PROJECT FINAL REPORT

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Executive summary

Natural Products from MARINE FUNGI for the treatment of cancer. The urgent need for novel substances for the treatment of severe human diseases such as cancer, combined with the recognition that marine organisms provide a rich potential source of such substances, support the intensive exploration of new substances from marine organisms. Marine fungi are such a prolific group of secondary metabolite producers, but are still poorly characterised and underutilised for biotechnological application. Though intensive research is necessary to unravel the important resources of the ocean, marine biotechnology has now started to develop integrated strategies: therefore, MARINE FUNGI was initiated with a focus on the development of anti-cancer drugs from marine fungi.

Characterisation of candidate structures up to the stage of *in vivo* proof of concept. The development of clinically relevant lead structures requires more than just screening. An interdisciplinary approach was necessary to characterise hit candidates to the stage of *in vivo* proof of concept, ready to enter further drug development, in order to valorise the results of the project. This included the development of robust, sustainable processes for the production of these compounds.

Marine fungi – a good source for the sustainable production of new bioactive natural products. We demonstrated sustainable exploitation by developing appropriate culture conditions for marine fungi, thus enabling efficient production of marine natural products in laboratory and large scale cultures, avoiding harm to the natural environment. Two approaches were used to generate effective production strains

a) Candidate strains, originating from our unique strain collection of marine fungi, were characterised and optimised using molecular methods. The genomes of these strains were sequenced.

b) New fungi were isolated from special habitats, i.e. tropical coral reefs, macroalgae endemic to the Pacific Ocean and Mediterranean sponges. Culture conditions for these new isolates were optimised for the production of new anti-cancer metabolites.

A transdisciplinary consortium was assembled expedite a fast track approach to the project. A group of eleven institutions from seven countries joined forces to establish an appropriate consortium of experts to cooperatively conduct the MARINE FUNGI project.

Training of the next generation of marine biotechnologists. The consortium leveraged its multidisciplinary, interdisciplinary and intersectoral network to train young scientists and to encourage lifelong learning of experienced researchers. Soft skill training, student exchanges and summer schools were organized on various aspects of drug discovery, commercialization requirements for small molecule drug discovery, the role of open innovation and ethical and IPR issues, as well as on entrepreneurship.

Multiple outcomes. The main valorisable outcomes of the project were structures of lead compounds for anti-cancer therapeutics; robust, sustainable processes for the production of the bioactive compounds; and a strain collection of more than 600 new marine isolates. This strain collection was constructed along with a corresponding database of the fermentation extracts generated and the compounds characterised from them, which can be used for many other purposes in the future. On the scientific level, fungal genomics has been expanded to encompass unique representative marine fungi, and their metabolic pathways have been opened to exploration.

Sustain marine biotechnology in Europe. The lead users of MARINE FUNGI results will be pharmaceutical companies that will take anti-cancer substances derived from marine fungi into further drug development. The project strengthened the competitiveness of European marine biotechnology by providing a scientific and technical basis for the exploitation of marine fungi. Additionally, MARINE FUNGI activities increased the visibility of marine biotechnology as a key technology in Europe: MARINE FUNGI launched a public website offering non-confidential project information, with a weekly blog to provide news. To increase the impact of the results, the partners have been active in dissemination, participating in established conferences, trade fairs and specific project meetings, e.g. with interested pharmaceutical companies, and will continue to do so.

Summary description of project context and objectives

Context and background

The urgent need for novel substances for the treatment of severe human diseases such as cancer and inflammatory disorders, combined with the recognition that marine organisms provide a rich potential source of such substances support the intensive exploration of new substances from marine organisms. Though intensive research is necessary to unravel the important resources of the ocean, marine biotechnology has only started to develop integrated strategies. The project consortium encompassed research on marine natural products by using microorganisms.

One of the most serious bottlenecks in developing natural products from marine sources during the past decades has been the availability of biomass and/or of optimised cultivation conditions to gain sufficient amounts of substances for preclinical and clinical studies. Prominent examples were the bryostatins, the halichondrins and other antitumoral or anti-inflammatory active substances from marine invertebrates. In these cases, the content in the animals was very low (less than 1 g per ton of biomass) and it was not possible to harvest such large amounts of organisms from nature without destroying their habitats, nor was it possible to cultivate the organisms or cell cultures thereof in sufficient scale and time. The project specifically used marine fungi, which can easily be grown in the laboratory and at large scale and that are potent producers of bioactive compounds, including anti-tumoral substances.

In recent years, much evidence has emerged demonstrating that many natural products extracted from marine animals and algae were in fact the products of associated microorganisms, mostly bacteria and fungi. This occurs because in early investigations in which extracts were obtained from macro organisms, the associated microorganisms were often included, partly because of their tight association. Because most of the macroorganisms contain a great variety and considerable amounts of associated microorganisms, the importance of studying the ability of these microorganisms to produce secondary metabolites is now recognised. Close interactions and chemical communication systems between the microorganisms and their hosts also mean that there is probably an important ecological role of the substances produced by associated microorganisms including beneficial aspects for the hosts. An important hint to this situation is the finding that a particular high percentage of microorganisms associated with various macro organisms (if compared to free living microbes in ocean waters) are able to produce antibiotic substances.

Marine microorganisms provide new molecules. The study of microorganisms associated with marine invertebrates and algae has been intensified in recent years, in the coordinator's as well as other laboratories. However, there is still a general neglect of this highly important field of research and development. Some studies have been made concerning the diversity and identity of marine bacteria. Studies on the biological role of marine fungi have largely focused on their ecology, pathogenicity, taxonomic relationships and exploitable hydrolytic enzymes, with limited exploration of secondary metabolites. However, there has been sufficient research to demonstrate that marine fungi have a high potential for producing new natural products relevant to the treatment of human diseases. Therefore and because of excellent experience over the past years in the coordinator's laboratory with the analysis of natural products from marine fungi, this project focussed on this highly important and still under-exploited group of marine microorganisms.

Less than 1% of the marine microbial diversity is known today, offering a huge potential of new structures and bioactivities to be exploited in a sustainable manner: The aim of MARINE FUNGI was to demonstrate the sustainable exploitation of underutilised marine natural resources. Despite marine fungi are a potent group of secondary metabolite producers; they are not well characterized and underutilised in terms of biotechnological application (Figure 1).

Avoid specimen collection and provide appropriate culture methods. Because marine fungi can be grown in laboratory cultures, it was only necessary to take samples from the marine environment at an early stage and in small amounts that were used for isolation of the associated microorganisms in laboratory cultures. In order to cover a wide range of habitats and host organisms and to consider geographically distant environments, marine fungi were isolated from coral reefs in Indonesia, from the algal forest off the Chilean coast and from marine sponges of the Mediterranean Sea. The fungal strains obtained from these natural sources were brought into pure culture in the laboratories and identified by classical and molecular methods and stored within existing culture collections to assure availability over long periods of time (decades), and thus the necessity for repeated sampling of the natural environment is avoided. We fully adhered to the international treaties related to biodiversity preservation and sustainable use of its components.

Figure 1. Marine Fungi represent an excellent source for new bioactives, which can be exploited for human health in a sustainable way. The oceans harbours many different habitat forms. This includes extreme habitats such as hot vents, deep sea, Wadden Sea and others. Within MARINE FUNGI, new isolates were obtained from corals, algae and sponges.

In addition to new isolates the project made use of strains available in the existing culture collection of marine fungi at the Kieler Wirkstoff-Zentrum at GEOMAR and included a number of preselected strains into strain improvement and optimisation of the production process. Within this collection a number of fungi producing anti-cancer compounds have already been identified and individual substances have either already been patented for this reason or are in the process of being patent-protected. Because laboratory cultivation in fermenters was employed for production of bioactive compounds by marine fungi, the processes facilitated an efficient and sustainable cultivation and had no impact on the natural environment even in large scale production. *In situ* harvesting of the producer or product was not involved during metabolite production, or at any other stage of the investigation.

Overcome bottlenecks and barriers currently encountered in culturing marine organisms with assumed or known bioactive properties. Many marine fungi with known secondary metabolite production can be cultivated in laboratory using standard techniques and have the potential to be scaled up to production scale. Culture conditions of marine fungi were optimised at the laboratory scale and scaled up in fermentation vessels of increasing size to develop sustainable production processes in order to produce large amounts of the bioactive substances for the purposes of this project and further investigations, which include chemical structure analysis, bioassays for antitumoral activities and further evaluation of drug development potential. By use of various classical and molecular genetic techniques the biosynthetic production of bioactive

compounds can be increased to levels appropriate for the use in large scale biotechnological processes. Bottlenecks related to the problematic culture of marine organisms and to the production of only minor amounts of desired substances can thus be effectively overcome. This greatly improved the availability of secondary metabolites from marine fungi for the development of industrial applications.

Identify possible applications as a prerequisite for the design of sustainable production pathways. Secondary metabolites of marine fungi with a wide spectrum of biological activities are known, and these can be used within a wide field of applications (Figure 2). Within the project, we focused specifically on metabolites with antitumoral properties. Despite former efforts, cancer mortality remains unacceptably high. In Europe, cancer is second only to heart disease as a cause of death and many cancer types are not treatable with available therapies. Thus, the demand for new anti-cancer therapeutics is high and is likely to remain so for the foreseeable future. In recent years, marine fungi have been shown to represent an excellent source of such new anti-cancer compounds, but are still a seriously underrepresented group of organisms particularly in applied marine biotechnology.

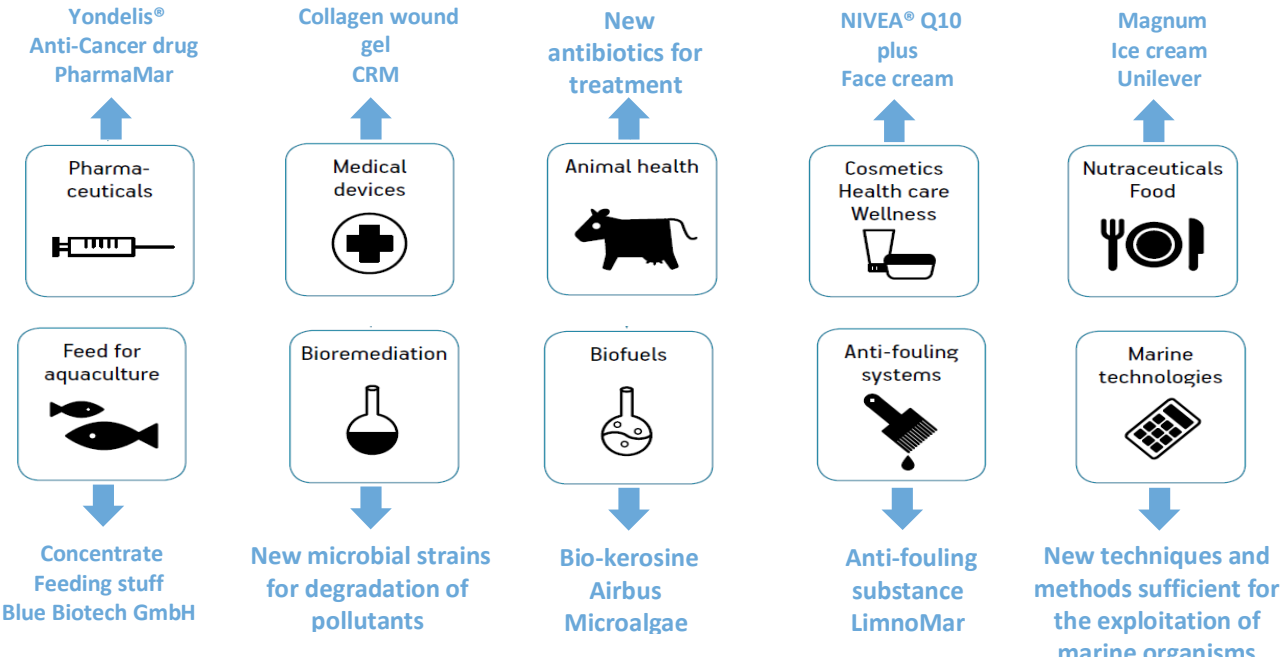


Figure 2. Marine Biotechnology: First Achievements show the application potential for marine natural products. Source: SUBMARINER compendium.

Specifically selected bioassays for testing anti-cancer potential were used in several of the participants' laboratories and strains identified as producers of promising metabolites were subject to different approaches of strain improvement and/or biotechnological process development. This ensured that production by marine fungi of compounds identified as suitable in anti-cancer therapies were scalable to pilot scale. Respective selective downstream processing techniques were developed for purification of antitumoral substances with high application potential and used for the purification of large amounts of these substances.

MARINE FUNGI demonstrated the sustainable exploitation of marine natural resources providing appropriate culture conditions for the group of marine fungi, thus enabling efficient production of marine natural products in the laboratory and also in large scale cultures, avoiding harm to the natural environment. The therapeutic focus of MARINE FUNGI was the development of novel

anti-cancer compounds. The development of clinically relevant lead structures required affords beyond screening: An interdisciplinary approach was applied to characterise hit candidates to the stage of in vivo proof of concept ready to enter further drug development. This included the development of robust, sustainable processes for the production of these compounds.

Valorise the existing knowledge by disseminating it widely to private and public researchers. The promotion of new natural products with high potential for application in anti-cancer therapies will be possible within the established network of highly competent partners of this project from public research institutions and private companies as outlined below. There will be a fair and equitable sharing of all benefits of results, patents and licenses arising from living and genetic resources. The sustainable exploitation of marine resources has been identified as a task in the integrated maritime policy for the European Union. Marine Biotechnology has started to develop relevant strategies, but intensive research is necessary to unravel these important resources of the ocean. This was a good reason to initiate this project which specifically deals with the exploitation of the anti-cancer potential of marine-derived fungi starting from the habitat and reaching a high added value product. Due to the time and cost intensive nature of this type of research, European pharmaceutical companies are not able to operate large screening efforts and currently seek the input of lead compounds ready for clinical testing.

Project objectives

Within MARINE FUNGI, we considered all aspects of the process, from the ecological considerations and specific habitat properties to high added value products for medical treatments. By doing so, we enhance the general understanding of the biology of marine fungi including global distribution and genetic inventories as well as its biotechnological potential. Through the genomic approach the results provided insight into fungal genome structure and evolution, pathogenicity and synthesis of biotechnologically relevant compounds, as well as insights into gene regulation.
The main objectives of the projects were:

- Genome analysis of three marine fungi and identification of biosynthetic genes and regulators
- Isolation of 600 new fungal strains from marine habitats in Chile, Indonesia and the Mediterranean and screening of these isolates for secondary metabolites
- Structural elucidation of new marine derived secondary metabolites and demonstration of their anti-cancer potential
- In vivo efficacy determination for five selected compounds in cancer models
- Process concepts for sustainable processes for the production of the bioactive compounds
- Dissemination and public relationship for marine Biotechnology in Europe

Description of the main S&T results/foregrounds

Multiple outcomes of a transdisciplinary project

The urgent need for novel substances for the treatment of severe human diseases such as cancer, combined with the recognition that marine organisms provide a rich potential source of such substances, support the intensive exploration of new substances from marine organisms. Marine fungi are a prolific group of secondary metabolite producers, but are still poorly characterised and underutilised for biotechnological application. Although intensive research is necessary to make use of the important resources of the ocean, marine biotechnology has now started to develop integrated strategies: therefore, MARINE FUNGI was initiated with a focus on the development of anti-cancer drugs from marine fungi. A transdisciplinary consortium was assembled to expedite a fast track approach to the project. A group of eleven institutions from seven countries joined forces to establish the necessary consortium of experts to cooperatively conduct the MARINE FUNGI project.

The main **valorisable outcomes** of the project were structures of lead compounds for anti-cancer therapeutics; robust, sustainable processes for the production of the bioactive compounds; and a strain collection of more than 600 new marine isolates. This strain collection was constructed along with a corresponding database of associated extracts and compounds, which can be used for many other purposes in the future. **On the scientific level**, fungal genomics has been expanded to encompass unique representative marine fungi, and their metabolic pathways have been opened to exploration. MARINE FUNGI delivered a huge diversity of S/T results – given in detail in the next section - and contributed to the **training of the next generation of marine biotechnologists.** The consortium leveraged its multidisciplinary, interdisciplinary and intersectoral network to train young scientists and to encourage lifelong learning for experienced researchers. Soft skill training, student exchanges and summer schools were organized on various aspects of drug discovery, commercialization requirements for small molecule drug discovery, the role of open innovation, and ethical and IPR issues, as well as on entrepreneurship.

One aim of MARINE FUNGI was to contribute to the **sustainability of marine biotechnology in Europe.** The lead users of MARINE FUNGI results will be pharmaceutical companies that will take anti-cancer substances derived from marine fungi into further drug development. The project strengthened the competitiveness of European marine biotechnology by providing a scientific and technical basis for the exploitation of marine fungi. Additionally, MARINE FUNGI activities increased the visibility of marine biotechnology as a key technology in Europe: MARINE FUNGI launched a public website offering non-confidential project information with a weekly blog. To increase the impact of the results, the partners have been active in dissemination, participating in established conferences, trade fairs and specific project meetings, e.g. with interested pharmaceutical companies, and will continue to do so.

Overview on S/T results

An interdisciplinary approach is necessary to characterise hit candidates to the stage of *in vivo* proof of concept ready to enter further drug development. We demonstrated sustainable exploitation by providing appropriate culture conditions for marine fungi, thus enabling efficient production of marine natural products in the laboratory and also in large scale cultures, avoiding harm to the natural environment. MARINE FUNGI covered two approaches to gain effective

producer strains, which led to the stage of *in vivo* proof of concept, building towards a robust platform for clinical development.

First, a molecular based approach using promising strains of marine fungi that were already known to produce cytotoxic compounds and which were taken from the culture collection of the Kieler Wirkstoff-Zentrum at GEOMAR was applied. Second, a culture based approach was used to increase the number of fungi isolated from marine macrobes and from different habitats of geographically distant regions.

In the first approach, three fungi were selected and sequenced to establish genome sequences and genetic systems. These three candidate strains were selected due to their production of new compounds active against cancer cell lines. Three different next generation sequencing methods were used. Three genomes were searched for biosynthetic genes, allowing the discovery of new compounds as well as the underlying regulation patterns for previously identified hit-structures. Using random (UV) and targeted (molecular genetic tools) mutagenesis, the strains were optimised for the production of the target anti-cancer compounds.

During the course of the project, we initiated three fungal genome projects. Concerning fungi, more than 100 whole genome sequences are available, and many more sequencing projects are ongoing (GOLD Genome Server, www.genomesonline.org). These advancements in genomic information gathering facilitate the search for secondary metabolite producers and help unravel the potential of secondary metabolite production in the relevant fungal strains. Analyses of the increasing number of sequenced genomes indicate that fungi encode the genetic information for the biosynthesis of many, as yet unknown, compounds.

By the completion of the project, three fungal genomes had been sequenced. We discovered the biosynthetic and regulatory genes by scanning the genome for the presence of characteristic biosynthetic genes. The biologically active compounds are produced by multifunctional enzymes, namely polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs).

Within the second approach, new marine fungal strains were isolated from key environments, such as Mediterranean hard rock habitats colonised by sponges, Indonesian coral reef habitats and Chilean macroalgal forests. Over 600 new fungal isolates were obtained and have been stored in the existing culture collections in order to ensure their long term availability. Taxonomic classification was achieved by molecular and microscopic methods. All these new fungal strains were identified and cultivated in a screening approach. Culture conditions for the new isolates were optimised for the production of anti-cancer metabolites. Nearly 1250 extracts were screened in the preliminary panel of cancer cell lines. Active extracts of these cultures were fractionated for chemical identification using state of the art technologies. Promising compounds were tested in an extended panel of relevant cancer cell lines and additional bioassays for compound characterisation available in different laboratories of the consortium. Out of a selection of more than 50 compounds that were screened in the full panel, a selection of eighteen compounds entered the liability testing. From these, three lead structures for further optimisation and tests of in vivo efficacy of the chosen compounds with respect to anti-cancer activity were chosen.
The culture conditions for the fungal strains were improved regarding the product profile and the amount of secondary metabolites produced. Three candidate strains were taken into controlled cultivation processes in submerged culture fermentation to establish conditions ready to be used in industrial scale-up. The resulting data of various fermentation techniques and the subsequent downstream purification was compiled into a process concept for these compounds.

Specific S/T results in the different scientific parts of the project

MARINE FUNGI was organized workpackages along the different scientific objectives. The workpackages had to interact in a very intense manner (Figure 3). In the following sections, the project achievements are given for each workpackage (WP).

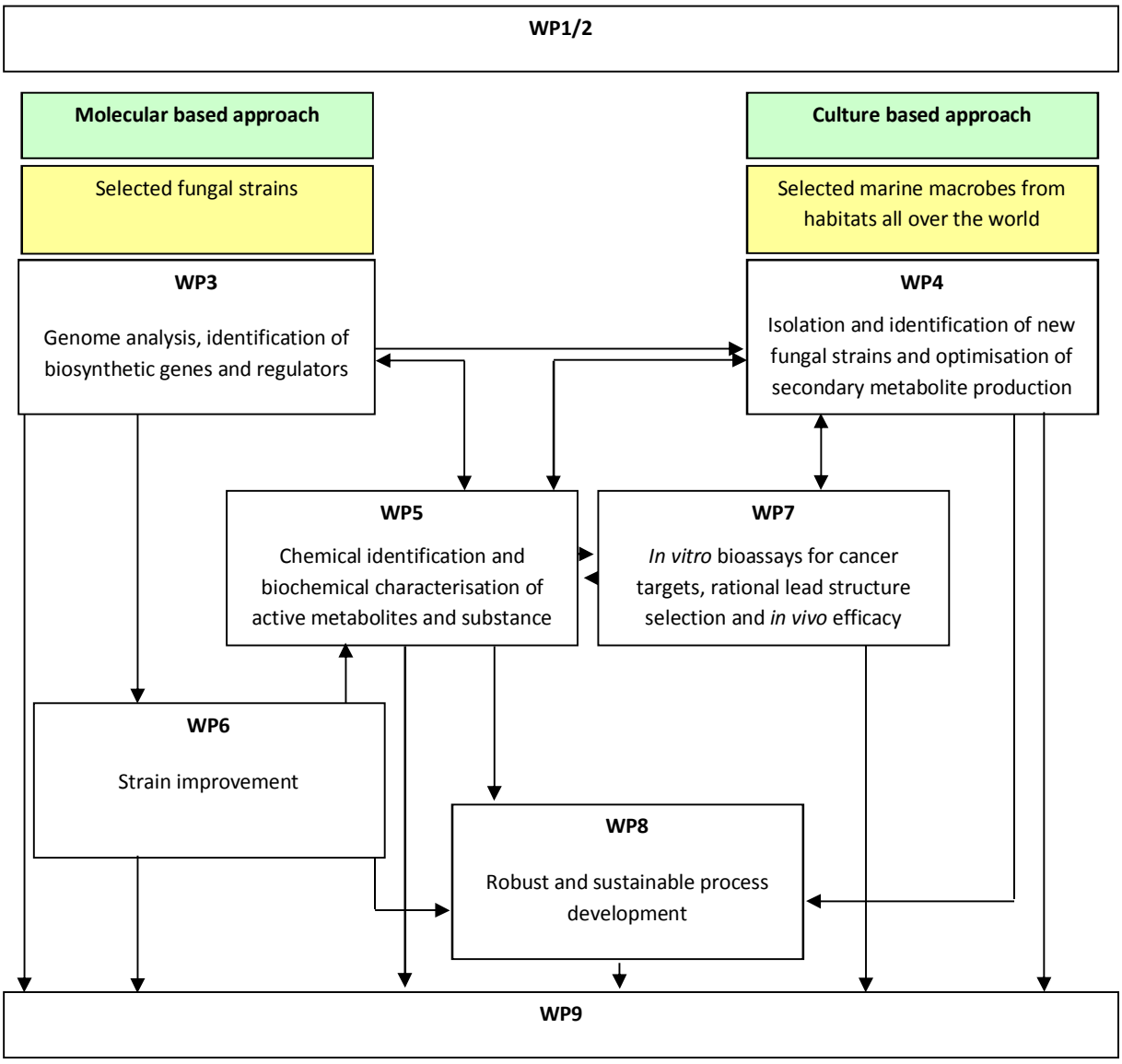
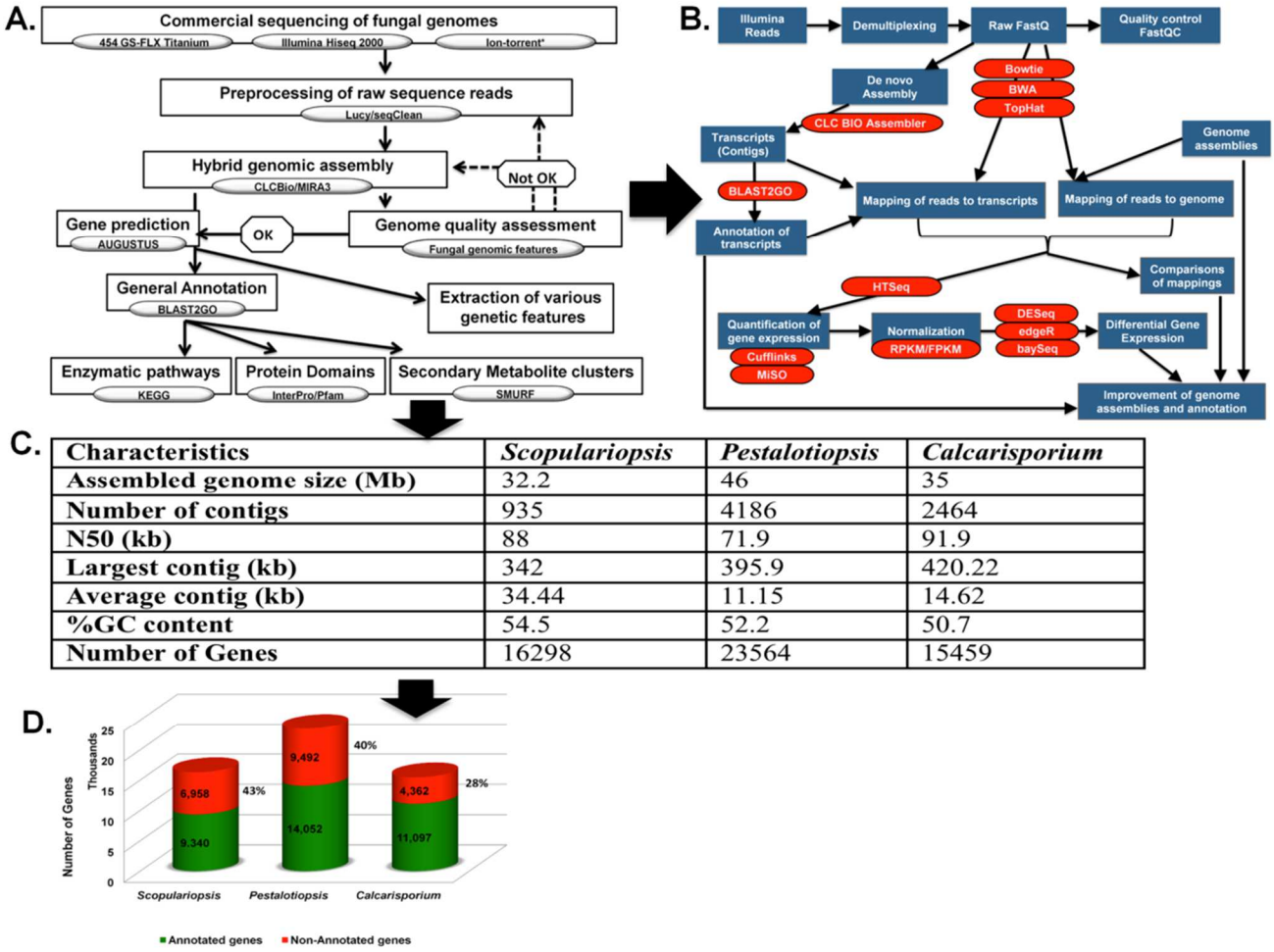


Figure 3 PERT chart of MARINE FUNGI illustrating the interconnections between the Workpackages.

Genome analysis, identification of biosynthetic genes and regulators (WP3)

Genome sequencing of marine fungi

We have selected three marine derived fungi namely *Scopulariopsis brevicaulis*, *Pestalotiopsis* and Calcarisporium sp. for exploring secondary metabolite (SM) encoding genes using next-generation genome sequencing and assembly protocol by 454 GS-FLX, Illumina HiSeq2000 and Ion-torrent methods (Figure 4A). Furthermore, expression profiling of these genes has been investigated with the help of RNA-Seq as described in Figure 4B.



Characteristics	*Scopulariopsis*	*Pestalotiopsis*	*Calcarisporium*
Assembled genome size (Mb)	32.2	46	35
Number of contigs	935	4186	2464
N50 (kb)	88	71.9	91.9
Largest contig (kb)	342	395.9	420.22
Average contig (kb)	34.44	11.15	14.62
%GC content	54.5	52.2	50.7
Number of Genes	16298	23564	15459

Figure 4.Overview of genome sequencing and RNA-Seq methods used during this study.

A. Protocol used in three fungal genome sequencing, annotation and analysis. * indicates that ion torrent was only used for sequencing of Scopulariopsis genome.

B. Protocol of Illumina based RNA-Seq approach used in our gene expression analysis. Blue box illustrates steps carried out. Red capsules indicate tools that were used on corresponding steps. RPKM/FPKM - reads or paired-end fragments per kilobase of exon model per million mapped reads.

C. Summary of genome assemblies for these three fungal genomes

D. Summary of annotation analysis reveal species-specific annotation level**.**

Genome of *Scopulariopsis brevicaulis*. We first sequenced the genome of *Scopulariopsis brevicaulis* strain LF580 using three different genome sequencing methods namely Roche 454, illumina and ion-torrent. Upon assembling the different type of sequencing reads, we achieved ~32 Mb genome with N50 equals to 88 kb and 935 contigs containing 16298 genes (Fig. 1C), which is on the high side for reported ascomycete genomes. The average intron length (=129.4) reflects the accuracy of gene prediction by the Augustus gene prediction tool as fungal genomes are known to possess small introns. During the annotation process, we were able to annotate 9340 genes (57.31 %) while 6958 genes (43.69 %) remained non-annotated in this genome. This genome has 17 genes (Table 1) encoding for non-ribosomal peptide synthetases (NRPSs), which is a typical fungal NRPS gene content. Additionally, this genome has 18 polyketide synthases (PKSs) and one gene is a hybrid NRPS-PKS (Table 1).

Table 1. Summary of secondary metabolite encoding genes in these three fungal genomes

Secondary Metabolite encoding genes	*Scopulariopsis*	*Pestalotiopsis*	*Calcarisporium*
Non-ribosomal peptide synthetase (NRPS)	17	44	52
Polyketide synthase (PKS)	18	62	66
Hybrid NRPS/PKS	1	7	7
Fatty acid synthase (FAS)	2	3	1
Dimethyl-allyl-tryptophan synthase (DMATS)	0	5	0
Sesquiterpene cyclase (SesCyc)	0	1	1

Genome of *Pestalotiopsis* sp. We sequenced our second fungal genome from *Pestalotiopsis* sp. strain KF079 using two different genome sequencing methods (Roche 454 FLX+ and Illumina HiSeq2000). The assembled genome comprised around a ~46 Mb genome with N50 equals to 71.9 kb and 4186 contigs containing 23492 genes (Fig. 1C), which is surprisingly very high for a fungus. The average intron length (=126.8) reflects the accuracy of gene prediction by the Augustus gene prediction tool as fungal genomes are known to possess small introns. Average intron per gene (2.2) is also in co-agreement with other fungi. We annotated 60% of the total number of genes (Figure 4D) in this fungal genome. The *Pestalotiopsis* genome has 44 NRPSs, 62 PKSs and 7 hybrid NRPS-PKS genes (Table 1).

Genome of *Calcarisporium* sp: We sequenced the third fungal genome from *Calcarisporium* sp. strain KF525 using two different genome sequencing methods (Roche 454 FLX+ and Illumina HiSeq2000). We achieved the genome assembly of about 35 Mb genome with N50 equals to 91.9 kb and 2464 contigs containing 15459 genes (Figure 4C), which is high for fungi. The percentage GC% for this genome is 50.7%. Average intron length (=121) reflects the accuracy of gene prediction by the Augustus gene prediction tool as fungal genomes are known to possess small introns. Average intron per gene (2.1) is also in agreement with other fungi. We annotated 72% genes, while 28% genes remained non-annotated (Figure 4D). This genome has 52 NRPSs, 66 PKSs and 7 hybrid NRPS-PKS genes (Table 1).

Comparative genomic analyses of marine fungi

BLAST2GO based annotation analyses suggested that these three fungi are close to the *Fusarium/Nectria* clade under the *Sordariomycetes* class in the tree of life (Figure 5A). This finding was also supported by genome-wide phylogenetic analyses of these fungi (Figure 5B).

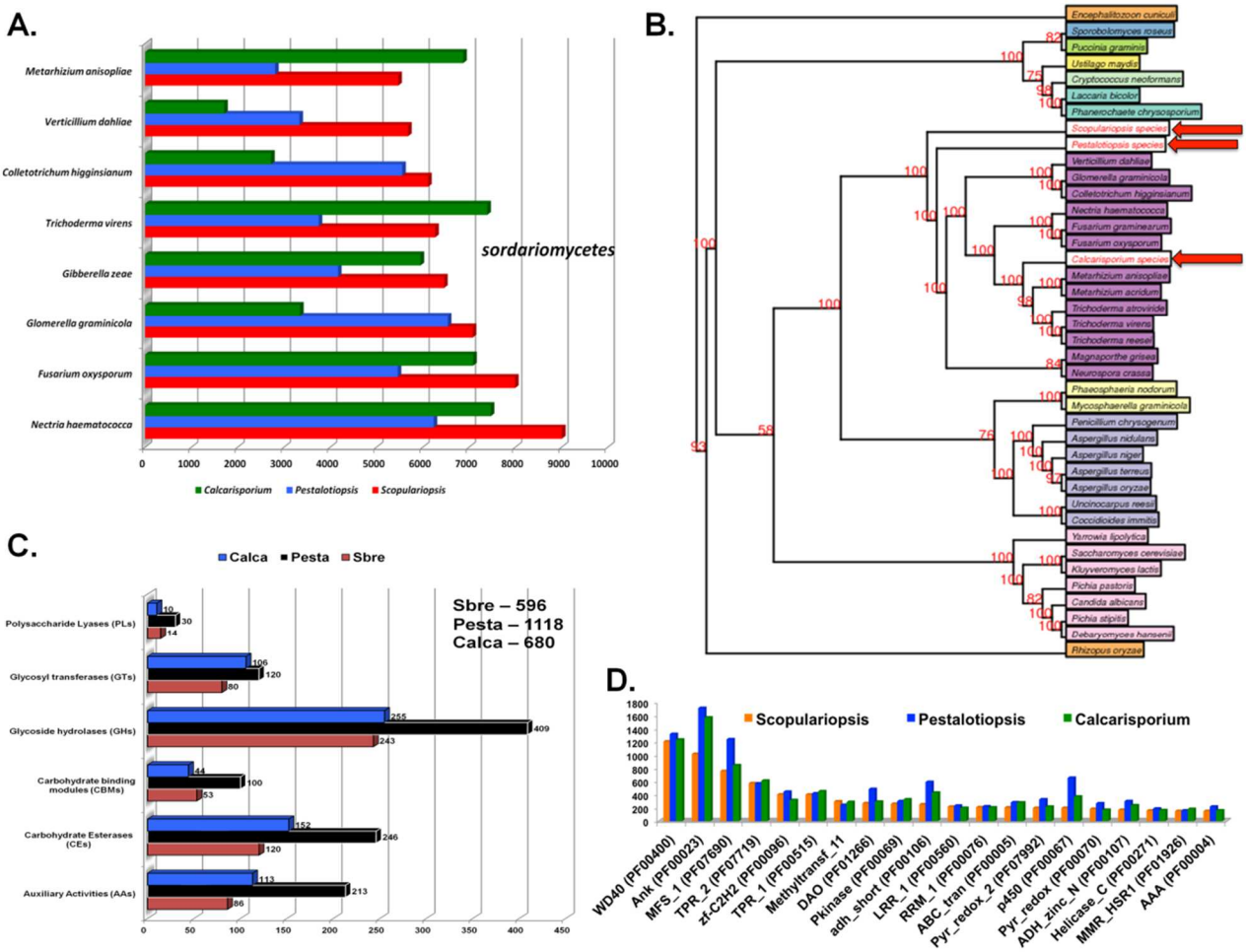


Figure 5.Overview of comparative genomic analyses of these three marine fungi.

- **A.** The three sequenced fungi belong to the sordariomycetes class, close to the clade of *Fusarium/Nectria* based on genome annotation using homology searches.
- **B.** Genome-wide phylogenetic analyses of three marine fungi with other ascomycetes supported the phylogenetic location to the clade of *Fusarium/Nectria*.
- **C.** Summary of carbohydrate active enzyme classes in three marine fungi.
- **D.** Top Pfam protein domains in three marine fungi.

Summary of RNA-Seq based expression profiling

Expression profiling was performed by *de novo* assembly of RNAseq reads for three organsims. Figure 6A provides summary of these assemblies. These analyses will help to understand regulation of secondary metabolite genes, as not all biosynthetic genes are expressed under a given condition. The number of transcripts per assembly is above 20000 with total sizes ranged from ~19-23 Mb, indicating that assembly is good as we expect a greater number of transcripts than the number of genes present.

A.

Characteristics	LF580-M26	KF079-wt	KF525-wt
N75	758	737	788
N50	1641	1616	1653
N25	2717	2656	2715
Minimum contig length	176	175	184
Maximum contig length	10517	10812	10553
Average contig length	972	944	981
Contigs (transcripts/EST)	20471	24886	22423
Total basepairs	19,895,119	23,493,681	22,002,705

B.

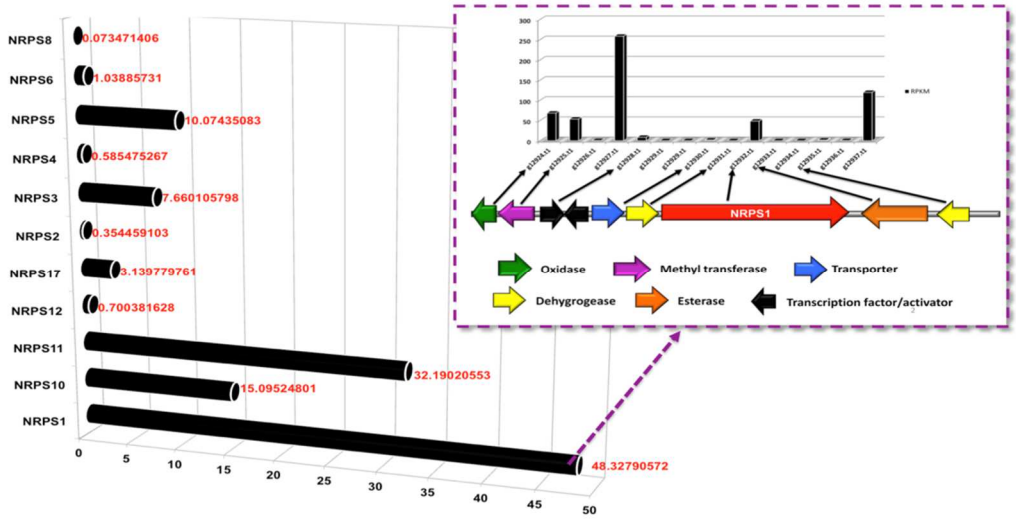


Figure 6 Overview of RNA-Seq analyses.

A. Summary of assemblies of the RNA-Seq reads for the three different transcripts representing the genes expressed by the 3 different organisms under the conditions studied .

B. Expression profile of NRPS genes from *S. brevicaulis* LF560-M26 mutant illustrates that NRPS1 gene has highest level of expression. Expression profile of NRPS1 gene cluster from *S. brevicaulis* LF560-M26 mutant reveals that majority of genes in this clusters are expressed (shown in box). RPKM = Reads per kilo base per million.

Summary of mass spectrometry and proteomics analyses of selected strains of marine fungi

Quantitative proteomics and analysis of significantly up- and down-regulated proteins by MS for LF580 and mutant M26 under the same conditions and Calcarisporium under different growth conditions were studied in detail and will form the basis of publication(s).

Isolation and identification of fungal strains and optimization of secondary metabolite production (WP4)

Exploring fungal diversity for cultivation

New fungal isolates were obtained from various marine macroorganisms and their identification was the first major objective. The second major objective was the establishment of effective and improved culture conditions that would ensure proper production of secondary metabolites. This included reproduction of secondary metabolite production in larger scales in Erlenmeyer flasks with the aim of providing sufficient amounts of material for the development of the lead

compounds (WP5, WP7). Additionally, conditions had to be identified that enabled production in stirred tank reactors for increased fermentation scale challenges in WP8.

Isolation and identification of fungi

More than 600 strains were obtained and prepared for conservation. In total, 605 strains were conserved and are available as active strains for all partners. From the Chilean algae, 259 strains, from Indonesian hard corals, 105 strains and from Mediterranean sponges, 241 strains were obtained and stored at two different sites. The isolated strains were stored in liquid nitrogen in order to generate a strain collection enabling strain constancy as well as conserving the strains for other research topics.

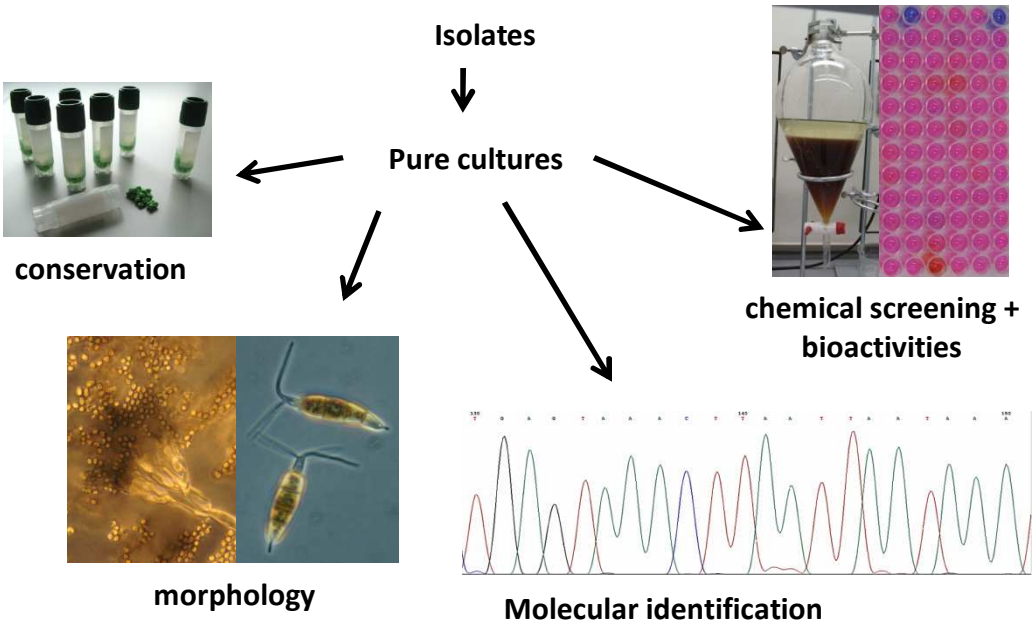


Figure 7. Strategy for identification of the newly obtained fungal isolates.

Pure fungal isolates were obtained by classical culturing techniques using a variety of media well suited for marine fungi. Identification of all the isolated pure cultures was realised by both gene sequence analysis (18S rDNA and ITS) and by classical morphological properties (Figure 7). The obtained fungal strains belong to more than 30 different genera, however, some difficulties occurred in identification of specific strains; sterile mycelia in combination with low similarity to known genera occurred for some strains. Those strains were listed as “unidentified“. The predominant genera were *Penicillium, Cladosporium, Fusarium, Aspergillus, Pleospora, Chaetomium, Acremonium, Hypocrea, Hamigera,* and *Hortaea*.

Laboratory culture of new isolates of marine fungi for preliminary screening and optimising conditions for secondary metabolite production

All strains were grown using four conditions each and subsequently extracted for submission anticancer screening (in WP5/WP7). Overall, 476 of the 605 strains were screened with a total number of 1273 extracts. The remaining 129 strains were not screened due to various reasons: some of the strains did not grow in the selected media, others were removed after contamination, some were identified as chemical “duplicates” (Figure 8).

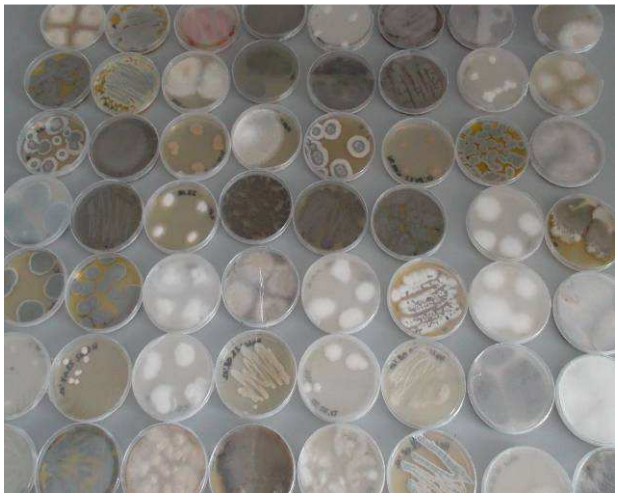


Figure 8. Agar plates of fungal isolates prepared for inoculation of screening cultures

Effective and improved culture conditions for new candidate strains, which were selected based on the feedback from the bioassays, i.e. describing their cytotoxic activity, and experiments to stimulate the biosynthesis of metabolites were established. A number of external stimuli, such as media composition, temperature and oxygen supply, which are known to influence the production of secondary metabolites, were assessed in order to establish effective and improved culture conditions: a cultivation protocol for efficient production of secondary metabolites from 30 new fungal strains was provided. These protocols were used for larger scale fermentations at the 10L scale in Erlenmeyer flasks for obtaining larger amounts of material for purification. Biomass was extracted and extracts were delivered to project partners for further purification of the targeted compounds. The most effective approach was to change the medium composition.

Strain management

The provision of the project's database (PORTAL) was an integral part of the project. This portal was maintained to record all relevant information on the isolation (source and location), identification of phylogenetic information, preservation and properties of strains isolated in the project. Beside the electronic data management, the establishment of conserved strain material of all new fungal isolates was achieved. The viability of the cryo conserves has been checked. For exchange of strains, active cultures have been used. All strains were replicated and are stored as a comprehensive strain collection at GEOMAR for further use by the consortium and via MTA by external users to address proper IP protection.

Chemical identification and biochemical characterization and substance purification (WP5)

To a large extent, work package 5 functioned as the chemistry laboratory of Marine Fungi. In this work package we received samples from other work packages, analysed and refined them chemically, and delivered refined samples and purified compounds to other work packages. In short, this work package was responsible for the following:

- Fermentations were converted to extracts,
- Extracts were analysed, profiled, and tested for anti-tumour activity in collaboration with WP7,
- Prioritised extracts were fractionated to enable purification of the active substances with assay-guidance,
- Active fractions were analysed and known compounds identified and dereplicated,
- Compounds with promising properties were purified,
- The chemical structures of purified compounds were elucidated,
- Metabolite profiles were established for marine fungal strains producing the most interesting compounds,
- Proteomes and cell signalling pathways of cancer cell lines treated with the most promising lead compounds were analysed to probe their mechanisms of action.

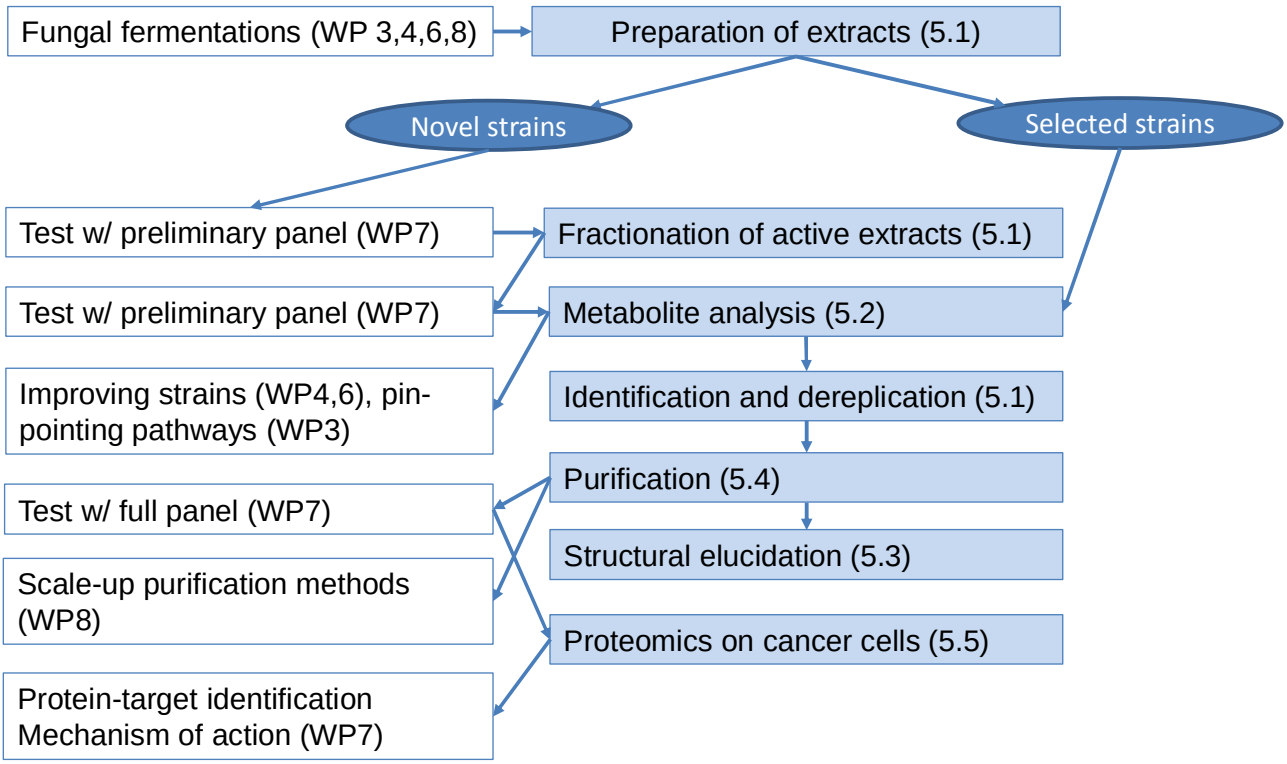


Figure 9. Work flow in work package 5 and interaction with other work packages.

As indicated in Figure 9, WP5 received and extracted samples from fungal fermentations from four different work packages. Extracts from newly isolated strains were subjected to activity testing against a preliminary panel of three different cancer cell lines in cooperation with WP7. We tested 1273 extracts in this manner. Forty two diverse strains (selected on the basis of both

taxonomic characteristics and genetic sequence information), that showed potent anticancer activity, were selected for assay-guided purification and the subsequent chemical identification of the active compounds. At this stage, many hits were dereplicated. That is, we found them to be caused by compounds with previously reported anticancer effects. Thus, they did not represent opportunities for the development of new IP-protected drug candidates, and therefore these compounds were of minor interest for the consortium. Novel compounds with anticancer effect (7), on the other hand, were produced at a larger scale (10+ litres, WP4) in order to obtain enough material for further testing of their anticancer effects, as described in WP7. This comprised both *in vitro* tests, liability assays and, for the most promising lead compounds, also *in vivo* experiments.

For a number (38) of *a priori* selected fungal strains with chromatographically interesting metabolite profiles potentially active and novel compounds were purified, identified, dereplicated and subjected to activity testing as pure compounds against the preliminary panel and, if successful, also against the extended panel of cancer cell lines (WP7). Identified lead compounds were purified from cultures grown at larger scales in WP4 for further testing in WP7, as described above for the best compounds purified from the freshly isolated strains.

A number of strains proved to be highly productive secondary metabolite producers and their metabolite profiles were characterised extensively. Of particular note was *Tolypocladium geodes* MF458, which was isolated from a Mediterranean sponge (*Tethya aurantium*) and identified by morphological and molecular characterisation (WP4). The strain was cultivated in a variety of media. The best antitumour activity in extracts was found from cells cultivated on Wickerham medium containing 30 g/L tropic marine salt using standing conditions. This activity was predominantly anti-proliferative rather than overtly cytotoxic. Chromatographic analysis of these extracts showed the presence of a rich variety of compounds as illustrated in Figure 10.

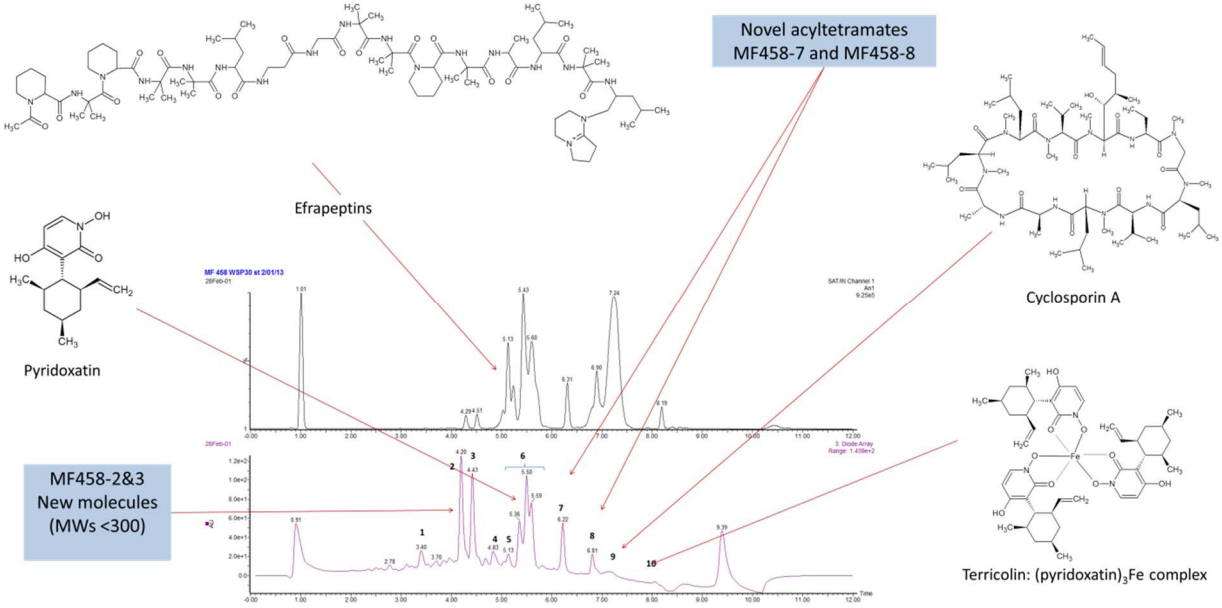


Figure 10. Establishing the metabolite profile of MF458 *Tolypocladium geodes*. Reversed phase HPLC analysis of ethyl acetate fermentation extract: evaporative light scattering detected chromatogram (top) and diode array UV-visual detected chromatogram (bottom).

Assay-guided fractionation revealed that anti-tumour activity was associated with a range of different compounds present in the extracts. The purification methods developed to isolate the active compounds used mainly reversed phase HPLC. Structure elucidation showed that the active compounds were the products of five different biosynthetic pathways. The most potent activities found were caused by compounds known to have anti-tumour effects: efrapeptins and pyridoxatin.

Tolypocladium spp. have attracted significant attention as producers of bioactive secondary metabolites. Efrapeptins, pyridoxatin and terricolin have previously been reported as products of terrestrial isolates of *T. geodes*, while production of the medicinally-significant cyclosporins is usually associated with other *Tolypocladium* spp. Metabolites from marine *Tolypocladium* spp., such as the new efrapeptin J, are also being reported. MF458, however, also produced new molecules with anti-tumour activity. The novel acyltetramates MF458-7 and MF458-8 only had very moderate anti-tumour potency. MF458-2 and MF458-3 have more potent effects and are being evaluated further. This work shows that new marine isolates of even previously well-researched species have the potential to produce new compounds with potentially useful biological activities.

The novel compounds identified during this project include the most promising lead candidates MFU-954, MFU_955 and MFU_108, which are currently undergoing lead evaluation as described for WP7. The structures of some of the novel compounds that proved to have less promising biological properties are shown in Figure 11. Calcaride A (Silber et al. 2013), from *Calcarisporium* sp. KF525, showed interesting effects on signal transduction in tumour cells (WP7) but proved to have undesirable properties on testing in liability assays (WP7). The novel acyltetramates MF458-7&8 (manuscript in preparation) had insufficient anti-tumour potency to merit further investigation.

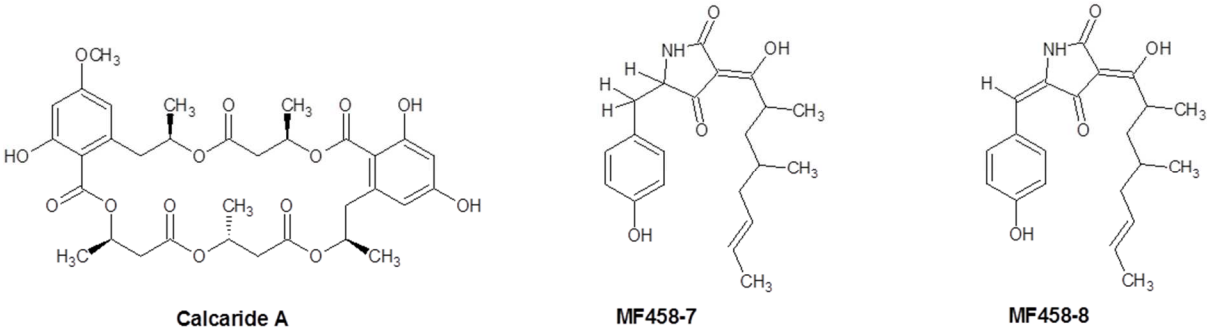


Figure 11. Structures of some of the novel marine fungal compounds characterised.

Additionally, WP5 attempted to elucidate the cell signalling pathways and proteome changes occurring in cancer cells upon exposure to our chosen lead compounds in time course experiments. Cell signalling was investigated by phosphoflow analysis while proteome changes were investigated by phosphoproteomics employing nLC-MS/MS methodology. These experiments did identify a number of pathways and phosphoproteins that were affected by our lead compounds. But on this basis alone no firm conclusions regarding likely mode-of-action of the chosen compounds can be made. Additional experiments reported in WP7 gave some indications to the possible targets and mode-of-action of one of the lead compounds.

Strain improvement (WP 6)

The main objective of WP6 was to create mutant strains with enhanced production of the target anticancer compounds. In the future, these optimized strains can be used to as production strains if the target compounds find their way into the pharmaceutical industry. Most of the work focused on production of scopularides by *S. brevicaulis*, where we applied both targeted and random mutagenesis approaches.

In the random mutagenesis approach we generated a UV mutant library by exposing 2000 *S. brevicaulis* spores to UV-radiation (312 nm) for 85 s. Single colonies were then isolated and 240 mutant strains tested for production of scopularides A and B. In this screen we identified one

mutant strain (M26), which had a two fold increase in production of scopularides compared to the wild type (Figure 12).

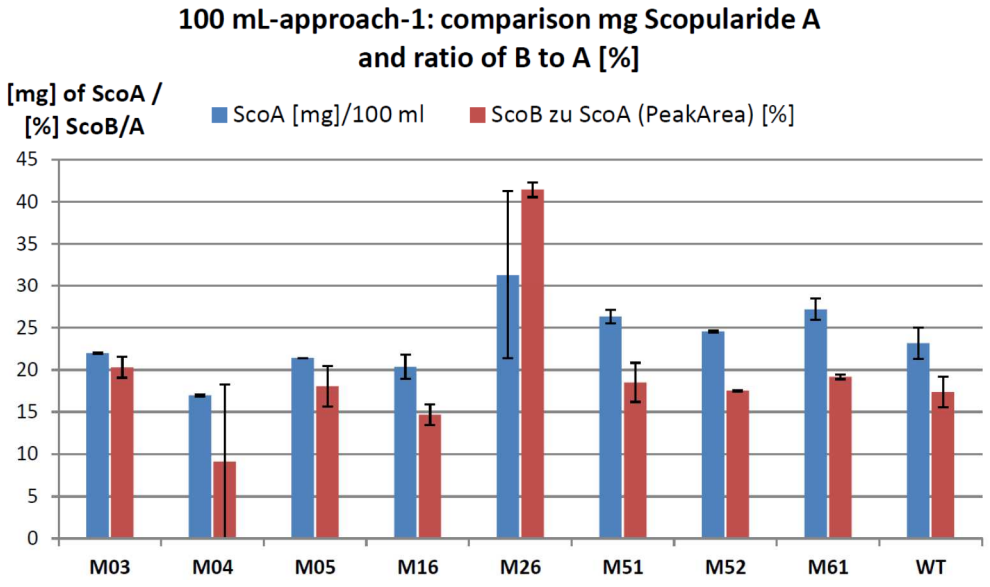


Figure 12. Production of scopularides by selected UV mutants.

Further analyses have shown that the increased production is due to improved growth and extraction properties and not because the scopularide biosynthetic pathway has been directly affected by the mutagenesis. The M26 strain was then used in an additional mutation round where 350 mutant strains were obtained, which however did not show any further enhanced production of the scopularides.

In the targeted mutagenesis approach we used the genomic sequence of *S. brevicaulis* provided by WP3. Scopularides consist of five amino acid residues and one hydroxycarboxylic acid with a long alkyl chain. These types of compounds are produced by nonribosomal peptide synthetases (NRPSs), which are large multidomain enzymes. Each domain in a NRPS consists usually of an adenylation domain which loads an amino acid; a carrier domain which holds the amino acid and a condensation domain for forming the amide bonds. For targeted up-regulation of the NRPS responsible for scopularide synthesis the gene has to be identified and the genome sequence of *S. brevicaulis* revealed 19 NRPSs and NRPS-like genes (Figure 13) of which NRPS1 is the most likely candidate as it is the only NRPS which contains the five adenylation domains needed for production of scopularides.

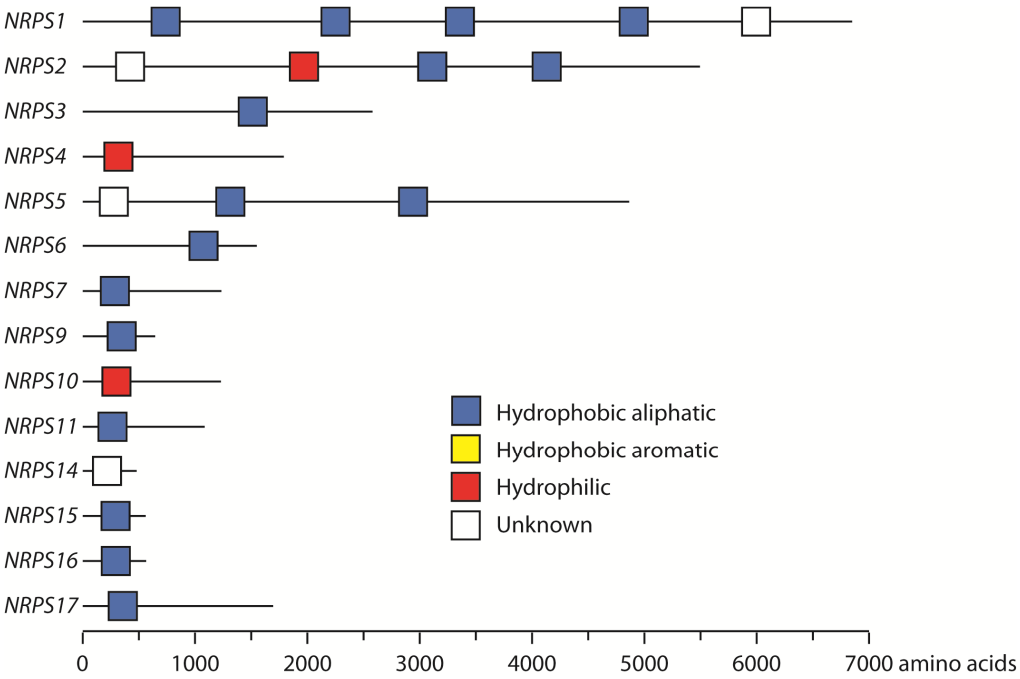


Figure 13. Illustration of adenylation domains in NRPSs in *S. brevicaulis* and their predicted amino acid substrate.

To verify that NRPS1 is the scopularide synthetase we first had to establish a transformation protocol for *S. brevicaulis* and we adapted the *Agrobacterium tumefaciens* transformation method which is in place at Aalborg University. Growth conditions, nutrition and spore inoculum were optimized for *S. brevicaulis* and we were able to introduce the transformation cassette into the fungus, which could be selected using hygromycin. The method was used to target the NRPS1 for gene replacement and we successfully obtained >100 mutants that were resistant to the selection marker. All resistant strains produced scopularides and diagnostic PCR revealed that the cassette had been inserted randomly and that the fungus does not perform homologous recombination.

To circumvent this problem we targeted transcription levels of NRPS1 by RNA interference, by expressing an antisense fragment of the gene. This was achieved by developing a vector containing translation elongation factor 1α promoter for constitutive expression of the antisense fragment. Furthermore the vector contains multiple cloning sites which allow easy substitution of the fragment and can therefore be used to develop vectors for other fungi. The NRPS1 RNAi vector was transformed into *S. brevicaulis* and the resulting mutants were screened for production of scopularides on Czapek dox medium with yeast extract (CYA) where we identified one strain (AS3), which did not produce scopularides (Figure 14).

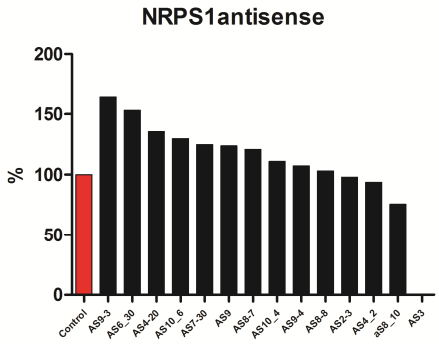


Figure 14. Production of scopularides by NRPS1 RNAi mutants compared to wild type (control)

The knock down effect on NRPS1 was, however, only temporary as the mutant regained the ability to produce scopularides, but the results suggests that NRPS1 is responsible for scopularide production. To enhance the production of scopularides we used the previously mentioned vector to constitutively express a transcription factor (TF) which is located near NRPS1. The obtained mutants were grown on CYA and screened for scopularide production (Figure 15).

A B

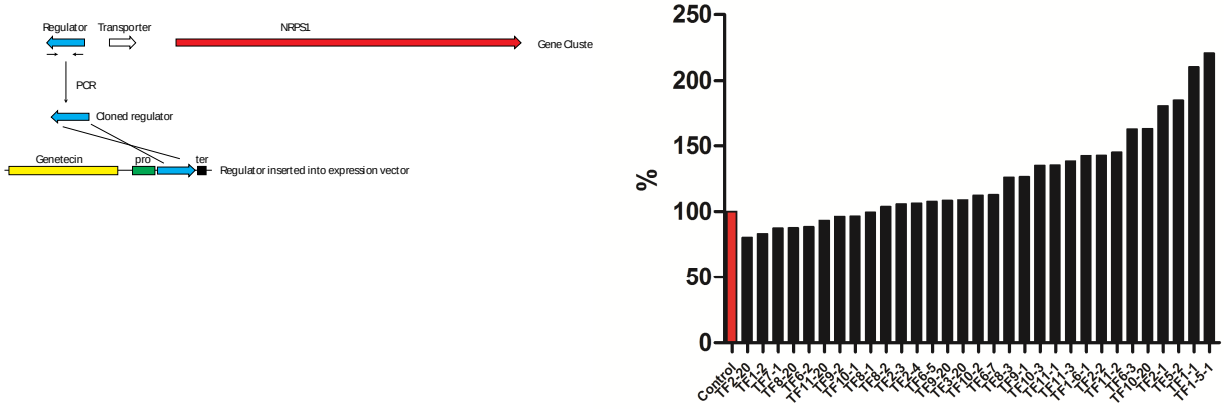


Figure 15. A. Cloning and insertion of the NRPS1 transcription factor into an expression vector. B Production of scopularides by the 31 obtained mutants.

The transformation resulted in 31 mutants of which 22 produced more scopularide A than the wild type. The highest production was observed in TF5-1, which produced 2.2 fold more than the wild type (Figure 15B). This mutant (together with TF5-2) was passed on to WP8 for comparable analyses against the wild type, where it again exhibited more than 2 fold increased production of scopularide A.

The proteome of the wild type *S. brevicaulis* strain was compared to the mutant with increased levels of scopularide (OE-TF5-1) and an RNAi silencing mutant (AS3) producing low amounts of scopularide. Neither NRPS1 or the TF were, however, identified in the samples and optimization of the protocol will be a task for the future.

In vitro bioassays, rational lead structure selection and in vivo efficacy determination (WP7)

The objectives of WP7 were to create a platform to identify molecules from marine sources, which demonstrate activity in *in-vitro* models of cancer, and then to further characterise the most promising compounds to a point where their efficacy in *in-vivo* disease models could be established. The long term aspiration is that the compounds or their derivatives would then enter the drug development process and ultimately provide new therapeutic options for patients suffering from cancer.

Screening panels

The first stage involved establishing a broad panel of cancer relevant bioassays and using these to screen crude extracts from fermentations of new fungal isolates and subsequently pure compounds. In this project, we have maximized the opportunity for anticancer agent discovery by using the human tumour cell line panel from the US National Cancer Institute (NCI). The approach, when combined with bioinformatic analysis of the activity pattern across the various cell lines, phospho-flow and fluorescent barcode analyses, gives access to information on selectivity and specificity with respect to particular tumor types and a preliminary assessment of the potential compound mode of action. Demonstration of anti-cancer activity of a given compound can involve a range of assays monitoring proliferation, viability, apoptosis, angiogenesis, anti-tumor immunity as well as target specific effects, signal pathways and biomarkers. The full NCI panel comprises 60 cell lines from different tissue origins, which represent a wide variety of cancer phenotypes. The screening procedure performed by the Developmental Therapeutics Program of the NCI and NIH is based on a colorimetric protein quantification assay using sulforhodamine B. The assay is performed in 96-well plates with a total volume of 200 µL and involves sophisticated staining and dye solubilisation procedure otrss. By assessing cell viability before compound exposure it is possible with this assay to differentiate cytostatic and cytotoxic effects. In this study, the protocol has been adapted to a luminescence format using ATP detection as a surrogate readout for cell number, allowing miniaturization and associated reduced usage of compound, cellular and detection reagents (Ellinger *et al.* 2014).

A comparison with the characteristic growth inhibition pattern of known standard compounds over the complete panel of cell lines can enable a preliminary assignment of the mode of action of a novel compound. The NCI has established the online tool COMPARE to facilitate this analysis. The COMPARE analysis can allow the early removal from the Drug Discovery workflow of novel compounds with less “desirable” modes of action, e.g. which are associated with significant side effects (e.g. DNA crosslinking) or are well catered for in the current pharmacopeia (e.g. selectively potent against cells which carry mutations in the B-raf gene). In theory, the opportunity exists to promote, at an early stage, compounds which have modes of action associated with reduced side effects and a lower risk of inducing resistance. The wealth of proteomics and transcriptomics data associated with the NCI60 panel also provides additional benefits in compound profiling decision making and prioritization.

Crude extracts obtained were profiled in singlicate against HL-60, MCF-7 and M14 cell lines as part of a bioactivity guided fractionation approach. Active fractions were purified to single compounds and characterized in a wider range of cell lines from the NCI60 panel (Figure 16). Detailed screening on isolated compounds was conducted on a fully automated cell::explorer system (Perkin Elmer, USA) and the Cell-Titre-Glo (CTG) protocol (Promega Inc., USA). The relative growth at 72 h (versus 24 h baseline) was used to calculate cell viability and proliferation parameters (GI50, LC50 and TGI), as described previously. Representative results can be seen in Figure 7.1. Within the project, over 270 compounds entered dose response profiling against the

preliminary panel of 4 cell. A total of 18 compounds were selected for detailed profiling against the extended panel.

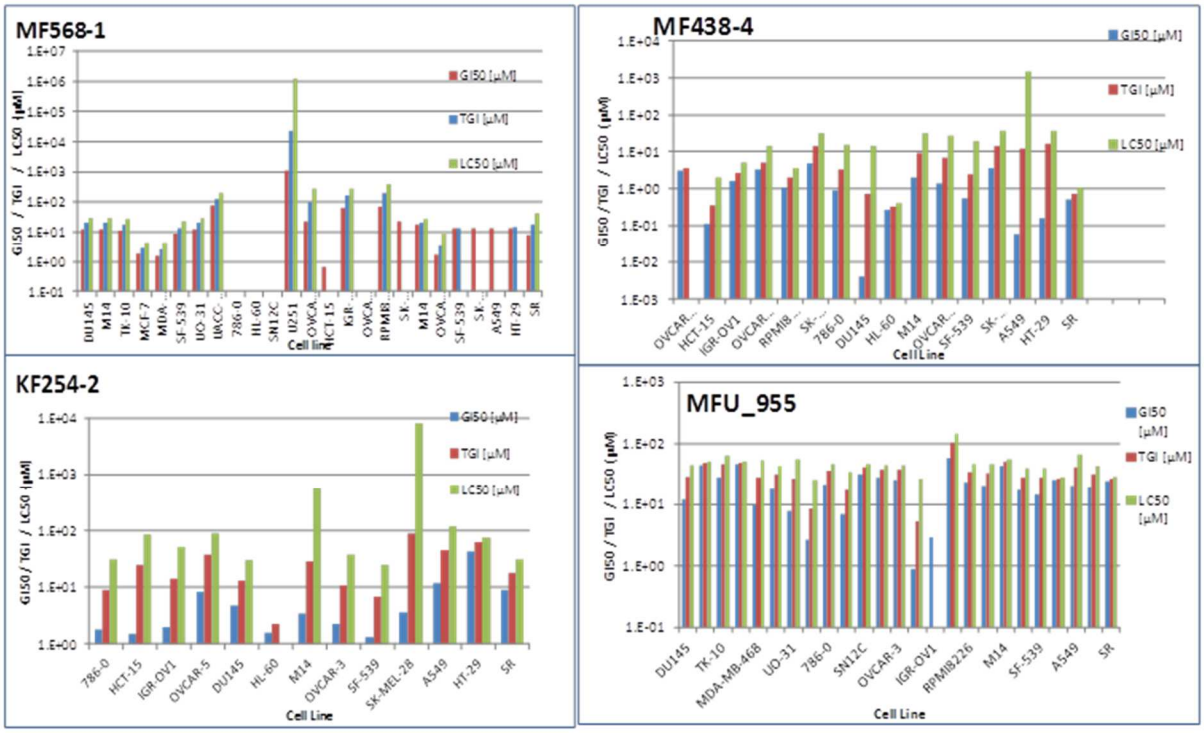


Figure 16. Bioactivity profiles (GI50, TGI and LC50) across members of the NCI panel for compounds MF568-1, MF438-4, KF254-2 and MFU-955.

Target identification

Target deconvolution following an observation of a phenotypic effect is one of the most technically challenging aspects of drug discovery with a typically low chance of success, due to potential issues of polypharmacology (i.e. the test compound hits several targets and pathways simultaneously) as well as the large number of candidate targets that can emerge, and where the false positive results need to be efficiently eliminated. Our aim was to elucidate the major molecular mechanisms which gave rise to changes in cell viability or proliferation. The approach taken was to combine affinity, screening and bioinformatics methods to identify putative molecular mechanisms of action. Some 4 compounds entered affinity based target identification using proteomic techniques. Compound MFU-954 was successfully analysed and two mitochondria associated targets with strong links to cancer were identified (VDAC1 and Prohibitin1/2). The protein function of VDAC-1 is to form a channel through the mitochondrial outer membrane and the plasma membrane to allow diffusion of molecules over the mitochondrial membrane and in and out of the cell. Interestingly, VDAC-1 has been implicated with cell death and apoptosis. The function of Prohibitin is to inhibit DNA synthesis and also prohibitin has a role in regulating cell proliferation. Moreover, defects in the prohibitin gene has previously been implicated with sporadic breast cancer and with somatic mutations in human tumours. Thus, MFU_954 may exert its anti-cancer activity through binding to two proteins implicated with apoptosis and cancer, namely VDAC and prohibitin. The COMPARE analysis using the NCI database of standard compound activities suggested that MFU-954 activity profile was most closely correlated (R^2 = 0.61) with dichloroallyl lawsone, an inhibitor of an enzyme that catalyses nucleotide biosynthesis in human mitochondria.

We also applied phospho-flow analyses as a complementary tool to provide a guideline for downstream target deconvolution and mode of action studies. Briefly, cells are stimulated in absence or presence of different concentrations of test compounds of interest for various time spans. Intra-cellular phosphorylation events are then analysed using phosphor epitope specific antibodies. In addition fluorescent cell barcoding (FCB) provides further information as it enables combinations of up to 48 samples from different conditions to be assessed within a single experiment. A subset of 5 compounds of interest was chosen for phosphoflow analysis and these were examined in detail for their effects on cell survival and proliferation linked signalling pathways. Cells were stimulated in the absence or presence of test compounds, and intracellular phosphorylation events are then analyzed using phosphor epitope-specific antibodies. In our studies, calcaride A inhibited the prosurvival and proproliferation signalling usually induced by TCR stimulation, as could be clearly observed at the highest compound concentration (0.1 mM). The activation of the MAP kinases Erk1/2 in the mitogenic Ras-Raf-MEK-Erk signalling pathway displayed delayed phosphorylation kinetics and a lower phosphorylation maximum of T202/Y204.

Compound Tox and Pharmacokinetic profiling

Priority compounds entering detailed profiling received a prediction of their physico-chemical and liability associated properties using the StarDrop software (Optibrium). Overall, the *in-silico* predictions appeared to tally with the observed properties, particularly with respect to solubility, CyP450 interactions and plasma protein binding. Priority compounds entering the *in-vitro* ADME profiling stages underwent systematic microsomal stability testing. Compounds were tested using mouse and human derived microsomes. Priority compounds were successfully tested against the following key CYP family members: CYP2C19; CYP2C9; CYP2D6; CYP1A2 and CYP3A4. Collectively these enzymes are involved in the metabolism of the great majority of marketed drugs. Standard compounds were used to confirm the pharmacological properties of the assays. Plasma protein binding studies were conducted on all priority compounds. Appropriate analytical methods were set up to quantify bound and unbound fractions. Due to the limited aqueous solubility of some of the priority compounds, the experimental scope was extended to include additional formulation optimisation step and *in-vivo* pharmacokinetic investigations. The *in-vivo* PK properties of 5 selected compounds were determined. Maximum tolerable dose studies were performed on 3 compounds (MFU_954, MFU_955 and MFU_108). Compounds were administered to non-tumour bearing athymic nude mice as a single dose by intraperitoneal injection. The compounds were evaluated at 4 different doses, however if a dose proved toxic the study was repeated at a lower dose but if a dose was tolerated the study was repeated at a higher dose. For each compound, the optimal dosing regimen for downstream Xenograft compounds was then selected. In collaboration with WP5, compound production was bulked up to support *in-vivo* studies, with up to 500mg of the lead compound being produced for each study.

Efficacy profiling in Xenograft models

By the completion of the project, some three compounds were progressed to Xenograft studies. The compound MFU_954 was analysed in a xenograft model using the NCI colon cancer cell Colo205. Compound MFU_955, a close analogue of MFU_954, was also analysed in Colo205. MFU_108 was analysed in A549 cells, a human lung adeno-carcinoma epithelial cell line. The protocol involved multiple dose administration of test compound by intraperitoneal injection (ip) over an extended period. Three groups, each with 10 mice per group, were treated as follows: untreated/vehicle control; positive control at MTD; test compound at MTD. The tumour measurements and animal observations were recorded 5 times per week for up to 4 weeks

following treatment (dependent on tumour growth). One compound (MFU_954) has so far shown statistically significant *in-vivo* efficacy in a colon cancer model (Table 1). Data showed significant growth delay for both the positive control, doxorubicin ($p<0.05$) and the test compound MFU_954 ($p=0.01$). Results from two other studies (MFU_955 and MFU_108) are pending.

Table 2. Statistical analysis of animal observation data for treatment with vehicle, Doxorubicin and MFU_954 in mouse xenograft study (colon cancer cell Colo205). N=10 mice per study arm, see above for experimental protocol. RTV – Relative Tumour volume. Time to RTV2, is Time taken to double relative tumour volume

Group	Time to RTV2 (days)	Growth delay (days)	Significance	Maximum % weight loss
Solvent control (20%DMSO/Oil, IP, day 0-4, 7-11, 14-18, & 21-25)	5.3	-	-	1 (day 1)
Doxorubicin, 10 mg/Kg, IV, day 0	8.5	3.2	p<0.05	5 (day 3)
MFU_954, 6 mg/Kg, IP, d0-4, 7-11, 14-18, & 21-25	10.1	4.8	p=0.01	0

The overall flow of compounds through the Marine Fungi screening platform is shown in Figure 17.

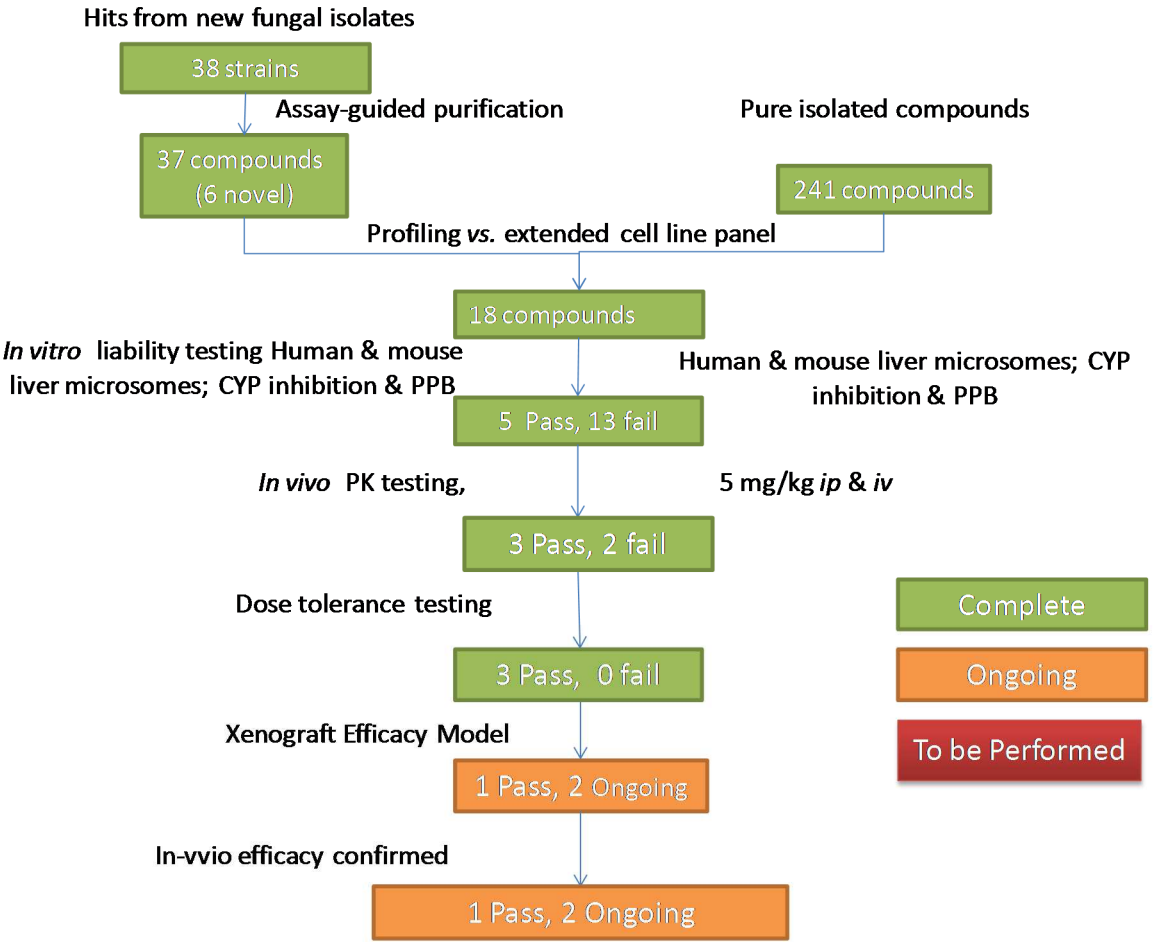


Figure 17. MARINE FUNGI pipeline of compounds. A total of 5 compounds entered in-vivo studies (PK testing) with 3 reaching Xenograft studies.

Summary on work on anti-cancer activities

The team was successful in identifying a compound (MFU_954) of marine fungal origin which has efficacy in *in-vivo* models of colon cancer. The working hypothesis from the COMPARE and affinity studies is that MFU-954 acts *via* a mitochondria related targeting mechanism, most likely by modulating the function of VDAC1 and /or Prohibitin1/2. Several compounds have entered drug discovery pipelines which target mitochondria (e.g. alpha-tocopheryl Succinate and menadione). The identification within the MARINEFUNGI project of compounds with a potential mitochondrial targeting mechanism might be of significant interest to pharmaceutical partners. Such mitochondrial mechanisms may provide an alternative to existing targeting mechanisms such as alkylating agents, anti-metabolites, anti-microtubule agents etc., which are all associated with significant side-effect profiles and limited efficacy in many cases.

Robust and sustainable process development (WP8)

The objective of WP8 was to develop processes which would ensure that production of anti-cancer therapeutic compounds produced by marine fungi would be scalable to pilot and industrial scale (Figure 18). This involved developing strategies to produce promising compounds at reasonable titers in scalable stirred tank reactors (STRs) and scaling up purification methods. Based on the results generated a process concept was described. It is important to consider strategies for scaling up compound production because during product discovery, these compounds are usually identified as extracts from strains growing in non-agitated or poorly agitated conditions, which are convenient for large scale product discovery, but not well suited for large scale production. The mechanisms regulating production are not known at early stages of product discovery, making the transition to STRs more challenging, and lack of access to sufficient quantities of some compounds may limit their progress through a drug development pipeline. The process is made more challenging by the slow growth of some marine fungi.

Figure 18. A major aim of MARINE FUNGI was to develop a process concept providing the technological basis for a sustainable use of marine microbial products in oncology.

WP8 focused on production of compounds from four genetically distinct fungi, LF580 (*S. brevicaulis*), KF970, KF525 (*Calcarisporium* sp.) and MF458 (*Tolypocladium geodes*), plus some mutants of LF580 generated by either classical or genetic engineering techniques in WP6. Strategies for STR production of the relevant compounds were developed for each of these strains, in collaboration with WP4, with production of three compounds being increased more than 8-fold in the process Figure 19 and the time scale being reduced for products from all four fungi.

Products from LF580 were readily produced in STR, but process optimisation led to large increases in total production and reduced the scale needed to produce sufficient compound for all other studies carried out in the project (WPs 5 and 7). Several hundreds of milligrams of bioactive compounds were produced and purified. LF580 could be grown to high biomass concentrations (> 30 g l-1) and grew at relatively high specific growth rates (> 0.2 h-1, depending on the medium), making it a convenient fungus for process development. Standard conditions were developed for comparing various strains. These helped to identify useful regulatory mutants (transformants), which had been developed in WP6.

MF458 also grew well in bioreactors. MF458 produced a range of metabolites and it was possible to improve both the titre and time of production of the bioactive compounds of interest. pH regulation in particular, provided a means of reducing the time of maximal production.

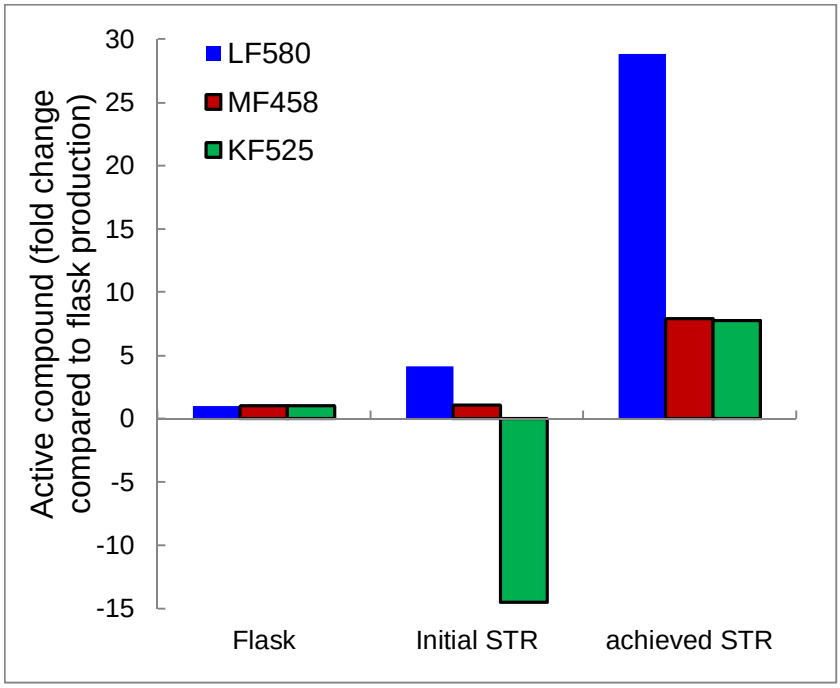


Figure 19. Production of bioactive compounds in LF580, MF458 and KF525 in STRs, relative to the production observed in flasks during the process of biodiscovery. The production level achieved in initial STRs is shown, as well as the levels eventually achieved within the project.

KF525 grew well in STRs, but did not produce bioactive compounds under the same conditions which had been used in flasks. Thus it initially appeared that KF525 could not produce these metabolites in STRs. The carbon source and pH of the environment were found to be key factors which enabled production in bioreactors. Surprisingly, given the low initial production, agitation was less important. KF525 is a slow growing fungus, however, and large scale production of its metabolites would benefit from further understanding of the mechanisms regulating production or transfer of the metabolic pathways to a faster growing host strain.

KF970 grew faster in STRs than in flasks and also produced the metabolites of interest. The faster growth allowed earlier harvesting of the compound(s).

More than 100 mg of some compounds were purified (>99.5% purity) using scalable purification methods. Sufficient material was available to further explore the bioactivities of some compounds using biotransformation to modify them.

The process concept takes into account the improved bio- and downstream processes to describe a concept for production of sufficient product for pre-clinical toxicology testing. Further scaling of the production process would not proceed for products which fail at this step, but it would be beneficial to understand the effort and costs involved in generating sufficient product at this point before proceeding. Further optimisation of the production process would be expected for products which successfully pass the toxicology tests. Figure 20 illustrates how the largest costs are associated with the downstream processing and that solvent recycling can significantly reduce the costs in the process.

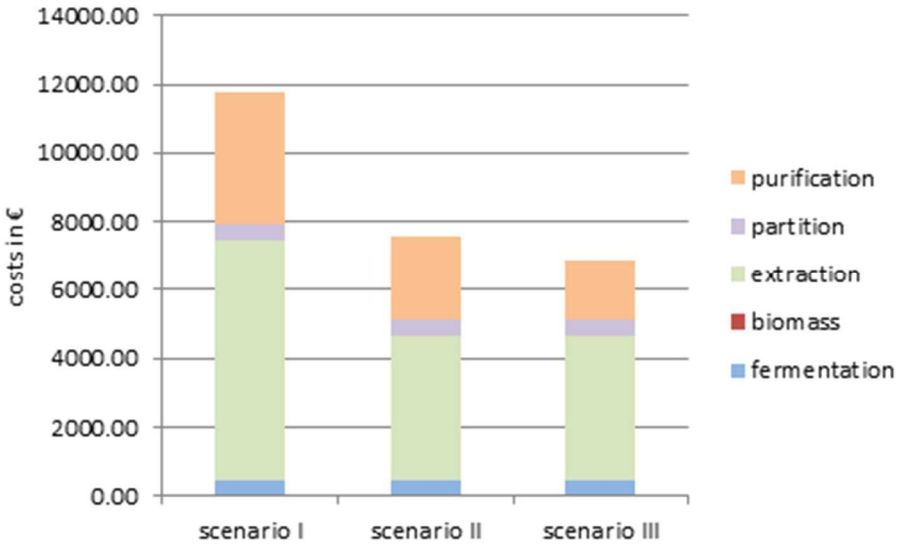


Figure 20. Estimated costs for the production of 50 g metabolite. Scenario I: no recycling of reactants; Scenario II: recycling of acetone in the extraction step; Scenario III: recycling of both acetone for extraction and acetonitrile in the purification step

Conclusions and recommendations

Metabolite production in bioreactors was successful with LF580, KF525, KF970 and MF458 and purification steps were scalable, providing adequate supplies of compounds to WP5 and WP7. Work with these strains highlighted the following recommendations for achieving bioreactor-based production of specific (potential) anti-cancer compounds from marine fungi:

i) Establish reproducible conditions in shaken flasks for production of the compound(s) of interest and a reproducible time profile for product formation. Problems associated with transition from static to shaken flask culture provide important background to understanding the regulation of product synthesis.

ii) Understand the nature of the compound of interest, and exploit conditions which have been useful in production of compounds which may utilise related biosynthetic routes. Pay particular attention to possible consequences of nitrogen, oxygen and carbon limitations and carbon catabolite repression, especially from glucose.

iii) Establish reproducible pre-cultures, preferably with filamentous mycelium to obtain homogeneous, reproducible pre-cultures. Various strategies have been developed to generate filamentous mycelia in shaken flasks. Take note of whether filaments or pellets are more productive, and consider the consequences of culture heterogeneity (e.g. because of pellet formation) on reproducible product profiles.

iv) Develop high biomass cultivations for products which remain in the biomass so that the total volume of the process can be reduced. Fed-batch processes may be useful.

It is also important to work with molecular biologists and biochemists to develop a full understanding of the biosynthetic pathway and if necessary re-engineer the regulation, generating strains which are more suitable for large scale production processes.
The process concept is available for marketing and provides a useful instrument for estimating costs associated with taking a compound to the next steps of drug testing.

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

Forming a sustaining network. MARINE FUNGI focused on a "sustainable use of the oceans and seas", as demanded by the EU Integrated Maritime Policy statement, by exploiting marine fungi, which can be sampled from the marine environment, but then cultivated under defined conditions without disturbance of or harm to the marine environment. This is accomplished by the formation of a strongly interacting research network comprising the scientific and technological actors, including 3 SMEs and 2 ICPC partners, necessary to move along the added-value chain from the marine habitat to the preclinical candidate and process concept.

Proper and fair sharing of intellectual property rights. Intellectual property rights in MARINE FUNGI are managed in accordance with the regulations in the EU contract. Specifically detailed arrangements related to IPR were made in the MARINE FUNGI Consortium Agreement. The protection of knowledge resulting from the project is controlled by a specific Intellectual Property Committee constituted from project partners ensuring a good balance between academic and industrial partners. There will be a fair and equitable sharing of all benefits of results, patents and licenses arising from living and genetic resources.

Training of the next generation of marine biotechnologists. The consortium leverages its multidisciplinary, interdisciplinary and intersectoral network to invest in dedicated training of young people and to encourage lifelong learning of experienced researchers engaged to disseminate their results and their expertise. Soft skill trainings, student exchange and summer schools are organized on various aspects of drug discovery, commercialization requirements needed for small molecule drug discovery, the role of open innovation and ethical and IPR issues, as well as on entrepreneurship. A training concept for marine biotechnology was compiled, a training workshop for soft skills was organised and the total of training activities was recorded and is provided in a separate document.

Novel molecules to treat cancer. The lead users of MARINE FUNGI will be pharmaceutical companies taking characterised anti-cancer substances derived from marine fungi into further drug development. The uptake and final use of the future products by the pharmaceutical industry has been promoted via networking meeting, presentations on partnering events and scientific dissemination. To raise the interest of industrial partners outside of the project for the development of new drugs to meet patient needs, a good network between the SMEs and the major pharmaceutical industries is required to generate partnering opportunities. Towards this end, the consortium presented its portfolio at partnering events and discussed the respective demands of the market with pharmaceutical companies and other players in drug development. For the initiation of discussions with Pharma Companies, patent applications are prepared for filing.

Sustain marine Biotechnology in Europe. The project strengthened the competitiveness of European marine biotechnology by providing scientific and technical grounds for the exploitation of marine fungi. The valorisation will help to ensure Europe's involvement in the quickly increasing world market of biotech products. The success of MARINE FUNGI will help to sustain and develop the business of the involved SMEs. It may also contribute to the employment opportunities in the Blue Biotech and Pharmaceutical sector in Europe in the future.

Visibility of European Blue biotechnology. Additionally, MARINE FUNGI aimed to raise the visibility of marine biotechnology as a key technology in Europe: MARINE FUNGI launched a public website offering non-confidential project information, and a weekly blog informs about news. To increase the impact of result dissemination, the partners participated and will continue to present MARINE FUNGI and its outcomes at established conferences, trade fairs and specific

project meetings arranged for e.g. pharmaceutical companies. During the first phases of the project dissemination of information was limited to the distribution of controlled publishable abstracts, in order to respect confidentiality for patent applications. Recently, the first of a series of articles were published in scientific journals. A number of follow up publications in scientific and in trade magazines, including specifications, results and achievements, is planned to publicise the discoveries of the programme and the potential technology transfer to users outside the consortium. A special emphasis was placed on the realization of the "End User meeting" at the end of the project life time, at which it was anticipated to make leads for the valorisation of the results of MARINE FUNGI. It was planned and organized by a committee formed of the following consortium partners. To reach the major industrial and academic actors in the field of anti-cancer drug development, the ELRIG –Research and Innovation 2014 conference, a well established meeting series, was chosen to serve as platform for technology transfer. Due to the involvement of the MARINE FUNGI consortium in the organization of the meeting, it specifically focused on providing insights, how cutting-edge technologies are utilised in the study of cancer, which as well was reflected in the conference subtitle: "Technology's Impact Into Cancer Research and Therapeutics". The MARINE FUNGI consortium has been active in 3 areas at the "Research and Innovation 2014" Conference: (i) Conferences program: The MARINE FUNGI Consortium was working in partnership with ELRIG to provide the Natural Product Screening and Development track. (ii) Poster session: The MARINE FUNGI consortium contributed with 10 posters to the poster session of "ELRIG - Research and Innovation 2014". (iii) Networking. the MARINE FUNGI consortium was represented with a booth within the exhibition area. The goal was to attract potential End Users, who wish to receive further information, beyond to what was given in the presentations and to exchange business cards for follow-up of the leads subsequent to the conference. All dissemination activities beyond the consortium and the project's life time are scheduled in a "Dissemination, exploitation, communication and knowledge transfer plan".

Target European citizens. Beside valorisation partners, a target group for MARINE FUNGI are European citizens. MARINE FUNGI aimed to increase the visibility of marine biotechnology as a key technology in Europe. Therefore, MARINE FUNGI launched a public website offering non-confidential project information. A weekly blog informed about news. Both will be continued as well as the presence in social media.

Further information about the project can be obtained at http://www.marinefungi.eu/.

The MARINE FUNGI consortium

Eleven institutions from seven different countries have joined forces building a new strongly interacting research network comprising scientific and technological partners, including two SMEs and two partners from ICP countries.

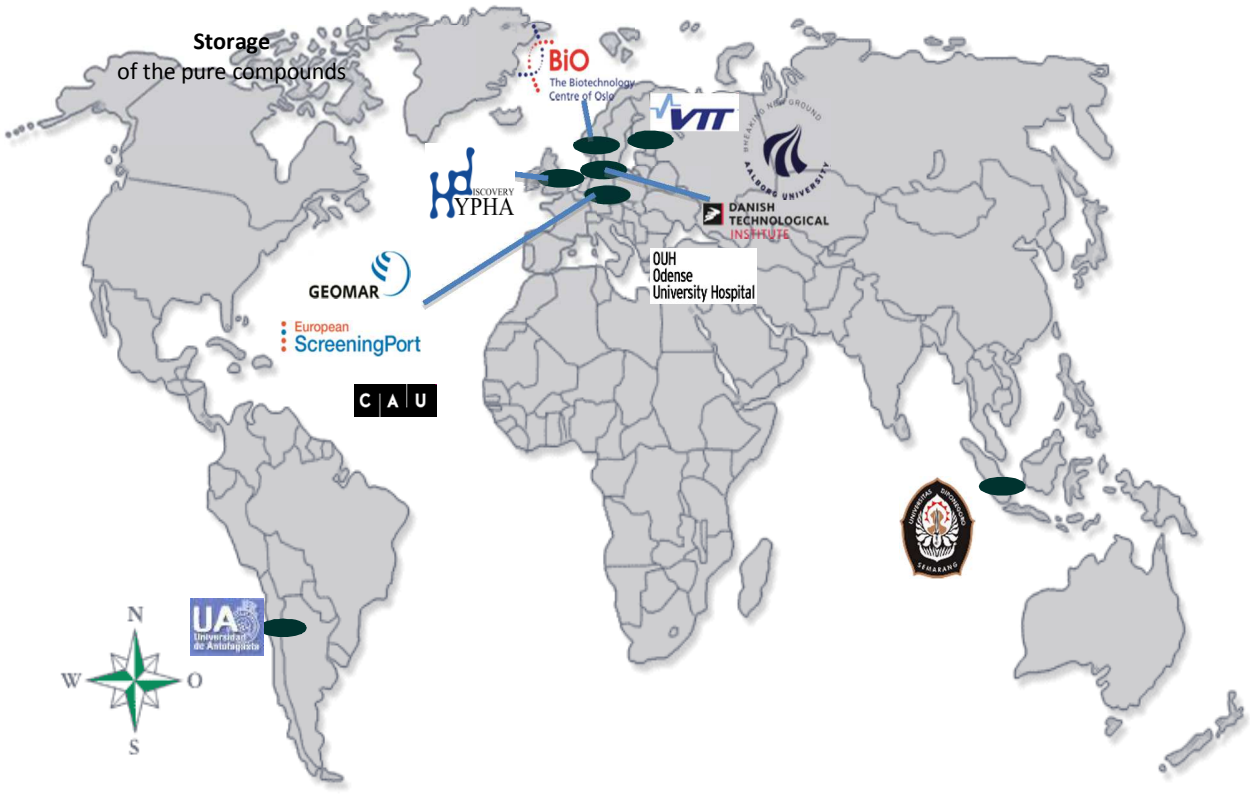
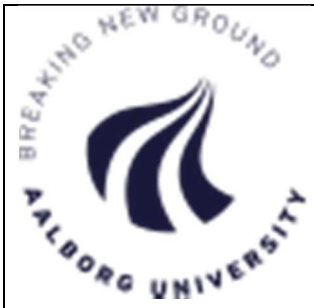


Table 3**Fehler! Verweisquelle konnte nicht gefunden werden.** summarises the affiliations of the partners and their specific role in the project. For each partner, the webadress and a specific scientific contact was provided.

Table 3 MARINE FUNGI partner.

Partner	Description
GEOMAR	Center for Marine Natural Product Research (KiWiZ) at GEOMAR: focused on identification of new natural products from marine microorganism and evaluation for applications in medicine, cosmetics, agriculture and food products. Role in Project: Coordinator of MARINE FUNGI, physiology and systematic of marine fungi, management of strain collections /strain delivery and improvement (WP4-lead, WP8), natural compound chemistry WP5, process optimization (WP8) PI contact: Prof. Johannes Imhoff , Am Kiel-Kanal 44, 24106 Kiel, Germany Tel. +49 (0)431-600 4450, jimhoff@geomar.de, Dr. Antje Labes, Tel. . +49 (0)431-600 4439, alabes@geomar.de, www.kiwiz.org, www.geomar.de
HYPHA DISCOVERY	Hypha Discovery Ltd: a UK-based microbial biotechnology SME helping partners in pharmaceutical and agrochemical R&D worldwide succeed through the production of mammalian and microbial metabolites, and through bioactive compound discovery from its MycoDiverse™ screening libraries. Role in Project: strain cultivation and stimulation (WP4), structural elucidation, chemical substance identification and purification (WP5), optimisation of purification processes (WP8), controlling the preliminary panel and preparing compound selection for detailed characterisation of anti-cancer activities (WP7-lead). IPP and business aspects (WP9) PI contact: Dr. Liam Evans, The Russell Building No.1, Brunel Science Park, Uxbridge, Middlesex UB8 3PQ, UK, Tel. +44 1895 814585, liam.evans@hyphadiscovery.co.uk, www.hyphadiscovery.co.uk
VTT	VTT Technical Research Centre of Finland (VTT): has a strong focus on the use of fungi in the development of biorefinery concepts, including biodiesel, bioethanol, chemicals and materials from renewable resources. Role in Project: VTT has contributed to comparative genomics (WP3) and in cultivation optimization (WP4). VTT provided one of the premier pilot facilities of northern Europe, for culture optimisation and scaling-up operations (WP8-leader). **PI contact:** Dr. Marilyn Wiebe, VTT Technical Research Centre of Finland, FI-02044 VTT, Tel. +358 20 722 5139, marilyn.wiebe@vtt.fi, www.vtt.fi
C A U	Christian-Albrechts-University of Kiel (CAU): department of Genetics and Molecular Biology in Botany focuses on fungal molecular genetics especially elucidation of the biological function of secondary metabolites Role in Project: fungal genome sequencing, annotation and their evaluation (WP3-lead), molecular information has been used in WP6 for molecular strain optimisation. **PI contact:** Prof. Frank Kempken, Christian-Albrechts-Platz 4, 24118 Kiel, Germany, Tel. +49 (0) 431 880 4274, fkempken@bot.uni-kiel.de, www.uni-kiel.de
DANISH TECHNOLOGICAL INSTITUTE	Danish Technological Institute (DTI): is a not-for-profit institute approved by the Danish Ministry of Science, Technology, and Innovation as a technological services provider. The Life Science Division, DTI offers professional and dedicated services to the Danish and international Life Science industry. Role in Project: proteomics methods for the molecular characterization of biological systems(WP3, 6), mode of action studies, cell signalling studies, and

	drug target identification (WP5-lead, WP7) **PI contact:** Dr. Jan Lorenzen, Kongsvang Allé 29, 8000 Aarhus C, Denmark, Tel. +45 72 20 20 00, jnl@teknologisk.dk, www.dti.dk
	University of Diponegro (UD): focus of thenCenter for Tropical Coastal and Marine Studies (CTCMS) is on bioprospecting of microbial symbionts of marine invertebrates/plants as source of marine natural products. Role in Project: UD identified biosynthetic genes in new fungal isolates (WP3), provided access to tropical coral reefs and has used its experience with the respective macrobes for the isolation of marine fungi (WP4). PI contact: Prof. Ocky Karna Radjasa, 50275 Semarang, Central Java, Indonesia Tel. +62 24 7460047, ocky_radjasa@undip.ac.id , www.undip.ac.id
	Universidad de Antofagasta (UA): The Bioinnovation Centre at Universidad de Antofagasta focuses on the study of microbial biodiversity in aquatic coastal marine system in the north of Chile and saline waters of the Altiplano in the Andes and the Atacama Desert. Role in Project: UA identified biosynthetic genes in new fungal isolates (WP3), provided access to Chilean macroalgae and has used its experience with the respective macrobes for the isolation of marine fungi (WP4) **PI contact:** Dr. Cristina Dorador Ortiz, Angamos 601, 1240000 Antofagasta, Chile, Tel. +56 55 657701, cdorador@uantof.cl, www.uantof.cl
	European ScreeningPort GmbH (ESP): SME provides scalable Drug Discovery Services to the academic, biomedical research community. ESP operates its own Hit Discovery centre offering assay development, screening, hit confirmation, selectivity, liability/ efficacy profiling, compound handling functions and biomarker discovery studies for clinical and pre-clinical research projects. It is supported by a comprehensive bioinformatics and IT infrastructure to aid rapid decision making. Role in Project: screening of fractions and metabolite profiles (WP7). ESP has direct access to pharmaceutical-scale medicinal chemistry and bioinformatics infrastructure and pharmaceutical stakeholders (WP9-lead). **PI contact:** Dr. Philip Gribbon, Schnackenburgallee 114, 22525 Hamburg, Germany, philip.gribbon@screeningport.com, Dr. Mira Grättinger, Tel. +49 (0)40 3037640, mira.graettinger@screeningport.com, www.screeningport.com
	University of Oslo (UIO), Biotechnology Centre: UiO has a very strong life sciences sector with excellence in molecular mechanisms of disease (cancer, infection and inflammation, neurology), drug target discovery, experience in translating discoveries into clinical practise, national coordinating role in translational medicine through a Nordic EMBL partnership Role in Project: UIO performed the phosphoflow analysis (WP5). The screening facilities of UiO provide a basis for the screening of fractions and metabolite profiles. **PI contact:** Prof. Kjetil Tasken, Biotechnology Centre Oslo, Gaustadalleen 21, 0349 Oslo, Norway, kjetil.tasken@biotek.uio.no, Dr. Johannes Landskron, Tel. +47 228 405 09 0047, johannes.landskron@biotek.uio.no, www.uio.no

	Aalborg University: The research areas of the Fungal Biotechnology group at Aalborg University evolve mainly around fungal secondary metabolites with a focus on linking secondary metabolites to their responsible gene clusters and discovering novel secondary metabolites through genetic engineering. Role in Project: evaluation of biosynthetic pathways in the fungal genomes (WP3), forecast of compounds from genomic (WP5), whole methodological set for strain improvement using classical and molecular methods (WP6; leader). **PI contact:** Dr. Jens Laurids Sørensen, Department of Biotechnology and Chemistry, Sohngaardsholmsvej 49, 9000 Aalborg, Denmark. Tel. +45 99408 524, jls@bio.aau.dk, www.en.aau.dk
OUH Odense University Hospital	Odense University Hospital (OUH): The Department for Clinical Biochemistry and Pharmacology at OUH focuses on various aspects of the development, use of biochemical assays for clinical research/ diagnostic purposes incl. drug metabolism. Role in Project: enhancement of compound characterisation, performing CYP-assays in the frame of ADME studies (WP7), development and use of proteomics methods for the characterization of novel mechanisms for fungal production of metabolites with anti-cancer activities. **PI contact:** Dr. Hans Christian Beck, Sønderboulevard 29, DK-5000 Odense C, Denmark, Tel. +45 29 647470, hans.christian.beck@rsyd.dk, www.ouh.dk
ProbioDrug	Terminated participation 31 Dec. 2012.