Final publishable summary report

(Text only – without figures)

Executive summary

Marine organisms, in particular sponges and their associated microorganisms, are an inexhaustible source of novel bioactive compounds for biomedical application. Industrial exploitation of this natural resource using traditional approaches is, however, hampered by supply problems - despite of numerous efforts in the past. Therefore, there is only one way: to start from the genes, or their biosynthetic pathways, to sustainably obtain the active molecules in sufficient amounts. The aim of this industry-driven integrating project was to combine the knowledge in marine genomics, chemogenetics and advanced chemistry to produce novel secondary metabolite (lead) compounds, as well as pharmacologically active peptides, and to bring them up to pre-clinical, and finally, after the end of the project, to clinical studies. This ambitious approach was based on breakthrough discoveries and the results of previous EU projects of members of the consortium, including European leaders in marine (sponge) genomics, metagenomics (polyketide synthase clusters), combinatorial biosynthesis and marine natural product chemistry/ structure elucidation.

The main results of the project in the different workpackages can be summarized as follows. *(i)* Bioprospecting and Sample Preparation – HIGHLIGHTS were the preparation and successfully testing of more than 400 sponge and bacterial extracts, and of a collection of >140 strains of marine bacteria from extreme marine environments (ISCAR collection) for antioxidant, antimicrobial, immunomodulatory, neuroprotective, antikinase, anti-osteoporosis and UV-protective activities. *(ii)* Screening and Bioactivity Identification – HIGHLIGHTS included the setting-up of a protein kinase platform, an Alzheimers' disease cellular screening platform and a screening platform for antiprotozoan activity, the discovery and elucidation of the synergistic effect of quercetin and polyphosphate (polyP) on bone hydroxyapatite formation, the identification of triazines and pyrazoles as Amyloid beta-42 inducers, the identification of a new target, the carbonic anhydrase, for compounds affecting bone formation and of a first activator of this enzyme, quinolinic acid, a sponge compound, and its combination with polyP-calcium as a regenerative material for treatment of osteoporosis-caused bone damage. *(iii)* Isolation/Structure Elucidation – HIGHLIGHTS were the discovery of smenamide A and B, two highly cytotoxic hybrid peptide/polyketide marine sponge compounds, and of incisesterol A5 and A6, potent sponge agonists of PXR receptor, as well as of halomerol A, biosynthesized by a novel metabolic pathway, the isolation of new cytotoxic compounds from ascidians (phosphoeleganin and conithiaquinones A and B), and of two new depsipeptides, sulfinyltheonellapeptolide and theonellapeptolide, and a series of cyclotoxic macrolides, including the new compounds isoswinholide B and swinholide K, from *Theonella swinhoei,* the isolation, sequencing and NMR structure of the peptides, barrettide A and B, from the cold water sponge *Geodia barretti*, the isolation of a new antibiotic cyclic hexapeptide from two Icelandic thermophilic bacterial strains, the identification of a thiazinoquinone antimalarial lead compound, the discovery of spiroplakortone, plakortone Q and plakdiepoxide, novel polyketide-based metabolites from the sponge *Plakortis simplex*, active on nuclear receptors, the isolation of lacunalides A and B, novel *trans*-AT PKS derived polyketides, and the discovery of a new antibacterial

lipophilic cyclopeptide from a thermophilic bacteria. *(iv)* Genomics – HIGHLIGHTS were the identification of new genes/cDNAs involved in carotenoid pathway, the demonstration of the interaction of retinal with the sponge peptide suberitine, the identification/characterization of two molecules of the sponge cryptochrome photoreception system, as well as of two marine sponge toxins of potential biomedical interest: a complement protein c8-alpha-like polypeptide from the hexactinellid *Crateromorpha meyeri* and a cytolysin from the demosponge *Dysidea avara*, the isolation and expression of a sulfatase from *D. avara*, the cloning of the first poriferan laccase from *Suberites domuncula*, a copper-containing enzyme involved in the anti-bacterial defense system of sponges. *(v)* Metagenomics and Gene Mining – HIGHLIGHTS included the identification of >50 gene clusters from uncultivated symbionts of marine sponges, the assembly of the misakinolide and psymberin gene cluster, the production of a new proteusin from a sponge symbiont and of polytheonamide analogs by heterologous expression, and the identification of Swf, a new group of mono-modular type I PKS/FAS, which appears to be specifically associated to sponge symbionts. *(vi)* Chemical Synthesis and Chemogenetics – HIGHLIGHTS were the identification of the 3D structural features of synthetic analogues of marine natural compounds, including plakortins (endoperoxides) and aplidinones (thiazinoquinone) with antimalarial activity, the elucidation of the mechanism of hydrolysis of 2-halotryptophans, the identification of the gene cluster responsible for the biosynthesis of merosterol, the chemical optimisation of the Leucettine scaffold, the establishment of the proof-of-concept in two mouse models for Leucettine L41 as an agent able to correct cognitive deficits associated with Down syndrome, and the synthesis and successful folding of barrettide A. *(vii)* Bioprocess Development and Scale-Up - HIGHLIGHTS included the successful establishment of the SustainCycle system for sponge culture, the successful scale-up of sponge primmorph culture, the development of a screening protocol for culturable marine microbial strains, and the implemention of a protocol for the culture of *Rhodothermus marinus* in bioreactor with higher productivities. *(viii)* Preclinical Studies – HIGHLIGHTS were the development of a novel microparticulate material with morphogenetic activity consisting of amorphous Ca-polyP, the development of electrospun fiber mats with this material and further bioactive components (retinoids), the selection for further preclinical testing of the most advanced compounds of BlueGenics project: (i) retinol/polyP for wound healing and (ii) Leucettines for Alzheimer's disease, the demonstration of activity of the electrospun mats with Ca-polyP and retinol on wound healing in mice (full excisional wound model); the demonstration of the synergistic effect of retinol and Ca-polyP on collagen type III gene expression, as well as on the expression of leptin, leptin receptor and FABP4, and the pharmacokinetics and biodistribution of Leucettine L41 in Down syndrome mice model. *(ix)* Intellectual Property – HIGHLIGHTS included seven patent applications, an enzyme website, a marine natural product database and a signature database. *(x)* WP10 Demonstration Activities – HIGHLIGHTS were several demonstrations at industrial fairs, a product website and the organization of special demonstrations events in Europe and in oversea. *(xi)* Dissemination, Communication – HIGHLIGHTS included >100 publications in high-impact journals, including Nature, a Book series, >140 presentations at national/international conferences, as well as numerous presentations to the general public.

Summary description of the project context and the main objectives

The objectives of this multidisciplinary project were: (1) To combine the knowledge in marine genomics with advanced methods in target-oriented drug screening and sustainable development for the discovery of novel natural products of biomedical /

biotechnological interest from sponges, associated microorganisms and marine bacteria, living in temperate or in extreme marine environments; (2) To use this knowledge for an efficient primary and secondary screening, including molecular-biology-based approaches, and identification of bioactive metabolites and peptides/proteins, which besides neuroprotective, antimicrobial, and anti-ageing activities will include hitherto neglected activities, such as anti-osteoporotic, anti-protozoan/anti-plasmodial, and innate-immune-response-modulating bioactivities; (3) To use advanced molecular biological and chemical techniques for the efficient isolation, structure elucidation and characterization of novel natural products, including secondary metabolites and bioactive peptides; (d) To develop and to introduce methods for fast identification of known compounds (dereplication) and for identification and characterization of minute amounts of natural products predicted by genome mining; (e) To identify, isolate and express new cDNAs (genetic “blueprints”) of commercial interest from sponge genomes, coding for enzymes/proteins involved in biosynthesis/biotransformation of bioactive molecules of therapeutical and/or biotechnical use; (f) To identify and characterize of cDNAs/proteins of commercial interest (such as light responsive cryptochromes, heat/cold shock proteins, and enzymes/proteins involved in accumulation of rare earth elements), isolated from extreme (darkness, high temperature, high pressure) aquatic environments; (g) To prepare metagenomic DNA libraries to access the whole aquatic bacteria diversity for genome mining (in silico identification of novel enzymes or other bioproducts for biomedical application); (h) To apply advanced metagenomic and biosynthetic prediction techniques to specifically target genes and compounds of biomedical interest for sustainable production in a heterologous cultivable host (special focus: *trans*-AT polyketide synthases and proteusin enzymes); (i) To harness the natural biosynthetic machinery in a combined biotechnological and synthetic approach to generate natural product analogues for structure-activity relationship studies (precursor directed / combinatorial biosynthesis – mutasynthesis – modification of natural / unnatural products); (j) To identify the three-dimensional structural features of the bioactive natural and unnatural compounds synthesized through molecular modeling studies; (k) To develop, scale-up and optimize the productivity of systems and fermentation processes for the sustainable large-scale production of selected bioactive compounds; and (l) To undertake preclinical studies of selected potential drug leads identified in the course of the project.

The final aim of this integrating project was the creation of a durable, industry-driven, molecular-biology-based marine drug discovery and production unit for the sustainable exploitation of aquatic molecular biodiversity, which will continue after the lifetime of the project and further strengthen the international position and effectiveness of European (SME-based) blue biotechnology industry.

To achieve this goal, the following specific objectives had to be accomplished:

- To sample aquatic invertebrates (primarily sponges) and aquatic microorganisms and to provide extracts for screening for bioactivities, genomics/metagenomics approaches, and isolation, purification and structure elucidation of the bioactive compounds, in addition to existing collections of extracts owned by the partners. The ISCAR collection, a collection of 150 marine bacteria from extreme marine environments has been made available by MATIS as a suitable source for the discovery of new potentially bioactive molecules. This objective included *(i)* sampling of lower aquatic invertebrates (primarily sponges), *(ii)* preparation of crude extracts and screening of bioactivity, *(iii)* isolation of cultivable strains potentially producing of bioactive secondary metabolites, *(iv)* phylogenetical identification of associated sponge microorganisms, and *(v)* cultivation of sponge associated bacteria and bacteria from extreme and non-extreme marine environments.

- To evaluate the bioactivity of extracts primarily from sponges, sponge-associated microorganisms and marine bacteria, in order to identify new compounds of potential biomedical interest, including: *(i)* UV protecting (anti-ageing) and anti-oxidant and antiinflammatory compounds, *(ii)* neuroprotective compounds, *(iii)* antimicrobial compounds, *(iv)* antiprotozoan/anti-plasmodial compounds, *(v)* immunomodulatory compounds and *(vi)* anti-osteoporotic compounds. To apply screening systems based on, among others, the use of disease-relevant kinases, as a target for identification of inhibitors that can be developed into new therapeutic drugs, cellular assays to identify potential anti-Alzheimer drugs, the OPG/RANKL/RANK system, as well as the carbonic anhydrase as a novel drug target, for the identification of potential anti-osteoporotic compounds. To provide bioactive extracts for further development by bioassay-guided fractionation, purification and structure elucidation.

- To isolate secondary metabolites and peptides and to determine the full structure of novel compounds from whole marine organisms (primarily sponges), cultures of associated bacteria, bioactive extracts, or genome mining, including *(i)* isolation of the active compounds from bioactive extracts by assay-guided fractionation, *(ii)* elucidation of the structure of novel natural products/peptides, *(iii)* fast identification of known compounds (dereplication), *(iv)* characterization of natural products predicted by genome mining, and *(v)* quantitative analysis of metabolites in extracts.

- To combine knowledge in marine genomics with advanced methods in target-oriented drug discovery and development, available within the consortium, in order to obtain new molecules of commercial interest by identification and cloning of enzymes/proteins from sponges involved in biosynthesis of bioactive compounds, as well as bioactive peptides or other products for therapeutical or biotechnical use. This objective involves the use of the poriferan EST database “spongebase” (UMC-Mainz), the analysis of the elaborated sponge ESTs for genes/cDNAs with potential applications in human therapy and biotechnology, and the expression, affinity purification and refolding of the recombinant proteins.

- To identify two classes of genes in sponge metagenomes as well as cultivated bacteria that are relevant for the biosynthesis (and sustainable biotechnological production) of pharmacologically active natural products: The first class encodes trans-AT polyketide synthases (PKSs) and the second is represented by proteusin biosynthetic enzymes. This task includes *(i)* development of PCR-based methods to target trans-AT PKSs and proteusin gene clusters, *(ii)* identification of the biosynthetic genes for bioactive natural products (PKS and proteusin), *(iii)* genome mining for the discovery of new natural products (PCR-based identification of coding sequences for putative biosynthetic enzymes), and *(iv)* preparation of a marine strain collection containing trans-AT PKS and proteusin genes for further cultivation and compound isolation. In addition, to perform expression profiling with high throughput sequencing in order to monitor the response of sponges grown in culture during the growth period and to prepare and sequence metagenomic DNA from extreme environments, including geothermal sources, for a gene mining approach.

- To develop and apply genetic methods for the engineered biosynthesis of analogues of the bioactive compounds (Chemogenetics-Bioengineering part), and to perform the chemical synthesis of the bioactive compounds (Chemistry part). The aim of the Chemogenetics-Bioengineering part is the generation of series of analogues of the medicinally significant natural products discovered in

WP3, in order to carry out pharmacological evaluation and structure-activity relationship (SAR) studies. The aim of the Chemistry part is on the synthesis of pharmacological inhibitors of disease-relevant protein kinases and of pharmacological modulators/inhibitors of amyloid beta-42 production, as well as the synthesis of disulfide-rich and cyclic peptides of sponge origin. This task includes *(i)* the application of precursor directed biosynthesis/mutasynthesis, *(ii)* combinatorial biosynthesis to generate analogues, *(iii)* synthetic modification of natural and unnatural products, *(iv)* development of specific protocols for the synthesis of small libraries of marine sponge-derived kinase inhibitors, *(v)* synthesis of antagonists/modulators of amyloid beta-42 production, *(vi)* establishment of methods for synthesis of disulfide-rich and cyclic peptides of sponge origin, and *(vii)* identification of three-dimensional structural features of bioactive natural and unnatural compounds through molecular modeling studies.

- To optimise and scale-up the SustainCycle system for the cultivation of sponges, to scale-up sponge primmorph cultures, to screen for strains showing promising processing features and optimize the fermentation process for the selected strains, in order to maximize the productivity of the process and minimize the formation of site products or maintain the profile of bioactive products across different production scales, to adapt the already optimized lab scale standardized culturing protocol of ISCAR marine bacteria to a large-scale production, and to scale-up of production of recombinant proteins.

- To select bioactive compounds arising from this project that are promising drug candidates and to progress at least a few towards preclinical development; candidates: marine biotechnology-derived beta-carotene and retinoid derivatives for anti-ageing ointments and gel preparations, as well as wound healing (applying advanced methods in 3D printing/electrospinning), and leucettines, derived from Leucettamine B, a natural product (kinase inhibitor) from the marine sponge *Leucetta microraphis* showing pronounced neuroprotective activity. This objective includes detailed *in vitro* and *in vivo* profiling of the potential candidate compounds, including *(i)* envisaged field of application (target disease, therapeutic area), *(ii)* potency and target selectivity, *(iii)* cytotoxicity of the compound, *(iv)* physico-chemical properties (solubility, stability and solid state characteristics), *(v)* pharmacokinetics *in vitro*, (vi) preliminary genotoxicity (Ames test), (vii) potential cardiac toxicity liability (hERG assay), *(viii)* pharmacokinetics *in vivo*, *(ix)* identification of a suitable biomarkers, *(x) in vivo* activity in relevant animal disease model, *(xi)* PK/PD relationship, *(xii)* preliminary repeat dose toxicity in the rat, *(xiii)* clinical dose prediction and therapeutic index, and *(xiv)* scale-ability of the production process. Based on the results of these studies, the compound may be selected as a preclinical candidate.

- To establish an integrated management of intellectual property generated within this project, including the filing of patents, and to promote an efficient transfer of technology, to identify products of potential commercial interest and to analyse their potential market, and to set-up a marine natural product database and enzyme website**.**

- To carry out demonstration activities of selected products and technologies developed in this project in order to inform and attract potential endusers, including, in particular, the platform technologies of molecular-biology-based biodiscovery, the SustainCycle system and sponge cell/tissue cultures for the production of bioactive secondary metabolites and selected sponge-derived recombinant enzymes/proteins.

- To disseminate and communicate the results generated in this project and to train the project team members in the technical skills and know-how available within the consortium, including publications in refereed scientific journals, active participation in national and international conferences and workshops, public presentations to non-expert audiences, preparations of leaflets and brochures, contributions to the media, and project website.

- To establish and maintain an efficient management structure, including the organisation of project meetings, workshops and summer schools, and the assessment of progress of the project.

Description of the main S&T results/foregrounds

The main S & T results of the project can be summarized as follows:

1. BIOPROSPECTING AND SAMPLE PREPARATION

Summary:

The aim of this work package was to provide, besides existing collections of extracts owned by the partners, new aquatic sources of compounds for the development of novel drugs for human therapy (neuroprotective, immunomodulatory, anti-inflammatory, antimicrobial, anti-oxidant, antimalarial and anti-osteoporotic compounds), as well as therapeutically relevant bioproducts/enzymes from aquatic organisms, based on a molecular biology approach. The specific objectives were: (i) Sampling of lower aquatic invertebrates (primarily sponges); (ii) Preparation of sponge crude extracts and screening of their bioactivities, (iii) Isolation of cultivable strains potentially producing of bioactive secondary metabolites; (iv) Phylogenetical identification of associated sponge microorganisms; (v) Cultivation of sponge associated bacteria from French coasts and of extremely diverse marine bacteria from extreme and non-extreme marine environments from Icelandic waters; and (vi) Production of crude extracts from those marine bacteria and/or aquatic invertebrates that can be used to isolate molecules of interest.

In detail:

Sampling aquatic organisms to be studied as possible sources of new compounds with potential biological activity: MATIS maintained the Icelandic Strain Collection and Records (ISCAR) with thousands of bacterial strains from Icelandic marine and terrestrial sources, including extreme environments like hot springs. 144 aqueous and organic extracts each from this collection were delivered for screening to the partners. **UMC-Mainz**, in collaboration with **RBI**, has undertaken several sampling excursions (29 sponge species; more than 100 extracts) in the Northern Adriatic Sea (Rovinj area; Istria). The collection of **MNHN** comprised 160 sponge samples and 27 sponge-associated bacterial strains. **UNINA** provided 96 new-made extracts of 24 species of marine sponges, and 23 new-made extracts of 8 species of ascidians. **UU** has extracted 25 sponge samples.

Classificatory assignment: Classifactory assignments was based on a combination of phylogenetic analysis of DNA sequence data and traditional systematic methods (e.g. morphological work). For the discovery of new sponge peptides and the metabolic

profiling study, **UU** has identified a total of 100 sponge samples using morphology and molecular markers.

Production of biomass of 150 ISCAR marine microorganisms in standard culturing conditions: Based on the ISCAR collection, marine bacteria were chosen to represent a broad range of environments and taxa, which promised the best chance of finding novel, interesting bioactive compounds. Strains were cultivated under a limited number of different conditions and biomass was harvested for crude extracts. 144 strains were chosen and processed.

Aqueous and organic crude extracts preparation from marine bacteria: Bacterial biomass was extracted to obtain crude aqueous and organic extracts. In total, 222 organic and aqueous extracts were sent to the partners and have been successfully tested in WP2.

Aqueous and organic crude extracts preparation of sponge samples and their associated bacteria: A total of 316 sponge and bacterial extracts has been prepared by **MNHN** and evaluated for their antioxidant activity and antimicrobial activity against the bacterial strains *Staphylococcus aureus* and *Escherichia coli* and the yeast *Candida albicans*, as well as their antikinase activity. **ManRos**, using extracts sent by **UNINA**, has (i) evaluated 116 extracts from sponges and ascidians for cell survival and protein kinase inhibition, (ii) identified 10 extracts displaying cytotoxicity effects, (iii) identified 7 extracts displaying some inhibitory activity on one or more kinase, (iv) evaluated 116 extracts from sponges and ascidians for Alzheimer's disease cellular assay (Amyloid beta-42 production), (v) identified 2 extracts/fractions triggering an enhanced production of amyloid beta-42, and (vi) identified 12 extracts preventing the enhanced production of amyloid beta-42 induced by Aftin-5.

Highlights:

- **Preparation of a total of 316 sponge and bacterial extracts and evaluation for their antioxidant, antimicrobial and antikinase activity (MNHN).**

- **Aqueous and organic extracts from over 140 strains of marine bacteria from extreme (e.g. marine geothermal springs) and cold-water environments in and around Iceland (ISCAR collection) were delivered for screening and successfully tested (MATIS).**

- **Sampling and preparation of 100 extracts (ethyl acetate) from sponges in the Northern Adriatic Sea, Croatia for testing for immunomodulatory, neuroprotective, anti-osteoporosis and UV-protective activity (UMC-Mainz).**

2. SCREENING AND BIOACTIVITY IDENTIFICATION

Summary:

The aim of this work package was to evaluate the bioactivity of extracts derived from sponges, sponge-associated microorganisms and marine bacteria, in order to identify metabolites with potential application in the field of neuroprotection, immunomodulation, antimicrobial and antiprotozoan/antiplasmodial activity, osteoporosis and anti-ageing. The specific objectives were: (i) Screening for the identification of compounds with UV protection, anti-oxidant and neuroprotective activities; (ii) Identification of bioactive extracts for further development; (iii) Use of

disease-relevant kinases as targets to identify, among sponge produced metabolites, new inhibitory scaffolds than can be developed into therapeutic drugs; (iv) Use of a cellular assay to identify potential anti-Alzheimer drugs; (v) Screening of extracts for interactors/modulators of OPG/RANKL/RANK system (anti-osteoporotic compounds); (vi) Screening for neuroprotective compounds using sponge system; (vii) Screening for antiprotozoan natural products; (viii) Screening for antimicrobial natural products; (ix) Screening for osteogenetic compounds using new assays/bone markers; (x) Screening for bioactive peptides; and (xi) Process-oriented screening to evaluate which candidate biological systems would be transferrable to higher scale and suitable for robust industrial operation platform

In detail:

Screening for the identification of compounds with UV protection activity: Ethyl acetate extracts from twenty sponges species (collected in the Northern Adriatic) were tested, two of them (*S. domuncula* and *Crella rosea*) yielded the most promising results. The extracts from these two sponge species were rich in carotinoids. The protective activity of the extracts was tested in assays with HeLa cells and NIH/3T3 fibroblasts, using *(i)* a full solar spectrum lamp (wavelengths higher than 320 nm or higher than 295 nm); and *(ii)* monochromatic UV-B lamp with a peak of emission at 312 nm. DNA damage was determined using Fast Micromethod, a sensitive assay for DNA single strand breaks.

Screening for antimicrobial and antioxidant compounds: Out of the 132 organic and aqueous extracts prepared by **MATIS**, 33 bacterial extracts revealed a weak antimicrobial activity and one showed a better, 49%, inhibitory activity against *S. aureus*. No compound revealed antimicrobial activity against *E. coli*. One novel lipophilic cyclopeptide, isolated by **UNINA** and obtained from thermophilic bacteria, *Thermoactinomyces vulgaris* (**MATIS**), from marine hot springs, revealed antimicrobial activity against *S. aureus*. From the bacterial strain *Sulfitobacter* sp. SJ1796, three known antibacterial compounds were isolated: the monoglyceride palmitoyl glycerol, the fatty acid palmitoleic acid, as well as lauramide diethanolamine. From the calcareous sponge *Clathrina clathrus*, studied by **MNHN**, none of the 12 bacterial strains of the *Vibrio* order showed any significant activity. From the organic extract of the marine sponge *Smenospongia conulosa,* numerous known antibacterial brominated alkaloids were identified. Agelasine D, isolated from the bioguided purification of the marine sponge *Agelas nakamurai*, revealed a significant antimicrobial activity against *S. aureus*. Another known compound, halicyclamine B, isolated from the sponge *Acanthostrongylophora ingens* showed a selective activity against *S. aureus* and the tetracyclic diamine alkaloid SS24 13-2, a selective activity against *E. coli*. Seven known diketopiperazines were also isolated but none of them revealed any antimicrobial activity against *S. aureus* and *E. coli*.

Screening for antiprotozoan natural products: Using the pLDH assay, the aqueous/organic crude extracts from 80 bacterial strains from the **MATIS** collection plus 12 synthetic thiazinoquinone compounds have been tested for their *in vitro* antimalarial activity against both chloroquine-sensitive and -resistant strains of *P. falciparum*. Nine extracts (all organic extracts) active on both strains with IC_{50} < 75 µg/ml are were selected for further studies. Some of the synthetic thiazinoquinone compounds showed a significant pharmacological activity. A screening method (Medium throughput method) has been developed by **UU** and used for screening of marine sponge extracts delivered by **MATIS** against *Trichomonas vaginalis.*

Screening for immunomodulatory compounds: UMC-Mainz screened for immunomodulatory compounds using an assay based on inhibition of aminopeptidases (APs) present on the cell surface of L5178Y mouse lymphoma cells. Inhibitors of cell

surface associated APs such as bestatin, a dipeptide produced by *Streptomyces olivoreticuli*, count to the most efficient drugs for cancer chemotherapy. This drug is a competitive inhibitor leucine aminopeptidases (microsomal Leu-APm and cytosolic Leu-APc), as well as of the aminopeptidases on the cell surface of L5178Y cells, with respect to the substrate Leu-NA. Comparative inhibition studies with sponge extracts/pure compounds and bestatin were performed with the enzyme on the surface of L5178Y cells, using Leu-NA or Arg-NA as substrate. The K_i:K_m ratios determined with sponge extracts/pure compounds in comparison to bestatin were used as a measure for the relative affinities of the enzymes to inhibitor (sponge extracts/pure compounds or bestatin) and substrate (Leu-NA). Two compounds among of 25 compounds tested reached a K_i: K_m ratio that was significantly lower than that of the average, although bestatin showed by far the highest affinity.

Screening for, optimizing, and characterizing pharmacological inhibitors of disease-relevant kinases: The screening methods have been extensively described in past papers from **ManRos**. In brief, purified recombinant and native kinases are incubated with ^{33}P-ATP and appropriate peptide substrates and the ^{33}P-phosphate incorporated in the peptide is monitored as a measure of the kinase activity. Extracts or purified products are added to reaction mix and their potential inhibitory activity is detected. **ManRos** used the following 13 mammalian kinases: CDK2/cyclin A, CDK5/p25, CDK9/cyclin T, CK1, CLK1, CLK2, CLK3, CLK4, DYRK1A, DYRK1B, DYRK2, DYRK3, GSK3. They also cloned and expressed 12 orthologues of DYRKs/CLKs kinases from various unicellular parasites. These are: *Leishmania major*: LmCLK1, LmDYRK2, LmCK1, *Leishmania donovani*: LdDYRK1B, LdDYRK3, LdDYRK4, *Plasmodium falciparum*: PfCLK1, *Trypanosoma brucei*: TbCLK1, *Trypanosoma cruzi*: TcCLK1, *Cryptosporidium parva*: CpLAMMER, *Toxoplasma gondii*: TgCLK, and *Giardia lamblia: GlCLK.* Since the beginning of the BlueGenics project, 88 pure products and 825 extracts have been evaluated. Only a few active products/extracts have been identified in the general screen, highlighting the specificity of the assays.

Screening for, optimizing and characterizing potential anti-Alzheimer compounds: ManRos's screening activity related to Alzheimer's disease is based on a dual approach: identification of potential 'Alzheimerogenic' compounds and identification of potential 'anti-Alzheimer' compounds. The assays use the established cellular screening system based on cell lines expressing APP (amyloid precursor protein). Briefly N2a-APP695 cells are exposed to a reference compound, Aftin-5, and test compounds and as a response, produce a large quantity of the 'Alzheimerogenic' product, amyloid beta-42 (A42). **ManRos** is thus able to detect (i) A42 inducers in the 'human chemical exposome' (potential 'Alzheimerogenic' products), and (ii) products which could prevent the production of A42 (potentially anti-Alzheimer products). In terms of discovering inhibitors of A42 production (potentially anti-Alzheimer products), the method has been validated using reference beta- and gamma-secretases inhibitors. However no inhibitor has been discovered in the screening campaign among the 825 extracts or 88 pure products tested. **ManRos** has screened over 3,500 products from the 'human chemical exposome' and identified the triazine herbicides and the pyrazole pesticides as A42 inducers. These molecules are potentially 'Alzheimerogenic'. **ManRos** has been focusing on one pyrazole to develop it as a new tool to develop a chemically induced animal model of Alzheimer's disease.

Screening for antiosteoporotic compounds: interactors/modulators of OPG/RANKL/RANK system: 120 substances have been tested by **UMC-Mainz** in cell culture (SaOS-2 cells). Their effects on mineralization were determined in the absence and in the presence of polyP applying the Alizarin Red assay. Based on these results, the increase in hydroxyapatite formation was calculated. **UMC-Mainz**, together with **NRGC-CAGS** and **NANOTEC**, identified a potential stimulator of bone mineralization

(quercetin), which is of interest for prophylaxis of osteoporotic disorders. A study of the combined effects of this natural product and polyP (Ca^{2+} complex) revealed that both compounds induce the mineralization process of SaOS-2 cells in a synergistic manner. Further studies revealed that the biomineralization process induced by these compounds is based on two differential modes of action. Both compounds quercetin (or isoquercitrin) and polyP cause a significant upregulation of the expression of the transcription factor RUNX2. The expression of the two co-activators of RUNX2, ATF6 and Ets1, becomes strongly increased in cells after exposure to quercetin/isoquercitrin. The assumption that the activating effect of quercetin/isoquercitrin occurs via a signal transduction pathway involving ATF6 that is independent from that induced by polyP is supported by the finding that quercetin/isoquercitrin but not polyP upregulates the expression of the gene encoding OCAL. PolyP, on the other hand, strongly increases the expression of gene encoding for Ets1, as well as the expression of alkaline phosphatase, an enzyme involved in hydrolysis of polyP.

Screening for neuroprotective compounds using sponge system: UMC-Mainz screened a series of extracts/purified compounds for potential neuroprotective activity. The following strategy was chosen. *(i)* Determination of the (differential) effect of the extracts/purified compounds on rat pheochromocytoma PC12 cells in comparison to L5178y mouse lymphoma cells; *(ii)* Correlation of the effects with the expression of the NMDA receptor subunits in the PC12 cell line used; *(iii)* Determination of the effect of the compounds positively evaluated in the PC12 cells / L5178y systems (compounds revealing the strongest differential effects – highest neuroprotective activity) on generation of A-beta-42 peptide. The results revealed that the extracts/compounds tested differentially affected the growth of L5178y cells and PC12 cells. Seven marine extracts/compounds that showed a pronounced differential effect on growth of L5178y cells and PC12 cells were evaluated for their potential protective effect against NMDA-induced cytotoxicity (PC12 cells) and neuroprotective, anti-Alzheimer activity. The compounds positively evaluated in the PC12 / L5178y cells tests, were found to be able to reduce A-beta-42 generation, using APP751 overexpressing CHO cells and an A-beta specific sandwich ELISA. These studies allowed the identification of extracts that are able to modulate gamma-secretase activity by decreasing the A-beta-42 levels without affecting the A-beta-40 levels.

Screening for osteogenetic compounds using new assays/bone markers: The carbonic anhydrase (CA) presents a novel target of potential drugs for treatment of bone disorders/defects, including osteoporosis. **UMC-Mainz** and **NRCGA-CAGS** demonstrated that bone formation starts with the formation of calcium carbonate bio-seeds that are then transformed into hydroxyapatite (HA)/calcium phosphate. The formation of the calcium carbonate bio-seeds is mediated by CA. Activation of that enzyme should lead to an increased bone formation. Until now only a few CA activators have been identified but none of them has been tested for its potential in the treatment of bone disorders. **UMC-Mainz** and **NRCGA-CAGS** succeeded to demonstrate that the CA-driven $CaCO_3$ formation *in vitro* is activated by organic extracts from the demosponge *S. domuncula* as well as by quinolinic acid, one compound isolated from these extracts. The effect of polyP, administered as Ca-polyP complex, on HA formation was found to be amplified by addition of the sponge extract or quinolinic acid. Quinolinic acid comprises with its *N*-heteroatom in the pyridine backbone, as well as the dicarboxylic acid side chains two potential interacting groups with the Zn-containing CA.

Highlights:

- **Two sponges producing promising compounds with UV-protective activity identifies (UMC-Mainz)**

- **Assay for detection of Amyloid beta-42 (A42) inducers (potential 'Alzheimerogens') (ManRos)**

- **Identification of some triazines and pyrazoles as Amyloid beta-42 inducers (ManRos)**

- **Selection of first scaffold to be developed (Leucettines) (ManRos)**

- **Bacterial strains from the MATIS collection tested for anti-cancerogenic, antimicrobial and antiprotozoan activity (UMC-Mainz, MNHN, UNINA)**

- **Establishment of assays to determine the effect of extracts/compounds on the expression of Alzheimer A-beta-40 and A-beta-42 peptides (ManRos)**

- **Among 120 substances tested 15 compounds turned out to be most promising in induction of mineralization of bone forming cells (SaOS-2 cells) (UMC-Mainz)**

- **Discovery of two molecules produced by marine organisms, quercetin and polyP, that act in a highly synergistic manner in stimulating hydroxyapatite formation (UMC-Mainz)**

- **Elucidation of the synergistic mode of action of quercetin with inorganic polyphosphate (Ca-polyP) (UMC-Mainz and NRCGA-CAGS)**

- **Identification of 7 extracts with potential neuroprotective activity (rat pheochromocytoma PC12 cells compared to L5178y mouse lymphoma cells); 2 extracts were found to display a significant protective effect against NMDA induced cytotoxicity in PC12 cells (UMC-Mainz)**

- **The carbonic anhydrase turned out to be a novel target for drugs stimulating bone hydroxyapatite formation (therapy and prophylaxis of osteoporosis) (UMC-Mainz)**

- **The first activator a marine (sponge) organisms that acts on carbonic anhydrase has been identified: quinolinic acid (UMC-Mainz)**

- **Identification of a highly simplified analogue of plakortin with antimalarial activity against chloroquine-resistant compounds strains of *P. falciparum* (UNINA)**

3. ISOLATION – STRUCTURE ELUCIDATION OF BIOACTIVE COMPOUNDS

Summary:

The aim of this work package was the efficient isolation of bioactive natural products, fast identification of known compounds, and full structure elucidation of novel compounds, irrespective of their origin from whole marine organisms, cultures of associated bacteria, bioactive extracts from other WPs, or genome mining. The specific objectives were: (i) Isolation of the active compounds (secondary metabolites, peptides) from bioactive extracts by assay-guided fractionation; (ii) Structure elucidation of novel natural products/peptides isolated from bioactive extracts; (iii) Fast identification of known compounds (dereplication); (iv) Characterization of the natural

products predicted by genome mining; (v) Identification of antiprotozoan natural products; (vi) Identification of neuroprotective natural products; (vii) Identification of antimicrobial compounds; (viii) Identification of antioxidant, antiinflammatory and antimicrobial marine compounds for cosmetic applications; and (ix) Quantitative analysis of metabolites in extracts.

In detail:

Isolation of secondary metabolites in bioactive extracts by assay-guided fractionation: From the marine sponge *Theonella swinhoei*, two new cytotoxic depsipeptides, sulfinyltheonellapeptolide and theonellapeptolide, and a series of cyclotoxic two macrolides, including the new compounds isoswinholide B and swinholide K, were isolated. Smenothiazole A and B from the marine sponge *S. aurea* are further examples, after smenamide A and B, of mixed biogenesis chlorinated PKS/NRPS compounds from this sponge (**UNINA**). Bioassay (cytotoxicity) guided fractionation of the extracts of the ascidian *Ciona edwardsii* led to the isolation of three new phosphate-containing linear polyketides, structurally related to phosphoeleganin, a cytotoxic compound acting as protein tyrosine phosphatase (PTP) 1B inhibitor (**UNINA**). Bio-guided fractionation of the sponge ***Stylissa flabelliformis*** led to the isolation of 11 compounds, 10 of them revealed antikinase activity (**MNHN**). Bio-guided purification of the antimicrobial sponge ***Xestospongia* sp.** led to the isolation of 14 compounds of the adociaquinone family. Seven new adociaquinone derivatives, xestoadociaquinones A, B, 14-carboxy-xestoquinol sulfate and xestoadociaminals A–D, together with seven known compounds were identified. Two compounds revealed a selective inhibitory activity towards CDK9/cyclin T and CDK5/p25, respectively. Bio-guided purification of the sponge ***Iotrochota purpurea*** extract led to the isolation of 3 known bromoindole compounds as well as a new compound named Matemone B (**MNHN**, **UNINA**). The bromoindole revealed antimicrobial activity against *S. aureus* and selective antikinase activity against CDK9, CLK1 and DYRK1. From the sponge ***A. ingens***, seven known diketopiperazines have been identified as well as the known halicyclamine B and six new derivatives, which were identified through extensive 1D and 2D NMR data. In addition, seven known diketopiperazines were also isolated. The diketopiperazine cyclo-(Pro-Phe) showed a selective antikinase activity against CDK2/cyclin E. Its stereochemistry was determined (**UNINA**). Bio-guided purification of the marine sponge ***Agelas nemoechinata*** extract led to the isolation of only 3 known compounds, identified as sceptrin, oxysceptrin and nakamuric acid. From *A. nemoechinata,* sceptrin revealed a significant antimicrobial activity against *S. aureus.* Agelasine D, isolated by **MNHN** from *A. nakamurai*, revealed a significant antimicrobial activity against *S. aureus*. The chemodiversity of *Spongia officinalis*, a Demospongiae from the Mediteranean Sea that accumulates high levels of heavy metals, was investigated by metabolic fingerprinting of samples over three years at two sites near Marseille **(MNHN)**. The known furospongin-1, demethylfurospongin-4, furospongenone and the isomers of γ-hydroxy-alpha,beta-butenolide furospongin-1 were identified through a comprehensive structural analysis of intensive explorations by LC-MS/MS analyses in positive and negative ion modes. Three new analogues were identified: the furanoderivative named furofficin and the two furanopyrroloterpenes named pyrrolospongicin-1 and -2. In addition, clathrilectin, a new lectin from the marine sponge *C. clathrus* has been isolated **(MNHN, UMC-Mainz and RBI).** Clathrilectin, a protein complex of 3200 kDa, is composed of monomers with apparent molecular masses of 208 and 180 kDa. The clathrilectin is the first lectin isolated from a calcareous sponge and displays homologies with predicted sponge proteins potentially involved in cell aggregation and interaction with bacteria.

Structure elucidation of novel natural products isolated from bioactive extracts: UNINA determined the structures of the isolated compounds by spectroscopic and chemical means. For smenamides A and B, a new class of highly cytotoxic hybrid

polyketide/peptides with no close structural analogues, complete elucidation of planar structure was performed. **MNHN** investigated the Indonesian sponge of *Xestospongia* sp., whose crude extract showed moderate antimicrobial, antioxidant and significant antiplasmodial activities. Successive chromatographies of the EtOAc extract led to the isolation of 9 pure compounds. Their structures were elucidated by extensive 1D and 2D NMR spectroscopic data and high-resolution electroscopic ionization mass spectrum. Conulothiazole A and B, two further members of the strongly cytotoxic chlorinated non-ribosomal peptide/polyketide (NRP/PK) hybrids that are found in the Caribbean sponges of the genus Smenospongia, were isolated from *S. conulosa*. Conulothiazoles provide a novel NRPS/PKS-derived skeleton to natural product scaffolds. Conulothiazoles are members of a class of secondary metabolites sharing a hybrid peptide/polyketide biogenesis pathway that is frequently found among cyanobacteria (**UNINA-MNHN**). The structure of phosphoeleganin B, a new phosphorylated polyketide isolated from the Mediterranean tunicate *Sidnyum elegans* was elucidated using mass spectrometry and NMR experiments. In light of the close structural relationship of phosphoeleganin B with the phosphoeleganin, a protein tyrosine phosphatase 1B (PTP1B) inhibitor, further studies are in progress to test the possible role of phosphoeleganin B to inhibit PTP1B activity (**UNINA**). Analysis of a Chinese specimen *of P. simplex* led to the isolation of a novel polyketide-based metabolite, which was named spiroplakortone, featuring a spiroketal lactone group. The structures of the new plakortone Q and plakdiepoxide have been determined on the basis of a combination of spectral and computational data. In addition, all the isolated polyketides have been evaluated for their agonistic effect on PPAR-gamma and PPAR-alpha, transcription factors involved in the regulation of cellular differentiation, development, and metabolism (**UNINA**)

Fast identification of known compounds (dereplication): UNINA performed dereplication of the extract of the sponge *S. aurea* using high resolution HPLC-MS analysis on an Orbitrap instruments. Analysis of the HPLC-MS spectra allowed to identify not only a number of brominated and non-brominated alkaloids, but also several known brominated alkaloids. The early identification of known metabolites saved useless efforts for their isolation and structure elucidation, and allowed to focus analytical efforts to the isolation of the new poliketides/peptide hybrids, smenamides A and B.

Isolation and characterization of natural products predicted by genome mining: The following natural products were discovered at **ETH Zurich** by genome-based predictions. Four new polyketides were discovered from cyanobacteria, comprising three new phormidolide congeners from a *Leptolyngya* sp., one new luminaolide congener from a *Scytonema* sp. Luminaolide A was previously only known from a marine coralline alga. In addition, the *Scytonema* sp. yielded merosterol A (also named halomerol A), a dichlorinated steroid-like meroterpenoid that exhbits an unprecedented skeleton and is biosynthesized via a new type of pathway. The absolute configuration was determined. Merosterol-like compounds are so fare unique in free-living bacteria but well-known from sponges and other marine macroorganisms. Identification of the halomerol pathway therefore also provides access to the other pathways that likely belong to symbiotic bacteria. In the course of the search for merosterol-type compounds, we could also isolate new analogue, merosterol B. A new proteusin was isolated and characterized following heterologous expression of a gene cluster from the sponge symbiont "Entotheonella factor TSY1". Marine bacterium *Gynuella sunshinyii* contained a giant *trans*-AT PKS gene cluster, and from the extract, novel *trans*-AT PKS derived polyketides lacunalides A and B were isolated.

Identification of antiprotozoan natural products: Analysis of the endoperoxide fraction of a *P. simplex* specimen collected in the South China Sea afforded five new polyketide endoperoxides belonging to the class of 1,2-diox-4-enes, along with two

known analogues (**UNINA**). This isolation offered an interesting opportunity to further extend the structure-activity relationships and refine our knowledge on the mechanism of action of this class of simple antimalarials. The obtained structure-activity relationships (SARs) evidenced that, overall, the derivatives characterized by 1,2-diox-4-ene rings resulted more active than those possessing the 1,2-dioxane ring, but less active than plakortin and dihydroplakortin. The quinone scaffold of marine secondary metabolites was used as a chemical starting point to synthesize a new series of thiazinoquinone compounds. Some of the synthetic derivatives showed an interesting *in vitro* antiplasmodial activity on strains of *P. falciparum* (IC_{50} < 1μM) with >10-fold selectivity for cytotoxicity using a mammalian cell line (e.g. HepG2), representing new antiplasmodial thiazinoquinone hits for drug discovery in antimalarial research. The regiochemistry of the dioxothiazine ring and the nature of the substituent on the quinone ring both play a crucial role in the antiplasmodial effect together with thiazinoquinone ring system planarity; moreover, in the active regioisomers, the length of alkyl side chain distinguishes the antiplasmodial from the cytotoxic activity. The whole of our results supports the hypothesis that the antiplasmodial activity of thiazinoquinones is related to their capacity to form toxic semiquinone species, with the thiazinoquinone ring system planarity favoring the reductive activation of parent quinone (**UNINA**). 19 new polyketide endoperoxides with an amine chain at C4 of the parent 1,2-dioxane scaffold were prepared, including new endoperoxide-quinoline hybrids. The inclusion of a substituted amine moiety on our easily accessible 3-methoxy-1,2-dioxane scaffold improved significantly the antimalarial activity (**UNINA**)

Identification of neuroprotective natural products: As a result of a close collaborative work between **MNHN**, **UNINA**, and **MANROS**, a novel halogenated protein kinase inhibitor, chloromethylhalicyclamine B, together with the known natural compound halicyclamine B, was isolated from the extract of the sponge *A. ingens*. Structure of chloromethylhalicyclamine B was determined by spectroscopic means, and it was shown that chloromethylhalicyclamine B is produced by reaction of halicyclamine B with CH_2Cl_2 used for extraction. Absolute configuration of chloromethylhalicyclamine B was determined by quantum mechanical calculation of its CD spectrum, and this also determined the previously unknown absolute configuration of the parent natural compound halicyclamine B. Chloromethylhalicyclamine B is a selective CK1-delta/epsilon inhibitor at low micromolar concentrations, while the natural compound halicyclamine B is inactive. Docking of chloromethylhalicyclamine B in the ATP-binding site of CK1-delta rationalized this finding suggesting two possible binding modes, in both of which the chloromethyl group is surrounded by lipophilic side chains and provides several additional hydrophobic interactions. More generally, docking studies showed that chloromethylhalicyclamine B can efficiently interact with the ATP-binding site of CK1-delta in spite of its globular structure, very different from the planar structure of known inhibitors of CK1-delta. This opens the way to the design a new structural type of CK1-delta/epsilon inhibitors (**UNINA**, **MNHN**, **MANROS**).

Identification of antioxidant, antiinflammatory and antimicrobial marine compounds for cosmetic applications: Investigation of the Mediterranean ascidian *Phallusia fumigata* by **UNINA** led to the isolation of compound, phallusiasterol A, capable to induce PXR transactivation in HepG2 cells. Supporting the role of phallusiasterol A as PXR regulator, **UNINA** has observed that it effectively increases the expression of two PXR target genes, CYP3A4 and MDR1, in a human hepatocyte cell line. Thus, phallusiasterol A could represent a potential lead for the treatment of liver and intestinal disorders. During the chemical analysis of the sponge *Plakortis* cfr. *lita*, two new highly degraded steroids, incisterols A5 and A6, have been obtained. They were shown to be potent agonists of the PXR receptor, and insights on the structural requirements for their activity have been found. Chemical reinvestigation of the Mediterranean ascidian *Phallusia fumigata* resulted in the isolation of a further sulfated sterol, phallusiasterol C. The possible role of phallusiasterol C as modulator of

the pregnane-X-receptor (PXR) has been investigated, but it turned out to be inactive. Although negative, this result C could have interesting implication in terms of structure-activity relationship; if integrated with previously reported SAR data, our results seem to suggest that the presence of a double bond at B ring in phallusiasterol C could be a detriment for the PXR regulating activity. Research carried out in close cooperation by **MATIS**, **UNINA**, and **MNHN**, led to the discovery of a new antibiotic cyclic hexapeptide, representing a new structural type for antibiotic cyclic peptides, from *Thermoactinomyces vulgaris* strain ISCAR 2354 (isolated from costal marine hot spring) and *Thermoactinomyces vulgaris* strain ISCAR 2850 (isolated from deep sea hot spring at 400 m depth). Its structure was determined by NMR and MS analysis and advanced Marfey's degradation. This compound represents a new structural type for antibiotic cyclic peptides.

Quantitative analysis of metabolites in extracts: UNINA developed, among others, a method for quantitative analysis of neurosporaside (a glycolipid from the fungus *Neurospora crassa* isolated by the **UNINA**) and other cerebrosides. The method was used to evaluate the glycolipid content in wild-type and mutate strains of *N. crassa*.

Isolation of bioactive peptides: The polar fractions of the different selected bioactive extracts did not reveal any occurrence of bioactive peptides.

Structure elucidation of bioactive peptides: This part has not been investigated because of the reason mentioned above.

Search for (cyclic) peptides from the cold water marine sponges: LC-MS was used to create a molecular fingerprint of the different sponge samples for the identification of new bioactive peptides. Two disulfide-containing peptides, barrettides A and B, from the cold water marine sponge *G. barretti* were sequenced using mass spectrometry methods and structurally characterized using NMR spectroscopy. The two peptides were found to only differ at a single position in their sequence. The 3D structure of barrettide A revealed that these peptides possess a unique fold consisting of a long beta-hairpin structure that is cross braced by two disulfide bonds in a ladder-like arrangement. The peptides are amphipathic in nature with the hydrophobic and charged residues clustered on separate faces of the molecule. The barrettides were found not to inhibit the growth of either *E. coli* or *S. aureus* but displayed antifouling activity against barnacle larvae (*Balanus improvisus*) without lethal effects in the concentrations tested. These peptides have a unique three- dimensional structure when compared to previously described disulfide-containing peptides.

Chemical structures features: Testing of methods developed to define multi-dimensional space has been performed and evaluated by comparison with simple plotting (**UU**). In a parallell study it has been indicated that Euclidean distances in chemical property space exceeding 1 distance unit indicates a significantly lower chance of obtaining biologically meaningful data. Data in the form of 37 chemical structures submitted by partners has been included in databases, in addition to 14667 compounds retrieved from litterature data-mining including a ca 70% taxonomical coverage to be used as a reference set. Additional structures can very rapidly be included and their positions in chemical property space calculated. Compounds included thus far have positions in chemical property space published at the ChemGPS-NP web service under the title 'BlueGenics v1'.

Highlights:

- **Discovery of smenothiazoles, new highly cytotoxic mixed biogenesis chlorinated PKS/NRPS compounds from *Smenospongia aurea* (UNINA)**

- **Chemical analysis of the Indonesian sponge *Plakortis* cfr*. lita* afforded two new analogues of the potent trypanocidal agent manadoperoxide B, namely 12-isomanadoperoxide B and manadoperoxidic acid B**

- **Discovery of incisesterol A5 and A6, potent agosist of PXR receptor, from the sponge *Plakortis cfr. Lita* (UNINA)**

- **Three new cytotoxic compounds were isolated from two species of ascidians: conithiaquinones A and B from *Aplidium conicum*, and phosphoeleganin from *Sidnyum elegans* (UNINA)**

- **From the marine sponge *Theonella swinhoei*, two new cytotoxic depsipeptides, sulfinyltheonellapeptolide and theonellapeptolide, and a series of cyclotoxic two macrolides, including the new compounds isoswinholide B and swinholide K, were isolated (UNINA)**

- **Two sponge peptides, barrettide A and B, have been isolated and sequenced from the cold water sponge *Geodia barretti*, and the NMR solution structure has been solved for one, barrettide A (UU)**

- **Chloromethylhalicyclamine B, a novel halogenated alkaloid was isolated from the extract of the sponge *A. ingens*, and is a selective CK1-delta/epsilon inhibitor in spite of its globular structure, very different from the planar structure of known inhibitors of CK1-delta (UNINA)**

- **A new antibiotic cyclic hexapeptide, representing a new structural type for antibiotic cyclic peptides, was isolated from two two Icelandic thermophilic bacterial strains.**

- **Discovery of phosphoeleganin B, a new phosphorylated polyketide from the Mediterranean tunicate *Sidnyum elegans*, which is a close structural analogue of phosphoeleganin, a protein tyrosine phosphatase 1B inhibitor (UNINA)**

- **Studies on 13 further thiazinoquinone compounds led to the identification of a thiazinoquinone antimalarial lead compound active on strains of *P. falciparum* with IC_{50} < 1µM (UNINA)**

- **Discovery of conulothiazole A and B, two further members of the strongly cytotoxic chlorinated non-ribosomal peptide/polyketide (NRP/PK) hybrids that are found in the Caribbean sponges of the genus *Smenospongia* (UNINA)**

- **Discovery of spiroplakortone, plakortone Q, and plakdiepoxide, novel polyketide-based metabolites from the sponge *Plakortis simplex*, active on the nuclear receptors PPAR-gamma and PPAR-alpha (UNINA)**

- **Discovery of merosterols, biosynthesized by a novel type of metabolic pathway (UNINA, ETHZ)**

- **Lacunalides A and B, novel *trans*-AT PKS derived polyketides, as well as a new analogue of phormodolide were isolated from the marine bacterium *Gynuella sunshinyii* (ETHZ)**

- **Identification of a tetracyclic diamine alkaloid as a selective antimicrobial compound against *E. coli* from the marine sponge *Acanthostrongylophora ingens* (MNHN and UNINA)**

- **Discovery of a new antibacterial lipophilic cyclopeptide compound obtained from thermophilic bacteria *Thermoactinomyces vulgaris* (MATIS, UNINA, MNHN)**

4. GENOMICS

Summary:

The aim of this work package was to combine the knowledge in aquatic (marine) genomics, achieved by the molecular biology groups of the consortium, with advanced methods in target-oriented, efficient and sustainable drug discovery and development from aquatic invertebrates and (associated) microorganisms. The specific objectives were: (i) Identification of cDNAs/genes involved in the carotenoids/retinoids biosynthetic pathways; (ii) Production of novel carotenoid cleavage products using these enzymes; (iii) Identification and cloning of bioactive peptides from sponges; (iv) Identification and isolation of cDNAs/proteins of commercial interest from deep sea sponges; (v) Identification and isolation of cDNAs/proteins involved in accumulation of rare earth elements from deep-sea manganese nodules and seamount crusts; and (vi) Expression, affinity purification and refolding of recombinant proteins.

In detail:

Carotenoids – Retinoids: Identification of cDNAs/genes involved in biosynthetic pathways: The genes/cDNAs encoding for the two key enzymes of carotinoid metabolism, the beta,beta-carotene-15,15-dioxygenase and the retinal dehydrogenase/reductase have been cloned from the marine demosponge *S. domuncula.* The expression of both genes is strongly up-regulated by retinoic acid. Experiments with sponges and sponge primmorphs kept under controlled conditions in the absence or presence of antibiotics revealed that the levels of beta-carotene are correlated with the bacterial load. In addition, **UMC-Mainz** identified two further (potential) carotenoid oxygenases that, however, after expression, did not cleave beta-carotene. The complete cDNAs, encoding the *S. domuncula* related beta-carotenoid oxygenases (*SDrBCO1* and *SDrBCO2*), were isolated.

Production of carotenoid cleavage products: The function of the *S. domuncula SDCDO* and the *S. domuncula SDrBCO* could be demonstrated in *E. coli*, which had been transformed with the gene clusters maintaining the accumulation of either beta-carotene or lycopene in the microorganisms.

Identification and cloning of bioactive peptides from sponges: UMC-Mainz could present evidence that retinal interacts with a toxic peptide in *S. domuncula*, with Suberitine. **UMC-Mainz** found that Suberitine in *S. domuncula* is inactivated after binding to retinal. This was the first time that it was shown that in sponges a low molecular bioactive metabolite can interact with a bioactive peptide and thus alters its function. In addition, **UMC-Mainz** identified and characterized two further toxins from marine sponges of potential biomedical interest: First a complement protein c8alpha-like polypeptide (toxin) from the hexactinellid sponge *Crateromorpha meyeri* and second a cytolysin from the demosponge *D. avara.* Modelling studies revealed that the *D. avara* cytolysin has the typical structure of the sea anemone actinoporins with a tightly folded sandwich of several beta-strands, which are embedded by the alpha-helices A and B. The 3D structure of the *D. avara* cytolysin shows an extremely tight

packing of the side chains in the hydrophobic core of the molecule typical to the actinoporins. In addition, the RGD motif is exposed and accessible for cell interaction.

Identification and isolation of cDNAs/proteins of commercial interest from deep sea sponges: NRCGA-CAGS in cooperation with **UMC-Mainz** identified/cloned two potential molecules of the sponge cryptochrome photoreception system the guanine nucleotide-binding protein beta subunit, related to beta-transducin, and the nitric oxide synthase-interacting protein. In addition, another sponge protein of biotechnological interest, which is involved in the cycling of sulphur, attracted our attention. **NRCGA-CAGS** and **UMC-Mainz** succeeded to identify to express in *E. coli* a sulfatase gene from marine sponge *D. avara.* The expressed recombinant enzyme might be useful for enzymatic modification of biopolymers for diverse biotechnological. Sulfation reactions are involved, for example, in the synthesis of carrageenans, sulfated polysaccharides that are found in the cell walls of red algae. They are widely used in the food industry because of their gelling, thickening and stabilizing properties.

Identification and isolation of cDNAs/proteins involved in accumulation of rare earth elements from deep-sea manganese nodules and seamount crusts: In addition to ferritin, **NRCGA-CAGS** together with **UMC-Mainz** focused on another sponge enzyme of commercial interest: Laccase, a metal-containing enzyme involved in the anti-bacterial defense system of sponges. Laccases are copper-containing enzymes that catalyse the oxidation of a wide variety of phenolic substrates and, in the presence of a mediator, also non-phenolic compounds. **NRCGA-CAGS** and **UMC-Mainz** succeeded to describe the first poriferan laccase from the marine demosponge *S. domuncula*. The sponge enzyme comprises the three characteristic multicopper oxidase homologous domains. This enzyme could be applied for detoxification and elimination of lignin-derived products, but also very likely in combination with a mediator(s) for killing bacteria. The *S. domuncula* laccase was heterologously expressed in *E. coli*. The recombinant sponge enzyme is significantly more active if the mediators ABTS and syringaldazine are added. The level of tissue expression of the laccase is strongly upregulated if the animals are exposed to bacterial LPS. This finding underscores our hypothesis that the sponge laccase has a role in host-defense against microorganisms.

Expression, affinity purification and refolding of recombinant proteins: This project resulted in the identification and characterization of a number of cDNAs encoding for proteins of biotechnological or biomedical interest. **NANOTEC** selected the right hosts for the expression and fermentation of the enzymes. Expression in *E. coli* was performed, e.g., using the oligohistidine expression vector pQ30 A. Sample lysis was performed, e.g., in lysis reagent Bugbuster with benzonase, and lysis of insoluble fraction in buffer containing urea. The lysates were purified and refolded using an automated chromatography system. The proteins expressed included, among others: Ferritin 1 (*S. domuncula*), complement protein c8-alpha-like polypeptide (*C. meyeri*), cytolysin (hemolytic toxin) (*D. avara*), sulfatase (*D. avara*) and laccase (*S. domuncula*).

Highlights:

- **Identification of new genes/cDNAs involved in carotinoid pathway**
- **Production of carotenoid cleavage products using the recombinant proteins**
- **Two further marine sponge toxins of potential biomedical interest have been identified and characterized (UMC-Mainz)**

- **The first toxin, a complement protein c8-alpha-like polypeptide, has been identified in the hexactinellid *Crateromorpha meyeri* and expressed as recombinant protein (UMC-Mainz)**

- **The second toxin is a cytolysin from the demosponge *Dysidea avara* which is related to the sea anemone group II cytolysins (UMC-Mainz)**

- **Modelling studies revealed that the *D. avara* cytolysin has the typical structure of the sea anemone actinoporins with a tightly folded sandwich of several beta-strands, which is embedded by the alpha-helices A and B (UMC-Mainz)**

- **Isolation and expression of a sulfatase gene from demosponge *D. avara*, which is of interest for enzymatic removal of negatively charged sulphate groups from biopolymers for diverse biotechnological applications (NRCGA-CAGS and UMC-Mainz)**

- **Cloning of the first poriferan laccase from the marine demosponge *S. domuncula*, a metal (copper)-containing enzyme involved in the anti-bacterial defense system of sponges (NRCGA-CAGS and UMC-Mainz)**

- **Demonstration that the laccase can be applied in combination with a mediator(s) for killing bacteria (NRCGA-CAGS and UMC-Mainz)**

5. METAGENOMICS AND GENE MINING

Summary:

The aim of this work package was to use state-of-the-art metagenomic and biosynthetic prediction techniques to specifically target genes and compounds of biomedical interest for sustainable biotechnological production. The specific objectives were: (i) Development of PCR-based methods to target *trans*-AT polyketide synthase (PKS) and proteusin gene clusters; (ii) Identification of the biosynthetic genes for bioactive natural products (PKS and proteusin); (iii) Genome mining for the discovery of new natural products (PCR-based identification of coding sequences for putative biosynthetic enzymes); (iv) Preparation of a marine strain collection containing *trans*-AT PKS and proteusin genes for further cultivation and compound isolation; (v) Preparation of metagenomic DNA from extreme environments; (vi) Expression profiling with high throughput sequencing; and (vii) Metagenomic studies of sponge microbiota and water microbes, and on extremophilic bacteria, using V6 tag pyrosequencing (FLX+454 sequencing).

In detail:

Development of PCR-based methods to target *trans*-AT PKS and proteusin gene clusters: For *trans*-AT PKS clusters, a large number of validated specific primer pairs were developed. Using these primers we can now detect PKS genes that correspond to specific structural moieties in polyketides, including tetrahydropyran rings, exomethylene groups, acetyl starter groups (1 primer pair each), amino acids and methyl groups (2 primer pairs each). In addition, we have generated three primer pairs with general specificity for *trans*-AT PKS genes. These primers have been successfully used to detect several gene clusters in sponge metagenomes. For proteusin clusters, **ETH Zurich** developed one new primer pair that specifically targets peptide-associated radical SAM methyltransferase genes.

Identification of the biosynthetic genes for bioactive natural products: **ETH Zurich** has identified a large number of biosynthetic gene clusters from sponge symbionts (sponges: *Discodermia kiiensis*, three different chemotypes of *Theonella swinhoei*) that encode the production of sponge-associated bioactive compounds. These include clusters for the following nonribosomal peptides: keramamides, orbiculamides, konbamides, nazumamide A, cyclotheonamides and pseudotheonamides, theonellamides, discodermins, novel cytotoxic peptides termed lipodiscamides. They have studied substrate specificity for several A domains in the clusters and characterized their functions. For polyketides they have isolated the misakinolide A and swinholide cluster and a remaining portion of the onnamide cluster that was not available before. In addition, >50 PKS, NRPS, proteusin, and alkaloid clusters with as-yet unknown function were identified from sponge symbionts. More than 60 new proteusin clusters were identified in various cyanobacteria and other bacteria, including two polytheonamide-like gene clusters. From further cyanobacteria, three new *trans*-AT PKS gene clusters were identified. Also, numerous *trans*-AT PKS gene clusters were identified from bacteria. Finally, one entirely new type of pathway that is derived from plastoquinone biosynthesis was discovered. The misakinolide and psymberin gene clusters, spanning ca. 80 and 60 kb, were assembled in yeast as a basis for heterologous expression. The polytheonamide cluster was assembled and transferred to several host, resulting in production of a range of polytheonamide analogs.

Genome mining for the discovery of new natural products: An automated prediction software (running title: TrAPPt) was developed that proposes polyketide structures from sequences of trans-AT PKSs. The products of all three new *trans*-AT clusters from cyanobacteria were identified. One encodes the known polyketide tolytoxin, the other clusters yielded several new compounds that were characterized. The product of the plastoquinone-derived pathway was identified as an unprecented and structurally unusual chlorinated steroid-like compound that is, however, biosynthesized via a steroid-independent route. From the *trans*-AT PKS gene cluster in *Pseudomonas syringae* B728a, the structure of the product was predicted. Using this information we could isolate the natural product from the extract, which was in good agreement with the predicted structure. *Gynuella sunshinyii* contained 6 different trans-AT PKS gene clusters, and two novel macrolides were isolated by genome mining. From the same bacterium **ETH Zurich** could also isolate a new phormidolide analogue and two tartrolon-type products, which were predicted from the genome sequence as well.

Preparation of a marine strain collection containing trans-AT PKS and proteusin genes for further cultivation and compound isolation: At **ETH ZURICH,** a cyanobacterial collection of 33 strains was prepared that contains trans-AT PKS and proteusin gene clusters. In addition, ca. 20 non-cyanobacterial strains carrying such clusters were added to the collection.

Gene mining for genes/proteins from extreme marine environments: At **ETH Zurich** several highly unusual enzymes from uncultivated bacterial symbionts were isolated and characterized. These include members of the new family of radical SAM peptide epimerases, which catalyze irreversible L- to D-amino acid conversions, a peptide N-methyltransferase that iteratively *N*-methylates asparagine residues, two iterative radical SAM C-methyltransferases, a peptide hydroxylase that converts several Asn residues, and a new polyketide synthase domain that catalyzes the stereoselective formation of di- and tetrahydropyran rings. In addition, a new type of terpene cyclase that forms benzannelated steroid rings was identified. Samples for metagenomic analyses taken at marine thermal springs in Iceland were analysed through high-throughput Next Generation Sequencing (NGS) (**MATIS** and

PROKAZYME). Following improved bioinformatic analyses, additional genes/enzymes of potentially commercial interest to those reported in the previous reporting period were chosen for further characterization. In addition, genomes of seven bacteria isolated from these extreme environments and the open ocean were mined for such genes as well. In total, over 200 different genes for carbohydrate enzymes were identified in the genomes of which selected ones were further characterized.

Expression profiling with high throughput sequencing of selected sponge species: The purpose of this task is the analysis of the impact that an *ex situ* culture environment has on sponges and their associated microbes, and to compare this to samples from their natural habitat. Sponges of the species *Halichondria panicea* Pallas have been sampled and brought into culture at partner **SAEBYLI**. Material from cultivated sponges was selected based on observed morphological changes in culture, as well as representing different treatments and culture conditions at Saebyli. All samples had mRNA extracted, using the Transplex Whole Transcriptome Amplification kit (Sigma-Aldrich), which was translated into cDNA according to the manufacturer's instructions. The chosen method allows the extraction and analysis of both the eukaryotic mRNAs from the sponge host as well as the prokaryotic mRNAs of the associated bacteria in one experiment. Multiple methodological difficulties were encountered during the analysis, i.e. delay through problems to obtain stable sponge cultivation, inability to extract high quality mRNA from the sponges and resulting inadequate NGS sequencing results of the cDNAs. Despite multiple tries these problems could not be solved during the period of the project. To obtain some information about the potential expression activity of sponge-associated bacteria and mitigate the lack of transcriptomic data, bacterial metagenomes were sequenced by Illumina NGS. Bioinformatic analysis, e.g. gene assembly and annotation, is currently ongoing.

Metagenomic studies of sponge microbiota and water microbes using V6 tag pyrosequencing: Shifts in the microbial diversity of the sampled tissue could be observed between sponges from their natural environment and upon transfer to the cultivations system, as well as between different water exchange regimes. The microbial community in sponge tissue from all collected individuals from their natural environment showed a similar relative abundance of dominant phylotypes with the order *Rhodobacterales* being the most represented. Upon transfer to the *ex situ* culture environment a relative increase of *Gammaproteobacteria* could be observed with the dominant phylotype being affiliated with the order *Alteromonadales*. In the recirculating system without any sea water exchange the shift toward *Alteromonadales* affiliated bacteria as dominant phylotype was accelerated compared to the flow-through regime were the shift took longer time. A general loss of bacterial diversity could however be observed in sponge tissue from the *ex situ* culture environment from both sea water exchange regimes.

Identification and isolation of beta-glucan active enzymes from extreme aquatic sources in Iceland (thermophilic and psychrophilic bacteria): beta-glucan active enzymes that were identified in bacterial metagenomes and genomes were isolated and further characterized through expression analyses. Of around 30 enzymes chosen for cloning and expression analysis, 9 tested positive. All these enzymes will be made commercially available through the PROKAZYME "enzyme boutique".

Highlights:

- **Identification of >50 gene clusters from uncultivated symbionts of marine sponges (ETH Zurich)**
- **Assembly of the misakinolide and psymberin gene cluster (ETH Zurich)**

- **Production of a new proteusin from a sponge symbiont and of polytheonamide analogs by heterologous expression (ETH Zurich)**

- **Large collection of strains carrying proteusin gene clusters (ETH Zurich)**

- **Enzymes from uncultivated sponge symbionts catalyzing new transformations (ETH Zurich)**

- **Swf, a new group of mono-modular type I PKS/FAS, which appears to be specifically associated to sponge symbionts (UNINA and ETH Zurich)**

- **Comparative metagenomic studies of sponge microbiota between natural and cultivated sponges show differences in the diversity and dominance of microbial taxa (MATIS)**

- **Nine novel beta-glucan active enzymes have been identified in microbial genomes and metagenomes, were cloned in expression vectors and had their activity confirmed (MATIS and PROKAZYME)**

6. CHEMICAL SYNTHESIS AND CHEMOGENETICS

Summary:

This WP comprised a Chemogenetics-Bioengineering part, describing the development and application of genetic methods for the engineered biosynthesis of analogues of the bioactive compounds for structure-activity relationship studies, and a Chemistry part describing the chemical synthesis of the bioactive compounds. The final aim of these research activities was to link highly advanced chemical strategies with genomic approaches for the synthesis and semisynthesis of analogues of bioactive natural products for pharmacological evaluation. The specific objectives were: (i) Biosynthetic elucidation of novel natural products with significant antiprotozoan activity or neuroprotective activity or with significant antioxidant/antiinflammatory activity (sequence and isotope guided); (ii) Precursor directed biosynthesis/mutasynthesis. Synthesis and feeding of analogues of biosynthetic precursors in order to generate series of compounds for structure activity relationships; (iii) Combinatorial biosynthesis to generate analogous; (vi) Synthetic modification of natural and unnatural products; (v) Development of specific, environment-friendly chemical protocols for the synthesis of small libraries of kinase inhibitors derived from marine sponge metabolites indentified in the screens of WP2; (vi) Synthesis of antagonists/modulators of amyloid beta-42 production induced in a cellular system, based on initial hits discovered in the bioactivity screening (WP2); (vii) Establishment of methods for synthesis of disulfide-rich and cyclic peptides of sponge origin; and (viii) Identification of three-dimensional structural features of bioactive natural and unnatural compounds through molecular modeling studies.

In detail:

Gene sequence guided biosynthetic elucidation of medicinally significant novel natural products: The 90 kb pathway for the production of the actin inhibitor misakinolide was assembled on a yeast-*E. coli* shuttle plasmid (**ETH Zurich**). New gene clusters for the biosynthesis of cytotoxic lipodiscamides were identified from an "Entotheonella" symbiont of the sponge *D. kiiensis*. The sequence of the discodermin cluster from *D. kiiensis* was completely assembled, revealing a radical SAM enzyme as only candidate for a t-Leu residue in the peptide. Components of the gene cluster for

the biosynthesis of a newly identified cyanobacterial meroterpenoid with pseudosteroidal structure were heterologously expressed in *E. coli*. The cluster encodes two functionally distinct halogenases that are promising tools for the biotechnological generation of novel halogenated steroids. All biosynthetic enzymes in the polytheonamide pathway were biochemically characterized, revealing numerous novel or unusual peptide modifications. Genome scans have been aquired for a marine actinomycete, and the biosynthetic cluster, of an unusual polyketide that is encoded within the genome, analysed *in silico*. Gene knock and gene complementation studies have enabled us to identify a cluster involved in the biosynthesis of a potentially important antibiotic (**USTAN**). The putative terpene cyclase of merosterol was also heterologously expressed in *E. coli*. Function of this enzyme was proofed by the co-incubation of the enzyme and its predicted substrate 5-geranylgeranyl-3,4-dihydroxy benzoic acid. The analysis of the assay extract with LCMS showed an additional peak, and NMR analysis of the isolated peak confirmed the proposed cyclized product.

Using isotopically labelled putative precursors to enable biosynthetic elucidation of novel natural products of interest: USTAN has investigated the biosynthesis of the antibiotic vD844 using isotopically labeled methionine precursors. Our findings are consistent with the *N*-methyl group being derived from methionine. Isotope labelling studied provided intriguing results surrounding the origin of the *N*-formyl group in this system. The most obvious origin for the formyl group in vD844 was expected to be through transfer from N^{10}-formyltetrahydrofolate, however, no gene encoding for a tetrhydrofolate-dependent transferase was located near the cluster. The biosynthetic gene cluster for antibiotic vD844 reveals two putative *N*-methyl transferases, H12 and H16, that could be responsible for the late stage modification of this antibiotic. In this pathway, holothin would be methylated twice on its primary amine and one of methyl groups would subsequently be oxidised to the formyl group. Informed by our observed labelling patterns, we have advanced this aspect beyond the isotopic labelling studies and we have cloned, expressed and purified the two methyl transferases and are in the process of synthesising the holothin core in order to provide substrate for the further assessment of these enzymes.

Generating series of natural product analogues through synthesising and feeding biosynthetic precursor mimics to wild type and heterologous expression strains, and gene disruption strains (Precursor directed biosynthesis and mutasynthesis): USTAN has successfully conducted precursor-directed biosynthesis on a *Streptomyces* strain. Having first successfully completed the synthesis of a library of putative precursor analogues including 5-bromo-L-tryptophan, 6-bromo-L-tryptophan, 7-bromo-L-tryptophan, 4-iodo-L-phenylalanine, 4-ethynyl-L-phenylalanine and butynoic acid. Using a genome reading approach to identify clusters and their putative precursors we selected an actinomycete that was most likely to incorporate these precursors. Precursor directed biosynthesis with this strain has excitingly unearthed unexpected catabolism with the potential for biotechnological applications.

Combinatorial biosynthesis to access series of new natural product analogues: The aim of this task was to introduce halogenase genes into bacterial hosts to work in concert with endogenous biosynthetic pathways to produce new, halogenated natural products with improved bioactivity and bioavailiability. Series of halogenases have been cloned and investigated by **USTAN**. HalKer, identified from a metagenome by the lab of **ETH-Zurich**, has been investigated. **USTAN** have achieved the heterologous production of this protein in soluble form and have demonstated its brominase and chlorisae activity on a broad range of substates *in vivo,* this provides the first functional evidence for this a new keramamide biosynthetic pathway identified in an entotheonella metagenome by **ETH Zurich**. Though HalKer has innately broad substrate specificity, unusually it utilizes 5-hydroxy tryptophan as its preferential substrate. Cystargamide contains a 5-hydroxy tryptophan. To explore whether cystargamide might incorporate

halotryptophan **USTAN** has synthesized and fed series of halotryptophans to cultures of the producing microbe. These experiments have resulted in new halogenated metabolites. Transformation procedures for the cystargamide producer and the strains received from **MNHN** have been explored by **USTAN**. Transformations have been achieved for some of the strains but not for the cystargamide producer. An alternative approach for this strain, is to explore cosmid library generation, heterologous expression of the entire pathway and complementing the biosynthetic pathway with the halogenase in the heterologous host. **USTAN** has utilized such a synthetic biology approach in order to access analogues of other less tractable biosynthetic pathways.

Synthetic modification of natural products, and of unnatural products generated through mutasynthesis and combinatorial biosynthesis: USTAN has developed series of mild conditions for the modification of halotryptophans embedded within natural products and peptides. **USTAN** carried out test-bed studies to explore cross coupling of halogenated pyrolles, prior to investigating the cross coupling of Stevensin (halopyrolle containing natural products), cross coupling has been successfully achieved, we now work to explore conditions to enable the selective cross coupling of dihalo containing pyrolles. **USTAN** has successfully applied cross coupling modifications to new to nature halometabolites that we have generated including bromocystargamide.

Synthesis of pharmacological inhibitors of disease-relevant protein kinases: ManRos has pursued our screen of extracts from marine sponges and bacteria, and purified products (provided by **MNHN**, **MATIS**, and **UNINA**) on disease-relevant kinases. Only a few active products/extracts have been identified in this screen, highlighting the specificity of the assays. In the current absence of promising hits from the past screens, we have focused our chemistry efforts on Leucettines, derived from the marine sponge natural product Leucettamine B as well as on the closely related marine sponge Polyandrocarpamines. More than 550 analogues have been synthesized over the past 9 years, some of which under the BlueGenics contract. All of them have been tested on 13 mammalian kinases, and some on 11 orthologues in unicellular parasites. Two sub-families of Leucettines are being developed taking into account pharmacological considerations and druggability. Besides no new promising scaffold has being identified and we have therefore decided to go ahead and further towards clinical trials with the most promising scaffold, the Leucettines. So far the reference Leucettine, L41, displays rather promising effects on two animal models of Down syndrome, and more recently on a genetical model of Alzheimer's disease.

Synthesis of pharmacological modulators of amyloid beta-42 production: The assay to detect inducers and inhibitors of Amyloid beta-42 (A42) is a cellular assay based on N2a-APP695 or CHO-7PA2-APP cells expressing APP (amyloid precursor protein). These cells are exposed to a reference compound, Aftin-5, and as a response, produce a large quantity of the 'Alzheimerogenic' product, A42. **ManRos** is thus able to detect: (1) A42 inducers in the 'human chemical exposome' (potential 'Alzheimerogenic' products), and (2) products which could prevent the production of A42 (potentially anti-Alzheimer products). **ManRos** has screened extracts and purified products derived from sponges and associated microorganisms for such biological activities. So far we not have identified any bioactivity, from sponges/associated microorganisms, which would be worth pursuing. Only two aqueous phases of fractions among 160 extracts reduced A42 production induced by Aftin-5. However in parallel **ManRos** has continued to work at the evaluation of new aftins, and we have identified some pesticides among the human chemical exposome. These results show that we may be exposed to 'Alzheimerogenic' products in our environment, although we need much more data to confirm this hypothesis. These products are in fact triazine herbicides and one of them (cybutryne) is an anti-fouling agent. Amyloids have been analyzed by immunoprecipitation/mass spectrometry and quantified. One of the

triazines was injected in WT and Alzheimer's disease model mice but did not trigger an increase in A42 levels. This is most likely due to their short half-life and rapid disappearance. In contrast, pyrazoles have a longer half-life (especillay the metabolites), they accumulate in adipose tissue and brain and the main metabolite of the main pyrazole we studied is extremely stable. These pyrazoles thus also constitute outstanding molecular tools for the study of Alzheimer's disease onset.

Establishment of methods for peptide synthesis of disulfide-rich and cyclic peptides of sponge origin: Synthesis and folding of the novel 31 amino acid residue barrettide A has been successfully done using microwave-assisted Fmoc-SPPS. Using this method, the peptide was synthesized on chlorotrityl chloride resin using microwave heated coupling and deprotection steps at a temperature of 60°C. DIC was used as the coupling agent together with the base ethyl (hydroxyimino)cyanoacetate. The purified reduced peptide was then oxidized under optimized folding conditions for 48 h and re-purified on RP-HPLC. Folded peptide was identified by MS, and the oxidized peptide co-eluted with native peptide on analytical RP-HPLC, confirming that the native disulfide connectivity was maintained in the synthetic peptide. A comparison of the H-alpha chemical shift values of synthetic and native peptide also confirmed that the correct disulfide isomer was obtained. In addition, the sialidase inhibiting sponge peptide asteropine A has been targeted for synthesis (**UU**). This peptide contains three disulfide bonds (barrettides have two disulfide bonds) carries a high negative charge because of the presence of multiple glutamic acid residues. Disulfide bonds are arranged in a cystine knot. This peptide has now been successfully been synthesized and folded, although at moderate yields. Hence, within the Bluegenics project **UU** has demonstrated methods for the synthesis and folding of both classes of disulfide rich sponge peptides currently known.

Molecular modeling studies: UNINA performed, among others, computational studies on thiaplidiaquinone B as well as on other marine-derived meroterpenes, conicaquinone A, conithiaquinones A and B, all isolated from *A. conicum*, and their reduced species in order to identify new key physical chemical properties related to their effects on cells growth and viability, also considering that subtle structural differences can determine significant variations in the biological activity of these compounds. The computational methodology previously used to study the conformational and electronic properties of several synthetic thiazinoquinone analogues was expanded by combining empirical and semi-empirical calculations with DFT methods specific for the calculation of the redox properties. The computational results indicate as chemical-physical properties possibly involved in the cytotoxic mechanism of action of natural thiazinoquinones: i) their electrophilicity index, ii) their ability to undergo a one electron reduction, and iii) the electron affinity of their protonated semiquinone species. In summary, computational studies performed on four bioactive natural thiazinoquinones led to the identification of the pharmacophoric features responsible for their cytotoxicity activity, and, combined with the previous results obtained on synthetic thiazinoquinones, pave the way to the rational design of selective (semi)synthetic quinones characterized by anticancer or antiplasmodial activity.

Highlights:

- **Identification of the gene cluster responsible for the biosynthesis of merosterol (ETH Zurich)**
- **Determination and application of a new halogenase (USTAN)**
- **New to nature halo natural products generated (USTAN)**

- **Mild aqueous conditions for synthetic diversification of halotryptophans and halopyrolles developed and applied (USTAN)**

- **Further chemical optimisation of the Leucettine scaffold (kinase inhibitors derived from the marine sponge natural product Leucettamine B) (ManRos)**

- **Proof of concept established in 2 mouse models for Leucettine L41 as an agent able to correct some of the cognitive deficits associated with triplication of DYRK1A seen in Down syndrome (ManRos)**

- **Screening for amyloid beta-42 inducers in the 'human chemical exposome' allows identification of triazine herbicides and pyrazole pesticides (ManRos)**

- **Identification of the three-dimensional structural features of synthetic analogues of marine natural compounds, such as plakortins (endoperoxides) and aplidinones (thiazinoquinone) responsible for their antimalarial activity (USTAN and ETH Zurich)**

- **Computational studies performed on four bioactive natural thiazinoquinones led to the identification of the pharmacophoric features responsible for their cytotoxicity activity (UNINA)**

- **Synthesis and folding of barrettide A successfully done (UU)**

7. BIOPROCESS DEVELOPMENT AND SCALE-UP

Summary:

The aim of this work package was (i) set-up and optimisation of the SustainCycle system for cultivation of sponges (although not prime focus of this project but needed to obtain sponge / sponge-associated bacteria material under defined conditions); (ii) scale-up of sponge primmorph cultures, (iii) high-throughput screening of processing conditions and optimization of fermentation process; (iv) scale-up of production of ISCAR marine bacteria extracts; and (v) scale-up of production of recombinant proteins. The specific objectives were: (i) Optimisation of the conditions in the SustainCycle system (bacteria, algae, pH and minerals) to maximize the growth of sponges; (ii) Upscaling of SustainCycle for sponge cultures to pilot scale; (ii) Screen at a very early stage which strains show promising processing features; (iv) Optimize the composition of the culture media in order to avoid high salt concentrations; (v) Optimize the reproducibility and productivity of the fermentation processes; (vi) Perform the scale-up of the processes for process technical and economical validation and for producing small batches of product; (vii) Adaptation of the already optimized lab scale standardized culturing protocol of ISCAR marine bacteria to a large scale production; (viii) Large scale production of selected ISCAR marine bacteria aqueous extracts; and (ix) Scale-up of production of recombinant proteins.

In detail:

Set-up and optimisation of conditions of the SustainCycle system to maximize sponge growth: An *ex situ* sponge culture system was developed in cooperation between the project partners **SAEBYLI** and **MATIS** to enable long-term cultivation of different sponge species and optimize culture conditions towards enhanced growth and

metabolite production while maintaining the sponge specific microbiota. *H. panicea* Pallas, 1766, an endemic sponge species to Iceland, was chosen as a target species due to its accessibility and production of pharmaceutically interesting bioactive compounds. The current sponge system based on the SustainCycle system has proven to be capable of keeping sponge individuals in captivity while maintaining pumping activity and observable growth of selected individuals.

Scale-up of sponge primmorph cultures: Sponge primmorphs provide a system or the production of sponge secondary metabolite. Using conventional sponge cell culture methods only small primmorphs (1-2 mm in diameter) are obtained. **UMC-Mainz** and **NANOTEC**, in cooperation with **NRCGA-CAGS** succeeded in cultivating primmorphs for several weeks in a special, newly constucted reusable 3-L bioreactor (upscaling factor 1:500). Using this bioreactor, after 2 days primmorphs with size of about 2 cm from the demosponge *S. domuncula* were obtained. These are the largest primmorph until now reported. It possible to further enlarge the primmorphs over several days by repeated addition of more cells from the same sponge individual.

High-throughput screening of processing conditions: A screening protocol for culturable marine microbial strains was implemented at shaken flask and using parallel controlled bioreactors. Further, medium enrichment proved to enable to obtain higher biomass levels and provide higher sensitivity in the screening test for strains fit for process development. These results had been obtained using the commercial Marine Broth, Difco™ MB 2216. It is an expensive medium, with a high salt concentration mimicking that of seawater. The screening protocol was used to assess different strategies to simplify the medium in order to enhance the growth potential while lowering the associated bioprocessing costs. Next, an alternative medium was used at **BIOTREND** which normally yields very good results in prolonged cultures combining high cell densities with the production of secondary metabolites. Further, based on the composition of MB from Difco, an alternative medium was produced at **BIOTREND**. The use of the **BIOTREND** MB medium resulted in a maximum OD of 7.02 at 40h. An alternative sea salts medium was prepared by **BIOTREND** in order to assess the effect of salts present in small amounts in commercial MB, called Main salts medium. The comparison between the growth curves of MainSalts and **BIOTREND** MB cultures shows that the salts present in smaller amounts in MB are probably not significant, allowing simplifying the medium preparation without adverse effects on growth. The extensive tests performed confirm that the screening platform used at **BIOTREND** is highly successful for detecting the impact of different processing conditions on process performance parameters, such as growth rates, titres and yields.

Optimization of fermentation process: The work was focused on *Rhodothermus marinus* strains provided by **MATIS**. A first challenge presented by this strain was the fact that it would aggregate in liquid culture, which will prevent obtaining high cell density cultures easy to control and process. In order to circumvent this problem, a strain adaptation trial was carried out through successive transfers in liquid culture, MB and M166 media, from the seed culture until less cell aggregation and higher OD were obtained. High concentrations of sodium chloride, such as those in seawater, are detrimental to the lifetime of stainless steel equipment such as standard bioprocessing equipment, particularly if the culture medium is sterilized in the bioreactor since the sterilization causes the release of Cl_2 which is corrosive. One approach is to replace the chloride ion in the medium composition while keeping an adequate concentration of essential ions such sodium. Various *Vibrio alginolyticus* and *Vibrio sp.* strains were received from **UMC-Mainz**. Preliminary studies were carried out using Marine Broth and when growth, as assessed by the evolution of optical density, was stopped, glucose was added to obtain a glucose concentration of 10 g/L. Most strains did increase significantly the biomass concentration after adding glucose, showing potential for the implementation in higher productivity processes. However, since

various *Vibrio* strains belong to the biosafety level 2 and the strains received were not yet fully characterized in terms of biosafety no further trials were carried out as Biotrend cannot work with strains belonging to biosafety level higher than 1 in larger scale. In addition, **BIOTREND** has worked with strains of the ISCAR collection from **MATIS**, which had shown promising activities. ISCAR 138 was successfully grown in fermenter and a fed-batch strategy was established.

Process scale-up: BIOTREND has implemented fermentation-based production of some of the promising bacterial extracts tested in BlueGenics. The first goal of the productions performed was to obtain sufficient amounts of extracts for a full chemical study. To attain this goal, diverse fermentation strategies were tested and implemented which also aimed at improving the productivity of the process, meaning, produce more bio-active, per reactor capacity and per processing time, which would be very relevant for an up-scaled application and to avoid requiring the processing of 200L of fermentation broth with a very low cell density. Some of the parameters that were tuned in this work involved the reduction of salt concentration, the use of temperatures which would result in higher growth rates and lower processing costs (ex. increase of fermentation temperature to avoid cooling costs when processing psychrophylic microorganisms), and the establishment of adequate nutrient feeding rates. One critical observation during these trials was the variation, sometimes very significant, of activity profiles during the fermentation and the triggering of the production of specific metabolites which depended on the carbon uptake rate, carbon/nitrogen/phosphorous ratios and influence of the presence/absence of specific oligo-elements. The detailed process design would need to take into account in-depth physiological information of the strains and if possible the knowledge of the specific pathways which would be required to be active for the production of the compounds of interest. Since in most cases the specific compounds responsible for the identified activities are not know yet, nor if just one compound or the synergistic action of multiple compounds is involved, it would not be feasible to perform the transition to 50L scale at the current stage, as it would entail a significant effort without an appreciable likelihood of success.

In the frame of BlueGenics project, **UMC-Mainz** succeeded to develop a novel biomimetic toothpaste containing morphogenetically active amorphous polyP microparticles enriched with retinyl acetate. The polyP ingredient strongly and specifically inhibited the growth of the cariogenic bacterium *Streptococcus mutans*, while triclosan, a component of many other toothpastes, inhibits the growth of widespread bacteria, e.g. *S. aureus*. In the frame of the preclinical studies, we had to upscale *S. mutans* (fermentation under S2 conditions in the **UMC-Mainz** laboratory, which has the required biological safety level and permission). The fermented bacteria were used for the isolation and purification of the bacterial phosphatase involved in degradation of the tooth enamel. The results revealed that our novel type of toothpaste is particularly suitable for prevention/repair of cariogenic damages of tooth enamel/dentin caused by *S. mutans.*

Scale-up of production of recombinant proteins/enzymes: Three proteins have been selected for large-scale production: *(i)* Bone morphogenetic protein from the demosponge *S. domuncula*; *(ii)* Laccase from *S. domuncula*; and *(iii)* Sulphatase from the demosponge *D. avara.* The cDNAs spanning the mature segments were inserted into the oligohistidine expression vector pQ30 A (Qiagen). The *E. coli* host strains BL21 (Novagen) was transformed with the plasmids. For large-scale production of the recombinant proteins, the bioreactor BIOSTAT Aplus was used. The expression of the recombinant protein was induced by addition of isopropyl beta-D-thiogalactopyranoside (IPTG). The recombinant protein were purified on PROFINIA Protein Purification system. Refolding of the recombinant proteins was performed by “dilution” method. In the first stage the protein samples in the elution buffer with high denaturant concentration (6 M urea) was diluted (1:10) in Tris-HCl buffer containing NaCl,

including one of the thiol/disulfide redox reagents, either glutathione [reduced (GSH) and oxidized (GSSG) glutathione] or cysteine/cystine. The final concentration of the urea in the refolding buffer was 0.6 M. For suppressing aggregate formation, L-arginine was added to a final concentration of 0.4 M in the renaturation mixture.

Highlights:

- **SustainCycle system successfully established (SAEBYLI)**
- **Successful scale-up of sponge primmorph culture (NANOTEC and UMC-Mainz)**
- **The screening protocol for culturable marine microbial strains implemented (BIOTREND)**
- **A protocol for the culture of *R. marinus* in bioreactor was implemented with higher productivities than those observed in shake flask (BIOTREND)**
- **Sufficient biomass of *Rhodothermus marinus* (BIOTREND)**
- **Increased production of biomass of *Vibrio* strains (BIOTREND)**
- **Successful scale-up of production of recombinant proteins/enzymes (NANOTEC)**

8. PRECLINICAL STUDIES

Summary:

The aim of this work package was the selection of bioactive compounds arising from this project that are promising drug candidates and the performance of additional studies required to justify their progression into pre-clinical development. The specific objectives of this work package were to select the most promising candidates from the pool of bioactive compounds identified and to progress at least a few towards preclinical development.

In detail:

Characterization of the anti-senescence properties of beta-carotene and retinoid derivatives obtained from marine biotechnology: Two natural compounds have been discovered by **NANOTEC** that inhibit senescence and therefore prevent aging. Both compounds can be applied either alone or in combination. One of these compounds is a marine sponge carotinoid with a known toxicity profile that blocks senescence on the level of the TOR/ageing pathway. The other compound is also effective in decreasing senescence induced by UV irradiation and in replicative senescence. Combinations of both compounds synergize in inhibiting senescence by reducing oxidative stress. Studies on the effect on proliferation revealed that both compounds increase cell proliferation and show a significant anti-senescence effect.

Topical formulation of active leads: The retinoid is also suitable for cosmetic applications either alone or in combination. This combination can be formulated for anti-ageing ointments and gel preparations. One striking advantage of these compounds/combination compared to existing products is the fact that they act on the level of the PI3K/AKT/mTOR pathway and replicative senescence.

***In vivo* analyses of topical preparation containing beta-carotene and carotenoid cleavage products: UMC-Mainz** and **NANOTEC** developed a novel microparticulate material that is bioactive. This material consists of a calcium salt of inorganic polyP, a natural nontoxic linear polymer. The Ca-polyP microparticles that form the novel material are amorphous, biodegradable and retain the morphogenetic activity of the inorganic polymer. These particles have an unusual hardness (e.g. elastic modulus of 1.3 GPa), can be prepared at mild conditions, are morphogenetically active (e.g., induction of alkaline phosphatase activity and bone HA formation), and are biodegradable. In addition, **UMC-Mainz** and **NANOTEC** succeeded to develop, based on the Ca-polyP-microparticles, amorphous Ca-polyP microspheres containing encapsulated retinoids. The size of the microspheres can be adjusted to a size range which is optimal for endocytotic cellular uptake. **NANOTEC** together with **UMC-Mainz** demonstrated that in MC3T3-E1 cells retinol/Ca-polyP microspheres cause an increase in collagen types I, II and III gene expression at concentrations at which the single components, retinol and Ca-polyP microspheres, are inactive. This synergistic effect was particularly pronounced for the collagen type III expression. The increase in collagen type III expression was blocked by triflupromazine, an inhibitor of clathrin-mediated endocytosis.

Testing active leads in a model of reconstructed human epidermis: UMC-Mainz and **NANOTEC** demonstrated that co-incubation of MC3T3-E1 cells with the retinol-Ca-polyP microspheres resulted in a significant synergistic effect on cell growth compared with particle-free polyP complexed with Ca^{2+} ions or amorphous Ca-polyP microparticles and retinol alone. RT-qPCR experiments revealed that addition of Ca-polyP microparticles to MC3T3-E1 cells causes a significant upregulation of the steady-state-expression of the genes encoding for the fatty acid binding protein 4 (FABP4), leptin and the leptin receptor. The increased expression of these genes was further enhanced if retinol-Ca-polyP microspheres were added to the cells. Furthermore, **UMC-Mainz** and **NANOTEC** developed electrospun poly(lactic acid) and polycaprolactone fiber mats. Besides Ca-polyP, further bioactive components can be incorporated into these PLA-based fiber meshes. **FIDELTA** tested the effect of the polycaprolactone-integrated compounds obtained from **UMC-Mainz** / **NANOTEC** on wound healing in C57BL/6N and db/db mice. Our results demonstrated the morphogenetic properties of PLA fiber mats, supplemented with amorphous Ca-polyP microparticles or, even more, retinol-Ca-polyP microspheres. **FIDELTA** tested the effect of the polycaprolactone-integrated compounds obtained from **UMC-Mainz** / **NANOTEC** on wound healing in C57BL/6N and db/db mice (see below).

Envisaged field of application of the compound (target disease): The most advanced compounds identified in BlueGenics project fall into the following two therapeutic indications: a) Retinol for wound healing; b) Leucettines for Alzheimer indication. Both areas of application are also of high commercial interest.

Evaluation of leads and pre-clinical candidate compounds and decision-making: One lead had been identified and evaluated by **FIDELTA** targeting wound healing indication. Polycaprolactone-integrated retinoic acid has demonstrated interesting wound healing potential that has been profiled further. The other lead was identified and tested by **ManRos** for Alzheimer indication. The lead molecule L41 has been selected as a biological proof of concept molecule. The second lead molecule L888 still needs to be selected as potential candidate molecule among two sub-families of Leucettines.

Potency of the compound: UMC-Mainz and **NANOTEC** performed dose-response experiments to determine the ED_{50} concentrations of the materials included in the new products / dressings for wound healing, including different polyP – divalent metal ion complexes and microparticles and retinoids. In addition, the ED_{50} concentrations of the

individual components of the quercetin/polyP combinations, as well as the carbonic anhydrase activator quinolinic acid used as potential compounds for treatment of osteoporotic bone disorders were investigated. **ManRos** studied the effect of different Leucettines in various kinase assays. Dose-response experiments were performed to determine the IC_{50} values. The drug passed the blood brain barrier, but not the intestinal barrier. It thus needs to be modified.

Transcriptional profiling: Proteomics and phosphoproteomics profiling have been carried out with Leucettine L41 in the hippocampal cell line HT22 and in WT and Down syndrome mice (**ManRos**). FFPE material from wound healing studies both with C57bl6 and db/db mice was used for transcriptional analysis. Significant transcriptional changes have been observed with respect to Collagen 1, Collagen 3, PAI-1 and alpha-SMA expression with C57bl6 mice on day 7 and 13 days.

Selectivity of the compound: ManRos has expanded the selectivity study strategy using Leucettine L41 as an example.

Cytotoxicity of the compound: Two studies have been performed with products aimed at wound healing indication: a) Cytotoxicity on HepG2 cells – Tested compounds did not affect cell viability of Hep-G2 cells (IC50 > 40 µg/mL). b) Effect on proliferation of normal human fibroblasts (NHLF) – Tested compounds did not affect NHLF proliferation, whereas staurosporine inhibited cell growth. c) Cytotox of Sceptin, Stevensen and Latonduin A on HepG2 and THP-1 - Among the three compounds tested, one compound showed some cytotoxicity effect, but only on THP-1 cell line (Latonduine A, IC_{50} = 9.2 µM), while two other tested compounds (Sceptrin and Stevensine) did not showed significant inhibitory activity on THP-1 cell line (IC_{50} > 100 µM). All three tested compounds did not showed any inhibitory activity on HepG2 cell line (IC_{50} > 100 µM).

Physic-chemical properties of the compound: Physicochemical properties of new chemical entities are extremely important for prediction of their drug-like properties. Studies on Leucettines analogues has led to a series of 14 inhibitors which have been extensively characterized.

Pharmacokinetics *in vitro.* Lead compound L41 demonstrates high protein binding and low permeability. Those properties will be worked on during lead optimisation phase. Detailed PK and biodistribution have been carried out in mice (ManRos) and rat (**ManRos**, **FIDELTA**).

***In vivo* pharmacokinetics.** Pharmacokinetic profiling of Leucettine L41 has been performed in male Sprague Dawley rats following intraperitoneal dosing. Plasma and brain concentrations of Leucettine L41 were determined in male Sprague Dawley rats following intraperitoneal administration at a target dose of 20 mg/kg. A total of 24 animals were dosed, with plasma and brain tissue collected up to 24 hours post dose. Leucttine L41 was quantifiable up to 24 hours post dose in plasma and up to 4 hours post dose in brain tissue. Maximum plasma levels were achieved at 0.5 h with a mean Cmax of 30.7 ng/mL, and oral exposure, expressed as AUC0-last, of 155±21.4 ng.h/mL. Maximum brain levels were achieved at 0.5 h with a mean Cmax of 40.9±32.2 ng/g, and oral exposure, expressed as AUC0-last, of 51.3±12.6 ng.h/g. The brain to plasma Cmax ratio was 1.3 and AUC0-last ratio was 0.332.

Demonstration of activity in *in vivo* models and establishment of PK/PD relationship: A) Wound healing experiments. The aim of the studies performed was to perform analysis of effects of potentially active compounds integrated in polycaprolactone nanoscaffolds and polyP on wound healing in C57BL76N and db/db mice. A recognized model for impaired wound healing, mouse strain T/BKS.CG-M+/+LEPR

DB/J (db/db) is homozygous for the diabetes spontaneous mutation (Leprdb) which leads to development of obesity and insulin-resistant diabetes at approximately four to eight weeks of age. Experiments with **C57Bl/6N** mice have resulted with recognition of beneficial effects of polyP and polyP-retinol on granulation tissue formation. B) mCIA model – link between inflammation and depression. Chronic inflammation and depression-like behaviour was investigated in a mouse model of collagen induced arthritis (CIA) in DBA/1J mice. Vehicle-treated CIA mice were compared to healthy controls and animals treated with imipramine and fluoxetine. Depression-like behaviour was assessed via sucrose preference test (SPT) and novelty suppressed feeding test (NSF). Serum levels of three biomarkers previously found to be correlated with major depressive disorder (prolactin; myeloperoxidase, MPO; alpha-1-antitrypsin, a1AT) were also evaluated.

Identification of biomarker(s) to evaluate the effect of the compound: Three sets of investigation were performed related to mechanism-related biomarkers for wound healing stream of products: a) Study on MC3T3- E1 cells. b) Identification of local wound healing marker in vivo. c) Identification of wound healing marker in systemic circulation. We have identified collagen 1 and collagen 3 as clear mechanistically linked markers of improved wound healing process caused by applied polyP products both in our in vitro cell culture system and complex in vivo wound healing experiments. Collagen 1 and collagen 3 are essential extracellular matrix proteins expressed during proliferative and matrix formation phase of wound healing which indicate progression towards healing. Initial injury is initially reflected by inflammation which is necessary for normal wound healing process. However, due to the additional factors, such as infection, inflammation can get augmented and human non-healing wounds stay trapped in that self-perpetuating phase. Accelerated formation of pre-fibrous granulation tissue and collagen 1 and 3 expression, observed in our experiments, are good prognostic markers that could be also monitored locally in humans.

Preliminary (Non-GLP) toxicity studies: Two studies were performed: a) Estimation of hERG binding affinity for 2 compounds aiming at Alzheimer's indication using the fluorescence polarization assay. Leucettine L41 and Fipronil did not inhibit hERG in the tested concentration range (IC50 > 30 µM). b) Acute dermal irritation of products aimed on wound healing indication on New Zealand White Rabbits. Based upon the result obtained, polyP-retinol and polyP-$CaCO_3$-Ca would not be considered a primary skin irritated ("non-irritant"). The test items would be further classified based upon Primary Irritation Index as negligible irritating to the skin.

Highlights:

- **Development of amorphous Ca-polyP microspheres that can be used for the encapsulation of bioactive molecules, e.g. retinol (UMC-Mainz and NANOTEC)**

- **Demonstration that retinol/Ca-polyP microspheres cause a synergistic effect in particular on collagen type III gene expression (NANOTEC and UMC-Mainz)**

- **Demonstration of the morphogenetic activity of electrospun PLA fiber mats with embedded retinol-Ca-polyP microspheres (NANOTEC and UMC-Mainz)**

- **Demonstration that polyP and retinol packed into fibrous mats causes a strong synergistic effect on the expression of genes, encoding for leptin, leptin receptor and FABP4 (UMC-Mainz and NANOTEC)**

- **Demonstration of the wound healing potential of polycaprolactone-integrated amorphous retinol-Ca-polyP microspheres in genetically diabetic mice (db/db) and C57BL/6N mice (full excisional wound model) (FIDELTA and UMC-Mainz)**

- **Demonstration of the wound healing potential of several additional polycaprolactone-integrated compounds on wound healing (FIDELTA and UMC-Mainz)**

- **Transcriptional profiling of FFPE sections from wound healing model in mice (FIDELTA and ManRos)**

- **Study of the effect of different Leucettines in various kinase assays (ManRos), selection of two sub-families**

- **Proteomics and phosphoproteomics profiling with Leucettine L41 (ManRos)**

- **Analysis of pharmacokinetics and biodistribution of Leucettine L41 in WT mice, in Down syndrome mice model and in rats (ManRos, FIDELTA)**

- **Analysis of pharmacokinetics and biodistribution of pyrazole pesticide in WT mice (ManRos) and in rats (ManRos, FIDELTA)**

- **Mechanistic biomarkers identified (UMC-Mainz, ManRos and FIDELTA)**

- **Determination of hERG liability for Leucettine L41 and pyrazole pesticide (FIDELTA and ManRos)**

9. INTELLECTUAL PROPERTY, TECHNOLOGY TRANSFER, AND MARKET ANALYSIS

Summary:

The objective of this work package was to establish an integrated management of knowledge and intellectual property within the consortium. The specific objectives were: (i) Analysis and management of knowledge and intellectual property; (ii) Analysis of the patentability of most promising natural products; (iii) Writing and filing patents; (iv) Analysis of the potential use and market of selected lead compounds, help in technology transfer; (v) Setting-up a website for enzymes developed in this project; (vi) Setting-up a marine natural product database; and (vii) Setting-up a signature database.

In detail:

Integrated management of knowledge and intellectual property: The protection of the know-how generated within BlueGenics was an important task of this project. All results obtained by the various partner groups of the consortium were checked for their commercial value. If these results were found to be relevant for future exploitation / introduction into the market patent application has been considered. In the search for the potential patentability of the products generated within this project, the partners were supported by a patent law firm. This task also included the search in the available patent data bases.

Focus existing portfolios and generate additional IP based on expertise in the project: The BlueGenics SME & IPR Board supported the partners in intellectually property matters. The consortium members followed the guidelines for the partnership's policy in respect of securing patents and granting licenses that had been described in the Consortium agreement.

Building-up a network structure: Patentability studies have been performed for each new and promising compound or novel bioactivity discovered in the frame of this project. The main focus of the BlueGenics partners **UMC-Mainz**, **NANOTEC** and **NRCGA-CAGS** was on compounds for treatment of osteoporosis and bone regeneration, as well as on compounds promoting wound healing.

Filing of patents and granting licenses: Seven new patent applications have been submitted.

Technology transfer and market analysis: The main target markets of the marine-derived products developed in BlueGenics project are in the area of therapy of osteoporosis, wound healing, immune modulation, kinase inhibition, cancers and Alzheimer's disease. The members of the BlueGenics consortium from academia were supported by the technology transfer departments of their respective institutions to find potential cooperation partners from industry. Several cooperation agreements with industrial partners have been concluded or are in preparation. In order to assess the marketing potential of products and exploration of potential user groups, **PROKAZYME**, for example, has made a market introduction in Japan for its enzymes.

Website for enzymes developed in this project: PROKAZYME has promoted the use of marine-derived biocatalysts, both as stand-alone products that can be used for different biotechnological applications, but also so that they can be used as processing aids for making higher value products from existing or new marine raw materials. For this purpose the Enzyme Homepage on www.prokazyme.com has been continuously updated.

Set-up of a marine natural product database: A primary database structure was defined in an ODBC framework utilising the software FileMaker Pro® (FMP), which was used for completing missing entries, enable sorting, and editing taxonomic assignements. Additionally, FMP also contain a module which could allow for direct publication of the entire, or selected parts, of the database via a web (HTTP) interface. This database, initially named BG-DNPT (BlueGenics Database of Natural Products and Taxonomy) was subsequently used for handling chemical property space data and preparing various plots. One of the key features is the attempt to have an updated and consistent taxonomic assignement which gives the possibility to sort or select compounds known from different evolutionary groups. Among the results obtained with support from BG-DNTP is the grapical visualization of compounds retrieved in the BlueGenics project and their chemical diversity in relation to reference datasets – one of which is the BG-DNTP, and another is the Maybridge screening compound library. In addition, a database has been set up by **RBI. RBI** selected sponges according bioactive compounds data mining, focussing on the sponge fauna of the Northern Adriatic Sea.

Set-up of a signature database: This data base is based on the extensive sequencing program of the expressed sponge genome by **UMC-Mainz** and **NANOTEC**. The collected data are deposited in an EST sponge data base ("SpongeBase") which comprises representatives of all poriferan classes. The actual data in the system at the end of BlueGenics project are distributed over **1.215 x 10-6 ESTs** of **13 poriferan species**, from demosponges, hexactinellid sponges, and

calcareous sponges, with an average length of 600 nt. The Signature database turned out to be a useful tool in the investigation and search for novel sponge genes/proteins of biotechnological interest.

Highlights:

- **Website for enzymes developed in this project (PROKAZYME)**
- **7 patent applications in this project (NANOTEC, MATIS, UNINA, MNHN and PROKAZYME)**
- **Marine natural product database (RBI)**
- **Signature database (UMC-Mainz)**

10. DEMONSTRATION ACTIVITIES

Summary:

The aim of this work package was to include, in parallel to the R&D activities, demonstration activities of selected parts of the biodiscovery pipeline (that are at a stage allowing such activities) to inform and to attract potential endusers about the achievements and products developed in the proposed project. The specific objectives were: (i) Demonstration of protein kinase platform; (ii) Demonstration of biotechnological applications of thermophilic bacteria/enzymes; (iii) Demonstration of application of sponge-derived recombinant proteins in dentistry; (iv) Demonstration of primmorphs for production of sponge-derived bioactive secondary metabolites; and (v) Demonstration of sustainable sponge production.

In detail:

Protein kinase platform: in-house demonstration of the platform: ManRos has demonstrated its protein kinase platform to several persons and instructed these persons in the use of this platform, which requires a specific training.

Aquatic enzymes with unique properties for biotechnological applications: demonstration at industrial fairs: PROKAZYME has promoted the use of marine-derived biocatalysts, both as stand-alone products that can be used for different biotechnological applications, but also so that they can be used as processing aids for making higher value products from existing or new marine raw materials. For this purpose the Enzyme Homepage on www.prokazyme.com has been continuously updated. This activity has been in line with the main goals of **PROKAZYME** to build up a webstore on the internet that sells enzymes, reagents and biochemicals from novel sources, in particular from extremophilic microorganisms.

Application of sponge-derived recombinant proteins in dentistry: Evaluation by potential endusers: This demonstration activity has been performed by UMC-Mainz and NANOTEC. Demonstration 1 (Title: "Display and exhibition of scaled-up material for caries prophylaxis") has been performed by UMC-Mainz in July 2013 in Beijing, China (30 participants). Demonstration 2 (Title: "Protection of teeth and surrounding tissue by bioorganic membranes") has been performed by NANOTEC also in July 2013 in Beijing, China (20 participants).

Primmorphs for production of sponge-derived bioactive secondary metabolites: in-house demonstration: NANOTEC undertook **in-house demonstration activities**

of the sponge primmorph system as a source for carotinoids / retinoic acid for cosmetics. These demonstration activities have been performed in Mainz at various dates in 2014. In addition, the following two demonstration activities were performed by **NANOTEC** and **UMC-Mainz,** in cooperation with **NRCGA-CAGS**. Demonstration I ("Quercetin and polyP: combination of a Chinese traditional drug and polyP for treatment of bone disorders – Potential compound to be printed into implants") in Beijing, China, May 2014. Demonstration II ("BlueGenics-derived products**")** in Shenyang, China, December 2014 (together with 10 invited experts). Topics: (a) Bioactive Peptides and proteins/enzymes from sponges for application in biomedicine and biotechnology. (b) Secondary marine metabolites: Carbonic anhydrase activators – quinolinic acid. (c) Sponge-derived recombinant proteins in dentistry.

Demo system for sustainable sponge production: SAEBYLI demonstrated its SustainCycle system for sustainable sponge production to selected experts. The aim was to get the feedback from these experts in order to further develop the system. The experts chosen included, among others, specialists in the field of sponge culture from Scripps Institution of Oceanography UCSD, California, and Wageningen University..

Highlights:

- **In-house demonstration of the Protein kinase platform platform (ManRos)**
- **Promotion of enzymes via web (PROKAZYME)**
- **Demonstration activity on BlueGenics-derived products in Beijing and in Shenyang, China (NANOTEC and UMC-Mainz in cooperation with NRCGA-CAGS)**
- **In-house demonstration of sponge primmorph system for production of sponge-derived bioactive secondary metabolites (NANOTEC and UMC-Mainz)**
- **Demo system for sustainable sponge production (SustainCycle system) (SAEBYLI)**

Description of the potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and the exploitation of results

This project significantly contributed to strengthening the competitiveness of the European Marine Biotechnology Industry through the introduction of advanced molecular-biology and chemogenetics methods for the development of bioactive compounds. New marine-derived drug candidates and biomedical materials have been developed that can enter or have already entered pre-clinical studies in the course of the project. The advanced technologies developed in this project are expected to have a great socio-economic and wider societal impact, as follows.

SOCIO-ECONOMIC IMPACT:

The socio-economic impact of this project can be summarized as follows:

1. The research activities of BlueGenics project, through the introduction of new technologies in marine biodiscovery and sustainable development of marine-derived bioactive compounds resulted in the development of new drug candidates and other products with a high potential to be introduced into clinics or biomedicine/biotechnology, in particular in the following areas:

    - Osteoporotic diseases, a major health threat worldwide, and other bone disorders

    - Neurodegenerative diseases, in particular Alzheimer disease that has become increasingly important, like osteoporosis, due to the demographic development

    - Wound healing, a market which is in rapid development

    - In addition, this project resulted in products for the growing cosmeceutical market (cosmetic products with biologically active ingredients)

2. The main bottleneck in marine drug development, the supply problem, can only be solved by molecular-biology-based approaches – the technologies/solutions demonstrated in the BlueGenics project contribute to mitigate or abolish this bottleneck in the marine biodiscovery pipeline.

3. The development of molecular-biology-based methods for the sustainable production of marine products will make “Blue Biotechnology” more attractive to investors.

4. The advanced technologies introduced in the frame of this project for drug discovery/development will help to strengthen both the EU leadership in science and technology and the competitiveness of European Biotechnology industry.

WIDER SOCIETAL IMPLICATIONS:

The wider societal implications of this project are the result of:

1. The new drug candidates, biologically active materials and peptides/proteins from marine resources and the methods for their sustainable production developed in this project will contribute to the prosperity of the European drug discovery and biotechnology industry.

2. The introduction of advanced techniques of molecular biology and biotechnology in the field of marine drug discovery and development will enhance the competitiveness of European industry in the emerging market of marine genomics/metagenomics, and also contribute to enhance EU scientific and technical excellence in this field.

3. The advanced genetically based techniques developed and introduced in this project will provide the basis for the development of innovative products for

future biomedicine/biotechnology – this can only be achieved by application molecular biological/biotechnological techniques as successfully demonstrated in this project.

4. This project has demonstrated the feasibility of the only “right” strategy for a future target-oriented and sustainable drug development: the application of a genomics approach, including the identification of the genes involved in a biosynthetic pathway and the heterologous expression of these genes in an easily culturable host - it is expected that the new techniques developed in this project will contribute to a long-term innovation in European marine drug discovery industry.

MAIN DISSEMINATION ACTIVITIES:

The aim of this work package is the dissemination and communication generated in the frame of the project, and the preparation of the Plan for Using and Disseminating the Knowledge (PUDK). The specific objectives are: (i) Dissemination and communication activities; and (ii) Plan for Using and Disseminating the Knowledge.

In detail:

- **Expo 2012 in Yeosu, Korea**

The coordinator had been selected in a competitive call as exhibitor in the German Pavilion at the Expo 2012 in Yeosu, Korea (“The Living Ocean and Coast”). One central topic of this Expo were novel technologies / applications of materials from marine organisms. This allowed the communication of the results of the project to a broader public.

- **Spanish-German Innovation Forum**

Foro de Innovación Hispano – Alemán, 20.03.2013, Madrid, Spain Public lecture of W.E.G. Müller UMC-Mainz.

- **Thailand's national agency for S&T Meeting**

The BlueGenics coordinator has been invited by the Thailand's national agency for S&T as an advisor to present his experience in European research programmes in the meeting “Research Management in Europe and Research Topics in Thailand of European Interests” of the National Science and Technology Development Agency (NSTDA) (7 – 9 May, 2013).

- **Workshop I**

The first BlueGenics Workshop I was held in the Muséum National d'Histoire Naturelle in Paris from July 8 - 9, 2013.

- **Horizon 2020 Info day**

FIDELTA presented its positive experience of participation in BlueGenics at the Horizon 2020 Info day in Zagreb December 18, 2013.

- **Workshop II**

The BlueGenics Workshop II was organized by **MATIS** and held in Reykjavik, Iceland, from September 4 - 5, 2014.

- **Summer school I**

The BlueGenics Summer school I was held in the Center for Marine Research in Rovinj, Croatia, from September 16 - 22, 2014. This Summer school was jointly organized by the EU FP7 projects BlueGenics and CoreShell. Partners from both projects participated in this event.

- **9th European Marine Natural Products Conference and pre-conference workshop**

The 9th European Marine Natural Products Conference in Glasgow, Scotland, 30th August – 2nd September 2015 has been organized as the main **common dissemination and communication** of the three EU consortia, BlueGenics, SeaBioTech and PharmaSea. The BlueGenics partners, W.E.G. Müller (**UMC-Mainz**) and H.C. Schröder (**NANOTEC**) were members of the Scientific Committee of this Conference.

This Conferences was associated with **a Pre Conference Workshop**, organized by the three consortia. The BlueGenics partners involved in this Pre Conference Workshop were Marie-Lise Bourguet-Kondracki (**MNHM**) and H.C. Schröder (**NANOTEC**). They gave two special teaching units / sessions in this Workshop.

- **Summer school II**

The 2nd BlueGenics Summer school was organized in Rovinj, Croatia (RBI) from September 17th – 22nd, 2015.

- **Biomarine Convention 2015, Wilmington, NC, USA**

B. Sommer-Ferreira (**BIOTREND**) gave a session about “Commercial trends of marine biotechnology”, during the Biomarine Convention, October 2015 in Wilmington, NC, USA, where he presented commercially relevant results from the BlueGenics project.

- **1st International Conference on 3D Printing in Medicine**

W.E.G. Müller and X.H. Wang were co-organizer 1st International Conference on 3D Printing in Medicine in Mainz, Germany, April 15-16, 2016.

- **2016 MRS Spring Meeting & Exhibit**

The results of BlueGenics project have been disseminated by W.E.G. Müller and XH Wang in the 2016 MRS Spring Meeting & Exhibit in Phoenix, Arizona, USA, from March 28-April 1, 2016.

- **WMF Forum 2016**

W.E.G. Müller and X.H. Wang participated and were speakers in the WMF Forum 2016 (“From Global Challenges to Grand Manufacturing Opportunities: Leading towards Growth and Sustainability”), Barcelona, Spain, 3-4 May 2016

- **Summer school III and Final symposium:**

The BlueGenics Summer school III and the Final symposium of BlueGenics project was held in the Center for Marine Research in Rovinj, Croatia, from May 29th – June 1st, 2016.

- **BlueGenics Dissemination Events in Chengdu and in Shanghai, China 2016**

The BlueGenics coordinator, W.E.G. Müller, and project partner X.H. Wang performed two Dissemination events in Chengdu and in Shanghai, China, in cooperation with the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, where they presented the products, developed in the frame of the **BlueGenics project**, in particular the products developed by the partners **UMC-Mainz**, **NRCGA-CAGS** and the SME partner **NANOTEC** to an audience consisting of industrial researchers, researchers from academia and potential customers. These dissemination events have been performed in the period from 9-17 July 2016. Special focus in Chengdu: Electrospun wound dressings for wound healing, products developed for treatment of bone disorders and novel compounds for the therapy of osteoporosis.

Dissemination to the broader public:

A series of activities have been undertaken to disseminate the results of the project to a broader public. These activities included, among others:

- **Public lecture during BueGenics Summer Schools 2014 and 2016**

In the frame of the BlueGenics Summer schools 2014 and 2016, W.E.G. Müller (**UMC-Mainz**) gave a Public lecture in the Multimedia Center Rovinj about the topics: “Treasures of the Adriatic Sea: Towards Sustainable and Innovative Bio-Economy” (2014) and “What we learnt from marine animals: From evolution to biomedical applications” (2016).

- **Medtech Rhineland-Palatinate**

NANOTEC and **UMC-Mainz** presented the products obtained in the frame of BlueGenics project to a broader audience during the exhibition Medtech Rhineland-Palatinate, Mainz, on 18 June 2014, on 8 July 2015 and on 20. July 2016 (see also below).

- **Germany/MPI-exhibition: Moscow, St. Petersburg, Beijing, Sao Paolo**

The BlueGenics coordinator W.E.G. Müller (**UMC-Mainz**) disseminated results obtained in BlueGenic project during the Germany/MPI-exhibition: Moscow, St. Petersburg, Beijing, Sao Paolo 2014.

- **Croatian Economic Society Reception Mainz**

W.E.G. Müller (**UMC-Mainz**) presented results and explained the aims of BlueGenics project to the Croatian delegation in the frame of the Croatian Economic Society Reception in Mainz, 20 February 2015 (Croatia – **RBI** and **FIDELTA** are partners of BlueGenics project).

- **Germany/MPI Max Plancks Science Tunnel Bilim Tüneli Sergisi exhibition Konya, Turkey**

W.E.G. Müller (**UMC-Mainz**) participated in the Germany/MPI Max Plancks Science Tunnel Bilim Tüneli Sergisi exhibition, 13.3. - 31.5.2015 (Konya, Turkey), showing results of the BlueGenics project.

- **Biomarine convention 2015**

Bruno Sommer Ferreira (BIOTREND) actively particpated in the Live Session/Panel discussion about the theme “What commercial trends for Marine compounds?” in the frame of the 6th BioMarine International Business Convention, 12-14 October 2015, in Wilmington, NC, USA (see also below).

- **Night Lecture 2016**

Together with colleagues from the university center BioMATiCS, W.E.G. Müller (**UMC-Mainz**) gave a public lecture about “From Molecule Biology to 3D Printing” in Mainz, Germany, May 12, 2016.

- **Publications in refereed scientific journals**

  >100 publications in high-impact journals, including Nature

- **Presentations at scientific meetings**

  > 140 presentations at international and national conferences, workshops etc.

- **Publication of a book within a progress series**

The BlueGenics partners finalized the preparation of a new volume of the Progress series on **“Marine Molecular Biotechnology”** (Subseries of the Springer Series: Progress in Molecular and Subcellular Biology). Several partners contributed a chapter.

A first volume of had already been published: W.E.G. Müller, X.H. Wang and H.C. Schröder (eds.) Biomedical Inorganic Polymers: Bioactivity and Applications of Natural and Synthetic Polymeric Inorganic Molecules. Springer-Press, Berlin, pp. 1-303 (2013).

The title of the new volume is: **“Blue Biotechnology – From gene to bioactive product”** (Editors: W.E.G. Müller, X.H. Wang and H.C. Schröder)

- **German-Chinese Joint Center for Bio-inspired Materials:**

There are close links between the BlueGenics project and the German-Chinese Joint Center for Bio-inspired Materials ("Biogeomaterials and Marine Sources for Innovative Applications in Biomedicine and Biogeosciences") which is also coordinated by the BlueGenics’s coordinator W.E.G. Müller and X.H. Wang (scientific coordinator).

Presentations at business congresses and industrial fairs:

The partners actively participated in the following business congresses and industrial fairs:

- **BIO 2013, Biotechnology Convention,**

The SME partner ManRos particpated in the BIO 2013, Biotechnology Convention, Chicago, USA, April 22-25, 2013.

- **CHINA HI-TECH FAIR**

The SME partner **NANOTEC**, in cooperation with **UMC-Mainz**, participated in the CHINA HI-TECH FAIR, November 16-21, 2014, in Shenzhen, China, Convention & Exhibition Center. Both institutions are also winners in the national competition "Germany – Land of Ideas".

- **Medtech Rhineland-Palatinate 2014, 2015 and 2016**

UMC-Mainz and **NANOTEC** presented the results of BlueGenics to the public in the frame of the exhibition Medtech Rhineland-Palatinate (see above).

- **6th BioMarine International Business Convention**

Bruno Sommer Ferreira (**BIOTREND**) summarized the achievements of BlueGenics project in the Live Session/Panel discussion about the theme "What commercial trends for Marine compounds?" in the frame of the, 12-14 October 2015, in Wilmington, NC, USA (see above).

Distribution of leaflets, brochures, and CD-Roms

The partners, in particular the SME companies, produced a number of leaflets and brochures with information about BlueGenics and distributed these leaflets/brochures during conferences, business meetings and industrial fairs.

Contributions to press, TV, broadcast

Several contributions to press and broadcast; for example, project goals and activities were disseminated in various newspapers and journals as well as in radio interviews.

Examples:

- **Espace Mathurin Méheut, Roscoff** L. Meijer (invited speaker; **ManRos**) gave a general public presentation - February 22, 2013.
- **ETH Zurich - News and views article in Nature.** Press release on Nature publication, extensive news coverage in the leading newspapers of Switzerland, Austria, and Brazil (Neue Züricher Zeitung, Der Standard, Folha de S. Paulo) and other publications.
- **German Entrepreneurs Travel/Meeting to China (2015).** Press contributions of the Coordinator, W.E.G. Müller, in the frame of the German Entrepreneurs Travel/Meeting to China, guided by the former German Minister Rudolf Scharping, from 31.05.-06.06.2015, and the German-Chinese Conference "One Belt, One Road" in Taicang (01.-02.06.2015).
- **TV contribution in ARTE.** W.E.G Müller (**UMC-Mainz**) contributed to the TV series X:enius – special production: "Überlebenskünstler Schwamm" ("survival artist: sponge"), shown in German TV – ARTE on 1. September 2015; 8:25 Uhr (, and repeated on Tuesday, 01.09.2015; and Wednesday, 16.09.2015.
- **Press coverage about scientific conferences. 1st International Conference on 3D Printing in Medicine -** W.E.G. Müller and XH Wang were co-organizers and chairmen of the 1st International Conference on 3D Printing in Medicine in Mainz, Germany, April 15-16, 2016. Press coverage in TV: swr.de/landesschau-aktuell (14.04.2016).

- **Radio interview with W.E.G. Müller (UMC-Mainz) in Bayerischer Rundfunk (Bavarian Broadcast).** W.E.G. Müller (**UMC-Mainz**) gave an interview to R. Schrott (journalist), Bayerischer Rundfunk (Bavarian Broadcast) about the application of marine-derived products in particular in the field of therapy of bone fractures of osteoporotic patients. This interview has been broadcasted in radio (Bayerischer Rundfunk) on 26 April 2016.

Highlights (Dissemination and Communication activities):

- **>100 publications in high-impact journals, including Nature, coverage in leading national newpapers (All)**
- **Book series in Springer-Press (All)**
- **>140 presentations at international and national conferences, workshops etc (All)**
- **Presentations to the general public (All)**

EXPLOITATION OF RESULTS:

In this project a number of breakthrough discoveries have been achieved, in particular in the field of bone disorders, wound healing and neurodegenerative diseases. The participation of the industrial/SME partners in the consortium was a high value for the development of strategies for the future commercialization of these results.

The R&D results of this project included both commercially exploitable results and contributions to the general advancement of knowledge.

The main commercially exploitable foreground achieved in this project can be summarized as follows:

- Marine-derived compounds that induce new bone formation: quercetin and polyphosphate (**UMC-Mainz**, **NANOTEC** and **NRCGA-CAGS**)

We discovered two compounds from marine sources, which are of interest for therapy of osteoporosis and related diseases. These compounds are quercetin and bio-polyP. Both compounds act synergistically on bone formation. They are also of interest for treatment of bone fractures in osteoporotic patients For further development, additional investments are needed. We are looking for VC money or cooperation with a larger company. Commercial use is planned in 2-3 years.

- Marine-derived compounds that induce new bone formation: quinolinic acid (**UMC-Mainz**, **NANOTEC** and **NRCGA-CAGS**)

We succeeded to identify a new drug target of compounds affecting bone formation: Carbonic anhydrase. A first activator of this new drug target has been identified: quinolinic acid a sponge compound. Both compounds act. Additional investment is needed. Commercial use is planned in 2-3 years.

- Dressings for bioactive wound healing (**UMC-Mainz**, **NANOTEC** and **NRCGA-CAGS**)

This exploitable foreground consists of novel wound healing nets into which morphogenetically active polymeric calcium phosphate particles or spheres with bioactive compounds, e.g. retinol, can be incorporated. These wound healing nets can be prepared using electrospinning procedures. The matrices can be optimized with regard to the desired mechanical properties, degradability and release kinetics of the incorporated bioactive molecules. Additional investment is needed. Commercial use is planned in 2-3 years.

- Morphoactive amorphous Ca-polyphosphate microparticles (**UMC-Mainz**, **NANOTEC** and **NRCGA-CAGS**)

Amorphous calcium phosphate microspheres that can be used for the encapsulation of bioactive natural compounds for skin regeneration / anti-aging. Ointments based on the developed UV-protective and anti-senescence calcium phosphate microspheres for treatment or prophylaxis of dermatological conditions such as inflammatory skin disorders, as well as disorders of increased cell turnover like psoriasis, and for photoaging. The commercial use is planned in 2018 and depends on further investments (VC or from other industrial partners).

- Marine-derived enzymes of biotechnological interest (**UMC-Mainz**, **NANOTEC** and **NRCGA-CAGS**)

The commercializable products are recombinant sponge enzyme for special biotechnological applications in food industry, as well as in detoxification reactions. For example, these enzymes include a sulfatase from a marine sponge, which is of interest for enzymatic removal of negatively charged sulphate groups from biopolymers for diverse biotechnological applications (application in food industry). Further investments are required (VC or from other industrial partners). Commercial use planned in 3-4 years.

- Marine sponge toxins of potential biomedical interest (**UMC-Mainz**, **NANOTEC** and **NRCGA-CAGS**)

We succeeded to identify and characterize marine sponge toxins of potential biomedical interest from the hexactinellid Crateromorpha meyeri and the demosponge *D. avara*. The intended commercial use is in 2019/2020.

- Sponge primmorph culture system (**NANOTEC** and **UMC-Mainz**)

Sponge primmorphs cultures are a system for the sustainable production of sponge secondary metabolites. We could scale-up the system for cultivation of primmorphs using a special bioreactor. Application in biomedicine, production of pharmaceuticals and cosmetics. We are looking for potential investors. The commercial use may also be possible in 2018/2019.

- Development of optimised bioprocesses from marine microorganisms (**BIOTREND**)

BIOTREND offers specialised bioprocess development services tackling the specificities of marine-derived microorganisms for the production of a diversified range of products, including, microbial biomass, cell extracts, enzymes and metabolites. This novel capability is being advertised in various events and in direct contacts with current and potential users.

- Sustain-Cycle system (**SAEBYLI** and **MATIS**)

SAEBYLI and **MATIS** established conditions to maintain and grow sponge cultures, i.e. of the cold-water sponge *H. panacea*, in aquaculture using the Sustain-Cycle system. The system is already in use at Sæbýli.

- Enzymes involved in beta-glucan pathways from marine and extremophile bacteria (**MATIS** and **PROKAZYME**)

Nine genes/enzymes involved in beta-glucan pathways have been identified and characterized from marine and extremophile bacteria genomes and metagenomes. Potential application in biomedicine, nutraceuticals, biotechnology and scientific research. The enzymes will becommercially available through website of **PROKAZYME** soon (until end of 2016).

- Antimicrobial compound from a bacterium isolated from Icelandic waters (**MATIS**, **MNHN** and **UNINA**)

We identified a strong antimicrobial activity in a protein extract derived from a bacterium isolated from Icelandic waters. Further characterization is currently ongoing to identify and isolate the compound and determine its structure and activity range. Commercial use (pending results of further characterization) is planned in 3-4 years.

- Compounds with potentially tumour-suppressing activity from bacteria isolated from Icelandic waters (**MATIS**, **UMC-Mainz** and **UNINA**)

We identified a strong inhibition of a cancer cell line in two protein extracts derived from bacteria isolated from Icelandic waters. Further characterization is currently ongoing to identify and isolate the compound and determine its structure and activity range. Commercial use (pending results of further characterization) is planned in 3-4 years.

- Lipophilic cyclic peptides from thermophilic marine bacteria (**MATIS**, **MNHN** and **UNINA**)

A new class of lipophilic cyclic peptides was discovered in the extracts of thermophilic marine bacteria from Icelandic waters. These extracts also showed antibacterial activity. Commercial use (pending results of further characterization) is planned in 3-4 years.

- Enzyme website (**PROKAZYME**)

The special focus of this website is on enzymes active on beta-glucans and other polysaccharides from marine sources discovered and developed in the project. The enzymes offered for sale for the food, neutraceutical and health products markets. Commercial use already ongoing.

- Biosynthetic gene cluster discovery for sustainable production of biosynthetic enzymes (**ETH Zurich**)

This exploitable foreground concerns the discovery of novel biosynthetic gene clusters based on homology to gene clusters from the talented producer Entotheonella sp. to allow for sustainable production of enzymes capable of unique chemical transformations. Commercial use is planned in 3-4 years.

- Proteusin modifying enzymes (**ETH Zurich**)

This exploitable foreground consists of proteusin modifying enzymes for synthetic biology applications. Application in pharmacology, biomedicine and toxicology. Commercial use is planned in 3-4 years.

- Marine sponge derived drug candidate: Leucettine L41 (**ManRos** and University of Rennes)

ManRos has progressed in the characterization of the DYRK1A kinase inhibitors Leucettines in terms of synthesis, selectivity, cellular assays. They have also made a first proof of concept of the beneficial effects of our first lead Leucettine L41 on the cognitive defects seen in 2 animal models of Down syndrome and two animal models of Alzheimers disease. The introduction into clinics might be possible in 3-4 years.

- Marine-derived herbicides/pesticides (**ManRos** and C.RIS Pharma)

ManRos has also progressed in our characterization of Amyloid A42 inducers found among herbicides/pesticides. The company wants to select one and develop it as a chemical tool to induce Alzheimers disease pathology in animals as new models of the disease. Commercial use is planned in 2 years.

There is also a number of exploitable foreground that has brought a significant progress in general advancement of knowledge, but is not foreseen for direct commercial application. These techniques comprise, for example, the assessment of the diversity of bacteria associated with the cold-water sponge *H. panacea* (**MATIS**), a dimeric actin inhibitor (**ETH Zurich** and Institut Pasteur), diverse metabolites from the sponge *S. officinalis* (**MNHN**), diverse metabolites from the Indonesian marine sponge *A. ingens*, including tetracyclic diamine alkaloids (**MNHN**, **UNINA** and **ManRos**), and from the Caribbean marine sponge *S. conulosa* (**MNHN** and **UNINA**), as well as from the Indonesian marine sponge *I. purpurea* (**MNHN**, **UNINA** and **ManRos**). Future applications are analyzed.

Website address:

http://www.bluegenics.eu/cms/

Contact details:

UMC-Mainz - Coordinator:
Prof. Dr. Werner E. G. Müller
ERC Advanced Investigator Group
Universitätsmedizin der Johannes Gutenberg Universität
Institut für Physiologische Chemie
Duesbergweg 6, 55128 Mainz, Germany
Phone: +49-6131-3925910, E-mail: wmueller @uni-mainz.de

ManRos:
Dr. Laurent Meijer
ManRos Therapeutics
Centre de Perharidy, Hôtel de Recherche
29680 Roscoff, France

Phone: +33 2 98 72 94 92 / +33 6 08 60 58 34, E-Mail: meijer@manros-therapeutics.com

NANOTEC:
Prof. Dr. Dr. Heinz C. Schröder
CEO
NanotecMARIN GmbH
Duesbergweg 6, D-55128 Mainz, Germany
Phone: +49 6131 3925791 / +49 6131 3924577, E-mail: hschroed@uni-mainz.de

SAEBILY:
Asgeir Gudnason
CEO
Saebyli ehf
Budarstig Number 23, 820 Eyrarbakki, Iceland
Phone: +354 8674433, E-Mail: asgeir@saebyli.is

PROKAZYME:
Dr. Jakob Kristjansson
CEO
Prokazyme ehf.
Vinlandsleio Number 14, 113 Reykjavik, Iceland
Phone: +354 664 7908, E-Mail: jakob@arkea.is

FIDELTA-GALAPAGOS:
Prof. Dr. Vesna Erakovic Haber
VP Biology
Galapagos istrazivacki centar d.o.o.
Prilaz baruna Filipovica Number 29, 10000 Zagreb, Croatia
Phone: +385 1 8886 320, E-mail: Vesna.ErakovicHaber@glpg.com

BIOTREND:
Dr. Bruno Sommer Ferreira
CEO
Biotrend S.A.
Biocant Park Núcleo 04 – Lot, 3060-197 Cantanhede, Portugal
Phone: +351 231410940, E-mail: bsf@biotrend.biz

MATIS:
Dr. Ragnar Jóhannsson
Research Group Leader
Matís ohf. / Icelandic Food and Biotech R&D
Vínlandsleið Number 12, 113 Reykjavík, Iceland
Phone: +354 422 5106 / +354 858 5106, E-Mail: ragnar.johannsson@matis.is

UNINA:
Prof. Dr. Alfonso Mangoni
Universita degli Studi de Napoli Federico II
Dipartimento di Farmacia
Dipartimento di Chimica delle Sostanze Naturali
Via Montesano 49, 80131 Napoli, Italy
Phone: +39 081 678 532, E-mail: alfonso.mangoni@unina.it

MNHN:
Dr. Marie-Lise Bourguet-Kondracki
Muséum national d'Histoire naturelle

Directeur de Recherches CNRS
Molécules de Communication et Adaptation des
Micro-organismes - MCAM, UMR 7245 CNRS
Rue Cuvier, CP 57, 75005 Paris, France
Phone: +33 1 40795606, E-mail: bourguet@mnhn.fr

UU:
Prof. Dr. Lars Bohlin
Head of Division
Uppsala Universitet
Div. of Pharmacognosy / Dept. of Medicinal Chemistry
Husargatan Number 3, S-751 23 Uppsala, Sweden
Phone: +46 18 4714492, E-mail: Lars.Bohlin@fkog.uu.se

RBI:
Prof. Dr. Renato Batel
Director
Ruder Boskovic Institute
Center for Marine Research
Laboratory for Marine Molecular Biology,
Giordano Palliaga Number 5, 55210 Rovinj, Croatia
Phone: +385 52804700, E-mail: batel@cim.irb.hr

NRCGA-CAGS:
Prof. Dr. Xiaohong Wang
National Research Center for Geoanalysis
Department of Science and Technology Research
Baiwanzhuang Dajie Number 26, 100037 Beijing, China
Phone: +86 10-68999596, E-mail: wxh0408@hotmail.com

USTAN:
Prof. Dr. Rebecca Goss
The University Court of the University of St Andrews
School of Chemistry
Biomolecular Sciences Building
North Haugh Number, KY16 9ST St Andrews, Fife, United Kingdom
Phone: +44 1334 463856, E-mail: rjmg@st-andrews.ac.uk

ETH-Zurich:
Prof. Dr. Jörn Piel
ETH Zurich
Institute of Microbiology
HCI G431, Vladimir-Prelog-Weg 1-5/10, CH-8093 Zurich, Switzerland
Phone: +41 44 633 07 55, E-mail: jpiel@ethz.ch