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## **High-Throughput Screening of marine resources**

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**Abbreviations:**

BRET: bioluminescence resonance energy transfer, CDKs: cyclin-dependent kinases, CK1: casein kinase 1, CytP450: cytochrome P450, Da: daltons, DMSO: dimethyl sulfoxide, EC<sub>50</sub>: half maximal effective concentration, ELISA: enzyme-linked immunosorbent assay, FRET: fluorescence resonance energy transfer, GPCR: G-protein coupled receptor, GSK3 $\beta$ : glycogen synthase kinase 3 $\beta$ , HCS: high-content screening, HMD: Hymenialdisine, HTRF: homogeneous time resolved fluorescence, HTS: high-throughput screening, IC<sub>50</sub>: half maximal inhibitory concentration, NMR: nuclear magnetic resonance, PK: pharmacokinetics, PKC: protein kinase C, PKIs: inhibitors of protein kinase, PPIs: protein-protein interactions, RNAi: ribonucleic acid interference, S/B: signal-to-background, SAR: structure activity relationship, SPA: scintillation proximity assay, TR-FRET: time resolved-FRET, SW: signal window, Y2H: yeast two-hybrid.

## 1. ***Introduction***

Oceans cover 70% of our planet and contain the greatest diversity of living organisms on earth. Considering the critical importance of natural products derived from living organisms in the discovery and development of bioactive agents of medical interest, the marine environment represents a vast resource to discover novel chemical structures with novel modes of action. For instance, 47% of drugs used in anticancer treatments are natural products and/or derivatives (1). In recent years, a growing number of new chemical entities from marine organisms with potent pharmacological properties have been described (2-4) (Figure 1). Noteworthy, bryostatin-like compounds are under current investigations for anti-cancer therapies (for details see part 4.4). It is worth mentioning a growing interest for the development of marine natural products and chemical derivatives with applications in agrochemical and cosmetics industries bearing great potential of economical impact.

The stagnation of the market in terms of newly approved drugs along with an increase in research and development expenses (so called “big pharma crisis”), creates an urgent need to speed-up the discovery of new natural chemical scaffolds (5) (Figure 2). To be effective, the exploration of marine natural products should be developed in parallel to high-rate analysis of bioactivity.

One of drug discovery process critical step is the determination of a **therapeutic target**, which can be defined as a cellular component (protein, DNA) or a “biological pathway” clearly involved in a specific pathology. Importantly, the therapeutic target must be **“druggable”**, compatible with the treatment of patients (e.g. small chemical compounds, antibodies.) in order to inhibit/activate its cellular function. According to Hugues *et al.*, a “druggable” target is accessible to putative drug molecules and elicits a biological response which may be measured both *in vitro* and *in vivo* (6).

**High-throughput screening (HTS)** consists in testing compounds in high numbers in order to determine if they can modulate a given molecular pathway. HTS relies on two major classes of assays: *in vitro biochemical assays* which are developed to select compounds with activities on purified molecular targets (e.g. disease-related enzymes as protein kinases, ligand-receptor interactions); and *ex vivo cell-based assays* developed in mammalian cells, yeast or bacteria engineered to overexpress the target protein (GPCRs, ion-channels and others membrane receptors...) or express a reporter gene (for review see (7)). **High-Content Screening (HCS)**, in comparison with HTS, is based on the analysis of specific cellular phenotypes through automated image capture and analysis by heavy computing systems (8; 9). This chapter will focus on HTS applied to the identification and selection of marine natural products and derivatives with biological activities of medical interest.

## ***2. High-Throughput Screening and Drug Development***

HTS is based on the development of automated and reproducible assays sufficiently robust to be miniaturized and fit different microtiter plate formats (96 to 3456 wells). The HTS approach is fully integrated in a drug development strategy (Figure 3). The central role of HTS and notably in basic research is to allow pharmaceutical companies to save time and money. The purpose of HTS assay is to select the optimal chemical structures that will be analyzed during pre- and clinical studies.

### ***2.1. Screening assay development and validation***

As mentioned above and as indicated on Figure 3, identification of therapeutic targets is an essential step prior to screening development. Several targets can be identified and validated for a single disease (10). Tools available to identify therapeutic targets include mRNA/protein levels analyses, genetic associations, bioinformatics (data mining) (6) or RNAi technology (11). The next step is to determine whether the molecular pathway requires

inhibition or activation in order to produce significant beneficial effects. Strategies for *in vivo* and *ex vivo* drug target validation include notably gene knock-out/in, dominant negative mutant or RNAi (6).

Biochemical assays are well adapted to assess effects on enzymatic reactions or binding interactions, whereas cell-based assays are preferred for molecular pathways, multiple targets or cell membrane compartments analysis. Current available methods are **(i) Colorimetric** (12) (e.g. Enzyme-Linked ImmunoSorbent Assay (ELISA) ((13)); **(ii) Fluorescent**, (e.g Fluorescence Resonance Energy Transfer, (FRET) (14; 15), Dissociation-Enhanced Lanthanide Fluorescent Immunoassay, (DELFI) (16)), fluorescence polarization (17), Time Resolved-FRET (TR-FRET) or Homogeneous-TRF, (HTRF) (18; 19), fluorogenic substrates (20), calcium flux (21), membrane translocation (22)); **(iii) Luminescent** (e.g. Bioluminescence Resonance Energy Transfer, BRET (23), bioluminescent substrates (24) or Luciferase reporter gene (25)); **(iv) based on the use of radioactive elements** (e.g Scintillation Proximity Assay, SPA (26)).

Screening assays are considered as **heterogeneous** (e.g. ELISA) or **homogenous** (e.g. HTRF). Heterogeneous assays (multistep) require steps that go beyond simple reagent additions, incubations, and reading (e.g. filtration, centrifugation, and plate washing steps), whereas homogeneous assays require only additions and incubations followed by reading. Multistep assays prevent the tested compounds to interfere with the readout and usually produce a higher signal-to-background ratio (S/B ratio) whereas homogeneous assays are more amenable to high-throughput analysis. Sensitivity, cost, automation, speed, robustness, reliability are important criterions to be taken into consideration. It is to be noted that downscaling of assays, reducing the volumes of material used, can significantly reduce total cost of HTS screens (Table 1).

## 2.2. Statistical tools for quality assessment of HTS assays

Generally, screening evaluation is based on the percentage of inhibition/activation obtained by the tested molecule on a specific target. Experimental data analysis requires their normalization in order to remove systematic plate-to-plate variation and standardize measurements across plates. Quality indicators such as reproducibility and accuracy are essential to assess the suitability of the assay for HTS. Many factors can affect the reproducibility of microtiter plate assays, such as “edge-effect” in cell-based assays. A widely used way to determine the suitability of an assay, prior to launch a full-scale high-throughput screen, is the calculation of the  $Z'$ -factor coefficient. The  $Z'$ -factor is a statistical parameter calculated as follow (originally described in (27)):

$$\mathbf{Z'\text{-factor}} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

$\mu$  and  $\sigma$  are the means and standard deviations of the positive ( $p$ ) and negative ( $n$ ) sets of data obtained with dedicated controls, respectively. A proper  $Z'$ -factor should be higher than 0.5 as described in Table 2. In the context of an enzyme inhibition-based assay, the positive control is defined as the signal obtained when the enzyme is incubated with its substrate and a known inhibitor, while the negative control is defined as the enzyme incubated with its substrate in the absence of inhibitor. Since most chemical compounds are dissolved in dimethyl sulfoxide (DMSO), controls should contain the same amount of DMSO, and its potential effect evaluated. The use of more than 1% DMSO (final concentration) is not recommended for cellular-based assay.  $Z'$ -factor is totally dependent on the HTS-assay conditions. Therefore, a new  $Z'$ -factor must be determined whenever changes are implemented in the assay (including reagents variability). Statistical tools are available to achieve quality assessment including percentage of control, median absolute deviation (MAD), signal-to-background ratio (S/B) and signal window (SW) (for review see (28) and (29)).

### **2.3. Choice of screening strategy**

In a typical HTS assay, a **primary screen** is performed at a single dose for all compounds. Results are then validated in a second round of screening. Other strategies can be used, namely **molecular pool** and **replicate screening**. The first one consists in testing a pool of compounds in one reaction. The advantage of this technique is to increase the screening output but it requires sorting steps for every positive reaction in order to extract and identify the interesting molecule. This strategy is somehow reminiscent of natural extracts screening where several rounds of fractionation/screening are necessary before approaching the positive compound. Replicate screening strategy (number of independent experiments,  $n>3$ ) allows direct elimination of false positives, the probability of reproducing experimental error decreasing with the number of replicates (see below for false positive definition). Inevitably, the chosen strategy will affect directly the screening cost and speed and should be carefully evaluated.

Whatever the strategy chosen, it is necessary to determine an activity threshold to identify “hits”. The threshold can be set using various methods such as percent inhibition cut-off or Bayesian methods (described in (29)).

### **2.4. Data analysis: from hits to leads**

#### **2.4.1. Hits**

The products tested during screening and having a positive action on the therapeutic target are called “**Hits**” (Figure 3). Valuable hits are potent and specific; they have a strong effect on the target of interest and minor off-target effects. However, even if the screening is based on a reliable technique and all quality controls validate the assay, the possibility of having identified a **false positive or negative compound** remains. False positive can be identified by preliminary analysis of the compounds to be screened. For instance, it is well



known that some dyes can interfere with activity readout or that alkylating agents can alter the target irreversibly and have no specific inhibitory activity. Reproducible hits without activity on the therapeutic target can be safely regarded as false positives and eliminated. When required (e.g. targeted pathology), cell mortality can be determined in parallel using cell-based assay to discard cytotoxic compounds.

Finally, remaining false positive compounds can be identified in subsequent screening rounds performing a dose effect (serial dilutions of the compound) where no effect will be observed whereas, for a “confirmed” hit, an **IC<sub>50</sub>** (concentration needed to inhibit half of the maximum biological response, Figure 4) or **EC<sub>50</sub>** (compound concentration where 50% of its maximal effect is observed, commonly used for agonist/stimulator assays) value can be determined. It is important to indicate here that various factors may influence **IC<sub>50</sub>** value. For instance, there is an interdependency between the **IC<sub>50</sub>** value and adenosine tri-phosphate (ATP) concentration for ATP-dependent enzymes such as kinases. **IC<sub>50</sub>** values can be used to compare antagonists’ efficiency and the most potent hits will be identified and selected. However, it is important to select molecules with chemical structures representative of each family identified in order to keep the **highest degree of chemical diversity** during the screening process.

To continue hit validation, selected structures can be analyzed using various other bioactivity assays to evaluate the relative **selectivity** of the molecules to their primary targets. In the case of protein kinases inhibitors (PKIs), large panels of kinases (>89% of the whole kinome), can be tested to obtain a comprehensive view of the molecule’s selectivity (KINOMEscan® program, DISCOVEREx, Fremont, USA).

A chemical scaffold has to be analyzed according to the **Lipinski’s Rules or Rule of 5 (RO5)**, all numbers are multiples of five) before further studying its mechanism of action.

These rules are used to evaluate if a chemical compound has properties that would make it a likely orally active drug in humans. They were formulated by Christopher A. Lipinski in 1997 (30) and are based on the observation that most medication drugs are relatively small and lipophilic molecules. These characteristics are associated with 90% of orally active drugs that have achieved Phase II clinical status and states that, in general, an orally active drug has no more than one violation of the following criteria: (i) molecular weight < **500** daltons (Da); (ii) hydrogen bond donor groups < **5** (nitrogen or oxygen atoms with one or more hydrogen atoms); (iii) hydrogen bond acceptor groups < **10** (nitrogen or oxygen atoms); (iv)  $\log P$  < **5** (octanol-water partition coefficient).

However, these rules are not always applicable to every drug. Indeed, Abbott/Genentech's oral Phase II candidate Navitoclax<sup>®</sup>, violates three of Lipinski's Rules for oral-drug-likeness (it has a molecular mass of 975 Da, a  $\log P$  of 12, and 11 hydrogen-bond acceptors) (31). Therefore, one should keep an open mind when screening complex and highly diverse marine natural chemical scaffolds (32).

The next step in hit validation consists to evaluate its molecular properties such as solubility and stability and, using a panel of *in vitro* assays, testing intestinal and hemato-encephalic permeability, metabolic stability, activity on cytochrome P450 (CytP450), or cell permeability,... (6).

#### **2.4.2. Leads**

Following the screening campaign, evaluation of selectivity and pharmacokinetics properties of the selected hits, the more potent molecules, will reach “**leads**” status (Figure 3). Medicinal chemistry is then applied in order to obtain derivative structures exhibiting optimized bioactivities. Thus, the lead structure is a starting point for improvement by chemical modification. In this perspective, dose response curves are generated using primary

screen assays for each pharmaco-modulation to determine activity through  $IC_{50}$  or  $EC_{50}$  evaluation to achieve Structure Activity Relationship (SAR: relationship between the chemical or tridimensional structure of a molecule and its bioactivity). Additional screens can be done to measure selectivity and pharmacokinetics properties. Overall the **drug candidate** will be a compromise between all these parameters. It will modulate the target efficiently and specifically, have good metabolic stability and bioavailability, be soluble and slightly lipophilic (33).

At this stage, further experiments might help to characterize the leads and especially their mechanism of action. Different affinity-based analysis (34) or other “omics”-based methods can be used to carefully and comprehensively determine the cellular targets of the new chemical scaffold. Unexpected cellular targets can explain potential side effects that could appear during preclinical or clinical phases.

## **2.5. From HTS assay to market: the drug development process**

After selection, drug candidates are submitted for clinical trials (Figure 3). The first step of these trials is **pre-clinical stage**, which consists in studying on animals various parameters such as genotoxicity, drug absorption, metabolism and metabolites elimination, as well as toxicity of the drug and its metabolites (ADME-tox). The clinical stage is divided in four phases: (i) **Phase I** is carried out on a small number of healthy human volunteers. During this phase, metabolic and pharmacological effects of drugs are determined along with associated side effect. The purpose of phase I studies is mainly to determine safety profiles; (ii) **Phase II** consists in further evaluation of safety together with efficiency in patients, and in the selection of dose regimen; (iii) **Phase III** is intended to gather additional information about efficiency and safety to evaluate the overall benefit-risk relationship of the drug; (iv)

**Phase IV** is long term monitoring for adverse reactions reported by pharmacists and doctors (35).

Overall, drug development is a very expensive and lengthy process. It may cost up to US \$ 1.7 billion and take between 12 and 15 years. The efficiency of drug development from lead compounds selected by screening is estimated at 1/5,000 (36).

#### ***4. Examples of High-Throughput Screening***

Many HTS assays have been developed depending on the therapeutic targets, some of which helped to highlight potential of marine products for pharmaceutical.

##### ***4.1. Chemical libraries: the fuel of HTS***

Along with HTS development, many molecules libraries have been compiled: e.g. ICSN-CNRS' (Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France) library constituted of +4000 compounds from synthetic and natural origin and Greenpharma Natural Compound library constituted of 240 known phytochemicals (Greenpharma SAS, Orléans, France). Development of these libraries is a long and tedious process. Indeed, each compound has to be controlled (mass spectrometry and/or NMR), diluted to specific concentration, archived and stored. Thus, screening requires organization, storage capacity and sometime laboratory information management system (LIMS). In addition, "working" microtiter-plates (also known as "daughter" plates) will be generated to limit freeze-thaw cycles and manipulation of "mother" plates. A LIMS solution will allow inventory tracking, especially if multiple users are involved on the screening platform.

##### ***4.2. Biochemical assay, the example of protein kinases***

A protein kinase is an enzyme that transfers a phosphate group from ATP to a specific substrate; this process is called “phosphorylation”. The 518 human protein kinases play an essential role in regulating cellular growth and survival, differentiation or membrane transport. Deregulations of kinases have been notably described in cancer (since 1978 and the discovery of the first oncogene, Src, a tyrosine kinase), inflammatory or Alzheimer’s disease and related neuronal disorders. This led to a growing interest in protein kinases and the development of specific and potent small molecule inhibitors of kinase activity, historically targeting ATP binding pocket. This class of enzymes has emerged as the most important class of targets in oncology drug discovery. More than 20 compounds are already on the market, such as Imatinib (Gleevec<sup>®</sup>, Glivec<sup>®</sup>) targeting Bcr-Abl (37). The market for kinase inhibitors is expected to reach \$11.6 billion in US and \$40.3 billion globally by 2016 (38) and so far only a slight part of the kinome is targeted by already approved-drugs. Kinase inhibitors have great potential. In light of this, HTS assays have been developed using multiple technologies including radioactivity-based assays, luminescent technology, TR-FRET, fluorescent polarization and scintillation proximity assay (SPA).

Screening campaigns have identified several marine compounds as protein kinase inhibitors (39-42). One of them, **Hymenialdisine** (HMD) (Figure 5), originally identified based on its antiproliferative effects on cultured lymphocytic leukemia cells, was characterized using Scintillation Proximity Assay (SPA), a homogeneous method to measure kinase activity where the amount of ATP left after a kinase reaction is determined. The luminescent signal is proportional to the amount of ATP and inversely proportional to the amount of kinase activity (43).

HMD is a bromopyrrole alkaloid isolated from a variety of marine sponges including *Hymeniacidon aldis* (or *Stylissa massa*), *Axinella verrucosa* (Figure 5), *Acanthella aurantiaca* in the early 1980s. It is a potent inhibitor of several closely related Cyclin Dependent Kinases

(CDKs), such as CDK1/cyclin B ( $IC_{50} = 22$  nM), CDK2/cyclin A ( $IC_{50} = 70$  nM), CDK2/cyclin E ( $IC_{50} = 40$  nM), and CDK5/p25 ( $IC_{50} = 28$  nM), as well as against more distantly related kinases such as GSK3 $\beta$  ( $IC_{50} = 10$  nM), Casein Kinase 1 ( $IC_{50} = 35$  nM) or Mek1 ( $IC_{50} = 6$  nM) (44). Kinetic analysis showed that HMD acts in an ATP-competitive fashion, and the co-crystal structure with CDK2 revealed that HMD occupies the ATP site and shows many of the hydrogen bonding interactions seen in other CDK-inhibitor complexes (45).

### 4.3. *Protein-protein interactions, PPIs*

Interactions between proteins are at the center of the interactomic of any living cell. This makes the **PPI modulation** one of the most challenging tasks in drug discovery. Despite their structural diversity, protein-protein interfaces are in many cases large and compact hydrophobic and relatively flat surfaces and thus can be considered as poorly druggable (46; 47). Nevertheless, only few residues on protein-protein interfaces show a dominant contribution to the binding free energy. These residues are known as “**hot spots**” and their discovery by crystallographic analysis and directed mutagenesis is a major breakthrough in the development of small molecule inhibitors for PPIs (31).

Extreme complementarities of protein-protein interfaces allows development of specific inhibitors and offers some advantages such as: lower drug resistance emergence due to binding of the inhibitor outside of the active site of the targeted proteins compare for example to the ATP-mimetic inhibitors of protein kinases described above) known to decrease side effects observed on human patients.

Despite these advantages, identification of small molecules that modulate protein-protein interactions remains largely untapped by pharmacological research (48). Few natural compounds were nonetheless identified such as microtubule-stabilizing agents (*e.g.*, taxanes,

and the marine compounds discodermolide, eleutherobin or laulimalide) (49) and -destabilizing molecules (*e.g.*, vinca alkaloids, and the marine compounds halichondrin B, dolastatin 10 or hemiasterlin) with important applications notably in cancer treatment (50).

In the last decade, following spectacular development of genomics and proteomics, a growing number of potentially druggable protein-protein interactions have been identified pressing the development of HTS assays in this area. Several technologies have been developed such as: yeast two-hybrid (Y2H), reverse yeast two-hybrid (51), bacteria two-hybrid (52), Mammalian Protein-Protein Interaction Trap (MAPPIT) (53), reverse MAPPIT (54), Bimolecular Fluorescence Complementation (BiFC), FRET- and BRET-based methods (55); (56).

One of the most promising research fields, in the design and development of PPI new therapeutics, is related to the human immunodeficiency virus (HIV) propagation. Indeed, the viral infection relies heavily on protein-protein interactions, especially between virus and host proteins, in almost every step of the virus lifecycle. A Y2H screening assay lead to the discovery of a hexapeptide from the marine worm, *Eunicidae gen. sp.*, showing potent inhibition properties on the 3'-processing activity of HIV-1 integrase through an unusual homoserine residue (57).

Finally, as an emergent and promising concept, study of PPI is still at an early stage and has not yet allowed the discovery of numerous interesting drugs. However, developing HTS in this application field will probably allow identification of potentially interesting new molecules and offer innovative treatment opportunities. In this respect, and due to their high chemical diversity, marine molecules will certainly play a major role.

#### **4.4. Cell-based assay, the example of Bryostatins**

In 1965–1966, **Georges Pettit** and his collaborators began the first systematic study of marine invertebrates and vertebrates as potential sources of new and potentially useful cancer chemotherapeutic drugs using the NCI's P388 *ex vivo* lymphocytic leukemia screening system to measure antineoplastic activity (58). In 1981, fractionation and purification of California *Bugula neritina* extracts led to characterization of a macrocyclic lactone, the Bryostatin-1 (Figure 5) showing cell growth inhibition properties at subnanomolar concentrations (58). Subsequently, additional researches led to the isolation of 20 new bryostatins from *B. neritina* collections from the Gulf of Mexico, Gulf and coast of California and Japan (Gulf of Sagami) (58). Interestingly, a recent study has reported that bryostatins are produced by symbiotic bacteria from *B. neritina* (59). Bryostatin-1 is one of the most abundant and best-studied compounds of this structural family. It was found to target protein kinase C (PKC) through high-affinity binding to the diacylglycerol-binding site of the C-1 regulatory domain of PKC.

While it was originally described to inhibit cell growth (58), Bryostatin 1 has subsequently shown a wide range of properties including anticancer activity, synergetic effect with other anticancer agents, reversal of multidrug resistance, stimulation of the immune system, improvement of learning and memory, neuroprotection after stroke, reduction of amyloid plaque formation, and activity against HIV (60). After more than thirty phase I and II clinical trials (61) in a variety of cancers, alone and in combination with other chemotherapy agents, bryostatin 1 failed to reach phase III due to a lack of efficiency on patients. Bryostatin-1 is still in clinical trial phase II for treatment against Alzheimer's disease (62).

## **5. Conclusions and Perspectives**

HTS assay development has supported an increased rate of discovery of marine bioactive compounds with various medical applications (63). Recent methodological screening developments, allowing the identification of protein-protein interaction modulators or the development of cell-based high-content screening assays will certainly lead to the discovery



of new marine-derived medicines. One of the current challenges in pharmaceutical is to increase the access to new chemical scaffolds in order to widen the diversity of chemical libraries: the key for success in drug discovery. Nature is an amazing medicinal chemist providing surprisingly innovative chemical scaffolds of which marine biodiversity is a vast, yet under-explored, supplier with many great discoveries to come, bearing hope for new potent human therapies.

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 Plate Format	Nb of tested compounds per plates	Nb of plates	Average volume of target added/well (μl)	Assay volume (μl)	Total volume of target added (ml)	Cost (\$)
96	88	2273	10	100	2182	436 400
384	352	568	2	20	436	87 200
1536	1408	142	0,4	4	87	17 400
3456	3168	36	0,08	0,8	17,4	3 480

**Table 1:** Impact of miniaturization on the cost of HTS assays. A library containing 200,000 compounds is evaluated in this example.

Z'-factor	Interpretation
1.0	Ideal. Z'-factors can never exceed 1.
$0.5 > Z' < 1.0$	An excellent assay.
$0 > Z' < 0.5$	A marginal assay.
Under 0	There is too much overlap between the positive and negative controls for the assay to be useful.

**Table 2:** Interpretation of the Z'-factor value: one of the crucial step of the preparatory phase of a HTS campaign (27).

**Figure 1:** Temporal evolution in the number of novel chemical entities characterized from marine organisms (adapted from (3)).

**Figure 2:** The “R&D productivity crisis” in pharmaceutical. *NME*, New Molecular Entities; *FDA*, US Food and Drug Administration (5). The curve in light grey (top left) represents the ratio NME/R&D expenses per year.

**Figure 3:** Drug development: from bench to the market, a long adventure. Drug development is used to define the process of bringing a new drug to the market. The screening is one of the major initial steps.

**Figure 4:** Determination of an  $IC_{50}$  value on a dose-response curve.

**Figure 5:** Images of marine producers of bioactive molecules. Left, *Bugula neritina* (© V. Lamare for the whole organism, © T.H. Ermak for the picture inset) for Bryostatin-1 and right, *Axinella verrucosa* (©, Oceanopolis, Brest, France) for Hymenialdisine. The corresponding chemical structures are depicted on the pictures.