Integrated Approach To Explore the Potential of Marine Microorganisms for the Production of Bioactive Metabolites

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Dedicated to Prof. Dr. Wolf-Dieter Deckwer on the occasion of his 60th birthday

During the last 10 years marine organisms have provided a large number of new natural products. Interesting compounds have mainly been derived from macroorganisms such as sponges, ascidians, corals and bryozoans. The number of secondary metabolites from marine microorganisms is smaller, but rapidly increasing. Because of the enormous difficulties involved in harvesting products from marine animals, and the fact that some of the bioactive compounds are produced by associated bacteria, the advantages of sustainable production of bioactive metabolites by bacteria or fungi, under the protection of natural resources, seem to be very attractive for the future. This review describes current progress in the isolation and identification of novel marine microorganisms, the discovery of new secondary metabolites, the biotechnological approaches to overproduce them, as well as the evaluation and characterization of their bioactivity.

Keywords. New bacteria from the North Sea, Secondary metabolites, Bioactivity, Biotechnological production, Structure elucidation, Phylogenetic screening

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1 Introduction

1.1 Marine Bacteria as Sources of New Bioactive Compounds

The search for bioactive compounds is presently reaching a new dimension – with such diverse approaches as genomics, proteomics, bioinformatics, combinatorial biosynthesis, combinatorial chemistry, targeted drug development, directed evolution of key enzymes, phage display libraries, automation and high throughput screening [1–4]. What role can traditional natural product screening play in today's drug development approaches [5], and what in particular

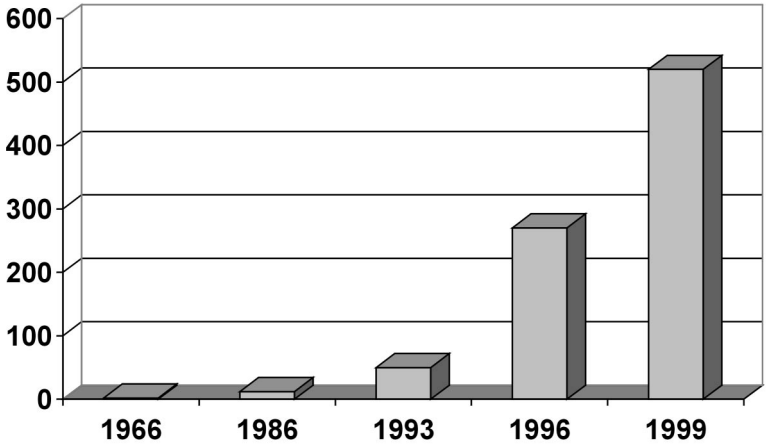


Fig. 1. Total number of metabolites from marine microorganisms (1966–1999)

can be expected from marine microorganisms [6, 7] as sources of novel compounds?

Marine organisms have provided a seemingly endless parade of novel structures. New carbon skeletons were described with a frequency that exceeded all expectations. Several structural features are uniquely or predominantly marine (reviewed by Faulkner since 1986 [8–10]). Thus, ample raw material was provided for combinatorial chemistry, synthetic chemistry, and biocatalysis [11]. The most striking change in the direction of marine natural product chemistry since 1993 was reflected in the sudden increase in reports of new metabolites from marine microorganisms (Fig. 1): Whereas less than ten compounds had been known 20 years ago [12], the number of structures has exponentially grown since then. Although a total number of around 400 compounds is still small in comparison with more than 15,000 structures from terrestrial microorganisms, new compounds are presently almost exclusively found in marine organisms (W Fenical, personal communication).

A number of invertebrate phyla is restricted to the marine environment, with no or few representatives in aquatic and terrestrial habitats, e.g., the Ascidia, Porifera, Coelenterata and Bryozoa. Consequently, from these organisms, a whole range of new chemical structures has been discovered that are not found in terrestrial organisms. However, in many cases, it is not yet clear whether the bioactive compound is produced by the invertebrate, by endosymbiontic or epiobiotic bacteria, or in a cooperative way by both. There is increasing evidence for an important role of the bacterial endosymbionts in the bryostatin-producing bryozoon *Bugula neritina* [13, 14] or for metabolite production in demospongiae [15].

1.2 The Phylogenetic Tree and the Production of Bioactive Compounds

What are marine bacteria? Defining them as bacteria with an absolute requirement for sodium chloride is not a practical solution, because many marine isolates may tolerate quite a wide range of salinities, prompting speculation that they are in fact terrestrial organisms that have been swamped into the oceans from rivers, estuaries and sewage outfalls. Pragmatically, marine microorganisms are therefore defined as bacteria that have been isolated from marine sources on marine media [16].

However, one has to bear in mind that the microbial ecology of marine habitats has been revolutionized by cultivation-independent analyses based on 16S rRNA. It is now well documented that only a fraction of the marine microbial diversity has been cultivated, presumably far less than 1% [17], no more than the "tip of the iceberg" [18]. Clone libraries of marine bacterioplankton 16S rRNA genes are dominated by a few phylotypes that have not been cultivated to date, and which are distributed globally [19, 20]. It can therefore be concluded that the "true" marine microorganisms are in most cases presently not known.

Within the operationally defined "marine bacteria", i.e., bacteria isolated from marine samples on marine media, bioactive compounds have been reported from *Pseudoalteromonas*, *Cytophaga*, *Alteromonas*, *Micrococcus*, *Bacillus*, *Acinetobacter*, *Agrobacterium* and *Pseudomonas* or from unidentified bacteria (Fig. 2).

Cultivated marine bacteria are scattered throughout the phylogenetic tree of the domain Bacteria. However, at lower phylogenetic levels, clusters of marine bacteria have been found which are distinct from those of terrestrial origin. One example is the so-called α3-subgroup of the α-Proteobacteria subclass of the division Proteobacteria, the *Roseobacter* clade [20]. A marine group of *Actinobacteria* [21] has been described, which has, to date, however not been cultivated.

Presently, there are three microbial phylogenetic hot spots known for the production of secondary metabolites:

1. The Streptomycetes, a group of filamentous Gram-positive bacteria (Actinomycetes) that are the work horses of natural product isolation [22];
2. the Myxobacteria, motile bacteria with a complex life cycle which form a distinct cluster within the δ-subclass of the Proteobacteria and have shown to be a rich source of novel structures and biological activities [23, 24];
3. the Cyanobacteria, the former bluegreen algae, photosynthetic bacteria which are distributed globally and produce extremely potent toxins (e.g., [25–27]).

In addition, the antibiotics and other bacteriocins were originally detected in lactic acid bacteria, but were later also found in other Gram-positive microorganisms [28]. Lactic acid bacteria are a group of non-spore-forming, anaerobic fermentative bacteria within the Gram-positives with low GC content.

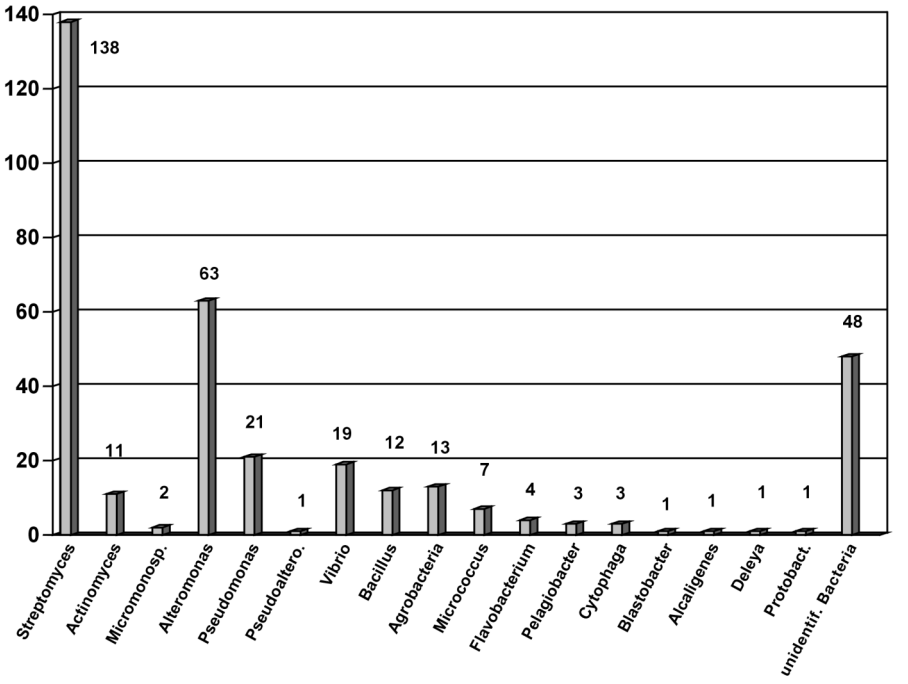


Fig. 2. Number of secondary metabolites isolated especially from marine bacteria until 1999

1.3 Research Network Marine Biotechnology in Lower Saxony

Within the network Marine Biotechnology in Lower Saxony [29] an integrated approach between research groups in microbiology, natural product chemistry, biotechnology, and medicine is underway to systematically explore the metabolic capabilities of North Sea bacteria for the production of bioactive compounds with respect to their phylogenetic position.

Cultivated marine bacteria are notorious for their slow growth and their preference for low nutrient media. Extract yields are therefore often small, resulting in problems with detection limits for natural product chemists. Moreover, it is known that growth media and growth conditions have a profound effect on the production of secondary metabolites and may be different for different strains [30]. It is our working hypothesis that interesting activities that are only expressed under specific conditions, and structures present in low concentrations, may not be detected during conventional mass screening of marine bacteria.

The tools of molecular biology and the phylogenetic framework which is now available as 16S rDNA sequence alignments allow a complementary strategy to be pursued. It is based on a phylogenetic screening of marine isolates and the in-depth investigation of selected phylogenetic groups in order to identify a new hot spot for the production of bioactive compounds.

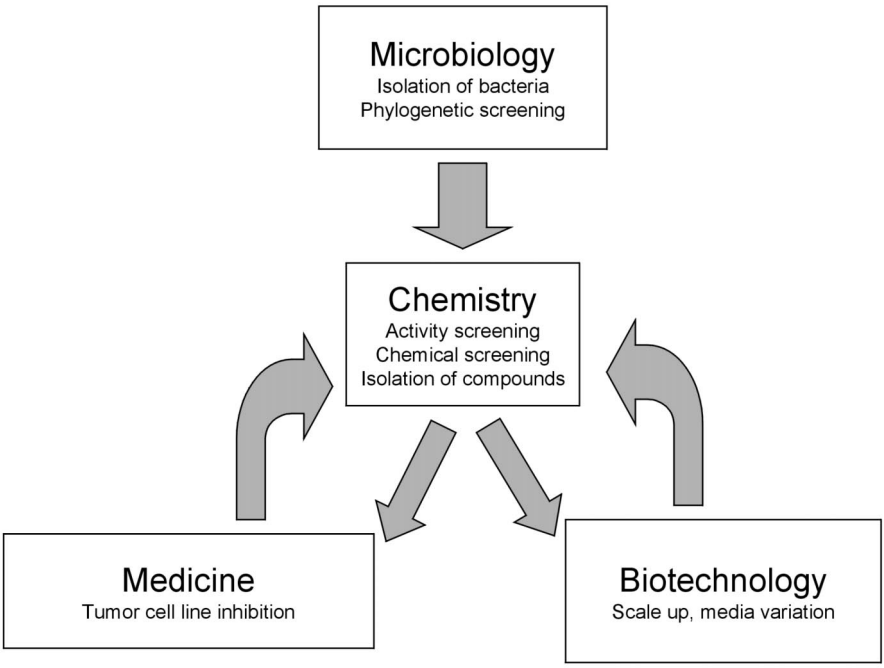


Fig. 3. Integrated approach to explore the metabolic capabilities of North Sea bacteria for the production of bioactive compounds

Isolates from interesting phylogenetic groups (Microbiology) are screened for their biological activity and chemical diversity (Natural Product Chemistry, Medicine). Selected isolates are then cultivated on a large scale and under a variety of cultivation conditions (Biotechnology). Interesting compounds are isolated and their structures are elucidated (Natural Product Chemistry). The concept is shown schematically in Fig. 3.

2
Molecular Tools for Phylogenetic Screening of the Marine Microbial Diversity and First Results on an Indigenous Group of Marine Microorganisms

2.1
Isolation of Marine Bacteria

Cultivation and isolation of bacteria are in some respects more similar to an art than to a scientific method, since there is a strong element of subjectivity involved. Many diverse parameters influence the result of cultivation experiments, including the precise source of the sample (it is probably almost impossible to extract identical samples from nature), the pre-treatment of the sample (storage, cooling, transport, mixing, sieving, filtering, etc.), the enrichment pro-

cedure, if any, and last, but not least, the media and incubation conditions used to cultivate the bacteria. Given the huge diversity of bacteria present in any natural sample and their ability to quickly grow under the right conditions, it is not surprising that totally different subsets of bacteria can be cultivated from a given sample by different cultivators or cultivation methods.

One of our strategies was to isolate marine bacteria which are abundant in the pelagial of the North Sea using "natural" conditions which do not select for the fastest growing strains. We used an unfiltered North Sea water sample obtained with a Ruttner-Schöpfer. The sample was processed within an hour after taking it. No enrichment was performed, but individual colonies were obtained by serially diluting the sample and spread-plating appropriate dilutions on agar plates containing a variety of marine media. Plates were incubated at room temperature for up to 4 weeks. The growth of eukaryotes and protozoan grazing were prevented by the addition of the antibiotic cycloheximide. The isolated bacteria were compared to the total community structure of the sample determined by the small subunit rDNA approach. Such combined approaches have been shown to be successful in retrieving numerically abundant bacteria from anoxic rice paddy soil [31] or oil reservoirs [32].

Other cultivation strategies which were followed were the enrichment of picoplankton bacteria under a wide range of nutrient and incubation conditions [33], and isolation of biofilm bacteria that had grown in situ on artificial surfaces.

2.2 Phylogenetic Screening of Marine Bacteria

Classification of isolates into the main taxa is usually performed using ribosomal probes, either by dot blot hybridization of extracted DNA or by whole cell hybridization using fluorescent probes [34]. However, since we processed a large number of isolates (approx. 900 strains), a polymerase chain reaction (PCR) approach based on crude DNA extracts of individual colonies was a faster and more versatile method. A so-called signature PCR was developed, which is based on amplification of 16S rDNA fragments of taxon-specific lengths using a mix of 16S rDNA targeted primers (Fig. 4) [33]. While hybridization with ribosomal probes requires several hybridization steps with a suite of probes, the signature PCR approach used here allows the diagnosis of the phylogenetic affiliation of a strain with one PCR reaction, since multiple primers are used simultaneously. If the result is ambiguous, it can be clarified using a second PCR reaction with a second set of primers. Moreover, the level of resolution of the signature PCR can be tailored to the phylogenetic groups under investigation, e.g., by including specific primers for uncultivated marine clones or groups of ecological interest (*Verrucomicrobia*, *Planktomycetes*, phototrophic α-*Proteobacteria*).

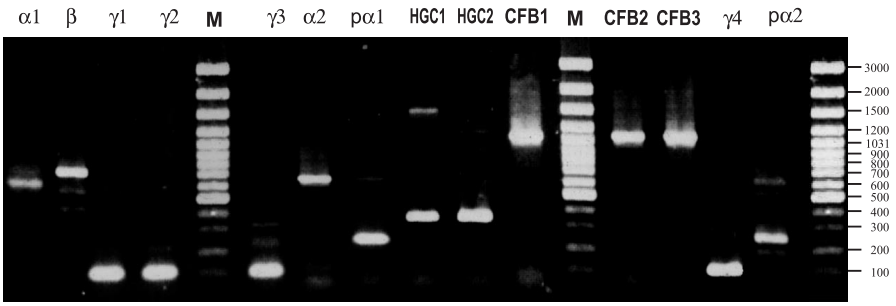


Fig. 4. Phylogenetic fingerprints of reference organisms obtained by signature PCR (SIG-PCR). An oligo primer mixture of nine taxon-specific 16S rDNA targeted primers yielded PCR products with taxon-specific lengths. α1 *Sphingomonas* sp. Hel9, α2 *Sphingomonas* sp. Hel42, pα1 *Erythrobacter* sp. Hel2, pα2 *Sulfitobacter* sp. Hel77, β *Burkholderia* sp. Hel3, γ1 *Halomonas aquamarina* Hel4, γ2 *Pseudoalteromonas carrageenovora* Hel5, γ3 *Pseudomonas sp.* Hel7, γ4 *Vibrio sp.* Hel11, CFB1 *Cytophaga hutchinsonii* (DSM 1761), CFB2 *Weeksella virosa* (LMG 8349), CFB3 *Cytophaga* sp. Hel21, HGC1 coryneform strain Hel1, HGC2 coryneform strain Hel12. The approx. lengths of the expected PCR products are as follows: 650 bp for α-Proteobacteria, 700 bp for β-Proteobacteria, 100 bp for γ-Proteobacteria, 1000 for CFB, 350 for high GC Gram-positives

2.3 Screening of Genetic Diversity – Genomic Fingerprints

Production of secondary metabolites is usually a strain-specific trait. Thus, typing of the isolated bacteria with a high resolution is necessary to assess the genetic diversity of the strains within a given phylogenetic group. The resolution limit of rRNA-targeted methods is not high enough for this purpose, since strains of the same species have the same 16S rRNA sequence. Even different species may sometimes have nearly identical 16S rDNA sequences. Using the variability in length and sequence in the region between the 16S and 23S rRNA operon, the so-called interspacer region polymorphism is a possibility, since this is a very stable but species- to strain-specific trait. However, an even higher resolution is obtained by genomic fingerprint methods. Here, we used a RAPD (random amplified polymorphic DNA) technique with arbitrary primers.

Figure 5 presents genomic fingerprints of 36 α-*Proteobacteria* isolates that had been enriched from a North Sea picoplankton sample. Only four of the bands (lanes 11 and 12, lanes 33 and 34) showed identical patterns. Thus, there were 32 different α-*Proteobacteria* strains present. The data show that a large diversity of bacteria can be enriched from a North Sea picoplankton sample, using a range of incubation and nutrient amendment conditions. The large diversity of the cultivated picoplankton isolates screened may be expected to house numerous phylogenetically and physiologically interesting microorganisms for future investigations [33].

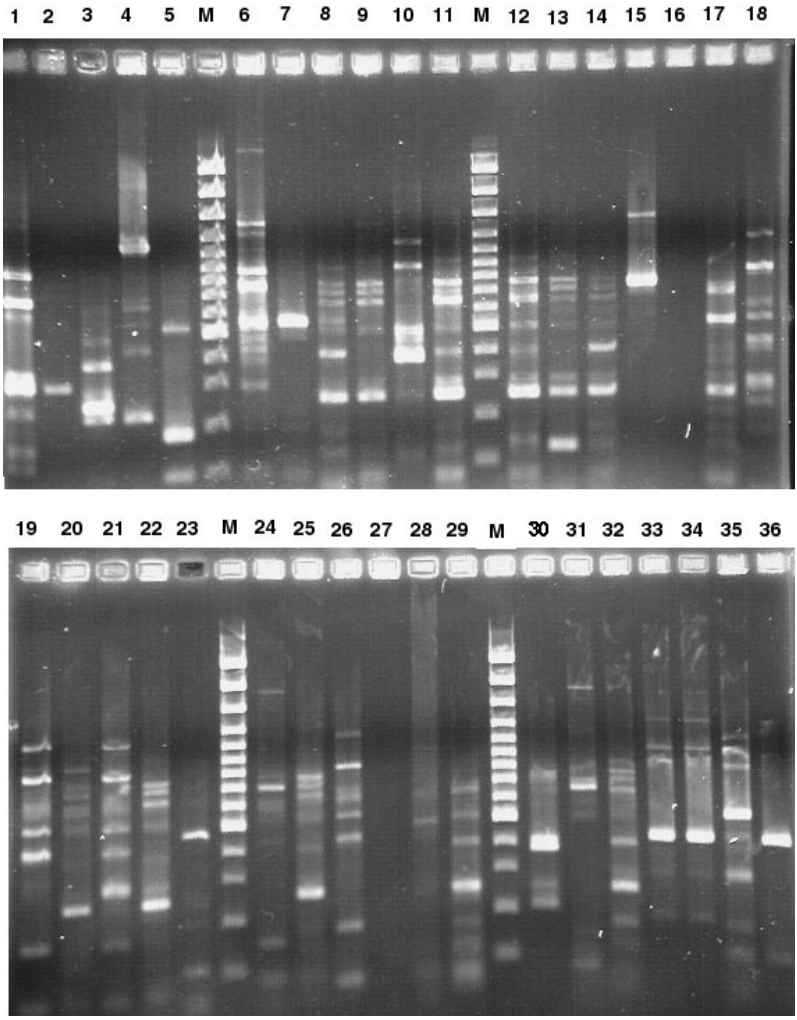


Fig. 5. Genetic diversity of marine picoplankton bacteria from the α-subclass of the Proteobacteria. Determination by random amplified polymorphic DNA (RAPD) fingerprinting of colonies from marine Cytophaga medium (lanes 2–32) and from Hg medium (lanes 33–35). Identical strains are present in lanes 33 and 34 and in lanes 11 and 12, while all other lanes represent different isolates

2.4 The Marine *Roseobacter* Clade of the *Proteobacteria*

One of the largest collections of published environmental small subunit rRNA gene clones from any source is that from marine prokaryotic plankton [20]. This dataset currently includes more than 600 identified bacterial clones. A comprehensive analysis of these sequences has shown that identical sequences falling into similar clades are recovered from geographically and ecologically

diverse regions of the oceans and thus represent global constituents of marine bacterioplankton communities. *Proteobacteria* from the α-subclass represent the largest group of bacterial clone types recovered from marine bacterioplankton and fall into three clusters:

1. The SAR116 cluster, whose cultivated members belong to the α1-subclass of the *Proteobacteria* but have a similarity of less than 90%.
2. The SAR11 cluster with no cultivated counterparts.
3. The *Roseobacter* clade in the α3-subclass of the *Proteobacteria*.

While cultivated microorganisms are not known for any of the two other clades, and indeed for none of the other marine bacterioplankton clades (with some notable exceptions, e.g., the detection of clones of *Pseudomonas putida*), the *Roseobacter* clade is the exception to the rule. Cloned sequences and cultivated organisms form a mixed phylogenetic assemblage and are closely related. Cloned *Roseobacter* sequences have been recovered from the oligotrophic Sargasso Sea, the Eastern Atlantic coast of the USA, the highly productive Oregon coast, and in shallow, enclosed lagoons off the Atlantic and Mediterranean coasts of France. Thus, the *Roseobacter* clade contains globally distributed members of bacterioplankton communities both in the oligotrophic open ocean and in marine coastal environments.

Cultivated members of the *Roseobacter* clade are almost exclusively found in marine or hypersaline habitats and have an absolute requirement for sodium chloride for growth. Their physiological characteristics are diverse. Two genera, *Erythrobacter* and *Roseobacter*, belong to the obligate aerobic phototrophic bacteria which possess bacteriochlorophyll *a* [35] and are capable of aerobic photosynthesis. However, bacteriochlorophyll synthesis only occurs in the dark and is completely inhibited even by low light intensities. Thus, photoautotrophic growth is not possible for these bacteria, only a transient enhancement of growth.

Representatives of the *Roseobacter* clade use thiosulfate as an electron donor, but are unable to grow autotrophically. By contrast, they are chemolitoheterotrophs, i.e., heterotrophic growth is enhanced by reduction of thiosulfate. Some strains can grow on diverse aromatic carbon sources [36] including lignin [37]. *Roseobacter* strains are also able to grow on dimethyl sulfopropionate (DMSP) [38, 39] that is produced by algal blooms at certain seasons in the Atlantic ocean. Consequently, *Roseobacter* strains comprised >20% of the 16S rDNA sampled during the bloom. Recently, *Roseovarius tolerans* was described [40], a budding bacterium isolated from a hypersaline lake in Antarctica. The production of bacteriochlorophyll *a* was apparently genetically remarkably variable in these isolates. A bacteriochlorophyll *a* producing new isolate was even obtained from a deep-sea hydrothermal vent [41]. Other members of the *Roseobacter* clade are entirely non-photosynthetic, e.g., *Sulfitobacter pontiacus* [42–44], *Marinosulfonomonas methylotropha* [45], *Antarctobacter heliothermus* [46], *Octadecabacter* [47], *Sagittula* [37], and *Ruegeria atlantica* [48].

A striking feature of the *Roseobacter* clade is that many isolates are symbionts of marine organisms, e.g., algae, diatoms, dinoflagellates, or have been obtained from surfaces of marine macrophytes. For example, gall inhabitants of

the marine algae *Prionis lanceolata* were closely related to *Roseobacter* [49]. Organisms belonging to the *Roseobacter* subgroup are ubiquitous and rapid colonizers of surfaces in coastal environments [50], and have been found in the accessory nidamental glands of *Sepia officinalis* [51], on the scallop *Pecten maximus* [52, 53], and on the seagrass *Halophila stipulacea* [54]. The dinoflagellate *Prorocentrum lima* is known to produce diarrhetic shellfish poisons. However, it is not yet clear if the dinoflagellates themselves or the *Roseobacter* strains associated with them produce the toxins [55, 56].

The *Roseobacter* clade thus comprises an ecologically interesting phylogenetic group of marine microorganisms that are distributed globally in coastal and open ocean marine bacterioplankton as judged from culture independent analyses. Cultivated isolates and bacteria known from environmental rDNA sequences form a mixed clade with high similarities between both. The *Roseobacter* clade thus forms the exception to the rule, being both abundant in the environment and readily cultivable. The isolates studied to date exhibit several physiological features which can be interpreted as adaptations to the marine environment, e.g., an absolute requirement for sodium chloride and the presence of an oxygenic photosynthesis apparatus, whose occurrence is however variable and which yields only additional energy. *Roseobacter* clade bacteria are able to use a wide spectrum of carbon sources, including aromatic compounds. Additional energy is gained by using oxidized sulfur compounds as electron acceptors, which are important components of the marine sulfur cycle

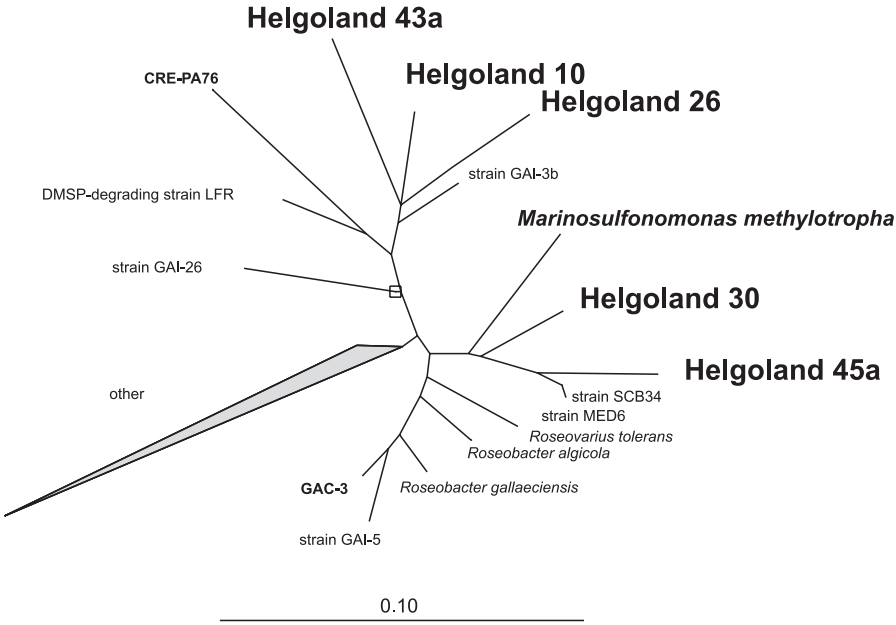


Fig. 6. Phylogenetic tree of the *Roseobacter* clade and the new strains Hel10, Hel26, Hel43 and Hel45

and in some cases coupled to algal blooms. Symbiotic relationships with marine organisms are frequently observed and may point to specific chemical defense mechanisms of these bacteria.

When screening 75 isolates from a water sample taken at a depth of 10 m about 2 km off the coast of Helgoland for phototrophic α3-subclass *Proteobacteria* (the *Roseobacter* clade) using the signature PCR approach, approx. 10 strains were found. From approx. 400 picoplankton isolates, 78 phototrophic α-Proteobacteria (*Roseobacter* clade), and in biofilms, 20 have been found. Several of those were characterized in depth and belong to new species and a new genus (Fig. 6). Descriptions of these new organisms are presently underway. The *Roseobacter* clade will also be investigated for its ability to produce bioactive compounds, since these organisms are phylogenetically unique, clearly marine, readily colonize surfaces and have co-evolved with marine invertebrates in close symbiotic relationships.

3
Screening for Novel Products and New Capabilities

A search for bioactive compounds in nature is a multistep procedure which begins with the selection of suitable sources. Biological, chemical or physical interactions of metabolites with test systems are then qualitatively or quantitatively evaluated [57]: This is the so-called screening which results in arrays of "interesting" or "less-interesting" cultures in the case of microorganisms. Whereas industrial high-throughput systems (100,000 samples and more per year) mostly use highly selective target-oriented test systems, screening systems with lower selectivity are advisable for the manual primary screening: This is dictated mainly by the low hit rate of industrial test systems which is close to 1:10,000 and requires high sample numbers. It is obvious that with capacities of less than 1000 strains per year at universities the success rate would be disappointingly low.

For a first orientation, crude extracts are investigated using the agar diffusion or serial dilution test to evaluate antibacterial, antifungal or – using microalgae – phytotoxic activity. Brine shrimp toxicity has a strong correlation with cytotoxicity and is therefore a good indicator for potential anticancer activity. Strongly positive hits have to be screened further in detail using cancer cell lines or target-oriented systems.

Thirty percent of 500 marine *Streptomyces* strains showed interesting properties in some respect. From 100 selected strains more than 50 new compounds were isolated in a previous project. Their structural diversity, however, was similar to that from terrestrial sources.

In parallel to the biological screening, a search for chemical and physical properties is widely used: This so-called *chemical* screening takes into account that only an unknown fraction of the total metabolite spectrum shows bioactivity in a given test system, but other potential drugs do not respond.

A mass-spectrometric screening for molecular ions using electrospray ionization techniques or matrix-assisted laser desorption/ionization time-of-

flight (MALDI-TOF) needs only microgram amounts and has been used successfully even with intact bacteria from slant agar cultures [58]. However, if a whole array of tests is to be served, much higher amounts of extracts are needed: Experience shows that in most cases bacteria produce secondary metabolites with yields of only 1 mg per liter of culture broth or less.

In chemical screening in its simplest version, extracts are evaluated by thin layer chromatography (TLC) using chloroform/methanol for development, and unspecific spray reagents like anisaldehyde, Ehrlich's reagent, or iodine vapor for detection. Combinations of high-performance liquid chromatography (HPLC) and ultraviolet (UV), nuclear magnetic resonance (NMR) or mass spectroscopy have a much higher resolution and may allow the dereplication of known compounds or even structure determinations. HPLC/UV or HPLC/MS combinations are widespread and are powerful tools in combination with databases.

Chemical and biological screening complement each other very well: The sensitivity of biological methods is much higher than that of chemical analyses and can detect even trace amounts, whereas the chemical screening targets new structures even if they are not obviously bioactive.

4
Strategies for Identifying Anticancer Compounds

During the past five decades of research in anticancer drug discovery about 100 products have been provided for the clinical treatment of malignancy. Important progress has been made in the chemotherapeutic management of hematologic malignancies. On the other hand, more than 50% of patients with tissue tumors either fail to respond or will relapse from the initial response and die from their metastatic disease. In particular, the status of the p53 gene that is mutated in a high percentage of human cancers (particularly lung, breast, colorectal, prostate, gastric and brain tumors) is an important determinant of the efficacy of chemotherapeutic agents [59]. In response to chemotherapeutic agents p53 protein levels rise and produce cell-cycle arrest and apoptosis [60]. Thus, the absence of p53 expression leads to an increase in cellular resistance to these agents. Other mechanisms of drug resistance include enhanced drug metabolism, altered drug accumulation, mutation of topoisomerase 2 and the presence of P-glycoprotein. Hence, the discovery of new cancer therapeutic agents remains critically important.

Marine organisms represent a largely unexplored source of unique toxic chemicals. These toxins are produced by the organisms as defense weapons against their predators. Several potent compounds demonstrating antitumor activity in vitro and in vivo have been isolated from marine organisms, e.g., the bryostatins (from *Bugula neritina*), dolastin 10 (from *Dolabella auricularia*) and halichondrine B (from *Halichondria okadai*) [61].

4.1
Anticancer Drug Development

The steps in the development of anticancer drugs from (marine) organisms are schematically shown in Fig. 7. The most important step in the selection process is initial mass screening. The screening methods can either be simple, such as tumor cell line or enzymatic (e.g., topoisomerase inhibition, microtubule assembly/dissassembly) tests, or more complex, such as an animal tumor in vivo.

4.1.1
In Vitro Tests

Current efforts favor tumor cell line tests, conducted by the National Cancer Institute (NCI) drug development program [62]. In the current NCI anticancer screen, each compound is tested against 60 human tumor cell lines derived from several cancer types (lung, colon, melanoma, kidney, breast, ovary, brain, leukemia). The tumor cells are seeded on 96-well microtiter plates and pre-incubated for 24 h. The test agents are then added to the wells (five 10-fold dilutions; 0.01 – 100 μmol/l) and are incubated for 48 h with the tumor cell lines. At the termination of the assay, the cells are fixed in situ with trichloroacetic acid (TCA), washed and dried. Sulforhodamine B (SRB), a dye that binds to the basic amino

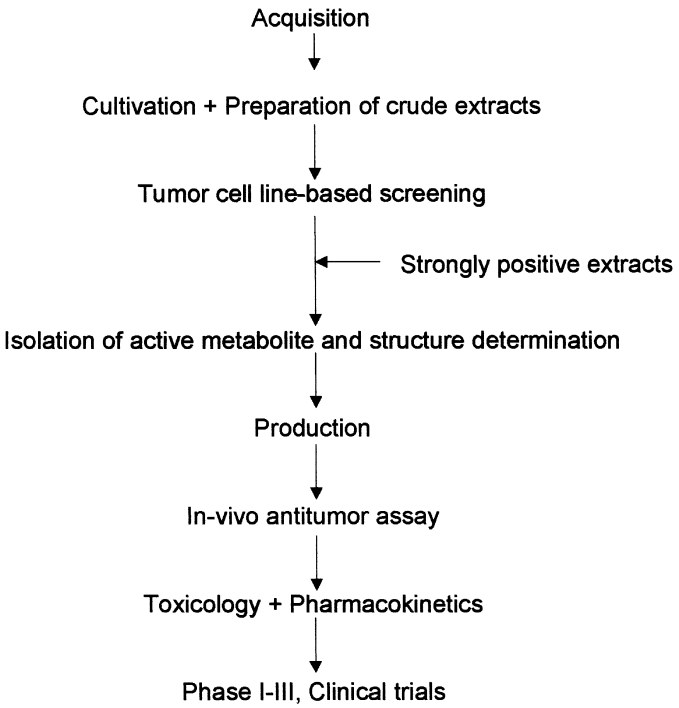


Fig. 7. Steps in cancer drug development from marine organisms

acids, is added. The cells are washed again and the remaining dye which is a function of the remaining adherent cell mass is solubilized and measured spectrophotometrically [63]. From these data the response parameters GI50 (drug concentration causing 50% growth inhibition), TGI (drug concentration causing 100% growth inhibition) and LC_{50} (drug concentration causing 50% reduction of the cell mass present before adding the test agents) are calculated. The individual response of each cell line to the test agent is then compared with a computer program (COMPARE) using a simple algorithm for aligning and contrasting the pattern of the test compound with those of known anticancer drugs. A similar pattern of cellular responsiveness implies a common intracellular target or mechanism of action. Active compounds are then selected for further testing for the following criteria: (i) potency, (ii) cell-type specificity, (iii) unique structure, and (iv) unique mechanism of action. An agent selected for further studies is then subjected to pharmacological studies in animals (pharmacodynamics, pharmacokinetics, toxicology).

4.1.2
In Vivo Antitumor Tests

Compounds selected from the cell line screen are tested in nude mice bearing the tumor cell line which is most sensitive in the in vitro screen. The tumor cell line is introduced subcutaneously or intraperitoneally in a minimal-stage xenograft model for initial testing. The test compound is administered continuously by osmotic mini-pumps or repeated intravenous injection and is initiated on the day of tumor injection. Agents that show significant tumor growth inhibition are then selected for further in vivo evaluation against more advanced stage tumors. Failure of in vivo efficacy for an agent should prompt additional studies to determine whether there is a pharmacokinetic explanation for loss of activity.

4.1.3
Preclinical Pharmacokinetics

Pharmacokinetic studies (absorption, bioavailability, distribution and excretion) in different animal species (e.g., mice, rat and dog) are of fundamental importance for the interpretation of animal pharmacology and toxicology data and the extrapolation of these data to humans. One goal of phase I clinical trials is to determine the maximally tolerated dose (MTD) of a new cytotoxic drug. The dose-limiting toxicity is a function of drug exposure, as measured by the area under the drug concentration versus time curve (AUC). Therefore, animals and humans are predicted to experience toxicity when the AUC of the drug is quite similar. With this assumption a simple method for escalating the dose in phase I clinical trials can be used. The steps are: (i) determine the LD_{10} in the mouse and the AUC of the drug at LD_{10}; (ii) start human testing with one-tenth of the mouse-equivalent LD_{10} dose and determine the AUC of the drug; (iii) compare the AUC at the starting dose with the target AUC (the AUC in mouse at LD_{10}) and increase the dose based on this ratio [64].

4.1.4
Preclinical Toxicology

The vast majority of anticancer drugs are cytotoxic compounds which have significant side-effects and a very small therapeutic index. The objective of preclinical toxicologic studies is to find a safe initial dose for clinical phase I studies and to define the qualitative and quantitative organ toxicities. The current toxicological investigations involve a two-step procedure. First, the acute toxicity in mice and rat is determined. The endpoint is the determination of the LD_{10} value, the dose that causes lethality in approximately 10% of the animals. The major objective of the subsequent second phase is to give qualitative and quantitative characterization of the organ-specific toxicities which can be observed at doses slightly higher than the highest nontoxic dose in acute toxicity. At best, these evaluations can establish data for a safe starting dose for human trials and predict organ toxicity.

4.2
The Screening of Extracts from North Sea Microorganisms

Several groups in the Marine Biotechnology Network of Lower Saxony are studying the cultivation of novel organisms isolated from the North Sea. Crude extracts of these organisms are used to determine antibacterial, antifungal, phytotoxic and cytotoxic activity. In our screening for cytotoxic activity we use routinely three tumor cell lines (HM02 cells derived from human gastric adenocarcinoma; HepG2 cells derived from a human hepatoblastoma; and MCF7 cells derived from human breast carcinoma). In addition we test the extracts in Huh 7 cells (hepatocellular carcinoma), a cell line which expresses p53 with increased half-life as a result of a point mutation at codon 220 [65]. This cell line is insensitive to vinblastine (Fig. 8) and other anticancer drugs such as cisplatin and methotrexate [66], and therefore provides the potential to identify new agents with specific activity against p53-negative tumor cell populations.

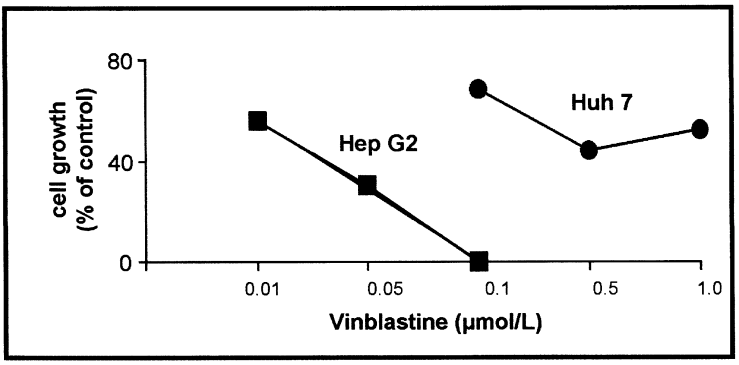


Fig. 8. Effect of vinblastine in hepatoma cells carrying wild-type p53 (HepG2) or in hepatoma cells with mutated p53 (Huh 7). Cells were incubated for 48 h with indicated concentrations of vinblastine

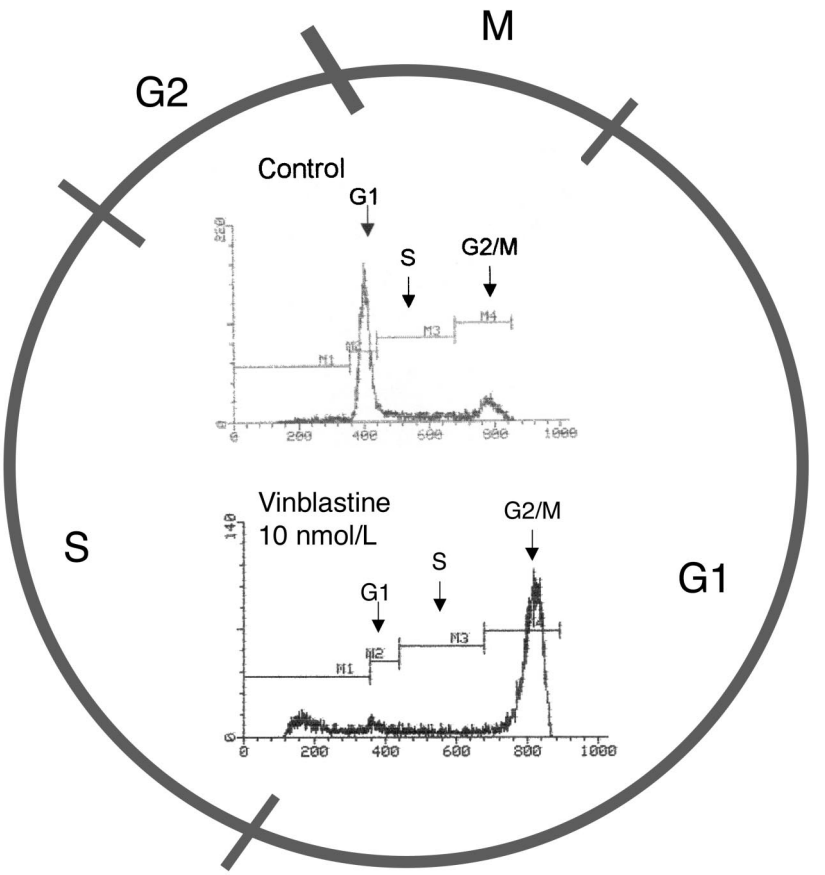


Fig. 9. The cell cycle. *M* mitosis; *G1+S+G2* interphase, i.e., the period between cell division. During G1 (G for gap) the cellular genome is in the diploid state. This is followed by the S-phase (S for synthesis) during which the DNA is replicated, and finally G2. *Insert* Cell-cycle analysis of HM02 cells cultured with and without vinblastine. Cell-cycle distribution was determined by staining DNA with propidium iodide, and the number of cells in different phases of the cell cycle was measured with a FACStar flow cytometer. Vinblastine, which disrupts the formation of microtubules, causes cell-cycle arrest in the G2/M-phase

We have examined approximately 1000 extracts over the past two years. Fifteen extracts (e.g., from the bacteria Hel3, Hel25, Hel37, Hel38 and strain Hel115aa) were highly active with GI50 values <1 µg/ml. To obtain information on the mechanism of action, highly active compounds (and extracts) are subjected to cell-cycle analysis. Continuously dividing tumor cells go from one mitosis (M) to the next, passing through G1-, S- (DNA synthesis phase) and G2-phases. Phase-specific anticancer drugs alter the cell cycle in a specific manner. Vinblastine, which disrupts the formation of microtubles, causes cell arrest in the G2/M-phase (Fig. 9). The antimetabolite 5-fluorouracil (5-FU), which alters RNA processing, inhibits DNA elongation and alters DNA stability, causes cell cycle arrest in the S-phase and induces apoptosis.

Thus, cell-cycle analysis can be used as a first indicator to identify the mechanism of action of a new compound (or extract). Using this strategy we were able to define rapidly the intracellular targets of ratjadone. This compound was isolated in 1994 by Höfle et al. from *Sorangium cellulosum* collected as a soil sample at Cala Ratjada (Mallorca, Spain) [67]. In an initial biological evaluation it was found that ratjadone exhibits high cytotoxicity in the mouse cell line L929 and the HeLa cell line KB 3.1 [67]. In our cell lines ratjadone inhibited cell growth with GI50-values ranging from 0.38 (HM02 cells) to 1.1 ng/ml (MCF7 cells). In contrast, Huh 7 cells were remarkably insensitive to ratjadone. Cell-cycle analysis revealed prominent G1 arrest, which was accompanied by a dramatic decrease in the S-phase. The G1 arrest was associated with elevation of p21 protein. This protein is an inhibitor of the cyclin E/cyclin dependent kinase 2 complex, involved in the control of the G1/S transit in eukaryotes. These results suggest that ratjadone inhibits tumor cell growth via G1 arrest in association with p21 induction.

5
Biotechnological Studies To Overproduce Metabolites by Marine Microorganisms

5.1
Overview of Biochemical Engineering Approaches

To date, the technical approaches used to cultivate marine bacteria and fungi have reached different standards. For example, marine barophiles or hyperthermophiles require high pressure (40–80 MPa) or high temperatures (70–110°C), raising a number of engineering challenges for bioreactor operation. These include vessels/equipment that are resistant to extreme conditions, maintenance of high temperature and reduction of evaporative loss of liquid media containing high salt concentrations. For instance, in the case of hyperthermophiles, several bioreactor configurations, such as gas lift bubble columns, continuously stirred tank reactors, three-phase fluidized beds and an oscillary-baffled reactor, have been tested [68–70]. Products of interest from the above microorganisms are thermostable enzymes. To date, the most familiar commercial application for such an enzyme is the use of Taq DNA polymerase, from *Thermus aquaticus* (not a marine microorganism), in the polymerase chain reaction. Fortunately, these "thermozymes" can now be produced by recombinant DNA technology.

As for the cultivation of other types of marine microorganisms, e.g., those with a specific potential for the production of biologically active metabolites, predominantly small-scale experiments (shake flasks) have been described. Alternatively, artificial seawater or 25:50:75:90% natural seawater has served as a basis for nutrient media. The concentrations of carbon and nitrogen sources reached up to 2% (w/w): starch, glucose, molasses, glycerol, soybean oil, yeast extract, malt extract, beef extract, peptone, cornsteep liquor and NZ-amine. In the absence of artificial or natural seawater, high concentrations of

$Na^+/Mg^{2+}/Cl^-$ ions were used instead. Temperatures in the range 20–28 °C and a pH value of 7.5 were favored. Jensen and Fenical [71] recommended replacing the above traditional nutrient components by natural C and N sources, polysaccharides and proteins from marine eukaryotic systems.

Compared to the number of publications on the structural analyses of marine microbial metabolites, those on quantitative biochemical engineering studies are negligible in the literature. There are only the following exceptions: The time courses of pH, biomass and bioactive product concentration were reported for three different *Micromonospora* species, isolated from a soft coral, a sponge and from seawater, respectively. Using 250-ml cultures in Erlenmeyer flasks, thiocoraline (depsipeptide), the macrolide IB-96212 and the tetrocarcin antibiotics arisostatins A and B were produced [72–77]. Both the depsipeptide and the macrolide were extracted (organic solvent) from the biomass; the tetrocarcin antibiotics were extracted from the supernatant. For cultivation on a larger scale and under more controlled conditions in bioreactors, the parameters of interest are summarized in Table 1. In most cases, for primary separation of all products, an extraction with organic solvents, such as chloroform/methanol or ethyl acetate, was the method of choice. Using this method, bioxalomycin and exophilin were isolated from the supernatant, agrochelin and the glucosylmannosyl glycerolipid from the cells and the lipodepsipeptides/po-

Table 1. Literature data on bioreactor cultivations of marine bacteria and fungi with respect to some important bioprocess conditions and metabolites

Strain; cultivation conditions	Product	Yield (mg/l)	Ref(s)
Streptomyces sp. (from sediment sample); 300 l, tap water, glucose, 28 °C, 200 rpm, 0.67 v/vm, 50 h	Bioxalomycins	10[a]	[78]
Exophiala pisciphila (from *Mycale adherens*); 15 l, potato/glucose, pH 7[b], 25 °C, 0 rpm, 0.67 v/vm, 10 d	Exophilin A	2	[79]
Hypoxylon oceanicum (from mangrove wood); 300 l, glycerol/soy peptone, pH 7[b], 22/28 °C, 250 rpm, 1 v/vm, 6–8 d	Lipodepsipeptide Polylactones	400 50	[80–82]
Agrobacterium sp. (from *Ecteinascidia turbinata*); 50 l, instant ocean salts, glucose, pH 7.2[b], 28 °C, 350 rpm, 0.67 v/vm, 30 h	Agrochelin	5	[83]
Microbacterium sp. (from *Halichondria panicea*); 40 l, artificial seawater salts, glucose, pH 7.5, 30 °C, 800 rpm, 0.4 v/vm, 27 h	Glucosylmannosyl glycerolipid	200	[84]

[a] Relative units.
[b] Initial value.

lylactones from the whole broth. When the bacteria do not form pellets or mycelia, we have applied successfully the solvent-free floating bed extraction technique using adsorber resins.

Some recent results on marine biochemical engineering were presented at the International Marine Biotechnology Conference (IMBC) 2000 in Townsville, Australia (29.09.–04.10.2000) dealing with the cultivation of microalgae and the development of photobioreactors [85, 86]. Low concentrations of biomass and metabolites are regarded as a bottle-neck in the elucidation of structures in marine microbial biotechnology, which can, however, be regarded as minor problems compared with the major problem of the marine microorganisms that have so far proved elusive in terms of cultivation [87].

5.2 Cultivation of North Sea Bacteria

In the North Sea project, the results from primary screening for biological activity or new compounds guide the selection of strains for upscaling and finally isolation and structural elucidation. Since even modern methods for structure determination and an initial biomedical evaluation require 10 mg of every compound or more, a scale-up fermentation is necessary. With the aid of biotechnical methods, fermentation conditions have to be optimized to achieve maximum yield of metabolites, to increase the genetic stability of the producer or to improve other parameters.

After reproduction of 100-ml flask experiments under the aspects of growth and metabolite spectra (TLC), from the end of 1999 the cultivation experiments were transferred to a 50-l scale in glass or stainless steel bioreactors. Preliminary results on nutrient components (mainly 10 g/l tryptone and 5 g/l yeast extract in 50% artificial seawater), pH (7–7.5) and temperature (30°C) were strictly considered. The bioreactor design, aeration and agitation were chosen depending on the individual strain requirement: 200–300 rpm, 0.1–0.3 v/vm. The higher aeration rates recommended in the literature (Table 1) have proved unnecessary until now. Additionally pO_2, qO_2 and qCO_2 were determined/calculated on-line, whereas optical density or biomass and natural products concentration (crude organic extract) were determined off-line. For downstream processing, cells and supernatant were separated by centrifugation (16,000 rpm) and extracted separately with ethyl acetate/acetone (cells, after freeze-drying) or ethyl acetate (supernatant). To date, many fermentations of new bacteria with a high potential to produce bioactive metabolites have been carried out. Depending on the strain the cultivation time has been in the range 24–72 h. The yield of crude organic material reached 150 mg/l at maximum. These experiments provided material for high-performance chromatography and subsequent structural elucidation and studies on bioactivity.

For strain Hel45 (*Roseobacter* sp.), some of the details regarding a 40-l cultivation, using a Rushton disc turbine for agitation, are presented in Fig. 10. Following the steps of the primary downstream processing, in total 70 mg/l of crude products were isolated from the cells and the supernatant [88]. More recently, by varying the carbon and nitrogen sources, the pattern of TLC spots

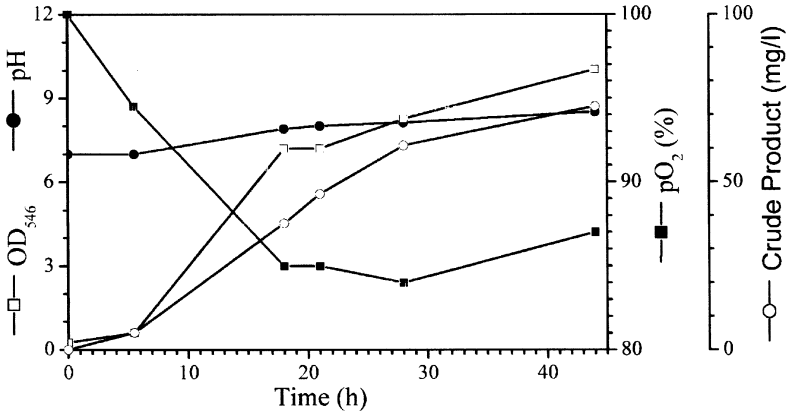


Fig. 10. Growth and metabolite production of the marine strain Hel45. OD_{546} Optical density at 546 nm; pO_2 oxygen partial pressure

showed another composition and additionally new TLC spots were visible. The constituent of one of them was identified as indole-3-carboxylic acid thiomethyl ester (**23**), a compound that has not been described as a natural product previously.

Except for providing chemists and pharmacists with material, further tasks of the biotechnology groups are as follows: (1) The product-oriented improvement of basic microbial cultivation, including the variation of nutrients (genetic algorithms), the quantitative estimation of single metabolites (HPLC) and the precursor-directed biosynthesis, (2) studies on the induction of special pathways for secondary metabolites, (3) studies on the mode of fermentation (batch, fed-batch, continuous culture), the aeration and agitation rates, as well as (4) the biocatalytical modification of naturally produced metabolites.

Because degraded components of dead organic matter are widely distributed in the sea, marine microorganisms mostly live in an environment of highly diluted nutrients. Following this observation, a minimal supply may be more important for marine microorganisms than for their terrestrial counterparts. A minimal medium was defined based on artificial seawater containing glucose as carbon and energy source, ammonia as nitrogen source, phosphate and trace elements. First results with a number of marine bacteria indicated that the addition of selected amino acids and vitamins increased their growth rate significantly [89]. For example, the growth of Hel42 is strongly enhanced by addition of vitamin B12 and two amino acids. All other strains tested needed at least two amino acids.

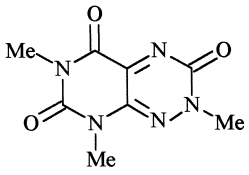
6
Chemical Structures

6.1
Dereplication

The isolation and structural determination of natural products is a time-consuming and expensive process, even using modern methods. It is most important therefore to recognize and exclude known compounds at the earliest possible stage, a process which is called *dereplication*.

For this task, easily accessible properties of mixtures or pure metabolites are compared with literature data. This may be the biological activity spectrum against a variety of test organisms. Widely used also is the comparison of UV [90] or MS data and HPLC retention times with appropriate reference data collections, a method which needs only minimal amounts and affords reliable results. Finally, there are databases where substructures, NMR or UV data and a variety of other molecular descriptors can be searched using computers [91]. The most comprehensive data collection of natural compounds is the Dictionary of Natural Products (DNP) [92], which compiles metabolites from all natural sources, also from plants. More appropriate for dereplication of microbial products, however, is our own data collection (AntiBase [93]) that allows rapid identification using combined structural features and spectroscopic data, tools that are not available in the DNP.

A yellow, strongly blue fluorescent metabolite from a marine streptomycete gave a very unusual proton NMR spectrum with only three heteroatom-bound methyl signals in the region of $\delta = 3.5 - 3.9$; C-Me, CH_2 or CH signals were not visible. A search for compounds with three or more hetero-methyl groups gave 1120 hits (out of 25,000 entries), and, on subtraction of all compounds with C-Me, CH_2 or CH groups, only four entries remained. Two had to be removed because of symmetry reasons. The experimental data matched those of 2-methylfervenulone (**1**), a structure which is difficult to solve on the basis of NMR data alone.



2-Methyl fervenulone (**1**)

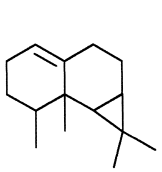
In the case of new compounds a database search is also helpful, because novel skeletons are rare and usually related compounds are already known which are easily revealed by a database search, thus identifying at least the compound class.

6.2 Structural Elucidation and Results

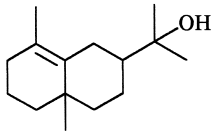
The only method for structural elucidation that is also applicable to mixtures is mass spectrometry, mainly when electrospray ionization (ESI) is used and precursor ions are investigated. Not all types of compounds, however, are accessible by this method, and, due to insufficient charge stabilization, compounds like labilomycin are not visible in the ESI spectra of crude extracts. As pure compounds are needed for further biological testing anyway, mixtures have to be separated before analysis.

The purification of bacterial constituents usually starts in a very conventional way with an extraction step of the crude broth at neutral or slightly acidic pH. Mycelium-forming organisms are separated by filtration, and the cell mass and the filtrate are extracted separately. For the liquid phase, adsorber resins allow high recovery rates of metabolites and low process costs due to repeated use of the resins. If liquid-liquid extraction has to be applied, medium or highly polar solvents are favored. Ethyl acetate is the solvent of choice, and only in few cases is butanol superior. To extract the moist cell material, ethyl acetate, acetone or dichloromethane/methanol can be used.

In the crude extracts, triglycerides, long-chain acids, hydrocarbons and some other nonpolar compounds dominate which require different separation techniques. The majority of these compounds are removed from the methanolic solution by cyclohexane extraction. Antibiotic activity is seldom found in the lipophilic phase; however, using modern gas chromatography/mass spectrometry (GC/MS) methods and cellular tests for cytotoxicity, we have found now strong cytotoxicity in some cases and a wide range of unexpected compounds: Whereas isoprenoids are common constituents of fungi [94], it was not known that bacteria also contain a rich variety of low-molecular terpenes, among them calarene (**2**), eudesmole (**3**) and many others [95].



Calarene (**2**)



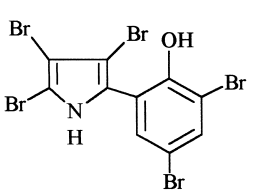
Eudesmole (**3**)

For the final purification, a sequence of normal phase chromatography, size exclusion chromatography, reversed-phase (RP)-HPLC and other techniques are used. There are no general rules as to how to proceed but, due to the high capacity and low irreversible absorption of Sephadex, size exclusion should be used in the very beginning, whilst HPLC is better employed for the final purification steps.

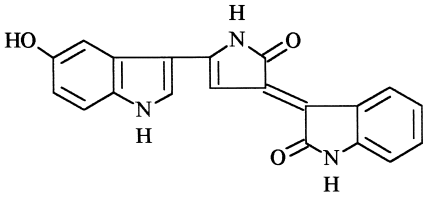
Gram-negative *Pseudomonads* and *Bacilli* from terrestrial sources do not produce many secondary metabolites. This seems to be similar in the sea; however, some of the compounds found there have extraordinary structures. The

antibiotic and cytotoxic pentabromopseudiline (**4**) was the first marine bacterial metabolite to be reported. It was isolated from a bacterium first assigned as *Pseudomonas bromoutilis* (later re-assigned to the genus *Alteromonas*) [96]. Related strains producing the same antibiotic have subsequently been repeatedly isolated from seawater samples.

A closer inspection of *A. luteoviolaceus* by different groups provided a further variety of highly active metabolites, tetrabromopyrrole, hexabromo-2,2′-bipyrrole, tetrabromobiphenol, several simple phenols including 4-hydroxybenzaldehyde and *n*-propyl-4-hydroxybenzoate, violacein (**5**) and related pigments, and the extremely strong siderophore alterobactin [97].

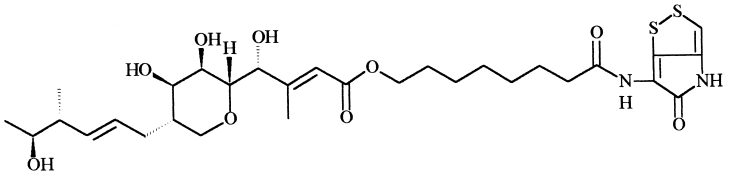


Pentabromopseudiline (**4**)

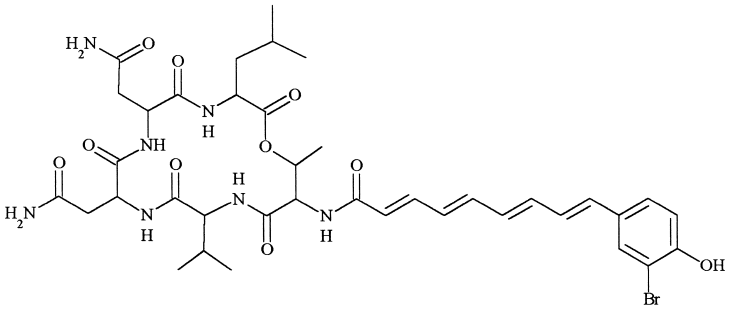


Violacein (**5**)

Other outstanding structures from marine *Alteromonads* are thiomarinol [98] (**6**) and the related holothines, or the bromoalterochromides (**7**) [99]. The latter are yellow chromopeptides due to their polyene chain; however, simple peptides (e.g. the leupeptins) have also been described.



Thiomarinol (**6**)

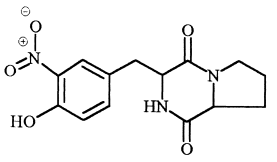


Bromoalterochromide A (**7**)

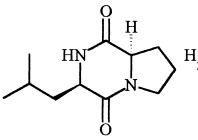
Diketopiperazines are rather abundant in marine bacteria. In spite of their simple structures, some are reported to have herbicidal [100] (**8**, **9**), chitinase

inhibitory [101] (**10**), phytotoxic and antifungal [102] (**11**) activity. A weak cytotoxicity [103] was reported for cyclo(D-Pro, L-Tyr) (**12**).

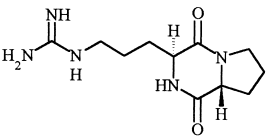
From the North Sea strain Bio39 we have isolated the α,β-unsaturated diketopiperazine **13**. The same metabolite has been isolated very recently from a *Penicillium* sp. [104]; however, the NMR data are different. Compounds of this type [105] (**14, 15**) show pronounced antitumor activity; however, compound **13** is inactive. Only restricted information is available for similar structures, as these compounds have not been reported often.



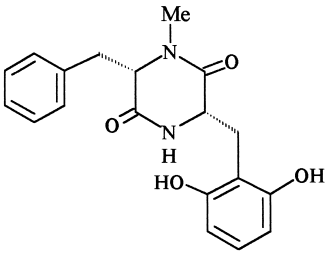
Pyriculamide (**8**)



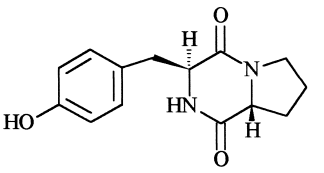
Maculosin 6 (**9**)



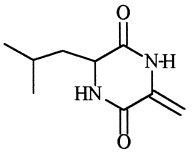
cyclo(L-Arg,D-Pro) (**10**)



Mactanamide (**11**)



cyclo(D-Pro,L-Tyr) (**12**)



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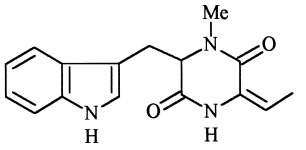
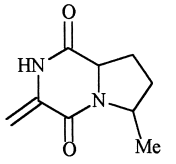


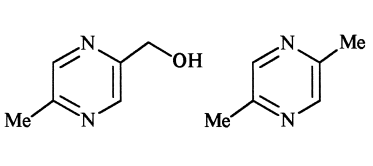
TDD (**14**)



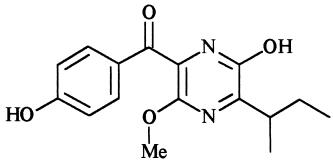
SF-2771 (**15**)

In the case of diketopiperazine **13** and related compounds, dehydrogenation of the preceding diketopiperazine occurs in the side chain. A shift of the double bond into the central ring and dehydration may result in the formation of substituted pyrazines. Simple pyrazines are known as signaling compounds from animals. The pyrazines **16** and **17** have also been isolated from marine Streptomycetes [106]. GC/MS investigations of bacterial flavor components [95] indicate that these and others are very wide-spread.

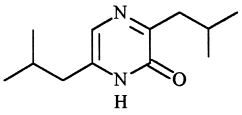
More complex pyrazines, however, are rare, and again a decreasing oxygen content of the aromatic system seems to indicate an origin from diketopiperazines (**18** [107], **19** [108]). We have now isolated another fully deoxygenated new



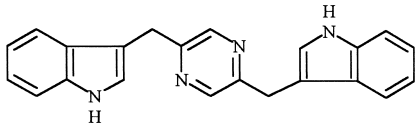
16 **17**



Septorin (**18**)



Flavacol (**19**)



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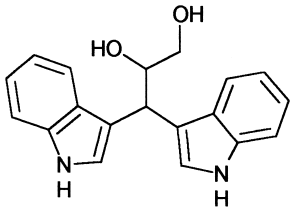
pyrazine **20** from a strain AM13,1 which belongs to the Cytophaga/Flexibacteria cluster.

Whereas simple indole derivatives are rare in marine Streptomycetes, they are rather common amongst the North Sea bacteria so far investigated. The extracts of strain Hel 45 that contain the diketopiperazines cyclo(Phe, Pro) and cyclo(Tyr, Pro), however, are dominated by large amounts of unsubstituted indole, the known dimer 3-(3,3′-diindolyl)propane-1,2-diol [109] (**21**) and various other, still unidentified, indole derivatives.

The indole **22** was previously isolated from the sponge *Dysidea etheria* [110] and has now been obtained from the Antarctic ice bacterium ARK 13-2-437. The lipid phase of Hel45 delivered additionally *N*-(2-hydroxyethyl)-11-octadecenamide and the new natural products 17-methyl-16-octadecenoic acid [95] and indole-3-carboxylic acid thiomethyl ester (**23**).



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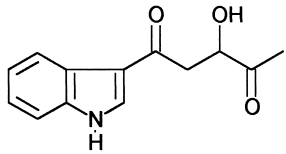
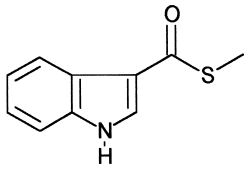


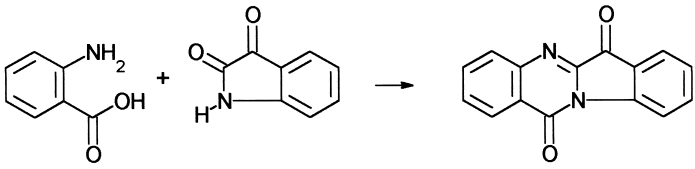
22



23

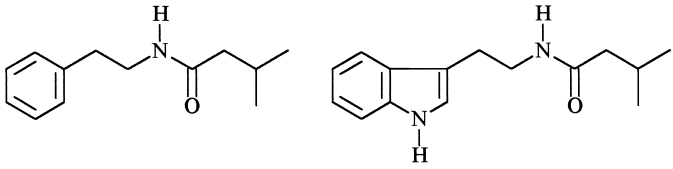
AM13,1, a microorganism from the Cytophaga/Flexibaterium cluster, is one of the few 'talented' strains amongst the North sea bacteria so far investigated: In addition to the indole **20**, the culture yielded phenylethyl acetate, indole-3-carboxylic acid, indolyl-3-acetic acid, uracil, anthranilic acid (**24**) and the new compounds *i*-valeryl-β-phenylethylamide (**27**) and N^{β}-*i*-valeryltryptamine (**28**). Very unexpected, however, was the isolation of yellow tryptanthrin (**26**) which is probably responsible for the broad but moderate antibiotic activity. The antifungal and antimicrobial pigment **26** is a biocondensation product of anthranilic acid (**24**) and isatin (**25**) that was isolated originally from the pathogenic yeast *Candida lipolytica*; however, it has also been found in plants [*Couroupita guianensis* (Lecythidaceae), *Isatis indigotica*]; an occurrence in bacteria has not yet been reported.

The yellow color of the AM13,1 colonies is due to their content of compound **26**. In most other cases, yellow cultures owe their color to the carotenoid zeaxanthin (Hel21) or one of the many vitamin K derivatives (e.g., menaquinone MK6 in Hel21).



Anthranilic acid (**24**) Isatin (**25**) Tryptanthrin (**26**)

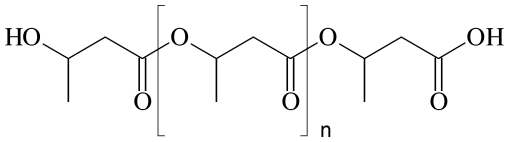
The activity of extracts against microalgae has led to the isolation of a large group of simple phenylethyl amides and various indolylethyl amides (e.g., **27**, **28**). We have obtained some of these compounds also from limnic bacteria, and, although their activity is low, it seems plausible that they play a role in the competition of bacteria with microalgae for free surfaces, perhaps on seaweed or other sessile organisms.



27 **28**

Polyhydroxybutyric acid (PHB) is a bacterial biopolymer which has gained much interest because of its potential use as a biodegradable plastic material. This compound is produced by various terrestrial bacteria and serves as an energy reservoir. PHB is usually highly polymeric (10,000 monomer units) and is stored in the bacteria as an insoluble material in inclusion bodies that are visible with an electron microscope [111]. Although PHB has been inten-

sively investigated, it was not known that also very low oligomers (OHB) occur. We were able now to isolate an OHB mixture **29** with n = 8 – 20 from the marine bacterium *Alteromonas distincta* strain Hel69, and from marine streptomycetes. Whether inclusion bodies are also present in Hel69 has still to be explored.



Oligohydroxybutyric acid (OHB; n = 8 – 20) (**29**)

7 Concluding Remarks

Culture-independent investigations of marine communities have provided a wealth of information on the phylogenetic positions and, in some instances, also on enzymes and pathways of uncultivated marine microorganisms. Clone libraries of amplified 16S rDNA fragments from marine habitats are dominated by sequences which have no match in cultivated bacteria. Judging from the extent of sequence differences observed, entirely new subdomains (*Crenarchaeota*), divisions (termed "candidate divisions") and genera, and an almost unlimited amount of species of Bacteria and Archaea, have thus been detected and represent a completely untapped source of new metabolic diversity awaiting successful cultivation attempts [112] and culture-independent characterization using tools of molecular biology [113 – 116].

The search for new chemical metabolites in marine microorganisms is a multistep procedure which starts with the selection of suitable sources and cultivation. Screening of crude extracts of North Sea bacteria using the agar diffusion method and a variety of test organisms has yielded inhibition zones of 15 – 25 mm diameter, whilst highly active strains gave inhibition diameters of up to 50 mm. Tests with brine shrimps and human cell lines in screens for antitumor activity have given surprisingly often positive results on the nanogram scale (Hel3, Hel38, 115a). In addition, high leishmaniacidal or antimalarial activities [117] in the range of a few μg crude extract per ml were found (Hel12, Hel38, GW135a), and it is certainly advisable therefore to extend the number and character of the test models. A p53 negative cell line, e. g., should be included in the initial screening process to provide the potential for identifying new agents with activity against p53-negative tumor cell populations.

Strong biological activities are obviously widespread amongst bacteria from the North Sea (Fig. 11). For the ongoing isolation and structural determination of the active constituents, conventional methods are suitable, but very low yields and genetic instabilities are causing time-consuming technical problems and have dictated long investigation times. We are confident, however, that marine bacteria are rewarding targets, and the strong bioactivities are an encouraging signal.

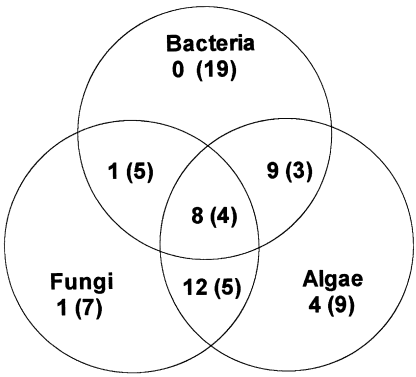


Fig. 11. Activity pattern of extracts from bacteria from the North Sea. Among 188 tested strains, 35 (19%) showed high and 52 (28%) moderate activity (values in *brackets*), 35 (19%) of them with selective and 17 (9%) with multiple activity (values in *overlapping areas*)

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