

ESI-MS: positive ion: m/z = 362 $[M+Na]^+$;

negative ion: m/z = 338 $[M-H]^-$.

IR (KBr, cm^{-1}): $\tilde{\nu}$ = 3421, 1655, 1456, 1366.

UV [$\lambda_{max,}$ nm (lg ε)] **:** MeOH: 249(4.27);

MeOH/HCl: 252 (4.27);

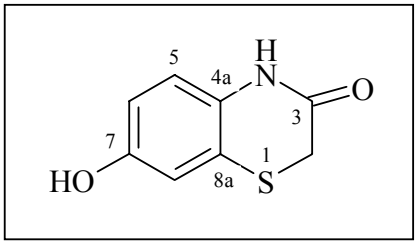
MeOH/NaOH: 251(4.28).

^{1}H-NMR (300 MHz, CD_3OD): δ = 6.92 (4H, 2′/6′-H), 6.56 (4H, 3′/5′-H).

^{1}H-NMR (300 MHz, d_5-pyridine): δ = 11.3 (1H, br, N-H), other data are omitted.

^{13}C-NMR (75.5 MHz, CD_3OD): δ = 167.1 (2C, s, C-6/7), 156.5 (2C, s, C-4′), 133.4 (4C, d, C-2′/6′), 130.5 (2C, s, C-3/4), 127.8 (2C, s, C-1′), 125.0 (2C, s, C-2/5), 114.9 (4C, d, C-3′/5′).

7-Hydroxy-2*H*-benzo[1,4]thiazin-3-one (37)



$C_8H_7NO_2S$ (181.21)

R_f-Value: sol. I: 0.44.

Color-reaction: light-brown (anisaldehyde).

UV [$\lambda_{max,}$ nm] : ACN/H_2O/H_3PO_4 (from HPLC-DAD): 245, 296.

EI-MS (70 eV): m/z (%) = 181 (100) $[M]^+$, 152 (38).

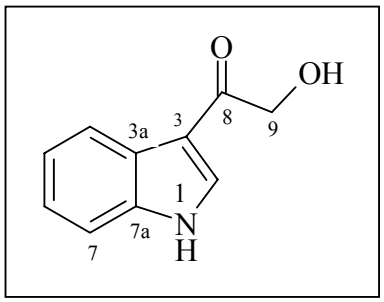
HREI-MS: for $C_8H_7SNO_2$: calculated: 181.0199, found: 181.0199 ± 2 ppm.

^{1}H-NMR (600 MHz, CD_3OD): δ = 6.79 (1H, d, J = 8.6 Hz, 5-H), 6.72 (1H, d, J = 2.6 Hz, 8-H), 6.61 (1H, dd, J = 8.6, 2.6 Hz, 6-H), 3.36 (2H, s, 2-H).

^{1}H-NMR (300 MHz, d_6-acetone): δ = 9.34 (1H, br, N-H), 2.95 (1H, br, O-H), other data are omitted.

^{13}C-NMR (150.8 MHz, CD_3OD): δ =167.8 (s, C-3), 154.9 (s, C-7), 130.8 (s, C-4a), 122.6 (s, C-8a), 119.6 (d, C-5), 115.3 (s, C-6), 114.8 (d, C-8), 30.7 (t, C-2).

3-Hydroxyacetylindole (38)



$C_{10}H_9NO_2$ (175.18)

R_f-Value: sol. I: 0.38.

Color-reaction: brown (anisaldehyde).

UV [$\lambda_{max,}$ nm]: ACN/H_2O/H_3PO_4: 241, 260, 300.

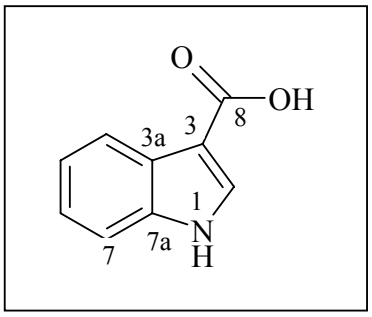
EI-MS (70 eV): m/z (%) = 175 (30) $[M]^+$, 144 (100), 116 (10).

^{1}H-NMR (600 MHz, CD_3OD): δ = 8.22 (1H, dd, J = 7.0, 1.0 Hz, 4-H), 8.19 (1H, s, 2-H), 7.45 (1H, dd, J = 7.5, 1.5 Hz, 7-H), 7.17 (2H, m, 5/6-H).

^{1}H-NMR (300 MHz, d_6-acetone): δ = 11.18 (1H, br, 1-H), 3.88 (1H, br, O-H), other data are omitted.

^{13}C-NMR (150.8 MHz, CD_3OD): δ = 196.0 (s, C-8), 138.2 (s, C-7a), 134.1 (d, C-2), 126.9 (s, C-3a), 124.4 (d, C-6), 123.3 (d, C-5), 122.7 (d, C-4), 114.8 (s, C-3), 112.9 (d, C-7), 66.3 (t, C-9).

Indole-3-carboxylic acid (39)



$C_9H_7NO_2$ (161.16)

R_f-Value: sol. I: 0.43.

Color-reaction: brown (anisaldehyde).

UV [$\lambda_{max,}$ nm]**:** ACN/H_2O/H_3PO_4 (HPLC-DAD): 226, 282.

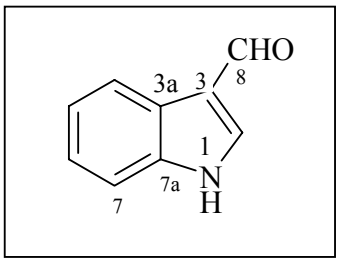
EI-MS (70 eV): m/z (%) = 161 (100) $[M]^+$, 144 (66), 116 (16), 89 (14).

^{1}H-NMR (300 MHz, CD_3OD): δ = 8.08 (1H, m, 4-H), 7.94 (1H, s, 2-H), 7.42 (1H, m, 7-H), 7.17 (2H, m, 5/6-H).

^{1}H-NMR (300 MHz, d_6-acetone): δ = 10.97 (1H, br, 1-H), 4.70 (1H, br, 8-H), other data are omitted.

^{13}C-NMR (75.5 MHz, CD_3OD): δ = 169.3 (s, C-8), 138.2 (s, C-7a), 133.4 (d, C-2), 127.6 (s, C-3a), 123.6 (d, C-5), 122.3 (d, C-6), 122.0 (d, C-4), 112.9 (d, C-7), 108.8 (s, C-3).

Indole-3-carboxaldehyde (40)



C_9H_7NO (145.16)

R_f-Value: sol. I: 0.47.

Color-reaction: brown (anisaldehyde).

UV [$\lambda_{max,}$ nm]**:** ACN/H_2O/H_3PO_4 (HPLC-DAD): 244, 261, 299.

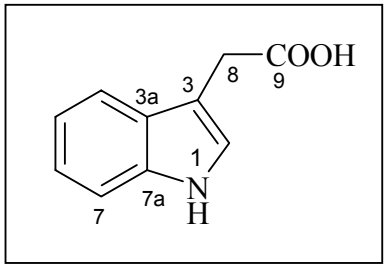
EI-MS (70 eV): m/z (%) = 145 (95) $[M]^+$, 144 (100) $[M-H]^+$, 116 (26) $[M-CHO]^+$, 89 (22).

^{1}H-NMR (300 MHz, CD_3OD): δ = 9.88 (1H, s, 8-H), 8.16 (1H, dd, J = 6.5, 1.5 Hz, 4-H), 7.46 (1H, dd, J = 6.5, 1.5 Hz, 7-H), 7.24 (2H, m, 5/6-H).

^{1}H-NMR (300 MHz, d_6-acetone): δ = 11.20 (1H, br, 1-H), other data are omitted.

^{13}C-NMR (75.5 MHz, CD_3OD): δ = 187.4 (s, C-8), 139.7 (d, C-2), 138.9 (s, C-7a), 125.7 (s, C-3a), 125.0 (d, C-6), 123.6 (d, C-5), 122.4 (d, C-4), 120.1 (s, C-3), 113.1 (d, C-7).

Indole-3-acetic acid (41)



$C_{10}H_9NO_2$ (175.18)

Mp.: 170 – 172 °C.

R_f-Value: sol. I: 0.47.

Color-reaction: brown (anisaldehyde)

EI-MS (70 eV): m/z (%) = 175 (40) $[M]^+$, 130 (100) $[M\text{-}COOH]^+$

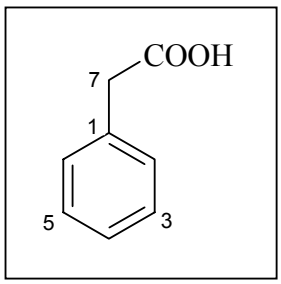
IR (KBr, cm^{-1}): $\tilde{\nu}$ = 3416, 2925, 2362, 1636, 1383, 743.

UV [λ_{max}, nm]: ACN/H_2O/H_3PO_4 (HPLC-DAD): 218, 279.

^{1}H-NMR (300 MHz, CD_3OD): δ = 7.54 (1H, d, J = 7.5 Hz, 4-H), 7.34 (1H, d, J = 7.5 Hz, 7-H), 7.12 (1H, s, 2-H), 7.00 (1H, ddd, J = 7.5, 7.0, 1.0 Hz, 5-H), 7.07 (1H, ddd, J = 7.5, 7.0, 1.0 Hz, 6-H), 3.69 (2H, s, 8-H).

^{13}C-NMR (75.5 MHz, CD_3OD): δ =176.5 (s, C-9), 138.0 (s, C-7a), 128.6 (s, C-3a), 124.6 (d, C-2), 122.4 (d, C-5), 119.8 (d, C-6), 119.4 (d, C-4), 112.2 (d, C-7), 108.9 (s, C-3), 32.0 (t, C-8).

Phenylacetic acid (<u>42</u>)



$C_8H_8O_2$ (136.15)

R_f-Value: sol. I: 0.66.

Color-reaction: negative (anisaldehyde, orcin, Ehrlich, $FeCl_3$).

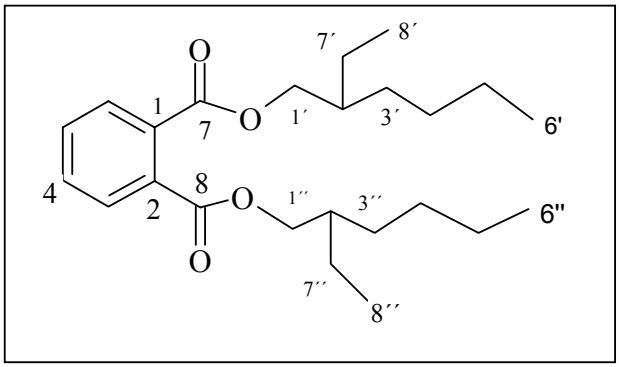
EI-MS (70 eV): m/z (%) = 136 (62) $[M]^+$, 91 (100) $[M\text{-}COOH]^+$, 65 (6).

UV [$\lambda_{max,}$ nm]**:** ACCN/H_2O/H_3PO_4 (HPLC-DAD): 258.

^{1}H-NMR (300 MHz, d_6-acetone): δ = 7.3 (5H, m, 2/3/4/5/6-H), 3.60 (2H, s, 7-H).

^{13}C-NMR (75.5 MHz, d_6-acetone): δ = 172.9 (s, C-8), 135.9 (s, C-1), 130.2 (d, C-2/6 or C-3/5), 129.1 (d, C-3/5 or C-2/6), 127.5 (d, C-4), 41.3 (t, C-7).

Bis(2-ethylhexyl)phthalat (<u>43</u>)



$C_{24}H_{38}O_4$ (390.56)

R_f-Value: sol. C: 0.58.

Color-reaction: brown (anisaldehyde).

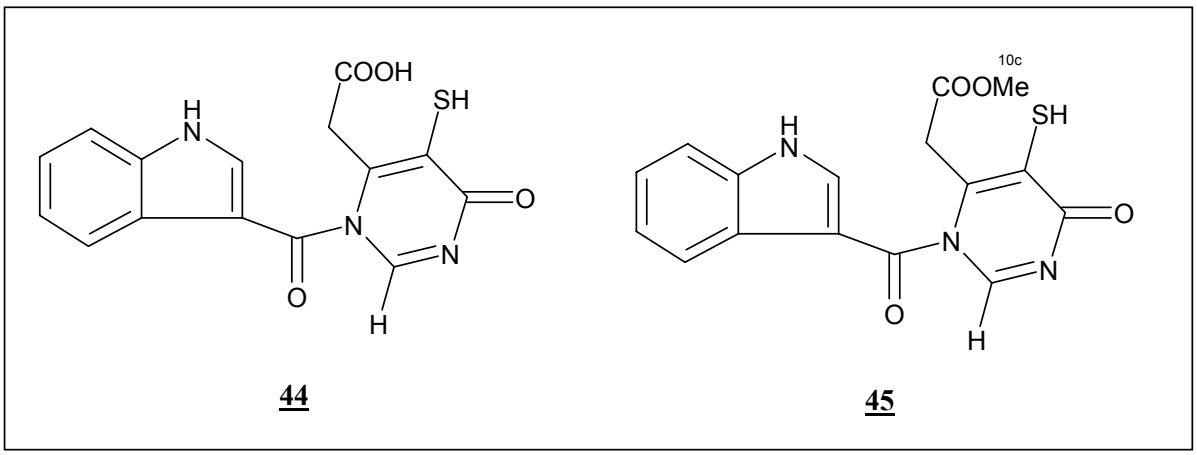
EI-MS (70 eV): m/z (%) = 390 (1) $[M]^+$, 279 (40), 167 (40), 149 (100).

ESI-MS: positive ion: m/z = 390 $[M+Na]^+$, 803 $[2M+Na]^+$.

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.70 (2H, m, 3/6-H), 7.51 (2H, m, 4/5-H), 4.20 (4H, m, 1′/1′′H_2), 1.66 (2H, m, 2′/2′′-H); 1.30 (16H, m, 3′/3′′/4′/4′′/5′/5′′/7′/7′′ H_2), 0.90 (12H, m, 6′/6′′/8′/8′′-H_3).

^{13}C-NMR (75.5 MHz, $CDCl_3$): 167.7 (s, C-7/C-8), 132.4 (s, C-1/C-2), 130.9 (d, C-3/C-6), 128.8 (d, C-4/C-5), 68.1 (t, C-1′/C-1′′), 38.7 (d, C-2′/C-2′′), 30.3 (t, C-7′/C-7′′), 28.9 (t, C-3′/C-3′′), 23.7 (t, C-4′/C-4′′), 23.0 (t, C-5′/C-5′′), 14.1 (q, C-8′/C-8′′) 11.0 (q, C-6′/C-6′′).

Glusun I (<u>44</u>) and its methyl ester (<u>45</u>)



<u>44</u> **<u>45</u>**

$C_{15}H_{11}SN_3O_4$ for **<u>44</u>** (329.33) **$C_{16}H_{13}SN_3O_4$** for **<u>45</u>** (343.36)

R_f-Value for <u>44</u>: sol. I: 0.44.

Color-reaction for <u>44</u>: brown ($PdCl_2$).

Mp. for 44: 150 °C (dec.).

ESI-MS for 44: positive ion: m/z = 374 $[M-H+2Na]^+$, 725 $[2M+3Na-2H]^+$;
negative ion: m/z = 328 $[M-H]^-$, 350 $[M-2H+Na]^-$, 679 $[2M-2H+Na]^-$.

EI-MS for 45 (70 eV): m/z (%) = 343 (54) $[M]^+$, 329 (4), 284 (10), 255 (24), 228 (16), 144 (100), 116 (23), 89 (18).

DCI-MS for 45: m/z = 344 $[M+H]^+$, 361$[M+NH_4]^+$.

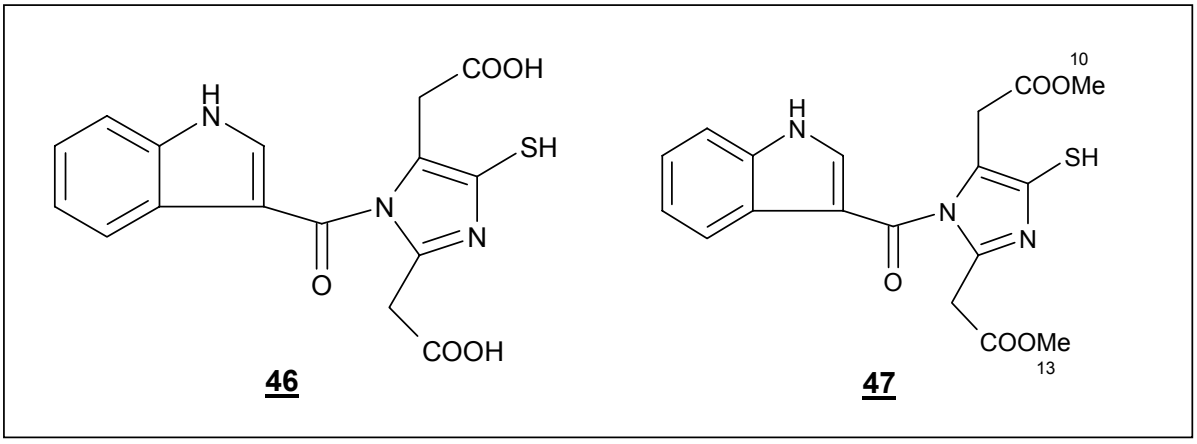
HREI-MS for 45: for $C_{16}H_{13}SN_3O_4$: calculated: 343.0629, found: 343.0629 ± 2 ppm.

IR for **44** (KBr, cm^{-1}): $\tilde{\nu}$ = 3381, 3162, 2937, 2511, 2380, 2309, 1729, 1658, 1597, 1548, 1514, 1420, 1387, 1236, 1061, 815, 753.

UV for 44 [$\lambda_{max,}$ nm (lg ε)]**:** MeOH: 271 (3.31), 278 (3.32), 356 (3.35);
MeOH/HCl: 272 (3.31), 278 (3.33), 356 (3.35);
MeOH/NaOH: 272 (3.32), 278 (3.34), 357 (3.34).

^{1}H-NMR and ^{13}C-NMR of 44 and 45: see Tab. 11

Glusun I (46) and its dimethyl ester (47)



$C_{16}H_{13}N_3O_5S$ for **46** (359.36) **$C_{18}H_{17}N_3O_5S$** for **47** (387.45)

R_f-Value for 46: sol. M: 0.35.

Color-reaction for 46: red-brown ($PdCl_2$).

EI-MS (70 eV) **for 47**: m/z (%) = 387 (100) $[M]^+$, 327 (28), 271 (26), 212 (32), 160 (79), 84 (80), 59 (37).

HREI-MS for 47: for $C_{18}H_{17}N_3O_5S$: calculated: 387.0889, found: 387.0889 ± 2 ppm.

ESI-MS for 46: positive ion: m/z = 382 $[M+Na]^+$, 741 $[2M+Na]^+$;
negative ion: m/z = 358 $[M-H]^-$, 717 $[2M-H]^-$.

IR for 46 (KBr, cm^{-1}): $\tilde{\nu}$ = 3441, 2925, 1651, 1634, 1617, 1557, 1541, 1383.

UV for 46 [$\lambda_{max,}$ nm (lg ε)]**:** MeOH: 272 (3.72), 365 (3.56);

MeOH/HCl: 268 (3.74), 370 (3.56);

MeOH/NaOH: 270 (3.70), 365 (3.55).

^{1}H-NMR and ^{13}C-NMR of 46 and 47: see Tab. 12.

V. Investigation of strain RK2207

1. 10 L-fermentation of strain

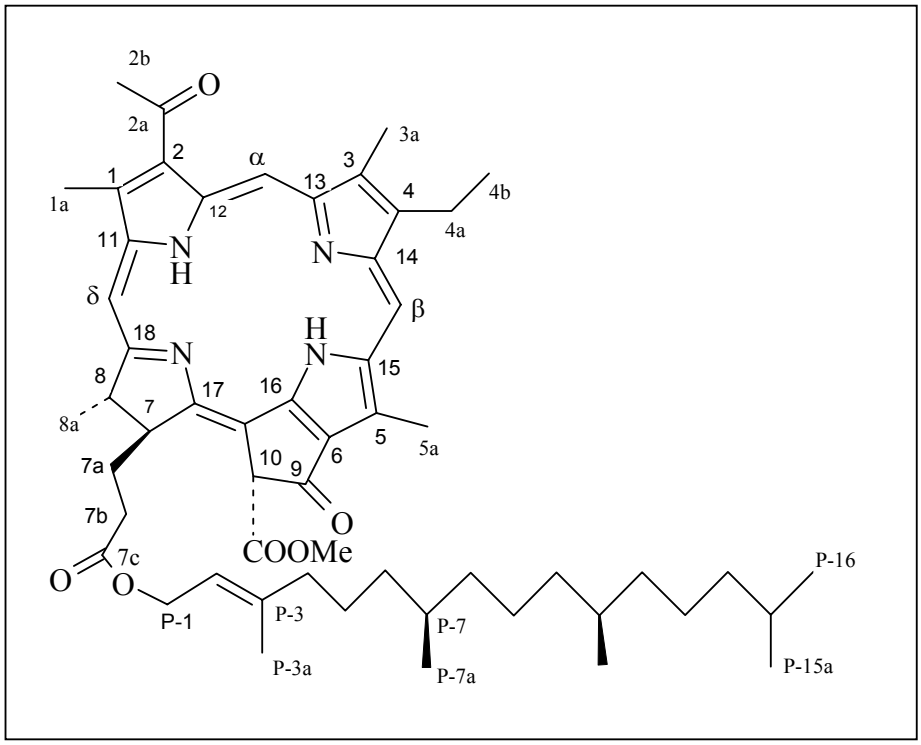
Five 250 mL-Erlenmeyer flasks (each containing 50 mL of SK medium) were inoculated with glycerin-culture (6 mL), respectively, and then cultivated at 25 °C and 180 rpm on a rotary shaker overnight. Five 1 L-Erlenmeyer flasks (each containing 200 mL of the medium) were inoculated with 50 mL of preculture, respectively. The resulting medium was cultivated at 25 °C and 120 rpm overnight.

9 L of SK medium were inoculated with 1 L of the preculture and fermented at 30 °C and 400 rpm for 64 h.

The culture broth was centrifuged. The biomass was extracted with acetone (4 × 100 mL) in an ultrasonic wave bath. The crude extract (327 mg) was obtained. The filtrate was extracted with ethyl acetate (2 × 100 mL). An additional crop of the crude extract (627 mg) was obtained.

2. Characterization of isolated secondary metabolites

Bacteriopheoohytin a_L (51)



$C_{55}H_{74}N_4O_6$ (887.20)

R_f-Value: sol. N: 0.33.

Color-reaction: natural green color.

ESI-MS: positive ion: m/z = 888 $[M+H]^+$;

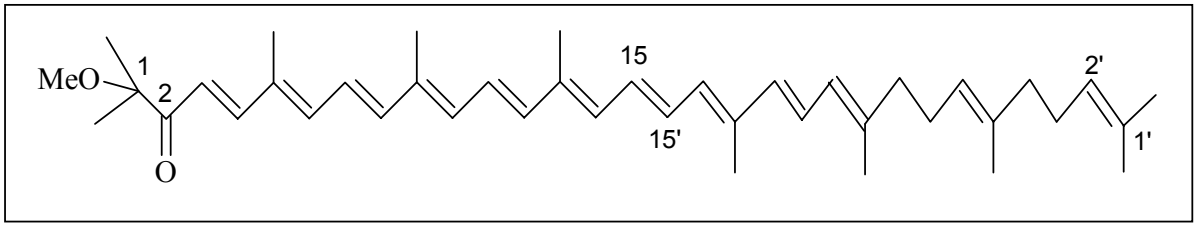
negative ion: m/z = 885 $[M-H]^-$.

HRESI-MS: for $[C_{55}H_{74}N_4O_6 + H]^+$: calculated: 887.57085, found: 887.57085 ± 2 ppm.

UV-Vis: [$\lambda_{max,}$ nm]**:** MeOH: 381, 408, 510, 544, 624, 681.

^{1}H-NMR and ^{13}C-NMR: see Tab. 11.

Spheroidenone (52)



$C_{41}H_{58}O_2$ (582.90)

R_f-Value: sol. P: 0.46.

Color-reaction: natural red color.

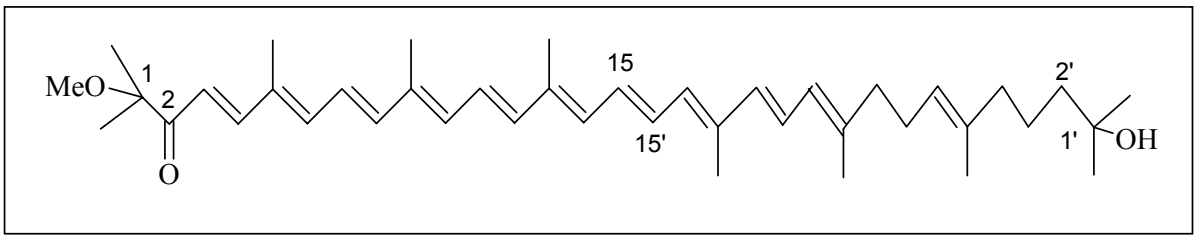
EI-MS: m/z (%) = 582 (14), 476 (10), 73 (100), 69 (36).

ESI-MS: positive ion: m/z = 605 $[M+Na]^+$, 1187 $[2M+Na]^+$, 1769 $[3M+Na]^+$**.**

HREI-MS: for $C_{41}H_{58}O_2$: cal: 582.4456, found: 582.4456 ± 2 ppm.

UV-Vis [$\lambda_{max,}$ nm]**:** MeOH: 457, 333, 284.

Hydroxyspheroidenone (53)



$C_{41}H_{60}O_3$ (600.91)

R_f-Value: sol. P: 0.24.

Color-reaction: natural red color.

EI-MS: m/z (%) = 600 (24) $[M]^+$, 494 (10), 73 (100), 69 (12).

ESI-MS: positive ion: m/z = 623 $[M+Na]^+$, 1223 $[2M+Na]^+$.

HREI-MS: for $C_{41}H_{60}O_3$: cal: 600.4540, found: 600.4540 ± 2 ppm.

UV-Vis [$\lambda_{max,}$ nm]**:** MeOH: 476, 368, 296.

VI. Using OSMAC method to activate the NRPS of strain H260 and identification of peptides

1. OSMAC method to activate the NRPS of strain H260

Tab. 16 gives the results of OSMAC approach to activate the NRPS of strain H260.

Tab. 16 Evaluation of OSMAC approach to activate the NRPS of strain H260

Cultivation mediums	Growth	Biol. activities	TLC	HPLC-MS-MS
MB, 3 d	++	B8: E+++, C+ A4: E++, S+ Z8: E++	A4: +	
MB, 4 d	++	B8: E+	A4: +	
MB, 5 d	++	B8: C++	A4: +	
SM, 3 d	++	B8: E++, S++ A4: E++ Z8: S++, C+		
SM, 4 d	++	B8: E++, C++ Z8: E+++, S+, C++		
SM, 5 d	++	B8: E++, C+ A4: E+++, C+ Z8: E+++, C++		
SM, NaCl 20g/L, 3 d	+	Neg.		
SM, NaCl 20g/L, 4 d	+	Neg.		
SM, NaCl 20g/L, 5 d	+	Neg.		
MB, ASW, 3 d	++	A4: S+, Z8: S+	A4: +	
MB, ASW, 3 d, 3.3% XAD	++	Neg.	A4: + Z8: +	
MB, ASW, 3 d, 4 mg/100mL N-octanoyl-DL-homoserinlactone	++	Z8: S+	A4: +	
MB, ASW, 3 d, 5 mg/100mL N-hexanoyl-DL-homoserinlacton	++	B8: S+ A4: S++ Z8: S+	A4: +	
SC, 3 d	++	B8: S+, B+ A4: S+, M+ Z8: E+++, S+, B+	A4: ++	Diketo-piperazines

Tab. 16 Evaluation of OSMAC approach to activate NRPS of strain (continued)

Cultivation medium	Growth	Biol. activity	TLC	HPLC-MS-MS
SD, 3 d	++	B8: S+, A4: S+ Z8: E+++, S+, B+	A4: ++	Diketo-piperazines
SE, 3 d	+	A4: S+, B+, C+		
SC, 3 d, 6 nM asparagine*	++	A4: M++, S++ Z8: S+, B+	A4: ++ Z8: ++	
SC, 3 d, 6 nM Prolin*	++	B8: B+ A4: M++, S++ Z8: S+, B+	A4: ++ Z8: ++	
SC, 3 d, 6 nM lysin*	++	B8: S+ A4: M++, S++, C+	A4: ++ Z8: ++	
SC, 3 d, 6 nM Hormaomycin*	++	A4: M+++, S++, C+ Z8: E+++, S+, B+, C+	A4: ++ Z8: ++	Diketo-piperazines

*: Its water solution (100 mL) was added into culture broth between 24 h and 28 h of cultivation.
B8: crude extract of filtrate at pH 8; A4: crude extract of filtrate at pH 4; Z8: crude extract of biomass.
E: *E. coli* agar diffusion test; S: *S. aureus* agar diffusion test; B: *B. subtilis* agar diffusion test; C: *C. albican* agar diffusion test; M: microalgae agar diffusion test.
+++: Inhibition zone larger than ϕ 3 cm; ++: Inhibition zone ϕ 2 – 3 cm; +: Inhibition zone about ϕ 1 cm; Neg.: no activity.

2. 50 L-fermentation of strain

The slant culture of H260 was inoculated into the Erlenmeyer flasks with MB medium and then incubated at 28 °C for 2 d. Fermentation was carried out in a 50 L-fermenter with SL medium at 28 °C for 3 d.

The culture broth was separated by an air press-filter. The biomass was extracted with different solvents in the following order: acetone (1 L), methanol (2 L), methanol/dest. H_2O (1 : 1, 1 L) and methanol/dest. water (2 : 8, 2 L). All extracts were evaporated *in vacuo* and freeze-dryer. The culture filtrate (100 mL) was extracted with ethyl acetate (2 × 100 mL), and the organic phase was concentrated *in vacuo*. The water phase was adjusted to pH 4 with HCl (2 M) and extracted with ethyl acetate (2 × 100 mL). The organic phase was concentrated *in vacuo*. Another culture filtrate (100 mL) was directly freeze-dried.

3. Isolation of bioactive compound

Mycelium extract (500 mg) was dissolved in redistilled water (1.5 mL) and centrifuged. The water phase was subjected to a RP-18 MPLC column and eluted with H_2O (100 mL), methanol/dest. H_2O (2 : 8, 100 mL), methanol/dest. H_2O (1 : 1, 100 mL) and methanol (100 mL). All collected fractions were dried and dissolved in methanol/dest. H_2O (1 : 1, 50 mg/mL). The solution (50 μL) of each fraction was subjected on a ϕ 9 mm filter-disc, respectively, and dried in sterile flow box. The dried filter-discs were tested against *E. coli*. The fraction 12 (93 mg) was found to be active.

The active fraction was separated by repeated preparative HPLC (prog. 6). Bioactive compound (3.9 mg) was obtained.

VII. Determination method and data of X-ray crystal structures

The crystals were removed from the flask and mounted on a glass fiber in a rapidly cooled perfluoropolyether[141] and then flash-cooled in a cold nitrogen stream.[142] Intensity data for compounds **11** and **12** were collected on a STOE-AED2 four-circle diffractometer. The diffraction data for the compounds **33** and **35** were measured on a STOE IPDS II diffractometer. The data for all compounds were collected at low temperature with graphite monochromated Mo-Kα radiation (λ = 0.71073 Å), performing φ and ω scans. All non-hydrogen atoms were refined anisotropically with similarity and rigid bond restraints. All hydrogen atoms bonded to carbon were included in the models at geometrically calculated positions and refined using a riding model. The other hydrogen atoms were located in difference Fourier synthesis and refined freely and – in the case of N–H bonds – with the help of distance restraints. The crystal data for all the compounds related to data collection, structure solution, and refinement are listed in Tab. 17 – 20.

Tab. 17 Crystal data and structure refinement of **11**

Empirical formula	$C_8 H_4 O_3 S_2$
Formula weight	212.23
Temperature	133(2) K
Wavelength	0.71073 Å
Crystal system	monoclinic
Space group	*P* 2(1)
Unit cell dimensions	a = 6.5207(9) Å
	b = 5.4515(6) Å β = 104.808(10)°
	c = 11.5610(15) Å
Volume	397.32(9) Å^3
Z	2
Density (calculated)	1.774 Mg/m^3
Absorption coefficient	0.632 mm^{-1}
F(000)	216
Crystal size	0.4 × 0.3 × 0.3 mm^3
θ range for data collection	1.82 – 24.56°
Index ranges	$-7 \le h \le 7$, $-6 \le k \le 5$, $-13 \le l \le 13$
Reflections collected	4809
Independent reflections	1246 [R(int) = 0.0841]
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	1246 / 1 / 122
Goodness-of-fit on F^2	1.125
Final R indices [$I>2\sigma(I)$]	$R1$ = 0.0413, $wR2$ = 0.1059
R indices (all data)	$R1$ = 0.0418, $wR2$ = 0.1105
Largest difference peak and hole	0.354 and –0.450 e Å^{-3}

Tab. 18 Crystal data and structure refinement of **12**

Empirical formula	$C_8 H_3 O_4 S_2$
Formula weight	227.22
Temperature	293(2) K
Wavelength	0.71073 Å
Crystal system	monoclinic
Space group	*C* 2/*c*
Unit cell dimensions	*a* = 25.214(5) Å
	b = 15.628(3) Å β = 101.92(3)°
	c = 6.9089(14) Å
Volume	2663.8(9) Å^3
Z	8
Density (calculated)	1.133 Mg/m^3
Absorption coefficient	0.387 mm^{-1}
F(000)	920
Crystal size	0.4 × 0.3 × 0.3 mm^3
θ range for data collection	2.61 – 24.65°
Index ranges	$-29 \le h \le 29$, $-18 \le k \le 18$, $-7 \le l \le 8$
Reflections collected	8524
Independent reflections	2247 [*R*(int) = 0.0912]
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	2247 / 0 / 128
Goodness-of-fit on F^2	3.720
Final R indices [$I>2\sigma(I)$]	*R*1 = 0.3169, *wR*2 = 0.6780
R indices (all data)	*R*1 = 0.3400, *wR*2 = 0.7018
Largest difference peak and hole	6.408 and –1.213 e Å^{-3}

Tab. 19 Crystal data and structure refinement of **33**

Empirical formula	$C_8H_5NO_2$
Formula weight	147.13
Temperature	203(2) K
Wavelength	0.71073 Å
Crystal system	monoclinic
Space group	*P*2(1)/*c*
Unit cell dimensions	*a* = 6.1857(10) Å
	b = 7.1259(12) Å β = 94.061(15)°
	c = 23.144(5) Å
Volume	641.30(19)
Z	4
Density (calculated)	1.524 Mg/m^3
Absorption coefficient	0.112 mm^{-1}
F(000)	304
Crystal size	0.70 x 0.50 x 0.40 mm^3
θ range for data collection	3.59 to 24.99°
Index ranges	$-7 \le h \le 7$, $-17 \le k \le 17$, $-8 \le l \le 8$
Reflections collected	3184
Independent reflections	1123 [R(int) = 0.0531]
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	1123 / 0 / 101
Goodness-of-fit on F^2	1.116
Final R indices [$I>2\sigma(I)$]	*R*1 = 0.0739, *wR*2 = 0.2254
R indices (all data)	*R*1 = 0.0789, *wR*2 = 0.2332
Largest difference peak and hole	0.499 and –0.313 e Å^{-3}

Tab. 20 Crystal data and structure refinement of **35**

Empirical formula	$C_{24}H_{31}NO_8S_3$ (including 3 molecules of DMSO)
Formula weight	557.68
Temperature	150(2) K
Wavelength	0.71073 Å
Crystal system	monoclinic
Space group	*C*2/*c*
Unit cell dimensions	a = 27.211(5) Å b = 9.5210(19) Å β = 119.77(3)° c = 23.144(5) Å
Volume	5204.7(18) Å^3
Z	8
Density (calculated)	1.423 Mg/m^3
Absorption coefficient	0.334 mm^{-1}
F(000)	2352
Crystal size	0.60 x 0.50 x 0.30 mm^3
θ range for data collection	3.53 to 22.48°
Index ranges	$-22 \le h \le 29$, $-10 \le k \le 10$, $-22 \le l \le 24$
Reflections collected	3444
Independent reflections	3385 [R(int) = 0.0494]
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	3385 / 4 / 343
Goodness-of-fit on F^2	1.066
Final R indices [$I>2\sigma(I)$]	$R1$ = 0.0599, $wR2$ = 0.1257
R indices (all data)	$R1$ = 0.1068, $wR2$ = 0.1514
Largest difference peak and hole	1.011 and –0.354 e Å^{-3}

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Lebenslauf

Am 26. September 1963 wurde ich als viertes Kind des Beamten Zhimin Liang und seiner Ehefrau Shuqing Chen, in Tianjin (China) geboren.

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Seit Mai 2000 arbeitete ich unter Anleitung von Prof. Dr. A. Zeeck an der vorliegenden Dissertation.