Stennett, H. L., Tiwari, K., Williams, S. E., Curnow, P., & Race, P. R. (2020). The extremophilic pharmacy: drug discovery at the limits of life. In N. M. Lee (Ed.), *Biotechnological Applications of Extremophilic Microorganisms* (pp. 43-72). de Gruyter. https://doi.org/10.1515/9783110424331-002

The extremophilic pharmacy: Drug discovery at the limits of life

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2.1. Introduction

Natural products have long been a cornerstone of human and veterinary medicine (1-3). Currently around 60% of all pharmaceuticals in clinical use are based on, or derived from, natural product scaffolds (3,4). These include a plethora of antibiotics, anti-cancer drugs, immunosuppressants, and perhaps most famously in recent years, the cholesterol-lowering statins. Microbial natural products in particular display a diverse array of biological activities, which are conferred by their often elaborate chemical architectures that can include multiple chiral centres and fused ring systems (4). This biosynthetic capability of microorganisms to produce natural product drug leads has been known for almost a century. However, it is only in the past two decades that an exponential growth in the cloning and characterisation of microbial secondary metabolic pathways has begun to reveal the vast untapped potential of natural products as a source of novel medicinal chemistry. These advances, in tandem with improvements in environmental sampling methods, are now allowing researchers to investigate the potential of extremophilic microorganisms as a source of new natural product drug leads. This approach is predicated on the premise that exposure to 'extreme' environmental conditions drives the evolutionary acquisition of metabolic innovations, which in turn translate into a capacity to biosynthesise unusual natural products with unprecedented bioactivities. In this chapter we summarise recent advances in the isolation, characterisation and clinical development of natural products from extremophiles, and chart the development of these molecules as pharmaceutical lead compounds.

2.2. Microorganisms as a source of pharmaceutical leads

Microorganisms are without equal in their capacity to fuse and tailor simple chemical building blocks into a vast array of complex chemical scaffolds. Because this comes at a metabolic cost to the organism, microbial natural products are selected through evolution for a specific bioactivity; that is, they are already 'pre-screened' for some useful biological function. As a consequence, microbial natural products have dominated the pharmaceutical industry since its inception. It is estimated that tens of millions of soil microorganisms were screened for their capacity to produce pharmaceutical lead compounds during the so-called "golden era" of natural product drug discovery between 1940–1970 (5-7). A significant proportion of the microbial natural products, and derivatives thereof, that are in clinical use today were discovered

as a consequence of the enormous efforts undertaken during this period (6,7). Examples include the antibiotics erythromycin (**1**), streptomycin (**2**), fosfomycin (**3**), and tetracycline (**4**), and the anti-cancer chemotherapeutic agent doxorubicin (**5**) (**Figure 2.1**). Of the >80,000 microbial natural products that have been reported to date, almost half (47%) have been shown to exhibit some form of biological activity (6), with natural products or their derivatives accounting for >40% of all Food and Drug Administration (FDA) approved medications since 2000 (8,9).

Figure 2.1. Examples of pharmaceuticals based upon natural products.

In certain categories of drugs natural products dominate. For example, 49% of anticancer drugs approved for clinical use between 1940-2014 were derived from natural products (8). Since 2000, 77% of all FDA approved antibiotics have been based on, or derived form, natural product scaffolds, of which all are of microbial origin (9); this is in the context that almost 70% of currently prescribed antibiotics hark back to the "golden age" of discovery (10,11). Focusing on polyketide metabolites alone, of 7000 known structures 20 have made it to the clinic (12). This successes rate of 0.3% compares very favourably to <0.001% for synthetic (xenobiotic) compounds. As recently as 2014 it was highlighted that only 2 drugs approved by the FDA could be categorised as being truly *de novo* (8).

Why are natural products such compelling leads for pharmaceutical development? As already discussed they have evolved to perform a specific function in living cells, and thus are generally biocompatible, bioavailable and bioactive. They are often optimised for a single specific function, with undesirable off-target effects being avoided. The chemical space explored by biology is large and chemical novelty is common, facilitated by highly efficient and dedicated catalysts (enzymes). Given these advantages over synthetic chemistry, it seems superficially surprising that natural product discovery has now been largely abandoned by the majority of pharmaceutical companies. This shift from natural product drug discovery has been driven in part by their non-compliance to Lipinski's rules (13). Though it should be noted that whilst 24 natural product drugs made it to market between 1981-2006 only 50% of these compounds obeyed these caveats, leaving the other half in a so-called 'parallel universe', in which natural products remain orally active but violate all but one of the rules of five (14). The defining characteristic of these drugs is the fact that they all exhibit a logP <5, and it is the ability of these often large and functionally diverse molecules to maintain a low logP that appears to be important (14). The second issue is the high frequency of repeated rediscovery of known strains and compounds from easily accessible environmental samples (15). It should, however, be possible to avoid rediscovering known natural products by targeting screening efforts on samples recovered from previously unexplored environmental niches. In particular, 'extreme' environments characterised by excessive pressure, temperature, salinity, and so on, predispose microbial life to the adoption of biosynthetic capabilities that facilitate survival under such challenging conditions. Such extremophilic microorganisms may therefore represent an exploitable and largely untapped resource of novel bioactive natural products. Given the increasing accessibility of molecular biology and other enabling technologies we suggest that bioprospecting of extreme environments could offer a productive new frontier in natural products drug discovery.

2.3. Categories of extremophiles

‘Extreme’ environments on planet Earth are those which experience physio-chemical conditions that are inhospitable to most forms of life. These include, for example, hydrothermal vents on the ocean floor, polar sea ice floes, sulphurous environments including soda lakes, and hypersaline environments. These niches all play host to diverse and complex polymicrobial communities (16,17). Microorganisms that are successfully adapted to such environments are termed extremophiles.

The extremophiles can be usefully considered in two broad categories: either as obligate extremophiles, which absolutely require one or more extreme conditions in order to grow, or as extremotolerant microorganisms, which retain the capacity to grow under standard laboratory conditions (17). This can be important in natural product discovery, and most of the examples given herein involve microbes that can be cultivated under mesophilic (non-extreme) conditions in the laboratory. Classifications of extremophiles include thermophiles and hyperthermophiles (microorganisms growing at high or very high temperatures, respectively), psychrophiles (microorganisms that grow best at low temperatures), acidophiles and alkaliphiles (microorganisms optimally adapted to acidic or basic pH values, respectively), barophiles or piezophiles (microorganisms that grow best under pressure), and halophiles (microorganisms that require NaCl for growth).

For the purposes of this chapter, we choose to focus upon extremophiles from marine environments. Marine environments are ‘extreme’ since they are high in salinity, often in combination with low temperatures and with variable acidity and pressure. An example of this is the deep ocean, whose microbial inhabitants are exposed to low temperatures and high pressures. Microorganisms that persist in such niches are classified as poly-extremophilic, reflecting the multiple adaptations required, and include microbial communities that persist at >10,800 m depth and under a hydrostatic pressure of >1000 atmospheres (18). Marine microbes are highly abundant, with the world’s oceans predicted to contain on the order of 10^{29} individual microbial cells (19). Estimates of the diversity of this marine life range from a few thousand species (20) to more than two million distinct taxa (21).

2.4. Life on the limit: How environmental conditions drive metabolic innovation

The mechanisms by which different organisms adapt to extreme environments provide a unique perspective on the fundamental characteristics of biological processes, such as the biochemical limits of macromolecular stability and the genetic instructions for constructing macromolecules that can function in one or more extreme conditions (22-28). Although much still remains to be learned about the strategies employed for survival under such conditions, what is known to us is that these microorganisms utilise novel biochemical pathways. Their stability and activity at extreme conditions make them useful alternatives to more labile molecules derived from mesophiles. This is particularly true for enzymes derived from extremophilic microorganisms, which remain catalytically active under extremes of temperature, salinity, pH, and in organic solvents. These properties make them attractive targets for use in industrial applications (29).

The biosynthetic potential of extremophiles, which are often difficult to isolate and study, is highly unexplored. Targeting these microorganisms as a novel source of natural product-based drug leads therefore represents an attractive route to mitigating the risk of rediscovering known natural products from microbial metabolomes.

Researchers have therefore in recent years turned their attention to the isolation and characterisation of extremophile microorganisms and a number of success stories are now beginning to emerge. Here we outline some notable examples of pharmaceutical leads identified from extremophile natural products that show promise for use in the treatment of a range of human and animal diseases.

2.5. Lomaiviticins

During a search for natural products with anti-cancer properties, McDonald *et al*., isolated the natural product compound namenamicin from the marine ascidian (sea squirt) *Polysyncraton lithostrotum*, which was collected off the coast of Fiji (30). **Figure 2.2** shows the chemical structure of namenamicin (**6**), which was shown to be a member of the enediyne family of anti-tumor natural products (**6-8**). These had previously been isolated exclusively from actinomycete bacteria (31) and so it was suggested that namenamicin was also of microbial origin. Attempts were made to isolate this supposed microorganism from the invertebrate (32). A phage lambda-based biochemical assay (33) was used to detect, identify, and purify DNA-damaging agents in the fermentation broth of cultured bacteria that were associated with the ascidian, and the halophile strain LL-371366 emerged as a promising candidate for enediyne production. This halophilic bacterium was originally classified as a new species, *Micromonospora lomaivitiensis*, but later phylogenetic analysis (34) revealed it to be a strain of *Salinispora pacifica*, an obligate marine actinomycete (35).

In addition to namenamicin, two novel glycosylated diazo benzofluorene dimers were also isolated from strain LL-371366 (32) and named lomaiviticin A and B (**9**,**10**) (**Figure 2.2**). Lomaiviticin A was tested for cytotoxicity against a panel of 24 cancer cell-lines and found to be extremely potent, with a half maximal inhibitory concentration (IC_{50}) down to 7 pM in some of the cell-lines. (32,36). The cytotoxicity profile of lomaiviticin A in these assays was unlike any other known DNA-damaging anticancer drug (32), implying that its mode of action was unique. Lomaiviticins A and B were also both found to have antibacterial activity in plate assays (32).

Figure 2.2. Chemical structures of namenamicin (**6**) and closely-related enediyne natural products (**7**,**8**), lomaiviticins (**9**,**10**), kinamycins (**11a-f**), and bleomycin (**12**).

The diazotetrahydrobenzo[b]fluorene moiety that forms the core of the lomaiviticins is highly unusual, reinforcing the idea that extremophiles will harbour unfamiliar metabolites. This tetracycle consists of a napthoquinone fused to a diazocyclopentadiene, which is in turn bonded to an oxidised cyclohexene ring, resulting in distinctive extended pi-conjugation. Diazo functions are rare in natural products, and little is known about their biosynthesis (37). The only other metabolites known to share the diazofluorene moiety are the kinamycins (**11a-f**), which were isolated from soil bacteria in Japan (**Figure 2.2**) (38). However, key structural differences differentiate these molecules. Kinamycins possess a single diazofluorene, whereas lomaiviticins contain two such residues linked *via* a carbon-carbon bond into a C_2- symmetric dimer. Kinamycins are less oxidised than lomaiviticins and are not functionalised by sugar residues. These differences appear critically important for activity, with kinamycins two orders of magnitude less cytotoxic than lomaiviticin A (39).

Attempts to achieve the total chemical synthesis of lomaiviticins have been impeded by two obstacles (40). Firstly, forming the carbon-carbon bond that links the two diazofluorene monomers with controlled stereochemistry is difficult due to the steric congestion around the dimer interface, which can impose unpredictable destabilising interactions (41). Secondly, the beta-hydroxy ketone at the dimer interface readily undergoes elimination and aromatisation reactions, resulting in a loss of stereospecificity. Although impressive progress has been made in synthesising substructures of lomaiviticins (41,42), including the core carbon skeleton of the molecule but lacking the sugar residues (43), complete synthesis of these natural products has not yet been reported. It has also proven challenging to isolate significant quantities of lomaiviticin A from fermentation broths. However, Woo *et al*., established that another bacterium, *Salinispora tropica* produces high levels of lomaiviticin C during fermentation (44). This metabolite is identical to lomaiviticin A, with the exception of the elimination of the diazo group on one of its diazofluorene moieties. The researchers developed a simple semisynthesis of lomaiviticin A from this more abundant natural product, allowing detailed studies on the activity of lomaiviticin A in parallel with lomaiviticin C and kinamycin C (39).

Kersten and co-workers identified the lomaiviticin biosynthetic cluster in *Salinispora tropica* (45). Designated the *lom* cluster, this region was predicted to encode 59 open reading frames. All of the genes thought to be involved in the biosynthesis of the diazofluorene core were identified, along with two glycosyltransferases consistent with the observed glycosylation pattern of the lomaiviticins. Significantly, a putative FAD-dependent monooxygenase gene (Lom19) shows 76% sequence identity to ActVA-Orf4 from the *Streptomyces coelicolor* A3(2) actinorhodin pathway (46). This enzyme catalyses carbon-carbon bond formation resulting in dimerisation of a multicyclic aromatic molecule, which suggests that Lom19 is the diazofluorene dimerase. The lomaiviticin cluster was later identified in *Salinispora pacifica*, and candidate enzymes involved in diazo synthesis and diazofluorene dimerisation were proposed by comparison with kinamycin clusters (34). Characterisation of the enzymes involved in lomaiviticin biosynthesis, in particular the dimerase and diazo synthases, is expected to provide new insight and novel tools for biocatalysis. Metabolic engineering of the *lom* cluster to produce new lomaiviticin analogues with improved properties is also an important goal.

The major activity of the lomaiviticin family appears to be introducing nicks into DNA. Lomaiviticin A nicks double-stranded DNA (dsDNA) and induces double-strand breaks (DSBs) in the presence of a reducing agent (39). The ratio of single-strand breaks (SSBs) to DSBs induced by lomaiviticin A (5:1) was much lower than expected if DSBs arose due to the cumulative effects of unrelated SSBs; this ratio is comparable to that of bleomycin (**12**) (6:1), which is known to effect two SSB events on complementary strands of dsDNA without dissociating (47). Taken together, this evidence supports a model in which lomaiviticin A binds dsDNA and nicks each strand of the duplex in a stepwise manner, producing extremely cytotoxic DSBs (48,49). The inability of lomaiviticin C and kinamycin C to do the same implies that both diazofluorene groups are required for this activity.

Subsequent studies by the same group established that vinyl radicals of lomaiviticin A can form by nucleophilic addition to the diazo moieties (50). These radicals are capable of abstracting hydrogen atoms from methanol and acetone. These data were used to

propose a model in which an unidentified nucleophile (possibly DNA itself) forms an adduct at the first diazo group, triggering the formation of the first vinyl radical which removes a hydrogen atom from the DNA backbone of one strand. This leads to a SSB by a well understood mechanism (51), and the process is repeated with the second diazo moiety and complementary DNA strand to produce a DSB.

Unexpectedly, the second nucleophilic addition was found to be far slower than the first, offering an explanation for the 5:1 ratio of SSBs to DSBs (50). This mechanistic model implies that lomaiviticin A adopts a DNA-bound conformation in which both diazofluorenes are simultaneously close to each dsDNA strand. This was confirmed by a combination of ^{1}H NMR spectroscopy of a palindromic DNA duplex bound to lomaiviticin A and molecular modelling of the complex based on NMR-derived constraints (52). The structure reveals that both diazofluorene groups are inserted into the minor groove of the DNA, forcing three bases out of the duplex. The energetic cost of this base flipping may be ameliorated by the favourable base-stacking interactions of the diazofluorenes and remaining DNA bases, and by electrostatic interactions between the amino sugar and the phosphate backbone. This binding mode places the diazo carbons of lomaiviticin A close enough to key deoxyribosyl hydrogen atoms for abstraction and strand scission. The model also accounts for the 10-100-fold reduced potency of lomaiviticin B, as its two additional ether linkages between diazofluorenes are expected to prevent it adopting the same mode of binding DNA. The diazofluorenes are a fascinating target for further research, with the potential to provide new insights in synthetic chemistry, enzymology, and cancer biology. Lomaiviticin A is being investigated as a monotherapy and combination therapy for DSB repair-deficient tumors (49,53).

2.6. Salinosporamide A

The halophile actinomycete *Salinispora tropica* is also the source of one of the most significant discoveries in extremophile natural product research in recent years: salinosporamide A (**13**) (54-58). Screening of organic extracts of cultured *Salinispora* strains by Feling *et al*., demonstrated a high rate of antibiotic and anticancer activity (59). The crude acetone fraction of *Salinispora* strain CNB-392 exhibited highly cytotoxic activity against a human carcinoma cell-line *in vitro*, with IC_{50} values of 80 ng/mL^{-1}. The active ingredient was identified as salinosporamide A (**Figure 2.3**), which contains a γ-lactam-β-lactone bicyclic core shared with Omuralide (**14)**, a compound produced by a terrestrial *Streptomyces* species (59,60). Salinosporamide A is elaborated by the addition of a methyl group at the C3 ring juncture, a chloroethyl group at C2 and a cyclohexane at the C5 position.

Figure 2.3. Chemical structures of salinosporamides A and B (**13**,**15**), and omuralide (**14**).

Omuralide was a milestone in cancer treatment as the first truly specific inhibitor of the proteasome, a catalytic complex responsible for non-lysosomal proteolysis in the cell (61-65). The modifications found in salinosporamide A caused the inhibition of proteasome activity with potency 35 times greater than omuralide (59). Structural biology revealed how salinosporamide A bound to the 20S proteasome subunit of yeast. The six catalytic subunits present in the proteasome contain an N-terminal nucleophilic threonine residue, which is essential for polypeptide hydrolysis and forms the target for a covalent bond with omuralide, affording the mechanism of inhibition (66). It was thus

postulated that the ester linkage between the Thr1OH$^{\gamma}$ and the β-lactone carbonyl would form an equivalent adduct in salinosporamide A. However, although nucleophilic addition of the β-lactone ring does occur, upon ring opening the C-3O displaces a key water molecule in the enzyme active site, hindering deacylation and forming a cyclic ether with the chloroethyl group of salinosporamide A. The resulting protonated threonine deactivates the catalytic N-terminus (67). The presence of the chlorine atom is thus vital for the potency of salinosporamide A and indeed salinosporamide B (**15**), a deschloro analogue of salinosporamide A, which is 500 times less potent than its chlorinated counterpart (68,69). The general relevance of halogen atoms in pharmaceuticals has long been recognised and marine environments represent an excellent source of novel halogenation pathways (68). Interestingly, it is clear that the ability of marine extremophiles to produce halogenated natural products represents a significant resource for promising drug leads.

The biosynthetic route to both salinosporamide A and B was first characterised by Moore and co-workers (69-71) who theorised the involvement of chloroethylmalonyl-CoA (**16**) in the addition of the unique polyketide extender unit chlorobutyrate (**Figure 2.4**). This halogenated precursor was shown to be formed by an *S*-adenosyl-L-methonine (SAM, **17**) dependent chlorinase, a new family of halogenase enyzmes that utilise a rarely observed nucleophilic substitution strategy, converting SAM to 5'-chloro-5'deoxyadenosine (5'-CIDA, **18**) (69).

Production of salinosporamide A from the producer microorganism was hampered by the unsuitability of large scale fermentation vessels for saline fermentation due to corrosive effects, and the inherent aqueous instability of the β-lactone ring. This was overcome by addition of a solid resin able to bind to the active product increasing production by sixty-nine-fold (72). Salinosporamide A was the first example of the use of saline fermentation to produce clinical trial material, paving the way for future natural products to be produced *via* the fermentation of halophilic bacteria (73). Total chemical synthesis was achieved only a year after salinosporamide A's chemical structure was first published (74,75). This synthesis is highly stereo-controlled and enantioselective, requiring the formation of five stereocenters within a fused γ-lactam-β-lactone bicyclic core.

Figure 2.4. Proposed biosynthetic pathway to chloroethylmalonyl-CoA (**16**) as a PKS extender unit in salinosporamide A (**13**) biosynthesis.

Salinosporamide A was licensed by Nereus Pharmaceuticals and is now showing great promise in clinical trials under the trade name Marizomib (76,77). The rise of salinosporamide A from the sea sediment to the clinic is an excellent example of the potential of the extremophile pharmacy (**Figure 2.5**).

Figure 2.5. Discovery and clinical development of salinosporamide A. Adapted from Fenical et al. *Bioorganic and Medicinal Chemistry*. 2009;17(6): 2175-80.

2.7. Marinostatin

Proteases are an abundant enzyme class involved in diverse and critically important biological processes. Proteolysis is required for cell cycle transitions, apoptosis, immune regulation, and cell signalling; accordingly, proteases are implicated in the pathogenesis of many diseases including inflammatory disorders, cancer, and infections

(78). Natural products that can act as protease inhibitors are highly valued both as potential therapeutics and as tools for basic science. Imada *et al*., (79) used an agar diffusion method to screen for protease inhibitors in bacteria from seawater and sediment samples from sites around Japan. Of the nearly 2000 isolates screened, three inhibited the hydrolysis of casein by the protease subtilisin. A strain termed B-10-31 produced the largest inhibitory zone and was selected for further study. This strain was eventually identified as *Pseudoalteromonas sagamiensis*, a halophile marine bacteria (80).

The protease inhibitor produced by *P. sagamiensis* was determined to be a twelve-residue peptide (81). This peptide, termed marinostatin (**26**), was the first peptide protease inhibitor isolated from a marine microorganism and is the smallest natural product member of the serine protease inhibitor family (82) (**Figure 2.6**). Early evidence indicated that marinostatin specifically inhibits serine proteases (with the exception of trypsin) and that this inhibition could be the result of unusual ester linkages (81,83). Subsequent analyses provided a detailed model of marinostatin inhibition, which we describe below.

Figure 2.6. Chemical structure and schematic representation of marinostatin (**26**).

The interactions of proteases and their protein inhibitors have been extensively reviewed (84,85). Canonical serine protease inhibitors bind the enzyme in a substrate-like manner, with inhibition thought to occur due to the inhibitor being 'too perfect' a substrate. The enzyme-inhibitor complex forms a classic lock-and-key interaction, with an optimal fit mutually stabilised by hydrogen bonds, electrostatic interactions and van der Waals forces. The K_i values of serine protease inhibitors are many orders of magnitude lower than the dissociation constant (K_D) values of their natural substrates. The residues of protease substrates and inhibitors are numbered according to distance from the scissile peptide bond between P_1 and P_1', as shown in **Figure 2.7**. The identity of residue P_1 is critical for binding and determines the specificity of the interaction. Different serine proteases have different amino acid preferences at this position, with proline often required within two positions of the reactive bond to enforce correct geometry.

Figure 2.7. General mechanism of polypeptide cleavage by a protease. The scissile peptide bond is shown in red. Substrate amino acid residues are labelled as P_1, P_1', P_2, P_2', and P_3, P_3'. Corresponding enzyme binding sites are labelled as S_1, S_1', S_2, S_2', S_3, S_3'.

Most serine protease inhibitors have a flexible binding loop containing the scissile bond, and a compact hydrophobic core stabilised by disulphide bridges, which were thought to be absolutely required for inhibition (85). However, the twelve residues of marinostatin are too few in number to pack into a hydrophobic core, and its sequence lacks cysteines. Kanaori *et al*., determined the solution structure of marinostatin by ^{1}H NMR spectroscopy and found that it could be superimposed on the structure of OMTKY3 (turkey ovomucoid third domain), a canonical serine protease inhibitor bound to subtilisin (86), and shared similar Ramachandran angles over the binding loop (87). Further work revealed how marinostatin achieves the structural rigidity required of canonical serine protease inhibitors. The peptide contains two ester linkages between

the beta-hydroxyl and beta-carbonyl groups of Thr3-Asp9 and Ser8-Asp11, which give it a bicyclic structure. The carbonyl of the 3-9 linkage is involved in a hydrogen bond with the backbone amide of Arg5, as evidenced by the greatly reduced hydrogen-deuterium exchange rate of this proton. This hydrogen bond is thought to suppress conformational fluctuation of marinostatin, helping to protect the reactive peptide bond from cleavage, and is essential for inhibitory activity. An analogue of marinostatin with cysteine residues at positions 3 and 9 forms a stabilising disulphide bridge but is significantly more flexible due to the loss of this key hydrogen bond. Consequently, it can only inhibit protease activity temporarily before its proteolytic inactivation (88). Further structure-activity studies shed light on the role of Pro7, which adopts a *cis* conformation to force the marinostatin backbone into a rigid beta-turn that promotes formation of the ester-amide hydrogen bond (89). Forcing this region into the *trans* conformation by replacing Pro7 with a *trans*-olefin abolished inhibitory activity and distorts the structure of marinostatin (82). The N-terminal Phe1 residue of marinostatin was also shown to be essential for activity due to predicted binding pocket interactions (89). Thus, it can be seen that mutually stabilising interactions between serine proteases and marinostatin confer rigidity to the inhibitor, ensure strong binding, and prevent deformation of the scissile peptide bond, protecting it from hydrolysis (85).

Most recently a total synthesis of marinostatin has been achieved by sequential esterification of the linear peptide using orthogonal protecting groups (90). This work paves the way for rational design of peptide analogues with different protease targets. The simplicity of marinostatin makes it an attractive target for modification. It is the smallest canonical protease inhibitor in nature (87) and the first nine residues comprise the minimal sequence required for activity (89). The specificity of marinostatin was successfully changed from subtilisin to trypsin by swapping residues four and five, and this modified peptide inhibited trypsin more potently than leupeptin, which is commonly used to prevent proteolytic degradation by endogenous proteases during recombinant protein purification (89).

The marinostatin biosynthetic gene cluster was first identified in 1998 (91) and since then it has become clear that marinostatin is a member of the ribosomally synthesised and post-translationally modified peptide (RiPP) family of natural products (92). RiPP genes are ubiquitous in microbial genomes. The immediate translation product is a precursor peptide that contains a leader sequence for recognition by biosynthetic enzymes. This precursor is post-translationally modified, and then undergoes proteolysis to remove the leader sequence before export from the cell. This pathway makes RiPPs prime candidates for metabolic engineering (93). The biosynthetic enzymes recognise the leader but are permissive of varying downstream sequences and biosynthesis relies on only a small number of enzymes, simplifying heterologous expression. Marinostatin belongs to a subgroup of RiPPs known as the microviridin family. The biosynthesis of microviridins is now well understood (94-96) and has been reconstituted *in vitro*, allowing the generation of diverse and highly relevant protease-targeted peptide inhibitor libraries (97).

Activity-based probes have recently emerged as enabling technologies for dissecting protease functions and identifying new drug targets (98-101). These small molecules consist of a recognition element that specifically binds a target protease, an electrophilic ‘warhead’ that reacts irreversibly with the protease active site to form a covalent adduct, and a tag that can be used to purify or track the probe by fluorescence or binding to a

partner. Activity-based probes have been extensively applied to the study of biological systems. In combination with super-resolution microscopy, these probes can non-invasively track single protease molecules in real time, allowing the dynamics and spatial organisation of protease activity to be visualised (102,103). Activity-based probes are particularly useful for systems in which genetic tools aren't well developed. Selective probes have been designed for *Mycobacterium tuberculosis* proteases to study their role in virulence and monitor infections (104), and activity-based probes have been used to identify macrophages as the cells that express cathepsin proteases in response to IL-4 secreted by cancer cells, promoting tumor growth and invasion (105). These specific tool compounds increase our understanding of complex biological processes and identify potential drug targets. As a simple and specific protease inhibitor with a well characterised structure, mechanism, and biosynthetic pathway, marinostatin is not only an exciting target for therapeutic development, but also a valuable starting point for the development of future biochemical probes.

2.8. Abyssomicin C

The marine environment has also been used as a source of novel antibiotics. This is demonstrated clearly by the discovery of the abyssomicins, which act as inhibitors of tetrahydrofolate (THF) biosynthesis. THF is required for several key metabolic reactions in bacteria, including the biosynthesis of amino acids and purine bases (106,107) (**Figure 2.8**). The production of THF is thus an essential pathway in many microorganisms but is absent in higher organisms, which instead obtain THF from their diet (107). This makes THF biosynthesis an attractive and well-established antibiotic target (106,107).

Bister *et al*., specifically focused upon a particular step in THF biosynthesis: the production of the precursor para-aminobenzoate (pABA, **27**) (**Figure 2.8**). They isolated 201 extremophile actinomycetes from deep sea sediment from the Sea of Japan and screened extracts from these bacteria for the ability to specifically inhibit the biosynthesis of pABA (108). This approach yielded a new compound abyssomicin C (**32**) from the halophile *Verrucosispora* strain AB 18-032 (**Figure 2.9**). Abyssomicin C was found to inhibit the growth of pathogenic strains of *Staphylococcus aureus* and *Mycobacterium tuberculosis* with MIC values in the low micromolar range but did not affect Gram-negative bacteria (108-110).

Figure 2.8. Biosynthetic route to the THF precursor pABA (**27**).

Figure 2.9. Chemical structures of selected abyssomicins (**32**-**36**), and cisoid and transoid enones.

The complex structure of abyssomicin C can be broken down into three structural motifs, as shown in **Figure 2.9**. These are (i) an oxabicyclo[2.2.2]octane core spiro-linked to (ii) a tetronic acid ring, fused to (iii) an eleven-membered ring including an α, β-unsaturated ketone. The similarity of the oxabicyclooctane system to a solution conformation of chorismate suggested that abyssomicin C might act as a substrate mimic for the enzyme ADC synthase (108) (**Figure 2.8**).

Additional members of the abyssomicin family (**33**-**36**) (**Figure 2.9**), were later discovered in related species of bacteria. Structure-activity relationship studies aided by various total syntheses of the abyssomicins helped to define the mechanism of

antibiotic activity (111-115). An enone group at position $C_{7\text{-}9}$ was found to be essential for antibiotic activity. This enone acts as a Michael acceptor which inhibits ADC synthase by covalently and irreversibly binding to a catalytic cysteine residue (108,109). Further structure-activity relationship studies with synthetic analogues of abyssomicin C have established that the C_{11} hydroxyl and C_3 carbonyl groups are not required for activity; that C_{11} benzyl ethers are more potent than the natural product; and that removing the three methyl groups lowers cytotoxicity by three orders of magnitude (116,117). However, the nonspecific reactivity of the enone group remains problematic, and desmethyl abyssomicin C remains cytotoxic at antibacterial concentrations (117). An interesting exception within this family is abyssomicin J (**38**), which lacks the Michael acceptor but retains antibiotic activity. It appears that this thioether-linked dimer undergoes a reverse Michael reaction *in situ* to generate active abyssomicin C, which would explain why its MIC value is half that of abyssomicin C (114). Delivering abyssomicin C in this form as a prodrug could enhance its bioavailability by stabilising the reactive enone.

During their total synthesis of abyssomicin C, Nicolaou and colleagues discovered that this molecule exhibits atropisomerism (111). Atropisomers are stereoisomers that arise due to restricted rotation around a single bond, with an energy barrier of at least 23.3 kcal/mol (118). The highly strained eleven-membered ring of abyssomicin C restricts the rotation of the C_7-C_8 bond, so that the C_7 carbonyl is either in a transoid (abyssomicin C) or cisoid (atrop-abyssomicin C) conformation. Nicolaou *et al*., found that atrop-abyssomicin C is 1.5 times more potent an antibiotic than its atropisomer and compared the X-ray crystal structures of the two compounds to propose a mechanism for this difference (115). The O=C_7-C_8=C_9 dihedral angle of abyssomicin C is 144.8°, whereas in atrop-abyssomicin C it is 26.4° (**Figure 2.9**). These two electron rich systems are forced closer together in the latter, increasing the degree of conjugation and making the enone a better Michael acceptor, which was later confirmed in kinetic experiments (111). The atropisomers can interconvert in the presence of acid, which explains why atrop-abyssomicin C was not identified in early studies that used acidic solvent for HPLC. A later study that avoided the use of acidic HPLC solvents found that atrop-abyssomicin C is the major biosynthetic product of fermentation (113).

The biosynthetic gene cluster for the abyssomicins (*aby*) was identified in *Verrucosispora maris* (119-121). Abyssomicins belong to the spirotetronate polyketide family of microbial metabolites, defined by a cyclohexene ring spiro-linked to a tetronic acid group (122). The expected PKS and tetronate biosynthesis genes were identified, along with various putative oxygenases, regulators, and exporters. Feeding experiments with radiolabeled precursors demonstrated that the linear abyssomicin C polyketide is built from acetates, propionates, and an unidentified glycolytic metabolite. The authors proposed a biosynthetic scheme including a [4 + 2] cycloaddition to form the spiro-link but could not identify a candidate enzyme for the transformation. Later studies on the related spirotetronate versipelostatin identified a small enzyme VstJ which catalysed a stereoselective [4 + 2] cycloaddition and found a small homologous 429 bp ORF in the *aby* cluster which has not previously been recognised as a gene (123). The predicted protein product of this gene, later named AbyU, was identical to a 141-residue protein from another *V. maris* strain in which abyssomicin production had not been reported (124).

The Diels-Alder reaction is a [4 + 2] cycloaddition reaction that involves concerted reorganization of a six-electron system to form a cyclohexene (125). Most enzyme-catalysed reactions involve either ionic two-electron or radical one-electron processes, and pericyclic reactions are extremely rare. For a long time, it was unknown if any enzyme catalysts of Diels-Alder reactions (Diels-Alderases) existed (126). While a small number of enzymes have been shown to catalyse [4 + 2] cycloadditions, whether they proceeded *via* a concerted Diels-Alder route with a single cyclic transition state was unknown. Biocatalysis of the Diels-Alder reaction is a major goal as it would allow new, more efficient, and more environmentally friendly routes to valuable bioactive compounds. Byrne *et al*., (127) confirmed that AbyU catalyses [4 + 2] cycloadditions with substrate analogues and determined the crystal structure of the recombinant enzyme. These findings enabled the establishment of a formalised biosynthetic route to the compound (**Figure 2.10**). AbyU was shown to be a homodimer comprising a pair of eight-stranded antiparallel beta barrels, each of which possesses a central hydrophobic channel forming the active site. Molecular dynamics simulations of the enzyme with substrate showed that AbyU catalysed a *bona fide* Diels-Alder reaction. The active site has excellent complementarity to its natural substrate and holds the molecule in a reactive conformation from which the Diels-Alder reaction can occur with a lowered free energy barrier. The simplicity of AbyU makes it an attractive candidate for reengineering, *via* mutation of key amino acid residues within the barrel core . It is hoped that such an approach could lead to the development of functionally optimised non-natural abyssomicins with improved clinical utility.

Figure 2.10. Biosynthesis of the abyssomicin C spirotetronate core. The linear polyketide **37** is condensed with a glyceryl unit to form **38**. Subsequent enzyme catalysed tailoring reactions give **40**, the substrate for the AbyU catalysed Diels-Alder reaction to give **41**.

2.9. Macrolactins

In 1989, as part of a research program to culture and characterise marine microorganisms, Gustafson *et al*., isolated a Gram-positive bacterium from a sediment core 980 m below sea level off the coast of San Francisco (128). Using the biochemical methods available at the time, the group found the strain taxonomically undefinable, but noted that it was a halophile with a strong salt requirement for growth and likely a piezophile due to the hydrostatic pressure of the deep-sea environment. When grown in liquid culture, this bacterium produced a novel family of macrolides with 24-membered macrocyclic lactone rings named macrolactins **(42-49**) (**Figure 2.11**).

The identity and abundance of the macrolactins produced by this strain was dependent upon the culture conditions used, and six individual macrolactins were isolated (128). Macrolactin A is considered the archetype of the family, with characteristic polyenes, a lactone linkage, three hydroxyl groups, and a single methyl substituent. The hydroxyl moieties are modified by dicarboxylic acids and sugars, and geometric isomerism occurs around the alkene groups to generate the structural diversity of the family. Later analyses established the absolute and relative stereochemistry of macrolactins, and a total chemical synthesis was established to compensate for the unreliable fermentation of the original producing strain (129,130).

In the original study, macrolactin A was reported to possess an extraordinary range of bioactivities. It inhibited growth of *B. subtilis* and *S. aureus*, murine melanoma cells,

herpes simplex virus, and HIV replication at the microgram scale in *in vitro* assays (128). Subsequent work established that macrolactin A could protect neurons from L-glutamate toxicity in a model of brain ischemia (131) and act as a biological pest control agent in potato scab disease (132). Furthermore, macrolactins A and F were rediscovered in a screen for squalene synthase inhibitors and proposed as potential therapeutics for hyperlipidemia (133). Other macrolactins were subsequently discovered in marine and soil *Bacillus* species, and to date the family has expanded to include macrolactins A-W and their derivatives (**42-49**) (**Figure 2.11**) (134-142).

Unlike the rest of the family, macrolactins S, V, and W exhibit antibacterial activity against both Gram-negative and Gram-positive bacteria. Macrolactin V exhibits a potent MIC of 0.1 $\mu g ml^{-1}$ against *E. coli*, *B. subtilis*, and *S. aureus*. Its epimer macrolactin S has similar activity but with a different profile, being ineffective against *B. subtilis* (141). Macrolactin W was much less active, but could additionally inhibit the growth of *P. aeruginosa*, an opportunistic Gram-negative pathogen, which is a frequent cause of hospital-acquired infections (142,143).

Figure 2.11. Chemical structures of selected macrolactins.

In the search for new antibiotics, the increasing number of sequenced bacterial genomes has allowed for rational selection of conserved bacterial genes as antibacterial targets. If a gene is essential in bacteria but no human homologue exists, it should in principle represent a good target for inhibition. Peptide deformylase (PDF), an essential bacterial enzyme which removes formate from the N-terminus of proteins, is one such target (144). PDF inhibitors have reached clinical development (145,146) but were discontinued due to issues with their selectivity and *in vivo* stability, which is a general issue with peptide therapeutics (147). During a screen for novel PDF inhibitors, macrolactin N was isolated from a soil-dwelling *B. subtilis* strain and found to inhibit *S. aureus* PDF with an IC_{50} of 7.5 μM (137). The same group identified macrolactins O-R as weaker PDF inhibitors and found that the PDF-inhibiting macrolactins have antibacterial activity against both Gram-negative and Gram-positive species (138). Computational studies (147) showed that macrolactins probably bind to the same site as unrelated PDF inhibitors, and that hydrophobic and van der Waals forces provide most of the binding energy. This provides a robust starting point for the rational redesign of macrolactins as antibiotics.

Macrolactin A and its 7-*O*-succinyl and 7-*O*-malonyl derivatives (SMA and MMA respectively) have attracted most interest as potential therapeutics. In 2013, Young-Hoon *et al*., were granted a patent for the use of these three compounds as anti-inflammatory drugs, taking advantage of their ability to inhibit the production of proinflammatory cytokines and nitric oxide (148). In 2014, they were also granted a patent for the use of macrolactin A derivatives as anti-angiogenic compounds (149). It transpired that SMA could inhibit a cell signalling component common to inflammation and angiogenesis, phosphatidylinositide 3-kinase (PI3K) (150,151). SMA inhibits PI3K *in vitro*, and suppresses phosphorylation of Akt, a downstream PI3K target *in vivo* (152). SMA strongly suppresses angiogenesis in a standard chick embryo assay and displays no cytotoxicity to human cells at concentrations up to 50 μM (151). SMA has also demonstrated anti-tumor activity in mouse models of cancer, and appears to act by inhibiting PI3K and tankyrase, a component of the Wnt pathway which inappropriately promotes cell survival and proliferation in many cancers (153,154). SMA shows

synergistic effects with common anticancer agents like cisplatin, allowing them to be used at lower doses to minimise side effects (155,156). An appropriate commencement dose for humans (~1.5 mg/kg) has been calculated for SMA (157), and the route towards clinical trials seems clear.

The macrolactin biosynthetic cluster (*mln*) was identified in the soil bacterium *Bacillus amyloliquefaciens* and characterised as a *trans*-AT type I modular polyketide synthase (PKS) system (158). This cluster has several unusual characteristics including split PKS modules, the absence of enoyl reductase and dehydratase domains predicted from the chemical structure of the macrolactins, and duplicated acyl-carrier protein domains, several of which lack the universally conserved serine residue essential for function. Feeding experiments with radiolabeled substrates indicated that acetate is the sole precursor of the macrolactin carbon skeleton and, in combination with the operon structure, these were used to propose a biosynthetic route to the compound.

One particular key enzyme required for macrolactin biosynthesis is the glycosyltransferase designated BmmGT1 (159). Intriguingly, this enzyme is not specific to macrolactin biosynthesis, but also acts on the polyketide antibiotic bacillaene. Overexpression of BmmGT1 resulted in the production of two novel macrolactin A derivatives glycosylated at positions 13 and 15, and incubation of the recombinant enzyme with UDP-*N*-acetylglucosamine produced a further new derivative. The substrate promiscuity of BmmGT1 could be exploited to generate new macrolactin analogues with improved bioavailability or reduced toxicity. Zotchev *et al*., developed an approach for generating *in silico* libraries of macrolactins with predicted bioactivities to guide metabolic engineering efforts (160). Their program, Bio-generator, indicates the PKS modifications required to produce the selected macrolactins and aims to reduce the laborious process of combinatorial biosynthesis of polyketides. The wealth of information about macrolactin biosynthesis should encourage rational redesign studies to harness the full potential of these remarkable metabolites (161).

2.10. Conclusions and future prospects

The examples discussed above show how the novelty and diversity of extremophile natural products can provoke new directions in pharmaceutical development. Much of Earth's microbial life remains uncharacterised and there is clear potential for new discoveries from these microorganisms (162-165). There is plenty of historic precedent to suggest that the complexity and biological activity of these natural products will be beyond both our imagination and synthetic capability. The major barrier to progress, both historically and in the present day, remains the need to screen cultivated bacteria for the production of functional natural products. This has proven limiting since the majority of microbes apparently resist laboratory culture. However, new technologies that are now emerging may help to overcome this challenge or even obviate the need for cultivation altogether.

Recent advances in DNA sequencing and synthesis methods are now driving genomics-based natural product drug discovery from cultured and uncultured microorganisms. These approaches are becoming ever more accessible and affordable, and are underpinned by a well-developed bioinformatics toolkit. Advances in synthetic biology are facilitating the reconstitution of natural product pathways in model or industrial hosts, enabling the production and rapid characterisation of natural products from

extremophiles at scale. Such approaches can increasingly be undertaken using liquid-handling robotics, allowing natural product libraries to be screened in a high-throughput fashion. This is being supported by the increasing sensitivity, sophistication and automation of analytical methods and separation technologies. Extremophiles undoubtedly represent the planet's largest unexplored pool of biological and chemical novelty (166). Through continued characterisation and interrogation of this resource, it is hoped that it will be possible to identify the pharmaceuticals of the future.

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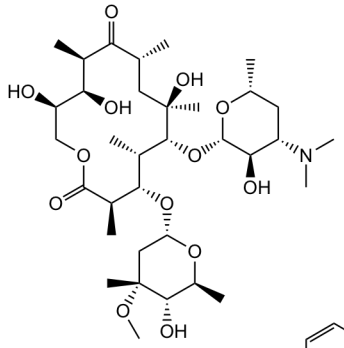
Index:

Figure 2.1



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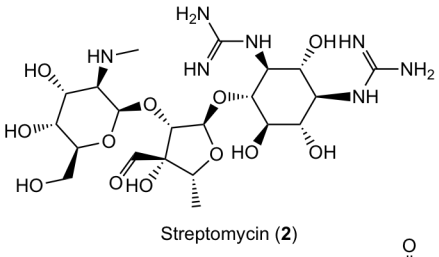

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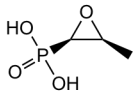

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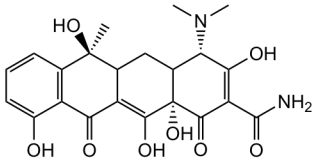
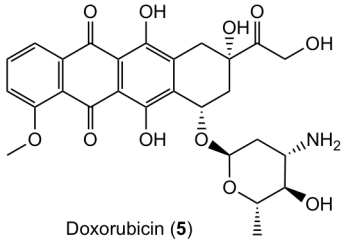
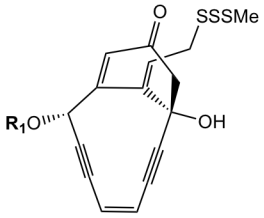


Tetracycline (**4**)



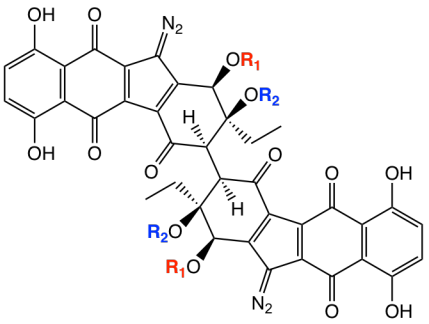
Doxorubicin (**5**)

Figure 2.2

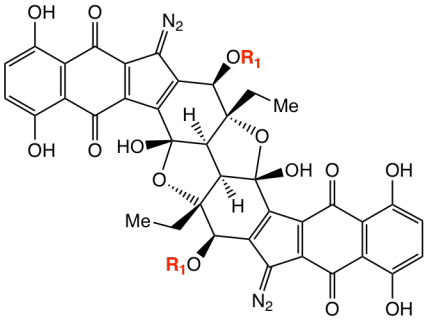


Enediyne warhead

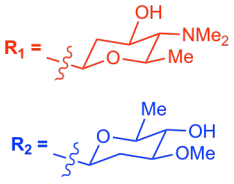
Namenamicin (**6**) R_1= sugar chain
Calicheamicin γ1 (**7**) R_1 = sugar chain + aromatic ring
Shishijimicin A (**8**) R_1= sugar chain + β-carboline

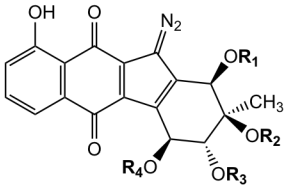


Lomaiviticin A (**9**)



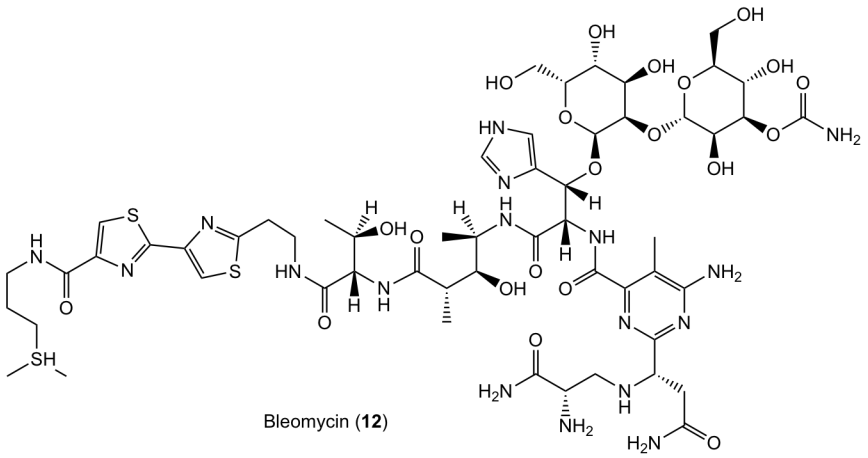
Lomaiviticin B (**10**)





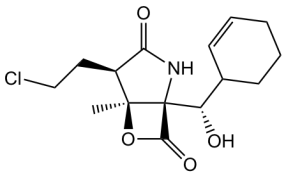
Kinamycins (**11a-f**)

(**11**)	R_1	R_2	R_3	R_4
A (**11a**)	H	Ac	Ac	Ac
B (**11b**)	H	Ac	H	H
C (**11c**)	Ac	H	Ac	Ac
D (**11d**)	Ac	H	Ac	H
E (**11e**)	Ac	H	H	H
F (**11f**)	H	H	H	H

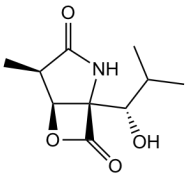


Bleomycin (**12**)

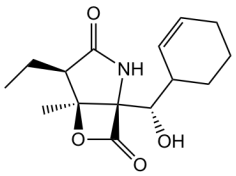
Figure 2.3



Salinosporamide A (**13**)



Omuralide (**14**)



Salinosporamide B (**15**)

Figure 2.4

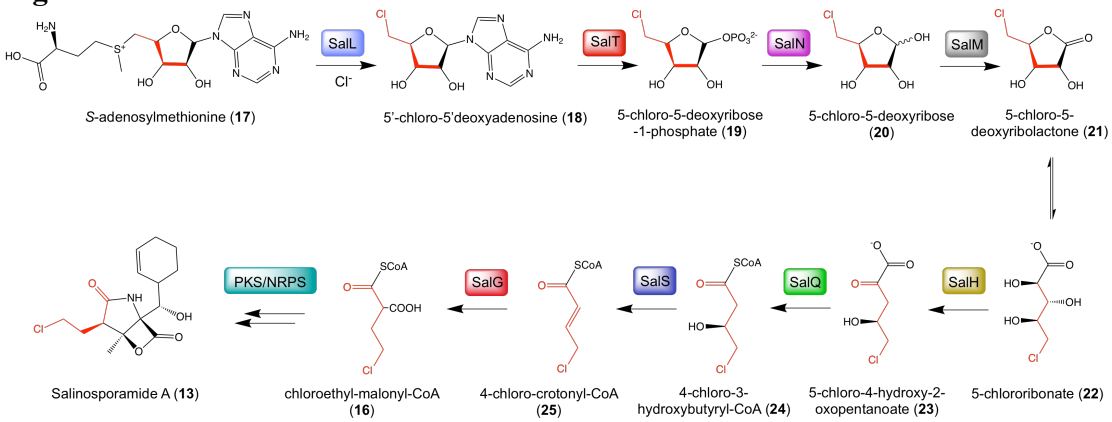

SalL
Cl-
SalT
SalN
SalM
PKS/NRPS
SalG
SalS
SalQ
SalH
S-adenosylmethionine (17)
5'-chloro-5'deoxyadenosine (18)
5-chloro-5-deoxyribose -1-phosphate (19)
5-chloro-5-deoxyribose (20)
5-chloro-5-deoxyribolactone (21)
Salinosporamide A (13)
chloroethyl-malonyl-CoA (16)
4-chloro-crotonyl-CoA (25)
4-chloro-3-hydroxybutyryl-CoA (24)
5-chloro-4-hydroxy-2-oxopentanoate (23)
5-chlororibonate (22)

Figure 2.5

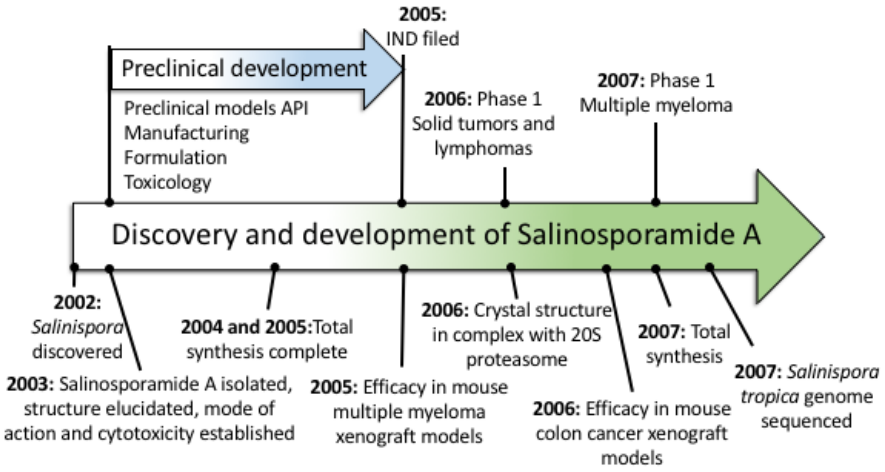
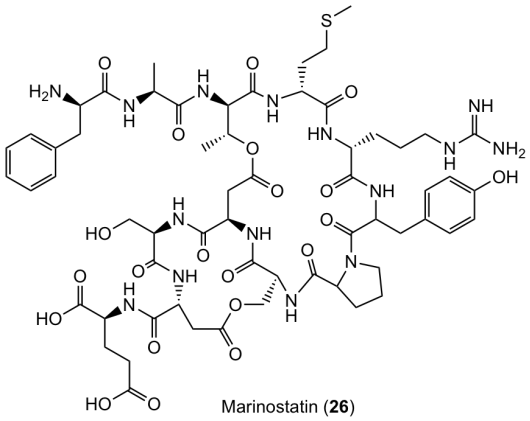
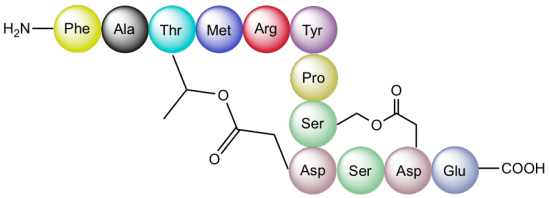

2005:
IND filed
Preclinical development
Preclinical models API
Manufacturing
Formulation
Toxicology
2006: Phase 1
Solid tumors and
lymphomas
2007: Phase 1
Multiple myeloma
Discovery and development of Salinosporamide A
2002:
Salinispora
discovered
2003: Salinosporamide A isolated,
structure elucidated, mode of
action and cytotoxicity established
2004 and 2005:Total
synthesis complete
2005: Efficacy in mouse
multiple myeloma
xenograft models
2006: Crystal structure
in complex with 20S
proteasome
2006: Efficacy in mouse
colon cancer xenograft
models
2007: Total
synthesis
2007: Salinispora
tropica genome
sequenced

Figure 2.6



Marinostatin (**26**)



Schematic representation of marinostatin (**26**)

Figure 2.7

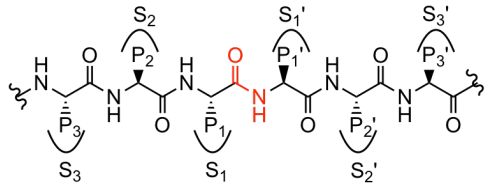


Figure 2.8

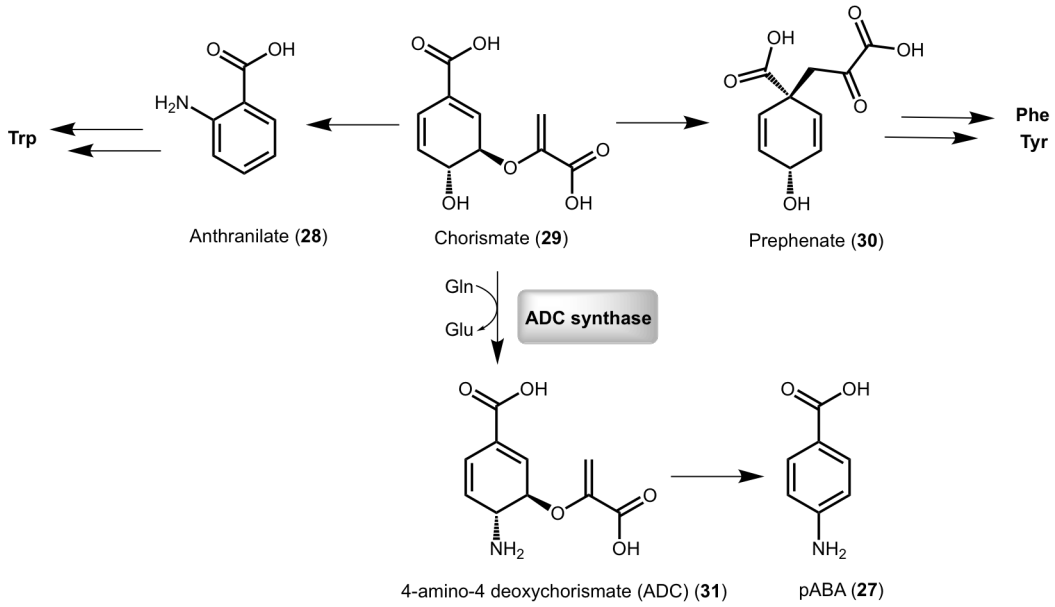
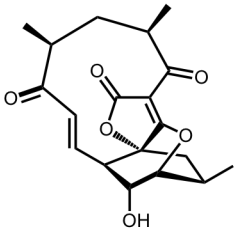
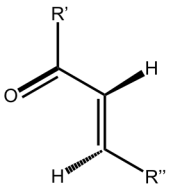

Trp
Anthranilate (28)
Chorismate (29)
Prephenate (30)
Phe
Tyr
Gln
Glu
ADC synthase
4-amino-4 deoxychorismate (ADC) (31)
pABA (27)

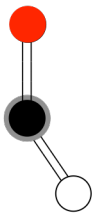
Figure 2.9



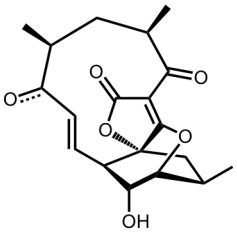
Abyssomicin C (**32**)



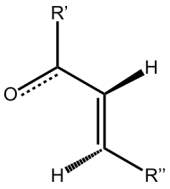
Transoid enone



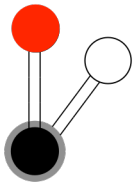
144.8° dihedral



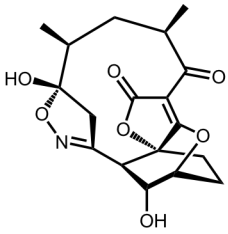
Atrop-abyssomicin C (**33**)



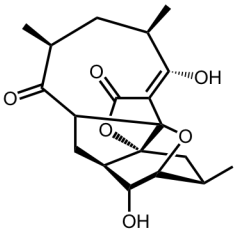
Cisoid enone



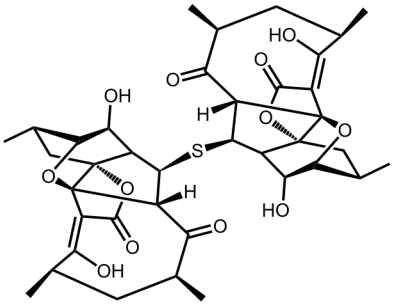
26.4° dihedral



Abyssomicin B (**34**)



Abyssomicin D (**35**)



Abyssomicin J (**36**)

Figure 2.10

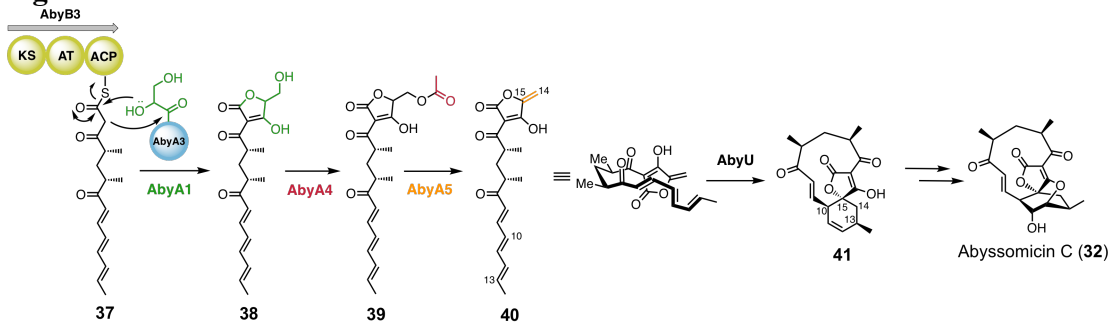
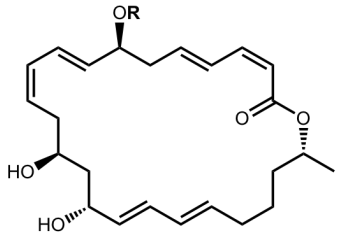
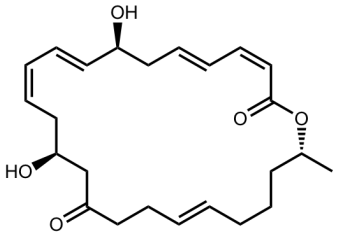

AbyB3
KS
AT
ACP
AbyA3
AbyA1
AbyA4
AbyA5
AbyU
37
38
39
40
41
Abyssomicin C (32)

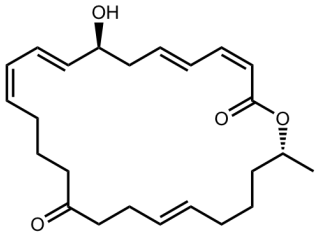
Figure 2.11



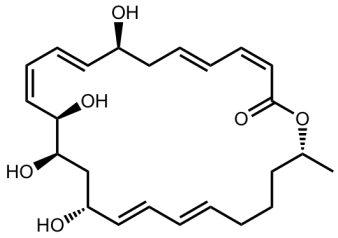
Macrolactin A (**42**), **R** = H
7-*O*-malonly macrolactin A (**43**), **R** = Malonyl
7-*O*-succinyl macrolactin A (**44**), **R** = Succinyl
Marinolactin W (**45**), **R** = Succinyl-glucosyl



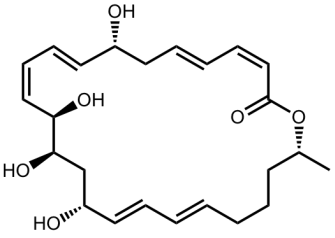
Macrolactin F (**46**)



Macrolactin N (**47**)



Macrolactin S (**48**)



Macrolactin V (**49**)