Natural products from marine bacteria

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Abbreviations

APCI	Atmospheric pressure chemical ionization
ATCC	American type culture collection (Rockland, Maryland/USA)
bp	Base pair(s)
CBS	Central office for fungal cultures, Baarn (Holland)
CFU	Colony forming units
CH	Cyclohexane
CPM	Counts per minute
Da	Dalton
DAD	Diode array detector
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DSMZ	German collection for microorganisms
EDTA	Ethylenediamine tetraacetic acid
EE	Ethyl acetate
ETH	Swiss Federal Institute of Technology, Zürich (Switzerland)
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
Fig.	Figure
for.	Forward
g	Gravity (relative centrifugal force)
GPS	Global positioning system
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
IC	Inhibitory concentration
IPTG	Isopropyl-β-D-thiogalactopyranoside
IR	Infrared
kb	Kilo base pairs
L	Mobile phase
LC-MS	Liquid chromatography-mass spectrometer

LD	Lethal dose
LMG	Laboratory for microbiology, Gent (Belgique)
mAU	Milliabsorption unit
max	Maximum
mCi	Millicurie
MDR	Multi-drug resistant
MeCN	Acetonitrile
MeOH	Methanol
MRSA	Methicillin resistant *Staphylococcus aureus*
No.	Number
nt.	Nucleotide
OD	Optical density
P	Buffer
p.a	pro analysis
PCR	Polymerase chain reaction
pers. comm.	Personal communication
PI	Propidium iodide
PLA_2	Phospholipase A_2
PMS	Phenazine-methosulfate (N-methylphenazinium-methylsulfate)
R	Chromogenic reagent
r	ribosomal (in connection with DNA or RNA)
rev.	Reverse
R_f	Rate of flow
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RP	Reversed phase
rpm	Revolution per minute
S	Solution
SDS	Sodium dodecylsulfate
SH-group	Thiol-group
sp.	Species
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
Tris	Tris (hydroxymethyl)-aminomethane

U	Unit
UV.	Ultraviolet
Vis.	Visible
w/v	Wight/Volume
X-gal	5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

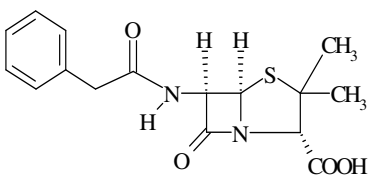
1. Introduction

1.1. Natural products

The story of bioactive natural products started more than 100 years ago. Their usual definition in the widest sense is chemical compounds isolated/derived from the nature i.e. living organisms such as plants, animals and microorganisms. These compounds may be derived from primary or rather secondary metabolism of these organisms (Bérdy, 2005). Chemistry of natural products is related to the isolation, biosynthesis and structure elucidation of new products that led to new medical and crop protection agents. Due to their chemical diversity and various activities against diseases, they have been playing an important role in pharmaceutical and agricultural research (Grabley & Thiericke, 1999).

The World Health Organization (WHO) estimated that 80% of the earth inhabitants mainly depend on traditional medicines for their health care (Farnsworth *et al*., 1985). Plants have been the roots of the traditional medicine that has existed for thousands of years starting from the first records about 2600 $_{BC}$. Some of these plants are still in use today for the treatment of ailments ranging from coughs and colds to parasitic infections and inflammation (Newman *et al*., 2000).

Since the discovery of penicillin (penicillin G, **1**) in 1928 (Fleming, 1929), intensive studies, mainly on soil derived bacteria and fungi, have shown that microorganisms are a rich source of structurally unique bioactive substances (Fenical, 1993). Penicillin represents the first antibiotic in the history of natural products from microorganisms despite that mycophenolic acid (**2**) was identified in the end of the nineteenth century by Bartolomeo Gosio (Bentley, 2000).



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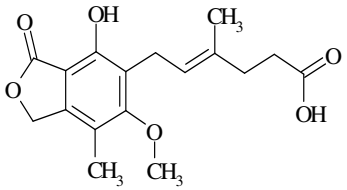


2

Fig. 1.1. Penicillin G (**1**) and mycophenolic acid (**2**), two natural products from *Penicillium* species.

1.2. Development of antibiotic history

The French word *antibiose* had been coined to describe antagonistic effects between microorganisms; it was the opposite of symbiosis. Selman Waksman, beginning in 1941, defined antibiotic as a "secondary metabolite, produced by microorganisms, which has the ability to inhibit the growth and even to destroy bacteria and other microorganisms, in a very low concentration" (Bentley, 2000). Not all secondary metabolites serve as antibiotics; many of them serve as plant growth factors and enzyme inhibitors (Ōmura, 1986) and as self-regulating factors in some bacteria (e.g. A-Factor, Beppu, 1992) and fungi (e.g. butyrolactone I, Schimmel *et al.,* 1998).

Zähner *et al.,* (1983) proposed the existence of a "playground" of secondary metabolism besides and closely connected to the five distinct primary cellular levels: intermediary metabolism, regulation, transport, differentiation and morphogenesis. As a result, the definition of secondary metabolites is not confined to antibiotics but should be extended to all those metabolites, which regulate all physiological and biochemical activities in the life cycle of organisms (Bérdy, 2005).

The development of penicillin by Florey and his colleagues opened the door to the "Golden Age of Antibiotics" that has dominated medical practice for several decades. It is an active agent produced by *Penicillium notatum* and has inhibitory effect against Gram-positive bacteria, among them the disease-causing species from the two genera *Streptococcus* and *Staphylococcus.* A post penicillin hunt for further antibiotics began in the 1940s and resulted eventually in the identification of thousands of microbial metabolites with a wide array of biological properties. Till the mid eighties, almost all groups of important antibiotic were discovered: the antibacterial cephalosporin C, streptomycin, tetracyclines, erythromycin (**3**), vancomycin, the antifungal amphotericin B (**4**), imidazoles, griseofulvin, strobilurins, the antiviral acicluvir (**5**), vidarabine and many other compounds that play a role in therapeutics and agriculture (Gräfe, 2000).

About one forth of more than one million known compounds from nature show bioactivity at least in one bioassay (Bérdy, 2005). Nearly 10% of the bioactive metabolites are antibiotics and antibiotics with other bioactivities from microbial origin. Actinomycetales were the most studied organisms by the scientists working in the field of natural products (Fenical, 1993; Bernan *et al.,* 1997; Pietra, 1997; Newman, 2000; Laatsch, 2000; Blunt *et al.,* 2005 and 2006). Over 10000 compounds were isolated from this group, mainly from species of the genus *Streptomyces* and rare actinomycetes. The most characteristic feature of the

recent years is the declining representation of metabolites from actinomycetales in microbial products, where they represent 30-35% of the all discovered compounds, and the increase in the representation of metabolites from fungi (for review see Bérdy, 2005).

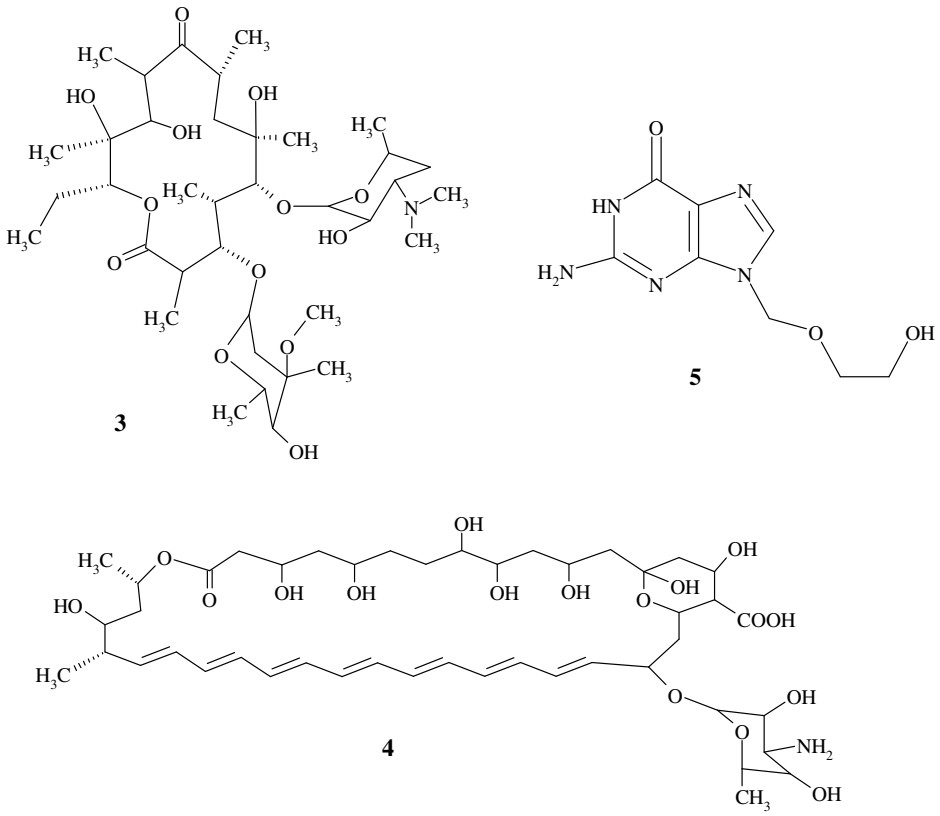


Fig. 1.2. Erythromycin (**3**), amphotericin B (**4**) and acicluvir (**5**). Two microbial antibiotics and one antiviral compound.

Although actinomycetes and fungi were studied extensively, it is clear that the rate of discovery of novel metabolites is decreasing and the number of antibiotics seems currently to approach a saturation curve (Laatsch, 2000; Faulkner, 2002a). With all these thousands of diverse known compounds, ranging from antiviral to antitumor agents and due to the appearance of a number of new diseases, the need for new therapeutic compounds is still urgent.

1.3. Looking for new therapeutically useful natural products

The appearance and escalation of clinical resistance, the acquired multi-drug resistance (MDR), the emerging of new pathogens, viral diseases *etc.,* all represent serious problems that cost millions of lives on earth. ß-Lactam antibiotics were used as the first defeating line against the pathogenic bacteria till the emergence of ß-lactamases. As a result, the search for more ß-lactamases-resistant compounds continued.

Throughout the years, extensive chemical programs were developed worldwide to synthesize bioactive compounds and to understand their mode of action. The increasing need for drugs able to control emerging diseases or resistant strains of microorganisms resulted in exploring the ocean by numerous scientists in the field of natural products. Since then great efforts have been accomplished aiming to isolate new metabolites from marine organisms.

1.4. Marine environment as a new source for bioactive metabolites

Marine organisms represent a promising source for natural products of the future due to the incredible diversity of chemical compounds that were isolated. The oceans, which cover almost 70% of the earth's surface and over 90% of volume of its crust (Fenical, 1993; Whitehead, 1999), contain a variety of species, many of which have no terrestrial counterparts. 34 of the 36 phyla of life are represented in oceans in contrast to 17 phyla representing the terrestrial environment (Faulkner, 2002b). The pioneers of marine microbiology, such as Claude Zobell, became active in delineating the vast numbers and diversity of true marine bacteria. One of the early isolations of secondary metabolites from marine sources was the isolation of cephalosporin in 1948 by Giuseppe Brotzu. Cephalosporin (cephalosporin C, **6**) was isolated from the fungus *Cephalosporium acremonium.* In the early 1950s, Bergmann and his colleagues isolated two compounds from a marine sponge, spongouridine (**7**) and spongothymidine (**8**) (Bergmann & Feeny, 1951, 1955). They were the first naturally occurring nucleosides with a sugar moiety other than ribose or deoxyribose. Later on Burkholder and his co-workers had isolated the first marine metabolite from the bacterium *Pseudomonas bromoutilis*, the highly brominated pyrrole antibiotic pentabromopseudiline (**9**) (Burkholder *et al.,* 1966).

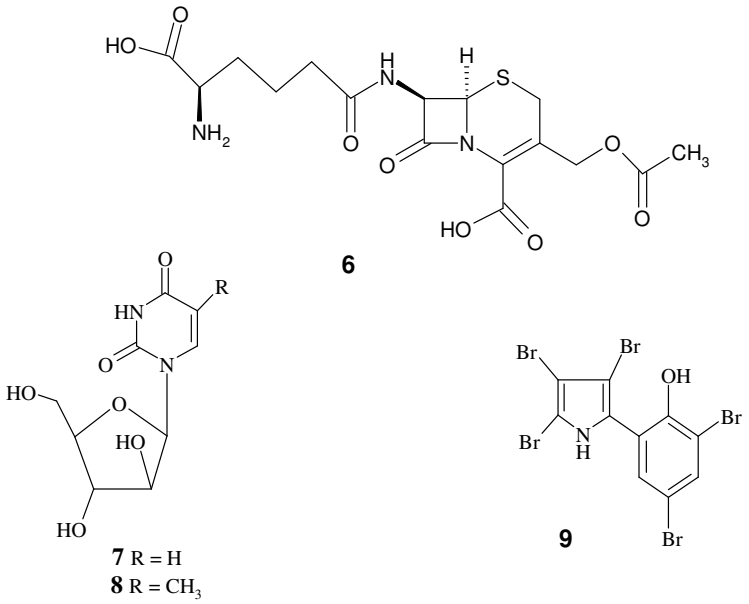


Fig. 1.3. Cephalosporin C (**6**), spongouridine (**7**), spongothymidine (**8**) and pentabromopseudiline (**9**) are the early marine derived secondary metabolites.

The systematic investigations of marine environment as sources of novel biologically active agents began intensively in the mid 1970s. Among the many phyla found in the oceans, bacteria (including cyanobacteria), fungi, certain group of algae, sponges, coelenterates, sea hares, bryozoans, tunicates and nudibranchs were the most studied organisms. During over 60 years of an increase in the number of natural products from marine resources, the number of publications in 2000 has declined slightly when compared with 1999 (Fig.1.4 a). Sponges followed by coelenterates were the most studied marine organisms. The share of microorganisms in marine studies since that time has not lost its significance and stayed more or less in a constant level in contrast to sponges that have lost the interest of the natural products' scientists (Fig. 1.4 b, c and d). The bioactivity profiles of marine metabolites include neurotoxic, antiviral, antitumor, antimicrobial or cytotoxic properties and are of considerable biotechnological interest.

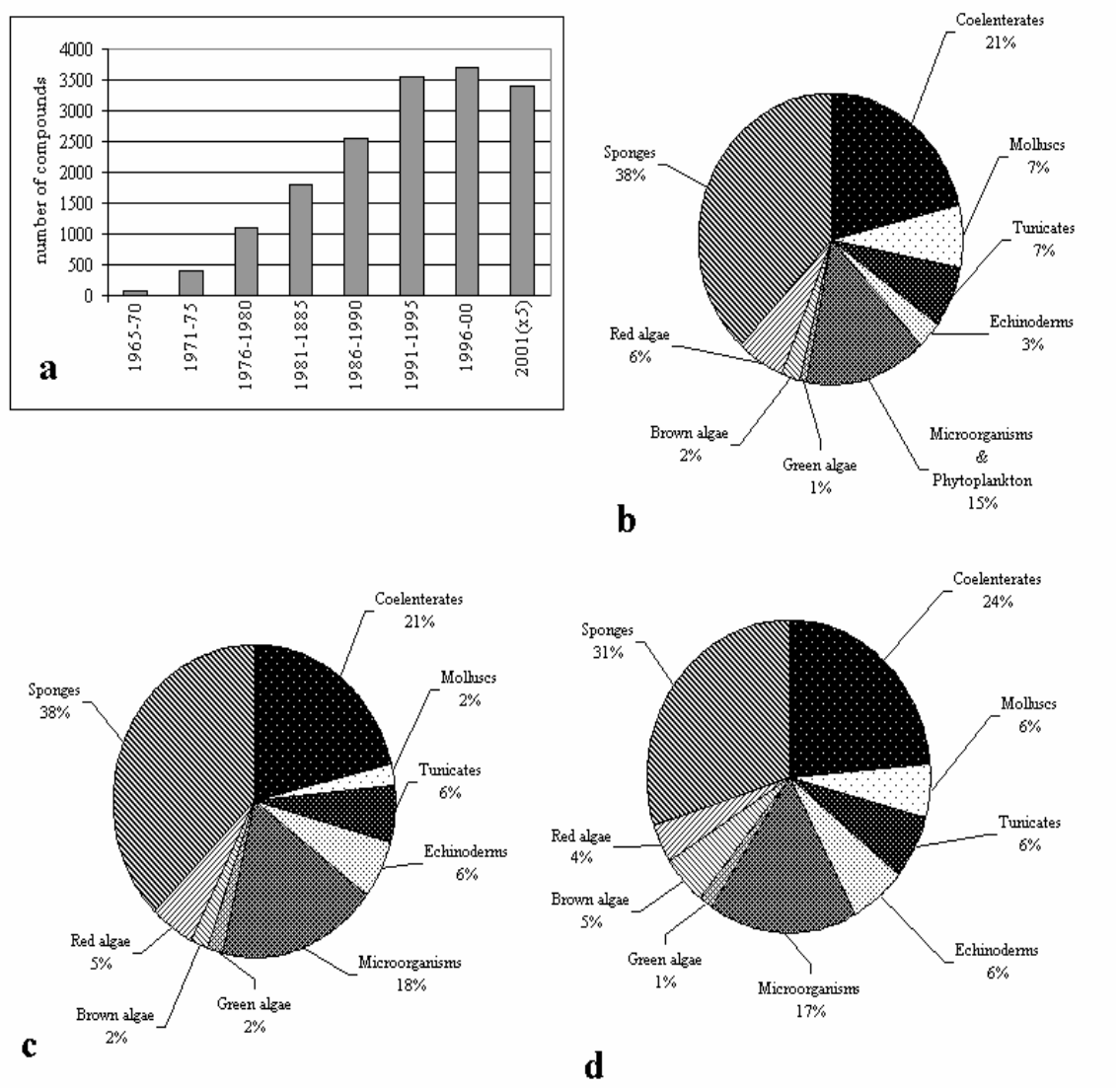


Fig. 1.4. Statistics of marine natural products. a) Since 1965 onwards, b) percentage of isolated compounds from different phyla till 2001, c) till 2002, d) till 2003 (modified from Blunt *et al.,* 2003, 2005).

1.4.1. Marine secondary metabolites with interesting activities

Many of the marine derived anti-inflammatory, neurotoxic and antitumor compounds had been included in clinical research in clinical trials. *Conus* venoms are small, highly constrained peptides, 10-30 amino acids in length. They target nicotinic acetylcholine receptors, voltage-sensitive calcium channels and sodium channels (Meyers *et al.,* 1993). A synthetic version of ω-contoxin MVIIA (ziconotide, **10**), the first isolated peptide from the venom of *Conus magus*, is used as a potential treatment for patients suffering from chronic pain. It is in the registration stage under the name Prialt in both US and Europe (Butler, 2005).

Marine compounds that interfere with protein kinase C (bryostatin 1, **11**) or inhibit the synthesis of macromolecules (didemnin B, **12**) in cancer cell lines were considered as promising antitumor drugs. Bryostatins, macrocyclic metabolites isolated from the bryozoans *Bugula neritina* (Petit *et al.,* 1982) and *Amathia convulata* (Hale, 2002), were used as a partial agonist of protein kinase C (PKC). Bryostatin 1 is currently in phase II clinical trials.

Didemnin B, a depsipeptide isolated from the Caribbean tunicate *Tridemnum solidum* (Rinehart *et al.,* 1981), inhibits the synthesis of RNA, DNA and proteins in various cancer cell lines.It shows anti-viral and immunosuppressive activities as well as being an effective agent in treatment of leukaemia and melanoma. Due to its toxicity, it was withdrawn from phase II clinical trials (Faulkner, 2000a; Amador *et al.,* 2003).

In 1997 a group from Spain found a novel bioactive depsipeptide, thiocoraline (**13**). It was isolated from the mycelial extract of the bacterium *Micromonospora marina* associated with a marine soft coral in the Indian Ocean. Thiocoraline showed potent cytotoxic activity at a nanomolar concentration against several tumor cell lines. It inhibits DNA polymerase-α (Romero *et al.,* 1997; Newman & Cragg, 2004). Thiocoraline is currently in preclinical phase by PharmaMar.

Manoalide (**14**), a sesterterpenoid isolated from the sponge *Luffariella variabilis* (de Silva & Scheuer, 1980) inhibits irreversibly the release of arachidonic acid from membrane phospholipids and subsequently inhibits the inflammatory reactions (Glaser & Jacobs, 1986, 1987). The work on this compound was discontinued in phase II clinical trial due to formulation problems (Newman & Cragg, 2004).

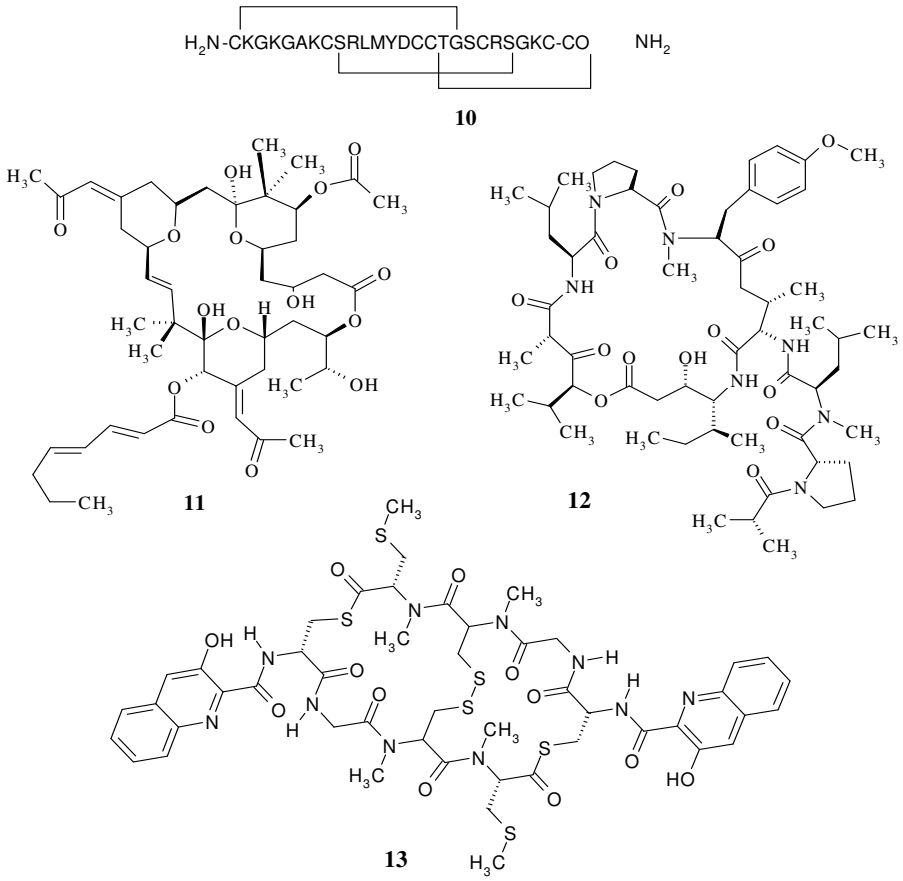


Fig. 1.5. Neurotoxic and antitumor marine compounds in clinical trials. Ziconotide (**10**), bryostatin 1 (**11**), didemnin B (**12)** and thiocoraline (**13**).

Pseudopterosins (**15**), tricyclic diterpene glycosides isolated from the Caribbean sea whip *Pseudopterogorgia elisabethae*, possess anti-inflammatory and analgesic activities as they inhibit PLA_2 and degranulation and leukotriene formation in human neutrophils, but do not affect eicosanoid biosynthesis in stimulated murine macrophages *in vivo* (Look *et al.,* 1986a, 1986b). Recently, it was reported that the real origin of this metabolite is the dinoflagellate symbiont *Sympoidinium* sp. localized within the tissues of the sea whip (Mydlarz *et al.,* 2003). Clinically it has not found its way yet as an anti-inflammatory drug, but it is used as an additive to prevent irritation caused by exposure to sun or chemicals under the name of the cosmetic care product, Resiliene®. Finally, scytonemin (**16**) isolated from the sheath of many cyanobacteria as a yellow-green pigment (Proteau *et al.,* 1993), has recently been patented as anti-inflammatory agent.

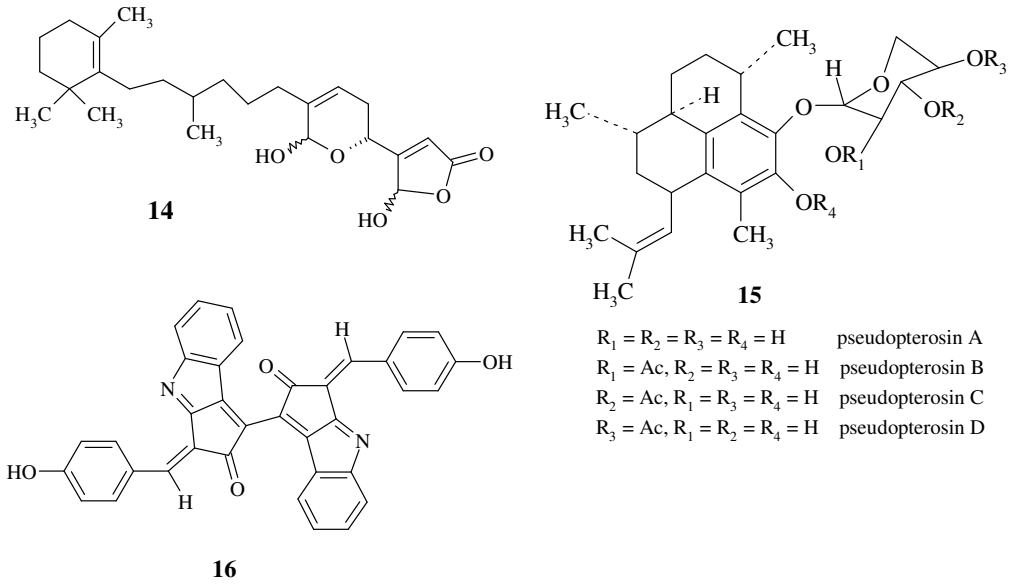
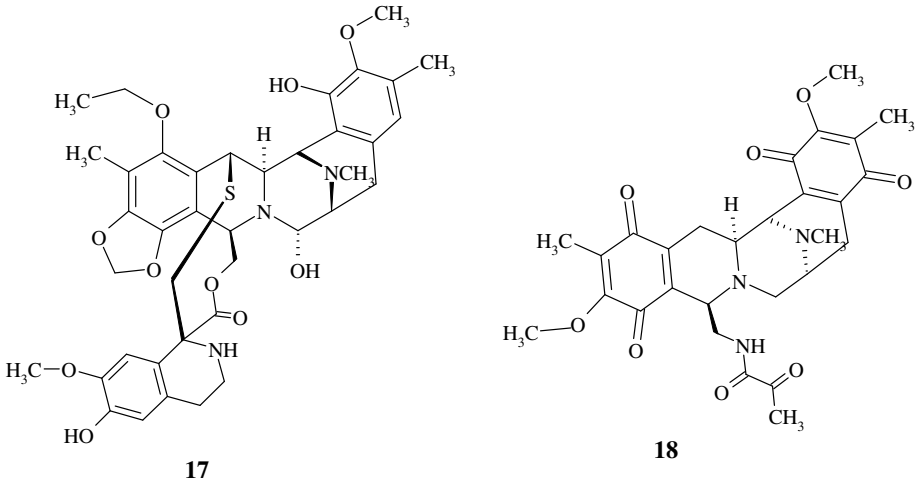


Fig. 1.6. Manoalide (**14**), pseudopterosins (**15**) and scytonemin (**16**) are anti-inflammatory marine compounds.

The low amounts produced from the above mentioned compounds as well as the striking structural similarities between some pharmaceutically active agents and known microbial metabolites addressed a question about their biosynthetic origin. Inspection of structural features of ecteinascidin- 743 (ET-743, **17**) from tunicate reveals similarities to saframycin B (**18**) isolated from *Streptomyces lavendulae* (Arai *et al.,* 1979) and safracin isolated from *Pseudomonas fluorescens* (Ikeda *et al.,* 1983). Such observation represents one of several clues on the microbial origin of these chemicals (for reviews see Moore, 1999; Proksch *et al.*, 2003; Piel, 2004).

Bacteria are regularly observed in unique microhabitats on surfaces and internal spaces of marine invertebrates. The cytotoxic macrolide swinholide 1, isolated from the sponge *Theonella swinhoei*, was found to be produced by the symbiotic unicellular bacteria inhabiting the endosome of this sponge (Lee *et al.,* 2001). Symbioses can range from relatively loose coexistence to highly interdependent interacellular associations. Sponge-bacterial association are probably the most thoroughly described. Several studies showed that the associated bacteria could be distinct from those in the surrounding seawater (specific association) (Jensen & Fenical, 1994). Association does not include just eubacterial groups; archaea, cyanobacteria and fungi are also sponge microsymbionts (Osinga *et al.,* 2001).



17 **18**

Fig. 1.7. Ecteinascidin-743 (**17**) and saframycin B (**18**) are examples on the structural similarities between invertebrate metabolites and microbial compounds.

1.4.2. Marine bacteria as a source for natural products

The oceans are massively complex and consist of diverse assemblages of life forms. The water column of the oceans contains approximately 10^6 bacterial cells per ml (Hagström *et al.,* 2002). Marine bacteria and other marine microorganisms develop unique metabolic and physiological capabilities. These capabilities enable them to survive in extreme habitats and to produce compounds that might not be produced by their terrestrial counterparts. Since 1990, the number of bioactive metabolites from marine bacteria has exponentially increased (Fig. 1.8) (Fenical, 1993; Kobayashi & Ishibashi, 1993; Bernan *et al.,* 1997; Faulkner, 1997, 1998, 1999, 2000 and 2001; Hill, 2003; Blunt *et al.,* 2003, 2004, 2005 and 2006).

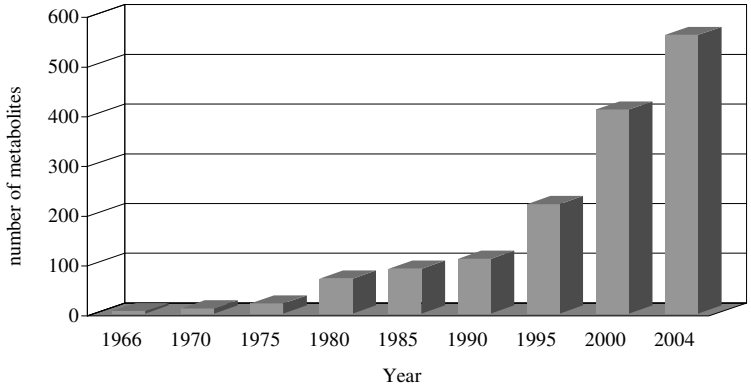


Fig. 1.8. Annual increase in the number of marine bacterial metabolites, according to AntiBase (Laatsch, 2005).

The search for new bioactive chemicals from marine organisms resulted in the isolation of about 10000 metabolites (Kelecom, 2002), many of which are potential biomedicals. These agents show a broad spectrum of biological activities.

Up to now, bioactive agents were isolated extensively from *Streptomyces*, *Altermonas/Pseudoalteromonas, Bacillus, Vibrio, Pseudomonas,* and *Cytophaga* (Fig. 1.9). These microorganisms were isolated from seawater, sediments, algae and marine invertebrates. They are able to produce quinones, polyenes, macrolides, alkaloids, peptides and to a lesser extent terpenoids.

Some of the first marine metabolites were isolated from seawater bacteria. The highly brominated pyrrole antibiotic (pentabromopseudiline) was active against Gram-positive bacteria. Its biosynthesis was not apparent from its structure, which led to studies by Laatsch and co-workers with *Alteromonas luteoviolaceus* on the biosynthesis (Laatsch *et al.,* 1994) as well as on the structure-activity relationships (Laatsch *et al*., 1995).

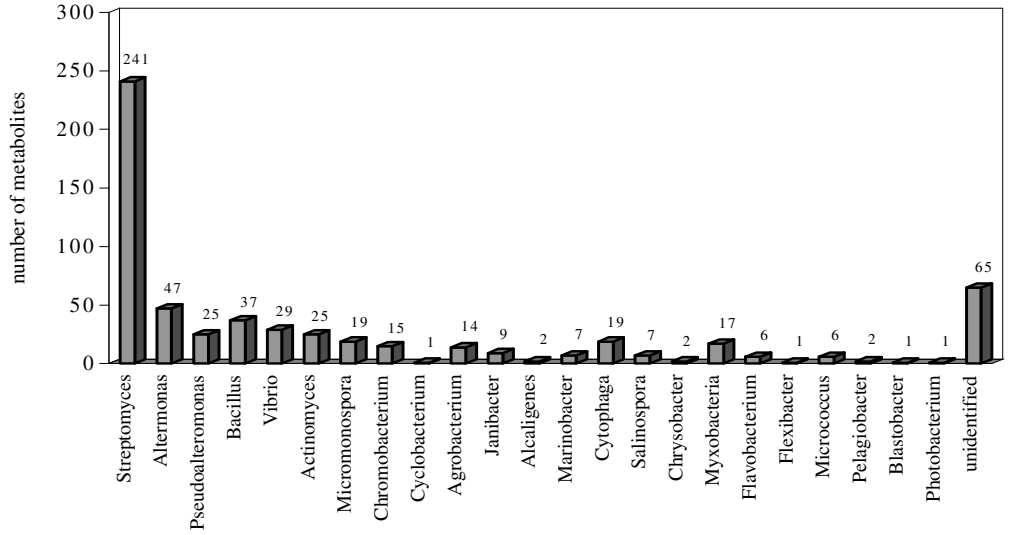


Fig. 1.9. Number of secondary metabolites isolated from some marine bacteria according to their taxonomic origin since 1966 till 2004 (modified from Laatsch, in preparation).

The pioneering work of Okami and co-workers represents the first building unit in the knowledge of the chemistry of marine derived bacteria, in particular of Actinomycetes. They have reported the isolation of a benzanthraquinone antibiotic from the actinomycete *Chainia purpurogena* (Okazaki, *et al.,* 1975) and istmycins A and B (**19**) antibiotics from the *Streptomyces tenjimariensis.*

One of the early marine metabolites is the 3,2-indolinedione (isatin, **20**). This compound is produced by a bacterium colonizing the surface of the embryos of the shrimp *Palaemon macrodactylus* and protects the eggs against the pathogen fungus *Lagenidium callinectes* (Gil-Turnes *et al.,* 1989). A new macrolide with antibacterial, antiviral and cytotoxic activities was isolated from a deep sea unidentified unicellular bacterium, macrolactin A (**21**) (Gustafson *et al.,* 1989). Two bicyclic depsipeptides, salinamide A and B (**22**), were isolated from a *Streptomyces* sp. from the surface of the jellyfish *Cassiopeia xamachana.* They exhibited moderate antibiotic activity, but were potent topical anti-inflammatory in chemically induced mouse ear edema assays (Trischman *et al.,* 1994; Moore *et al.,* 1999).

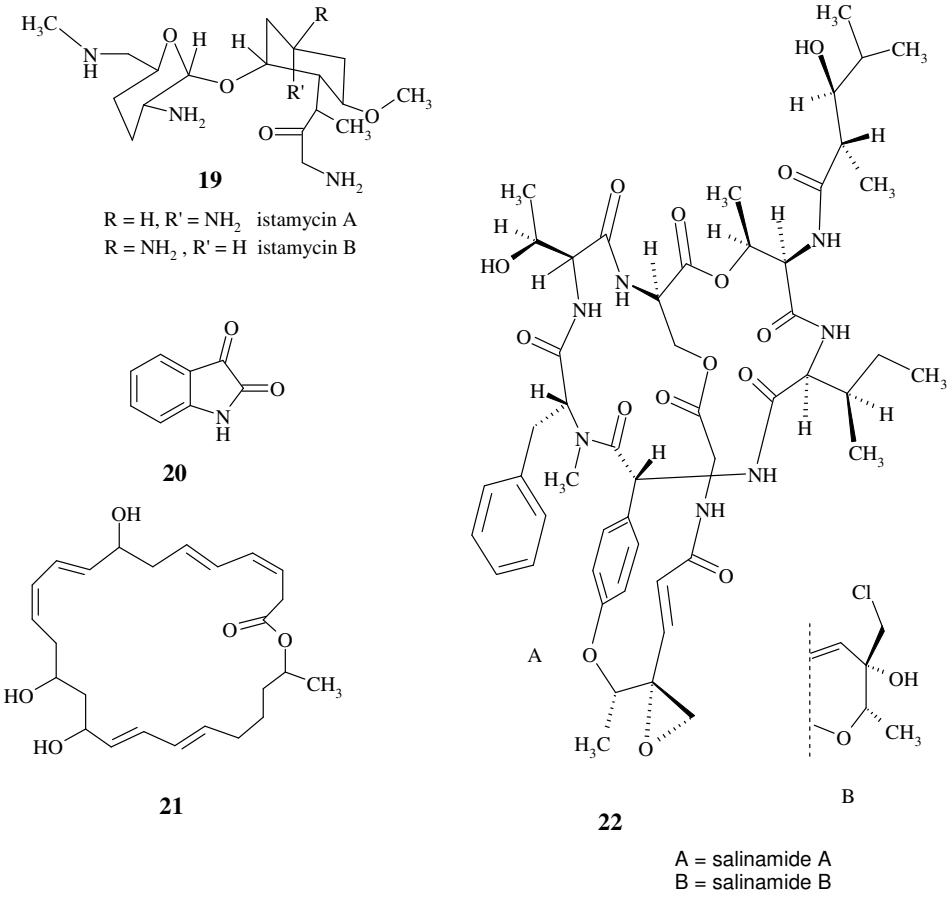


Fig. 1.10. Istamycins A and B (**19**), isatin (**20**), macrolactin A (**21**) and salinamide A and B (**22**) are examples on compounds isolated from marine derived bacteria during the last century.

1.4.2.1. Newly described metabolites from marine bacteria

Since the beginning of this century nearly 250-300 marine compounds have been described. Interestingly, within the same period the number of described metabolites

produced by terrestrial bacteria does not exceed 150 compounds (Laatsch, pers. Comm.). Nearly 100 marine compounds from bacterial origin were isolated within the year 2004. Actinomycetes were as usual the most studied group. A novel bioactive macrolide was isolated from the culture of the *Micromonospora* sp. IB-96212 (**23**) (Fernández-Chimeno, 2000). The group of Laatsch isolated novel anticancer compounds, chandrananimycins A, B and C (**24** and **25**), from *Actinomadura* sp. (Maskey *et al.,* 2003). These compounds exhibited antibacterial and cytotoxic activities.

A bactericidal antibiotic, MC21-A (3,3´, 5,5´-tetrabromo-2, 2´-biphenyldiol, **26**) was isolated from the new species *Pseudoalteromonas phenolica.* It is a brominated anti-MRSA substance that rapidly permeabilizes the cell membranes of MRSA, but it has no lytic activity against bacterial cells or human erythrocytes (Isnansetyo & Kamei, 2003). Recently, mechercharmycin A (**27**) and mechercharmycin B (**28**) were isolated the bacterium *Thermoactinomyces* sp. X-ray crystallographic analysis of these compounds showed that mechercharmycin A is a cyclic-peptide and mechercharmycin B is its linear congener. Mechercharmycin A showed cytotoxic activity against human lung carcinoma and human leukaemia (Kanoh *et al.,* 2005).

The isolation of highly cytotoxic proteasome inhibitor, salinosporamide A (**29**), from *Salinospora* sp. represents one of the remarkable studies in this century. This new genus was proposed by Fenical and his colleagues in 2002 to a group of rare obligate marine actinomycetes isolated from the ocean sediments (Mincer *et al.,* 2002). This compound displayed a potent *in vitro* cytotoxic activity against human colon carcinoma. This effect was due to the inhibition of the 20S proteasome (Feling *et al.,* 2003). Recently halogenated macrolides, sporolides A and B (**30**), were isolated from *Salinospora tropica*. Sporolides A and B were neither antibacterial nor cytotoxic (Buchanan *et al.,* 2005).

Despite this interest on metabolites from marine derived bacteria, studies in this field encounter some problems. Firstly, not more than 5% of the marine bacteria observed in marine samples are amenable to be cultured with the normal microbiological techniques (König & Wright, 1999). Secondly, the taxonomy of marine bacteria is very poorly defined and many publications describe compounds isolated from numbered strains of otherwise partially or totally unidentified bacteria. Thirdly, very low fermentation yields that may be in some cases in the range of milligrams per litre from dense culture are common.

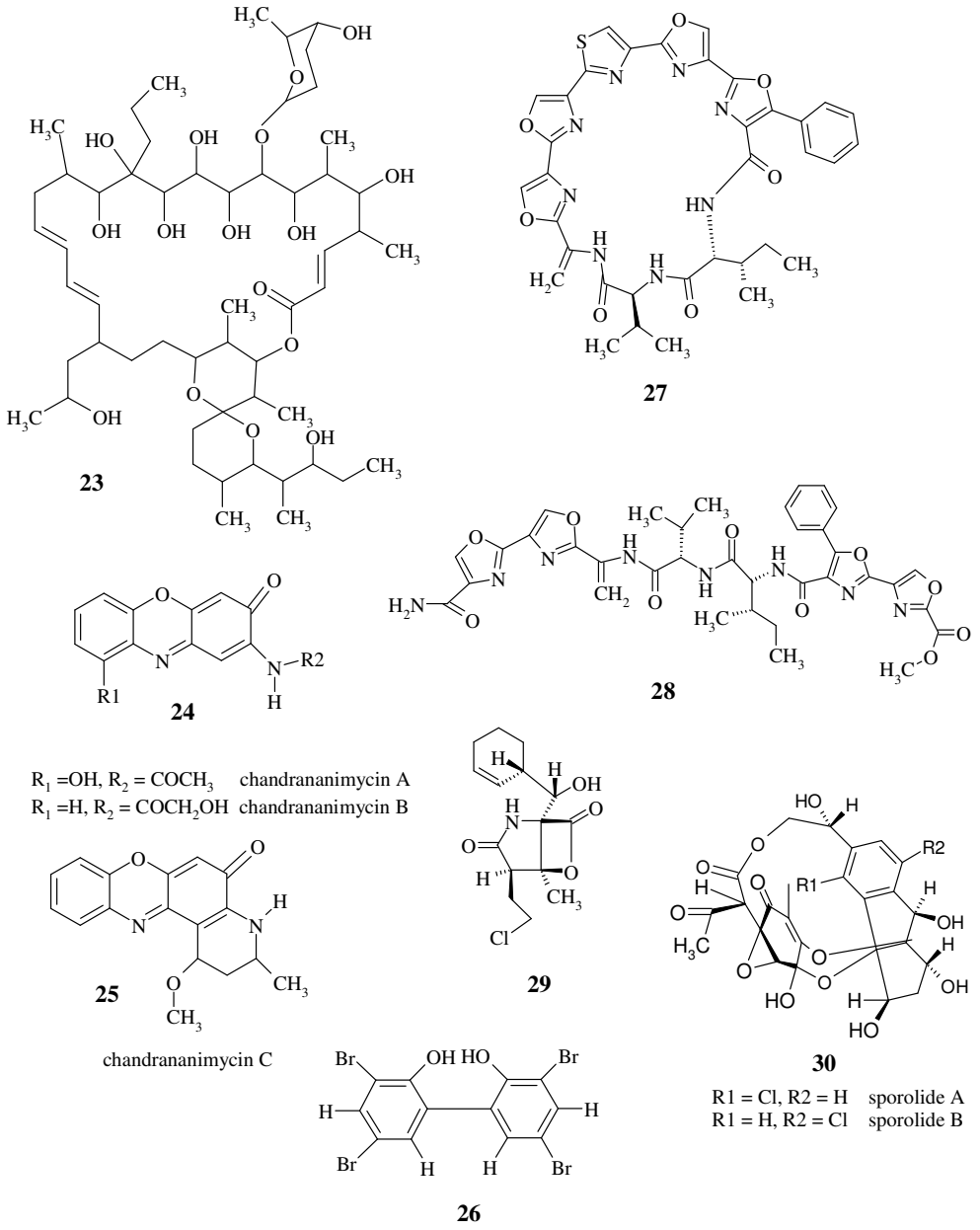


Fig. 1.11. Macrolide IB-96212 (**23**), chandrananimycins A and B (**24**), chandrananimycin C (**25**), antibiotic MC21-A (**26**), mechercharmycin A (**27**), mechercharmycin B (**28**), salinosporamide A (**29**) and sporolides A and B (**30**) are examples on the recently described compounds from marine derived bacteria.

In order to overcome such obstacles, microbiologists developed PCR-based screening assays that may increase the screening efficiency for bioactive compounds. Progression in the knowledge of the genes involved in the biosynthesis of secondary metabolites and the knowledge with different biosynthetic systems, e.g. polyketide synthetases (PKS), non-ribosomal polypeptide synthetases (NRPSs), halogenases, allow completely new approaches, such as combinatorial biosynthesis, to the discovery of novel antibiotics and add a another

source of data for the elucidation of metabolites structure (Carsten *et al.,* 2002; McAlpine *et al.,* 2005). Such knowledge has led to the discovery of the bacterial origin of bryostatins (Davidson *et al.,* 2001).

1.4.2.2. Marine metabolites from North Sea bacteria

The German North Sea is a special ecological area due to the dynamic tidal water. Therefore, its microbiological and chemical features should differ from those of other marine environments (Liand, 2003). Screening of numerous crude extracts of North Sea bacteria using agar diffusion tests and toxicity tests against brine shrimps and cytotoxicity tests against human cell lines was significant. The structures of most of the isolated metabolites are, however, not complex and seem to be derived from the amino acid pathways.

Laatsch and co-workers reported the isolation of a new nucleoside secondary metabolite from the strain Bio134, 3´-acetoxy-2´deoxythimidine (**31**), the isolation of the plant metabolite isoxanthohumol (**32**) from the bacterium Pic009 that was reported as anticarcinogenic and antifungal agent and the isolation of two new antibacterial agents, quinoline-2-one-4-carboxylic acid methylester (**33**) and 3-pyridinecarboxamide (**34**) from the strain Hel59b (Shaaban, 2004). This group also reported the isolation of a two new indole alkaloids from *Vibrio parahaemolyticus* Bio 249, 3,3-bis (3-indolyl)-butane-2-one (**35**) and 1,1,3-tris (3-indolyl)-butane (**36**) (Veluri *et al.,* 2003).

Zeeck and co-workers reported the isolation of the sulfur containing tropodithietic acid (**37**), the isolation of 3-(4´-hydroxyphenyl)-4-phenylpyrol-2,5-dicarcoxylic acid (**38**) and 3,4-di(4´-hydroxyphenyl) pyrrole-2,5-dicarcoxylic (**39**) from the strain RK377 and the isolation of bacteriopheophytin a_L(**40**) for the first time from marine source, from strain RK2207 (Liang, 2003).

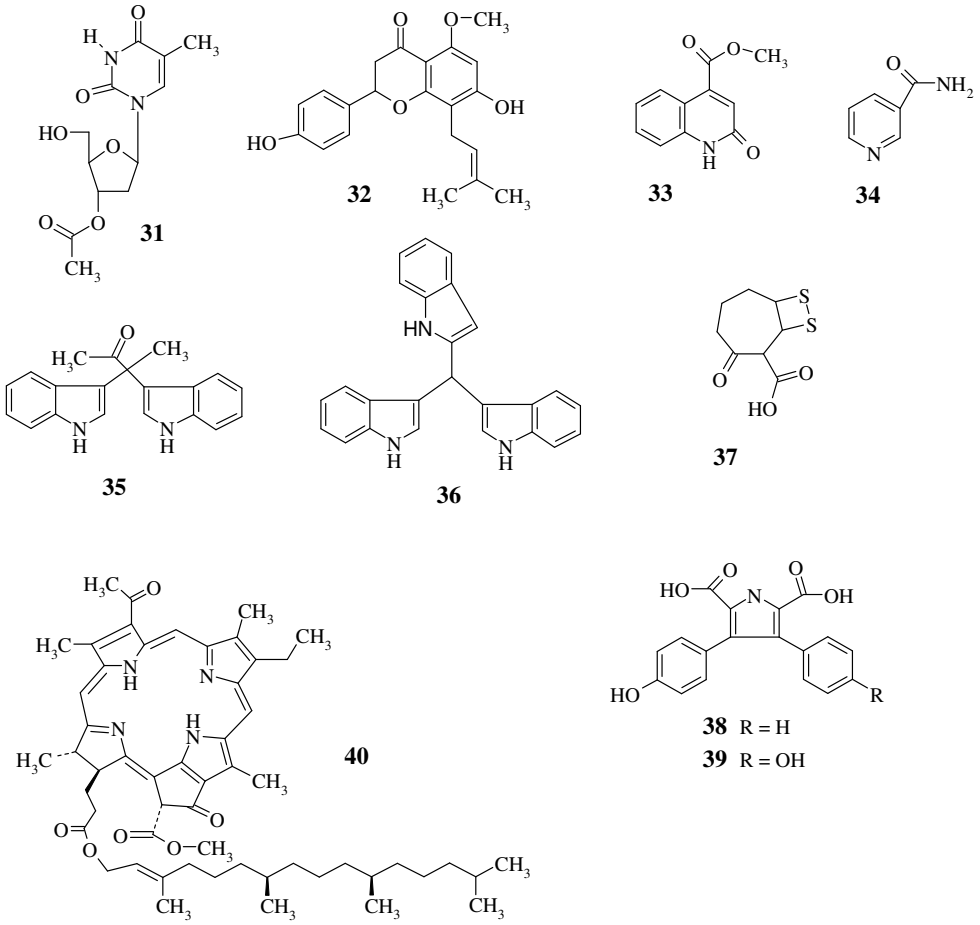


Fig. 1.12. Examples on marine compounds from North Sea bacteria. 3´-acetoxy-2´deoxythimidine (**31**), isoxanthohumol (**32**), quinoline-2-one-4-carboxylic acid methylester (**33**), 3-pyridinecarboxamide (**34**), 3,3-bis (3-indolyl)-butane-2-one (**35**), 1,1,3-tris (3-indolyl)-butane (**36**), tropodithietic acid (**37**), 3-(4´-hydroxyphenyl)-4-phenylpyrol-2,5-dicarboxylic acid (**38**), 3,4-di(4´-hydroxyphenyl) pyrrole-2,5-dicarboxylic acid (**39**) and bacteriopheophytin a_L(**40**).

Aim of the present study

The successful studies on compounds derived from marine sources, their activities and the unique structures that were previously described raised our interest in exploring and isolating bioactive chemicals from such habitats. Moreover, the accumulation of several bodies of evidences that the associated microorganism might be the real source of some of the previously ascribed metabolites to marine macroorganism promoted us to isolate a number of bacterial strains from soft corals in a try to isolate substances that may have similar structures to known marine metabolites from marine animals.

Therefore, this work was initiated to culture marine derived bacteria and optimising their growth and production of antimicrobial metabolites; to screen their crude extracts using different biological test systems. This was followed by isolation and elucidation of the new and desired biologically active secondary metabolites.

2. Materials and Methods

2.1. Chemicals and organic solvents

Organic solvents for HPLC, HPLC gradient grade

Acetonitrile	Scharlau Chemie S.A., Barcelona, Spain
Methanol	J.T. Baker, Deventer, Holland

Organic solvents, analysis grade

1-Butanol	Merck, Darmstadt
2-Propanol	Merck, Darmstadt
Acetone	J.T. Baker, Deventer, Holland
Cyclohexane	Merck, Darmstadt
Ethanol	Carl Roth, Karlsruhe
Ethyl acetate	J.T. Baker, Deventer, Holland
Methanol	J.T. Baker, Deventer, Holland
Toluene	Merck, Darmstadt

Other chemicals

A-Z-amine	Sigma, St. Louis, USA
α-Aminoisobutyric acid	Sigma, St. Louis, USA
L-Alanine	Sigma, St. Louis, USA
D-Aspartic acid	Sigma, St. Louis, USA
L-Asparagine	Fisher Scientific, USA
Bacto agar	Difco (Becton Dickinson), Heidelberg
Beef extract (powder)	Difco (Becton Dickinson), Heidelberg
Beef extract (desiccated, paste)	Difco (Becton Dickinson), Heidelberg
Chitin	Carl Roth, Karlsruhe
Corn starch	Sigma, St. Louis, USA

Marine salts mixture “instant ocean”	Tropic marine®, Dr. Biener, Wartenberg
Glycine	Serva, Heidelberg
D-Glucose	Riedel de Haen, Seelze
DL-Lactic acid	Carl Roth, Stuttgart
D-Lactose	Fluka, chemie-AG, Neu-Ulm
L-Lysine	Fluka, chemie-AG, Neu-Ulm
L-Lysine. HCl	Sigma, St. Louis, USA
Malt extract	Dr. Fränkle, Fellbach
Nutrient broth	Difco (Becton Dickinson), Heidelberg
Peptone from soymeal	Difco (Becton Dickinson), Heidelberg
Peptone from casein	Serva, Heidelberg
L-Phenylalanine	Serva, Heidelberg
Silicon antifoam	Merck, Darmstadt
Seaweed extract	Manŭfactum, Waltrop
Soluble starch	Carl Roth, Stuttgart
Sorbitol	Carl Roth, Stuttgart
Tryptone	Difco (Becton Dickinson), Heidelberg
Tween 20 and Tween 80	Carl Roth, Karlsruhe
Yeast extract (Difco)	Difco (Becton Dickinson), Heidelberg
Yeast extract	Hartge Ingredients, Hamburg
D-Xylose	Sigma, St. Louis, USA
DMEM-medium	Gibco-Invitrogen, Karlsruhe
HEPES buffer (1M, pH 7.2)	Gibco-Invitrogen, Karlsruhe
RPMI 1640 (with 25 mM HEPES)	Gibco-Invitrogen, Karlsruhe
Fetal calf serum	Gibco-Invitrogen, Karlsruhe
PMS	Serva, Heidelberg
Penicillin G	Serva, Heidelberg
Streptomycin sulphate	Merck, Darmstadt
Trypsin (3,6 U/mg, from bovine pancreas)	Serva, Heidelberg
DMSO	Fluka, chemie-AG, Neu-Ulm
NBT	Sigma, St. Louis, USA

TPA	Sigma, St. Louis, USA
Acetic acid 99-100%	Carl Roth, Karlsruhe
Hydrochloric acid, 32% p.a.	Merck, Darmstadt
Sulphuric acid, 97% p.a	J.T. Baker, Deventer, NL
Trichloroacetic acid, 99%	Acros, Geel, Belgien / New Jersey, USA
H_3BO_3	Carl Roth, Karlsruhe
$FeHPO_4 \cdot 7H_2O$	Riedel de Haen, Seelze
$(NH_4)_2HPO_4$	Riedel de Haen, Seelze
Na_2HPO_4	Riedel de Haen, Seelze
$ZnSO_4 \cdot 7H2O$	Riedel de Haen, Seelze
Agarose	Biozym,Oldendorf
Alugram Sil G/UV_{254} TLC-Plates	Macherey Nagel, Düren
Silica gel 60 (0,063-0,2 mm)	Macherey Nagel, Düren
Sephadex (LH-20)	Pharmacia, Uppsala, Sweden
Ampicillin	Sigma, St. Louis, USA
EDTA	Carl Roth, Karlsruhe
Ethidium bromide	Carl Roth, Karlsruhe
Loading dye (6x)	MBI Fermentas, St. Leon-Rot
Lysozyme	Sigma, St. Louis, USA
IPTG	MBI Fermentas, St. Leon-Rot
Phenol-chloroform (DNA-extraction)	Carl Roth, Karlsruhe
SDS	Carl Roth, Karlsruhe
Tris	Carl Roth, Karlsruhe
Triton X-100	Boeher, Manheim
X-gal	MBI Fermentas, St. Leon-Rot
KH_2PO_4	Carl Roth, Karlsruhe
K_2HPO_4	Merck, Darmstadt
Sodium acetate	Carl Roth, Karlsruhe
NaCl	Carl Roth, Karlsruhe

[8-^{14}C]-Adenine (50 mCi/mM)	NEN Du Pont, Bad Homburg
L-[1-^{14}C]-Leucine (50 mCi/mM)	NEN Du Pont, Bad Homburg
N-Acetyl-[1-^{14}C]-glucosamine (50 mCi/mM)	NEN Du Pont, Bad Homburg
[2-^{14}C]-Thymidine (50 mCi/mMl)	NEN Du Pont, Bad Homburg
[2-^{14}C]-Uridine (50 mCi/mM)	NEN Du Pont, Bad Homburg
Quickszint 454, 501	Zinsser Analytik, Frankfurt a. M.

All other chemicals were purchased from Merck (Darmstadt, Germany); otherwise their sources are specified in the text. Methanol (technical grade) and ethyl acetate (technical grade) were kindly supplied by BASF (Ludwigshafen/Rhein, Germany).

2.2. Photographic documentation

All photomicrographs showing the morphology of the bacterial strains were taken online with the phase-contrast microscope Nikon eclipse E600 (Model E-LP, Nikon, Japan). Inhibition of conidial germination in *Magnaportha grisea* was documented by using an inverted microscope Leica DM IRB (Leica microscopy, Wetzlar). Fluorescence of cells stained with acridine orange was documented with digital camera (Canon Powershot G2).

2.3. Media, buffers and solutions

2.3.1. Constituents of the complex media according to the manufacturer's recipe

Yeast extract:

Proteins	68%
Total nitrogen	10.9%
Amino nitrogen	5.2%
Loss on drying	4.3%
Ash	11%

Malt extract:

Total carbohydrate	64%
Glucose	5%
Maltose	36%
Sucrose	1%
Dextrin	15,6%
Others	6.4%
Proteins	2.5%
Fat	< 1%
Ash	1.1%
Dry substances	72%

Beef extract:

Total nitrogen	12.4%
Amino nitrogen	2.3%
Loss on drying	3.5%
Ash	9.3%
NaCl	0.3%

2.3.2. Media used for cultivation of microorganisms

During this study different media were used for cultivation of bacteria and fungi. The pH was adjusted with 1N NaOH or 1N HCl prior to autoclaving for 25 min (flasks till 5 L) or 40 min (fermentor) at 121 ^{o}C and 1 bar. For solid media, 2% agar was added before autoclaving.

2.3.2.1. Cultivation of bacteria

The following media were used for cultivation of bacteria. Unless otherwise mentioned, they were prepared in 1 L distilled water.

M_1 LBm-Medium (Luria-Bertani medium) modified for North Sea strains

Tryptone	5 g
Yeast extract	5 g
NaCl	10 g
H_2O dist	500 ml
Artificial seawater 1	500 ml
pH	7.2 ± 0.2

M_2 Modified LBm with glucose

Tryptone	5 g
Yeast extract	5 g
NaCl	10 g
Glucose	5 g
H_2O dist	500 ml
Artificial seawater1	500 ml
pH	7.6 ± 0.2

M_3 Modified LBm with glucose and marine salts

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Glucose	5 g
Marine salts mixture	33.3 g
pH	7.6 ± 0.2

M_4 Modified LBm with XAD-16 and seaweed

Tryptone	5 g
Yeast extract	5 g
NaCl	10 g
Glucose	5 g
Seaweed extract	2.5 ml
Dry XAD-16	500 ml
H_2O dist	500 ml
Artificial seawater 1	500 ml
pH	7.2 ± 0.2

M_5 Tryptone soya broth (TSB) with artificial seawater

Peptone from casein	17 g
Peptone from soymeal	3 g
Glucose	2.5 g
NaCl	5 g
K_2HPO_4	2.5 g
H_2O deionised	500 ml
Artificial seawater 2	500 ml
pH	7.3 ± 0.2

M_6 Half strength TSB with artificial seawater

Half strength of **M_5** was prepared.

M_7 SSW (Synthetic seawater medium)

NaCl	30 g
$MgCl_2 \cdot H_2O$	1.4 g
$MgSO_4 \cdot 7H_2O$	5 g
$CaCl_2 \cdot 2H_2O$	0.7 g
Peptone	0.5 g
Glycerol	3 ml
Yeast extract	0.5 g
pH	7.6 ± 0.2

M_8 Marine chitin medium 1

Chitin	2.5 g
Yeast extract	0.1 g
Peptone from casein	0.5 g
NaCl	10 g
H_2O dist	500 ml
Artificial seawater 1	500 ml
pH	7.4 ± 0.2

M_9 Marine chitin medium 2

Chitin	2.5 g
K_2HPO_4	0.7 g
KH_2PO_4	0.3 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$FePO_4 \cdot 7H_2O$	10 mg
$ZnSO_4 \cdot 7H_2O$	10 mg
H_2O dist	500 ml
Artificial seawater 1	500 ml
pH	7.4 ± 0.2

M_{10} B1-medium

Soluble starch	5 g
A-Z- amine	2.5 g
Beef extract (paste)	3.8 g
Soy meal	1 g
Yeast extract	2.5 g
KNO_3	1.5 g
Seaweed extract	2.5 ml
Marine salts mixture	33.3 g
pH	8.00 ± 0.2

M_{11} B2-medium

Corn steep solids	5 g
A-Z- amine	2.5 g
Beef extract (powder)	3.8 g
Soy meal	1 g
Yeast extract	2.5 g
KNO_3	1.5 g
Seaweed extract	2.5 ml
Marine salts mixture	33.3 g
pH	8.00 ± 0.2

M_{12} Marine broth (MB)

Tryptone	5 g
Yeast extract	1 g
Artificial seawater 2	1000 ml
pH	7.6 ± 0.2

M_{13} Nutrient broth medium

Nutrient broth	8 g
pH	7.6 ± 0.2

M_{14} Nutrient broth medium with sodium chloride

Nutrient broth	8 g
NaCl	1 g
pH	7.2 ± 0.2

M_{15} Complex medium

Beef extract	1.5 g
Yeast extract	3 g
Peptone	6 g
Glucose	1 g
H_2O dist	500 ml
Artificial seawater 2	500 ml
pH	7.6 ± 0.2

M_{16} Minimal medium in experiment of synthesis of macromolecules in bacteria

K_2HPO_4	7 g
KH_2PO_4	3 g
$(NH_4)_2HPO_4$	1 g
$MgSO_4 \cdot 7H_2O$	100 mg
NaCl	100 mg
Glucose	2 g
pH	7.2

M_{17} LB-medium

Tryptone	10 g
Yeast extract (Difco)	5 g
NaCl	10 g
pH	7.5 ± 0.2

M_{17a} LB-medium with antibiotics

100 µg/ml ampicillin was added to M_{17} after autoclaving and cooling to 55 °C.

M_{18} Medium for heterotrophic bacterium

Sucrose	10 g
K_2HPO_4	2.5 g
KH_2PO_4	2.5 g
$(NH_4)_2HPO_4$	1 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$FePO_4 \cdot 4H_2O$	10 mg
$MnSO_4 \cdot H_2O$	7 mg
Yeast extract	1 g
Tryptone	5 g
NaCl	20 g
H_2O dist	500 ml
Artificial seawater 2	500 ml
pH	7.0 ± 0.2

M_{19} BMS-N medium (Helmke & Weyland, 1984)

$(NH_4)_2HPO_4$	1 g
KCl	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	25 g
Marine salts mixture	16.7 g
YNB	10 ml
H_2O dist	990 ml
pH	7.2

M_{20} BMS medium (Helmke & Weyland, 1984)

KH_2PO_4	0.5 g
K_2HPO_4	1.5 g
KCl	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	25 g
Marine salts mixture	16.7 g
PH	7.2

M_{21} Oxidation/Fermentation agar (Hugh & Leifson, 1953)

Peptone from casein	0.5 g
Yeast extract (Difco)	0.2 g
NaCl	5 g
K_2HPO_4	0.2 g
Bromthymol blue	80 mg
Agar	2.5 g
Marine salts mixture	16.7 g
pH	7.1

M_{22} PY-medium (Lipski *et al.,* 1992)

Peptone from casein	2 g
Yeast extract (Difco)	1 g
NaCl	5 g
K_2HPO_4	0.3 g
Bromthymol blue	80 mg
Glucose	0.5 g
pH	6.7 ± 0.2

M_{23} SIM medium (sulphite, indole, motility)

30 g of a purchased SIM-agar (Difco) were dissolved in half strength artificial seawater 1, pH 7.2.

Yeast nitrogen base without amino acids (YNB)

KH_2PO_4	1 g
NaCl	0.1 g
$MgSO_4 \cdot 7H_2O$	5 g
$CaCl_2$ (anhydrous)	0.1 g
Inositol	2 mg

Trace elements solution (Hoagland-solution)

H_3PO_3	611 mg
$MgCl_2 \cdot 4H_2O$ (Fluka)	389 mg
$CuSO_4$ (Fluka)	56 mg
$ZnSO4 \cdot 7H_2O$	56 mg
$AL_2(SO_4)_3$ (Sigma-Aldrich)	56 mg
$NiSO_4 \cdot 6H_2O$ (Roth)	56 mg
$Co(NO_3)_3 \cdot 6H_2O$ (Sigma)	56 mg
$(NH_4)Mo_7O_{24} \cdot 4H_2O$ (Roth)	56 mg
TiO_2 (Sigma-Aldrich)	56 mg
LiCl (Roth)	28 mg
$SnCl_2$	28 mg
KI (Roth)	28 mg
KBr	28 mg

Stock solution

$SrCl_2 \cdot 6H_2O$ (ICN-biomedicals)	3.4 g (dissolved alone)
KCl	50 g
$NaHCO_3$	16 g
KBr	16 g
H_3PO_3	2.2 g
NaF	240 mg
NH_4NO_3	160 mg

Artificial seawater 1

NaCl	24 g
$MgCl_2 \cdot 6H_2O$	5 g
$MgSO_4 \cdot 7H_2O$	5 g
$CaCl_2$ (anhydrous)	0.5 g
KCl	0.5 g
KBr	0.1 g
$FePO_4 \cdot 4H_2O$	10 mg

Artificial seawater 2

Sodium sulphate (anhydrous)	3.44 g (dissolved in 500 ml dist. H_2O)
Ferric citrate	0.1 g
NaCl	19.45 g
$MgCl_2 \cdot 4H_2O$ (Fluka)	8.8 g
$CaCl_2$ (anhydrous)	1.8 g
Na_2HPO_4	8 mg
Trace elements	1 ml
Stock solution	10 ml
H_2O dist	489 ml

2.3.2.2. Cultivation of fungi

Fungi used in the susceptibility tests with the isolated bioactive compounds were cultivated in the following media

M_{24} Yeast extract-malt extract medium (YM)

Malt extract	10 g
Yeast extract	4 g
Glucose	4 g
pH	5.5 ± 0.2

M_{25} Yeast extract-malt extract glucose medium (YMG)

Malt extract	10 g
Yeast extract	4 g
Glucose	10 g
pH	5.5 ± 0.2

M_{26} Sabouraud 2% glucose agar for the cultivation of *Candida albicans*

47 g of purchased medium were dissolved in 1 L of distilled water, pH 5.5.

M_{27} Yeast medium in experiment of synthesis of macromolecules in yeasts

Malt extract	5 g
Yeast extract	1 g
Glucose	2 g
pH	5.5

2.3.3. Media used for cell lines

Before being used, cell line media (RPMI 1640 / DMEM) were warmed at 37 °C. Addition of the antibiotics solution was done by sterile filtration (sterile filter Typ FP 30/0.2, 0,2 µm CA-S, Schleicher & Schuell, Dassel).

M_{28} RPMI medium for suspension cell line

RPMI 1640 (with 25 mM HEPES)	89 ml
Fetal calf serum	10 ml
Antibiotics solution	1 ml

M_{29} DMEM medium for monolayer cell line

DMEM	89 ml
Fetal calf serum	10 ml
Antibiotics solution	1 ml

2.3.4. Solutions and buffers

Unless otherwise noted, buffers and solutions used for the preparation of competent cells and the extraction of genomic DNA were prepared according to Sambrook *et al.* (2001) in 1 L distilled water. The pH value was adjusted by using 1N HCl or 1N NaOH.

P_1 Phosphate buffered saline (PBS)

NaCl	8 g
$Na_2HPO_4 \cdot 2H_2O$	1.44 g
KCl	0.2 g
KH_2PO_4	0.2 g
pH	7.4

P_2 TE-buffer for genomic DNA extraction

Tris-HCl, pH 8.0	10 mM
EDTA	1 mM

P_3 Triton X-100 lysis buffer for DNA fragmentation assay

0.2% Triton X-100 was added to **P_2**.

P_4 10x TBE-buffer for gel-electrophoresis

Tris	108 g
H_3BO_3	55 g
Na_4EDTA	8.3 g
pH	8.0

P_5 TES-buffer (TE-saline) (Süßmuth *et al.,* 1999)

Tris-HCl, pH 8	30 mM
EDTA	5 mM
NaCl	50 mM

P_6 TES-sucrose Puffer

33.3 g sucrose were dissolved in 100 ml of **P_5**.

P_7 Lysoyzme mixture

Lysozyme	20 mg
15% sucrose in **P_2**	10 ml
pH	8.0

S_1 Antibiotics solution

Penicillin G	6.5 mg
Streptomycin sulphate	10 mg
H_2O deionized	1 ml

S_2 Acridine orange (AO) solution

Acridine orange	6 µg/ml
Citric acid	0.1 M
Na_2HPO_4, pH 2	0.2 M

Prepare 90 ml of citric acid; add acridine orange and 10 ml of Na_2HPO_4.

S_3 Giemsa-staining solution

Giemsa solution (azur eosin methylene blue solution for microscopy) was diluted 1:10 in normal saline solution **S_5**.

S_4 NBT-staining solution for HL-60 differentiation assay

NBT	1 mg
TPA	0.1 µg
PBS (P_1)	1 ml

S_5 Normal saline solution

NaCl	9 g
H_2O deionized	1000 ml

S_6 Propidium iodide staining master mix for cell cycle analysis

Rnase A (10 mg/ml)	10 µl
Propidium iodide (1 mg/ml)	40 µl
PBS (P_1)	950 µl

S_7 Trypsin solution

EDTA	0.02%
Trypsin	0.33 U/ml

The solution was prepared in PBS (P_1).

S_8 XTT-staining solution

PMS (0.383 mg/ml **P_1**)	0.1 ml
XTT (1 mg/ml **M_{28}**)	4.9 ml

2.4. Enzymes

Taq-polymerase and restriction enzymes were purchased from MBI Fermentas (St. Leon-Rot, Germany). RNase A was purchased from Sigma, St. Louis, USA. For RNase A preparation, 10 mg of the enzyme were dissolved in 1 ml of TE-buffer. Before storing at –20 °C, the enzyme was heated in a boiling water bath for 10 min to inactivate any contaminating DNase.

2.5. Primers

All primers used in the PCR-reactions for the amplification of 16S rDNA were synthesized by MWG-biotech (Ebersberg).

16SA (for): 5'-AGA GTT TGA TCC TGG CTC-3' (18 nt.)

16SB (rev): 5'-AAG GAG GTG ATC CAG CCG CA-3' (20 nt.)

2.6. Organisms

2.6.1. Screened bacterial strains

The following marine bacterial strains, isolated by Dr. Elisabeth Helmke and her colleagues in Bremerhaven, were screened during this work:

T 268 (*Pseudoalteromonas* sp.)	T 436 (*Salegentibacter* sp.)
T 402	T 445
T 233	T 396 (*Psychrobacter* sp.)

The following epibiotic bacterial strains were isolated from soft corals flourishing in the Red Sea/Gulf of Aqaba-Jordan:

WMBA1-2	WMBA2-29-2	WMBA5-100
WMBA1-3	WMBA2-31	WMBA11-44
WMBA1-4 (*Vibrio* sp.)	WMBA2-32	WMBA11-45
WMBA1-5 (*Ralstonia* sp.)	WMBA2-34	WMBG1-89
WMBA1-8	WMBA2-104	WMBG2-49
WMBA2-10	WMBA5-24	WMBG7-61
WMBA2-11	WMBA5-27	WMBG7-65

The following bacterial strains were chosen for the isolation of bioactive metabolites as they showed promising bioactivities in screening:

T 268 (*Pseudoalteromonas* sp.)	T 436 (*Salegentibacter* sp.)
T 396 (*Psychrobacter* sp.)	WMBA1-4 (*Vibrio* sp.)
WMBA1-5 (*Ralstonia* sp.)	

2.6.2. Test organisms for the biological characterization

2.6.2.1. Bacteria and fungi

Test organisms were kept on slants stored at 4 °C. They were suspended in agar medium after cooling to 55 °C and poured into Petri dishes (Ø 9 cm).

Table 2.1. Organisms and conditions of the agar-diffusion test.

Organisms	Strain No.	Temperature [°C]	Medium
Fungi:			
*Candida albicans**	ATCC 90028	37	M_{26}
Paecilomyces variotii Bainier	ETH 114646	37	M_{24}
Penicillium notatum	Dept. Biotech.	27	M_{24}
Mucor miehei	Tü 284	37	M_{24}
Nematospora coryli Peglion	ATCC 10647	27	M_{25}
Bacteria:			
Gram-positive:			
Bacillus brevis	ATCC 9999	37	M_{13}
Bacillus subtilis	ATCC 6633	37	M_{13}
Micrococcus luteus	ATCC 381	37	M_{13}
*Staphylococcus aureus**	ATCC 11632	37	M_{14}
Gram-negative:			
Enterobacter dissolvens	LMG 2683	27	M_{13}
*Proteus vulgaris**	DSM 30119	37	M_{14}
*Pseudomonas aeruginosa**	ATCC 15442	37	M_{14}

Strains marked with * are pathogenic. I would like to thank Sabine Pauls for performing the agar-diffusion assay with these strains.

Table 2.2. Organisms and conditions of the minimum inhibitory concentration (MIC) test.

Organisms	Strain No.	Temperature [°C]	Medium
Fungi:			
Paecilomyces variotii Bainier	ETH 114646	37	M_{24}
Penicillium notatum	Dept. Biotech.	27	M_{24}
Phytophthora infestans	CBS 366.51.	20	M_{24}
Mucor miehei	Tü 284	37	M_{24}
Nematospora coryli Peglion	ATCC 10647	27	M_{24}
Saccharomyces cerevisiae FL 200	Prof. Lacroute, Straßburg	27	M_{24}
Ustilago nuda	CBS 118.19	27	M_{24}
Bacteria:			
Gram-positive:			
Bacillus brevis	ATCC 9999	37	M_{13}
Bacillus subtilis	ATCC 6633	37	M_{13}
Micrococcus luteus	ATCC 381	37	M_{13}
Gram-negative:			
Escherichia coli K12	Dept. Biotech	37	M_{13}
Enterobacter dissolvens	LMG 2683	27	M_{13}

2.6.2.2. Bacteria for transformation

Escherichia coli Top 10F′ (Grant *et al.*, 1990) was used for cloning and amplification of plasmid-DNA.

Genotype: F′[*lac*qTn10(tetR)], *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), φ80*lac*Z ΔM15 Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L, *end*A1, *nup*G (Invitrogen, Karlsruhe)

2.6.2.3. Cell lines

Table 2.3. Cell lines used for the evaluation of the cytotoxic activity.

Cell line	Description	Source	Medium
Colo-320	Human colorectal adenocarcinoma	DSMZ ACC 144	M_{28}
HeLa S3	Human epithelial-cervix carcinoma	ATCC CCL 2.2	M_{29}
HepG2	Human hepatic carcinoma	DSMZ ACC 180	M_{29}
HL-60	Human promyelocytic leukaemia	ATCC CCL 240	M_{28}
Jurkat	Human acute T-cell leukaemia	DSMZ ACC 282	M_{28}
L1210	Mouse lymphocytic leukaemia	ATCC CCL 219	M_{28}
MCF-7	Human breast adenocarcinoma	DSMZ ACC 115	M_{29}
MDA-MB-231	Human breast adenocarcinoma	ATCC HTB-26	M_{29}

2.6.2.4. Nematodes

Caenorhabditis elegans	Dr. G. Rack, Tübingen
Meloidogyne incognita	Bayer AG, Monheim

2.6.2.5. Effects on seed germination and growth

Lepidium sativum L. (Brassicaceae)	Dicotyledon
Setaria italica L. (Poaceae)	Monocotyledon

Lepidium seeds were bought from Schürmann (Kaiserslautern) and *Setaria* seeds were bought from Dr. Bernd Honeburg (Göttingen).

2.7. Isolation of marine bacteria

2.7.1. Collection of soft corals

Soft corals were collected by SCUBA diving in the Red Sea/Gulf of Aqaba. They were collected from three main sites along the Jordanian coast (GPS readings: 29°27.865`N 34°58.865`E, 29°27.433`N 34°58.556`E and 29°21.605`N 34°57.815`E). A part from each colony was preserved in 70% ethanol for identification, a part was immediately used to isolate the associated epibiotic bacteria and a part was deep-frozen in a plastic bag at – 20 °C in order to be extracted in the future.

2.7.2. Isolation of epibiotic bacteria from the collected soft corals

Soft corals, once they were brought to the surface, were flushed with sterile artificial seawater. Parts of the colonies were vortexed three times in sterile seawater in order to remove loosely attached microorganisms. They were divided aseptically into small pieces from which the outer coenenchyme was aseptically detached from the inner tissues. The separated tissues (coenenchyme as well as the inner tissues) were suspended in test tubes containing sterile seawater. The suspensions were 3-5 fold diluted (1:10) with sterile artificial seawater. 50 µl from each tube was spread on plates with different agar media (M_1, M_8 and M_{12}). The plates were monitored for bacterial growth. New colonies were streaked on new agar plates for purification. This process was repeated several times till pure culture plates were obtained.

All media were supplemented with cycloheximide (50 mg/l) and nystatin (50 mg/l) to inhibit the growth of yeasts and fungi.

2.7.2.1. General storage of bacteria

All bacterial strains isolated from soft corals were lyophilised. The screened bacteria in this study were maintained on agar slants of M_1 under sterile paraffin oil. They were stored at 4 °C and at room temperature. These strains were subcultured every 3-4 months in order to keep them alive. I would like to thank Anja Schüffler for lyophilising the bacterial strains.

The *Escherichia coli* strain used for transformation was conserved in glycerol. 0.9 ml of a well grown overnight culture was mixed with 0.9 ml of sterile glycerol (80%) and stored at – 80 °C.

2.7.3. Identification of bacterial strains

To identify the isolated bacterial strains the following studies were performed:

2.7.3.1. Morphological characterization

Morphological characterization was performed with a stereomicroscope on bacterial strains grown for 48-72 hours on M_1 agar plates. Several characteristic features were examined, such as surface characteristics, consistency, type of margins and elevation. Whole colony form and colour were determined with naked eye.

Gram-staining was examined under a light compound microscope. Gram-reaction of a smeared colony was done by using 3% KOH as well as by staining with crystal violet, decolourisation with ethanol and counterstaining with safranin (Süßmuth *et al.,* 1999). Using a phase-contrast microscope, a wet mount preparation was examined to detect the motility, shape of sole bacterial cell and presence of endospores.

2.7.3.2. Biochemical and physiological characterization

Unless otherwise indicated, all physiological and biochemical characteristics were studied using standard procedures (Baumann *et al*., 1972; Bowman, 1997; Süßmuth *et al.,* 1999). Experiments were performed in sterile test tubes filled with 10 ml of test media. An inocubation temperature of 23 °C and test media containing equivalent salts content to half strength seawater had been chosen for the following tests:

Catalase activity was determined using 3% H_2O_2; presence of cytochrome oxidase was tested using oxidase-test strips (Bactident® oxidase, Merck, Darmstadt). Reduction of nitrate was determined in M_1 supplemented with 0.1% KNO_3. Formation of nitrite was detected with nitrate-test strips (Merck, Darmstadt) after 3, 7 and 14 days. Production of H_2S-gas and indole were tested using M_{23}. Indole production was detected with Kovac's reagent (Bactident® oxidase, Merck, Darmstasdt). Production of acid from different carbohydrates was done as

described by Helmke & Weyland (1984) and detected in M_{21}. The inoculated media were scored for changes in colour (indicating acid production) every week for two months.

The test for utilization of organic compounds as sole carbon source and as sole nitrogen as well as carbon source was conducted as described by Helmke & Weyland (1984) in M_{19} and M_{20} respectively. Hydrolysis of starch, gelatine, Tween 80 and esculin were detected in agar plate of M_1. Arginine dihydrolase, decarboxylase as well as lysine decarboxylase were detected using M_{22} according to Lipski *et al.* (1992). The ß-galactosidase activity was detected with Yeast ß-Galactosidase Assay Kit (Pierce, USA) following the manufacturer's instructions. The requirement for sodium ions, tolerance to temperature and salinity were deduced from 2.8.2.2.

2.7.3.3. 16S rDNA sequencing

The identification of bacterial strains based on the partial 16S rDNA sequence was carried out as described under 2.11.1. Data obtained from these studies were matched with two main literatures:

- Bergey's manual for systematic bacteriology Vol. 1 (Krieg, 1984) and

- The Prokaryotes (Starr *et al.,* 1981).

2.8. Cultivation of bacterial samples

2.8.1. Screening for suitable media for bacterial growth and production of bioactive metabolites

Several media were tested (M_1-M_{12}, M_{15} and M_{18}) in order to select the suitable media that allow the growth of the bacterial strains and high production of bioactive secondary metabolites. Fermentation took place in 1 l Erlenmeyer flasks containing 500 ml of culture fluid. The cultures were incubated at 4 °C, 18 °C and 25 °C with 121 rpm agitation. A single colony from a well grown agar plate was used as an inoculum. Immediately after the inoculation of each bacterial strain, 10-20 ml sample and thereafter-daily samples were taken for OD measurement (see 2.8.4.1). When the OD decreased, the culture was harvested by centrifugation for 10 min at 10000 rpm (16000x g, Roto Super 40, Hettich-Tuttlingen). The supernatant was divided into two equal volumes. The pH of one half was adjusted to pH 4 with 1 N HCl and the other half was adjusted to pH 8 with 1 N NaOH. The supernatants were extracted with equal volumes of ethyl acetate. After separation, the organic phases were dried over Na_2SO_4 (anhydrous) and concentrated *in vacuo* at 40 °C.

For strains cultured in M_4, the extract was obtained by eluting decanted XAD-16 with 400 ml methanol then 200 ml acetone. The resulting crude extracts were dissolved in 1 ml methanol and stored at 4 °C for further use.

Antimicrobial activity of each extract was determined using the agar diffusion assay. 100-300 µg of crude extract was applied onto 6 mm filter paper discs. Their activities were tested against the microorganisms listed in Table 2.1 excluding pathogenic strains.

2.8.2. Small-scale fermentation in Erlenmeyer flasks

2-L Erlenmeyer flasks containing 1l of medium M_1, M_{10} or M_{11} were inoculated with a single colony from a well grown agar plate. The flasks were incubated on a rotatory-shaker (Braun, Melsungen) with 121 rpm. Directly after the inoculation, 50 ml sample and thereafter-daily samples were taken. The growth was monitored by OD measurements of 10 fold-diluted samples at 580 nm and by changes in pH value (pH-meter, CG 825, Schott, Hofheim). As the OD decreased, the culture was harvested by centrifugation for 10 min at 10000 rpm (16000x g, Roto Super 40, Hettich-Tuttlingen).

The pH of the supernatant was adjusted to pH 4 and was extracted with an equal volume of ethyl acetate. After separation, the organic phase was dried over Na_2SO_4 (anhydrous) and concentrated *in vacuo* at 40 °C. The resulting crude extract was dissolved in 1 ml methanol and its antimicrobial activity was monitored using the agar-diffusion assay (2.10.1). The crude extracts were stored at – 20 °C.

The bacterial pellet was washed 2-3 times with sterile filtered artificial seawater and lyophilised. Extraction of lyophilised cells was carried out with methanol overnight at 4 °C. The organic phase was dried and stored at – 20 °C.

2.8.2.1. Determination of bioactivities of crude extracts

The antimicrobial activities of the crude extracts were determined by applying 200-300 µg onto filter paper discs as described in 2.10.1. Cytotoxic activities were evaluated with different concentrations up to 100 µg/ml against cell lines listed in Table 2.3.

2.8.2.2. Optimization of growth conditions

Optimization of culturing conditions was carried out for each bacterial strain depending on the screening results obtained in 2.8.1. Incubations were performed at the following temperatures: 4 °C, 10 °C, 21 °C, 28 °C, 35 °C and 37 °C. In order to optimise the salt content, different concentrations of marine salts mixture were used (0 g, 10 g, 33.5 g, 50 g, 75 g and 100 g per litre) and the cultures were incubated at 21 °C. Whenever M_{10} or M_{11} was used for bacterial cultivation, the strain was simultaneously cultivated in medium M_1 under the same conditions.

The crude extract from each culture was examined with analytic HPLC (see 2.9.3.2) to monitor changes in the metabolites chromatogram. The antimicrobial activities were tested by agar diffusion test as described in 2.10.1.

2.8.3. Large-scale fermentation of bacterial strains

Bacterial strains of interest were cultivated in 500 ml Erlenmeyer flasks containing 250 ml of M_1 for 24-48 hours. 50 ml of this culture were used to inoculate a 20 l fermentor. 3-5 ml

of silicon antifoam were added prior to autoclaving and whenever it was necessary during the fermentation process. Fermentations were carried out under the following conditions:

***Pseudoalteromonas* sp. T268**

20-L Fermentor:	Type C6, Biolafitte, Paris; 120 rpm, 3 l airflow/min, 20 °C ± 0.5 °C, Medium M_1.
20-L Fermentor:	Type Biostat U20, Braun & Diessel, Melsungen; the same conditions but with 4 l airflow/min.

***Salegentibacter* sp. T436**

20-L Fermentor:	Type C6, Biolafitte, Paris; 120 rpm, 3 l airflow/min, 21 °C ± 0.5 °C, Medium M_{10} and Medium M_{11}.
20-L Fermentor:	Type Biostat U20, Braun, Melsungen; 150 rpm, 4 l airflow/min, 21 °C ± 0.5 °C, Medium M_{10}.

***Psychrobacter* sp. T396**

20-L Fermentor:	Type C6, Biolafitte, Paris; 120 rpm, 3 l airflow/min, 4 °C ± 0.5 °C, Medium M_{10}.

***Ralstonia* sp. WMBA1-5**

20-L Fermentor:	Type C6, Biolafitte, Paris; 150 rpm, 3 l airflow/min, 22 °C ± 0.5 °C, Medium M_1.

***Vibrio* sp. WMBA1-4**

20-L Fermentor:	Type C6, Biolafitte, Paris; 150 rpm, 3 l airflow/min, 25 °C ± 0.5 °C, Medium M_{11}.
20-L Fermentor:	Type Biostat U20, Braun, Melsungen; the same conditions but with 4 l airflow/min.

During the fermentation process, 150-200 ml sample and thereafter daily samples, in case of WMBA1-4 in intervals of 6-12 hours, were taken and used to measure the OD (2.8.4.1) and pH values, to determine the antimicrobial activity (2.10.1) and to evaluate the cytotoxic activity (2.10.4) of the crude extract. In addition, changes in the weight of crude extract were recorded. The increase in the colony forming units (CFU) (2.8.4.2) was used as an indication for cell viability.

During fermentation in fermentor of the Typ Biostat U, the exhaust-gas was analysed as described under 2.8.4.3. Regulation of the airflow rate, speed of the propeller and temperature were achieved by the aid of a Multi-fermentor control system unit supported by the micro-MFCS 3.2 programme (Braun, Melsungen).

The cultivation was stopped directly after the OD value of the culture began to decrease. The culture was centrifuged for 30 min at 2700x g with Roto Silenta (Hettich-Tuttlingen). The aqueous supernatant was extracted two times with an equal volume of ethyl acetate. After separation, the organic phase was dried over Na_2SO_4 (anhydrous) and concentrated *in vacuo* at 40 °C. The resulting crude extract was dissolved in methanol to a final concentration of 10 mg/ml and stored at –20 °C.

2.8.4. Fermentation parameters

2.8.4.1. Culture turbidity

Growth of the bacterial cells indicated by the degree of turbidity was evaluated by measuring the optical density of the culture at 580 nm (OD_{580}). Measurements of the OD were done with 10-fold diluted samples with a spectrometer (UV. Spectrometer, Lambda 16, Perkin-Elmer, Langen).

2.8.4.2. Colony forming units (CFU)

The numbers of viable cells were determined by spreading 10 µl of diluted culture fluids (10^{-5}, 10^{-7}, 10^{-9}, 10^{-11} and 10^{-13}) on M_1 agar plates. This was used as an additional parameter for measuring the bacterial cell growth during fermentation process.

2.8.4.3. Exhaust-gas analysis

The metabolic activities of the cultivated bacterial strains in fermentor Biostat Typ U were monitored and recorded online by the aid of an automated process control system supported by the micro-MFCS 3.2 programme (Braun, Melsungen). The following parameters were registered online: oxygen partial pressure in the culture fluid (oxygen-electrode Type O_2-sensor, 25 mm diameter, Ingold, Steinbach/Ts); oxygen content (Magnet pneumatic oxygen-analyzer Type Magnos 4G, Hartmann & Braun, Frankfurt a. M.) and the carbon dioxide content (carbon dioxide analyzer Type SB-305, ADC, England) were analysed from the downstream air outlet. Data of the exhaust-gas analysis were used to calculate the respiration quotient (RQ) online.

2.8.4.4. Secondary metabolites determination using the analytical HPLC

Preliminary idea on the presence of known compounds was obtained by using analytical HPLC (see 2.9.3.2). Their UV-spectra were compared with the HPLC-DAD-data base of the Department of Biotechnology/Kaiserslautern.

2.9. Methods for purification and physico-chemical characterization of the secondary metabolites

2.9.1. Column chromatography (CC)

Preliminary separation of the different substances that constituting the active crude extract was achieved by using silica gel 60 (0.063-0.2 µm mesh, Merck, Darmstadt) as a stationary phase and elution with increasing polarity of the mobile phase (i.e. starting from 100% cyclohexane through cyclohexane-ethyl acetate mixture, ethyl acetate-methanol mixture till 100% methanol) under atmospheric pressure. Additional separation was done using size-exclusion chromatography (Sephadex LH-20) with 100% methanol as eluent.

2.9.2. Thin Layer Chromatography (TLC)

Aluminium plates impregnated with silica gel 60 (20 x 20 cm, Alugram® SIL G/UV_{254}, Macherey & Nagel, Düren) were used for TLC. The following mobile phases were used to develop the spotted samples on the plate:

L1:	Toluene : acetone : acetic acid	70 : 30: 1
L2:	Toluene : acetone	7 : 3
L3:	Cyclohexane : ethyl acetate	1 : 1
L4:	Methanol : acetone	1 : 1
L5:	Methanol : dichloromethane	1 : 9

The developed spots were detected with the following methods:

Fluorescence extinction at 254 nm

Fluorescence at 360 nm

Bioautography

Staining with the following spray-reagents (chromogenic reagents):

R_1: Anisaldehyde in ethanol (1 ml anisaldehyde, 1 ml H_2SO_4, 38 ml EtOH)

R_2: Anisaldehyde in acetic acid (0.5 ml anisaldehyde, 1 ml H_2SO_4, 50 ml acetic acid)

R_3: Vanilline-sulphuric acid (1% vanilline in H_2SO_4)

R_4: Rhodamin B (0.1%, Merck, Darmstadt; fluorescence extinction)

R_5: Potassium permanganate (0.05% in water, decolorization)

R_6: Molybdate-phosphoric acid (Merck, Darmstadt)

R_7: Ninhydrin (2,2-dihydroxyin-1,3-dandion; 0,1% spray-reagent, Merck, Darmstadt)

Colouration of spots on plates sprayed with R_1, R_2, R_3, R_6 and R_7 was detected by heating on a hot plate at 120 °C.

2.9.3. High performance liquid chromatography (HPLC)

2.9.3.1. Preparative HPLC

For purification of the intermediated fractions obtained from the preliminary separation steps, the following preparative systems were used:

- Jasco PU-1586 with multi-wavelength detector MD-910 (Jasco, Groß-Umstadt)
- Jasco PU-2087 Plus with UV detector UV-1570M (Jasco, Groß-Umstadt)

Separation and purification of the interesting compounds were achieved by using the following preparative columns:

- Hibar RT LiChrosorb RP 18, Merck, Darmstadt (7µm, 250 x 25 mm, 15-20 ml/min).
- Luna C 18 (2), Phenomenex, Aschaffenburg (10µm, 250 x 10 mm, 5 ml/min).
- Nucleosil 100-7 C-18, Macherey & Nagel, Düren (7 □m, 250 x 21 mm, 10 ml/min).

For separation, a mixture of 0.1% H_3PO_4 and methanol or Acetonitrile with gradients of decreasing polarity was used as an eluent. Before the application of samples, runs in analytical HPLC (2.9.3.2) were examined and then the samples were filtered (Chromabond® C18, Macherey & Nagel, Düren).

2.9.3.2. Analytical HPLC

The retention time and purity of the isolated substances were detected by the aid of analytical HPLC. The analytical HPLC (Model: Agilent 1100 series, Agilent, Waldbronn) is equipped with a diode array detector (G1315B) and a separation column (RP-18, LiChroCart®, 5 µm, 125 x 4 mm, Merck, Darmstadt). Phosphoric acid or formic acid (0.1% in H_2O) (eluent A) and methanol or acetonitrile (eluent B) were used as mobile phases in the following gradients:

Table 2.4. Gradients used in the analytical HPLC

Gradient	Column Material	Flow rate [ml/min]	Eluent A	Eluent B	Time [min]	Eluent B [%]
G_1	RP-18	1.5	0.1% H_3PO_4	MeOH	0	0
					20	70
					30	100
					35	100
					40	0
G_2	RP-18	0.8	0.1% HCOOH	MeCN	0	0
					0.5	0
					20	100
					21	100
					23	0
G_3	RP-18	1	0.1% H_3PO_4	MeCN	0	1
					20	100
					24	100
					25	1
G_4	RP-18	0.45	0.1% HCOOH	MeCN	0	1
					20	100
					24	100
					25	1

The HPLC runs took place at 40 °C and with 5-50 µl injection volumes. In order to compare different chromatograms with each other, G_4 was used as a reference gradient.

2.9.4. Spectroscopy

UV-Spectra were recorded in MeOH using the UV/VIS-spectrometer, Lambda 16 (Perkin-Elmer, Langen). IR-spectra were recorded in KBr pellets with a Bruker IFS-48 spectrometer (Bruker, Karlsruhe). Mass spectra were recorded with LC-MS (LC/MSD, Agilent series 1100, Agilent, Waldbronn). I would like to thank Anja Meffert for the LC-MS measurements.

Additional spectroscopic measurements for structural elucidation of the purified substances were done in the Institute of Organic and Molecular Chemistry-University of Göttingen. I would like to thank Prof. Dr. H. Laatsch and his co-workers for elucidation the structure of the isolated substances.

2.10. Biological characterization

2.10.1. Agar diffusion test and bioautography

The antimicrobial activities of the crude extracts as well as the isolated substances were determined according to Zähner (1965). Organisms used in this test and conditions of cultivation are listed in Table 2.1. Inhibition zones measurements and types of inhibition (diffused, incomplete or complete) were determined after 24-48 hours incubation.

Fungal plates were prepared in a one layer of M_{24} supplemented with 2% agar. After autoclaving and cooling to 50 °C, fungal spores or yeast cells were added to a final density of 10^5-10^6 spores or cells/ml. 20 ml of this preparation were poured into each Petri dish.

Bacterial plates were prepared in two layers of M_{13} supplemented with 2% agar. 10 ml layer of M_{13} without bacterial test organisms were overlaid with 10 ml of the same medium containing 10^6 bacterial cells/ml.

In bioautography, the bands developed by chromatographic running of the crude extract on TLC plates as described in 2.9.2 and after the evaporation of the organic solvent, were cut into small pieces (1 x 0.5 cm). Each piece was placed with the silica side on the agar test plate seeded with the test organisms.

2.10.2. Determination the minimal inhibitory concentration (MIC)

The minimal inhibitory concentration was determined by serial dilutions assay. Bacterial and fungal strains used for susceptibility test were incubated in a liquid medium containing different concentrations of the isolated substances.

The test organisms were incubated overnight (16 hours) prior to the assay. For bacterial strains, spores or cell suspension in a sterile solution S_5 were used as inoculum, while in case of fungi, spores suspension in Tween-80 solution (3 drops Tween-80/100 ml H_2O) were used as inoculum. Different concentrations of the pure substances in MeOH were pipetted into 96-well microtiter-plates (Cellstar®, Greiner, Frickenhausen) and left till the organic solvent evaporated. Then, 200 µl of cell suspension in M_{13} for bacterial strains/M_{24} for fungi and yeast (1 x 10^5 cells or spores/ml) were added to each well. Growth controls (wells without substances) and blanks (wells with uninoculated medium) were included.

Measuring of growth of the tested organisms and determination of the MIC was done after incubation for 24-48 hours on a plate-shaker (IKA, MTS4, Jank & Kunkel, Staufen) under the conditions listed in Table 2.2. The minimum inhibitory concentration is defined as the lowest concentration of the substance that inhibited the growth (visible turbidity) of the test microorganisms. This was determined by naked eye observation as well as by measuring the OD at 600 nm by EIA-Reader (Model 2550, Biorad, Düsseldorf). The type of inhibition, bacteriostatic or bactericidal/fungistatic or fungicidal was determined by platting the preparations, where there were no visible growth, on agar plates with the proper medium and with incubation for 24 hours.

Chloramphenicol and amphotericin B were used as a positive control.

2.10.3. Inhibition of germination in *Magnaporthe grisea*

The test was done according to Thines (1997) in 96-well microtiter plates (Cellstar®, Greiner, Frickenhausen). I would like to thank Wolfgang Schuck for the aid he presented in this test.

2.10.4. Cytotoxicity test

The cytotoxicity test was carried out according to Zapf *et al.* (1995). The activities were evaluated for crude extracts and pure compounds against suspension as well as monolayer cell lines from human and animal origin (see Table 2.3.). Handling with cell lines was carried out under a sterile Laminar-Flow Cabinet (Microflow Biological Safety Cabinet, Nalge Nunc, Wiesbaden). The cells were incubated in a humidified atmosphere containing 5% CO_2 (Heraeus, Karlsruhe).

2.10.4.1. Suspension cell culture

Cultivation of the suspension cell lines Jurkat, HL-60 and L1210 took place in 20 ml of M_{28} in filter-cap tissue culture flasks with growth areas of 75 cm² (Cellstar®, Greiner, Frickenhausen). Subculturing took place every 2-3 days. The culture flasks were left in upright position till the cells settled down on the bottom of the flasks. Then, 12-15 ml of the supernatant were carefully removed. 10 ml of a fresh medium were added and the cells were

suspended. The suspension was divided, depending on the cell density, into new flasks. The preparation was completed with a fresh medium to 20 ml total volume.

The test was performed with different concentrations of crude extracts or pure substances (in duplicates) against 0.5-1 x 10^5 cells/ml. The cytotoxic activity was evaluated after 24, 48 and 72 hours microscopically under an invert microscope (Olympus, Japan). Cell lysis, cell deformation, cell fragmentation and growth inhibition were indications of cytotoxic activity of the substances. In addition, the cytotoxicity was determined quantitatively by staining with a tetrazolium salt-XTT (Sodium 3,3´-(1-[(phenylamino)carbonyl]-3,4-tetrazolium)-bis (4-methoxy-6-nitro) benzene sulphuric acid hydrate).

100 µl of the medium were carefully removed from each well. Then, 50 µl of freshly prepared solution S_8, warmed at 37 °C (according to the working instructions, Cell-Proliferation-Kits II, Roche, Mannheim), were added to each well. The plate was incubated in humidified atmosphere with 5% CO_2 at 37 °C. Then, the absorbance was determined at 480 nm in EIA-Reader (Model 2550, Biorad, Düsseldorf).

The absorbance of the cultures in the plate was measured several times within 3-8 hours till the OD_{480} value did not change. The assay based on the cleavage of the yellow tetrazolium salt (XTT), by the dehydrogenase activity in mitochondria of living cells, to form an orange formazan dye (max. absorbance 450-500 nm).

2.10.4.2. Monolayer cell culture

Cultivation of the monolayer cell lines Colo-320, MDA MB-231, MCF-7, HeLa S3 and HepG2 took place in 40 ml of M_{30} in filter-cap tissue culture flasks with growth areas of 175 cm² (Cellstar®, Greiner, Frickenhausen). Subculturing was carried out every 2-3 day, in which the medium was removed and the cells adhering to the bottom of the flask were washed with sterile PBS. In order to detach the cells from the bottom of the flask, 2 ml of trypsin (S_7) were added and left for 2 min. The detached cells were thoroughly suspended in 10 ml of fresh medium, divided into new flasks and filled up to 40 ml total volume.

The test was performed with different concentrations of crude extracts or pure substances (in duplicates) against 5 x 10^4 cells/ml. The cytotoxic activity was evaluated microscopically at the time intervals mentioned in 2.10.4.1. In addition, the cytotoxicity was determined quantitatively by using Giemsa stain as described by Mirabelli *et al.* (1985).

2.10.4.3. NBT-differentiation

Different agents are able to induce the differentiation of human promyelocytic leukaemia cell line (HL-60) to granulocytes or monocytes/macrophages. This differentiation is associated with the ability of the differentiated cells to acquire the functional characteristics of the normal peripheral blood cells, including phagocytosis, complement receptors, chemotaxis and the ability to reduce nitro-blue tetrazolium (NBT) (Baehner *et al.,* 1968, 1976, Breitman *et al.,* 1980 and Seo *et al.,* 2004).

The ability of some of the isolated pure compounds to induce the morphological differentiation of HL-60 was detected following the method described by Kocksch (1992). Differentiated cells reduce the water insoluble NBT to dark-blue, cell-associated, nitro-blue formazan deposits. As a positive control 1.3% DMSO and 1 ng TPA were used. Cells without addition of the test substances were included as negative control.

2.10.4.4. DNA fragmentation

Some cell lines show signs of apoptotic reaction against certain tested substances. Therefore, DNA fragmentation analysis was done as described under 2.11.2.

2.10.4.5. Cell cycle analysis

Analysis of the cell cycle took place by flow cytometric determination of DNA content. Propidium iodide (PI) is one of the most commonly used dyes to quantitatively assess DNA content. The PI intercalates into the major groove of double-stranded DNA and produces a highly fluorescent adduct that can be excited at 488 nm with a broad emission centred around 600 nm. For DNA staining, a protocol was adopted from Ormerod (1992).

Briefly, HL-60 cells were cultivated (see 2.10.4.1) overnight in 24-well microtiter plate. 1-2 x 10^6 cells were washed in cold PBS, resuspended in 200 µl of the same buffer and vortexed. The suspended cells were slowly added to a plastic test tube containing 4 ml of ice-cold 70% EtOH with vortexing. Then, the preparation was incubated overnight at 4 °C. Cells were precipitated by centrifugation at 500x g for 10 min, after that the supernatant was carefully aspirated and the pellet was re-suspended in PI master mix to a final cell density of 0.5 x 10^6 cells/ml.

The suspension was then transferred into a Falcon tube and incubated at 37 °C for 30 min. Measurement of DNA content was done by flow cytometry (FACS-Calibur, Becton Dickinson Imunocytometry Systems) and the cell cycle distribution was calculated with the software Cell Quest Pro (BD Bioscience, Franklin Lake, Holland).

2.10.4.6. Acridine orange staining of apoptotic cells

Acridine orange (AO) is a metachromatic dye that differentially stains the double-stranded (ds) and single-stranded (ss) nucleic acids. When AO intercalates with dsDNA it emits green fluorescence upon excitation at 480-490 nm. On the contrary, it emits red fluorescence when interacts with ssDNA or RNA. The condensed chromatin in apoptotic cells is more sensitive to DNA denaturation than normal chromatin (Holz *et al.,* 1992). Therefore, apoptotic cells display an intense red fluorescence and a reduced green emission when compared to non-apoptotic interphase cells.

AO staining was done according to Gorman *et al.* (1994). Briefly, HL-60 cells were cultivated overnight in a 24-well microtiter plate containing the compounds to be tested (see 2.10.4.1). 1 x 10^6 cells (counting were done with haemocytometer) were washed with cold PBS and centrifuged at 4 °C and 500x g for 10 min (Eppendorf centrifuge 5417R, Eppendorf, Hamburg). The pellet was resuspended in 1 ml of cold PBS, to which 20 µl of RNase A were added and incubated for 30 min at 37 °C. Prior to the addition of 200 µl AO solution, the cell suspension was treated for 30-45 sec with 0.1 N HCl at room temperature. The cells were observed under fluorescent microscope (Zeiss, Jena) with a green filter.

2.10.5. Oxygen uptake in *Bacillus subtilis*, *Nematospora coryli* and human cell lines

The effect of substances on the oxygen uptake (respiration) by microorganisms and human cell lines was observed with *B. subtilis* cultivated in M_{13} at 37 °C, *N. coryli* cultivated in M_{24} at 27 °C and human cell lines in M_{28} (without fetal calf serum) at 37 °C. The cultivation of microorganisms took place overnight (16 hours) in water bath-shaker (Gyrotory Model G76, New Brunswick, USA). After calibrating the instrument, 2-3 ml of the culture (10^7 cancer cells or 10^9 cells of microorganisms/ml) were placed in a gas-sealed chamber with a magnetic stirrer. Oxygen uptake was measured polarographically with an oxygen electrode

(pO_2-analysator R55, Bachofer, Reutlingen). The culture was saturated with oxygen and after a short respiratory course (nearly till 50% oxygen uptake), different concentrations of the substance to be tested in a maximum volume of 10 µl of MeOH were added immediately and the inhibition in oxygen uptake was recorded. As a control, a run was done with 10 µl MeOH. During the test, the growth of microorganisms was monitored microscopically and by measuring the OD at 600 nm. Antimycin A and strobilurin A were used as positive controls in case of eukaryotic cells.

2.10.6. Synthesis of macromolecules *in vivo* in microorganims

2.10.6.1. *Nematospora coryli*

The effect of the isolated compounds on incorporation of 0.1 µCi from each of (8-^{14}C)-adenine, (2-^{14}C)-uridine, (1-^{14}C)-leucine, and N-acetyl-D-(1-^{14}C)-glucosamine radioactive precursors in macromolecules biosynthesis *in vivo* was examined.

The cell suspension was cultivated in 250 ml of M_{27} overnight (16 hours) at 27 °C in a water bath-shaker (Gyrotory Model G76, New Brunswick, USA). The culture was diluted 1:10 with the same medium and incubated again under the same conditions. The growth was monitored at 600 nm (UV/VIS-spectrometer, Lambda 16, Perkin-Elmer, Langen) till the OD reached 0.3-0.4. 25 ml Erlenmeyer flasks, containing different concentrations of the tested compounds, were filled with 5 ml of the culture. As a control, 10 ml of the same culture were placed in a 25 ml Erlenmeyer flask without the tested compound. After 15 min incubation in water bath-shaker (Gyrotory Model G76, New Brunswick, USA) at 27 °C, 1 ml from each flask was taken out and added to a plastic test tube containing one of the four radioactive precursors and incubated for further 90 min. The reaction was stopped by adding 1 ml of ice-cold 15% TCA. In case of adenine incorporation and before stopping the reaction, 100 µl 1 M NaOH were added and incubation for extra 30 min took place. Addition of NaOH aimed to hydrolyse RNA. The precipitate, resulted from stopping the reaction, was filtered through cellulose nitrate-filter (0.45 µm pore size, Schleicher & Schuell, Dassel) and washed two times with 5% TCA. The filters were then placed under red light until dryness.

The dry filters were placed in a plastic counting vials. Then, 5 ml of scintillation liquid (Quickszint 501, Zinsser Analytic, Frankfurt a.M) were added. Radioactivity was measured with a liquid scintillation counter (Betaszint BF5001, Wallac 1410, Wallac, Freiburg). A reaction of the radioactive precursors with the untreated cells represented a positive control.

For negative control, the reaction of the radioactive precursors with the untreated cells was stopped immediately by the addition of 15% TCA. Daunomycin, actinomycin D and cycloheximide were used as standards.

2.10.6.2. *Bacillus subtilis*

The effect of the isolated compounds on incorporation of 0.1 µCi from each of (2-^{14}C)-thymidine, (2-^{14}C)-uridine, (1-^{14}C)-leucine, and N-acetyl-D-(1-^{14}C)-glucosamine radioactive precursors in macromolecules biosynthesis *in vivo* was examined.

The spore suspension was cultivated in 250 ml of M_{16} overnight (16 hours) at 37 °C in a water bath-shaker (Gyrotory Model G76, New Brunswick, USA). The culture was diluted till OD_{600nm} of 0.15-0.2 with the same medium and incubated again at the same condition. The growth was monitored at 600 nm (UV/VIS-spectrometer, Lambda 16, Perkin-Elmer, Langen) till the OD reached 0.25-0.3. 25 ml Erlenmeyer flasks, containing different concentrations of the tested compounds, were filled with 5 ml of the culture. The steps after this cultivation were done as described in 2.10.6.1 excluding the hydrolysis step of RNA. A reaction of the radioactive precursors with the untreated cells represented a positive control. For negative control, the reaction of the radioactive precursors with the untreated cells was stopped immediately by the addition of 15% TCA. Daunomycin, actinomycin D and cycloheximide were used as standards.

2.10.7. Synthesis of macromolecules *in vivo* in human cell lines

1-2 x 10^5 cells/ml in M_{28} were cultivated in 24-well microtiter-plates (Cellstar®, Greiner, Frickenhausen). 0.1 µCi from each of the radioactive precursors (2-^{14}C)-thymidine, (2-^{14}C)-uridine, and (1-^{14}C)-leucine was added to the culture. In the positive control no substances were added. The culture was incubated overnight (16 hours). The reaction stopped by addition of 1 ml of 15% TCA. In the negative control the reaction was stopped immediately after the addition of each of the radioactive precursors. The precipitate was filtered through cellulose nitrate-filter (0.45 µm pore size, Schleicher & Schuell, Dassel) and washed two times with 5% TCA. The filters were then placed under a red light lamp until dried. Measurement of the radioactivity was done as described in 2.10.6.1.

2.10.8. Seeds germination and growth

The effect of the isolated compounds on seeds germination (shoots as well as root elongation) was investigated using seeds of monocotyledonous and dicotyledonous plants (see 2.6.2.5). Different concentrations (10 µg, 25 µg and 50 µg/disc) were applied onto 13 mm filter discs (Schleicher & Schuell, Dasseln) in 14 mm polyethylene tubes and allowed to dry. Seeds from each of the different plants (6 seeds) were placed on the filter disc which was then moistened with 150 µl tap water and incubated for 72 hours at 27 °C in dark followed by 24 hours incubation under artificial light (40 W). A test without the substances was done as a control.

2.10.9. Determination of the nematicidal activity

The nematicidal activities of the isolated compounds were evaluated as described by Stadler *et al.* (1993) and Anke *et al.* (1995). The saprophytic nematode *Caenorhabditis elegans* and the plant parasite *Meloidogyne incognita* were used as test organisms.

The compound had a nematicidal effect when nematodes lost their motility and upon transferring to new media their motility did not recovered.

2.10.10. Reaction with L- cysteine

To examine the inactivation of the bioactive metabolites by SH-group containing compounds, a test was done as described by Kupchan (1970).

2.11. Molecular biology part

2.11.1. Partial 16S rDNA sequence

2.11.1.1. Preparation of genomic DNA

Genomic DNA was prepared following the combined methods of Marmur (1961) and Süßmuth *et al.* (1999) with some modifications. Unless otherwise indicated, all steps were done in Eppendorf tubes and at 4 °C. Briefly, cells of an exponentially growing culture in M_1 (2 ml) were harvested by centrifugation at 11500 rpm (14000x g, Eppendorf centrifuge

5417R, Eppendorf, Hamburg) for 15 min. The pellet was suspended in 1.5 ml of TES-sucrose buffer with repeated pipetting and then centrifuged for 10 min. After removal of the supernatant, the pellet was completely resuspended in 200 µl of lysozyme mixture and incubated at 37 °C for 20-30 min. 10 µl of 0.5 M EDTA (pH 8) and 50 µl of 20% SDS were added and the preparation was mixed carefully. The preparation was completed to 0.5 ml with TE-buffer and extracted three times with phenol : chloroform : isoamyl alcohol (25 : 24 : 1, Carl Roth, Stuttgart). After each time, the preparation was centrifuged for 15 min and the supernatant was placed in a new Eppendorf tube.

25 µl of 5 M NaCl and 1 ml of 95% ice-cold ethanol were added to the preparation and left overnight at – 20 °C. After centrifugation for 15 min, the pellet was suspended in 100 µl of TE-buffer containing 50 µg RNase A. The reaction was incubated at 37 °C for 1 hour. The DNA was precipitated with 40 µl of 3 M ammonium acetate (pH 5.2) and 250 ml cold isopropanol. The preparation was incubated for 5-10 min at room temperature and centrifuged with washing two times with cold 70% EtOH. The pellet was left to dry, dissolved in 100-150 µl of TE-buffer and stored at 4 °C. Genomic DNA is stable for several months by storing at 4 °C.

2.11.1.2. Gel-electrophoresis and restriction of DNA fragments

2.11.1.2.1. Agarose-gel electrophoresis of DNA

In order to determine size and purity of the genomic DNA, an agarose-gel electrophoresis was carried out following the general methods of Sambrook *et al.,* (2001). The sample was run on a 1% agarose gel using 1x P_4 as a running buffer. 10 µl of DNA (300–500 ng) were mixed with 2 µl of 6x loading dye (MBI Fermentas, St. Leon, Germany) and placed into the gel-pocket. The size of the DNA fragment was determined by running a 1-kb DNA ladder (MBI Fermentas, St. Leon, Germany) with the samples simultaneously. Gel-electrophoresis was performed with 70-90 volt and the visualization was achieved by staining with ethidium bromide solution (5 µg/ml) for 10-15 min.

2.11.1.2.2. Restriction of DNA

Restriction of the plasmid-DNA was done according to the general methods of Sambrook *et al.* (2001). The restriction reaction was done using specific restriction enzymes and incubation according to the manufacturer's instructions.

2.11.1.3. Polymerase chain reaction (PCR)

16S rDNA genes were amplified from genomic DNA using 16SA (forward) and 16SB (reverse) primers in the PCR reaction. The reaction mixture contained, in a volume of 50 µl:

DNA	100-300 ng
$MgCl_2$ (25 mM)	8 µl
Primer 16SA (10 pmole)	5 µl
Primer 16SB (10 pmole)	5 µl
dNTPs (10 mM)	2 µl
PCR-buffer with $(NH_4)_2SO_4$ (10x)	5 µl
Taq-polymerase (1U/µl)	1 µl
Sterile H_2O dist	added to 50 µl total volume

PCR reaction was done in a "Master-cycler gradient" (Eppendorf, Hamburg) using the following programme:

Initial denaturation	3 min	94 °C
30 cycles :		
Denaturation	30 sec	94 °C
Annealing	30 sec	45-55 °C
Extension	30 sec	72 °C
Terminal extension	10 min	72 °C

For each prepared genomic DNA, conditions of the PCR-reactions were optimised. A band with 1.4-1.5 kb was eluted from the agarose-gel with NucleoSpin® Extract-Kit (Macherey & Nagel, Düren) following the manufacturer's instructions.

2.11.1.4. Cloning of DNA

The isolated PCR-product was cloned in vector pDrive using the "PCR Cloning"-kit (Qiagen, Hilden) according to the manufacturer's instructions.

2.11.1.5. Transformation of the *E. coli*

2.11.1.5.1. Preparation of competent cells

Competent cells were prepared as described by Hanahan (1985). 10 ml M_{17} were inoculated with a single colony from an agar plate with *E. coli*. The culture was incubated overnight at 37 °C and 150 rpm agitation. 4 ml of this culture were used as inoculum for 400 ml of the same medium that was incubated under the same conditions until OD_{600nm} reached 0.3-0.4. Then, the culture was incubated on ice for 5 min followed by centrifugation for 10 min at 4500x g and 4 °C (Eppendorf centrifuge 5417R, Eppendorf, Hamburg). The pellet was suspended in 100 ml of 0.1 M $CaCl_2$ by vortexing followed by incubation on ice for 2 hours. After incubation, the cells were centrifuged for 10 min (4500x g and 4 °C) and the pellet was resuspended in 16 ml ice-cold $CaCl_2$/glycerol solution (0.1 M $CaCl_2$, 30% glycerol). Sterile Eppendorf tubes (1.5 ml) were filled with 100 µl of the preparation and stored at – 80 °C.

2.11.1.5.2. Transformation of competent cells

Competent cells were transformed by addition of 10 µl of plasmid after thawing on ice. The preparation was carefully mixed and left for 30 min on ice. Following a heat shock for 1 min at 42 °C, 1 ml of medium M_{17} was added. The preparation was incubated for 1 hour at 37 °C with 150 rpm agitation and then centrifuged for 5 min (4500x g, room temperature, Eppendorf centrifuge 5417R). 900 µl of the supernatant were removed and the remaining of the preparation was carefully remixed and plated on agar medium M_{17a} supplemented with 0.1 M X-gal and 0.2 M IPTG for white/blue selection of recombinant colonies. The plate was incubated overnight at 37 °C. From these colonies, plasmid mini-preparation was performed using NucleoSpin® plasmid Kit (Macherey & Nagel, Düren) according to the manufacturer's instructions.

2.11.1.6. Determination of concentration of the DNA

Concentration and purity of the isolated genomic DNA and plasmid-DNA were determined with UV/VIS-spectrophotometer (NanoDropTM, NanoDrop, Wilmingon, USA).

2.11.1.7. Sequencing of partial 16S rDNA

30 µl of the vector-DNA (80-100 ng/µl) were sent to GATC Biotech (Konstanz) for sequencing of the 16S rDNA fragment. Nearly 750 bp were sequenced by each of M13 (forward) and M13 (reverse) primers.

2.11.2. DNA fragmentation

Fragmentation of DNA was detected by agarose gel-electrophoresis. Formation of a distinctive ladder pattern indicated an apoptotic event. The analysis was done as described by Nagy *et al*. (1995) with slight modifications. Briefly, HL-60 cells were cultivated in 24-well microtiter-plates (Cellstar®, Greiner, Frickenhausen) in M_{28} with different concentrations of the desired effectors and at a density of 5 x 10^5 cells/ml. After incubation, the cells were collected in 1.5 ml Eppendorf tubes, centrifuged at 4 °C and 250x g for 10 min (Eppendorf centrifuge 5417R). The pellet was washed with 0.5 ml of ice cold PBS, centrifuged, resuspended in Triton X-100 lysis-buffer by vortexing and placed on ice for 20-30 min. After centrifugation for 15 min at 4 °C and 4000x g, the supernatant was placed in a new Eppendorf tube and was extracted three times with phenol : chloroform : isoamyl alcohol (25 : 24 : 1, Carl Roth, Stuttgart). After each time, the preparation was centrifuged for 10 min, the supernatant was removed and placed in a new Eppendorf tube. The DNA was precipitate by addition of 100 µl of ice-cold 5 M NaCl and 700 µl of ice-cold isopropanol. The preparation was vigorously vortexed and left overnight at – 20 °C.

DNA was recovered by centrifugation for 10 min at 20000x g and 4 °C. The supernatant was removed and the pellet was left until dryness. After washing with 1 ml 70% EtOH followed by centrifugation (10 min, 20000x g, 4 °C), the tube was inverted for 30-45 min and the pellet was left to dry completely. DNA was dissolved in 25 µl TE-buffer and incubated at 37 °C for 1 hour with 5 µg RNase. 5 µl of loading dye were added to the reaction and electrophoresis was performed on 1.5-1.8% agarose-gel simultaneously with a 100 bp marker-plus (MBI Fermentas, St. Leon-Rot).

3. Results

During this work, twenty seven marine bacteria were screened for the production of antimicrobial metabolites. Six bacteria originated from different habitats in the North Sea and twenty-one strains from soft corals flourishing in the Gulf of Aqaba-Jordan. Three strains from the North Sea (T268, T396, and T436) and two from the surface of a soft coral (WMBA1-4 and WMB4A1-5) were selected as promising candidates for the isolation of bioactive compounds. Results of screening are shown in Table. 3.1.

Table 3.1. Summary of the results of selected bacteria in screening.

Bacterial strain	Duration (day)	Culturing media	Optimum medium	Temperature (°C)	Activity	
					Anti-bacterial	Anti-fungal
T 268	5-6	M_1-M_3	M_1	20	+	-
T 436	5-7	M_1, M_4-M_{10}, M_{15}, M_{18}	M_{10}	20	++	++
T 396	7-8	M_1, M_{10}	M_{10}	4	+	+
WMBA1-4	4	M_1, M_{10}, M_{11}	M_{11}	27	++	+
WMBA1-5	5	M_1, M_4, M_{11}	M_{11}	23	+	-

Inhibition zones: (-) not active (+) 10-12 mm (++) more than 12 mm

3.1. Secondary metabolites from *Pseudoalteromonas* sp. T268.

3.1.1. *Pseudoalteromonas* sp. T268

Strain T268 was isolated from the intestine of the Antarctic krill *Euphausia superba.* It grows as transparent, light brown colonies with wrinkled surfaces and wavy margins on M_1 agar. The cells are Gram-negative, nonsporogenic, motile, strictly aerobic and rod-shaped. They are 2.5-3.2 (3.8) µm long and 0.9-1.3 µm wide (Fig. 3.1). The strain does not accumulate poly-β-hydroxybutyrate and has no arginine dihydrolase system. It is oxidase and

catalase positive. It is psychrotolerant, i.e. it grows at 4 °C as well as 35 °C but not at 37 °C. Although it grows in M_1 without addition of marine salts, it requires Na^+ for growth and can endure different concentrations of marine salts up to 10% (w/vol). The biochemical characteristics of this strain are shown in Table 7.1 (see appendix).

The amplified 16S rDNA from this strain was compared with sequences in the GenBank nucleotide database using BLAST and in the Ribosomal Database Project-II (RDP). All sequences showing more than 99% similarity belonged to unidentified *Pseudoalteromonas* species in the gamma-proteobacteria-group.

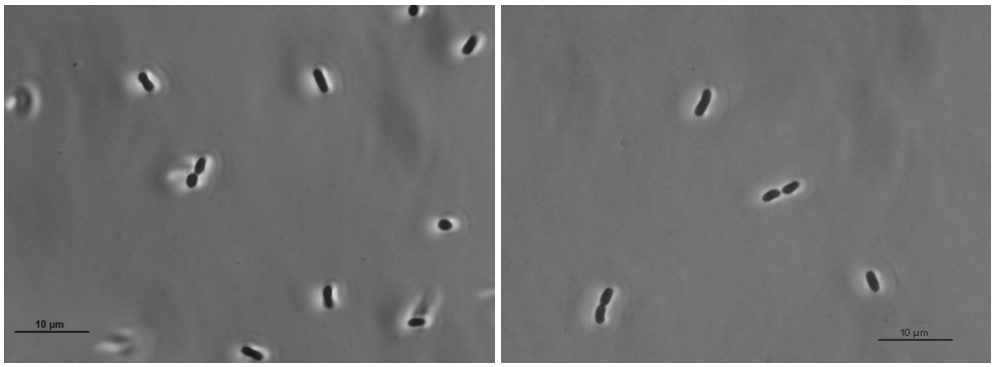


Fig. 3.1. Microphotographs of *Pseudoalteromonas* sp. T268 under the phase contrast microscope. Scale bars represent 10 µm.

3.1.2. Fermentation of *Pseudoalteromonas* sp. T268

Pseudoalteromonas sp. T268 grew equally well on media M_1-M_3, but the yield of the crude extract decreased from 100 mg/l in M_1 to 65 mg/l in M_2 and M_3. Addition of glucose to M_2 and M_3 had no beneficial influence either on the yield of crude extracts or on the antibacterial activities. The strain was fermented in 20 l of M_1-medium at 20 °C with 120 rpm agitation and an aeration of 3-4 l/min (see 2.8.3). Fig. 3.2 shows the typical fermentation scheme in a Biostat U20.

The optical density values were proportional to the numbers of colonies grown on M_1 plates from daily samples, which was at the maximum after 67 hours and remained in a plateau phase till the end of fermentation. The maximum activity was achieved after 67 hours of fermentation.

Five 20 l scale fermentors were carried out in order to isolate adequate amounts of the bioactive compounds.

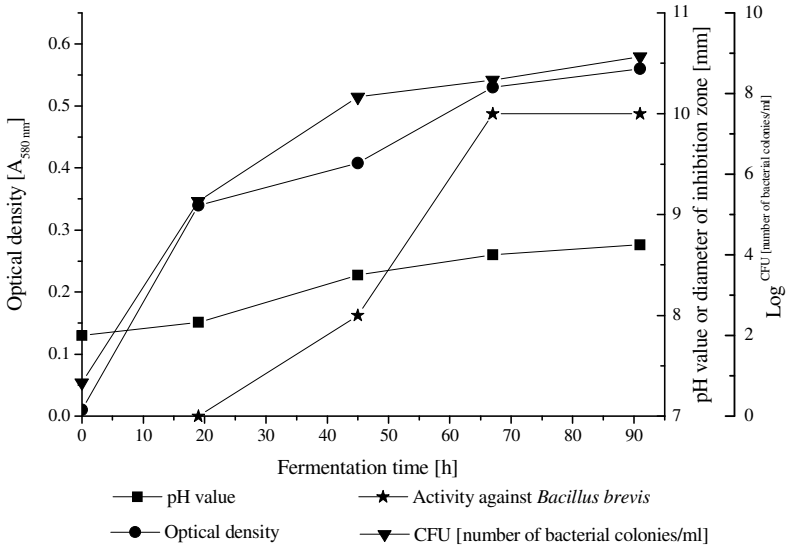


Fig. 3.2. Fermentation of *Pseudoalteromonas* sp. T268 in 20 l M_1-medium.

3.1.3. Purification of secondary metabolites from *Pseudoalteromonas* sp. T268

A total of 87 litres of culture fluid were obtained from five successive fermentations. The filtrate was adjusted to pH 4 and extracted with an equal volume of ethyl acetate. The crude extracts from all fermentors were combined to give 18 g of oily paste. This extract was handled as shown in Figs. 3.3 and Fig 3.4. Purification of secondary metabolites was done by bioactivity directed fractionation. The group of Prof. Dr Laatsch (University of Göttingen) elucidated the structures of the purified compounds.

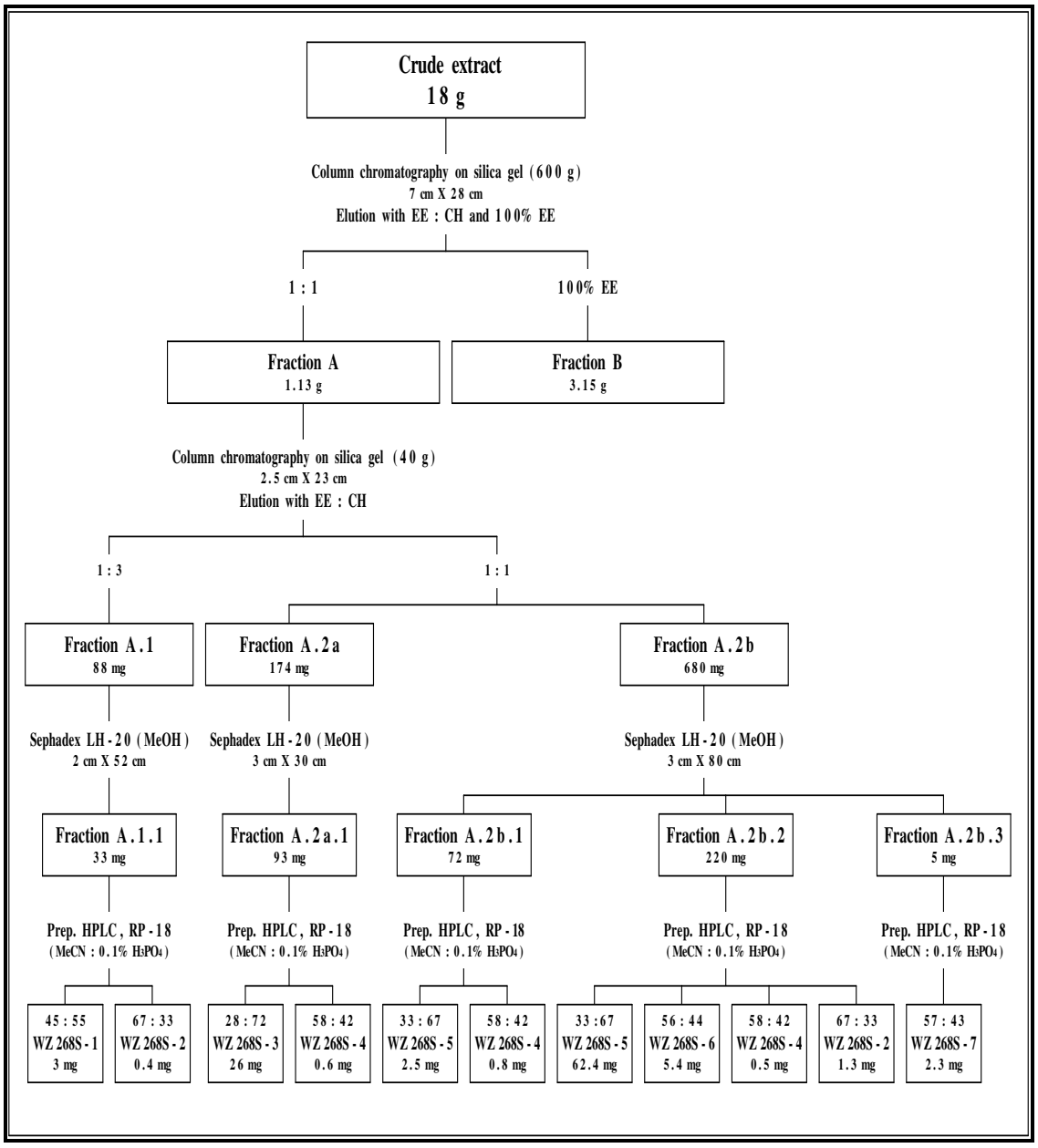


Fig. 3.3. Purification scheme of fraction A from the crude extract of *Pseudoalteromonas* sp. T268 fermented in M_1.

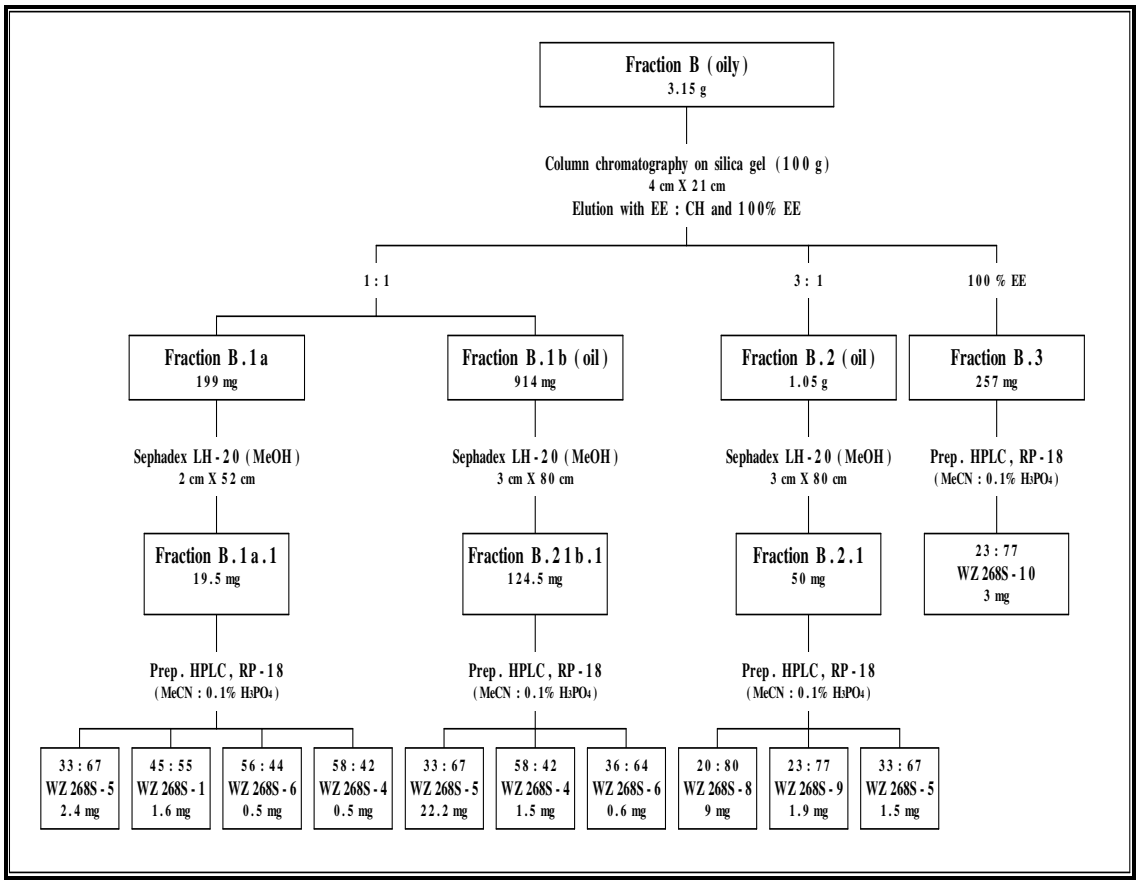
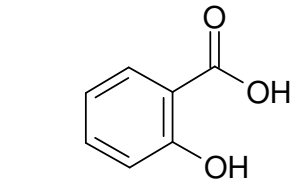


Fig. 3.4. Purification scheme of fraction B (see p. 67) from the crude extract of *Pseudoalteromonas* sp. T268.

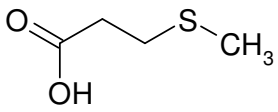
3.1.4. Physico-chemical characteristics

The isolated metabolites from *Pseudoalteromonas* sp. T268 are shown in Fig. 3.5. They are soluble in methanol, ethyl acetate, isopropanol and cyclohexane. Their physico-chemical characterizations were done as described in 2.9. Retention times of the isolated compounds in analytical HPLC and the R_f-values in various mobile phases are shown in Table 7.2 (see appendix). Their reactions with various chromogenic reagents are shown in Table 3.2. Their UV spectra, IR spectra and LC-MS mass spectra (APCI negative ionisation) are shown in 7.1. (see appendix).



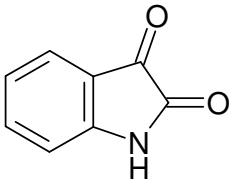
WZ 268S-1

(Salicylic acid)



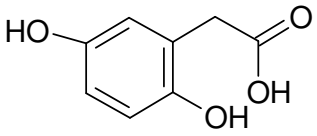
WZ 268S-3

(3-Methylthiopropionic acid)



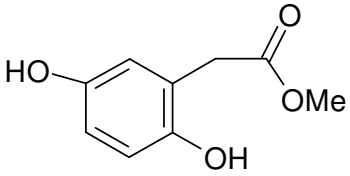
WZ 268S-5

(Isatin)



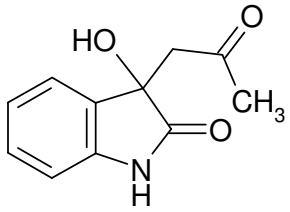
WZ 268S-8

(Homogentisic acid)



WZ 268S-9

(Homogentisic acid methylester)



WZ 268S-10

(3-Acetonyl-3-hydroxy-oxindole)

Fig. 3.5. Structures of the substances from *Pseudoalteromonas* sp. T268.

Salicylic acid was isolated as a white substance that is soluble in most organic solvents. It is known for a long time as one of plants phenolic derivatives (Loffredo *et al.,* 2005) as well as from bacteria (Gaille *et al.,* 2002). It was chemically characterized and synthesized since 1874 (RÖMPP chemical lexicon, 2006)

WZ 268S-2 and **WZ 268S-6** are light brown substances that are soluble in methanol, ethyl acetate, acetone, isopropanol and cyclohexane. Their molecular formulas are $C_{12}H_{16}O_4S_3$ and $C_{11}H_{14}O_4S_3$ with molecular weights of 320 Da and 306 Da respectively. Both have UV_{max} at 235 nm (log ε = 3.83), 280 nm (log ε = 3.3), and 336 nm (log ε = 3.63). They show fluorescent extinction at 254 nm. Due to low amounts of **WZ 268S-2**, it was not possible to perform additional physico-chemical characterizations. **WZ 268S-6** is still under elucidation at the time of writing this thesis.

3-Methylthiopropionic acid is a colourless substance that is soluble in most organic solutions, but fairly soluble in cyclohexane. It has the molecular formula $C_4H_8O_2S$ (M. wt. 120 Da). It is known as an intermediate substance from microbial metabolism of L-methionine (Surette & Vining, 1976). Our colleague in the group of Prof. Laatsch, University of Göttingen, isolated and chemically characterized this compound from the unidentified bacterium T846, which was fermented in IBWF (Schuhmann, 2005).

WZ 268S-4 is a pale yellow substance with a molecular weight 255 Da. It has UV_{max} at 221 nm (log ε = 3.87) and 290 nm (log ε = 3.4). It shows fluorescent extinction at 254 nm. The structure of the compound was not elucidated till the time of writing this thesis.

Homogentisic acid and its **methyl ester** derivative are white substances that are soluble in methanol, ethyl acetate, acetone and isopropanol. They are derivatives of the gentisic acid and formed as intermediate compounds in phenylalanine or tyrosine metabolism (Dai *et al.,* 1991; Arias-Baarau *et al.,* 2004). Both have UV_{max} at 228 nm (log ε = 3.22) and 295 nm (log ε = 3.08).

Fenical and his co-workers previously isolated isatin and 3-hydroxy-3-acetonyloxindole (AntiBase 2003). Comparing the spectroscopic data with that of the authentic isatin led to the identification of **WZ 268S-5** (Gil-Turner *et al.,* 1989). **WZ 268S-10** was known for a long time as the synthetic compound 3-hydroxy-3-acetonyloxindole that obtained from condensation of isatin with acetone (Braude & Lindwakk, 1933)

3.1.5. Other isolated compounds

During this work a number of frequently isolated compounds from microorganisms were purified from fraction A of *Pseudoalteromonas* sp. T268, such as phenyl acetic acid (6 mg), vanillic acid (2 mg), and 3-hydroxybenzoic acid (15 mg).

Table 3.2. R_f-value and colour reaction of the isolated metabolites from *Pseudoalteromonas* sp. T268 with different chromogenic reagents on TLC in mobile phase L_1.

Compound	R_f	Colour reaction with					
		R_1	**R_2**	**R_3**	**R_4**	**R_5**	**R_6**
WZ 268S-2	0.75	-	Yellow	Yellow	-	-	Grey
WZ 268S-4	0.64	-	-	-	-	-	-
WZ 268S-6	0.44	-	Yellow	Yellow	-	-	Grey
WZ 268S-8	0.16	Yellow	-	Green	-	Decolourisation	-
WZ 268S-10	0.2	Violet	Violet	Yellow	-	Decolourisation	Orange-brown

- : no change in the original colour

3.1.6. Influence of the salt concentration on the production of WZ 268S-6 and homogentisic acid

Production of **WZ 268S-6** and **homogentisic acid** was influenced by the concentration of the marine salts in medium M_1 (Fig. 3.6). Cultivation of *Pseudoalteromonas* sp. T268 in M_1 without marine salts resulted in the production of 5.9 mg/l of homogentisic acid instead of 0.1 mg/l with undetectable production of **WZ 268S-6**. In the contrary, 0.8 mg/l of **WZ 268S-6** (instead of 0.07 mg/l with undetectable production of homogentisate) was produced when the strain was cultivated in the medium with 10% (w/v) marine salts.

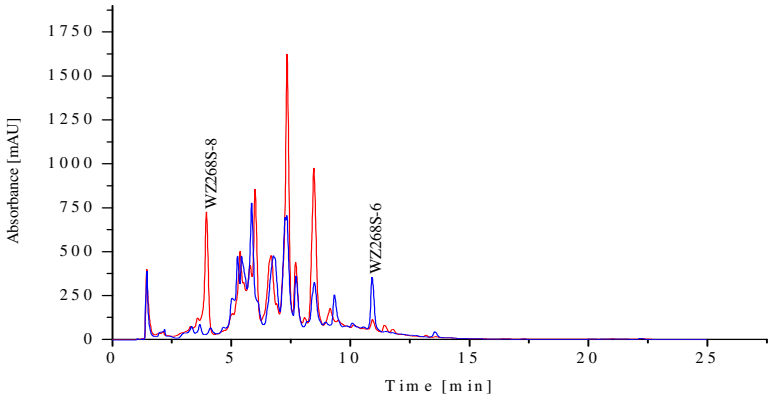


Fig. 3.6. Analytical HPLC chromatogram of the crude extract of *Pseudoalteromonas* sp. T268 in M_1 without marine salts content (red line) or with 10% (w/v) marine salts (blue line).

3.1.7. Biological characterization of the compounds

A number of tests were performed to evaluate the biological activities of the isolated substances from the crude extract of *Pseudoalteromonas* sp. T268. Salicylic acid was known for a long time as an intermediate compound in the metabolism of many plants and microorganisms, it shows antimicrobial activities and plays an important role as a signal molecule in the systemic acquired resistance (SAR) in plants (Spletzer & Enyedi, 1999). Therefore, only its influence on the conidial germination *M. grisea* was evaluated in this study. Since the antifungal and phytotoxic activities of **WZ 268S-3** were previously determined (Kim *et al.,* 2003b), the substance was only included in cytotoxicity and nematicide tests. **WZ 268S-5** (isatin) was described in literature as an antifungal agent (Gil-Turner *et al.,* 1989) and some of its derivatives as antimicrobial agents (Selvam *et al.,* 2004), nevertheless it was not documented if this compound *per se* had antibacterial activity. Thus, it was tested for its antibacterial activity.

3.1.7.1. Antimicrobial activities of the isolated compounds

Table 3.3 and Table 3.4 summarise the antimicrobial activities of the purified secondary metabolites from *Pseudoalteromonas* sp. T268. None of these compounds inhibited the growth of the tested fungi (*N. coryli*, *M. miehei*, and *P. variotii*) at 50 µg/disc.

Table 3.3. Antimicrobial activity of the compounds from *Pseudoalteromonas* sp. T268 in agar diffusion test.

Compound	Inhibition zone (mm) 50 µg/disc	
	Bacteria	
	E. dissolvens	***B. brevis***
WZ 268S-1	-	-
WZ 268S-2	-	18
WZ 268S-3	-	-
WZ 268S-4	-	-
WZ 268S-5	-	-
WZ 268S-6	-	16
WZ 268S-8	-	11i
WZ 268S-9	-	17
WZ 268S-10	-	-

- : no inhibition i : incomplete inhibition (presence of a number of single resistant colonies)

It's apparent that the isolated metabolites were active against Gram-positive bacteria. **WZ 268S-2**, **WZ 268S-6** and **WZ 268S-9** (homogentisate methylester) were the most potent antibacterial agents. **WZ 268S-8** (homogentisic acid) showed weak activity against Gram-positive bacteria in contrast to its related methylester derivative.

The minimal inhibitory concentrations (MIC) were determined for the compounds that showed antimicrobial activities in the agar diffusion test. The results are given in Table 3.4. Although **isatin** did not show antimicrobial activities in the agar diffusion test, it was included in this assay.

Table 3.4. Minimal inhibitory concentration (MIC) of the metabolites from *Pseudoalteromonas* sp. T268 in the serial dilution assay.

Organisms	**MIC [µg/ml]**			
	WZ 268S-2	**-5**	**-6**	**-8**
Fungi:				
Paecilomyces variotii	NT	-	50s	-
Penicillium notatum	NT	-	-	-
Phytophthora infestans	NT	-	100c	-
Mucor miehei	NT	-	-	-
Nematospora coryli	NT	100c	-	-
Saccharomyces cerevisiae FL 200	NT	-	-	-
Ustilago nuda	NT	-	-	-
Bacteria:				
Gram-positive:				
Bacillus brevis	100c	-	50s	-
Bacillus subtilis	100c	100c	100c	-
Micrococcus luteus	NT	100s	50s	-
Gram-negative:				
Escherichia coli K12	NT	100s	50s	-
Enterobacter dissolvens	NT	-	-	-

- : not active up to 100 µg/ml NT : not tested s : bacteriostatic/fungistatic
c : bactericidal/fungicidal

WZ 268S-6 showed moderate activity at 50 µg/ml against bacterial test strains and *Paecilomyces variotii* and weak activity against *Phytophthora infestans*. Although **WZ 268S-5** showed no antimicrobial activities in agar diffusion test, it showed weak activity against most bacterial test strains and *Nematospora coryli. Enterobacter dissolvens* was not sensitive to all tested substances from *Pseudoalteromonas* sp. T268.

As a positive control, chloramphenicol has a MIC value of 1.6 µg/ml against Gram-positive and Gram-negative bacteria. The MIC value of amphotericin B against the fungal strains was less than 0.4 µg/ml.

WZ 268S-6 was the only substance that was tested for its reaction with the SH-group of L-cysteine as described under 2.10.10. The reaction mixture after one hour still retained its activity and TLC separation did not reveal the formation of new spots.

Other antimicrobial tests: isatin showed weak activity against *Staphylococcus aureus* causing an inhibition zone of 12 mm at 100 µg/disc. **Salicylic acid** inhibited the conidial germination of *M. grisea* at 15 µg/ml.

3.1.7.2. Cytotoxic effects of the isolated compounds

Compounds that exhibited cytotoxic activity are listed in Table 3.5. IC-values represent the mean of three independent measurements.

Table 3.5. Cytotoxic activities of the compounds from *Pseudoalteromonas* sp. T268.

Compound	L1210		Jurkat		MDA-MB-321		MCF-7		Colo-320	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
	[µg/ml]									
WZ 268S-2	<20	20	<20	>100	100	>100	100	>100	35	50
WZ 268S–4	30	40	20	>100	>100	>100	>100	>100	>100	>100
WZ 268S–6	10	20	10	50	15	20	30	50	10	50
WZ 268S–8	26	>100	30	>100	55	60	80	>100	35	>100
WZ 268S–9	3	5	6	>100	100	>100	>100	>100	35	50
WZ 268S–10	100	>100	50	>100	>100	>100	>100	>100	>100	>100

IC_{50} : inhibition in proliferation of 50% of cells IC_{90} : inhibition in proliferation of 90% of cells

L1210 and Jurkat were more sensitive to the tested substances than breast cancer and Colo-320 cells. **Homogentisic acid methylester** showed higher cytotoxic effects on L1210 and Jurkat, while **homogentisic acid** was more effective against breast cancer cells (MDA-MB-321 and MCF-7).

As **WZ 268S-6** showed a potent cytotoxic effects against suspension cell lines (IC_{50} = 10-15 µg/ml), it was also tested against HL-60 cells. It showed a cytotoxic effect with IC_{50} = 5 µg/ml.

3.1.7.3. Influence of WZ 268S-6 on the synthesis of macromolecules *in vivo* in L1210 and HL-60 cell lines

The effect of **WZ 268S-6**, the most potent cytotoxic compound, on the incorporation of radioactive precursors into macromolecules (DNA, RNA and protein) was studied as

described in 2.10.7. The substance was tested up to a final concentration of 20 µg/ml. Fig. 3.7 shows the influence of this compound on the synthesis of macromolecules in L1210. IC_{50} ranged between 12 µg/ml and 16 µg/ml. Protein biosynthesis was the most sensitive, 11.6 µg/ml caused 50% inhibition of leucine incorporation. The complete inhibition in the synthesis of macromolecules at 20 µg/ml is in accordance with the IC_{90} value in cytotoxicity test.

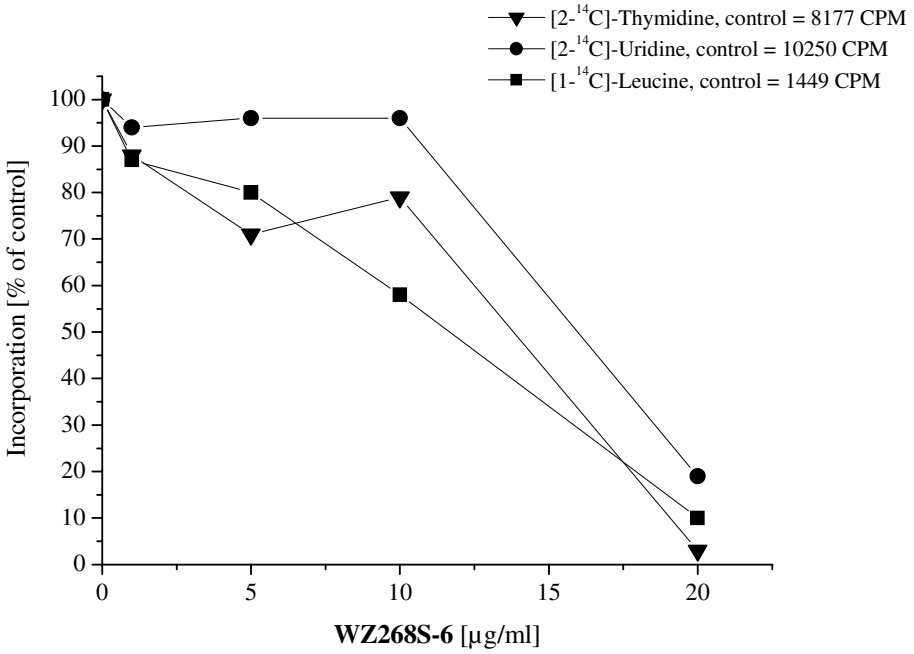


Fig. 3.7. Effect of **WZ 268S-6** on macromolecule biosynthesis in L1210 cell line.

Upon studying the influence of **WZ 268S-6** on the biosynthesis of macromolecules in HL-60 cells, up to 20 µg/ml, only leucine incorporation was weakly influenced at the highest concentration (25 % inhibition).

3.1.7.4. NBT-differentiation test

As **WZ 268S-6** was cytotoxic against HL-60 cells, its ability to induce the morphological differentiation of this cell line was observed for 96 hours as described in 2.10.4.3. It induced differentiation of 30-40% of HL-60 cells at a concentration of 5 µg/ml. 10-13% of HL-60 cells in the control underwent spontaneous differentiation, while 1.3% of DMSO and TPA (1 ng/ml) caused the differentiation of 80-90% and 70% of cells respectively.

3.1.7.5. Nematicide test

WZ 268S-3 (3-methylthiopropionic acid) was the only substance that showed a weak nematicidal activity against *Meloidogyne incognita* with LD_{50} of 50 µg/ml and LD_{90} of 70 µg/ml. *Caenorhabditis elegans* was not affected up to 100 µg/ml.

3.1.7.6. Inhibition of seed germination

Four substances were used to evaluate their effects on the germination of *S. italica* and *L. sativum* seeds. The results are shown in Table 3.6.

Table 3.6. Phytotoxic effects of the metabolites from *Pseudoalteromonas* sp. T268.

Compound	***Setaria italica* [µg/ml]**						***Lepidium sativum* [µg/ml]**					
	67		**167**		**333**		**67**		**167**		**333**	
	Rs	**Ss**	**Rs**	**Ss**	**Rs**	**Ss**	**Rs**	**Ss**	**Rs**	**Ss**	**Rs**	**Ss**
WZ 268S-1	-	-	-	-	(+)	-	(+)	-	+	-	+	(+)
WZ 268S-5	(+)	-	(+)	-	+	-	(+)	-	(+)	-	+	(+)
WZ 268S-6	-	-	-	-	-	-	E+	-	E	-	+	-
WZ 268S-8	-	-	-	-	(+)	-	(+)	-	+	-	+	-

Rs : root system Ss : shoot system E : enhancement: < 50% E+ : enhancement > 50%
- : 0-25% inhibition (+) : 25%-50% inhibition + : 50%-75% inhibition

The compounds showed weak influence on the growth of the root system and shoot system (stem and leaves) of the germinating seeds. The inhibitory effect of these substances was more evident for roots than for shoots. The dicotyledonous roots were more sensitive than that of the monocotyledon. **Salicylic acid** and **homogentisic acid** exhibited phytotoxic activities on the root of the monocotyledonous and dicotyledonous seeds with 333 µg/ml and 67 µg/ml respectively. **WZ 268S-5** (isatin) was phytotoxic for both seed types at a concentration of 67 µg/ml. Although

WZ 268S-6 had no effect on the germination of *S. italica* seeds; it enhanced the growth of the root system in *L. sativum* at concentrations less than 167 µg/ml.

The other isolated substances were not tested due to the low amounts available.

3.2. Secondary metabolites from *Salegentibacter* sp. T436.

3.2.1. *Salegentibacter* sp. T436

Strain T436 was derived from a bottom section of a sea ice floe collected during the cruise ARKXIII/2 from the Eastern Weddell Sea. The strain grew after two days from plating on M_1 agar. During the first 3-5 days it formed circular, beige colonies with shiny smooth surfaces. The colonies became yellow in the well-grown plate. They are convex, 3-5 mm wide with smooth margins. The cells are Gram-negative ovoid rods, non-sporogenic, non-motile and strictly aerobic. The strain does not accumulate poly-β-hydroxybutyrate and has no arginine dihydrolase system. It is oxidase positive, catalase positive and β-galactosidase negative. It can reduce nitrate. The bacterial cells are 0.9-1.1 (1.4) µm long and 0.7-0.85 µm wide (Fig. 3.8). The strain is psychrotolerant. It is able to grow at a temperature range from 4-35 °C but not at 37 °C. Growth at 4 °C was weak as it took 10 days to be obvious and at 35 °C it grew in the form of filaments. The optimum temperature for growth was between 21-27 °C. It requires Na^+ for growth and withstands different concentrations of marine salts up to 10% (w/v). The growth was weak in M_1-medium without marine salts. The biochemical characteristics of this strain are listed in Table 7.1 (see appendix).

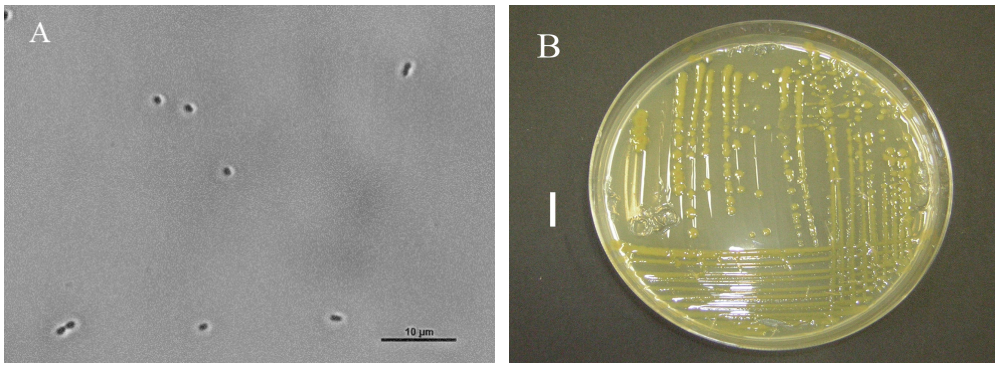


Fig. 3.8. Microphotograph of *Salegentibacter* sp. T436 under the phase contrast microscope (A) and photograph of the strain on M_1 agar plate (B). Scale bars are 10 µm and 1 cm respectively.

Comparing the amplified 16S rDNA from this strain to sequences in the GenBank nucleotide database using BLAST and in the Ribosomal Database Project-II (RDP) showed a 97-99% similarity level with unidentified Arctic sea ice bacterial strains listed in the paper of Brinkmeyer and Helmke (2003) and which were proposed to belong to the genus *Salegentibacter*. The nearest identified phylogenetic relatives were *Salegentibacter holothuriorum* and *S. salegens* (97% similarity level). Therefore, this strain is a species belonging to the genus *Salegentibacter* in the family *Flavobacteriaceae* of the phylum *Cytophaga-Flavobacterium-Bacteroides.*

3.2.2. Small scale fermentation of *Salegentibacter* sp. T436

Different media were utilized to cultivate *Salegentibacter* sp. T436 as shown in Table 3.7. Good growth was obtained in media M_1, M_4, M_{10} and M_{18}. The crude extract produced by strain T436 when cultivated in M_{10} showed the highest antimicrobial activity although the yield was equal to or half of the yield of the crude extracts obtained upon cultivation in the other media. Therefore, M_{10} was used for optimization of growth and crude extract production before it was used for large scale fermentations.

3.2.3. Optimization of growth and secondary metabolites production

Prior to fermentation of this strain in large scale, the optimum conditions that promote the bacterial growth and increase the amount of the produced crude extract were deduced as mentioned in 2.8.2.2. It was found that the optimum temperature range for growth of strain T436 was 21-27 °C and the optimum marine salts concentrations ranging from 33.5–75 g/l in M_{10}. There were no significant differences in the analytical HPLC chromatograms and activities of the crude extracts from the cultures of strain T436 under these conditions. The only difference was the higher amount of the crude extract produced at 21 ^{o}C with 33.5 g/l marine salts. Therefore, these conditions were applied in 20 l fermentations.

Table 3.7. Growth of *Salegentibacter* sp. T436 in different media and bioactivities of the crude extracts.

Medium	Growth	Duration (day)	Inhibition zone (mm) 300 µg/disc				
			B. brevis	***M. luteus***	***N. coryli***	***P. variotii***	***M. miehei***
M_1	++	7	-**	-	8	-	-
M_4	++	6	10	-	-	-	-
M_5	+	7	-	-	7	-	-
M_6	+	7	-	-	-	-	-
M_7	-*	-	-	-	-	-	-
M_8	+	6	10	-	10	-	-
M_9	+	6	9	-	10	-	-
M_{10}	**++**	**7**	**13**	**15**	**16**	**-**	**10d**
M_{15}	+	5	11	-	7	-	-
M_{18}	++	5	-	-	7	-	-

*- : no growth + : moderate growth ++ : good growth
**- : not active

3.2.4. Fermentation of *Salegentibacter* sp. T436 in 20 l fermentors

3.2.4.1. Fermentation in B1-medium (M_{10})

Salegentibacter sp. T436 was fermented in M_{10} at 21 °C with 120 rpm and aeration of 3-4 l/min (2.8.3). Fig. 3.9 shows the typical fermentation scheme in a Biostat U20.

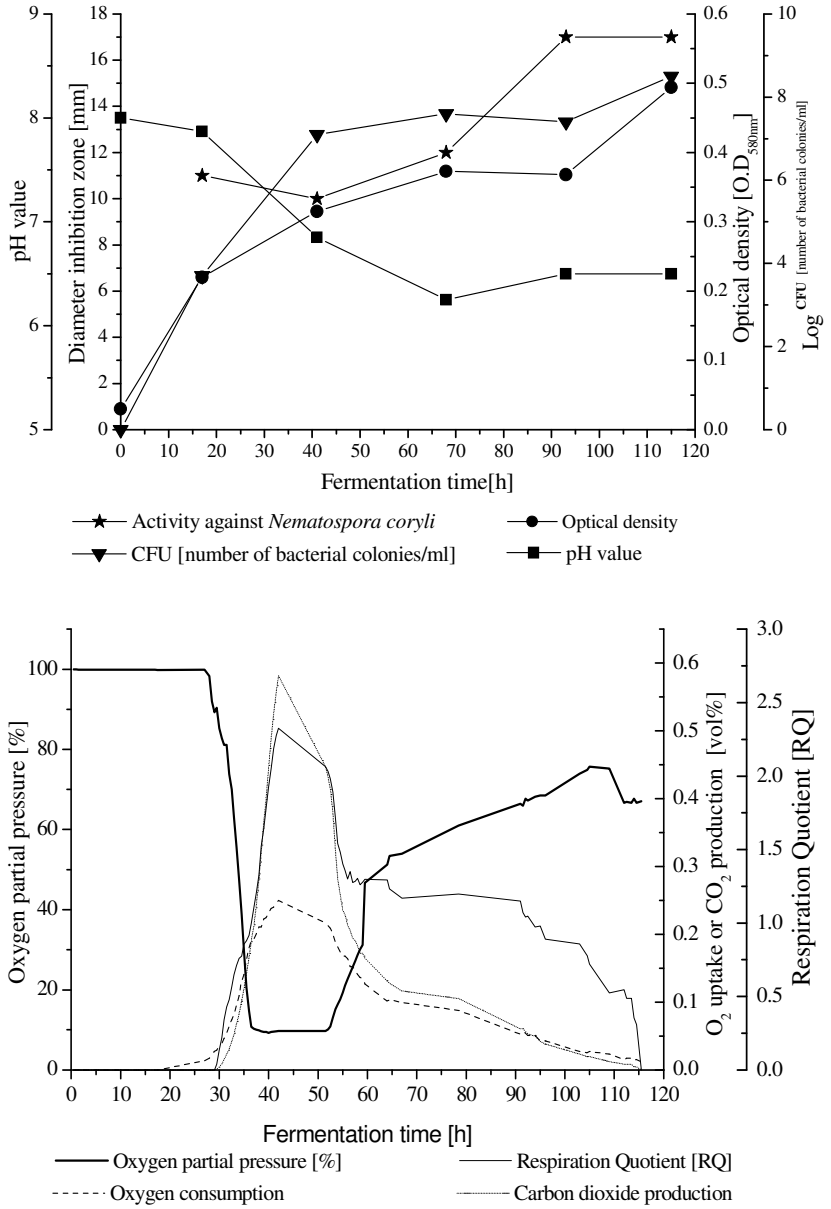


Fig. 3.9. Fermentation of *Salegentibacter* sp. T436 in 20 l M_{10}-medium. Growth, pH, and antifungal activity (upper diagram) and the exhaust gas analysis (lower diagram) during the fermentation process.

The duration of the fermentation process varied between 115 hours to 160 hours. The culture was harvested either when the OD began to decrease (in Biolafitte Type C6) or when the partial oxygen pressure began to increase (in Biostat U20). A characteristic feature in all fermentations was the decrease of pH value to a weakly acidic range (pH 6.5-6.7) and that the maximum antimicrobial activity was approached after 90 hours of cultivation. As shown in the lower diagram of Fig. 3.9, oxygen consumption started after 17 hours of cultivation, while

carbon dioxide production and the decrease in partial oxygen pressure were observed after 30 hours of fermentation. Although all these physical parameters started to decrease after 50-55 hours, the fermentation process was completed to 115 hours due to the increase in the OD and CFU. The culture was harvested as soon as the partial oxygen pressure recovered 80% of its original value. RQ value reached a maximum of 2.3 indicating an anaerobic like respiration and the utilization of organic compounds or NO_3^- as an electron acceptor in oxygen limitation state.

3.2.4.2. Purification of secondary metabolites from *Salegentibacter* sp. T436 fermented in M_{10}

An oily crude extract (8.97 g) was obtained upon extraction of 43 litres of the acidified (pH 4) culture fluid. The crude extract was processed as shown in Figs. 3.10 and 3.11. Purification of secondary metabolites was performed by bioactivity directed fractionation. The group of Prof. Dr Laatsch (University of Göttingen) elucidated the structures of the purified compounds.

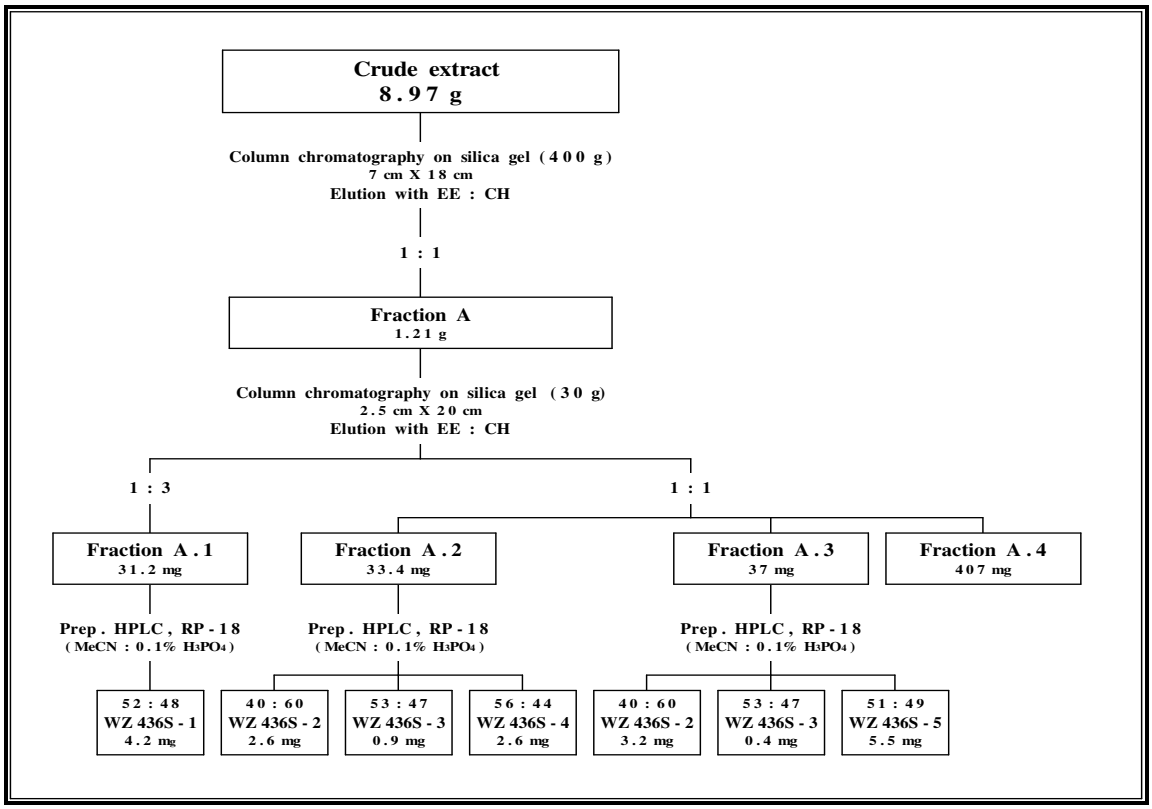


Fig. 3.10. Purification scheme of fraction A from the crude extract of *Salegentibacter* sp. T436 fermented in M_{10}.

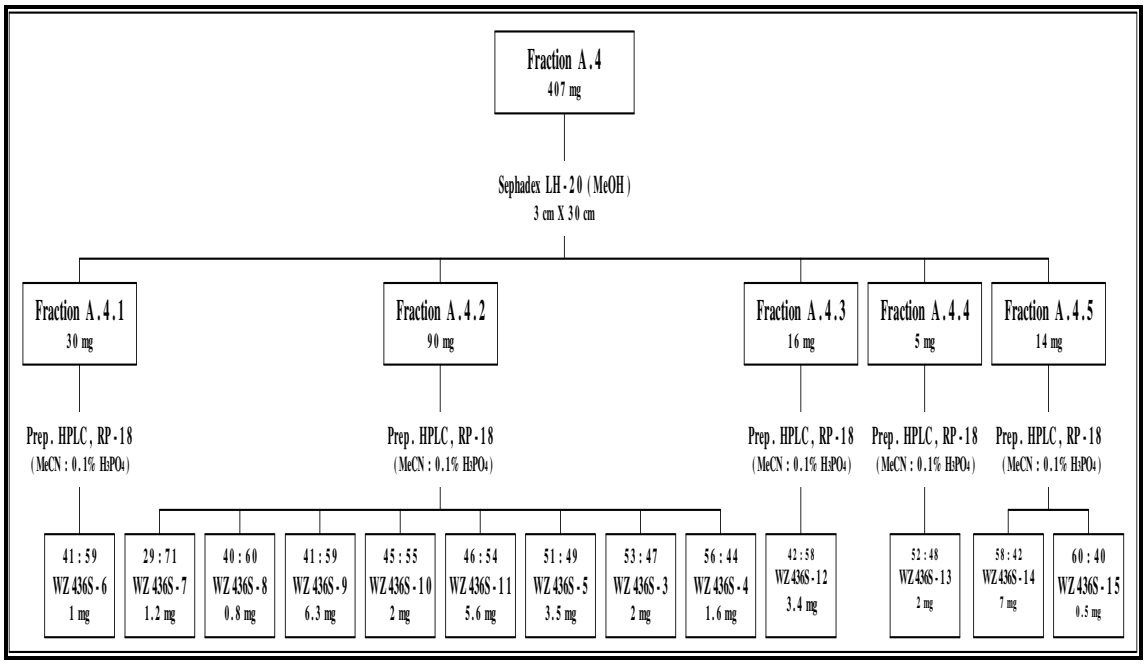


Fig. 3.11. Purification scheme of fraction A.4 (see p. 82) from the crude extract of *Salegentibacter* sp. T436.

3.2.4.3. Fermentation in B2-medium (M_{11})

This strain is unable to utilize carbohydrates as a carbon source (see Table 7.1, appendix). Therefore, we replaced the starch in M_{10} with corn steep solids as carbon and nitrogen source (M_{11}). It was interesting to examine if the spectrum of the produced compounds would be different or if new compounds would be produced. Three fermentations were carried out in M_{11}.

The duration of fermentation varied between 117 hours to 136 hours. During fermentation, the maximum bioactivity coincided with the highest value of the biomass production. The pH, after a slight decrease, increased again to reach 8.3 at the end of fermentation. The crude extracts from these fermentors were less active as compared to fermentation in M_{10}.

3.2.4.4. Purification of secondary metabolites from *Salegentibacter* sp. T436 fermented in M_{11}

A total of 15.4 g of combined crude extract was obtained upon extraction of 50 litres of culture fluid. The crude extract was handled as shown in Figs. 3.12 and 3.13. Purification of secondary metabolites was performed by bioactivity directed fractionation. The group of Prof. Dr. Laatsch, University of Göttingen, elucidated structures of the purified compounds.

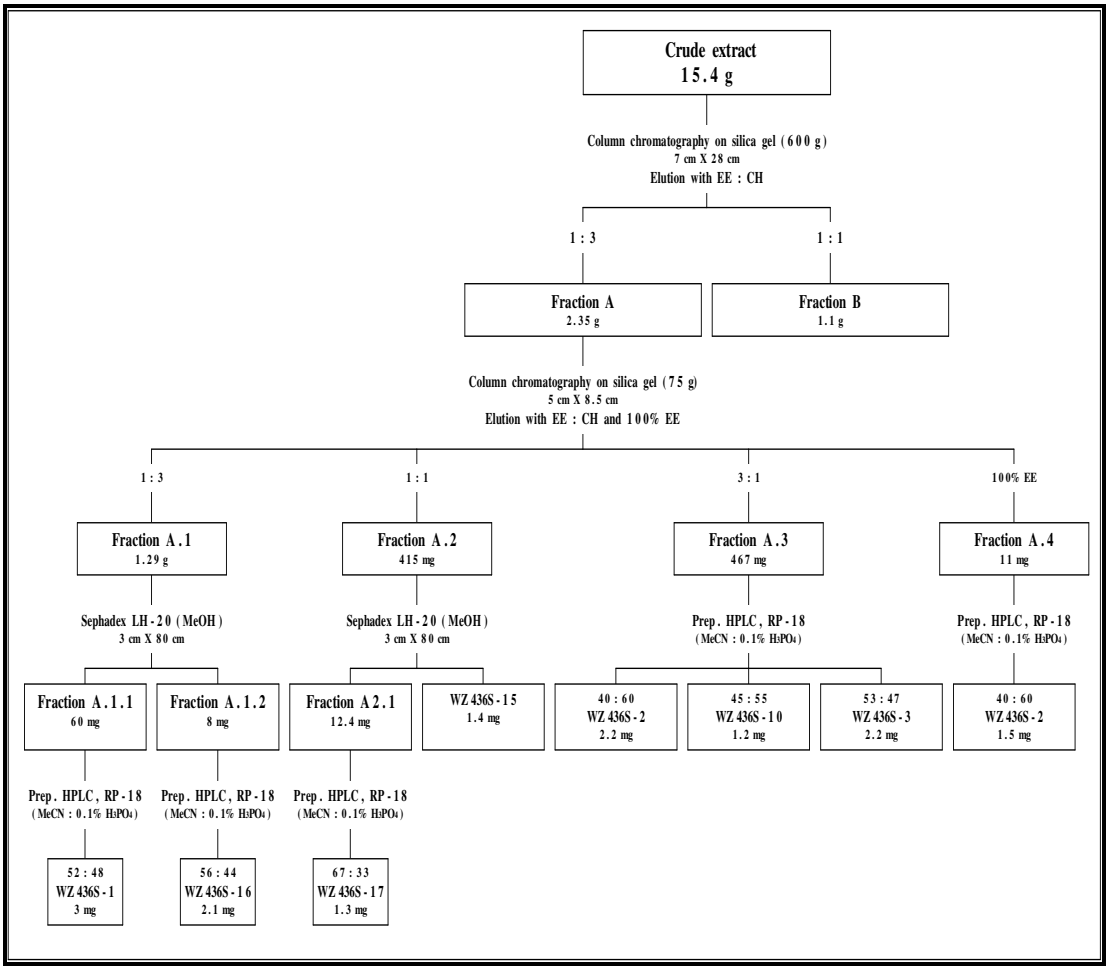


Fig. 3.12. Purification scheme of fraction A from the crude extract of *Salegentibacter* sp. T436 fermented in M_{11}.

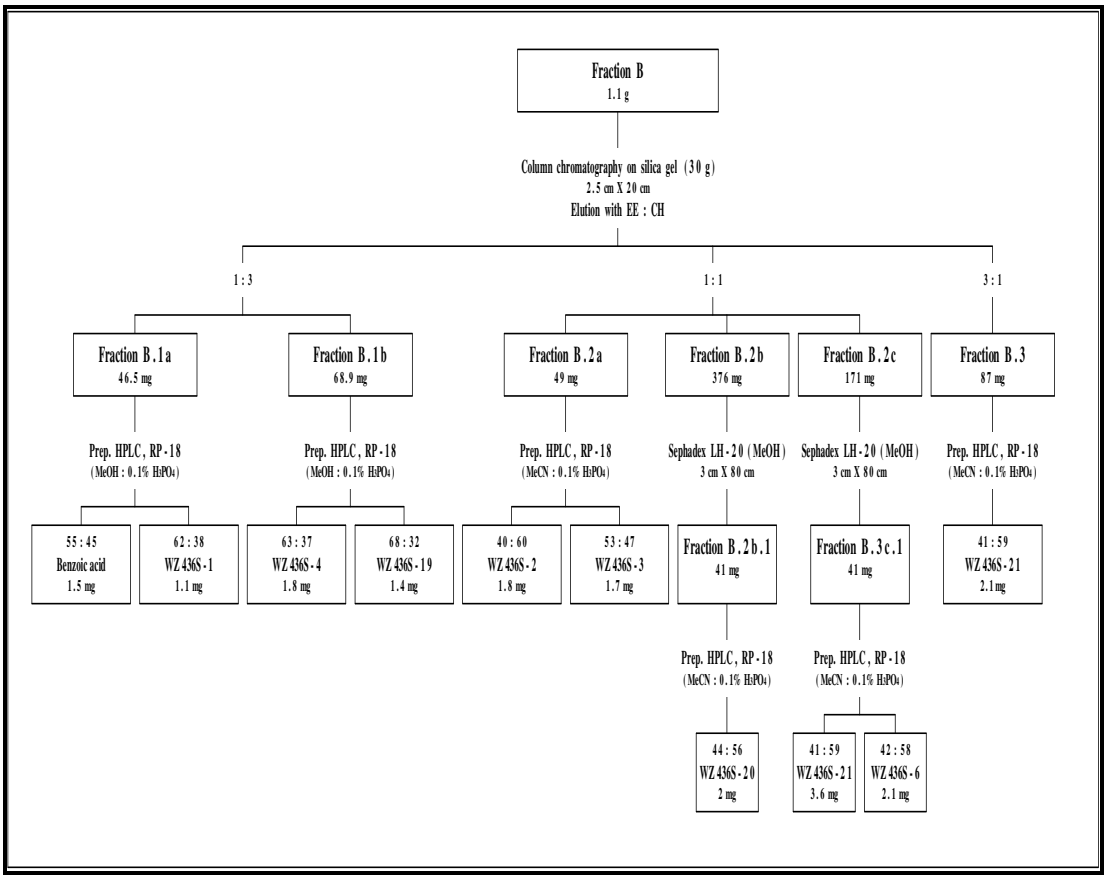


Fig. 3.13. Purification scheme of fraction B (see p. 84) from the crude extract of *Salegentibacter* sp. T436.

As shown in the purification scheme, cultivation of *Salegentibacter* sp. T436 in M_{11} gave rise to production of six additional substances, **WZ 436S-16** till **WZ 436S-21**. In this fermentation, **WZ 436S-5**, **WZ 436S-9** and **WZ 436S-11** till **WZ 436S-14** were not detected.

3.2.5. Physico-chemical characteristics

A total of 20 compounds were isolated from *Salegentibacter* sp. T436. They were mononitro- and dinitro-4-hydroxy-phenyl derivatives and mononitro- and dinitrogenistein (Fig. 3.14). Their chemical names are listed in Table 7.3 (see appendix). IR spectra shows bands at wave numbers (ν_{max}) 1626 cm^{-1}, 1574 cm^{-1} indicating an aromatic basic structure and the typical absorption bands of the nitro group, stretching bands between the ν_{max} 1570-1475 cm^{-1} (strong band) and 1360-1290 cm^{-1}. Physico-chemical characterizations were done as described in 2.9.

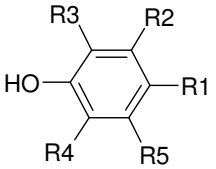
3.2.5.1. Nitrated 4-hydroxy-phenyl derivatives

All mononitro- and dinitrated derivatives have either yellowish or orange colour. Unless otherwise mentioned, they are soluble in methanol, ethyl acetate, isopropanol and acetone. Their colours after drying on TLC plate did not change on reacting with the chromogenic reagents. Their retention times in analytical HPLC and the R_f-values in various mobile phases are shown in Table 7.2 (see appendix). They show fluorescent extinction at 254 nm and no fluorescence at 360 nm on TLC plate. UV spectra, IR spectra, LC-MS mass spectra (APCI negative ionisation) are shown in 7.2.1 (see appendix). Mononitro-derivatives have UV_{max} at ~275 nm and ~350 nm in methanol, while the dinitro-derivatives have maxima at ~230, ~350 nm, and ~430 nm in methanol. In acidified methanol (0.1 µM HCl in methanol) the UV_{max} of dinitrated derivatives shifted to ~250 and ~350 (see Fig. 7.11, appendix). Molecular formulas, molecular weights, UV_{max} (log ε) and characteristic bands, other than nitro bands, in the spectroscopic measurements of the new isolated substances are listed in Table 3.8.

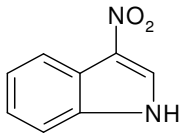
During purification, 4-nitrophenol was isolated as a pale yellow substance. It is a known intermediate substance and environmental contaminant in the degradation of many nitro-aromatic compounds and pesticides. It was used in a large scale in the synthesis of aspirin substituted acetaminophen and in the manufacture of pesticides such as parathion (Shinozaki *et al.,* 2002, Kitagawa *et al.,* 2004). Thus, no additional physico-chemical characterizations were performed on this substance.

3.2.5.2. Dinitro-methoxy-phenol derivatives

WZ 436S-5 was isolated as a yellow mixture of two isomers (**WZ 436S-5a** and **WZ 436S-5b**). Both have UV_{max} at 266 nm (log ε = 3.92) and 342 nm (log ε = 3.7) in methanol. It shows fluorescent extinction at 254 nm. IR-spectrum shows nearly identical bands for the nitro group and aryl-aromatic structure. **WZ 436S-5a** (4,6-dinitroguaiacol) is a known substance from the red algae *Marginisporum aberrans* together with 3,5-dinitroguaiacol and showed antibacterial activity against *B. subtilis* (Ohta, 1979), while **WZ 436S-5b** was never been reported previously. In this work they will be considered as one substance under the name of **WZ 436S-5**, since it was not possible to separate them.



R1: NO_2, R2= R3= R4= R5= H : **4-nitrophenol**

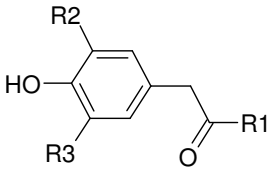


WZ 436S-13

R1: COOH, R2= R3= R5=H, R4= NO_2 : **WZ 436S-2**

R1: NO_2, R2= R5= H, R3= NO_2, R4= OMe : **WZ 436S-5a**

R1= R2: NO_2, R3= R4= H, R5= OMe : **WZ 436S-5b**

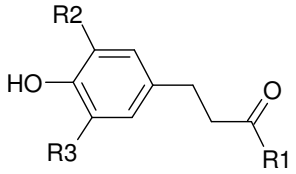


R1: OMe, R2= NO_2, R3= H : **WZ 436S-1**

R1: OMe, R2= R3= NO_2 : **WZ 436S-3**

R1: OH, R2= NO_2, R3= H : **WZ 436S-9**

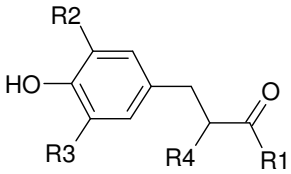
R1: OH, R2= R3= NO_2 : **WZ 436S-12**



R1: OMe, R2= R3= NO_2 : **WZ 436S-4**

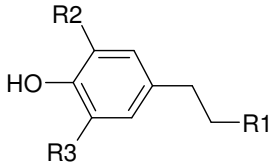
R1: OH, R2= NO_2, R3= H : **WZ 436S-10**

R1: OH, R2= R3= NO_2 : **WZ 436S-11**



R1: OMe, R2= R3= NO_2, R4= Cl : **WZ 436S-19**

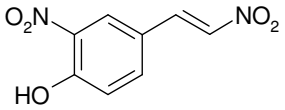
R1: OMe, R2= NO_2, R3= H, R4= OH : **WZ 436S-20**



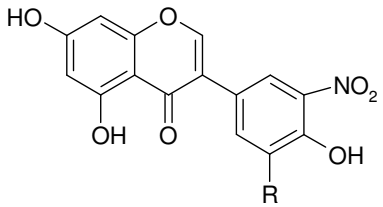
R1= OH, R2= NO_2, R3= H : **WZ 436S-6**

R1= Cl, R2= R3= NO_2 : **WZ 436S-17**

R1= OH, R2= R3= NO_2 : **WZ 436S-21**



WZ 436S-16



R= NO_2 : **WZ 436S-14**

R= H : **WZ 436S-15**

Fig. 3.14. Structures of the twenty substances from *Salegentibacter* sp. T436.

Table 3.8. Colours and spectroscopic data of the new substances from *Salegentibacter* sp. T436.

Compound	Colour	Molecular formula	Molecular weight (Da)	UV_{max} (log ε) in methanol	Characteristic band in IR spectrum (ν_{max})
WZ 436S-2	LY	$C_7H_5NO_5$	183	237 nm (4.38) 340 nm (3.51)	1685 cm^{-1} (C=O, acid)
WZ 436S-3	O	$C_9H_8N_2O_7$	256	348 nm (a, 3.03)	1738 cm^{-1} (C=O, ester)
WZ 436S-4	Y	$C_{10}H_{10}N_2O_7$	270	352 nm (3.41)	1736 cm^{-1} (C=O, ester)
WZ 436S-11	Y	$C_9H_8N_2O_7$	256	356 nm (a, 3.36)	1713 cm^{-1} (C=O, acid)
WZ 436S-12	Y	$C_7H_5N_2O_7$	242	350 nm (a, 3.57)	1695 cm^{-1} (C=O, acid)
WZ 436S-17	Y	$C_8H_7N_2O_5Cl$	246	352 nm (3.62)	-
WZ 436S-19	Y	$C_{10}H_9ClN_2O_7$	304	348 nm (a, 3.1)	700 cm^{-1} (Cl^-)
WZ 436S-20	Y	$C_{10}H_{11}NO_6$	241	274 nm (3.42) 354 nm (3.07)	1741 cm^{-1} (C=O, ester)
WZ 436S-21	Y	$C_8H_8N_2O_6$	228	351 nm (a, 3.17)	-

LY : pale yellow Y: yellow O : orange a : acidified methanol (0.1 µM HCl)

3.2.5.3. WZ 436S-13 (3-nitroindole)

The compound was isolated in the form of a yellow solid that is soluble in polar and non-polar organic solvents. It has the molecular formula $C_8H_6N_2O_2$ with a molecular weight of 162 Da. It has UV_{max} at 246 nm (log ε = 3.54), 266 nm (log ε = 3.42), 272 nm (log ε = 3.38) and 346 nm (log ε = 3.49). A fluorescent extinction at 254 nm on TLC plate was observed. Its retention times in analytical HPLC and the R_f-values in various mobile phases are shown in Table 7.2 (see appendix). It forms a brown spot on reacting with vanilline-sulphuric acid, while the other chromogenic reagents did not lead to change in the yellow colouration of the substance. UV spectrum, IR spectrum and LC-MS mass spectrum (APCI

negative ionisation) are shown in 7.2.1.2 (see appendix). This substance is known for a long time as a synthetic compound (Berti *et al*., 1968) and herein is reported, for the first time, from natural source.

3.2.5.4. Nitrated genistein

WZ 436S-14 (3,5-dinitrogenistein) and **WZ 436S-15** (3-nitrogenistein) are yellow to orange substances with the molecular formulas $C_{15}H_8N_2O_9$ (M. wt. 360 Da) and $C_{15}H_9NO_7$ (M. wt. 315 Da) respectively. They are sparsely soluble in methanol and insoluble in acetone, isopropanol, ethyl acetate or cyclohexane, but soluble in DMSO. They were previously reported (Shangguan *et al.,* 1999).

3.2.6. Other isolated compounds

Additional metabolites were isolated from fermentation of *Salegentibacter* sp. T436 in M_{10}. These compounds were phenyl acetic acid (0.8 mg), β-phenyl lactic acid (3 mg), 3-hydroxybenzoat (1.2 mg), and 3-indolylethyl-methylether (1 mg), a known synthetic compound.

3.2.7. Biological characterization of the compounds

Biological characterization of the isolated compounds was done as described in 2.10. Although most of the mononitro-aromatic compounds isolated in this work from *Salegentibacter* sp. T436 were previously reported either as phytotoxic (**WZ 436S-1** and **WZ 436S-6**) or as antibacterial agents (**WZ 436S-5** and **WZ 436S-10**), they were included in antimicrobial activity assays in order to compare their activities with that of the dinitro-derivatives. Neither of these known compounds was reported as an antifungal nor as a cytotoxic agent.

WZ 436S-14, **WZ 436S-15** and **WZ 436S-16** were reported earlier from natural sources (for literature see Table 7.3 in appendix), but they were not biologically characterized. Therefore, they were examined in this study.

3.2.7.1. Antimicrobial activities of the isolated compounds

Although all isolated compounds from *Salegentibacter* sp. T436 were examined in the antimicrobial assays, only the results of the active compounds are summarized in Table 3.9-Table 3.11.

Table 3.9. Antimicrobial activities of the compounds from *Salegentibacter* sp. T346 in the agar diffusion test.

Compound	Inhibition zone (mm) 50 µg/100 µg per disc				
	Bacteria		Fungi		
	E. dissolvens	*B. brevis*	*N. coryli*	*M. miehei*	*P. variotii*
WZ 436S-1	-	-/13	-	-/11	-
WZ 436S-2	-	-/11	17d/20d	-	-
WZ 436S-3	-	-/11	-	-	-
WZ 436S-4	-	-/20i	-	-	-
WZ 436S-5	-	-	-/10	-	-
WZ 436S-6	-	-/10d	-	-	-
WZ 436S-11	-	-	-/14d	-	-
WZ 436S-13	-	-	-/12	-	-
WZ 436S-15	-	-/10	-	-	-
WZ 436S-16	-	20/25	22/27	17/20	22/25
WZ 436S-17	-	-/20	-/30d	-	-
WZ 436S-19	-	-/17d	-	-	-
WZ 436S-21	-	-/18	-/16	-	-

- : no inhibition d : diffuse zone (very weak growth in the inhibition zone
i : incomplete inhibition (presence of a number of single resistant colonies).

The methyl esters of the nitro-aromatic compounds (**WZ 436S-1**, **-3**, **-4**, and **-19**) showed antibacterial activity, whereas the carboxylic acid derivatives of the nitro-aromatic compounds (**WZ 436S-2** and **-11**) showed antifungal activity, and the substituted ethyl-derivatives of the nitro-aromatic compounds (**WZ 436S-17** and **-21**) exhibited antibacterial as well as antifungal activities. Most of these compounds exhibited their activities at 100 µg/disc. Mononitrated derivatives were less active than dinitrated ones except in case of **WZ 436S-1** and **WZ 436S-3** where the dinitrated derivative was less active. These differences in potency against bacteria or fungi as well as between dinitro- and mononitro-derivatives may be attributed to the polarity and solubility of the tested compounds and their transport into the cells.

Minimal inhibitory concentration was evaluated for the substances that were active in the agar diffusion test and were reported as new compounds from natural source in this study. MIC values of most compounds were in the range of 50-100 µg/ml. The results are shown in Tables 3.10 and 3.11.

Table 3.10. MIC of the metabolites from *Salegentibacter* sp. T436 in the serial dilution assay (**part A**).

Organisms	**MIC [µg/ml]**				
	WZ 436S-1	**-2**	**-3**	**-4**	**-11**
Fungi:					
Paecilomyces variotii	-	-	-	-	-
Penicillium notatum	100s	-	-	-	-
Phytophthora infestans	NT	-	-	100s	-
Mucor miehei	100s	-	-	-	-
Nematospora coryli	-	100s	-	100s	100s
Saccharomyces cerevisiae FL 200	-	-	-	-	-
Ustilago nuda	-	-	-	-	-
Bacteria:					
Gram-positive:					
Bacillus brevis	100s	-	-	100c	-
Bacillus subtilis	100s	-	100s	100c	-
Micrococcus luteus	100s	-	-	100s	100s
Gram-negative:					
Escherichia coli K12	50s	-	-	50s	100c
Enterobacter dissolvens	-	-	-	-	-

- : not active up to 100 µg/ml NT : not tested s : bacteriostatic/fungistatic
c : bactericidal/fungicidal

In contrast to the agar diffusion test, **WZ 436S-13** exhibited weak activities against Gram-positive and Gram-negative bacteria. **WZ 436S-16** was the most potent antimicrobial agent. It was very active against *N. coryli* with a MIC of 0.8 µg/ml (see Table 3.11).

Table 3.11. MIC of the metabolites from *Salegentibacter* sp. T436 in the serial dilution assay (**part B**).

Organisms	**MIC [µg/ml]**			
	WZ 436S-13	**-16**	**-17**	**-21**
Fungi:				
Paecilomyces variotii	-	50s	-	-
Penicillium notatum	-	25s	-	-
Phytophthora infestans	100s	12.5s	-	NT
Mucor miehei	-	12.5s	100s	100s
Nematospora coryli	100s	0.5-1c	50s	-
Saccharomyces cerevisiae FL 200	-	12.5s	-	-
Ustilago nuda	100c	6-7s	-	-
Bacteria:				
Gram-positive:				
Bacillus brevis	100c	12.5s	50s	100s
Bacillus subtilis	100c	12.5s	50s	100s
Micrococcus luteus	100c	12.5s	50s	100s
Gram-negative:				
Escherichia coli K12	100c	6-7s	25s	100c
Enterobacter dissolvens	-	100s	-	-

- : not active up to 100 µg/ml NT : not tested s : bacteriostatic/fungistatic
c : bactericidal/fungicidal

Incubation with L-cysteine for one hour, as describes under 2.10.10, did not influence the bioactivities of these compounds or led to the formation of a new spots on TLC plate.

3.2.7.2. Influence of WZ 436S-16 on the the synthesis of macromolecules *in vivo* in *Nematospora coryli*

The effect of **WZ 436S-16** on the incorporation of radioactive precursors in the macromolecules in *N. coryli* is shown in Fig. 3.15.

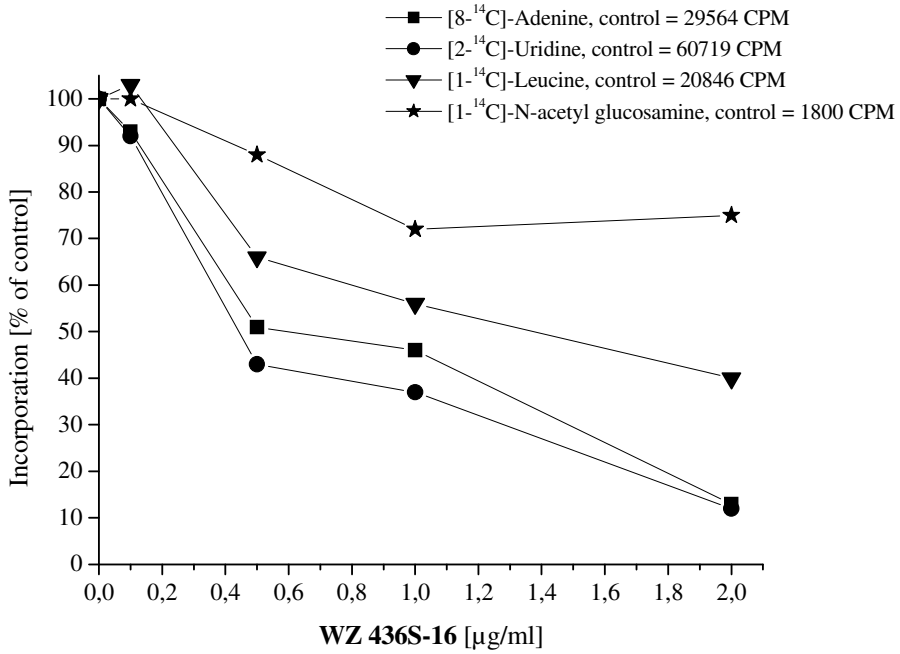


Fig. 3.15. Effect of **WZ 436S-16** on macromolecule biosynthesis in *N. coryli*.

WZ 436S-16 inhibited the biosynthesis of DNA and RNA to 50% at 0.5 µg/ml and to 90% at 2 µg/ml. Protein biosynthesis was inhibited to 55% at the highest applied concentration with no effect on chitin biosynthesis.

Other antimicrobial tests: compounds that exhibited antimicrobial activities at 50 µg/ml were examined for their effects on the growth of pathogenic microorganisms. **WZ 436S-16** was the only compound that at 25 µg/disc caused inhibition zones of 12 mm and 18i mm in *Candida albicans* and *Proteus vulgaris* respectively. Germination of *M. grisea* conidia was inhibited at 50 µg/ml of **WZ 436S-2**; at 25 µg/ml of each of **WZ 436S-3**,**WZ 436S-4** and **WZ 436S-17**; at 10 µg/ml of **WZ 436S-11**; and at 5 µg/ml of each of **WZ 436S-5** and **WZ 436S-16**.

3.2.7.3. Cytotoxic effects of the isolated compounds

All isolated metabolites from *Salegentibacter* sp. T436 were evaluated for their cytotoxic effects against suspension and monolayer cell lines. IC-values, the means from three independent experiments, for the cytotoxic substances are listed in Table 3.12.

Table 3.12. Cytotoxic activities of the isolated compounds from *Salegentibacter* sp. T436.

Compound	L1210		Jurkat		MDA-MB-321		MCF-7		Colo-320	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
	[µg/ml]									
WZ 4368S-4	100	>100	>100	>100	>100	>100	>100	>100	>100	>100
WZ 4368S-9	100	>100	>100	>100	>100	>100	>100	>100	>100	>100
WZ 4368S-11	>100	>100	60	>100	>100	>100	>100	>100	>100	>100
WZ 4368S-13	>100	>100	>100	>100	100	>100	>100	>100	100	>100
WZ 4368S-15	50	>100	60	>100	>100	>100	>100	>100	>100	>100
WZ 4368S-16	20	50	10	30	30	50	>100	>100	17	40
WZ 4368S-17	80	>100	>100	>100	>100	>100	>100	>100	80	>100

IC_{50} : inhibition in proliferation of 50% of cells IC_{90} : inhibition in proliferation of 90% of cells

The cytotoxic effects were pronounced for the dinitro-derivatives except **WZ 436S-9** and **WZ 436S-15** which are mononitro aromatic substances. They triggered inhibition in cell proliferation with IC_{50} of 50-100 µg/ml. Total inhibition (IC_{90}) in proliferation of cancer cell lines was not achieved at the highest applied concentrations.

WZ 436S-16 showed the highest activity towards most cell lines. It had no effect on MCF-7 cell line, though it showed cytotoxic activity against MDA-MB-321. Due to its cytotoxic effect, its influence on the proliferation of HL-60 cells was also evaluated. It was cytotoxic against HL-60 with an IC_{50} value of 5 µg/ml.

3.2.7.4. Influence of WZ 436-16 on the synthesis of macromolecules *in vivo* in Jurkat and HL-60 cell lines

The effect of **WZ 436S-16** on the incorporation of radioactive precursors in the macromolecules in HL-60 cells is shown in Fig. 3.16.

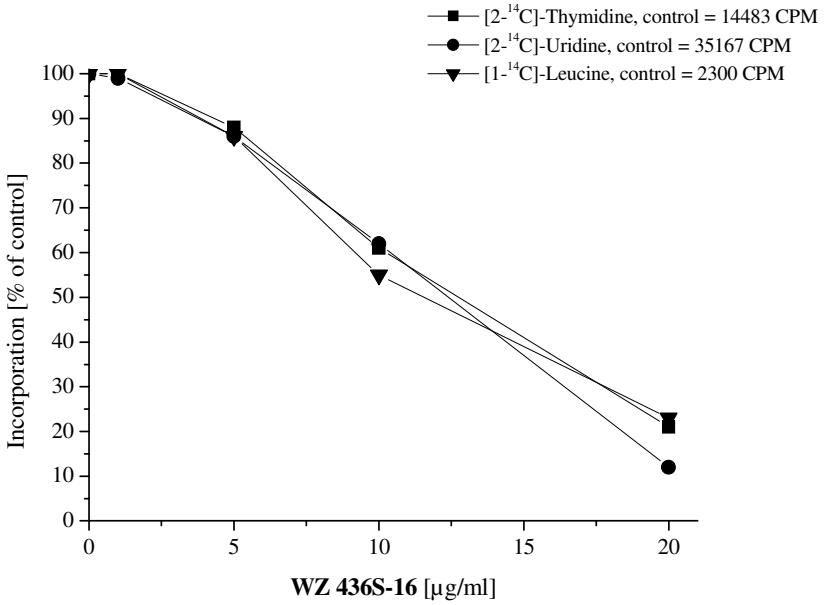


Fig. 3.16. Effect of **WZ 436S-16** on macromolecule biosynthesis in HL-60 cell line.

The biosynthesis of macromolecules in HL-60 cells was inhibited at 11-13 µg/ml to 50%, while in Jurkat cells DNA, RNA, and protein were inhibited to 25-35% at 20 µg/ml

3.2.7.5. Induction of HL-60 differentiation by WZ 436S-16

WZ 436S-16 (5 µg/ml) was able to induce the morphological differentiation of 50-60% of HL-60 cells. 10-13% of the untreated HL-60 cells underwent spontaneous differentiation, while 80-90% of cells were differentiated to monocytes/granulocytes upon treatment with 1.3% of DMSO.

3.2.7.6. Inhibition in oxygen uptake by microorganisms and HL-60 cells by WZ 436S-16

For more thorough investigation on the activity of **WZ 436S-16** against microorganisms and HL-60 cells, especially the inhibition in biosynthesis of DNA, RNA, and protein at the same time, the influence of this compound on oxygen uptake by these organisms was performed as described in 2.10.5.

As shown in Fig. 3.17, **WZ 436S-16** exhibited different effects on the oxygen uptake by the tested organisms. In contrast to microorganisms, it uncoupled the oxidative

phosphorylation from electron transport in HL-60 cells and stimulated the respiration process. It did not exert any effect on the oxygen uptake by *B. subtilis*, while it caused 50% inhibition of the respiration process in *N. coryli* at 3.6 µg/ml. Microscopic examinations and OD measurements (Fig.3.17 B) of the growing yeast cells with different concentrations of the inhibitor revealed that the decrease in oxygen uptake by *N. coryli* was not due to cell death or lysis.

As a positive control, antimycin A caused 86% inhibition in oxygen uptake by *N. coryli* and 75% inhibition in HL-60 cells at 5 µg/ml and 2 µg/ml respectively. It did not exert any effect up to 75 µg/ml on the respiration process in *B. subtilis.*

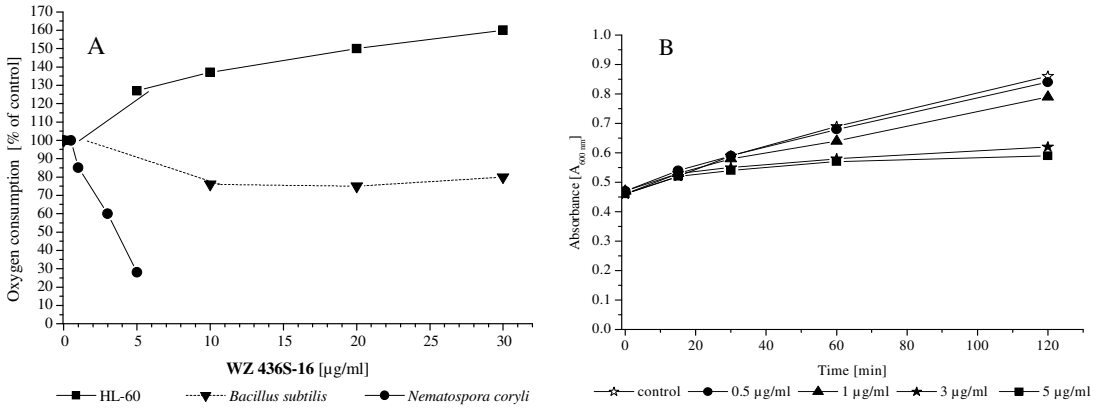


Fig. 3.17. Effect of **WZ 436S-16** on oxygen uptake in microorganisms and HL-60 cell line (A) and growth of *N. coryli* (B).

3.2.7.7. Induction of apoptotic reaction by WZ 436S-16

Due to the effect of **WZ 436S-16** on HL-60 cells, its effect on induction of apoptosis was investigated. A hallmark sign of apoptotic response is the fragmentation of DNA in the affected cells. Fig. 3.18 shows the induction of apoptosis in HL-60 by this compound.

HL-60 cells underwent a pronounced apoptosis (DNA fragmentation) upon treatment with 10 µg/ml of **WZ 436S-16** for 16 hours. Treatment with 20 µg/ml led to formation of a DNA smear that may be due to the early cell death and unspecific oligosome degradation.

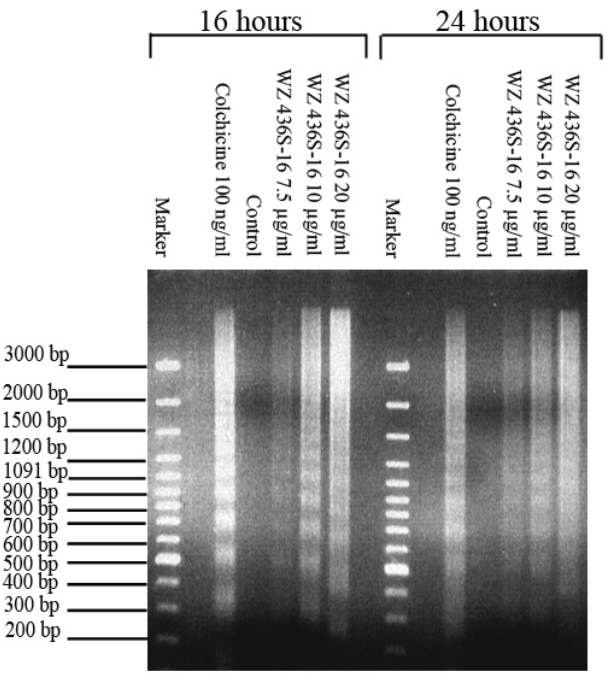


Fig. 3.18. DNA fragmentation by **WZ 436S-16** for 16 and 24 hours. Control: untreated cells and marker: 100 bp plus DNA-ladder.

In order to confirm this apoptotic action, two experiments were performed: cell cycle analysis and staining of apoptotic cells with acridine orange. Cell cycle analysis showed the presence of 9% and 32% of cells in sub-G1 phase upon treatment with 10 µg/ml and 20 µg/ml for 8 hours respectively. Fig. 3.19 shows the treated cells, for 16 hours, after staining with acridine orange.

Incubation of HL-60 cells under the same conditions used in DNA fragmentation test for 16 hours resulted in shrinkage of cells and accumulation of chromatin materials that acquired in some cases an orange-green colour due to accumulation of the lysozymes around the apoptotic bodies (Fig. 3.19 B). Figures 3.19 C and D show the clumping followed by fragmentation of the chromatin materials with shrinkage or deformation of cells due to **WZ 436S-16**.

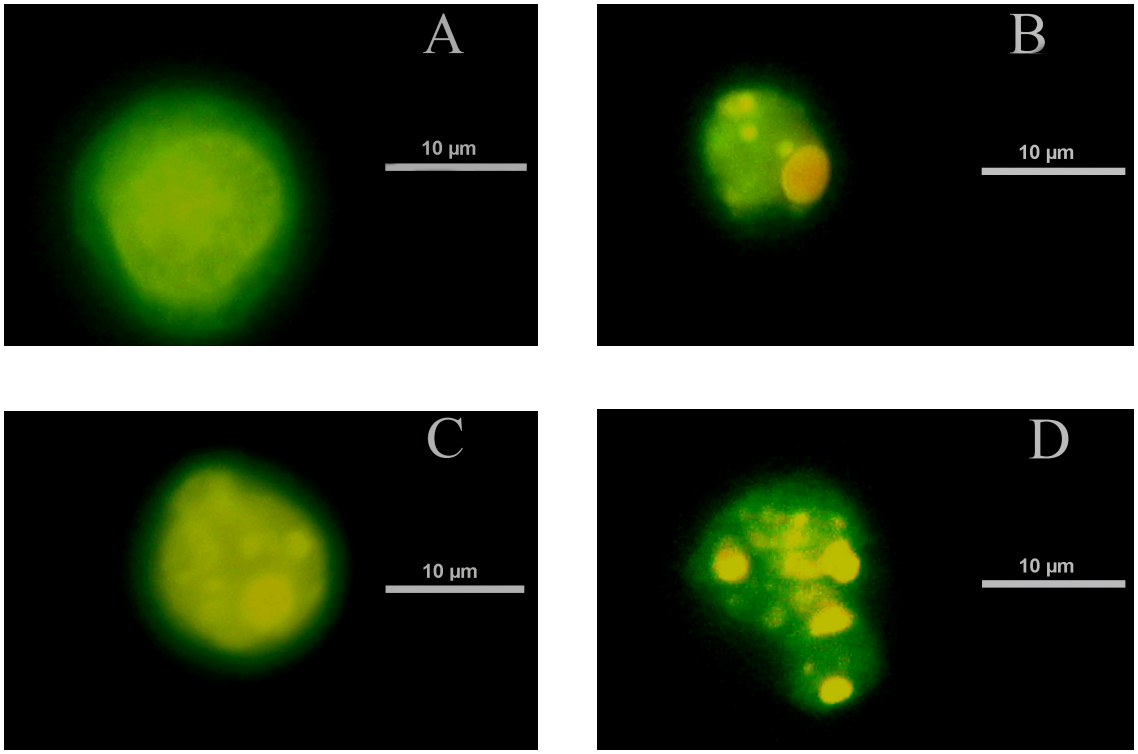


Fig. 3.19. Microphotographs of HL-60 cells under the fluorescent microscope showing the untreated control cell (A) and three forms of apoptotic reactions due to treatment with either colchicine (100 ng/ml, B) or **WZ 436S-16** (10 µg/ml, C and 20 µg/ml, D).

3.2.7.8. Nematicide test

Only three compounds from *Salegentibacter* sp. T436 exhibited nematicidal activities. **WZ 436S-5** showed a nematicidal activity against *Meloidogyne incognita* with LD_{90} of 25 µg/ml. Each of **WZ 436S-13** and **WZ 436S-16** triggered the death of 90% of *Caenorhabditis elegans* at 100 µg/ml.

3.2.7.9. Inhibition of seed germination

Despite the fact that nitrophenols were generally reported as phytotoxic substances, their phytotoxic effects were evaluated in this work in order to elicit the difference between the mononitro- and dinitro-derivatives. Results of this experiment are summarised in Table 3.13.

Table 3.13. Phytotoxic effects of the isolated metabolites from *Salegentibacter* sp. T436.

Compound	*Setaria italica* [µg/ml]						*Lepidium sativum* [µg/ml]					
	67		167		333		67		167		333	
	Rs	Ss	Rs	Ss	Rs	Ss	Rs	Ss	Rs	Ss	Rs	Ss
WZ 436S-1	(+)	-	(+)	-	(+)	(+)	E+	E	(+)	-	+	-
WZ 436S-2	-	(+)	(+)	(+)	(+)	(+)	E	E+	-	E	+	+
WZ 436S-3	-	-	(+)	-	(+)	(+)	-	-	(+)	-	++	++
WZ 436S-5	-	-	-	-	+	(+)	(+)	-	(+)	-	+++	+++
WZ 436S-9	(+)	-	+	-	++	(+)	(+)	-	+	-	+++	(+)
WZ 436S-10	(+)	-	+	-	+	-	-	-	-	-	-	-
WZ 436S-11	-	-	-	-	-	-	-	-	-	-	(+)	(+)
WZ 436S-12	-	-	-	-	(+)	-	-	-	(+)	-	(+)	-
WZ 436S-13	(+)	-	+	(+)	++	+	-	-	++	(+)	+++	(+)
WZ 436S-16	(+)	(+)	+	(+)	+++	(+)	+	-	+	-	+	-
WZ 436S-17	+	(+)	+	(+)	+	(+)	+	+	+++	+++	+++	+++
WZ 436S-21	-	-	-	-	(+)	-	-	-	-	-	-	-

Rs : root system Ss : shoot system E : enhancement: < 50% E+ : enhancement > 50%
- : 0-25% inhibition (+) : 25%-50% inhibition + : 50%-75% inhibition ++ : 75%-90% inhibition
+++ : > 90% inhibition

Analysis of these data revealed that nitrated compounds were, in general, plant regulator substances. Most of the tested substances exhibited significant phytotoxic activities at concentrations higher than 67 µg/ml, especially for the root system (e.g. **WZ 436S-9**, **-13**, **-16** and **-17**). Presence of a second nitro group, e.g. **WZ 436-12**, increased the concentrations needed to influence the germination of both seed types (**WZ 436S-12** in comparison with **WZ 436S-9**). Methylation of the carboxylic acid side chains in these substances exhibited controversial roles; although it decreased the phytotoxic activity in the mononitrated substances (e.g. **WZ 436S-9** in comparison with **WZ 436S-1**), it increased the phytotoxicity of their dinitrated congeners (e.g. **WZ 436S-3** in comparison with **WZ 436S-12**).

WZ 436S-21 did not show a significant phytotoxic activity. However, replacement of the OH-group in **WZ 436S-21** by (Cl^-) to form **WZ 436S-17** rendered the compound a phytotoxic activity. Some of the phytotoxic compounds showed a moderate inhibition in chlorophyll production, indicated by the pale green colouration of the leaves, in comparison with the control (e.g. **WZ 436S-9**, **WZ 436S-12**, **WZ 436S-13** and **WZ 436S-16**).

Two substances enhanced germination of the dicotyledonous seed, **WZ 436S-1** and **WZ 436S-2**. They promote the elongation of shoot and root systems with different extents at 67 µg/ml. Although **WZ 436S-2** promoted the growth of *L. sativum* seeds, it inhibited chloroplast formation (white leaves).

3.3. Secondary metabolites from *Vibrio* sp. WMBA1-4

3.3.1. *Vibrio* sp. WMBA1-4

The bacterial strain WMBA1-4 was isolated from the surface of the soft coral *Sinularia polydactyla* collected by SCUBA diving at a depth of 15 m in the Red Sea (GPS: 29°21.605`N 34°57.815`E). It was purified by serial dilutions and platting techniques. It forms circular, light beige colonies on M_1 agar plate. The cells are Gram-negative and do not form spores. They are motile and facultative anaerobic rods. The strain does not accumulate poly-β-hydroxybutyrate and has no arginine dihydrolase system. It is oxidase positive, catalase positive and β-galactosidase negative. It can reduce nitrate and produce indole. The cells are 2.3-2.8 (3.5) µm long and 1-1.2 µm wide (Fig. 3.20B and C). It is psychrotrophic, able to grow only between 10-30 °C. The optimum temperature for growth was 22-27 °C.

The strain does not require Na^+ for growth and can withstand higher concentrations of marine salts up to 75% (w/v). The growth was weak in M_1-medium without marine salts. The biochemical characteristics of this strain are listed in Table 7.1 (see appendix).

Analysis of the amplified 16S rDNA from this strain led to 99% similarity level with *Vibrio* species. The nearest identified phylogenetic relatives were *Vibrio splindidus* biovar II isolated from North-western Pacific Ocean and Otsuch Bay, Japan (Urakawa *et al.,* 1999) and *Vibrio shilonii*, a pathogen that causes bleaching of the Mediterranean coral *Oculina patagonica* (Kushmaro *et al.,* 2001). Therefore, this strain is a species belonging to the genus *Vibrio* in the gamma-proteobacteria-group

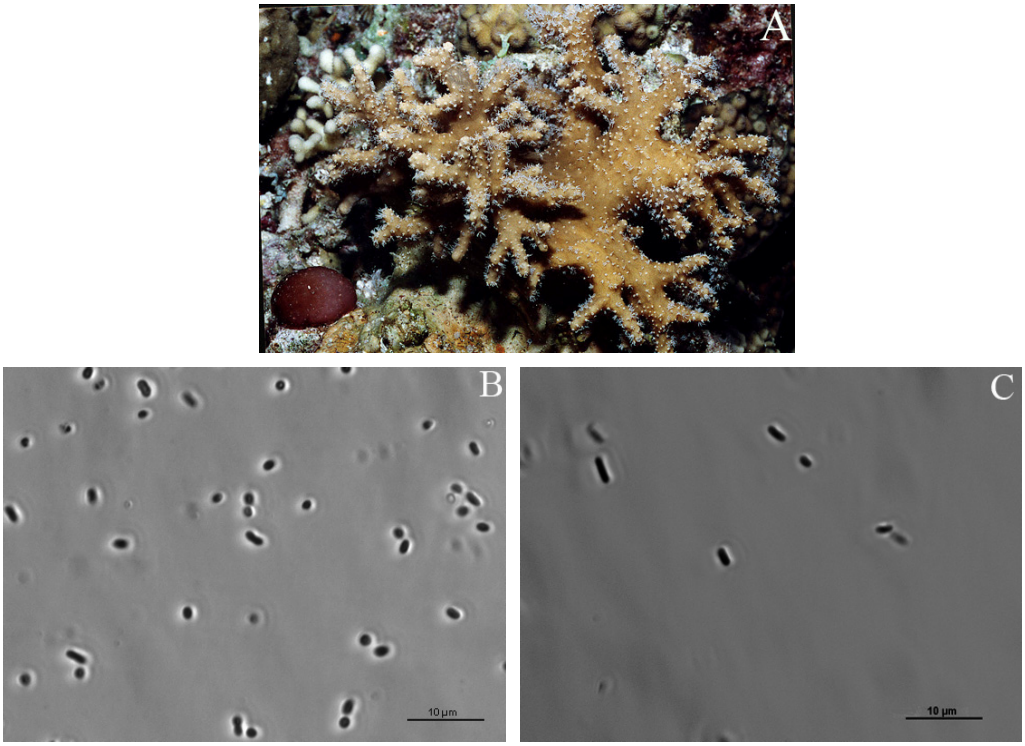


Fig. 3.20. Underwater photograph for *Sinularia polydactyla* (A) and microphotographs with phase contrast microscope for the isolated *Vibrio* sp. WMBA1-4 (B & C). Scale bars are 10 µm.

3.3.2. Small scale fermentation of *Vibrio* sp. WMBA1-4

Vibrio sp. WMBA1-4 was fermented in three media M_1, M_{10} and M_{11} in order to select the suitable media for the bacterial growth and the production of antimicrobial compounds (Table 3.14). Although M_{10} and M_{11} differ only by substituting starch with corn steep solids, the growth was better in the later medium with production of a more active crude extract. Thus, M_{11} was chosen for fermentation of this strain in a large scale.

Table 3.14. Growth of *Salegentibacter* sp. T436 in different media and production of antimicrobial compounds.

Medium	Growth	Duration (Day)	Inhibition zone (mm) 300 µg/disc				
			B. brevis	***M. luteus***	***N. coryli***	***P. variotii***	***M. miehei***
M_1	++	4	8	-	-	-	-
M_{10}	+	4	10	-	12	-	-
M_{11}	**++**	**4**	**13**	**10**	**20**	**-**	**12**

- : not active +: moderate growth ++ : good growth

3.3.3. Optimization of growth and secondary metabolites production

Cultivations in M_{11} with different marine salts concentrations and at different temperatures were carried out, as mentioned in 2.8.2.2, in order to select the optimum condition for the bacterial growth and the production of antimicrobial compounds. The optimum temperature was 22-27 °C with 33.5–75 g/l of marine salts mixture in M_{11}.

3.3.4. Fermentation of *Vibrio* sp. WMBA1-4 in 20-L fermentors

Five fermentations were carried out with *Vibrio* sp. in M_{11} at 25 °C with 150 rpm and aeration of 3-4 l/min (2.8.3). The duration of fermentation varied between 46-72 hours. The OD reached its maximum after 43-48 hours, while the maximum number of CFU was attained in 27-43 hours. The maximum activity was detected after 27 hours against the bacterial test strains and after 43 hours against the fungal strains. Fig. 3.21 illustrates the fermentation scheme of this strain in a Biostat U20 with exhaust gas analysis.

Fermentation process was ended when the oxygen partial pressure started to increase again.

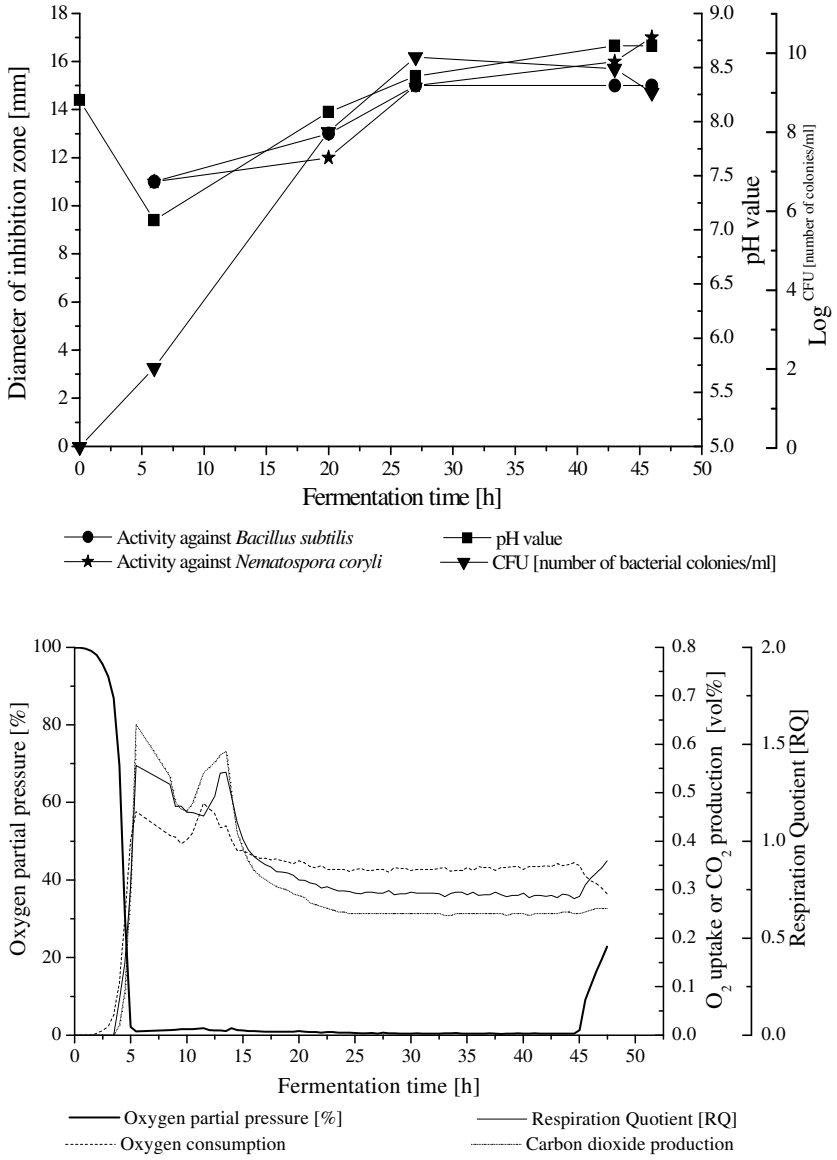


Fig. 3.21. Fermentation of *Vibrio* sp. WMBA1-4 in 20 l B2-medium (M_{11}). Growth, pH, and antibacterial activity (upper diagram) and the exhaust gas analysis during fermentation process (lower diagram).

3.3.5. Purification of secondary metabolites from *Vibrio* sp. WMBA1-4 cultivated in M_{11}

An oily brown crude extract (18 g) was obtained by extraction of 80 litres of acidified (pH 4) culture fluid with ethyl acetate. Purification of secondary metabolites was performed by bioactivity directed fractionation as shown in Fig. 3.22-Fig. 3.24. The group of Prof. Laatsch elucidated the structures of isolated compounds.

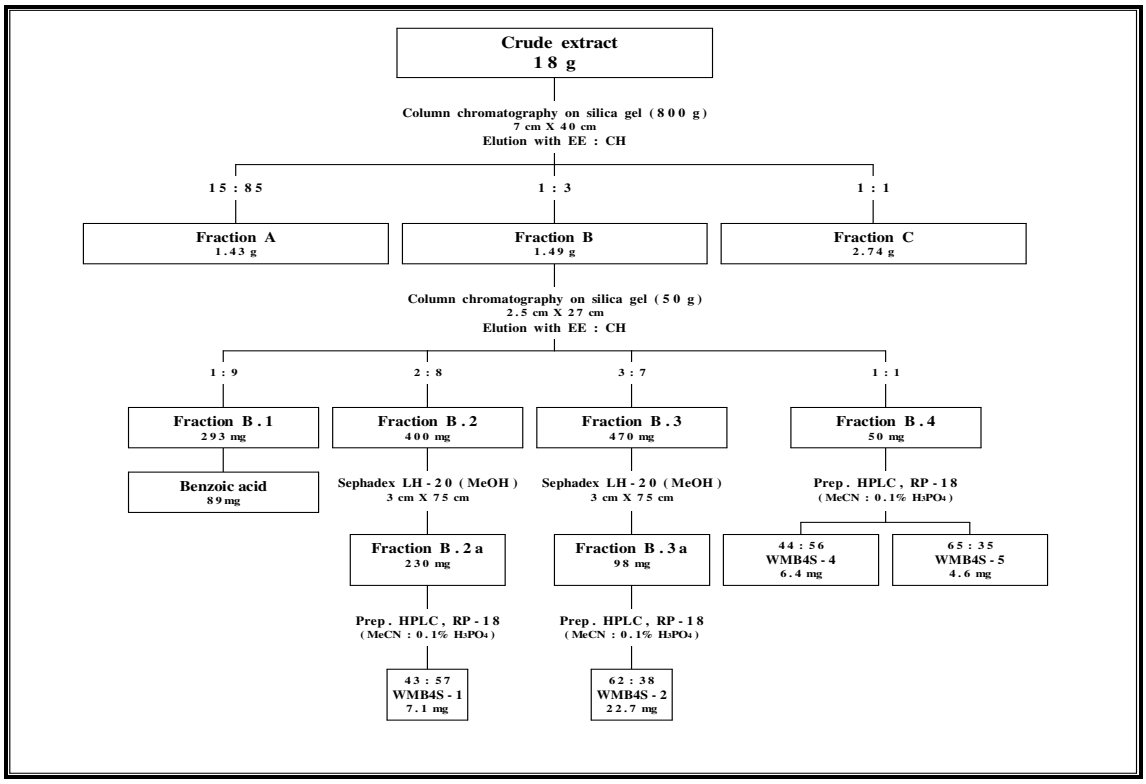


Fig. 3.22. Purification scheme of fraction B from the crude extract of *Vibrio* sp. WMBA1-4 in M_{11}.

From fraction A **WZ 436S-16** and benzoic acid were obtained (data not shown in Fig. 3.22).

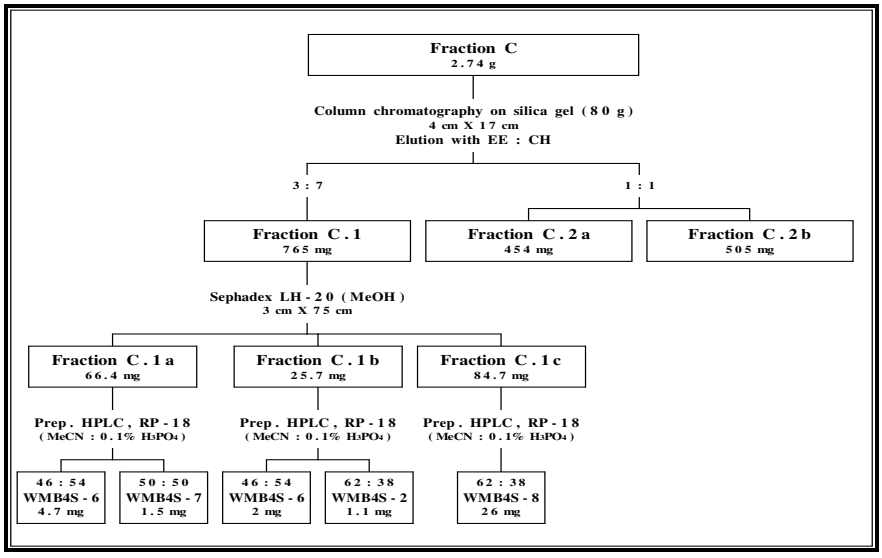


Fig. 3.23. Purification scheme of fraction C from the crude extract of *Vibrio* sp. WMBA1-4 (**part A**).

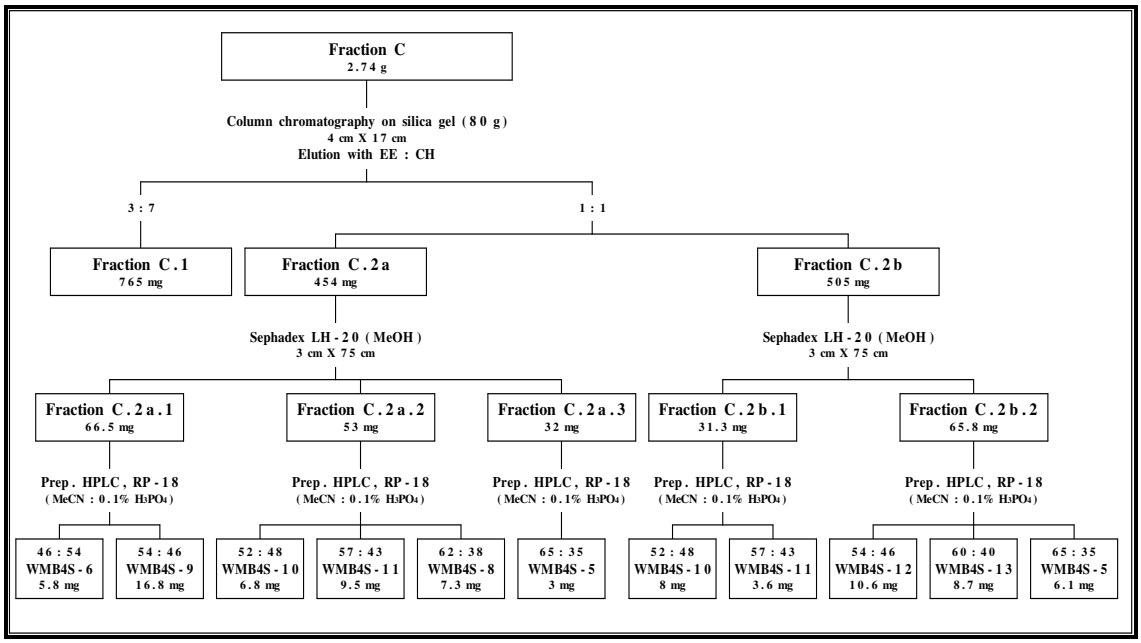


Fig. 3.24. Purification scheme of fraction C (see p. 104) from the crude extract of *Vibrio* sp. WMBA1-4 (**part B**).

3.3.6. Isolation of metabolites from *Vibrio* sp. WMBA1-4 fermented in M_1

The strain was fermented in a small scale (5 x 2 L of M_1), although its crude extract did not exhibit antimicrobial activity. The chromatogram of the crude extract of WMBA1-4 showed, in the non-polar range, a large number of peaks that have indole-like UV spectra in the analytical HPLC. The purified secondary metabolites are shown in Fig. 3.25.

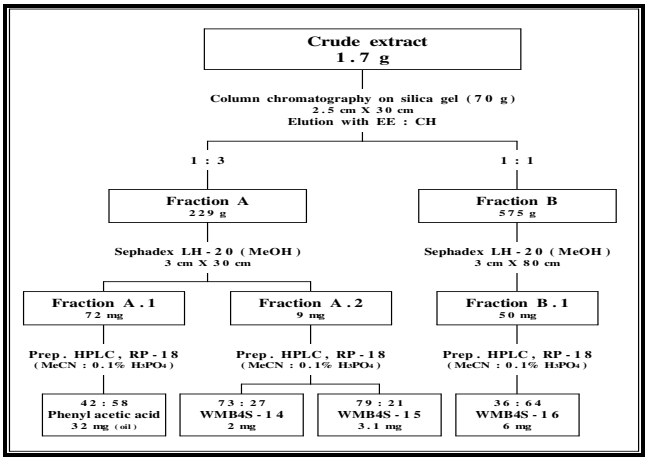


Fig. 3.25. Purification scheme of the crude extract of *Vibrio* sp. WMBA1-4 in M_1.

3.3.7. Physico-chemical characteristics

Thirteen nitrogen-containing compounds and three 4-hydroxy-phenyl derivatives were isolated from *Vibrio* sp. WMBA1-4. Most of these compounds are yellow to orange mononitro/dinitro-derivatives (Fig. 3.26). The chemical names of all isolated substances are listed in Table 7.4 (see appendix). IR spectra showed the typical absorption bands of the nitro group as mentioned under 3.2.5. Unless otherwise noted, their colours did not change on reacting with the chromogenic reagents. Their retention times in analytical HPLC and the R_f-values in various mobile phases are listed in Table 7.2 (see appendix). UV spectra, IR spectra, and LC-MS mass spectra (APCI negative ionisation) are shown in 7.3.1 (see appendix). Physico-chemical characterizations were done as described in 2.9.

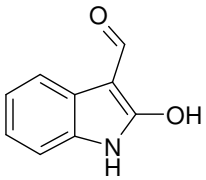
3.3.7.1. Nitrated 4-hydroxyphenyl-derivatives

WMB4S-1 (3-nitro-4-hydroxybenzaldehyde) is a yellow substance with the molecular formula $C_7H_5NO_4$ (M. wt. 167 Da). It is soluble in most organic solvents and shows a fluorescent extinction at 254 nm. UV-spectrum in acidified methanol shows UV_{max} at 264 nm (log ε = 4.13) and 346 nm (log ε = 3.65). IR spectrum shows a band at ν_{max} 1689 cm^{-1} for aldehyde group. In this work, it is isolated from natural source for the first time.

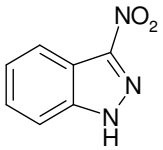
WMB4S-6 3-(3-nitro-4-hydroxyphenyl)-2-propenoic acid is a yellow substance with the molecular formula $C_9H_7NO_5$ (M. wt. 209 Da). It is soluble in methanol, acetone, ethyl acetate, cyclohexane and isopropanol. It shows a fluorescent extinction at 254 nm on TLC plate. It has UV_{max} at 278 nm (log ε = 4.31) and 360 nm (log ε = 3.4). IR spectrum shows bands at ν_{max} 1701 cm^{-1}, 1625 cm^{-1} for (C=C) alkenes. This compound was known as synthetic (see Table 7.4, appendix) substance and herein is reported for the first time from natural source.

3.3.7.2. 2-Hydroxy-1H-indole-3-carbaldehyde (WMB4S-4)

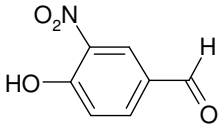
WMB4S-4 is a white substance that is soluble in most organic solvents and has the molecular formula $C_9H_7NO_2$ (M. wt. 161 Da). It shows a fluorescent extinction at 254 nm and blue fluorescence at 360 nm. It has UV_{max} at 236 nm (log ε = 4.02), 242 nm (log ε = 4.02), and 298 nm (log ε = 4.07). IR spectrum shows a band at ν_{max} 1697 cm^{-1} (C=O) and 2854 cm^{-1} for aldehyde group. It is a new substance from natural source.



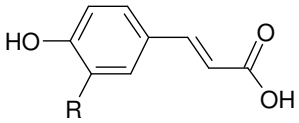
WMB4S-4



WMB4S-7

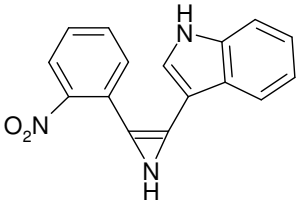


WMB4S-1

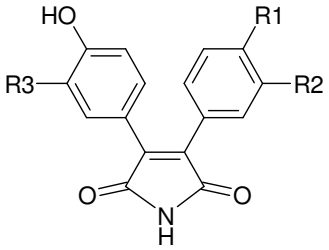


R = NO_2 : **WMB4S-6**

R = H : **WMB4S-16**



WMB4S-5

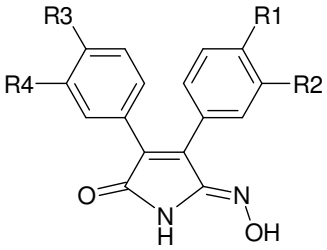


R1, R2 = H, R3 = NO_2 : **WMB4S-2**

R1 = OH, R2, R3 = NO_2 : **WMB4S-8**

R1, R2, R3 = H : **WMB4S-9**

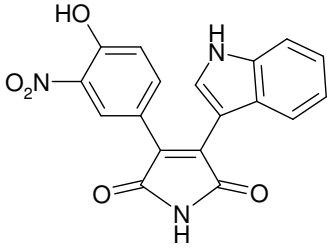
R1 = OH, R2 = H, R3 = NO_2 : **WMB4S-10**



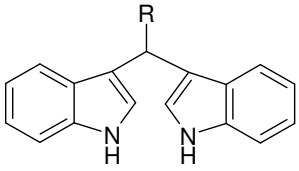
R1, R2 = H, R3 = OH, R4 = NO_2 : **WMB4S-11a**

R1 = OH, R2 = NO_2, R3, R4 = H: **WMB4S-11b**

R1, R3= OH, R2, R4 = NO_2 : **WMB4S-12**



WMB4S-13



R = Me : **WMB4S-14**

R = C_6H_5 : **WMB4S-15**

Fig. 3.26. Structures of the sixteen metabolites from *Vibrio* sp. WMBA1-4.

3.3.7.3. 3-Nitro-1H-indazole (WMB4S-7)

This compound was isolated as a pale yellow solid with the molecular formula $C_7H_5N_3O_2$ (M. wt. 163 Da). It is soluble in DMSO. It has UV_{max} at 234 nm (log ε = 3.6) and 326 nm (log ε = 3.5). It shows a fluorescent extinction at 254 nm. The IR spectrum shows the typical bands of nitro group. This substance was not reported previously from natural sources.

3.3.7.4. Nitrated pyrrol-2,5-dione-derivatives (maleimide derivatives)

They are yellow to orange substances. Some are soluble in methanol, acetone, ethyl acetate and isopropanol (**WMB4S-2**, **WMB4S-9**, and **WMB4S-11**) while the others are sparsely soluble in organic solvents except in DMSO (**WMB4S-8**, **WMB4S-10**, **WMB4S-12** and **WMB4S-13**). The IR spectra of these derivatives show bands at ν_{max} 1700-1730 cm^{-1}, 1760-1780 cm^{-1} indicating the presence of (C=O) of dione and at ~3420 cm^{-1} for an (N-H) containing compounds. Table 3.15 summarizes the new isolated maleimide derivatives with their colours, molecular formulas, molecular weights, UV_{max} (log ε) from UV spectrum and the characteristic IR bands, other than the typical bands mentioned above, in the spectroscopic measurements. In contrast to the other maleimide derivatives, **WMB4S-2**, **WMB4S-9**, **WMB4S-8** and **WMB4S-10** showed a yellow fluorescence at 360 nm in addition to a fluorescent extinction at 254 nm.

3.3.7.5. 3-[3-(2-Nitro-phenyl)-1H-azirin-2-yl]-1H-indole (WMB4S-5)

It is a dark yellow substance that is soluble in methanol, acetone, isopropanol and ethyl acetate. It has the molecular formula $C_{16}H_{11}N_3O_2$ (M. wt. 277 Da). It shows a fluorescent extinction at 254 nm. It has UV_{max} at 262 nm (log ε = 4.19), 286 nm (log ε = 4.28), 350 nm (log ε = 3.86), and 406 nm (log ε = 3.89). The compound forms a dark brown colour upon reaction with each of anisaldehyde in ethanol as well as in acetic acid and vanillin-sulphuric acid. It was neither reported previously as a synthetic compound nor as a natural product.

Table 3.15. Colours and spectroscopic data of the new substances from *Vibrio* sp. WMBA1-4.

Compound	Colour	Molecular formula	Molecular weight (Da)	UV_{max} (log ε) in methanol	Characteristic band in IR spectrum (ν_{max})
WMB4S-2	Y	$C_{16}H_{10}N_2O_5$	310	228 nm (4.43) 265 nm (4.24) 364 nm (3.96)	-
WMB4S-8	O	$C_{16}H_9N_3O_8$	371	232 nm (4.49) 265 nm (4.37) 362 nm (4.05)	-
WMB4S-9	Y	$C_{16}H_{11}NO_3$	265	232 nm (4.21) 384 nm (3.81)	-
WMB4S-10	Y	$C_{16}H_{10}N_2O_6$	326	232 nm (4.33) 253 nm (4.30) 386 nm (3.95)	-
WMB4S-11	Y	$C_{16}H_{11}N_3O_5$	325	312 nm (4.23)	960 cm^{-1}, 1627 cm^{-1} (oxime)
WMB4S-12	Y	$C_{16}H_{10}N_4O_8$	386	216 nm (4.36) 280 nm (4.19) 316 nm (4.22)	As **WMB4S-11**
WMB4S-13	O	$C_{18}H_{11}N_3O_5$	349	218 nm (4.37) 262 nm (4.10) 332 nm (3.52) 424 nm (3.75)	-

Y: yellow O : orange

3.3.8. Other isolated metabolites

A number of previously purified substances from strain T436 were also isolated from the crude extract of strain WMBA1-4 in M_{11}. From fraction B, benzoic acid (80 mg), nitrophenol (11 mg), **WZ 436S-1** (5 mg), **WZ 436S-4** (0.7 mg), and **WZ 436S-16** (8 mg) were obtained. From fraction C, **WZ 436S-2** (2.1 mg), **WZ 436S-5** (6.5 mg), **WZ 436S-9** (1.2 mg), and **WZ 436S-13** (6.8 mg) were isolated. 3-Hydroxy benzoic acid (12 mg) was isolated from the crude extract of strain WMBA1-4 in M_1.

3.3.9. Biological characterization of the metabolites

3.3.9.1. Antimicrobial activities of the isolated substances

Antimicrobial activities of the isolated metabolites from *Vibrio* sp. WMBA1-4 were determined using the agar diffusion and the minimal inhibitory concentration (MIC) assays. Only the active substances are listed in Table 3.16-Table 3.18.

Table 3.16. Antimicrobial activities of compounds from *Vibrio* sp. WMBA1-4 in agar diffusion test

Compound	Inhibition zone (mm) 50 µg /disc				
	Bacteria		Fungi		
	E. dissolvens	*B. brevis*	*N. coryli*	*M. miehei*	*P. variotii*
WMB4S-1	-	18	-	13	-
WMB4S-2	-	16	-	-	-
WMB4S-4	-	11	-	-	-
WMB4S-5	-	10	-	-	-
WMB4S-6	-	12	30d	-	-
WMB4S-7	-	-	12	-	-
WMB4S-8	-	15	-	-	-
WMB4S-9	-	10	12i	-	-
WMB4S-11	-	21	-	-	-
WMB4S-12	-	20	-	-	-
WMB4S-13	-	20	-	-	-

- : no inhibition d : diffuse zone (very weak growth in the inhibition zone
i : incomplete inhibition (presence of a number of single resistant colonies).

Most substances were active against Gram-positive bacteria. Three compounds, **WMB4S-6**, **WMB4S-7** and **WMB4S-9**, showed activity against *Nematospora coryli* while **WMB4S-1** was active against *Mucor miehei. E. dissolvens* and *P. variotii* were resistant for all tested compounds in agar diffusion assay.

All these active compounds retained their activities in agar diffusion test even upon incubation with L-cysteine for one hour.

Tables 3.17 and 3.18 show the MIC values of the substances against the different test microorganisms. *E. dissolvens* was not sensitive to all tested compounds. The antibacterial activities were more pronounced than the antifungal ones.

Table 3.17. MIC of the metabolites from *Vibrio* sp. WMBA1-4 in the serial dilution assay (**part A**).

Organisms	**MIC [µg/ml]**				
	WMB4S-1	**-2**	**-4**	**-5**	**-6**
Fungi:					
Paecilomyces variotii	100s	50s	-	-	-
Penicillium notatum	50s	-	-	100s	-
Phytophthora infestans	50s	100s	-	50s	NT
Mucor miehei	50s	50s	-	-	-
Nematospora coryli	100c	50s	50c	25c	50c
Saccharomyces cerevisiae FL 200	100c	-	-	-	-
Ustilago nuda	50c	100s	50c	50c	-
Bacteria:					
Gram-positive:					
Bacillus brevis	50s	25s	-	50c	-
Bacillus subtilis	50s	25c	100s	12.5s	-
Micrococcus luteus	50c	25s	-	12.5s	-
Gram-negative:					
Escherichia coli K12	50s	25c	-	12.5s	100s
Enterobacter dissolvens	-	-	-	-	-

- : not active up to 100 µg/ml NT : not tested s : bacteriostatic/fungistatic
c : bactericidal/fungicidal

Three compounds showed a specific moderate activity against *N. coryli*, **WMB4S-4**, **WMB4S-6** and **WMB4S-9**. Two substances showed antibacterial activity, **WMB4S-11** and **WMB4S-12**, at a concentration of 6.25-12.5 µg/ml. **WMB4S-5** exhibited weak antifungal (25-50 µg/ml) and moderate antibacterial (12.5-25 µg/ml) activities.

Table 3.18. MIC of the metabolites from *Vibrio* sp. WMBA1-4 in the serial dilution assay (**part B**).

Organisms	**MIC [µg/ml]**				
	WMB4S-8	**-9**	**-11**	**-12**	**-13**
Fungi:					
Paecilomyces variotii	-	50s	50s	-	-
Penicillium notatum	-	-	-	-	-
Phytophthora infestans	NT	-	100s	-	-
Mucor miehei	-	-	50s	-	100s
Nematospora coryli	100s	6-7s	50c	100s	50c
Saccharomyces cerevisiae FL 200	100c	-	-	-	-
Ustilago nuda	-	-	-	-	-
Bacteria:					
Gram-positive:					
Bacillus brevis	100s	100c	6-7s	12.5s	25s
Bacillus subtilis	50s	100s	6-7s	12.5s	25s
Micrococcus luteus	100c	100c	12.5s	12.5s	50s
Gram-negative:					
Escherichia coli K12	50s	100c	6-7s	12.5s	25s
Enterobacter dissolvens	-	-	-	-	-

- : not active up to 100 µg/ml NT : not tested s : bacteriostatic/fungistatic
c : bactericidal/fungicidal

From these data, the substances could be divided into three categories, those with antifungal activities (**WMB4S-4**, **WMB4S-6** and **WMB4S-9**), those with antibacterial activities (**WMB4S-11** and **WMB4S-12**) and the remaining substances showed a broad antimicrobial spectrum.

Other antimicrobial tests: compounds that were antimicrobial at 50 µg/ml were examined for their effects on the growth of pathogenic microorganisms. Only *Proteus vulgaris* was sensitive to some of tested substances. 5 µg/disc of each of **WMB4S-2** and

WMB4S-11 caused 15i mm and 21 mm inhibition zones respectively. **WMB4S-12** and **WMB4S-13,** at 50 µg/disc, caused 9 mm and 14 mm inhibition zones respectively. Conidial germination of *M. grisea* was inhibited at 50 µg/ml of each of **WMB4S-2**, **WMB4S-4**, and **WMB4S-11**; at 25 µg/ml of **WMB4S-6**; and at 5 µg/ml of **WMB4S-1**.

3.3.9.2. Effect of WMB4S-2, -9, -11, -12, and –13 on the oxygen uptake by microorganisms

The influence of each of **WMB4S-2**, **WMB4S-11**, **WMB4S-12** and **WMB4S-13** on the oxygen uptake by *B. subtilis* and **WMB4S-9** on the oxygen uptake by *N. coryli* was evaluated. **WMB4S-11** and **WMB4S-12**, at 30 µg/ml, inhibited the respiration of *B. subtilis* to 50%. **WMB4S-2** and **WMB4S-13** inhibited the bacterial respiration to a 50% at 50 µg/ml while **WMB4S-9** did not significantly influence the oxygen uptake, up to 15 µg/ml, in *N. coryli*.

OD measurements and microscopic observations of tested microorganisms revealed that the inhibition in respiration was not due to cell death or lysis.

As a positive control, antimycin A caused 86% inhibition in oxygen uptake by *N. coryli* at 5 µg/ml.

3.3.9.3. Influence of the active compounds on the synthesis of macromolecules *in vivo* in microorganisms

The most potent antimicrobial compounds were examined for their effect on the macromolecules biosynthesis in microorganisms. **WMB4S-11** and **WMB4S-12** were tested with *B. subtilis* while **WMB4S-9** was tested with *N. coryli*.

WMB4S-11 did not significantly inhibit the biosynthesis of various macromolecules in *B. subtilis*. A maximum inhibition of 45% was achieved at 10 µg/ml. **WMB4S-12** caused a 50% inhibition in RNA and protein biosynthesis at 17 µg/ml and 20 µg/ml respectively. 20 µg/ml reduced the biosynthesis of DNA and cell wall (incorporation of N-acetyl-glucosamine) to 60%.

6 µg/ml of **WMB4S-9** inhibited the biosynthesis of DNA in *N. coryli* to 55%, while 10 µg/ml reduced the biosynthesis of RNA and protein to 55% and cell wall to 65%.

3.3.9.4. Cytotoxic effects of the isolated compounds

Although all isolated compounds from *Vibrio* sp. WMBA1-4 were examined in cytotoxicity assay against monolayer and suspension cell lines, Table 3.19 shows only the IC-values (the mean from three independent experiments) of the cytotoxic substances.

Table 3.19. Cytotoxic activities of the isolated compounds from *Vibrio* sp. WMBA1-4.

Compound	L1210		Jurkat		MDA-MB-321		MCF-7		Colo-320	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
	[µg/ml]									
WMB4S-2	50	>100	>100	>100	>100	>100	>100	>100	>100	>100
WMB4S-4	40	>100	20	>100	>100	>100	>100	>100	>100	V
WMB4S-5	20	100	20	>100	25	90	>100	>100	30	100
WMB4S-7	50	80	100	>100	>100	>100	>100	>100	100	>100
WMB4S-8	15	>100	60	>100	>100	>100	>100	>100	>100	>100
WMB4S-9	30	>100	>100	>100	>100	>100	100	>100	40	>100
WMB4S-10	>100	>100	>100	>100	>100	>100	>100	>100	100	>100
WMB4S-11	15	>100	20	>100	25	>100	20	>100	50	>100
WMB4S-12	15	>100	25	>100	>100	>100	>100	>100	100	>100
WMB4S-13	15	>100	50	>100	>100	>100	>100	>100	100	>100
WMB4S-14	10	30	20	30	25	35	20	30	20	30
WMB4S-15	15	20	25	30	50	90	90	>100	40	50

IC_{50} : inhibition in proliferation of 50% of cells IC_{90} : inhibition in proliferation of 90% of cells

Most of the substances were cytotoxic for L1210, Jurkat, and Colo-320 cells. **WMB4S-5**, **WMB4S-11**, **WMB4S-12**, **WMB4S-14** and **WMB4S-15** were moderate cytotoxic compounds against L1210 and Jurkat cells. These compounds, excluding **WMB4S-12**, showed weak to moderate cytotoxic activities against Colo-320 and both of the breast cancer

cells. **WMB4S-14** and **WMB4S-15** were the only selective cytotoxic agents, e.g. without having antimicrobial activities.

3.3.9.5. Influence of the active compounds on the synthesis of macromolecules in L1210 cells

Since L1210 cells were more sensitive to the tested substances than the other tumor cells, they were used to depict the effect of the most cytotoxic compounds on the biosynthesis of macromolecules as shown in Table 3.20. Several concentrations were tested (1, 5, 10, 20, 30 µg/ml) and the IC_{50} was deduced from the inhibition curve of each compound.

Table 3.20. Effect of **WMB4S-5, -11, -12, -13** and **WMB4S-15** on the incorporation of radioactive precursors in biosynthesis of macromolecules in L1210 cells.

Compounds	Inhibition in incorporation of					
	[2-^{14}C]-thymidine DNA		[2-^{14}C]-uridine RNA		[1-^{14}C]-leucine Protein	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
	[µg/ml]					
WMB4S-5	18	> 30	18	> 30	21	> 30
WMB4S-11	30	> 30	24	> 30	12.5	> 30
WMB4S-12	> 30	> 30	20	> 30	21	> 30
WMB4S-13	> 30	> 30	19	> 30	28	> 30
WMB4S-15	4	22	11	25	7	23

The cytotoxic effect of **WMB4S-5** was due to the inhibition in the biosynthesis of all macromolecules at 18-21 µg/ml. Biosynthesis of RNA and DNA was inhibited to 50% at 18 µg/ml, while inhibition of 50% of leucine incorporation in biosynthesis of protein was achieved at 21 µg/ml.

WMB4S-11 inhibited mainly the biosynthesis of protein (incorporation of leucine) to a 50% at 12.5 µg/ml while synthesis of RNA and DNA was inhibited to 50% at higher

concentrations. Therefore, the cytotoxic effect of this compound is due to its influence on the protein biosynthesis. In contrast to **WMB4S-11**, its dinitrated congener (**WMB4S-12**) caused a 50% inhibition in synthesis of RNA and protein at 20-21 µg/ml. Biosynthesis of DNA was not affected up to 30 µg/ml.

WMB4S-13 triggered a 50% inhibition in RNA and protein biosynthesis at 19 µg/ml and 28 µg/ml respectively. DNA biosynthesis was not affected up to 30 µg/ml.

Biosynthesis of DNA (incorporation of thymidine) and protein (incorporation of leucine) was inhibited to 50% by 4 µg/ml and 7 µg/ml of **WMB4S-15** respectively. 11 µg/ml caused 50% inhibition in RNA biosynthesis.

3.3.9.5.1. Nematicidal effect of the isolated metabolites from *Vibrio* sp. WMBA1-4

Only **WMB4S-1** exhibited a nematicidal activity against *Meloidogyne incognita* with LD_{90} of 25 µg/ml while **WMB4S-16** and **WMB4S-6** were weakly nematicidal agents as they caused the death of 90% of *M. incognita* at 100 µg/ml.

3.3.9.6. Inhibition of seed germination

The compounds exhibited moderate activities on shoot and/or root system. At 67 µg/ml, seed germination was inhibited to 25-50%. However, this inhibition was not concentration dependent, e.g. higher concentrations did not lead to higher inhibition in seed germination. **WMB4S-4** and **WMB4S-5** did not influence the germination of shoot and root systems. Some substances exhibited a weak activity against *S. italica* at a concentration starting from 167 µg/ml (**WMB4S-12**) or against *L. sativum* at a concentration starting from 333 µg/ml (**WMB4S-11**).

Nitration of the diphenylmaleimide derivatives decreased the concentration needed to exhibit their activities (**WMB4S-2** in comparison with **WMB4S-9**). In addition, the dinitro-derivative (**WMB4S-8**) was more phytotoxic than its mononitro-congener (**WMB4S-10**) for monocotyledonous seeds. For the dicotyledonous seeds the mononitro-derivative was more phytotoxic than the dinitro-derivative. Presence of oxime group abolishes this difference, where mononitro-derivative was potent phytotoxic than the dinitrated ones. Only **WMB4S-6** and **WMB4S-11** triggered 50-75% inhibition in seeds germination at 333 µg/ml. **WMB4S-11** inhibited chlorophyll production in *L. sativum* seeds as the leaves were yellow coloured.

Table 3.21. Phytotoxic effect of the isolated metabolites from *Salegentibacter* sp. T436.

Compound	*Setaria italica* [µg/ml]						*Lepidium sativum* [µg/ml]					
	67		167		333		67		167		333	
	Rs	Ss	Rs	Ss	Rs	Ss	Rs	Ss	Rs	Ss	Rs	Ss
WMB4S-1	-	-	(+)	-	+	(+)	-	-	(+)	-	(+)	(+)
WMB4S-2	-	-	(+)	-	(+)	(+)	-	-	+	-	+	(+)
WMB4S-4	-	-	-	-	-	-	-	-	-	-	-	-
WMB4S-5	-	-	-	-	-	-	-	-	-	-	-	-
WMB4S-6	(+)	-	(+)	-	++	(+)	-	-	(+)	-	(+)	-
WMB4S-8	(+)	-	(+)	-	(+)	-	-	-	-	-	(+)	-
WMB4S-9	-	-	-	-	-	(+)	-	-	-	-	(+)	+
WMB4S-10	-	-	-	-	-	-	(+)	-	(+)	-	(+)	-
WMB4S-11	-	(+)	+	(+)	++	(+)	-	-	-	-	+	(+)
WMB4S-12	-	-	(+)	-	(+)	-	-	-	-	-	-	-
WMB4S-13	(+)	-	(+)	(+)	+	(+)	-	-	-	-	(+)	-
WMB4S-16	(+)	-	(+)	-	(+)	-	(+)	-	(+)	(+)	(+)	(+)

Rs : root system
Ss : shoot system
- : 0-25% inhibition
(+) : 25%-50% inhibition
+ : 50%-75% inhibition
++ : 75%-90% inhibition

3.4. Fermentation of two additional bacterial strains

During this work two additional bacterial strains were fermented in 20 L scale. **Strain T396** was isolated from the penguin faeces in the Antarctic sea. It is an ovoid rod, Gram-negative and strictly aerobic bacterium. The cells are 2-2.4 (3.8) µm long and 1.5-1.9 µm wide. It forms light brown, circular colonies with shiny smooth surfaces on M_1 agar. The colonies are 3-4 mm wide, convex with smooth margins. The strain is catalase positive, oxidase positive and β-galactosidase negative. Comparing the 16S rDNA sequence with sequences in the genes databank indicated that this strain belongs to the genus *Psychrobacter* in the γ-proteobacteria group.

Fermentation of *Psychrobacter* sp. T396 led to the production and purification of previously isolated substances from the other strains. Benzoic acid, salicylic acid, phenyl acetic acid, **WZ 436S-1** and **WZ 436S-9** were isolated from the crude extract of a 20 l fermentor of this bacterial strain.

WMBA1-5 was isolated from the surface of the soft coral *Sinularia polydactyla* flourishing in the south part of the Gulf of Aqaba/Red Sea. It is Gram-negative, rod-shaped facultative anaerobic bacterium. It forms transparent, irregular colonies with irregular margins on M_1 agar. The cells are 1.9-2.6 µm long and 1.1-1.5 µm wide. The strain is catalase positive, oxidase positive and β-galactosidase negative. Comparing the 16S rDNA sequence with sequences in the genes databank indicated that this strain belongs to the genus *Ralstonia* in the β-proteobacteria group

Fermentation of *Ralstonia* sp. WMBA1-5 led to the production and purification of previously isolated substances from the other strains. Phenyl acetic acid, phenyl lactic acid, **WZ 436S-13** and isatin (**WZ 268S-8**) were isolated from the crude extract of a 20 l fermentor of this bacterial strain.

4. Discussion

Emerging of new infectious diseases and resistant pathogens for which no effective therapies are available represent a serious problem for the human life (Needham, 1994; Cragg *et al.,* 1997). In the recent years, it was shown that the marine microorganisms are able to produce molecules with novel structures and biological activities (Bernan *et al.,* 1997; Thakur *et al.,* 2005). Marine bacteria acquired a particular interest since number of bodies of evidences proved that, in fact, associated microorganisms produced some highly bioactive metabolites that were previously ascribed to marine macroorganisms

Marine bacteria are most generally defined by their requirement of seawater, or most specifically sodium ions, for growth (Jensen & Fenical, 2000). They were typically cultured from seawater, animate and inanimate surfaces. Bacteria isolated from marine habitats are heterotrophic. Gram-negative fermentative strains belonging to the genera *Vibrio*, *Photobacterium*, *Colwellia*, and *Aeromonas* and the non-fermentative strains belong to *Alteromonas*, *Pseudomonas*, *Pseualteromonas*, *Marinomonas*, *Shewanella*, and *Flavobacterium* are especially common (Holmström *et al.,* 1998, Nichols *et al.,* 2005).

Traditional phenotypic identification of bacterial taxa is difficult and time-consuming. The interpretation of results from phenotypic methods can involve a substantial amount of subjective judgment (Drancourt *et al.,* 2000). Therefore, testing for multiple physiological and biochemical traits complemented with 16S rRNA genes (16S rDNA) analysis served as a basis for a sound classification and allowed a reliable identification of the bacterial strains in this study. The amount of evolution or dissimilarity between rRNA sequences of a given pair of organisms is representative of the variation shown by the corresponding genomes (Rosselló-Mora & Amann, 2001).

Previously there was less emphasis on biological testing, but increasingly there has been a focus on the biological properties of isolated compounds (Blunt, 2005). The probability of finding active metabolites is obviously dependent on the number of bacterial strains screened and on the primary tests. Therefore, the core of this work was to complement the continuing studies on bioactive compounds from marine bacteria and was directed toward substances showing antimicrobial activities. Twenty-seven bacterial strains were screening and the cultivation conditions for growth and crude extract production from the interesting strains was

carried out. This was followed by the purification of the bioactive compounds and examination of the activities of the new compounds using different biological systems.

During this discussion different aspects will be considered, the influence of the nature of the habitats from which these bacterial strains were isolated on the output of the fermentation process, the isolated compounds and their activities in respect to some related known compounds, the pathways through which some compounds might be produced and especially nitration of the aromatic compounds, and the pathway through which **WZ 436S-16** might induce apoptosis in the promyelocytic leukaemia cells (HL-60).

4.1. Salicylic acid-, oxindole-derivatives and WZ 268S-6 from *Pseudoalteromonas* sp. T268

The genus ***Pseudoalteromonas*** was created in 1995. It comprises most species that belonged to the genus *Alteromonas* (Gauthier *et al*., 1995) except the type species *Alteromonas macleodii*. Members of this genus are pigmented and non-pigmented bacteria that are frequently isolated from marine habitats (Holmström & Kjelleberg, 1999; Kalinovskaya *et al.,* 2004).

Pseudoalteromonas species produce extracellular agarases (Leon *et al.,* 1992; Vera *et al.,* 1998) and proteases (Chen *et al.,* 2003), antimicrobial protein (Longeon *et al.,* 2004), brominated compounds (Gauthier & Flatau, 1976; Laatsch & Pudleiner, 1989), antifungal (Gil-Turnes *et al.,* 1989), antibacterial (Sawabe *et al.,* 1998; Isnansetyo & Kamei, 2003), algicidal (Lovejoy *et al.,* 1998) and antifouling agents (Holmström *et al.,* 1998), and the neurotoxin tetrodotoxin (Simidu *et al.,* 1990).

Addition of glucose to the M_1-medium had no beneficial effect on the growth of T268 and repressed the production of bioactive crude extract. Although the presence of glucose can enhance the growth of some bacterial strains, it may suppress some enzymes that are important in biosynthesis of antibiotics. Gallo and Katz (1972), for example, observed that glucose inhibits the enzyme that catalyses the formation of the phenoxazinone ring of actinomycin.

Most of the metabolites from *Pseudoalteromonas* sp. T268 were simple phenolic derivatives, e.g salicylic acid (SA), homogentisic acid (HGA) and homogentisic acid methylester (HGM) were isolated from this strain. SA is known mainly as a plant metabolite,

but its production from different bacterial strains was evident (Ratledge & Winder, 1962; Gaille *et al.,* 2002) as a precursor of secondary metabolites and siderophores, e.g. pyochelin from *Pseudomonas aeruginosa* (Mercado-Blanco *et al.,* 2001). In plants, SA is produced through phenylpropanoid pathway, while several experiments proved the biosynthesis of SA from isochorismate in bacteria. A postulated pathway for synthesis of SA and HGA is shown in Fig. 4.1.

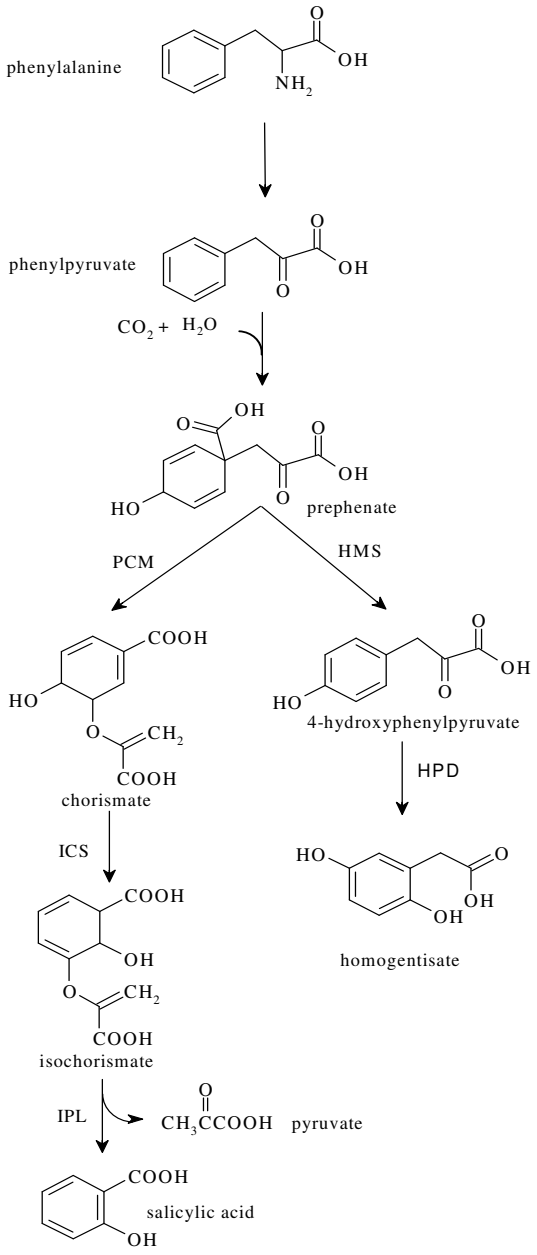


Fig. 4.1. Postulated biosynthesis of salicylic acid and homogentisic acid in *Pseudoalteromonas* sp. T268. HPD: 4-OH-phenylpyruvate dioxygenase, HMS: 4-OH-phenylpyruvate synthetase, IPL: isochorismate-pyruvate lyase, ICS: isochorismate synthetase, PCM: prephenate-chorismate mutase

Although SA did not exhibit antimicrobial activities either in agar diffusion test or in serial dilutions assay at 100 µg/ml, it inhibited the germination of *Magnaporthe grisea* at 15 µg/ml. SA is an ubiquitous mediator in the systemic acquired resistance (SAR) of plants against plant pathogens. Recently it was reported that SA affected the physiology and down regulated the virulence factors of *P. aeruginosa* and *Staphylococcus aureus* using *Arabidopsis thaliana* as a biological model. It disrupted the aggregation/biofilm formation and ability of these pathogens to attach to abiotic and biotic surfaces (Prithiviraj *et al.,* 2005a & b).

HGA was not significantly active at 100 µg/ml against bacteria in contrast to HGM. Herein HGM is reported, for the first time, as an antibacterial agent. HGA acid and its related HGM were cytotoxic. HGM was more cytotoxic than HGA with IC_{50} of 3-6 µg/ml. Methylation of the carboxylic side chain in HGA enhanced the antibacterial and the cytotoxic activities. The HGM isolated from the *Entada phaseoloides* inhibited the proliferation of P-388 cells with nearly the same IC value as in this study (Dai *et al.,* 1991). Moreover, HGA caused oxidative DNA damage through generation of active oxygen species, which explains the increase in the risks of different alimentary canal cancers observed with high consumption of high protein foods (Hiraku *et al.,* 1998).

Salicylic acid, HGA, and HGM were phytotoxic by inhibiting root elongation, especially for the dicotyledonous seed. This was also evident for the effect of SA on seedlings of lettuce and tomato (Loffredo *et al.,* 2005). The root systems of these plants were more sensitive than the shoot systems. Several phytotoxic phenolic compounds, including SA, inhibited the mitochondrial electron transport system causing oxidative stress by production of reactive oxygen species (ROS) and inactivation of catalase and ascorbate oxidase in plants (Rao *et al.,* 1997; Xie & Chen, 1999).

The production of HGA and **WZ 268S-6** was differentially affected by presence of salt in the culture medium. Absence of salts from the medium resulted in a 59 fold increase in HGA, but production of **WZ 268S-6** was no longer detected in M_1-medium. Increase in the amount of salts (salt stress) resulted in diminution of HGA and an increase in **WZ 268S-6** in comparisons to the normal culturing conditions. A similar effect was noticed in *Streptomyces coelicolor.* The production of actinorhodin was inhibited by high salt concentrations and production of undecylprodigiosin was increased by salt stress (Sevcikova & Kormanec, 2004).

WZ 268S-6 showed weak antimicrobial and moderate cytotoxic activities. It had no effect on the germination of monocotyledonous seeds but promoted the growth of

dicotyledonous seeds at concentrations less than 167 µg/ml. Unfortunately, its structure is not yet elucidated. The cytotoxic activity is not due to the inhibition in the synthesis of macromolecules (DNA, RNA and protein).

Isatin was isolated as a major compound (~1.2 mg/l) with **3-hydroxy-3-acetonyl-oxindole** as a minor substance (0.035 mg/l). **Isatin** was isolated for the first time in 1989 from marine *Pseudoalteromonas* sp. associated with crustacean eggs. It showed antifungal activity by protecting the crustacean eggs against the pathogenic fungus *Lagenidium callinectes* (Gil-Turnes *et al*., 1989). This compound was also isolated from other *Pseudoalteromonas* species (Kalinovskaya *et al.,* 2004) and from *Halomonas* sp. (Liang, 2003). Isolation of isatin in this study pointed out to the possibility of using this substance as a chemotaxonomic marker on the members of the *Alteromonas*-like bacteria.

3-Hydroxy-3-acetonyl-oxindole was isolated by the group of Fenical from natural source (unpublished data, AntiBase) and is known since a long time to be produced from condensation of isatin with acetone in presence of diethylamine (Braude, 1933). Acetone was not used during the purification steps and the substance was detected in daily samples by analytical HPLC. This excludes the possibility that this substance might be an artefact produced by purification procedures.

In this study isatin exhibited antimicrobial and phytotoxic activities at 100 µg/ml and 167 µg/ml respectively. In contrast, 3-hydroxy-3-acetonyl-oxindole showed only cytotoxic activity against L1210 and Jurkat cells with 50-100 µg/ml. According to my knowledge, there were no studies describing isatin as antibacterial compound. In similar studies, isatin-3-thiosemicarbazone derivatives (**41)** exhibited antiviral and tuberculostatic activity (Pandeya *et al.,* 2005), N-acyl-isatin derivatives (**42)** showed antibacterial activity against *S. aureus* at very high concentrations (Dilber *et al.,* 1990). In a study on isatin derivatives as γ-lactams that were proposed to mimic the classical β-lactam antibiotics, they showed no significant activity against the tested bacteria or inhibition of the transpeptidase (Hadfield *et al.,* 2002). In this study isatin exhibited activity, at 100 µg/disc, against the penicillin resistant *S. aureus* ATCC 11632. Therefore, it might have another role than inhibition of transpiptidase.

Interestingly, brominated derivatives of oxindole (convolutamydines A-D, **43**) were isolated from the marine bryozoan *Amathia convoluta* (Zhang *et al.,* 1995). Convolutamydine A and B were active against HL-60 cells (Kamano *et al.,* 1995). This activity is in agreement with the cytotoxic effect of 3-hydroxy-3-acetonyl-oxindole on L1210 and Jurkat cell lines.

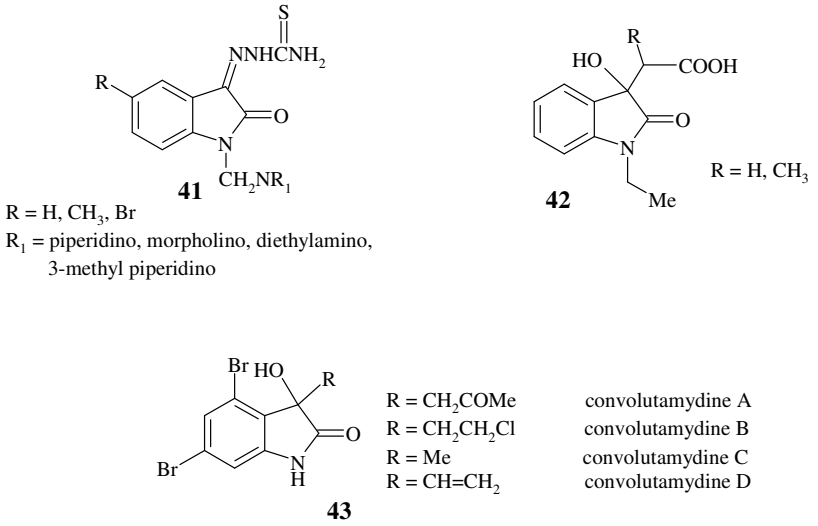


Fig. 4.2. Examples on oxindole derivatives.

The structural similarity between the compounds (**43**) from the *A. convoluta* and the oxindole in this study indicates that the microbial origin of convolutamydines could not be excluded.

4.2. Nitro-aromatic natural products

In this study, a large number of nitro-aromatic compounds were isolated. Although substances with a nitro group substitution have been reported from microbial origins (Carter *et al.,* 1989), they are relatively rare structural elements in nature (Winkler & Hertweck, 2005).

Only a small number of nitrated natural products were reported to have biological activities. They exhibited cytotoxic activities such as the polyketide aureothin (**44**) (Hirata *et al.,* 1961), antimicrobial activities such as chloramphenicol (**45**) (Rebstock *et al.,* 1949), pyrrolnitrin (**46**) (Arima *et al.,* 1964), pentabromopseudiline (Burkholder *et al.,* 1966), and dioxapyrrolomycin (**47**) (Carter *et al.,* 1987). Moreover, phytotoxicity of some substances with nitro substitutions might enhance the pathogenicity of several plant pathogens, e.g. *Pyricularia oryzae*. For example, nitro-phenyllactic acid derivatives (**48**) and nitro-phenyl acetic acid (**49**) (Evidente *et al.,* 1990, 1992), thaxtomin A (**50**) (King & Lawrence, 1995), and several *o*-nitrophenols (Sviridov & Ermolinskii, 1990) were phytotoxic.

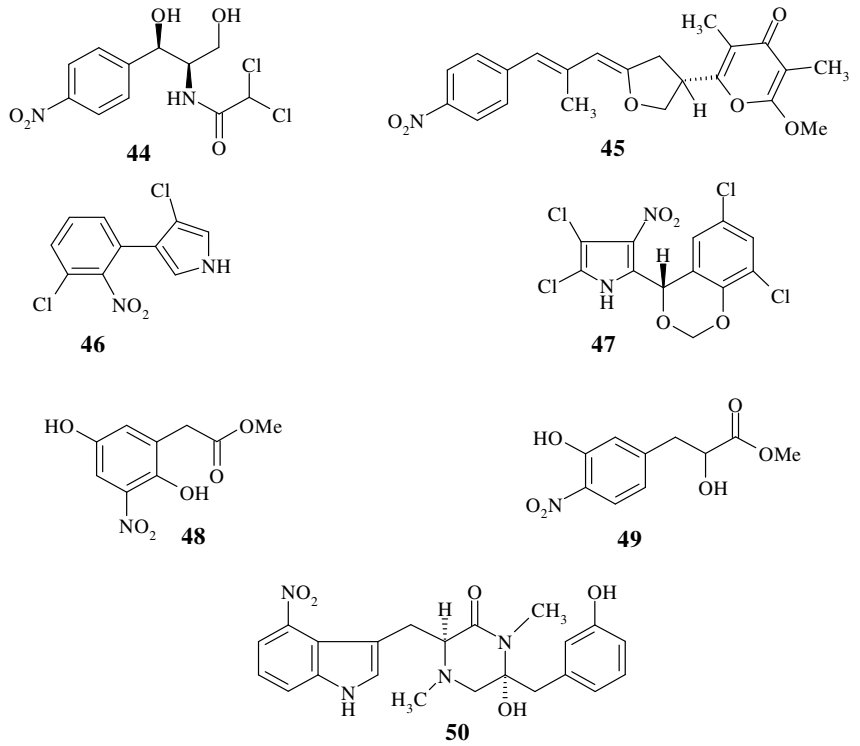


Fig. 4.3. Biologically active nitro-aromatic compounds. Chloramphenicol (**44**), aureothin (**45**), pyrrolnitrin (**46**), dioxapyrrolomycin (**47**), 2,5-dihydroxy-3-nitro-pheny acetic acid methyl ester (**48**), 3-hydroxy-4-nitro-phenyllactic acid methyl ester (**49**), and thaxtomin A (**50**).

From marine sources, nitro-containing compounds were represented by a few numbers of substances in the last 25 years. Antimicrobial activities were exhibited by the two nitro-purine derivatives from the bryozoan *Phidolopora pacifica*, phidolopine and desmethoxy-phidolopin (**51**) (Ayer *et al.,* 1984; Tischler *et al.,* 1986), and the isolated compound in this study, 4,6-dinitroguaiacol from the red algae *Marginisporum abberans* (Ohta, 1979).

Some of the nitrated substances were cytotoxic. The nitrotyramine derivative (**52**), from an unidentified anaerobic bacterium from marine sediments (Fu *et al.,* 1995), the sesquiterpenoid alcyopterosins C (**53**), E (**54**), and H (**55**), isolated from the marine soft coral *Alcyonium paeslleri* with other alcyopterosins (Palermo, 2000), were cytotoxic for several cell lines. Recently, lajollamycin (**56**) was isolated from *Streptomyces nodosus* as an inhibitor for the growth of B16-F10 tumor cells and Gram-positive bacteria (Manam *et al.,* 2005).

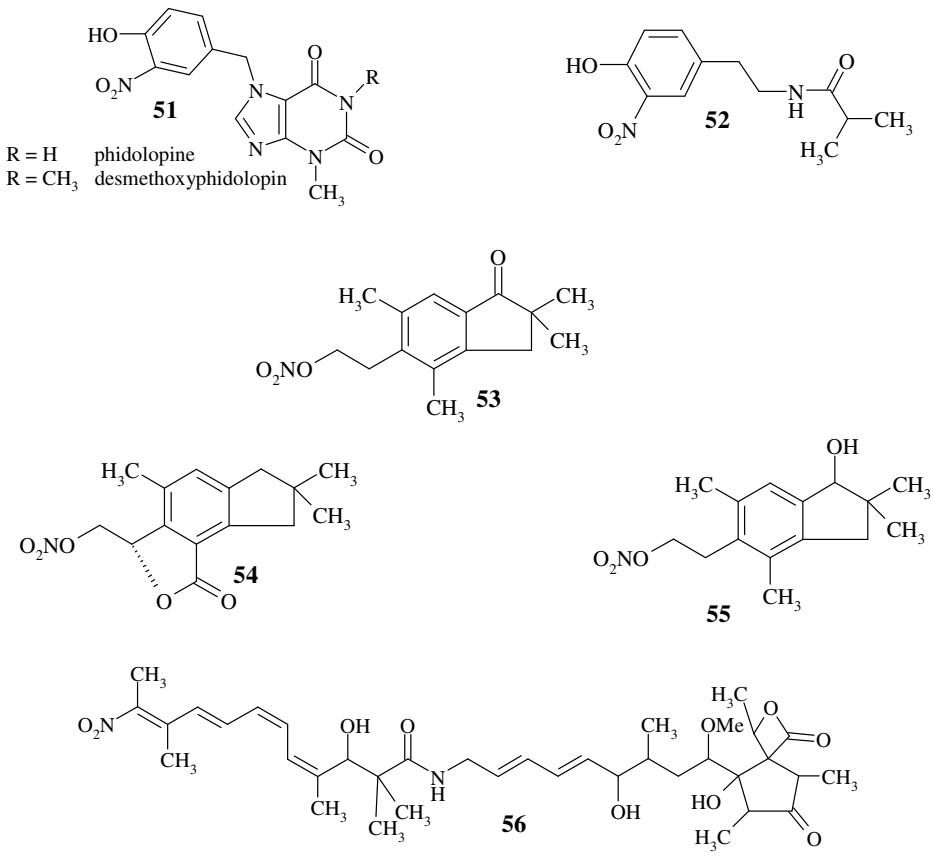


Fig. 4.4. Examples on bioactive compounds with nitro-substitution from marine habitats. Phidolopine and desmethoxy-phidolopin (**52**), N-(2-methylpropionyl)-3-nitrotyramine (**52**), alcyopterosin C (**53**), alcyopterosin E (**54**), alcyopterosin H (**55**), and lajollamycin (**56**).

4.2.1. Possible pathways for biosynthesis of nitro-compounds

The biotransformation of nitro-compounds to the corresponding amino-derivatives by degrading microorganisms was extensively studied (McCormick *et al.,* 1976; Hallas & Alexander, 1983; Haigler & Spain, 1993), but the biosynthesis of aromatic nitro group is not fully understood. In microorganisms, several pathways were proposed for nitration of organic compounds.

One possible pathway involves the aromatic substitution by direct nitration in the presence of KNO_3 in the growth medium. Such a pathway was evident for the biosynthesis of dioxapyrrolomycin by *Streptomyces fumanus* using the double-labelled $K^{15}N^{18}O_3$ (Carter *et al.,* 1989). Another possibility is the enzymatic oxidation of the amino group in aromatic compounds. This oxidation is the last step in the biosynthesis of most nitro containing antibiotics. Kirner and van Pée (1994) showed that pyrrolnitrin is formed from tryptophane

through several steps catalysed by a haloperoxidase, which ended with oxidation of the amino group of the intermediate metabolite, 3-(2-amino-3-chlorphenyl)-4-chlorpyrol, to the nitro group (Fig. 4.5).

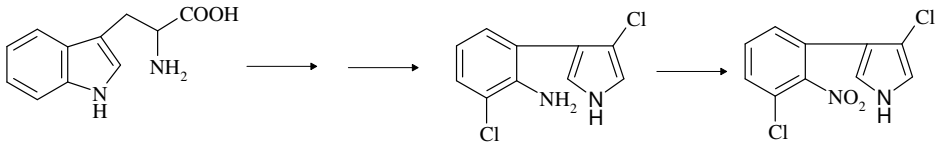


Fig. 4.5. Enzymatic oxidation of the amino group in biosynthesis of pyrrolnitrin (modified from Kirner and van Pée, 1994).

In this pathway, it was postulated that haloperoxidase, during the halogenation of the organic compounds, forms an acetate ester at the serine residue in the catalytic site. This ester is hydrolysed by hydrogen peroxide, resulting in the formation of peracetic acid as a strong oxidizing agent that unspecifically oxidizes the aromatic amino to nitro groups (van Pée, 1996).

A growing body of evidence supported that bacteria, have assimilatory nitrate reductase genes, use nitrate as a substrate to produce nitric oxide (NO) (Sakihama *et al.,* 2003). This introduced a new possibility for the biosynthesis of nitro compounds. 3-Nitrotyrosine was proposed as a diagnostic marker in oxidative pathology in tissues for the presence of peroxynitrite, radical nitrogen dioxide, and related reactive nitrogen species (Zhang *et al.,* 2001).

It was postulated that nitration of tyrosine-like organic compounds could be performed by the reactive nitrogen species (RNS). In normal physiological conditions, nitration may take place by two pathways. The first pathway depends on the production of peroxynitrite (Fig. 4.6). Superoxide radical, produced during the aerobic metabolic activities of microorganims, could react with a reactive nitrogen species to form a peroxynitrite radical. The high affinity of peroxynitrite to carbon dioxide results in the formation of a reactive intermediate (nitrosoperoxycarbonate).

The active unstable intermediate could nitrate tyrosine in physiological solutions (route B) or may decompose to the nitronium ion NO_2^+ and CO_3. Nitronium ion could nitrate tyrosine residues in proteins (route A). Moreover, this intermediate compound may decompose to NO_2 and CO_3^- radicals, which are able to oxidize tyrosine to the corresponding tyrosine radical that reacts with other molecule of NO_2 radical giving rise to 3-nitrotyrosine

(route C). Route (A) could be ruled out since the lifetime of nitronium ion is too short as it reacts with water to form nitrate (Squadrito & Pryor, 1998; Monzani *et al.,* 2004).

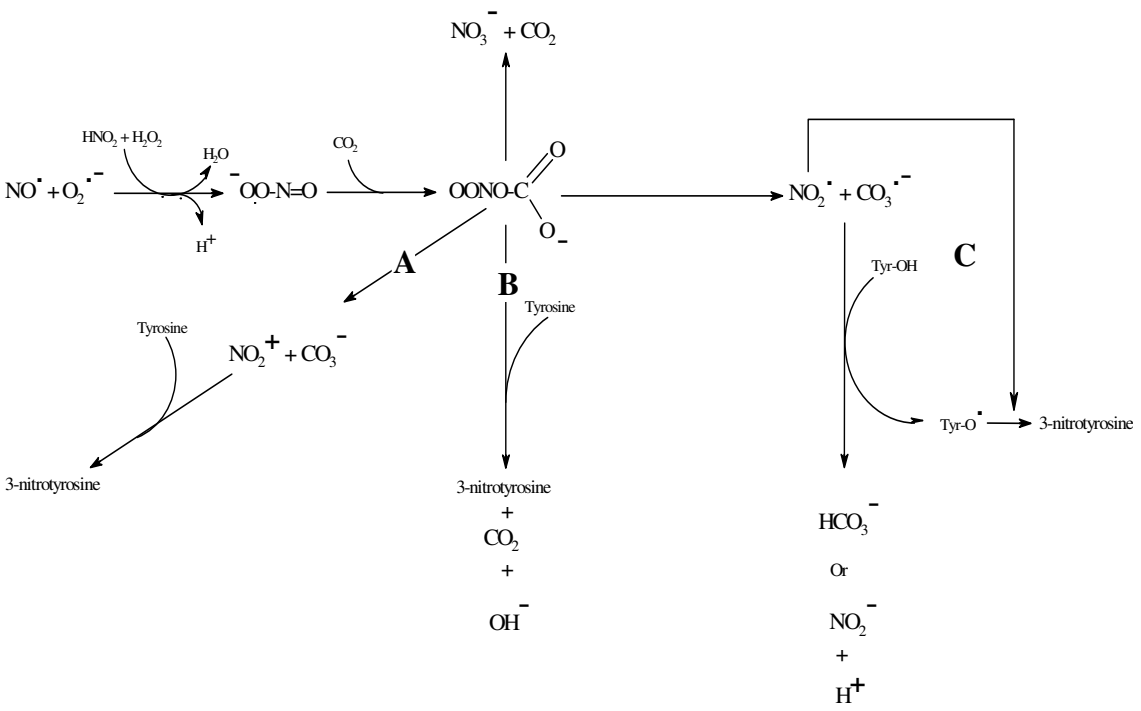


Fig. 4.6. Proposed peroxynitrite dependent pathways for the biosynthesis of 3-nitrotyrosine. (Modified from Kono *et al.,* 1998; Squadrito & Pryor, 1998; Lehnig & Jakobi, 2000; Zhang *et al.,* 2001; Sakihama *et al.,* 2003).

The second pathway is an enzymatic peroxynitrite independent nitration. Sakihama *et al.* (2003) proposed that nitration of tyrosine takes place by reaction of a tyrosine radical with a NO_2 radical; both resulted from oxidation with peroxidases in presence of H_2O_2 (reactions 1-3).

$$H_2O_2 + 2\ NO_2^- \xrightarrow{\text{peroxidase}} 2\ H_2O + 2\ NO_2^{\bullet} \qquad \text{(reaction 1)}$$

$$H_2O_2 + 2\ \text{Tyr-OH} \xrightarrow{\text{peroxidase}} 2\ H_2O + 2\ \text{Tyr-O}^{\bullet} \qquad \text{(reaction 2)}$$

$$2\ \text{Tyr-O}^{\bullet} + 2\ NO_2^{\bullet} \xrightarrow{\text{peroxidase}} 2\ \text{Tyr-NO}_2 \qquad \text{(reaction 3)}$$

It is mostly evident that formation of these mononitro/dinitroderivatives depends on the presence of potassium nitrate in the medium and the precursors used in the feeding experiments. The enzymatic determination for the presence of peroxidase activity was difficult as strain T436 was catalase positive. However, the production of a chlorinated compound in this study (**WZ 436S-17**) indirectly indicated the presence of a haloperoxidase. It is probable that biosynthesis of the mononitro/dinitrocompounds took place by one of the peroxinitrite dependent or independent pathways, especially as they are tyrosine-like derivatives. Production of nitrated isoflavonoids pointed out that nitration took place by direct substitution.

The group of Itoh, in 1993, transformed aniline to nitrobenzole using bromoperoxidase from *Pseudomonas putida*, however, this enzyme failed to oxidize arylamines such as *o*-chloraniline and *p*-toluidine to the corresponding nitro-derivatives (Kirner & van Pée, 1994). Peroxynitrite mediated nitration of 4-hydroxyphenylacetic acid catalysed by catalase was reported previously (Kono *et al.,* 1998).

4.2.2. Mononitro/dinitrosubstances from *Salegentibacter* sp. T436 are antimicrobial, phytotoxic and cytotoxic agents

Members of *Cytophaga-Flavobacterium-Bacteroides* division are common marine bacterioplanktons (Nedashkovskaya *et al.,* 2003) with a relative abundance that could reach to 72% in Antarctic Ocean (Glöckner *et al.,* 1999). The genus ***Salegentibacter*** comprises pigmented species that are phylogenetically related to *Flavobacterium* but their 16S rRNA sequence data indicated that these strains represent potentially distinct taxa within the *Flavobacteriaceae* (McCammon & Bowman, 2000). They are heterotrophic, motile by gliding or non-motile strains that are closely associated with live and dead phytoplanktons, i.e. they colonize living algae, and absorb nutrients produced during photosynthesis (Bowman & Nichols, 2002).

16S rDNA sequence analysis revealed that strain T436 is related to a number of unidentified species collected in cruises in Arctic and Antarctic sites. These species were collectively assigned to the genus *Salegentibacter*. Strain T436 differs from *S. holothuriorum* in five traits tested in this study. It reduces nitrate, does not grow at 37 °C, tolerates high salt concentrations (more than 10%), has no β-galactosidase activity, and is unable to utilize carbohydrates. It differs from *S. salegens* in requiring for yeast extract for growth in defined

mineral-salt medium, inability to hydrolyse starch, and absence of β-galactosidase activity. These traits and only a 97% similarity level of the 16S rDNA sequence of strain T436 to those of *S. holothuriorum* and *S. salegens* reveal that this bacterium represents a distinct species in the genus *Salegentibacter*.

Some heterotrophic bacteria, but not all, are able to grow on NO_3^- as a sole nitrogen source (Richardson *et al.,* 2001) and have functional assimilatory nitrate reductase genes (nasA) (Allen *et al.,* 2001). A remarkable feature in the exhaust gas analysis profile during fermentation of strain T436 was the double rate production of carbon dioxide in comparison with the consumed oxygen, indicating an anaerobic condition. This observation was correlated with the ability of this strain to reduce nitrate ions and to produce nitro-aromatic compounds. Moreover, survival (viability) of this bacterium in sea ice bottom, which represents a microenvironment with a low ambient oxygen concentration and abundant autotrophic microbiota, reveals the capability of this strain to utilize electron acceptor sources other than oxygen for energy and respiration during decomposition and mineralization of dissolved and particulate organic matters. Therefore, in oxygen limiting state, *Salegentibacter* sp. T436 was able to oxidize organic matters and produce carbon dioxide using nitrate as electron acceptor.

This is the first time in which a species from this genus is fermented and such a huge number (20 compounds) of nitrated substances are produced. Nitration took place in the ortho site in respect to the position of the hydroxyl group. It is known that most active dinitrated compounds have nitro-substitutions at the ortho- and para- positions. Presence of a side chain in the para-position relative to the hydroxyl group may result in nitration at position 3 and 5 (ortho-positions).

It is highly possible that these substances were produced from metabolism of phenylalanine. This is supported by the ability of strain T436 to utilize L-phenylalanine but not L-tyrosine as a carbon and nitrogen source (Table 7.1, appendix). These substances were nitrophenyl-acetate and -propionate derivatives. In general most of these derivatives were weakly antimicrobial, with biostatic effect, and weakly cytotoxic.

All mononitro-compounds were known from natural sources except **WZ 436S-20**. Although **WZ 436S-9** and **WZ 436S-10** were known as antibiotics T0007 B_1 and T0007 B_2 that showed specific activity against the Gram-positive *Clostridium difficile* (Zhang *et al.,* 1990). Herein, they were not active against bacteria and fungi up to 100 µg/ml. Another study reported **WZ 436S-9** as antibacterial agent against *E. coli* and *S. aureus* with MIC value of 2-

2.5 mM (~ 400-500 µg/ml) (Tesaki *et al.,* 1998). This indicates that **WZ 436S-9** could exhibit its activity at higher concentrations. In addition, this compound was used as a signal molecule in enhancing the ice-nucleation activity of *Xanthomonas campestris* (Watanabe & Watanabe, 1994; Watanabe *et al.,* 1994). It will be interesting in the future to answer the question, if the origin of strain T436 from sea ice is correlated with the production of such signal molecule.

Although **WZ 436S-1** and **WZ 436S-6** were previously reported as phytotoxic substances from *P. oryzae* (Sviridov & Ermolinskii, 1990), there were no data about their antimicrobial activities. **WZ 436S-1** showed, in contrast to **WZ 436S-6**, weak antimicrobial activity against *P. notatum, M. miehei,* and all bacterial strains other than *E. dissolvens.* It is possible that this activity was due to the methylester functionality in **WZ 436S-1** which makes it slightly neutral and lipophilic.

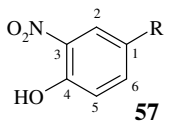
All 4-hydroxy-3,5-dinitrophenyl derivatives (seven compounds) are new from natural sources. Four of these derivatives were known as synthetic compounds. All these dinitrated derivatives were active in this study, at least in one biological system. Presence of a second nitro group enhanced the antibacterial activities of the dinitrated derivatives in comparison with their mononitrated analogues.

The antibacterial activities of the methylester derivatives of the nitro-substituted compounds (**WZ 436S-1**, **-3**, **-4**, and **-19**) were more pronounced than the antifungal ones in agar diffusion test, while **WZ 436S-2** and **WZ 436S-11**, nitrated derivatives with a carboxylic acid side chain, were antifungal. Correlation of these results with the behaviour of these compounds with different organics solvents in TLC-chromatography revealed that the presence of a second nitro group increases the polarity of these compounds and methylation decreases slightly this polarity. Therefore, all these differences in activities could be due to the differences in polarity and solubility of these analogues and subsequently their transport through the cell wall and membrane of the microorganism. Chloro-substitution in the ethyl side chain of the dinitrated **WZ 346S-17** enhanced its antimicrobial activity over the dinitrotyrosol **WZ 436S-21**. Halogen containing phenols were known for their antifungal activities (Gershon *et al.,* 1995).

Nitrated benzoic derivatives were known for their phytotoxic activities (Price & Wain, 1976). **WZ 436S-2** exhibited a selective activity against the yeast *N. coryli.* Although its activity was detected at 100 µg/ml in serial dilutions assay, but in agar diffusion test it inhibited the growth of this strain at 50 µg/ml. Generally the detected differences between the

activities in agar diffusion test and that in serial dilutions assay could be attributed to the solubility and diffusion of substances in the solid medium.

Several studies revealed that nitrophenyl derivatives are generally plant regulators. This is consistent with the results from the phytotoxicity test performed in this study. The majority of metabolites from *Salegentibacter* sp. T436 were phytotoxic at concentrations ranged from 67-333 µg/ml. Presence of a second nitro group in the phenyl derivatives decreased the activity of these compounds in contrast to their mononitro analogues. Presence of a nitro group in the 3-position and a hydroxyl group in the 4-position were found to be essential for the phytotoxic activity of the nitrophenol derivatives regardless of the function group in the 1-position (**57**) (Price & Wain, 1976; Tesaki *et al.,* 1998). Moreover, presence of a halogen substitution promoted the phytotoxicity of nitrophenols as in case of **WZ 436S-17** in comparison with **WZ 436S-21**. Halogen containing nitrophenols were known previously as herbicides (Scarno *et al.,* 2002).



57

Interestingly, **WZ 436S-2** (4-hydroxy-3-nitrobenzoic acid) promoted the elongation of root and shoot systems at a concentration less than 1 mM. However, it was phytotoxic at a higher concentration in accordance with the results obtained by Kisser-Priesack *et al.* (1990). Although it promoted seed germination at lower concentrations, it inhibited the chloroplast development (white leaves). Such observation was also reported in a study on the structure-activity relationships on nitrobenzoate derivatives (Price & Wain, 1976).

Isoflavonoids are known polyphenolic plant metabolites, especially from soy products (Verdrengh *et al.,* 2003). They are present in form of glucosidic conjugates that are hydrolysed by the glucosidase activity of microorganisms. Genistein is known as anticancer compound (Hedlund *et al.,* 2003), specific inhibitor for tyrosine kinase of the epidermal growth factor (Akiyama *et al.,* 1987), antiangiogenic agent (Büchler *et al.,* 2003), and anti-inflammatory substance and inhibitor of cycloxygenases (Lam *et al.,* 2004).

Mononitro- and dinitrogenistein (**WZ 436S-14** and **–15**) were previously known from a genetically engineered *Streptomyces* K3 (Shangguan *et al.,* 1999). They showed no significant antimicrobial or phytotoxic activities. Only mononitrogenistein was mildly cytotoxic for L1210 and Jurkat cells. Nitration of these isoflavonoids could lead to the inhibition in their activities against cancer cells. This was emphasized by the fact that mononitrogenistein was not potent as its parental genistein and dinitrogenistein was not active even in cytotoxicity test.

A potent activities were detected with **WZ 436S-16** (dinitro-β-styrene). Up to date, it was only reported from the Indian mangrove plant *Sonneratia acids* Linn. f. (Bose *et al.,* 1992) and was synthesized several times (Bose *et al.,* 2004). Herein, it was biologically characterized for the first time. It showed antibacterial (MIC 6.25-12.5 µg/ml) and antifungal (MIC 0.8-25 µg/ml) activities. The significant fungicidal activity of **WZ 436S-16** against *N. coryli* and the inhibition in the biosynthesis of macromolecules is due to the inhibition in oxygen uptake by this yeast (see 3.2.7.6). The decrease in cellular ATP influences all biological mechanisms, especially synthesis of DNA, RNA, and proteins. The position of the nitro groups in respect to the position of the hydroxyl group is as in 2,4-dinitrophenol (DNP), a known oxidative phosphorylation uncoupler. It cannot be ruled out that at lower concentrations (less than 0.5 µg/ml), **WZ 436S-16** stimulates the respiration in *N.* coryli, but at higher concentrations a total uncoupling is taking place concomitant with inhibition in respiration. The target for **WZ 436S-16** in bacterial cells is still to be explored.

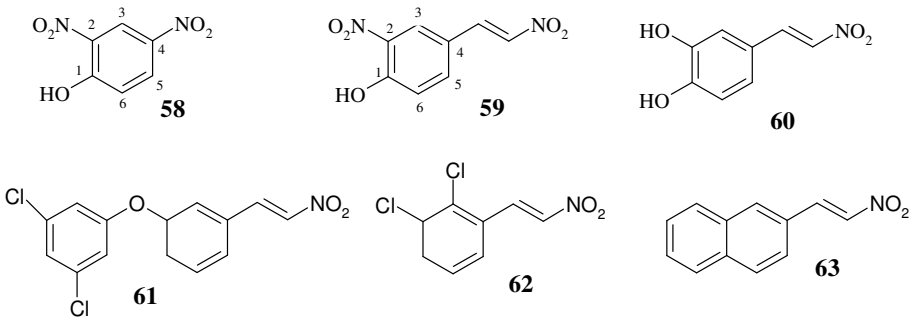


Fig. 4.7. 2,4-Dinitrophenol (DNP, **58**) and β-nitrostyrenes derivatives, WZ 436S-16 (**59**), SL-1 pigment (**60**), 3-(3,5-dichlorophenoxy)-nitrostyrene (**61**), 2,3-dichloro-nitrostyrene (**62**), and 2-(2-nitrovinyl)-naphthalene (**63**).

A similar β-nitrostyrene (SL-1, **60**) was isolated previously from *Streptomyces lavendulae*. It is a moderate antibacterial (MIC 12.5-25 µg/ml), weak antifungal (MIC 100 µg/ml) compound with specific activity against some dermatophytes species from the genus *Trichophyton* (MIC 6.25-25 µg/ml), and anti-proliferative agent against L1210 cells with IC_{50} of 1µg/ml (Mikami *et al.,* 1987; Mikami *et al.,* 1991).

WZ 436S-16 was cytotoxic for cell lines with IC_{50} of 5-30 µg/ml in contrast to 1 µg/ml of SL-1 with L1210 cells. That means, the presence of a nitro group in the adjacent position of the hydroxyl group enhances the antimicrobial activities of β-nitrostyrene but reduces its cytotoxic effect. A study by Kaap *et al.* (2003) revealed that a nitro group at the β-position of

the side chain in styrene derivatives is essential for their cytotoxic activity. Furthermore, substitutions at positions other than at the para-position improved this activity.

4.2.2.1. WZ 436S-16 causes cell differentiation, apoptosis and stimulates respiration process in the promyelocytic leukaemia (HL-60)

The cytotoxic effect of the dinitro-β-styrene, **WZ 436S-16**, against HL-60 cells was due to its effect on coupling of oxidative phosphorylation with electron transport in the electron transport system (ETS). The similarity in structural conformation between **WZ 436S-16** and DNP (position of nitro groups in respect to the hydroxyl group) explains this effect. Uncouplers have a hydrophobic character making them soluble in the bilipid membrane. They carry protons across the inner mitochondrial membrane into the matrix leading to alteration in membrane potential and cessation of ATP generation (Linsinger *et al.,* 1999). Although HL-60 cells and *N. coryli* are eukaryotic cells, the difference in their response to **WZ 436S-16** indicates that the reaction depend on the cell type and state as well as on the concentration of the effectors.

Prolonged exposure to uncouplers may lead to inhibition of respiration and the leakage of electrons from the ETS. These electrons react with oxygen to generate a ROS (Hao *et al.,* 2004). Formation of ROS induces the activation of signal proteins, such as p21, that inhibit cell cycle kinases. The p53 independent activation of p21 promotes the terminal differentiation of myeloid tumor cells (Erkle *et al.,* 2002). Differentiation of HL-60 cells with 5 µg/ml of **WZ 436S-16** might take place by the same pathway.

The results from the incorporation of radioactive precursors in synthesis of macromolecules and uncoupling of the oxidative phosphorylation from transport of electrons through the mitochondrial inner membrane in HL-60 cells indicated that these cells undergo programmed cell death. Differentiation of cells is frequently, at higher concentration of the inducers, followed by apoptosis (Erkel *et al.,* 2002; Rosato *et al.,* 2003). Cell-cycle state and availability of energy influence the commitment of cells to undergo apoptosis or necrosis (Richter *et al.,* 1996; Chernyak *et al.,* 2005). Apoptosis takes place in individual cells while necrosis involves group of cells. Morphologically, necrotic cells suffer from loss of homeostasis, swelling, undergo extensive membrane and intracellular organelle disruption and lysis. Formation of plasma membrane protrusions, cell shrinkage, chromatin condensation and formation of apoptotic bodies all are morphological indications on apoptosis (Fig. 4.8).

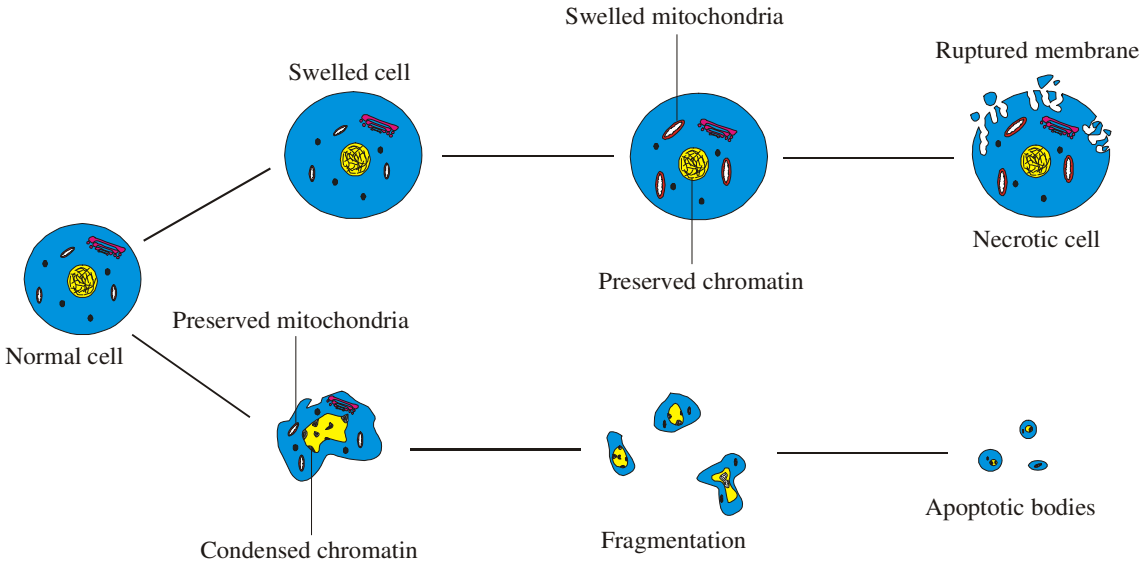


Fig. 4.8. Difference between morphological changes in apoptotic and necrotic cell.

Microscopic and molecular examinations (3.2.7.7) revealed that HL-60 cells underwent apoptosis. DNA fragmentation (DNA ladder), a hallmark sign of programmed cell death, was complemented with morphological examination of apoptotic cells stained with acridine orange. The acquired yellow to orange colouration was due to the accumulation of lysozymes around the apoptotic nucleus. Accumulation of masses of chromatin in the nuclear periphery, fragmentation, and the beginning of the formation of apoptotic bodies were evident.

Formation of DNA ladder indicated activation of endonucleases by cysteine proteases (caspases). They are enzymes that are activated by up-regulation of transcriptional signals and factors in the upstream of the apoptosis cascade. Since HL-60 cells are null-p53 (Pelicano *et al*., 2003), therefore, apoptotic induction and caspase activation took place independent of this transcriptional factor. Furthermore, the activation of these proteolytic enzymes due to alteration of mitochondrial membrane potential and release of cytochrome c, initiator of pro-caspase 9 activation, could take place.

Nitrostyrene derivatives were potent inhibitors of human telomerase (Kim *et al.,* 2003a). An enzyme that adds TTAGGG repeat onto the 3′ ends of chromosomes. Telomeres, which maintain chromosomal integrity, lose some of their repeats after each cell division because of the DNA end replication problem. Therefore, presence of telomerase in tumor cells and undifferentiated progenitors makes them immortals (Shay & Wright, 2002; Beitzinger *et al.,* 2006). Inhibition of telomerase makes the affected cells to enter a so-called cell senescence state. Upon senescing, cells suffer from DNA damage and undergo a temporarily

cell arrest in G_1 phase to allow the DNA repair machinery to fix the damage, or execute lethal programs such as apoptosis to retain the damaged cell from further expansion (Kahlem *et al.,* 2004). This was evident in the study of Kim *et al* (2003a). Screening of chemical libraries (compounds **61-63**) resulted in finding out a number of nitrostyrene derivatives that caused progressive telomere erosion followed by induction of senescence.

Theoretically, uncouplers induce maximal respiration rate at concentrations lower than that necessary for complete uncoupling. At these concentrations ATP synthesis is not completely blocked and steady state membrane potential is high. At higher concentrations of uncouplers, the membrane potential drops and ATP synthesis ceases. This was not the case in DNP that stimulated respiration at 0.1-0.2 mM but did not completely dissipate the membrane potential and inhibit the respiration even at a 20 fold higher concentration (Chernyak *et al.,* 2005). As DNP, **WZ 436S-16** stimulated the respiration rate in HL-60 cells at 5 µg/ml and did not inhibit it up to 30 µg/ml.

Uncouplers, by their own, have no effect on apoptosis or lead to DNA fragmentation over a wide range of concentrations (Moyes *et al.,* 2002). However, several studies showed that these compounds enhance the extrinsic apoptotic pathway as they sensitize the tumor cells to some apoptotic effectors in Jurkat and CEM cells (Linsinger *et al.,* 1999; Hao *et al.,* 2004). The fact that **WZ 436S-16** act as an uncoupler, which sensitize HL-60 cells suffering from replication crises due to telomerase inhibition, explains the induction of apoptotic behaviour in these cells.

In other words, the structural similarity of **WZ 436S-16** to β-nitrostyrenes (Fig. 4.7) that causing the formation of an aberrant DNA and leading to cell arrest, then its uncoupling ability of oxidative phosphorylation with ETS may promote tumor cells to execute apoptosis. The exact machinery through which apoptosis signalling took place is still unclear, but it is highly possible that it took place by an intrinsic mitochondrial dependent pathway. Further work is recommended with this compound to find out which signalling cascades are involved.

A study by Ramírez *et al.* (2003) demonstrated that telomere shortening was one of the early events that occurred in apoptosis prior to the activation of caspases. Moreover, they demonstrated that cells with short telomeres showed a drop in mitochodrial membrane potential and an increase in the ROS production leading to apoptosis.

Apoptosis is a highly dynamic process during which characteristic morphological and biochemical markers may be observed in cells for only a limited period of time. In this study, the maximum DNA fragmentation, on agarose gel, was not detected after the appearance of apoptotic morphological signs, but it was detected when cells showed signs of secondary necrosis under the inverted microscope. This indicated that internucleosomal DNA cleavage is a late event of apoptosis, which consistent with the observations of Kravtsov *et al.* (1999) in HL-60 cells.

4.2.3. Mononitro/dinitro-compounds from *Vibrio* sp. WMBA1-4

Large numbers of sedentary marine organisms are associated with bacteria. They produce secondary metabolites that play a role in protection against other pathogenic and fouling microorganisms. Vibrios include more than 60 species and comprise the major culturable bacteria in marine environment (Okada *et al.,* 2005). Several species in this genus are pathogens for fish, coral, and mammals (Arias *et al.,* 1999; Kushmaro *et al.,* 2001). I6S rDNA sequence indicated that strain **WMBA1-4** belongs to the genus *Vibrio* with *V. splindidus* biovar II and *V. shilonii* as the nearest relatives. However, it differs from *V. splindidus* in two traits in this study; namely, utilization of D-glucouronic acid and L-leucine. It differs from *V. shilonii* in the ability to grow with high salts concentrations (more than 6%), utilization of D-lactose and L-rhamnose. Therefore, strain **WMBA1-4** represents a distinct species in the genus *Vibrio*. Its inability to hydrolyse starch explains the weak growth in M_{10} in contrast to M_{11}.

Vibrio species were used several times as a model for studying the role of sodium and potassium ions in amino acids uptake by bacterial cell. Moreover, they were the origin of several secondary metabolites, such as tetrodotoxin and anhydrotetrodotoxin (Noguchi *et al.,* 1986), agarase (Sugano *et al.,* 1993), and siderophores (Jalal *et al.,* 1989).

4.2.3.1. Indole and 4-hydroxy-phenyl derivatives

Some of the nitrated 4-hydroxyphenyl derivatives from *Salegentibacter* sp. were also purified from *Vibrio* sp. Moreover, mononitro-hydroxybenzaldehyde and mononitro-cinnamate were isolated from this strain. Their activities did not differ significantly from those in 4.2.2. The IC_{50} value of 3-nitro-4-hydroxybenzaldehyde was less than that of the

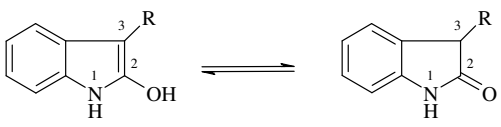
nitrophenyl derivatives reported from *Salegentibacter* sp. T436. The polarity of aromatic compounds with an aldehyde group side chain is less than the corresponding aromatic acids. Therefore, they are slightly lipophilic to the bilayer lipid membranes, especially if they are small molecules with low molecular weights. This explains the observed antimicrobial and nematicidal activities of **WMB4S-1** in this study.

WMB4S-6 (3-[3-nitro-4-hydroxyphenyl]-2-propenoic acid) showed a selective fungicidal activity against the yeast *N. coryli*. Taking into consideration that **WZ 436S-10** (3-nitro-4-hydroxyphenylpropionic acid) was not active up to 100 µg/ml, thus presence of the double bond in the cinnamate derivative promotes its antifungal activity. The analogue of **WMB4S-6** (**WMB4S-16**), that does not contain a nitro group, was not active up to 100 µg/ml, indicating the role of the nitro group in this antifungal activity.

The phytotoxic effect of these compounds was due to, like in the nitrated derivatives from *Salegentibacter* sp., the presence of a nitro group at 3-position and a hydroxyl group at 4-position. Absence of these groups in the mentioned positions was the cause of the inactivity of **WMB4S-16** in the phytotoxicity test. The aldehyde group in **WMB4S-1** might be either reduced to 3-nitro-4-hydroxybenzyl alcohol or oxidized to 3-nitro-4-hydroxybenzoic acid. Occurrence of such conversion should not alter the phytotoxic activity of the produced compounds as observed from incubation of pea and wheat shoot tissues in 3-nitro-4-hydroxybenzaldehyde (Price & Wain, 1976).

WMB4S-4 was known as a synthetic compound till this study. It is an oxindole derivative showing fungicidal activity, mainly against *N. coryli* and *U. nuda*. It was cytotoxic for L1210 and Jurkat cells as **WZ 268S-10** (3-hydroxy-3-acetonyl-oxindole) and convolutamydines. In literature there are no evidences for an active indole with OH-group on any position in the pyrrole ring.

Taking into consideration that **WMB4S-4**, as other oxindoles, might be present in two isoforms in physiological solution (**64** and **65**) and that isatin, in our test system, was not cytotoxic and weak antifungal, it could be deduced that presence of a hydroxyl group in 2-position is enhanced for the antifungal activity and a side chain of at least 1-carbon at 3-postion is essential for the cytotoxic activity against tumor cells. Moreover, comparing the activity of **WMB4S-4** with that of **WZ 268S-10** indicated that this hydroxy group at 2-position decreased the concentration needed from these compounds to trigger cytotoxic effects.



64 **65**

4.2.3.2. Maleimide and azirine derivatives

A number of nitrated bis-arylmaleimide and one diarylazirine compounds were purified from *Vibrio* sp. Compounds having a pyrrole ring system as a core in their structures are not frequently known as natural products. Several diarylmaleimide derivatives were isolated from fungal strains with their related succinimide derivatives (Fig. 4.9). The pigmented bis-indolylmaleimide compounds, arcyriarubins B and C (**66**) (Steglich *et al.,* 1980) and himanimides A-D (**67**, **68**) (Aqueveque *et al.,* 2002), diarylmaleimides with their related diarylsuccimide derivatives, isolated in our department, are examples of rarely occurring natural products with such a central ring system.

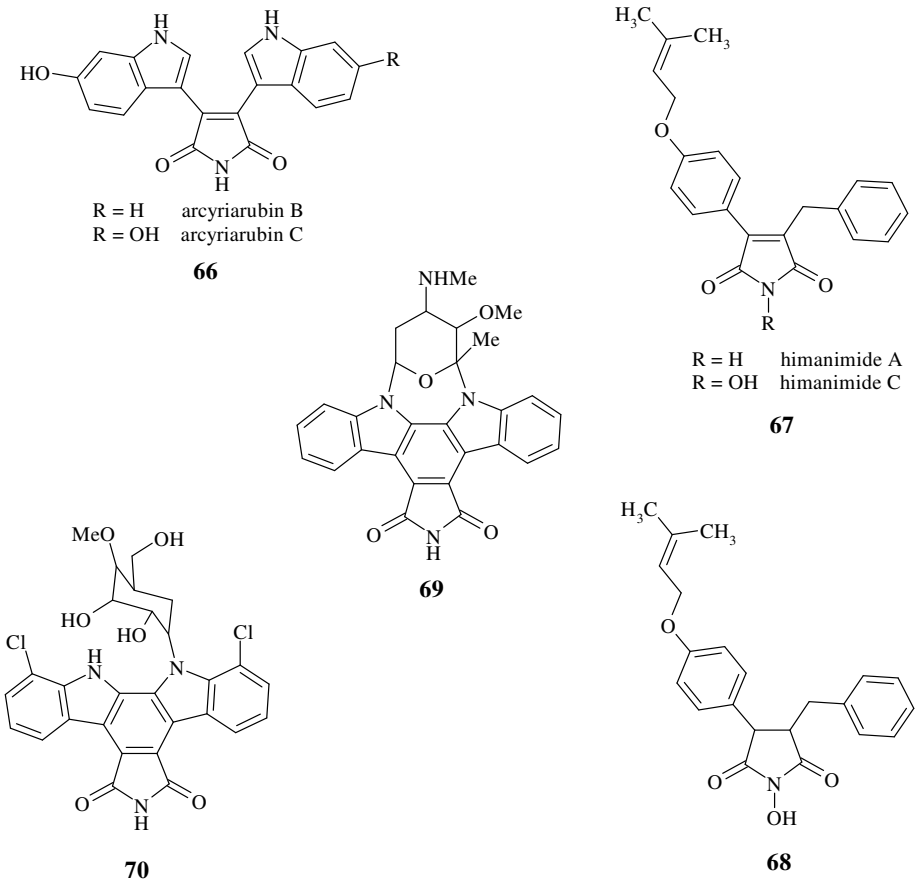


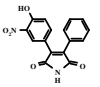
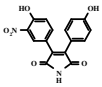
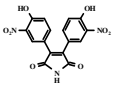
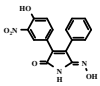
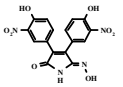
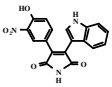
Fig. 4.9. Diarylmaleimide, diarylsuccimide, and diarylcarbazole metabolites from microbial origin. Arcyriarubin B & C (**66**), himanimide A & C (**67**), himanimide D (**68**), 7-oxostaurosporine (**69**), and rebeccamycin (**70**).

Several related bis-indolylmaleimide derivatives were synthesized as protein kinase inhibitors and antitumor agents (Davis *et al.,* 1992; Ohtsuka & Zhou, 2002) due to their

structural similarity with 7-oxostaurosporine (**69**) and rebeccamycin (**70**) isolated from streptomycetes (Bush *et al.,* 1987; Osada *et al.,* 1992). In all studies so far with these compounds, the double bond of the maleimide ring and the presence of a hydrogen bond donor ability in the maleimide unit appeared to be essential for their activities (Davis *et al.,* 1992; Kaletas *et al.,* 2005). Moreover, the general structure –CO-N(R)-CO- gives these compounds a hydrophobic and neutral nature and therefore they can cross biological membranes *in vivo* (Prado *et al.,* 2004).

The novel maleimide derivatives isolated in this study represent part of a series of alkaloids from marine bacteria. They are rarely encountered in nature and the presence of a nitro-substitution on the aryl ring gives them an extraordinary position. These compounds were, like their related alkaloids, active against bacteria and fungi. Moreover, they exhibited cytotoxic activities mainly against suspension cell lines. The variation in the activities within these substances might be attributed to the types and positions of substituted groups on the aryl ring and the hydrophobic nature of the aryl ring itself (Table 4.1).

Table 4.1. The most prevailed IC_{50} and MIC values of the maleimide derivatives

Activity	**Compounds**						
	 **WMB4S-9**	 **WMB4S-2**	 **WMB4S-10**	 **WMB4S-8**	 **WMB4S-11**	 **WMB4S-12**	 **WMB4S-13**
Antibacterial (MIC, µg/ml)	100	25	-	50-100	6-7	12.5	25
Antifungal (MIC, µg/ml)	50	50-100	-	100	50	100	50-100
Cytotoxic (IC_{50}, µg/ml)	30-40	50	-	15-60	15	15	15

In 3,4 diphenylmaleimides, presence of a nitro group adjacent to a hydroxyl group on one of the phenyl rings enhanced the antimicrobial activity. **WMB4S-2** was more active than **WMB4S-9**. Presence of a hydroxyl group on the second phenyl ring abrogated these activities, which is evident in case of **WMB4S-10** in contrast to **WMB4S-2**. The presence of a second nitro group in **WMB4S-8** retained the bioactivites and enhanced the cytotoxic

activity in comparison with **WMB4S-2**, **-9**, and **10**. This observation is consistent with investigations done by Davis *et al* on related compounds. In a structure-activity relationship, they showed that introduction of a nitro group in the phenyl ring of a synthetic diarylmaleimide (a maleimide moiety with a phenyl and indole rings) enhanced its inhibitory activity for protein kinase C (PKC) in comparison with related substances without nitro-substitution (Davis *et al.,* 1992).

Comparing the activities of the 3,4-bis-phenylmaleimide compounds, in which the imide group is flanked by two ketone groups, to those in which an oxime group replaces one ketone group, indicted that such substitution is essential for the enhancement of the antimicrobial activities. The antibacterial activity of **WMB4S-11** and **WMB4S-12** was more than that of **WMB4S-2** and **WMB4S-8** respectively. Formation of this oxime group resulted from reaction of a hydroxylamine with the side ketone in the maleimide moiety. Furthermore, presence of two 3-nito-4-hydroxyphenyl rings attached to the maleimide moiety in **WMB4S-12** reduced the antimicrobial activities in comparison with **WMB4S-11**.

Replacement of one of the phenyl rings with an indole ring enhanced the cytotoxic effect of the maleimide derivative (compare **WMB4S-13** with **WMB4S-2**). This might be attributed to the lipophilic nature of the indole ring in contrast to the phenol ring. This was also evident in investigations carried out by Davis *et al.* (1992).

To summarize the above analysis, presence of a mono-nitration in the phenyl group increase the antimicrobial activity, while presence of two 4-hydroxyphenyl groups or presence of two nitro groups (each on one phenyl group) reduced the antimicrobial activities. However, presence of two nitro groups enhanced the cytotoxic activity. Replacement of one of the ketones on the maleimide moiety with an oxime group enhanced the antibacterial and cytotoxic activities of the mononitrated derivatives and only the antibacterial activity of the dinitrated derivatives. Replacement of one of the phenyl rings with an indolyl ring increase the cytotoxic activity, which may attribute to the increase in the hydrophobicity of the compound.

The cytotoxic activity of **WMB4S-11** is attributed to the inhibition in the biosynthesis of protein in L1210 cells. This inhibition was reduced upon introduction of second hydroxyl and nitro groups in **WMB4S-12**. The antibacterial activity of these two compounds was not due to the inhibition in the biosynthesis of macromolecules or to the inhibition of oxygen uptake by *B. subtilis*. The other nitrated bis-arylmaleimide derivatives that showed potent

bioactivities had neither significant effect on the oxygen uptake nor on the biosynthesis of macromolecules in microorganisms and L1210 cells.

The azirine derivative (**WMB4S-5**) isolated in this study showed antibacterial and antifungal activities. Moreover, it exhibited a moderate cytotoxic activity against the different tumor cells. Presence of indolyl ring attached to the azirine ring renders a hydrophobic nature to this compound, which may be essential for its antimicrobial and cytotoxic activities. Occurrence of a bis-arylazirine is very rare in nature. To date, azirine derivatives are represented by one class of compounds in the marine environment, the cytotoxic azacyclopropenes (Molinski & Ireland, 1988; Salomon *et al.,* 1995). These compounds were isolated from the sponge *Dysidea fragilis.*

The phytotoxic activity of the nitrated alkaloids in this study could be attributed to the presence of the nitro group in a direct position adjacent to the hydroxyl group as the case in the other reported nitro-derivatives in this study. This was emphasized by the inactivity of **WMB4S-5** against the germinating seeds since it has only a nitro group attached to the phenyl ring in the bis-arylazirine. Moreover, the derivative (**WMB4S-9**), without nitrate substitution, showed a very weak phytotoxic activity only at the highest applied concentration (333 µg/ml). The monocotyledonous seed was more sensitive than the dicotyledonous seed to these compounds. The only maleimide derivative that exhibited a significant phytotoxic activity starting from 167 µg/ml was **WMB4S-11**. The presence of an oxime group in this compound enhanced the phytotoxic activity, compared with the other mononitrated maleimide derivatives, and inhibited the production of chlorophyll, which may indicate an effect on essential enzymes in the photosynthesis process.

In marine environment similar alkaloids were isolated from ascidians, sponges molluscs, and recently from bacteria. They are 3,4-diaryl-substituted pyrroles (**71**) with 2 or 2,5 functionalities. Lamellarins O, P, Q and R (**72**, lamellarins O and P), isolated from the sponge *Dendrilla cactos* (Urban *et al.,* 1994; Urban *et al.,* 1995), and polycitrins A and B (**73**), isolated from the ascidian *Polycitor* sp. (Rudi *et al.,* 1994), have the typical structural rearrangement of the maleimide derivatives isolated in this study. Moreover, Linag (2003) isolated similar bis-arylpyrroles from the bacterium *Halomonas* sp. (see compounds **38** and **39** in Fig.1.12).

They were all derived from DOPA (2-amino-3-[3´,4´-dihydroxyphenyl] propionic acid) or from tyrosine (Urban *et al.,* 2000). This is in agreement with the ability of strain **WMBA1-4** to utilize L-tyrosine but not L-phenylalanine as N and C source.

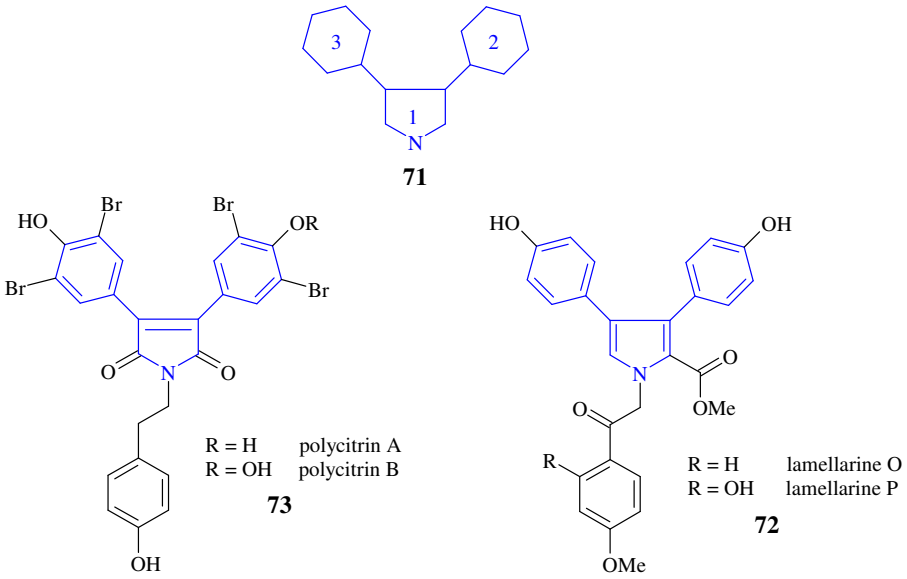


Fig. 4.10. Marine metabolites with similar structural arrangements to diarylmaleimide derivatives.

The structural similarity between the metabolites in Fig. 4.10 and the maleimide derivatives in this study points out to the role of the symbiotic organisms in the production of these compounds.

4.2.3.3. Bis-indolylalkane derivatives from *Vibrio* sp. WMBA1-4 cultivated in M_1

Although the crude extract of *Vibrio* sp. WMBA1-4 in M_1 medium did not exhibit antimicrobial activities in the preliminary screening, TLC chromatogram showed a series of chromophores. Mainly two bis-indolylalkanes were isolated, **WMB4S-14** (2-bis-indolylethan, vibrindole A) and **WMB4S-15** (*meso*-phenyl-2-bis-indolylmethan or its cationic form Turbomycin B). Vibrindole A is known since 1994 from the marine *Vibrio parahaemolyticus* (Bell & Carmeli, 1994), while *meso*-phenyl-2-bis-indolylmethan is so far known as a synthetic compound (He *et al.,* 2006). Turbomycin B was recently isolated from one of the *E. coli* clones containing DNA fragments from soil samples (Gillespie *et al.,* 2002).

Although vibrindole A and turbomycin B were known as compounds with antibacterial activities, they did not show antibacterial or antifungal activities in this study. In a similar study, Veluri *et al.* isolated a trimeric indole (1,1,1-tris [3-indolyl] methane) as a noncationic form of turbomycin A (**74**) (Veluri *et al.,* 2003). In contrast to turbomycin A, this trimeric indole was inactive against all tested bacterial strains. This finding enforced the assumption

that **WMB4S-15** is the synthetic *meso*-phenyl-2-bis-indolylmethan and not turbomycin B (**75**).

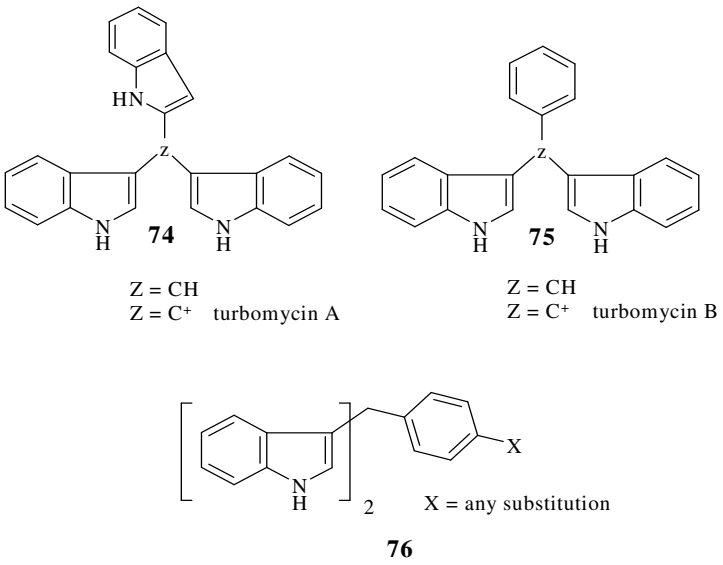


Fig. 4.11. Substituted bis-indolylmethane derivatives.

Despite their inactivity against tested microorganisms and plant seeds, they were cytotoxic compounds with IC_{50} of 10-15 µg/ml. This is the first time that these compounds are reported as anticancer agents. In previous studies with similar compounds, ring substituted diindolylmethane (**76**), bis-indolylalkanes proved to cause activation of PPARγ, inhibition of ERK1/2 and subsequently induction of apoptosis in various tumor cell lines (Qin *et al.,* 2004; Chintharlapalli *et al.,* 2005; Contractor *et al.,* 2005). Since not enough amounts of **WMB4S-14** and **–15** were isolated in this study, further fermentations are needed in the future to complement the results of cytotoxicity test with apoptotic experiments.

5. Summary

In this study, 27 marine bacteria were screened for production of bioactive metabolites. Two strains from the surface of the soft coral *Sinularia polydactyla*, collected from the Red Sea, and three strains from different habitats in the North Sea were selected as a promising candidates for isolation of antimicrobial substances.

A total of 50 compounds were isolated from the selected bacterial strains. From these metabolites 25 substances were known from natural sources, 10 substances were known as synthetic chemical and herein are reported as new natural products, and 13 metabolites are new. Two substances are still under elucidation. All new compounds were chemically and biologically characterized.

***Pseudoalteromonas* sp. T268** produced simple phenol and oxindole derivatives. Production of **homogentisic acid** and **WZ 268S-6** from this bacteria was affected by the salinity stress. **WZ 268S-6** shows antimicrobial and cytotoxic activities. Its target is still unclear. Isolation of isatin from this strain points out for the possibility of using this substance as a chemotaxonomical marker for *Alteromonas*-like bacteria.

A large number of nitro-substituted aromatic compounds were isolated from both ***Salegentibacter* sp**. **T436** and ***Vibrio* sp**. **WMBA1-4**. They may be derived from metabolism of phenylalanine or tyrosine.

From ***Salegentibacter* sp. T436**, 24 compounds were isolated, of which four compounds are new and six compounds were known as synthetic chemicals. **WZ 436S-16** (dinitro-β-styrene) is the most potent antimicrobial and cytotoxic compound. It inhibits the oxygen uptake by *N. coryli* and causes apoptosis in the human promyelocytic leukaemia (HL-60 cells).

From ***Vibrio* sp. WMBA1-4**, 13 new alkaloids were isolated, of which four were known as synthetic products and herein are reported as new substances from natural sources. The majority of these compounds show antimicrobial and cytotoxic activities. The cytotoxic activity of **WMB4S-11** against the mouse lymphocytic leukaemia (L1210 cells) is due to the inhibition in the protein biosynthesis, while the remaining cytotoxic alkaloids have no effect on the synthesis of macromolecules in this cell line. The antibacterial activity of **WMB4S-2**, **-**

11, **-12**, **-13** and the antifungal activity of **WMB4S-9** are not due to the inhibition in the macromolecules biosynthesis or in the oxygen uptake by the microorganisms.

The biological activity of these nitro-aromatic compounds from ***Salegentibacter*** **sp. T436** and ***Vibrio*** **sp. WMBA1-4** is influenced by the presence of a nitro group and its position in respect to the hydroxyl group, number of the nitro groups, and the type of substitutions on the side chain. In diaryl-maleimide derivatives, types and position of substitution on the aryl rings, on the maleimide moity, and the hydrophobicity of the aryl ring itself lead to variations in the extent of the bioactivity of these derivatives.

This is the first time that vibrindole (**WMB4S-14**) and turbomycin B or its noncationic form (**WMB4S-15**), isolated from ***Vibrio*** **sp.**, are reported as cytotoxic compounds. **WMB4S-15** inhibits the biosynthesis of macromolecules in L1210 cells.

The structural similarity between some of the metabolites in this study and previously reported compounds from sponges, ascidians, and bryozoan indicates that the microbial origin of these compounds must be considered.

Zusamenfassung

Im Rahmen dieser Arbeit wurde die Produktion der bioaktiven Metabolite von 27 marinen Bakterienstämmen untersucht. Zwei Stämme, die auf der Oberfläche der Weichkoralle *Sinularia polydactyla* im Roten Meer leben, und drei Stämme aus verschiedenen Habitaten der Nordsee wurden zur Isolierung der antimikrobiellen Substanzen ausgewählt.

Aus diesen Stämmen wurden insgesamt 50 Verbindungen isoliert. 25 dieser Substanzen sind als Naturstoffe bereits aus anderen Quellen bekannt. Weitere 10 Substanzen, die bisher nur als synthetische Verbindungen bekannt waren, wurden nun auch als Naturstoffe beschrieben. 13 Metabolite sind neu. Die Strukturaufklärung von zwei isolierten Substanzen steht noch aus. Die neuen Verbindungen wurden chemisch und biologisch charakterisiert.

***Pseudoalteromonas* sp. T268** produziert strukturell einfache Phenole und sich ähnelnde Oxindole. Die Konzentration von Meersalz steuert die Produktion der Homogentisinsäure und von **WZ 268S-6**. **WZ 268S-6** zeigt antibakterielle und zytotoxische Wirkung. Der Wirkort ist bisher noch nicht bekannt. Isatin, eine ebenfalls von diesem Stamm produzierte Substanz, könnte als ein chemotaxonomischer Marker für *Alteromonas*-ähnliche Bakterienstämme verwendet werden.

Aus zwei weiteren Bakterien, ***Salegentibacter* sp. T436** und ***Vibrio* sp. WMBA1-4**, konnte eine Vielzahl aromatischer Nitroverbindungen isoliert werden, die sich von Phenylalanin oder Tyrosin ableiten. Weiterhin wurden 24 Verbindungen aus ***Salegentibacter* sp. T436** isoliert, von denen vier Verbindungen bisher unbekannt waren und sechs weitere nur als synthetische Stoffe beschrieben wurden. **WZ 436S-16** (Dinitro-β-styren) zeigt in dieser Gruppe die stärkste Wirkung. Es hemmt bei *N. coryli* die Sauerstoffaufnahme und führt in der humanen promyelocytischen Leukämie-Zelllinie HL-60 zur Apoptose.

Aus dem Stamm ***Vibrio sp.* WMBA1-4** wurden 13 Alkaloide isoliert, von denen vier bisher nur als synthetische Substanzen bekannt waren. Für die Mehrheit dieser Substanzen konnte eine antimikrobielle und zytotoxische Wirkung nachgewiesen werden. Während für **WMB4S-11** mit der Hemmung der Proteinbiosynthese in L1210 Zellen der Wirkort eingegrenzt werden konnte, zeigten die übrigen zytotoxischen Verbindungen keinen Effekt auf die Makromolekülsynthese in L1210 Zellen. Die antibakteriellen Aktivitäten von

WMB4S-2, **-11**, **-12**, **-13** und die antifungische Aktivität von **WMB4S-9** sind ebenfalls nicht auf eine Hemmung der Makromolekülbiosynthese oder der Sauerstoffaufnahme durch empfindliche Mikroorganismen zurückzuführen.

Die biologische Aktivität der aromatischen Nitroverbindungen von ***Salegentibacter*** **sp. T436** und ***Vibrio*** **sp. WMBA1-4** wird durch die Anwesenheit einer Nitrogruppe und ihrer Position in Bezug auf die Hydroxylgruppe, die Anzahl an Nitrierungen und die Art der Substitutionen an der Seitenkette beeinflusst. Bei Diaryl-maleimid Derivaten führen Art und Position der Substitutionen an den Arylringen und an der Maleimid-Gruppe sowie die Hydrophobizität des Arylringes selbst zu Schwankungen in der Intensität der biologischen Wirkung.

In dieser Arbeit konnte zum ersten Mal die zytotoxische Wirkung der aus *Vibrio* sp. isolierten Substanzen, Vibrindol (**WMB4S-14**), Turbomycin B oder ihrer nicht kationischen Form (**WMB4S-15**), nachgewiesen werden. Es wurde gezeigt, dass **WMB4S-15** die Biosynthese der Makromoleküle in L1210-Zellen hemmt.

Die strukturelle Ähnlichkeit zwischen einigen Metaboliten dieser Arbeit und zuvor beschriebener Verbindungen aus Porifera, Ascidien und Bryozoen verlangt nach einer kritischen Betrachtung des mikrobiellen Ursprungs dieser Substanzen.

6. Literature

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7. Appendix

Table 7.1. Biochemical characterization of *Pseudoalteromonas* sp. T268, *Salegentibacter* sp. T436 and *Vibrio* sp. WMBA1-4.

Test	Bacterial strain		
	***Pseudoalteromonas sp.* T268**	***Salegentibacter sp.* T436**	***Vibrio sp.* WMBA1-4**
Oxid/Ferm :			
D-Glucose	+/-	-/-	+/+
D-Lactose	-/-	-/-	-/-
Sucrose	+/-	-/-	-/-
L-Arabinose	-/-	-/-	-/-
D-Xylose	-/-	-/-	-/-
Fructose	-/-	-/-	+/+
Maltose	+/-	-/-	+/+
Rhamnose	-/-	-/-	-/-
Manitol	-/-	-/-	+/+
Glycerol	+/-	-/-	+/+
Raffinose	-/-	-/-	-/-
Sorbitol	-/-	-/-	-/-
D-Galactose	-/-	-/-	+/+
Organic compounds as C-source :			
Sodium acetate	+	+	+

Oxid/Ferm: oxidation/fermentation C: carbon source N: nitrogen source

Continued

Test	Bacterial strain		
	***Pseudoalteromonas sp.* T268**	***Salegentibacter sp.* T436**	***Vibrio sp.* WMBA1-4**
D-Glucuronic acid	-	-	+
Succinic acid	+	-	+
Glycine	+	-	+
L-Tyrosine	-	-	-
Hydroxy butyric acid	-	+	-
α-Aminoisobutyric acid	-	+	-
p-Aminobenzoat	-	-	-
DL-Lactic acid	-	+	+
Oxalic acid	+	-	+
L-Alanine	+	+	+
Sodium glutamate	+	+	+
D-Aspartic acid	-	-	+
Sodium pyruvate	+	+	+
Organic compounds as C and N-source :			
L-Alanine	+	+	+
L-Leucine	+	-	+
L-Proline	+	+	+
p-Aminobenzoat	-	-	+
D-Aspartic acid	+	-	+
L-Lysine	-	-	-
L-Histidine	+	+	+

Oxid/Ferm: oxidation/fermentation C: carbon source N: nitrogen source

Continued

Test	Bacterial strain		
	***Pseudoalteromonas sp.* T268**	***Salegentibacter sp.* T436**	***Vibrio sp.* WMBA1-4**
L-Cysteine	+	-	+
L-Asparagine	+	-	-
L-Phenylalanine	+	+	-
L-Glutamine	+	+	+
L-Tyrosine	+	-	+
H_2S-production	-	-	-
Indole production	-	-	+
Nitrate reduction	-	+	+
Hydrolysis of :			
Starch	-	-	-
Gelatine	+	+	+
Esculin	+	+	+
Esterase activity (Tween 80)	+	+	+
Tyrosine hydrolysis	+	-	-
Arginine dihydrolase	-	-	-
ß-galactosidase	-	-	-
NaCl requirement	+	+	-
Motility	+	-	+
Spore	-	-	-
Capsule	-	-	-
Poly-β-hydroxybutyrate	-	-	-

Oxid/Ferm: oxidation/fermentation C: carbon source N: nitrogen source

Table 7.2. Rate of flow (R_f) and retention times of the isolated metabolites in various mobile phases.

Compound	R_f-value Mobile phase					Analytical HPLC Retention time [min]	
	L1	L2	L3	L4	L5	G_1	G_4
WZ 268S-2	0.75	0.69	0.53	0.75	0.91	18.03	13.25
WZ 268S-4	0.64	0.6	0.38	0.75	0.91	15.09	11.33
WZ 268S-6	0.44	0.19	0	0.65	0.5	15.71	10.95
WZ 268S-8	0.16	0.11	0.09	0.68	0.19	3.13	2.51
WZ 268S-9	ND	ND	ND	ND	ND	5.55	5.5
WZ 268S-10	0.2	0.16	0.1	0.73	0.5	6.02	5.95
WZ 436S-2	0.5	0.08	0.05	0.67	0.35	10.8	8.04
WZ 436S-3	0.56	0.25	0.11	0.81	0.51	12.41	10.55
WZ 436S-4	0.55	0.35	0.19	0.78	0.56	14.07	11.54
WZ 436S-11	0.48	0.08	0	0.69	0.29	11.45	9.28
WZ 436S-12	0.48	0.06	0	0.69	0.24	9.5	8.24
WZ 436S-13	0.53	0.55	0.35	0.79	0.69	14.16	10.41
WZ 436S-17	0.52	0.31	0.16	0.79	0.5	15.43	12.45
WZ 436S-19	0.5	0.16	0.16	0.78	0.41	15.82	12.53
WZ 436S-20	0.63	0.58	0.35	0.78	0.75	ND	9.11
WZ 436S-21	0.5	0.25	0.15	0.78	0.41	9.73	8.37
WMB4S-1	0.6	0.45	0.3	0.75	0.69	9.3	8.3
WMB4S-2	0.69	0,64	0.5	0.79	0.75	18.1	12.43
WMB4S-3	0.5	0.19	0.18	0.64	0.83	4.95	5.25
WMB4S-4	0.6	0.56	0.43	0.79	0.66	11.6	8.75

ND : not determined

Continued

Compound	R_f-value Mobile phase					Analytical HPLC Retention time [min]	
	L1	L2	L3	L4	L5	G_1	G_4
WMB4S-5	0.54	0.5	0.39	0.79	0.64	18.5	12.96
WMB4S-6	0.5	0.19	0.13	0.63	0.71	15.36	9.24
WMB4S-7	ND	ND	ND	ND	ND	ND	9.9
WMB4S-8	0.61	0.53	0.38	0.75	0.75	16.75	11.7
WMB4S-9	0.54	0.54	0.44	0.79	0.65	15.86	10.95
WMB4S-10	0.55	0.53	0.4	0.81	0.64	15.36	10.65
WMB4S-11	0.55	0.54	0.4	0.81	0.64	17.4	11.68
WMB4S-12	0.53	0.46	0.31	0.76	0.63	16.5	10.97
WMB4S-13	0.55	0.53	0.38	0.78	0.69	18.12	11.87
WMB4S-15	0.66	0.65	0.6	0.8	0.81	ND	15.67
WMB4S-16	0.46	0.25	0.19	0.7	0.44	ND	7.2

ND : not determined

7.1. UV-, IR-, and mass spectra of the compounds from *Pseudoalteromonas* sp. T268.

7.1.1. WZ 268S-2 and WZ 268S-6

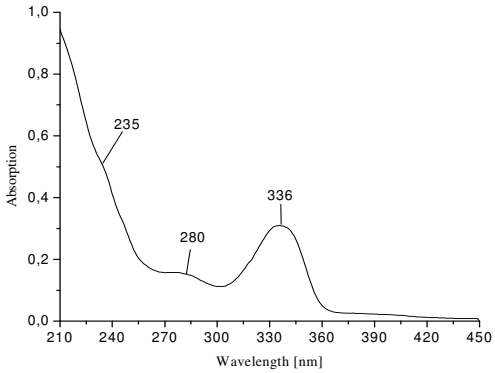

Fig. 7.1. UV-spectrum of **WZ 268S-2** and **WZ 268S-6** in methanol (42 µg/ml and 21.5 µg/ml). UV_{max} are 235 nm, 280 nm and 336 nm.

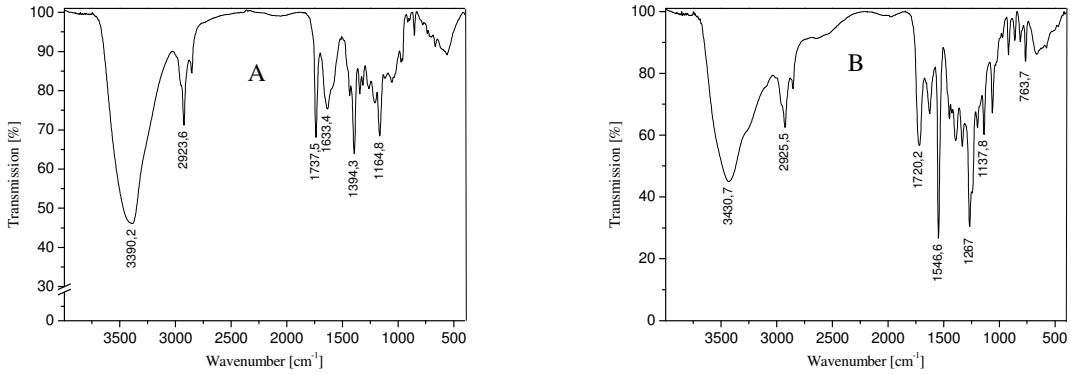

Fig. 7.2. IR-spectra of **WZ 268S-2** (100 µg/34 mg KBr) (A) and of **WZ 268S-6** (100 µg/35 mg KBr) (B).

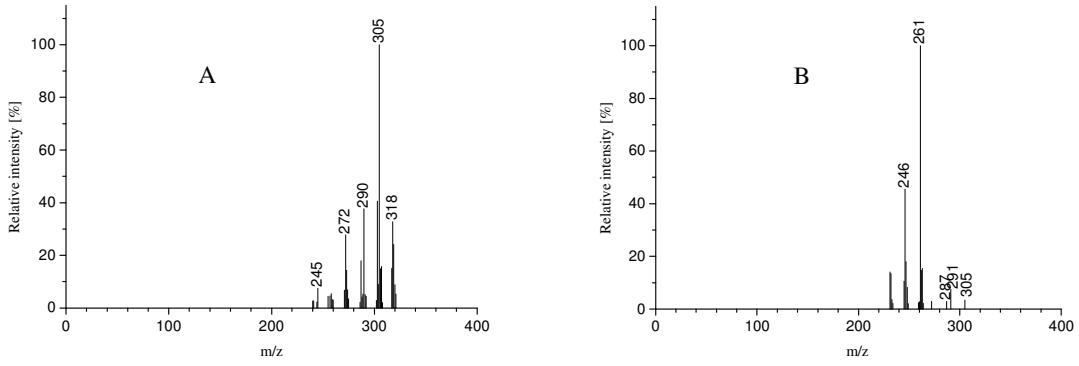

Fig. 7.3. Mass-spectra of APCI negative ionization of **WZ 268S-2** (A) and **WZ 268S-6** (B).

7.1.2. WZ 268S-4

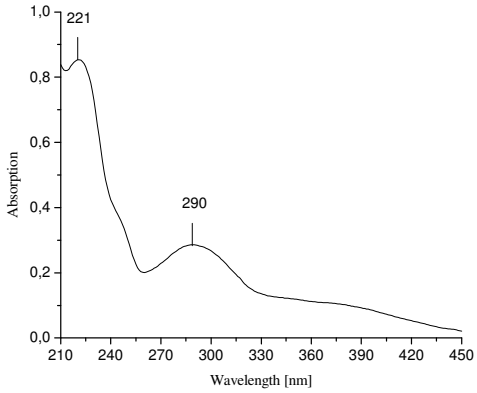

Fig. 7.4. UV-spectrum of **WZ 268S-4** in methanol (29.1 µg/ml). UV_{max} are 221 nm and 290 nm.

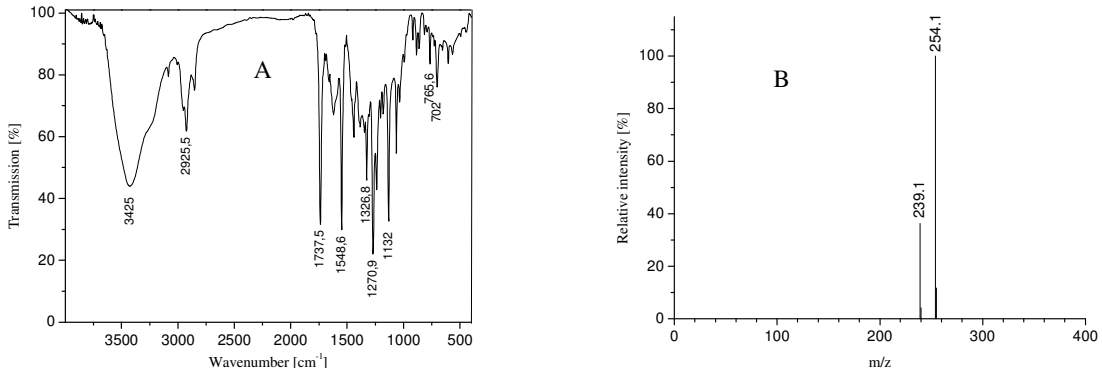

Fig. 7.5. **WZ 268S-4**, IR-spectrum (34 µg/100 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

7.1.3. WZ 268S-8 and WZ 268S-9 (homogentisic acid and homogentisate methylester)

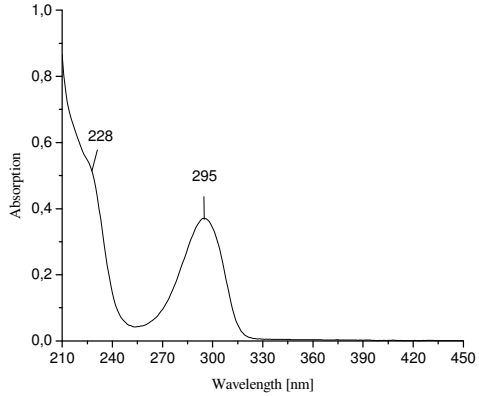

Fig. 7.6. UV-spectrum of **WZ 268S-8** and **WZ 268S-9** in methanol (52.1 µg/ml). UV_{max} are 228 nm and 295 nm.

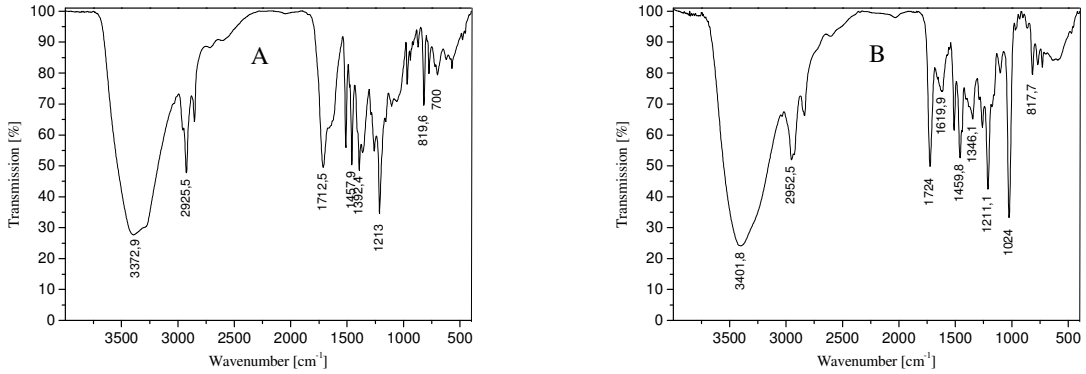

Fig. 7.7. IR-spectra of **WZ 268S-8** (100 µg/33.6 mg KBr) (A) and **WZ 268S-9** (100 µg/35 mg KBr) (B).

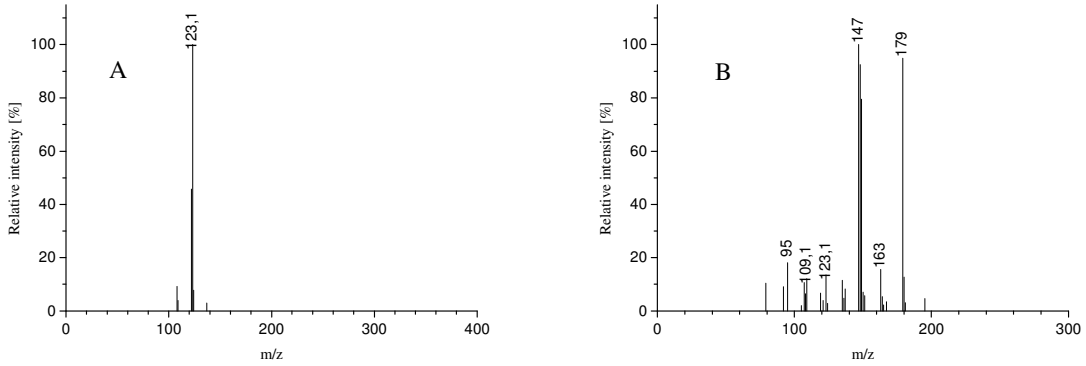

Fig. 7.8. Mass-spectra of APCI negative ionization of **WZ 268S-8**. (A) and **WZ 268S-9** (B).

7.2. Compounds from *Salegentibacter* sp. T436.

Table 7.3. Chemical names of the substances from *Salegentibacter* sp.

Compound	Chemical name	Note	Reference
WZ 436S-1	4-Hydroxy-3-nitrophenylacetic acid methyl ester	K	Sviridov & Ermolinskii, 1990
WZ 436S-2	4-Hydroxy-3-nitrobenzoic acid	NS	Bose *et al.,* 2006
WZ 436S-3	4-Hydroxy-3,5-dinitrophenylacetic acid methyl ester	NS	Commercial
WZ 436S-4	4-Hydroxy-3,5-dinitrophenylpropionic acid methyl ester	N	-
WZ 436S-5	Isomer of **WZ 436S-5a :** 4,6-Dinitroguiacol and **WZ 436S-5b :** 4,5-Dinitro-3-methoxyphenol	K	Ohta, 1979
WZ 436S-6	4-Hydroxy-3-nitrophenylethanol	K	Sviridov & Ermolinskii, 1990
WZ 436S-9	4-Hydroxy-3-nitrophenylacetic acid	K	Zhang *et al.,* 1990
WZ 436S-10	4-Hydroxy-3-nitrophenylpropionic acid	K	Zhang *et al.,* 1990
WZ 436S-11	4-Hydroxy-3,5-dinitrophenylpropionic acid	NS	Commercial
WZ 436S-12	4-Hydroxy-3,5-dinitrophenylacetic acid	NS	Commercial
WZ 436S-13	3-Nitro-1H-indole	NS	Berti *et al*., 1968
WZ 436S-14	3',5'-Dinitrogenistein	K	Shangguan *et al.,* 1999
WZ 436S-15	3'-Nitrogenistein	K	Shangguan *et al.,* 1999
WZ 436S-16	2-Nitro-4-[2'-nitroethenyl] phenol	K	Bose *et al.,* 1992
WZ 436S-17	2-[4-Hydroxy-3,5-dinitrophenyl] ethyl chloride	N	-
WZ 436S-19	3-[4-Hydroxy-3,5-dinitrophenyl]-2-chloropropionic acid methyl ester	N	-
WZ 436S-20	3-[4-Hydroxy-3-nitrophenyl]-2-hydroxy propionic acid methyl ester	N	-
WZ 436S-21	4-Hydroxy-3,5-dinitrophenylethanol	NS	Commercial

K: Known substance NS: reported as synthetic (not from natural source) N: Newly reported

7.2.1. UV-, IR-, and mass spectra of the isolated compounds

7.2.1.1. Nitrated 4-hydroxy-phenyl derivatives

7.2.1.1.1. WZ 436S-2 (4-hydroxy-3-nitrobenzoic acid)

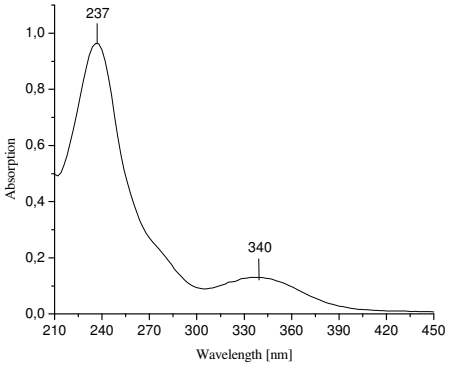

Fig. 7.9. UV-spectrum of **WZ 436S-2** in methanol (7.4 µg/ml). UV_{max} are 237 nm and 340 nm.

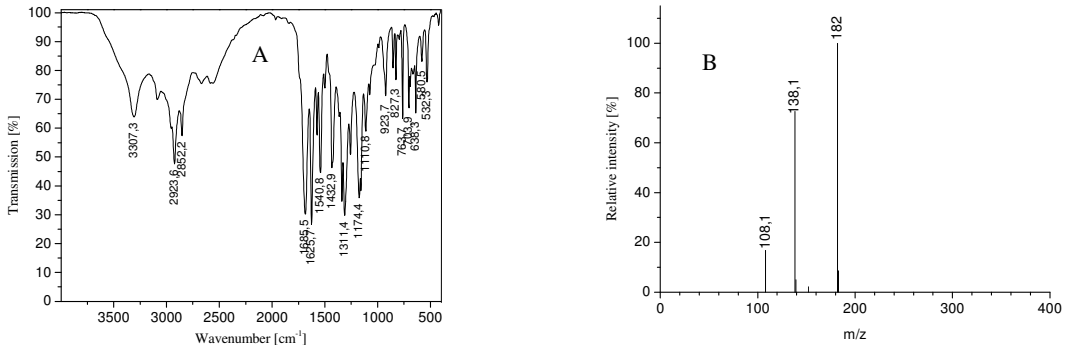

Fig. 7.10. WZ 436S-2, IR-spectrum (100 µg/33.3 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

7.2.1.1.2. 4-Hydroxy-3-nitrophenyl derivatives and 4-hydroxy-3,5-dinitrophenyl derivatives (compounds WZ 436S-3, -4, -11, -12, -17, -19-21)

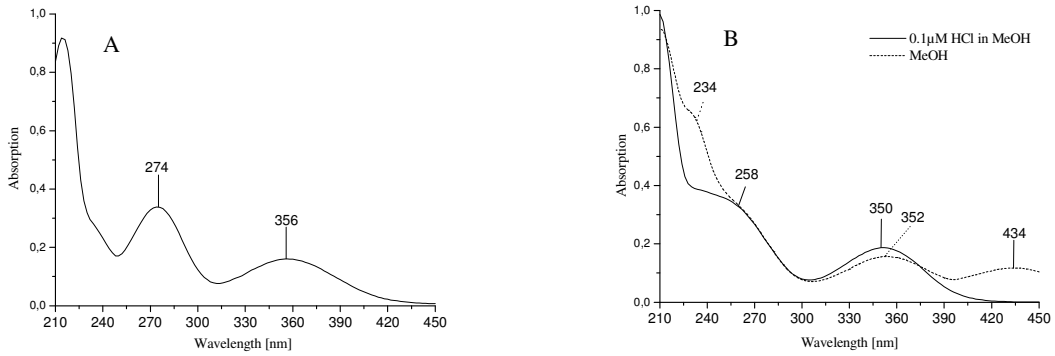

Fig. 7.11. UV-spectrum of 4-hydroxy-3-nitrophenyl derivatives (A) and 4-hydroxy-3,5-dinitrophenyl derivatives in methanol.

7.2.1.1.2.1. WZ 436S-3

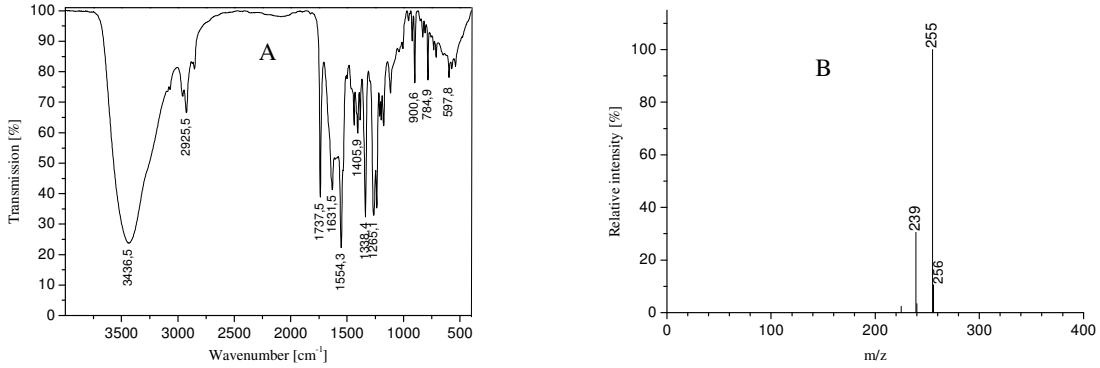

Fig. 7.12. WZ 436S-3, IR-spectrum (100 µg/34 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

7.2.1.1.2.2. WZ 436S-4

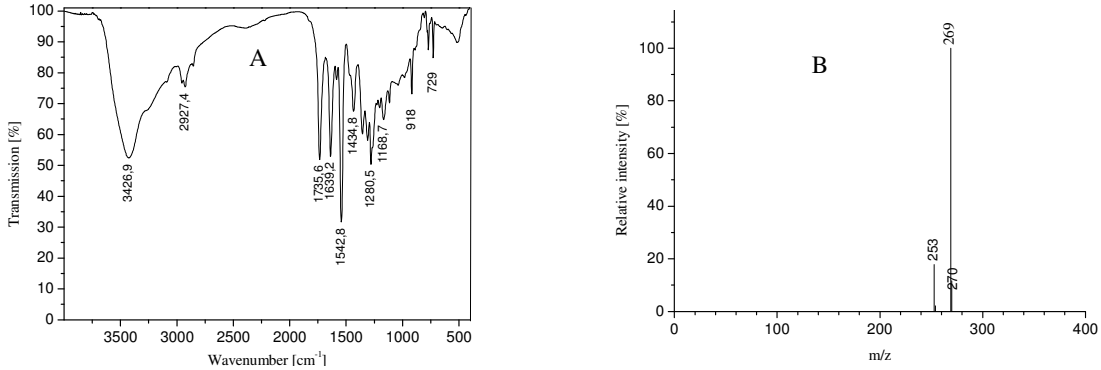

Fig. 7.13. WZ 436S-4, IR-spectrum (100 µg/34.5 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

7.2.1.1.2.3. WZ 436S-11

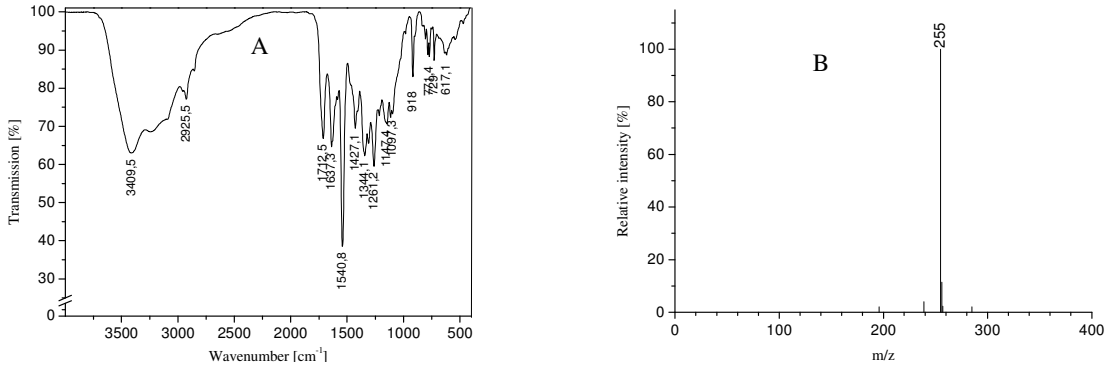

Fig. 7.14. WZ 436S-11, IR-spectrum (100 µg/34.8 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

7.2.1.1.2.4. WZ 436S-12

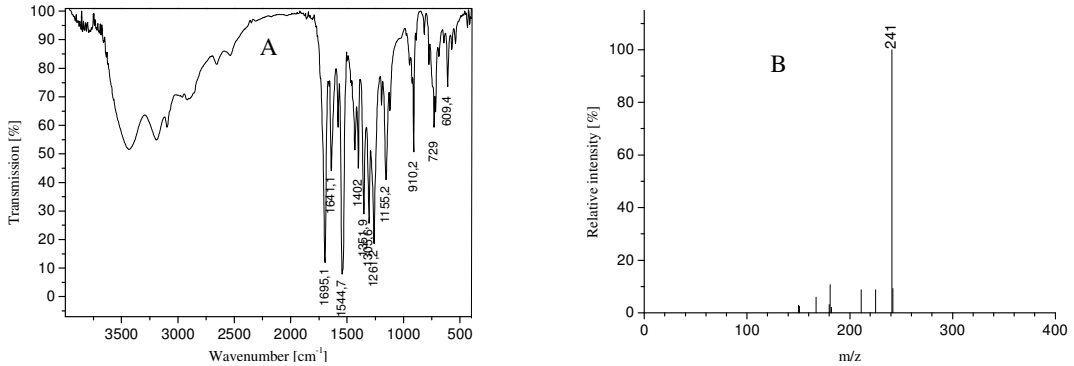

Fig. 7.15. WZ 436S-12, IR-spectrum (100 µg/34.7 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

7.2.1.1.2.5. WZ 436S-17

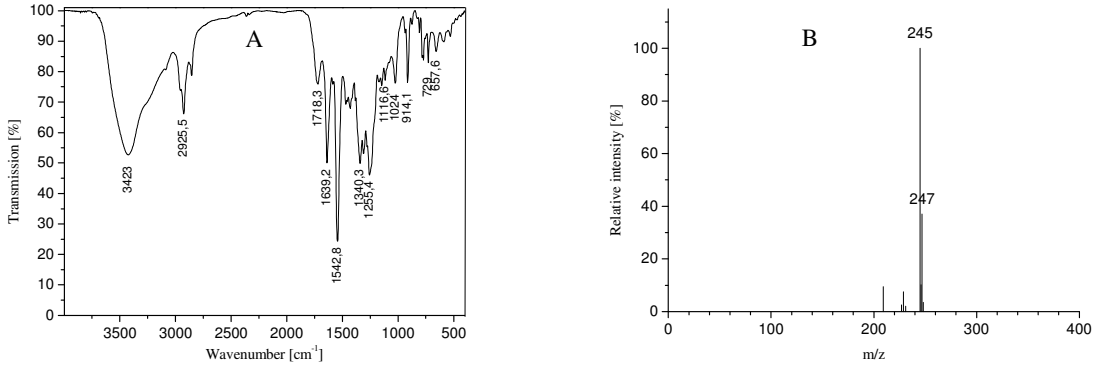

Fig. 7.16. WZ 436S-17, IR-spectrum (100 µg/34 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

7.2.1.1.2.6. WZ 436S-19

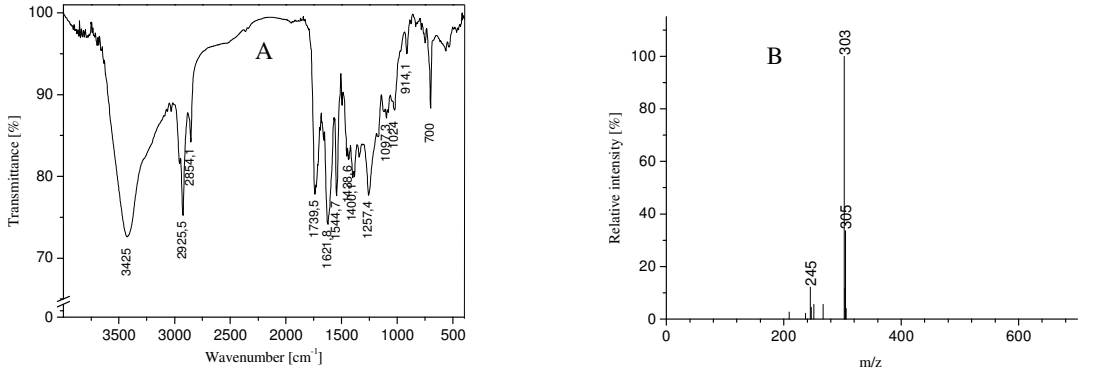

Fig. 7.17. WZ 436S-19, IR-spectrum (100 µg/33.5 mg KBr) (A) and mass-spectrum APCI negative ionization (B)

7.2.1.1.2.7. WZ 436S-20

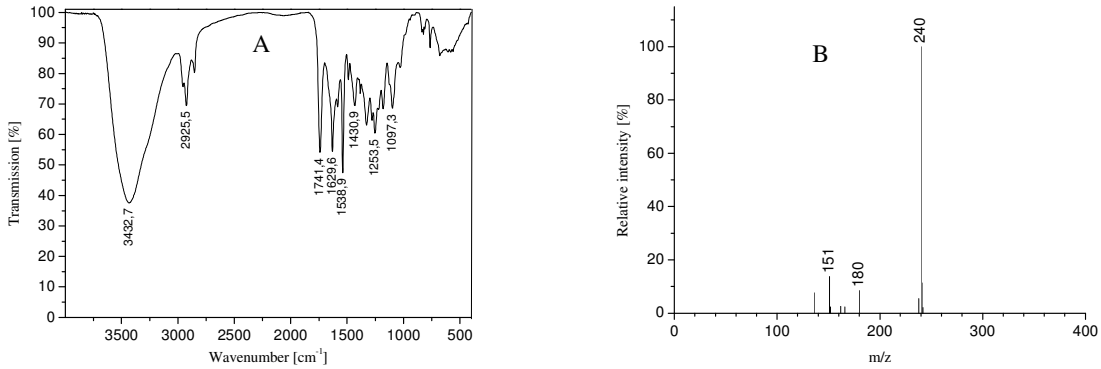

Fig. 7.18. WZ 436S-20, IR-spectrum (100 µg/36.8 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

7.2.1.1.2.8. WZ 436S-21

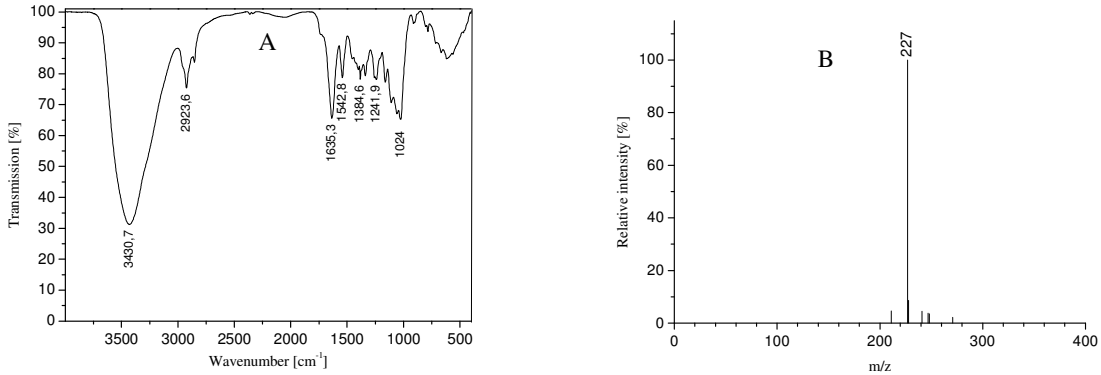

Fig. 7.19. WZ 436S-21, IR-spectrum (100 µg/35 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

7.2.1.2. WZ 436-13 (3-nitro-1H-indole)

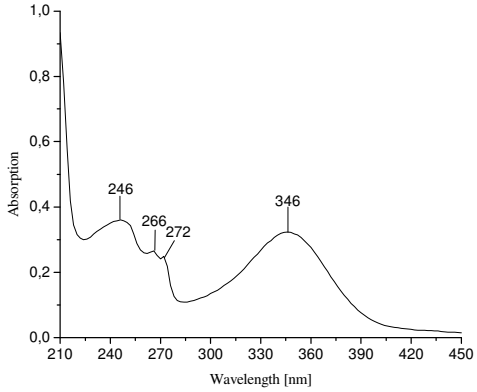

Fig. 7.20. UV-spectrum of **WZ 436S-13** in methanol (16.7 µg/ml). UV_{max} are 246 nm, 266, 272 and 346 nm.

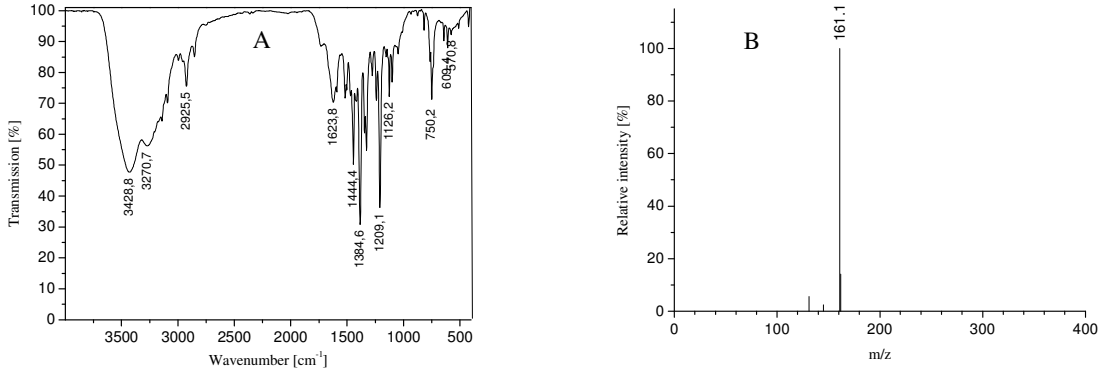

Fig. 7.21. WZ 436S-13, IR-spectrum (100 µg/33.6 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

7.3. Compounds from *Vibrio* sp. WMBA1-4

Table 7.4. Chemical names of the substances from *Vibrio* sp.

Compound	Chemical name	Note	Reference
WMB4S-1	3-Nitro-4-hydroxybenzaldehyde	NS	Sun *et al.,* 2005
WMB4S-2	3 [3-Nitro-4-hydroxyphenyl]-4-phenyl-pyrrol-2,5-dione	N	-
WMB4S-4	2-Hydroxy-1H-indole-3-carbaldehyde	NS	Not reported
WMB4S-5	3-[3-(2-Nitrophenyl)-1H-azirin-2-yl]-1H-indole	N	-
WMB4S-6	3 [3-Nitro-4-hydroxyphenyl]-2-propenoic acid	N	-
WMB4S-7	3-Nitro-1H-indazole	NS	Cohen-Fernandes & Habraken, 1971
WMB4S-8	3,4-Bis [3-nitro-4-hydroxyphenyl]-pyrrol-2,5-dione	N	-
WMB4S-9	3 [4-Hydroxyphenyl]-4-phenyl-pyrrol-2,5-dione	N	-
WMB4S-10	3 [3-Nitro-4-hydroxyphenyl]-4-[4-hydroxy-phenyl]-pyrrol-2,5-dione	N	-
WMB4S-11	Isomer of : **WMB4S-11a :** 3 [3-Nitro-4-hydroxyphenyl]-4-phenyl-pyrrol-2,5-dione-5-oxime and **WMB4S-11b :** 3 [3-Nitro-4-hydroxyphenyl]-4-phenyl-pyrrol-2,5-dione-2-oxime	N	-
WMB4S-12	3,4-Bis [3-nitro-4-hydroxyphenyl]-pyrrol-2,5-dione-monoxime	N	-
WMB4S-13	3 [3-Nitro-4-hydroxyphenyl]-4-[1H-indol-3-yl]-pyrrol-2,5-dione	N	-
WMB4S-14	2-Bis [1H-indolyl] ethane (vibrindole A)	K	Bell & Carmeli, 1994
WMB4S-15	Turbomycin B **or** *meso*-Phenyl-2-bis [1H-indolyl] methane	K **OR** NS	Gillespie *et al.,* 2002 **or** He *et al.,* 2006
WMB4S-16	3 [4-Hydroxyphenyl]-2-propenoic acid	K	Jiang *et al.,* 2003

K: Known substance NS: reported as synthetic (not from natural source) N: Newly reported

7.3.1. UV-, IR-, and mass spectra of the isolated compounds

7.3.1.1. Nitrated 4-hydroxyphenyl-derivatives

7.3.1.1.1. WMB4S-1 (-nitro-4-hydroxybenzaldehyde)

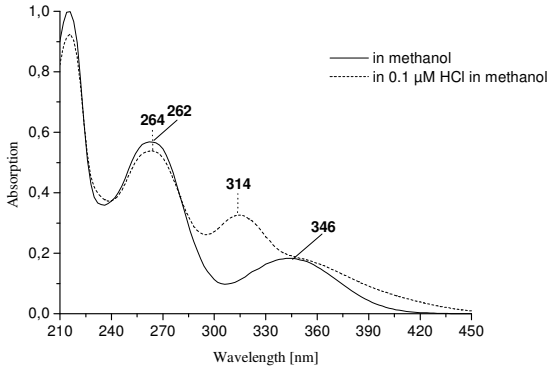

Fig. 7.22. UV-spectrum of **WMB4S-1** in methanol (7.1 µg/ml). UV_{max} are 262 nm and 346 nm.

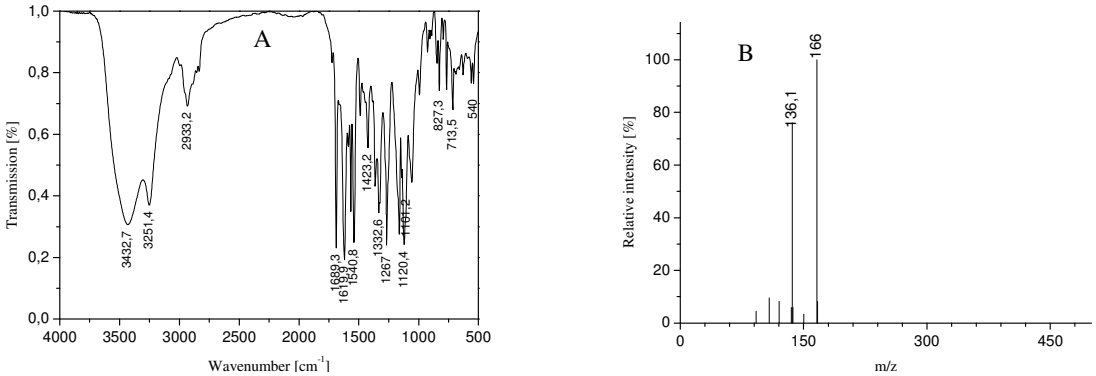

Fig. 7.23. WMB4S-1, IR-spectrum (100 µg/34 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

7.3.1.1.2. WMB4S-6 (3 [3-nitro-4-hydroxyphenyl]-2-proenoic acid)

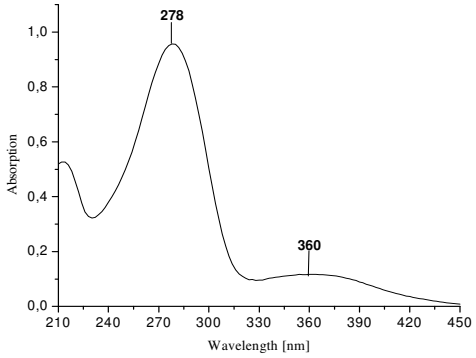

Fig. 7.24. UV-spectrum of **WMB4S-6** in methanol (9.9 µg/ml). UV_{max} are 278 nm and 360 nm.

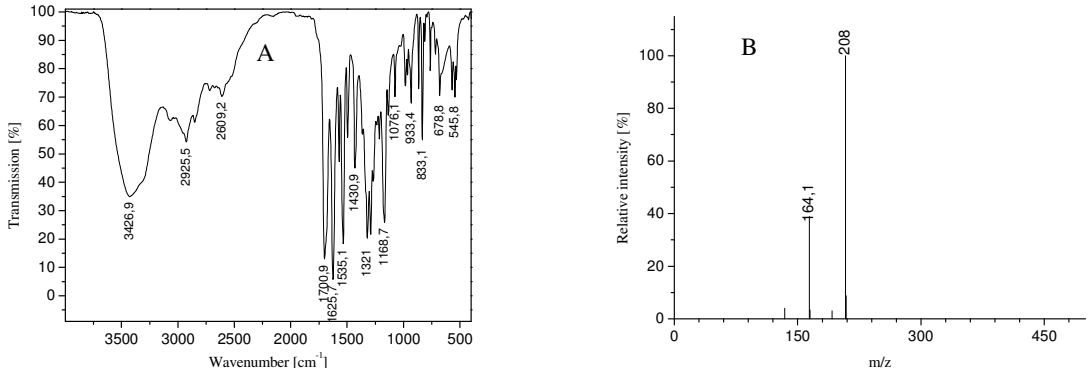

Fig. 7.25. WMB4S-6, IR-spectrum (100 µg/36 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

7.3.1.2. Indazole and oxindole derivatives

7.3.1.2.1. WMB4S-4 (2-hydroxy-1H-indole-3-carbaldehyde)

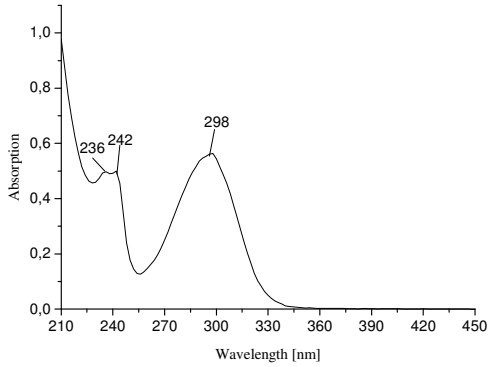

Fig. 7.26. UV-spectrum of **WMB4S-4** in methanol (7.6 µg/ml). UV_{max} are 236 nm, 242 nm and 298 nm.

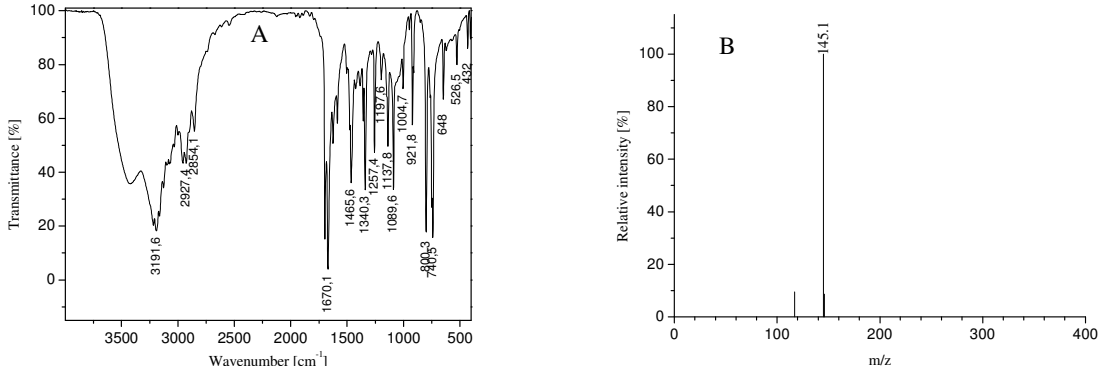

Fig. 7.27. WMB4S-4, IR-spectrum (100 µg/35 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

7.3.1.2.2. WMB4S-7 (3-indole-1H-indazole)

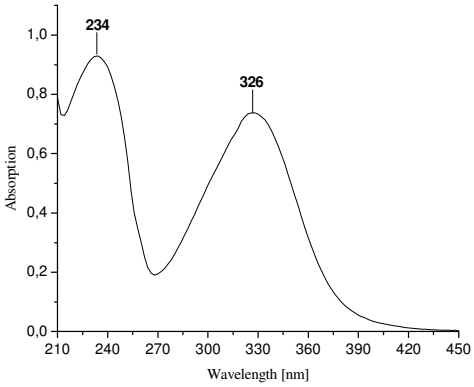

Fig. 7.28. UV-spectrum of **WMB4S-7** in methanol (38.5 µg/ml). UV_{max} are 234 nm and 326 nm.

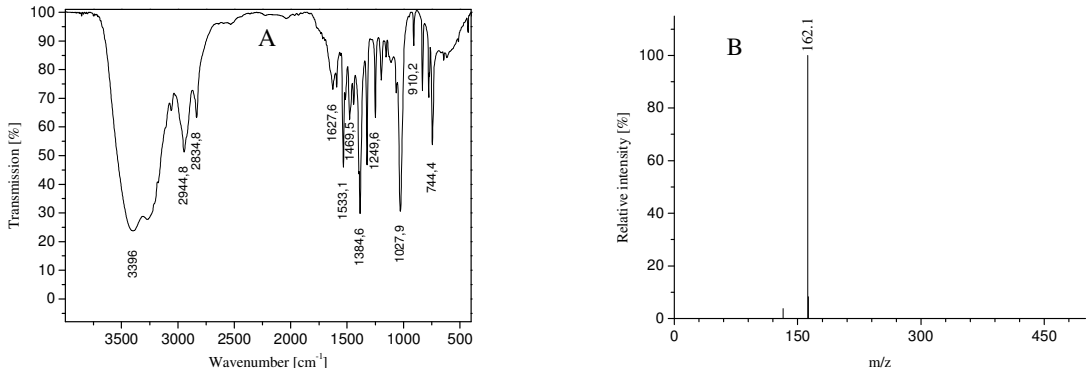

Fig. 7.29. WMB4S-7, IR-spectrum (100 µg/36 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

7.3.1.3. Pyrrol-2,5-dione-derivatives (compounds WMB4S-2, -8-13)

7.3.1.3.1. WMB4S-2 and WMB4S-9

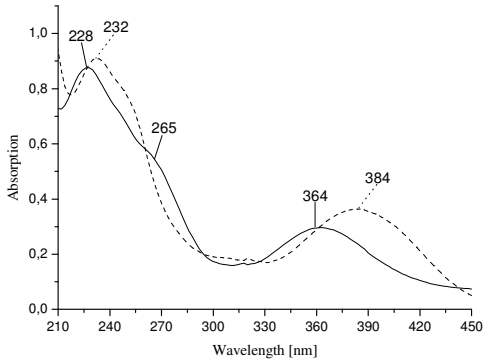

Fig. 7.30. UV-spectrum of **WMB4S-2** (solid) and **WMB4S-9** (dash) in methanol (9.9 µg/ml and 14.8 µg/ml). UV_{max} are 228 nm, 265 nm, 364 nm and 232 nm, 384 nm.

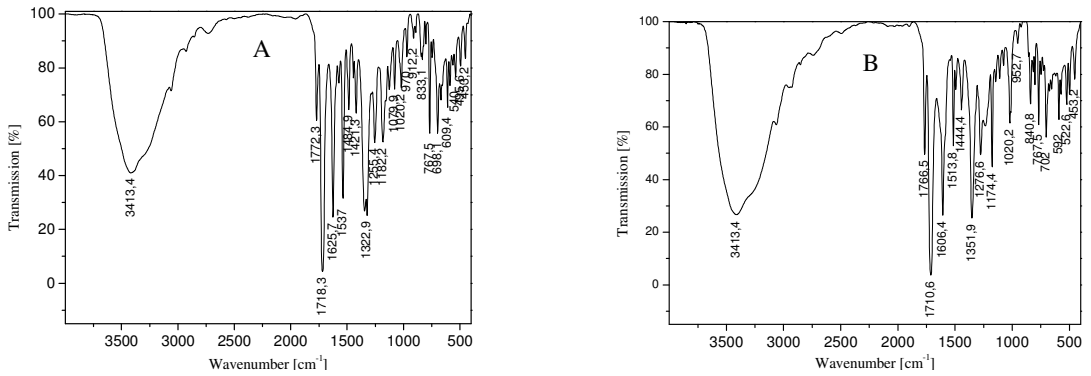

Fig. 7.31. IR-spectra of **W WMB4S-2** (100 µg/33 mg KBr) (A) and **MB4S-9** (100 µg/35 mg KBr) (B).

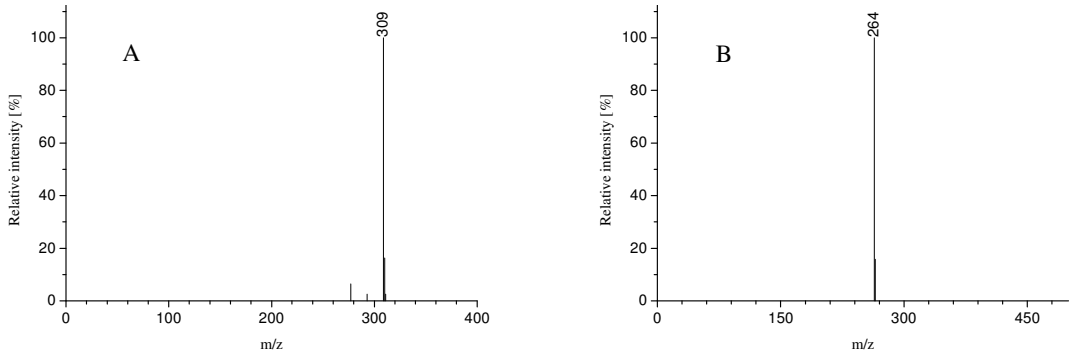

Fig. 7.32. Mass-spectra of APCI negative ionization of **WMB4S-2** (A) and **WMB4S-9** (B).

7.3.1.3.2. WMB4S-8 and WMB4S-10

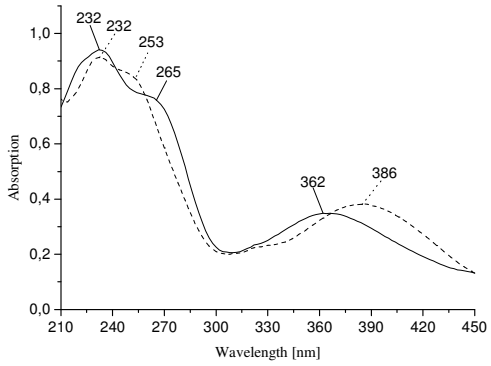

Fig. 7.33. UV-spectrum of **WMB4S-8** (solid) and **WMB4S-10** (dash) in methanol (11.5 µg/ml and 13.8 µg/ml). UV_{max} are 232 nm, 265 nm, 362 nm and 232 nm, 253 nm, 386 nm.

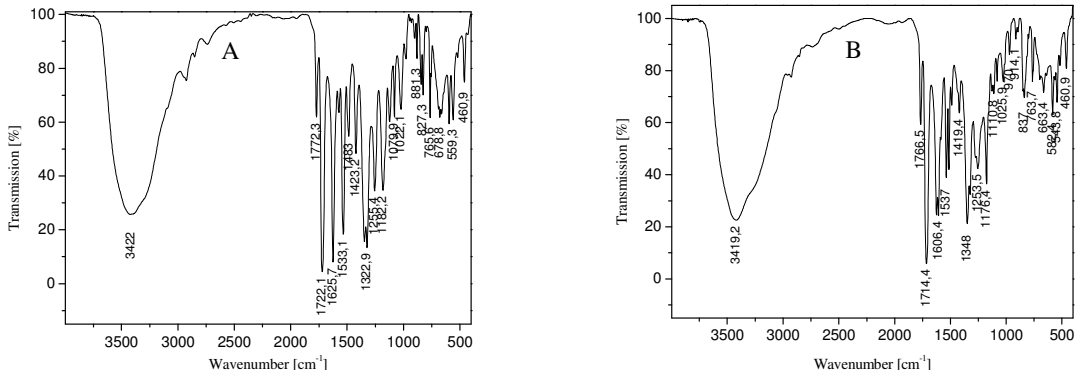

Fig. 7.34. IR-spectra of **WMB4S-8** (100 µg/35 mg KBr) (A) and **WMB4S-10** (100 µg/33 mg KBr) (B).

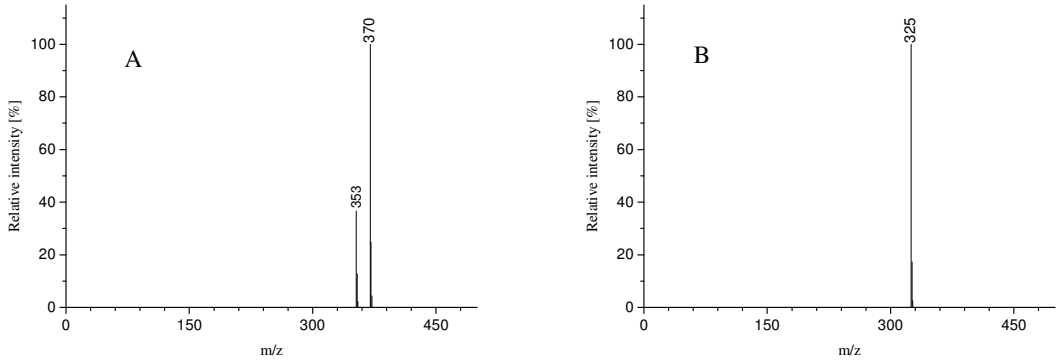

Fig. 7.35. Mass-spectra of APCI negative ionization of **WMB4S-8** (A) and **WMB4S-10** (B).

7.3.1.3.3. WMB4S-11 and WMB4-12

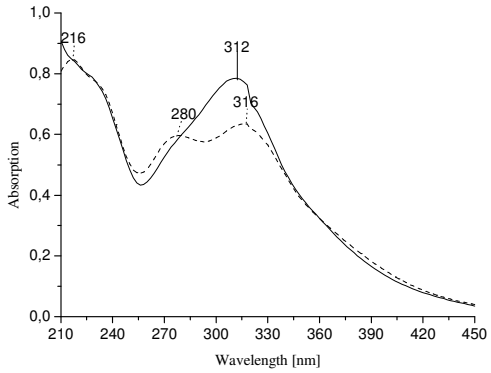

Fig. 7.36. UV-spectrum of **WMB4S-11** (solid), UV_{max} is 312 nm and **WMB4S-12** (dashed), UV_{max} are 216 nm, 280 nm, 316 nm in methanol (each 14.8 µg/ml respectively).

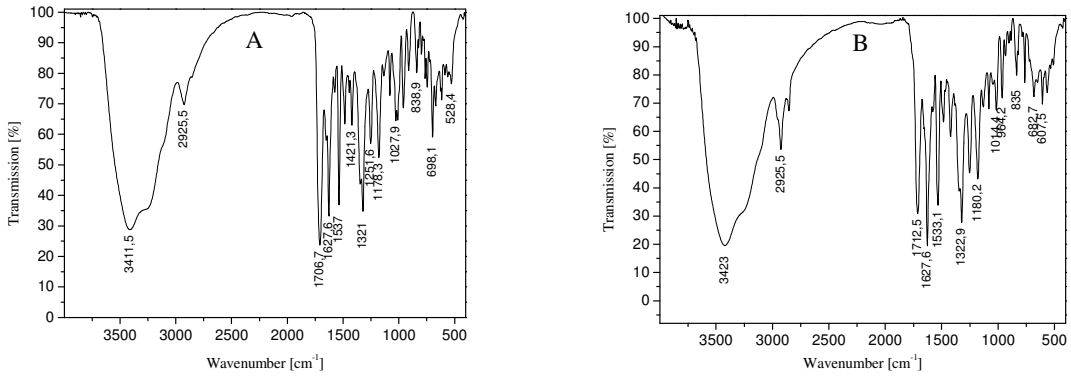

Fig. 7.37. IR-spectra of **WMB4S-11** (100 µg/34 mg KBr) (A) and **WMB4S-12** (100 µg/35 mg KBr) (B).

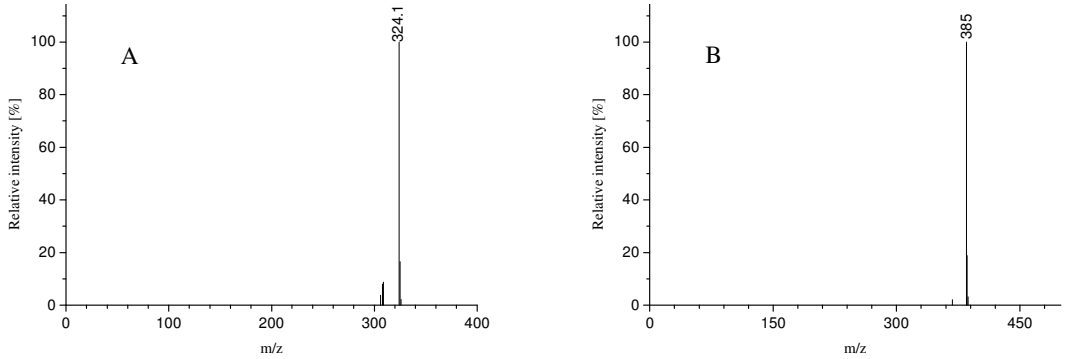

Fig. 7.38. Mass-spectra of APCI negative ionization of **WMB4S-11** (A) and **WMB4S-12** (B).

7.3.1.3.4. WMB4S-13

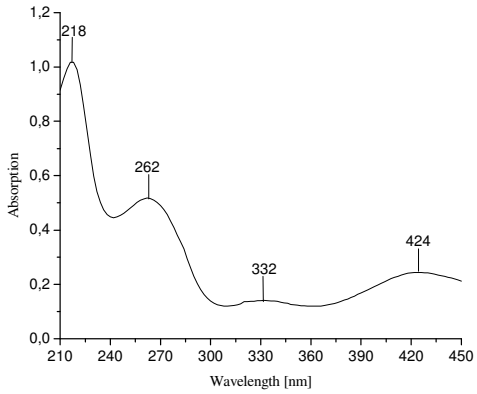

Fig. 7.39. UV-spectrum of **WMB4S-13** (14.8 µg/ml) in methanol. UV_{max} are 218 nm, 262 nm, 332 nm and 424 nm.

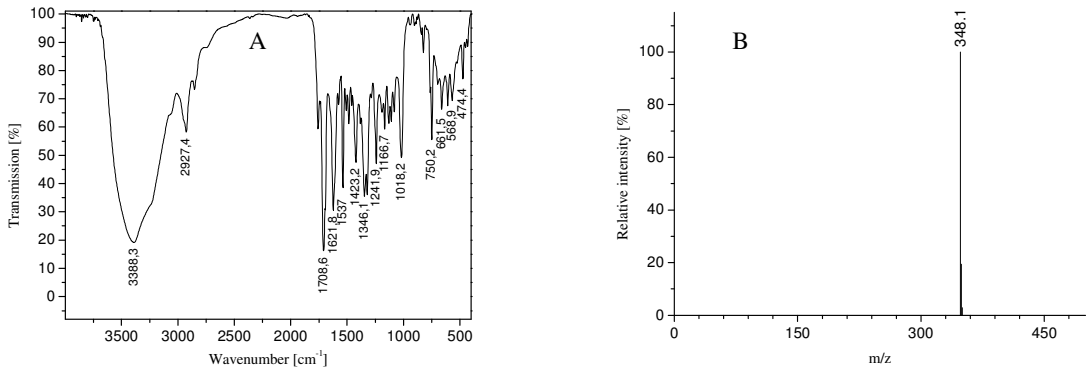

Fig. 7.40. WMB4S-13, IR-spectrum (100 µg/34 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

7.3.1.4. 3-[3-(2-nitro-phenyl)-1H-azirin-2-yl]-1H-indole (WMB4S-5)

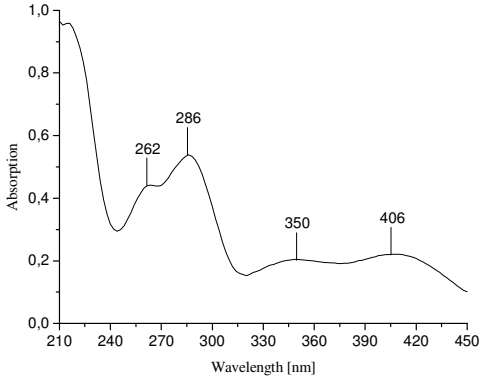

Fig. 7.41. UV-spectrum of **WMB4S-5** (7.9 µg/ml) in methanol. UV_{max} are 262 nm, 286 nm, 350 nm and 406 nm .

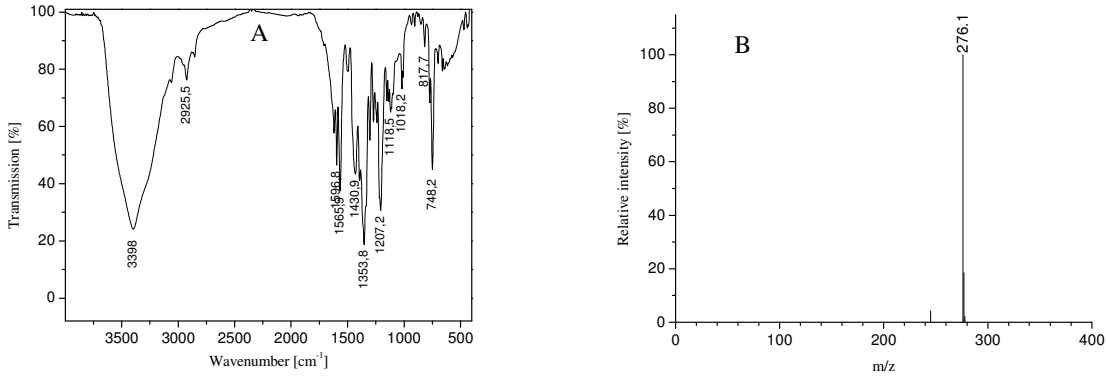

Fig. 7.42. WMB4S-5, IR-spectrum (100 µg/32.8 mg KBr) (A) and mass-spectrum APCI negative ionization (B).

CURRICULUM VITAE

PERSONAL DATA

Name	Wael Ali Al-Zereini
Date of Birth	30.05.1971
Place of Birth	Amman, Jordan.
Family status	Single

EDUCATION

1987 - 1988	General Secondary Education, Al-Hussein college higher School/Amman, Jordan.
1988 - 1992	B. Sc. in Biology, Major: Zoology, Cairo University/Egypt.
1993 - 1995	Diploma in educational Sciences, Mu'tah University/Karak, Jordan
1998 - 2001	M. Sc. in biology.
2002-	Ph.D student in Dept. Biotechnology, Technical University of Kaiserslautern/ Kaiserslautern, Germany.

WORKING EXPERIENCE

1993 - 1997	Laboratory supervisor and Teaching assistant, Dept. Biological sciences, Mu'tah University/Karak, Jordan.
1997 - 1998	Biological Sciences Teacher, Al-Etihad Secondary School/Amman, Jordan.