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The marine actinomycete genus *Salinispora*: a model organism for secondary metabolite discovery

Paul R. Jensen,*[a] Bradley S. Moore[ab] and William Fenical[a]

Covering: 2001 to 2014

This review covers the initial discovery of the marine actinomycete genus *Salinispora* through its development as a model for natural product research. A focus is placed on the novel chemical structures reported with reference to their biological activities and the synthetic and biosynthetic studies they have inspired. The time line of discoveries progresses from more traditional bioassay-guided approaches through the application of genome mining and genetic engineering techniques that target the products of specific biosynthetic gene clusters. This overview exemplifies the extraordinary biosynthetic diversity that can emanate from a narrowly defined genus and supports future efforts to explore marine taxa in the search for novel natural products.

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1 Introduction

Microbial-derived natural products represent a major component of today's pharmaceutical arsenal. Despite their historical importance, the world's major pharmaceutical companies moved en masse away from microbial natural products in favor of alternative discovery platforms such as combinatorial chemistry.[1] Contributing to this paradigm shift was the continued re-discovery of known compounds and a growing belief that microbial resources have been over-exploited. However, increased demand for new drugs to treat antibiotic resistant bacterial infections and other chronic diseases, coupled with the low returns from alternative discovery platforms, have led to a resurgence of interest in natural products research.[2] This renewed interest includes the exploration of bacteria from poorly studied environments, a concept based on the premise that adaptations to these environments include the production of new secondary metabolites.[3] Marine bacteria have become a particular focus in these efforts and have yielded many interesting new compounds.[4,5]

Actinomycetes are a major source of microbial-derived natural products[6] making marine-derived strains likely targets for natural product discovery.[7,8] Although it was revealed long ago that actinomycetes could be recovered from marine samples, including deep sea sediments,[9] it remains unknown to what extent these bacteria are ecologically or evolutionarily distinct from their terrestrial relatives. This uncertainty arises from the fact that spore-forming actinomycetes are abundant in soils and washed into the sea in large numbers where their metabolic activities remain largely unknown.[10] Although there is evidence that common soil genera such as *Streptomyces* can be metabolically active in the sea,[11] we have yet to gain a broader perspective on this subject. None-the-less, there is emerging evidence for marine adaptation even among streptomycetes[12,13] and a number of exclusively marine *Streptomyces* spp. have been described.[14] Furthermore, at least five marine actinomycete genera have been described[15–19] providing clear evidence that marine-derived actinomycetes can be taxonomically distinct from those occurring on land. Among these genera, *Salinispora* has proven to be a prolific source of novel natural products[4] and a model organism with which to address correlations between bacterial diversity and secondary metabolite production.[20,21]

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Here we review the discovery of the marine actinomycete genus *Salinispora* and its development as a model for natural product research. The focus is on new carbon skeletons with the discoveries presented largely in chronological manner. Some of these molecules have important biological activities, which have been summarized. Many have inspired synthetic, biosynthetic, and mechanistic studies, which have been highlighted. Early discovery efforts employed more traditional bioassay-guided approaches while some of the more recent discoveries result from the application of genome mining and genetic engineering approaches. We have also summarized the known compounds and new derivatives thereof that have been reported from this taxon. The major aim of this review is to encapsulate the remarkable biosynthetic capacities of a single marine actinomycete taxon and to emphasize how natural products chemistry has been merged with biological and biochemical studies in an interdisciplinary effort to develop more informed approaches to natural product discovery.

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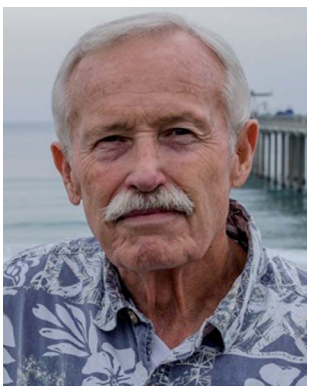
Bradley S. Moore is currently Professor of Oceanography and Pharmaceutical Sciences at the Scripps Institution of Oceanography and the Skaggs School of Pharmacy and Pharmaceutical Sciences at University of California, San Diego. He holds degrees in chemistry from the University of Hawaii (B.S. 1988) and Washington (Ph.D. 1994), was a postdoc at the University of Zurich (1994–1995), and held prior faculty appointments at the University of Washington (1996–1999) and Arizona (1999–2005). His research interests involve exploring and exploiting marine microbial genomes to discover new biosynthetic enzymes, secondary metabolic pathways, and natural products for their biomedical and biotechnological utility. Dr Moore is the Chair of the NPR *Editorial Board.*

2 Discovery of the genus

The cultivation of *Salinispora* strains was first reported in 1989 as part of a study addressing actinomycete distributions in marine sediments.[22] At the time, their morphological and chemotaxonomic characteristics indicated they were close relatives of the genus *Micromonospora*, and it was proposed they represented a new species within this genus based on the observation that they failed to grow when seawater was replaced with deionized water in the growth medium. Subsequent phylogenetic studies placed these bacteria in a clade that was distinct from the Micromonosporae, and it was suggested they represent a new genus for which the name "*Salinospora*" was originally proposed.[23] This taxon was formally described in 2005 as the first obligate marine actinomycete genus with the name revised to *Salinispora* to meet nomenclatural standards.[19] The original description included the species *S. tropica* and *S. arenicola* while a third species, *S. pacifica*, was subsequently proposed[24] and formally described.[25] The three species share approximately 99% 16S rRNA gene sequence identity and are not well resolved using this conserved phylogenetic marker.[25] However, less conserved loci have been used to generate well-supported phylogenies that clearly delineate the three species and reveal the sister relationship between *S. tropica* and *S. pacifica* relative to the more ancestral *S. arenicola* lineage.[24–27]

Salinispora spp. are most frequently reported from marine sediments, however this may represent sampling bias. They have also been reported from an ascidian,[28] seaweeds,[13] and marine sponges.[27,29] To date, there is no evidence that plant or invertebrate-associated populations are ecologically or evolutionarily distinct from those that occur in sediments. *Salinispora* strains have been cultured from depths as great as 1100 m (ref. 30) but have been detected using culture independent



William Fenical received his Ph.D. at the University of California, Riverside, in synthetic organic chemistry, in 1969. After a short investment in industrial research, he accepted a postdoc position with James J. Sims, also at UC-Riverside, to initiate studies of marine natural products chemistry. In 1973, Bill moved to San Diego to take up a position at the Scripps Institution of Oceanography (SIO), UC-San Diego, where he has resided ever since. Bill's early interests have spanned several disciplines including the chemistry and ecology of marine algae, and the chemistry of gorgonian corals and ascidians. In the mid 1990s, he moved toward developing studies of marine microorganisms, a field that had been largely overlooked. Bill has published 450 papers in marine natural products drug discovery and chemical ecology and is currently director of the Center for Marine Biotechnology and Biomedicine at SIO.

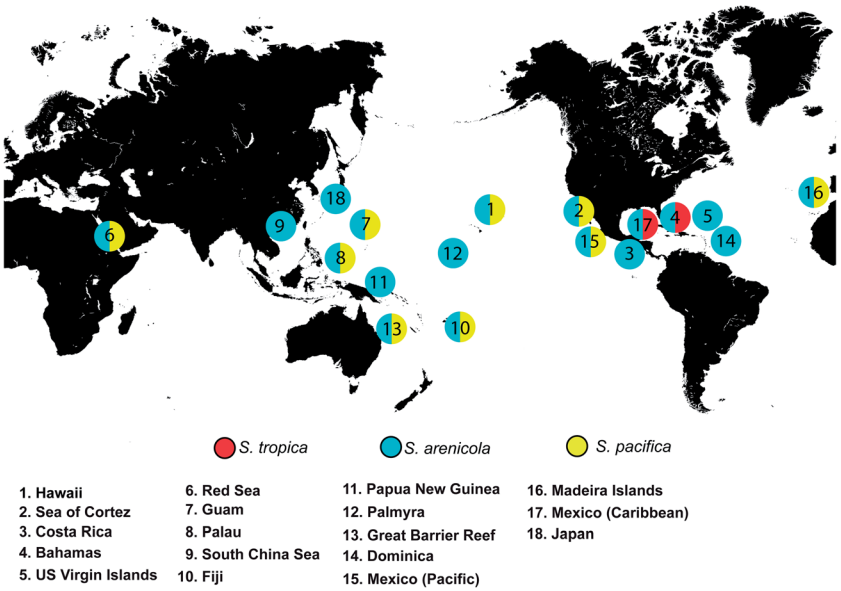


Fig. 1 Global locations from which the genus *Salinispora* has been reported. These reports originate from multiple research groups and are based on GenBank (http://www.ncbi.nlm.nih.gov/genbank/) 16S rRNA sequence deposits.

methods from much greater depths, the current record being 5669 m.[31] They have been cultured from tropical and sub-tropical sites around the globe,[32] with the most northern report coming from samples collected off Japan[33] (Fig. 1). The lack of reports from more northern and southern latitudes may be due to limited sampling from these regions or yet to be determined environmental variables that limit their distributions.

To date, reports of *S. tropica* have been restricted to the Caribbean, *S. pacifica* has been reported from numerous global sites except for the Caribbean, while *S. arenicola* has the broadest distribution and has been reported from all sites from which the genus has been recovered.[32,34] *Salinispora* spp. are heavily invested in secondary metabolism, with *ca.* 10% of their genomes devoted to this process.[35] The majority of their secondary metabolite biosynthetic gene clusters are located in

Table 1 Secondary metabolites reported from *Salinispora* spp.

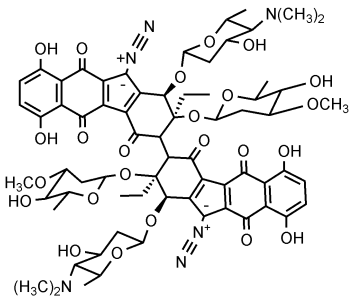
No.	Species[a]	Compound	Biosynthetic origin	Novelty	Activity (target)	References
1	*S. tropica*	Salinosporamide A	PKS-NRPS	New	Proteasome	47
2	*S. tropica*	Sporolide A	ePKS	New	Reverse transcriptase[d]	85
3	*S. tropica*	Salinilactam	Type I PKS	New	ND	35
4	*S. tropica*	Sioxanthin	Terpene	New	ND	123
5	*S. tropica*	Antiprotealide	PKS-NRPS	New	Proteasome	57
6	*S. pacifica*	Pacificanone A	Type I PKS	New	ND	121
7	*S. pacifica*	Salinipyrone A	Type I PKS	New	ND	121
8	*S. pacifica*	Cyanosporoside A	PKSe	New	ND	93
9	*S. pacifica*	Lomaiviticin A	Type II PKS	New	Cytotoxic (DNA)	28
10	*S. pacifica*	Enterocin	Type II PKS	Known	Antibiotic	129
11	*S. arenicola*	Saliniketal A[b]	Type I PKS	New	Ornithine decarboxylase	99
12	*S. arenicola*	Arenicolide A	Type I PKS	New	ND	96
13	*S. arenicola*	Saliniquinone	Type II PKS	New	Cytotoxic	116
14	*S. arenicola*	Cyclomarin A	NRPS	Known	Anti-inflammatory	117
15	*S. arenicola*	Cyclomarazine[c]	NRPS	New	ND	118
16	*S. arenicola*	Arenimycin	NRPS	New	Antibiotic	113
17	*S. arenicola*	Arenamide A	Type II PKS	New	Anti-inflammatory (NFκB)	98
18	*S. arenicola*	Staurosporines	Alkaloid	Known	Protein kinase	21
19	*S. arenicola*	Isopimara-8,15-dien-19-ol	Terpene	New	ND	124
20	*S. arenicola*	Rifamycin B	Type I PKS	Known	RNA polymerase	104
21	*S. arenicola*	Mevinolin	PKS	Known	HMG-CoA reductase	133
22	*St*, *Sa*, and *Sp*	Desferrioxamine B	NRPS	Known	Iron chelator	132
23	*St*, *Sa*, and *Sp*	Lymphostin	NRPS-PKS	Known	Immunosuppressant	127

[a] Original report of compound detection from *Salinispora* spp. [b] Rifamycin synthase intermediate. [c] Cyclomarin synthetase intermediate. [d] Predicted, e = enediyne, ND = not determined.

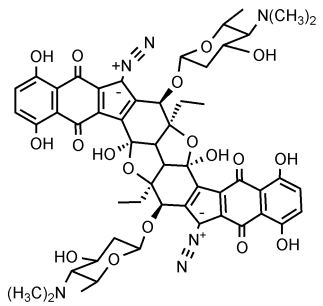
genomic islands, which was used to suggest the products provide ecologically relevant adaptive traits.[36] The genus is unique among the Micromonosporaceae in that all strains tested to date fail to grow when seawater is replaced with deionized water in the growth medium, which was subsequently linked to a variety of marine adaptation genes using both bioinformatic[37] and experimental approaches.[38] However, the primary interest in this taxon has focused on its ability to produce unique and biologically active secondary metabolites.

3 *Salinispora* natural products

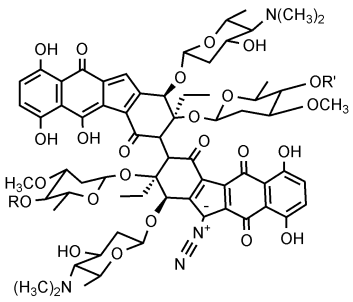
The secondary metabolites reported to date from *Salinispora* spp., are predominantly new (Table 1). This supports the concept that new taxa from poorly studied environments represent an important resource for secondary metabolite discovery. While not widely recognized, the first compounds described from the genus *Salinispora* were lomaiviticins A and B (**1**, **2**),[28] the structures of which were published in 2001. At the time, the producing strain was reported as a new *Micromonospora* species with the proposed name "*Micromonospora lomaivitiensis*". However, subsequent 16S rRNA gene sequence analysis identified this strain as *S. pacifica*.[39] The lomaiviticins were isolated by researchers at Wyeth (now Pfizer) as part of efforts to identify enediyne-producing bacteria from the marine ascidian *Polysyncraton lithostrotum*. Although the lomaiviticins do not belong to this structural class, they none-the-less possess powerful antibiotic activities and, in the case of **1**, nanomolar to picomolar cancer cell cytotoxicities that were ultimately linked to the induction of double-strand DNA breaks,[40] a mechanism of action similar to that exerted by enediynes.[41] Further studies by the Herzon group led to the isolation of additional compounds in this series (lomaiviticins C–E) (**3–5**) and the complete relative and absolute stereochemistry of **1**.[42] The gene cluster responsible for lomaiviticin biosynthesis (*lom*) was initially identified in *S. tropica* by deleting the beta-ketosynthase gene in the ST_PKS2 pathway and correlating its loss to the loss of biological activity associated with lomaiviticin.[43] The *lom* locus was subsequently shown to occur in most strains of *S. pacifica* in addition to all *S. tropica* strains for which genome sequences are available.[44] It was independently characterized in *S. pacifica* by the Balskus group[39] who established that the associated type II polyketide synthase (PKS) supports a new strategy for propionyl starter unit generation previously observed in type I PKS pathways.[45] Numerous groups have also established synthetic routes to different portions of the lomaiviticin aglycone with Herzon and coworkers completing the first enantioselective synthesis of the aglycone.[46] To date, the total synthesis of lomaiviticin has not been reported.



1, lomaiviticin A



2, lomaiviticin B



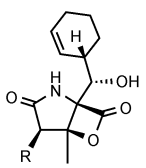
3, lomaiviticin C, R = R' = H
4, lomaiviticin D, R = H, R' = CH_3
R = CH_3, R' = H
5, lomaiviticin E, R = R' = CH_3

3.1 Salinosporamides

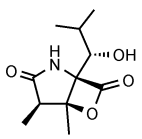
Two years after the discovery of the lomaiviticins, salinosporamide A (**6**) was reported from *S. tropica* strain CNB-392.[47] Salinosporamide A garnered immediate attention due to the rarity of the fused γ-lactam-β-lactone bicyclic ring system and its potent activity against the 20S proteasome, which became a validated target for cancer chemotherapy following the approval of bortezomib (Velcade®) for the treatment of multiple myeloma and other cancers.[48] At the time, the most closely related compound was *clasto*-lactacystin-β-lactone (**7**), also known as omuralide,[49] a transformation product of lactacystin, which was originally discovered by Ōmura and co-workers from a *Streptomyces* sp.[50] These compounds share the same ring system however **4** lacks the methyl group at the C-3 ring junction, has a methyl instead of a chloroethyl at C-2, and an isopropyl instead of a cyclohexene at C-5. A crystal structure of **6** bound to the yeast 20S proteasome revealed that the β-lactone carbonyl reacts with the catalytic N-terminal threonine to form an irreversible, covalent adduct.[51] A subsequent intra-molecular reaction between the C3–O and the C-2 side chain of **1** yields a cyclic tetrahydrofuran ring that blocks access to nucleophilic water into the binding pocket thus contributing to the irreversible binding of the compound to the proteasome. Subsequent studies revealed a redundant proteasome β-subunit within the salinosporamide gene cluster that confers resistance

to this compound in the native organism.[52] Salinisporamide A was developed by Nereus Pharmaceuticals (San Diego) under the names NPI-0052 and marizomib, undergoing extensive preclinical evaluation[53] and a variety of phase I and phase Ib clinical trials.[54] It is currently undergoing additional phase I clinical trials *via* a license from the University of California San Diego to Triphase Accelerator Corp. (http://triphaseco.com/pipeline/). Total syntheses were reported by both the Corey and Danishefsky groups in 2005,[55,56] and these were followed by numerous other synthetic routes. Despite the synthetic tractability of salinosporamide A, Nereus Pharmaceuticals produced the material used for clinical trails *via* fermentation.[57] The development of optimized fermentation protocols resulted in a number of publications addressing the ionic requirements for *Salinispora* growth and salinosporamide A production.[58–61]

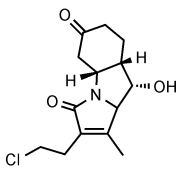
Subsequent studies of *S. tropica* strain CNB-392 led to the isolation salinosporamides B (**8**) and C (**9**) along with five related compounds that were determined to be artifacts of the isolation process.[62] The structure of salinosporamide B differs from A simply by the loss of chlorine, however the >500-fold loss in cytotoxicity associated with **8** provided the first evidence that the chloroethyl substituent plays a major role in the biological activity of **6**. During the course of purifying multi-gram quantities of **6** for clinical trails, researchers at Nereus Pharmaceuticals isolated seven additional compounds in the salinosporamide series (salinosporamides D–J, **10–16**) from *S. tropica* strain NPS000465 (CNB-476).[63] These compounds largely represent modifications to the C-2 chloroethyl substituent and include bromosalinosporamide (**17**), which was produced when synthetic sea salts were replaced with sodium bromide in the fermentation medium. A more detailed analysis of salinosporamide structure activity relationships revealed that replacement of the chloroethyl group with non-halogenated substituents was associated with a marked reduction in potency while halogen exchange was well tolerated.[64]



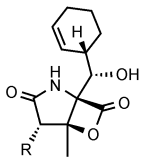
6, salinosporamide A, R = CH_2CH_2Cl
8, salinosporamide B, R = CH_2CH_3
10, salinosporamide D, R = CH_3
11, salinosporamide E, R = $CH_2CH_2CH_3$
17, bromosalinosporamide, R = CH_2CH_2Br
18, salinosporamide K, R = H
19, fluorosalinosporamide, R = CH_2CH_2F



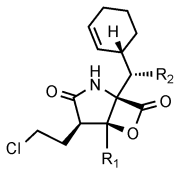
7, omuralide



9, salinosporamide C



12, salinosporamide F, R = CH_2CH_2Cl
13, salinosporamide G, R = CH_3
14, salinosporamide H, R = CH_2CH_3



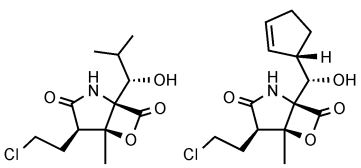
15, salinosporamide I, R_1 = CH_2CH_3, R_2 = OH
16, salinosporamide J, R_1 = CH_3, R_2 = H

As of this writing, the most recent natural product reported in the salinosporamide series from a *Salinispora* spp. is salinosporamide K (**18**). This compound was discovered by genome mining following the surprising observation of a biosynthetic pathway related to that reported for salinosporamide A in *S. pacifica* strain CNT-133.[65] This pathway lacked the genes associated with the biosynthesis of the chloroethyl substituent in salinosporamide A and as predicted yielded a product that lacked substitution at the C-2 position. In a follow-up analysis of 61 *S. pacifica* strains, 15 tested positive for the *sal* pathway and salinosporamide K production was confirmed in one additional strain.[66] Phylogenetic analyses were used to infer that the *sal* pathway was acquired prior to the *S. tropica*–*S. pacifica* split and subsequently evolved independently in these two species with gene deletion accounting for the loss of the chloroethylmalonyl-CoA pathway in *S. pacifica*.[66] These studies, along with the discovery of the related compounds cinnabaramides A–G and the associated biosynthetic pathway from a *Streptomyces* sp.,[67,68] provided some of the first evidence of the evolutionary complexity associated with secondary metabolism in *Salinispora* species. The *sal* pathway has more recently been detected in a limited number of *S. arenicola* strains[33,44] although compound production appears to be very low in this species (unpublished data).

The structure of salinosporamide A belies its biosynthetic complexity. While the C-2 ethyl group in the deschloro-analog originates from butyrate *via* ethylbutyryl-CoA,[69] the chloroethyl group in salinosporamide A is derived from a new chlorination mechanism driven by the *S*-adenosyl-L-methionine-dependent chlorinase SalL.[70] The halogenated product 5-chloro-5-deoxyadenosine is then converted in a seven-step route to chloroethylmalonyl-CoA, which acts as an unprecedented halogenated PKS extender unit in salinosporamide A biosynthesis.[71,72] The biosynthetic pathway to chloroethylmalonyl-CoA is unique to salinosporamide A and has not yet been observed in public DNA sequence databases, thereby supporting the notion that new microbial genera harbor novel biosynthetic processes. Subsequent biosynthetic studies revealed that salinosporamides D and E are alternatively accessed from methylmalonyl-CoA and propylmalonyl-CoA substrates, respectively, with the latter representing a new PKS extender unit derived from an α,β-unsaturated fatty acid.[73] Realizing that chloroethylmalonyl-CoA is a dedicated substrate in salinosporamide A biosynthesis, its selective overproduction was achieved by the genetic manipulation of the pathway specific regulatory gene *salR2* to increase the production yield of salinosporamide A.[74] Salinosporamide's cyclohexenylalanine residue is also unique among natural products and originates *via* a newly realized pathway from prephenic acid involving the prephenate decarboxylase SalX.[75] How dihydro-4-hydroxyphenylpyruvate is converted into cyclohexenylalanine and how salinosporamide's β-lactone-γ-lactam bicyclic ring system is enzymatically constructed remain outstanding questions. Resolving the complexities of salinosporamide biosynthesis has provided new insight into the mechanisms of natural product assembly and opportunities to generate new structural diversity *via* metabolic engineering.

3.2 Engineered salinosporamides

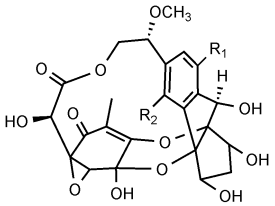
Metabolic engineering has provided unprecedented opportunities to generate new chemical diversity outside of the inherent capabilities of wild-type bacteria.[76] The first such efforts with a *Salinispora* sp. involved a combination of genetic engineering and precursor-directed biosynthesis to yield fluorosalinosporamide (**19**).[77] By inactivating the SalL chlorinase in *S. tropica*, which does not accept flouride, and adding synthetic 5′-fluorodeoxyadenosine (5′-FDA), a precursor of fluoroacetate production in *Streptomyces cattleya*,[78] it was possible to isolate **19** from a fermentation of the mutant strain. The proteasome inhibition of this compound was intermediate between that of salinosporamide A and the deschloro-analogue, with the increased energy required to break the C–F bond resulting in a reversible interaction with the active site threonine.[77] In subsequent studies, it was possible to generate **19** by replacing the *salL* chlorinase gene in *S. tropica* with the *S. cattleya* fluorinase responsible for generating the C–F bond in 5′-FDA.[79] Additional bioengineering efforts led to the production of antiprotealide (**20**),[80] originally produced as a synthetic hybrid between salinosporamide A and omuralide.[55] By deleting the *salX* prephenate decarboxylase, a series of salinosporamide derivatives with diverse natural and unnatural amino acid residues were engineered,[81] including antiprotealide and salinosporamide X7 (**21**), the latter of which displayed equal to slightly improved cytotoxic potency compared to salinosporamide A (**6**). Interestingly, **20** was subsequently shown to be produced as a natural product by *S. tropica* during the large-scale production of salinosporamide A for clinical trials.[57] Engineering approaches continue to hold great promise for the generation of additional new compounds in the salinosporamide series.[82] Detailed reviews covering various methods to produce salinosporamides including traditional fermentation, precursor-directed biosynthesis, mutasynthesis, semi-synthesis, and total synthesis provide detail on much of the work that has been done on these compounds.[83,84]



20, antiprotealide 21, salinosporamide X7

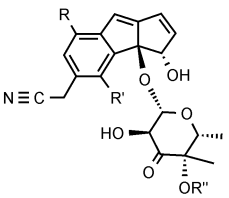
3.3 Other new compounds

Subsequent studies of *S. tropica* strain CNB-392 led to the isolation of sporolides A (**22**) and B (**23**).[85] It was proposed that these compounds are non-enzymatically derived *via* a Bergman cyclization reaction from an unstable nine-membered enediyne precursor,[86] which was subsequently supported by the analysis of the sporolide (*spo*) gene cluster.[87] Nine-membered enediynes are notoriously difficult to isolate in the absence of an apoprotein due to their lack of stability.[88] Although there was no biological activity reported for sporolides A and B, *in silico* target prediction showed a maximum docking score with HIV-1 reverse transcriptase, with the activity of sporolide B confirmed *in vitro* using a fluorescent assay.[89] A synthesis of the sporolide ring framework has been achieved[90] along with the total synthesis of sporolide B.[91,92]

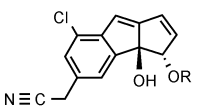


22, sporolide A, R_1 = H, R_2 = Cl
23, sporolide B, R_1 = Cl, R_2 = H

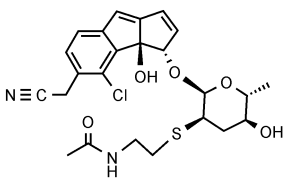
S. pacifica is the most diverse of the three species in terms of molecular systematics.[26] It also maintains considerably greater PKS and nonribosomal peptide synthetase (NRPS) diversity than the other two species.[44] In addition to the lomaiviticins, the second structurally novel series of compounds discovered from *S. pacifica* were the cyclopenta[*a*]indene glycosides cyanosporaside A (**24**) and B (**25**), with the producing strain CNS-103 isolated from a sediment collected in Palau.[93] The similarity of the cyanosporaside aglycone to the cycloaromatization product of the *Streptomyces*-derived nine-membered enediyne compound C-1027[94] led to the hypothesis that, like sporolides A and B, **24** and **25** are also derived from an enediyne precursor. Subsequent studies using a different *S. pacifica* strain (CNS-143) yielded four additional compounds in the series (cyanosporosides C–F) (**26–29**) and the first genetic evidence supporting the cyanosporaside's enediyne biosynthetic origin.[95] As might be expected, none of these enediyne cycloaromatization products have the potent cytotoxic activities associated with the predicted parent molecules, none of which have been isolated to date.



24, cyanosporaside A, R = H, R' = Cl, R'' = H
25, cyanosporaside B, R = Cl, R' = H, R'' = H
26, cyanosporaside C, R = H, R' = Cl, R'' = acetyl



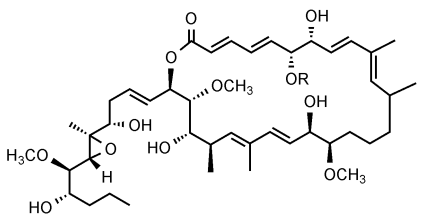
27, cyanosporaside D, R = acetyl
28, cyanosporaside E, R = H



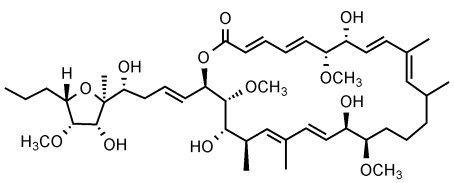
29, cyanosporaside F

S. arenicola is the most broadly distributed and abundant of the three species.[32] It has also been the source of a number of interesting new compounds in addition to some well-known actinomycete secondary metabolites. The first new structures reported from this species were the 26-membered ring macrolides arenicolides A–C (**30–32**) isolated from strain CNR-005.[96] These compounds were discovered using LC-MS based

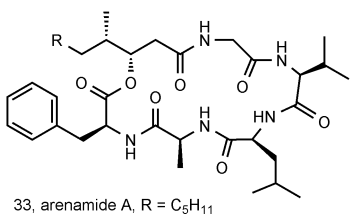
screening with A and B being simple methyl derivatives and C possessing a substituted tetrahydrofuran ring potentially generated from a cyclization of the epoxide. Three additional *S. arenicola* strains were found to produce arenicolide A and expression studies linked a specific ketosynthase sequence to its biosynthesis.[97] All four of the arenicolide-producing strains originated from separate samples collected around the island of Guam, supporting the concept that location plays an important role in secondary metabolism.[44] Genome sequence data and molecular networking (Duncan *et al.*, unpublished data) provided strong circumstantial evidence linking these compounds to the gene cluster identified as PKS28, which was only observed in one of 75 *Salinispora* genome sequences, further supporting the restricted distribution of this pathway among *Salinispora* strains. Another group of new compounds that also appear to be rare among *S. arenicola* strains are the arenamides. These cyclohexadepsipeptides (**33–35**) were discovered by comparative LC-MS analysis of crude extracts, which revealed that strain CNT-088 produced compounds not previously observed from this species.[98] Once isolated, arenamides A and B demonstrated NFκB inhibition and anti-inflammatory activity. To date, this is the only strain from which these compounds have been detected.



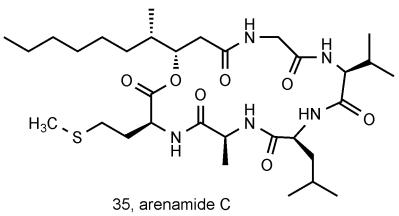
30, arenicolide A, R = CH_3
31, arenicolide B, R = H



32, arenicolide C

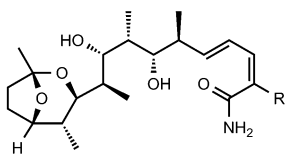


33, arenamide A, R = C_5H_{11}
34, arenamide B, R = C_3H_7

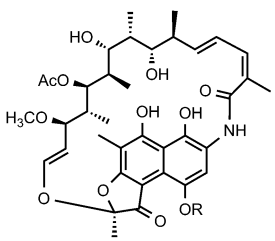


35, arenamide C

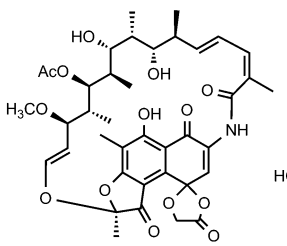
Continued studies of *S. arenicola* strain CNR-005 along with concurrent studies of strain CNR-059 led to the isolation of the bicyclic polyketides saliniketals A (**36**) and B (**37**).[99] Saliniketals A and B were found to inhibit ornithine decarboxylase induction, an important target for the chemoprevention of cancer, with IC50 values of 1.95 and 7.83 μg mL^{-1}, respectively. These compounds possess unusual structural features that have inspired at least three total syntheses.[100–102] Structural similarities between the saliniketals and the ansa chain of the rifamycin class of antibiotics, which co-occur in the fermentation extract, led to questions about the biosynthetic origin of these compounds, which were ultimately shown to be by products of the rifamycin biosynthetic pathway.[103] Rifamycins were first reported in *Salinispora* spp. from the sponge-derived strain M403, which was shown to produce both rifamycin B (**38**) and SV (**39**).[104] Subsequent studies included the development of an HPLC-MS-MS method capable of detecting picomolar concentrations of compounds in this class[105] and provided evidence that *S. arenicola* strains were also capable of producing rifamycins O (**40**) and W (**41**).[20] A new antibiotic in the rifamycin series, salinisporamycin (**42**), was also reported from a sediment-derived *S. arenicola* strain.[106] The ability of rifamycin-producing *S. arenicola* strains isolated from a marine sponge to inhibit *Mycobacterium* strains isolated from the same sponge was used to suggest that rifamycins may function in competition against sponge microbial community members.[107] Rifamycins were shown to be produced throughout the *S. arenicola* growth cycle, however the amount produced was time[108] and



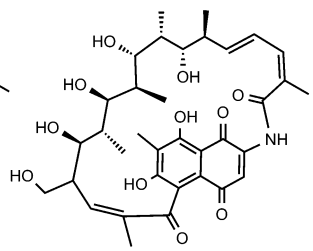
36, saliniketal A, R = H
37, saliniketal B, R = OH



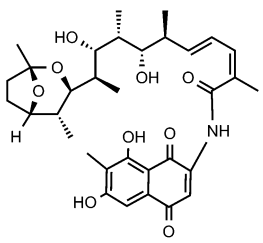
38, rifamycin B, R = CH_2COOH
39, rifamycin SV, R = H



40, rifamycin O



41, rifamycin W



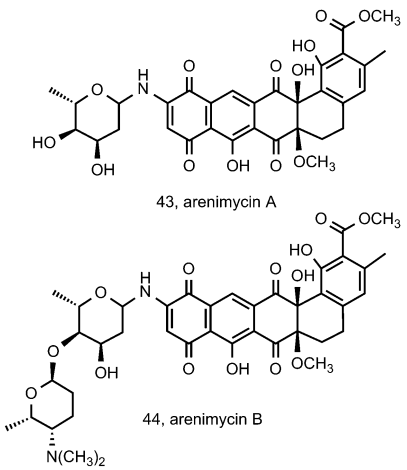
42, salinisporamycin

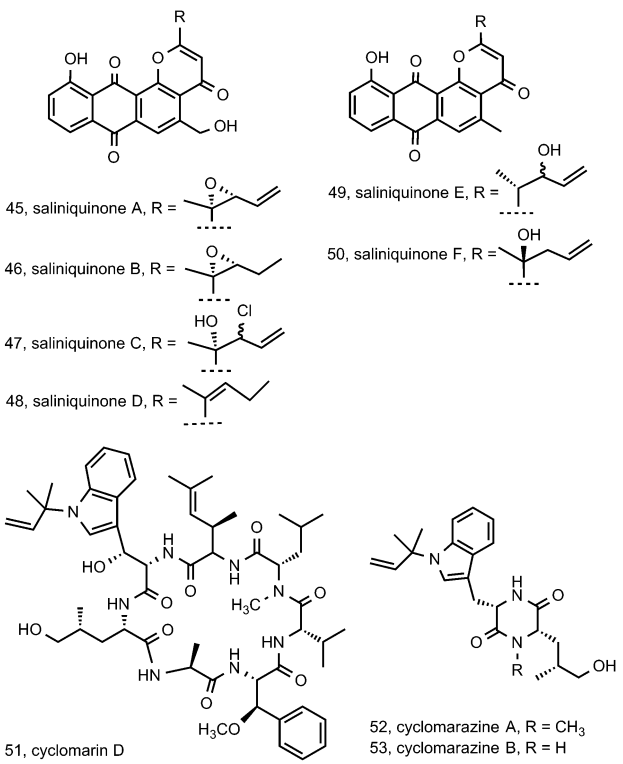
salinity[109] dependent with rifamycins S and W achieving maximum concentrations after 29 days.[110]

As part of our efforts to isolate new compounds from *Salinispora* species using traditional bioassay-guided approaches, we observed that certain compounds were produced in taxonomic-specific patterns. This concept was addressed in more detail and led to the conclusion that some compounds are "species specific", *i.e.*, they were consistently produced by members of the same species.[21] In the case of *S. arenicola*, species-specific compounds include rifamycins and staurosporines while *S. tropica* strains consistently produce salinosporamides.[21] Similar patterns have not been detected at the species level for *S. pacifica*. Subsequent analyses of *Salinispora* genome sequences support these observations[44] while studies of strains derived from Great Barrier Reef sponges confirmed the association between rifamycin production and *S. arenicola*.[20] The fixation of certain gene clusters at the species level, regardless of geographic origin, provides clear evidence of selection and implies that the small molecule products of these pathways are associated with important ecological functions that may help distinguish the three species. However, this is not to imply that other species cannot also produce either identical or related compounds, as is the case for the rifamycins, staurosporines, and salinosporamides.[67,111,112]

The consistent production of compounds in the rifamycin class by *S. arenicola* strains creates challenges when screening for antibiotic activity. In an effort to discover new antibiotics from *Salinispora* spp., an extract library exceeding 2000 testing units was screened against a rifamycin-resistant MRSA strain. Of the six strains that showed promising activity, *S. arenicola* strain CNR-647 was investigated further leading to the isolation of the new antibiotic arenimycin A (**43**).[113] This compound belongs to the benzo[α]naphthacene class of antibiotics with an *N*-linked 2-*O*-methyl-L-rhamnose residue and provides another example of a compound that is rarely observed among *Salinispora* isolates. The arenimycins were subsequently linked to the *arn* gene cluster in *S. arenicola* strain CNB-527 using a glycogenomic approach, which also led to the discovery of a second compound in the series, arenimycin B (**44**).[114] Its structure contains a disaccharide unit that is associated with improved MRSA activity. Experimental support linking the *arn* cluster to the arenimycins comes from the heterologous expression of the cluster from a desert soil eDNA sample, which led to the discovery of two additional compounds in the series.[115] Compounds such as the arenimycins, which are rarely observed among *Salinispora* strains, were originally termed "accessory" metabolites[21] and there was some evidence that their production is linked to specific geographic locations.[97] This ultimately led to the hypothesis that strains acquire pathways from the local gene pool and that sampling from diverse locations would increase the likelihood of discovering new secondary metabolites from otherwise highly similar strains.[44]

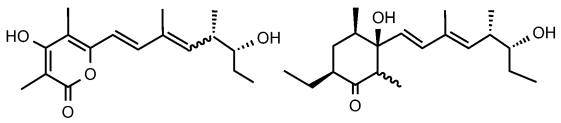
Another example of the metabolic diversity among strains that are clonal at the 16S rRNA level comes from *S. arenicola* strain CNS-325, which yielded saliniquinones A–F (**45–50**).[116] These six new anthraquinone-γ-pyrones are highly cytotoxic and represent the first members of the pluramycin class to contain both a terminal olefin and five carbons in the C-2 side chain. A final example of *S. arenicola* metabolic diversity was the isolation of compounds in the previously described cyclomarin class[117] including cyclomarin D (**51**), a new compound in the series, from strain CNS-205.[118] Cyclomarins were produced by only two of 46 *Salinispora* strains examined as part of a chemotyping study[21] and the pathway was observed in only one of 75 *Salinispora* genome sequences[44] indicating the rarity of this gene cluster. Interestingly, the cyclomarins were originally reported from a marine-derived *Streptomyces* sp.[117] suggesting the gene cluster may have been exchanged among these sediment-inhabiting taxa. In the course of studying cyclomarin biosynthesis, two new diketopiperazines cyclomarazines A (**52**) and B (**53**) were isolated from *S. arenicola* CNS-205 and shown to share a common biogenesis with the cyclomarins.[118] Functional





characterization of the prenyltransferase CymD in the cyclomarin (*cym*) pathway[119] provided a mechanism to generate unnatural *N*-alkylated tryptophan derivatives in the cyclomarin series.[120]

While the 16S rRNA gene has proven too conservative to delineate the three *Salinispora* spp. using phylogenetic approaches, it is well known that even minor differences in this locus can correspond to major differences in genome content, and thus detailed analyses have been performed to document all *Salinispora* 16S rRNA sequence variants reported to date.[27,32] These efforts have required a careful monitoring of the position of all variable nucleotides relative to the level of conservation for that region of the gene and have provided a method to distinguish among 16S rRNA sequence variants or "sequence types" based on single nucleotide polymorphisms, each of which has been assigned a letter. These sequence types have proven to be of value in terms of targeting strains for secondary metabolite production, with one of the first such applications coming from a study of *S. pacifica* strain CNS-237, which differed from the cyanosporaside-producing *S. pacifica* strains by three base pairs. CNS-327 was found to produce the new polyketides salinipyrones A (**54**) and B (**55**) and pacificanones A (**56**) and B (**57**).[121] The pacificanones bear a uniquely substituted cyclohexanone ring, while the similarity in the two structure classes suggests a common type I PKS biosynthetic origin with the differences potentially due to module skipping.[121]



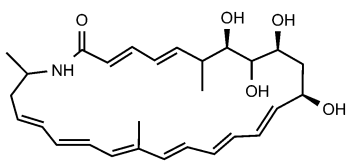
54, salinipyrone A, *Z* olefin
55, salinipyrone B, *E* olefin

56, pacificanone A, *alpha* Me
57, pacificanone B, *beta* Me

4 Genome-aided natural product discovery

Genome mining has added an important new dimension to the field of natural product discovery[122] and to our understanding of the ecology and evolution of secondary metabolism.[44] The first *Salinispora* genome sequence analyzed was that of *S. tropica* strain CNB-440, which revealed a total of 17 diverse biosynthetic pathways of which only four had been linked to their respective products.[35] Among these was a type I PKS that created problems with the genome assembly due to the highly repetitive nature of the modules comprising the pathway. Using reverse genome mining, the preliminary structure of the macrolactam salinilactam A (**58**), isolated from this strain, revealed a framework that was consistent with this pathway. Further structure elucidation revealed that salinilactam A was derived from a PKS with at least 10 extension modules, information that ultimately proved critical for the assembly of the pathway and closure of the genome. Once assembled, a bioinformatic analysis of the pathway facilitated the elucidation of the structure, which proved problematic due to its instability and overlapping olefinic NMR signals.[35]

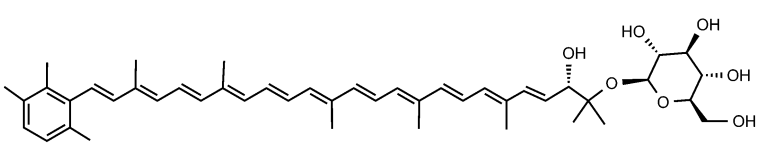
A dominant phenotypic trait associated with *Salinispora* cultures is their orange pigmentation. While this was assumed to be due to carotenoid production, the biosynthetic origin and structures of these compounds had not been defined. A bioinformatic search of the *S. tropica* strain CNB-440 genome revealed genes associated with carotenoid biosynthesis in four distinct chromosomal regions.[123] Genetic investigations confirmed that, contrary to what is typically observed in bacteria, carotenoid biosynthesis in *Salinispora* spp. is not due to a single gene cluster. The structure of this pigment was assigned as a new, C-40 carotenoid called sioxanthin (**59**), which is glycosylated on one end of the molecule and contains an aryl moiety on the other. Glycosylation is unusual among actinomycete carotenoids, and sioxanthin joins a rare group of compounds that possess both polar and non-polar head groups. Additional genome mining efforts targeted a second terpenoid gene cluster that was shared between CNB-440 and CNS-205.[36] Called *terp1* in *S. arenicola* strain CNS-205, recombinant expression studies were used demonstrate that this three-gene cluster produces the new diterpenoid isopimara-8,15-diene-19-ol (**60**).[124] This compound was not observed in cultures of the native strain suggesting the cluster is either inactive or expressed only under certain conditions.



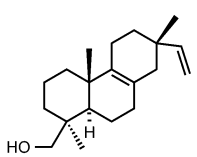
58, salinilactam

5 Previously described secondary metabolites and new derivatives

As already mentioned, previously described compounds reported from *Salinispora* strains include staurosporines and rifamycins, which have been discussed above in the context of species-specific production.[21] It is interesting to note that these

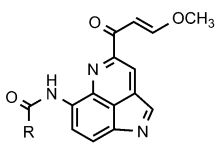


59, sioxanthin

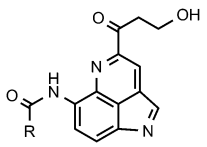


60, isopimara-8,15-diene-19-ol

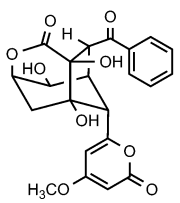
two secondary metabolites, which are consistently observed from *S. arenicola*, are among the most potent, biologically active compounds reported from the genus. Additional known compounds reported from *Salinispora* spp. include lymphostin (**61**), a potent immunosuppressant originally isolated from a *Streptomyces* sp.[125] Lymphostin shares structural similarities with the ammosamides, reported from a marine-derived *Streptomyces* sp.,[126] and belongs to the diverse class of pyrroloquinoline natural products. The molecular basis for lymphostin biosynthesis has been determined *via* interrogation of the *lym* gene cluster, which includes a uniquely organized modular synthetase.[127] Fermentation studies designed to induce lymphostin production also yielded the new derivative lymphostinol (**62**) along with the eight additional analogues neolymphostin A–D (**63–66**) and neolymphostinol A–D (**67–70**). The *lym* pathway represents a rare example of one that is common to the vast majority of *Salinispora* strains.[44] Another previously described secondary metabolite was predicted based on the detection of a gene cluster with a high level of homology to the enterocin pathway[44] in three *S. pacifica* strains. Interestingly, and as in the case of the cyclomarins, this pathway has also been observed in a marine-derived *Streptomyces* sp.[128] Given that enterocin (**71**) had not been previously reported from *Salinispora* strains, the pathway was instead heterologously expressed in two different *Streptomyces* hosts using the recently developed yeast-mediated transformation-associated recombination technique known as TAR cloning.[129] This experiment represents the first successful heterologous expression of a *Salinispora* secondary metabolite gene cluster and opens the door for future studies targeting orphan biosynthetic gene clusters in this genus.



61, lymphostin, R = methyl
63, neolymphostin A, R = isopropyl
64, neolymphostin B, R = ethyl
65, neolymphostin C, R = sec-butyl
66, neolymphostin D, R = isobutyl

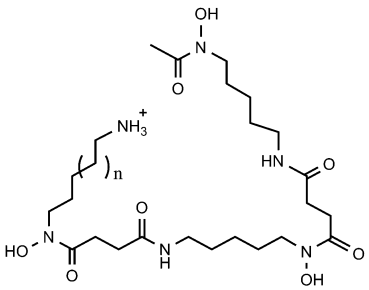


62, lymphostinol, R = methyl
67, neolymphostinol A, R = isopropyl
68, neolymphostinol B, R = ethyl
69, neolymphostinol C, R = sec-butyl
70, neolymphostinol D, R = isobutyl

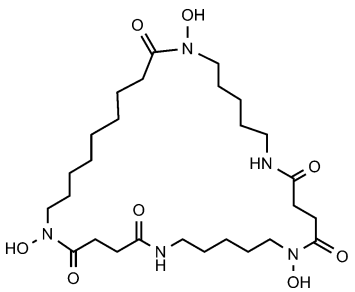


71, enterocin

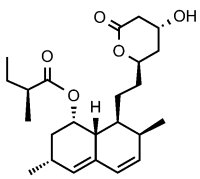
Salinispora genomes are also enriched in biosynthetic gene clusters predicted to encode the biosynthesis of siderophores,[36] small, high affinity, iron-chelating compounds secreted by bacteria.[130] Gene inactivation experiments suggested that the siderophore associated with the *des* pathway was the primary iron chelator in both *S. tropica* and *S. arenicola*.[131] Bioassay-guided fractionation confirmed the production of desferrioxamines B (**72**) and E (**73**) in both species, which were formally linked to the *des* pathway *via* gene inactivation. Subsequent studies using $Ni_{(II)}$-based immobilized metal ion chromatography led to the isolation of six additional desferrioxamine siderophores including the new analogue desferrioxamine N (**74**).[132] A recent report also describes the isolation of the known fungal metabolite mevinolin (**75**) from two different *S. arenicola* strains,[133] which led the authors to suggest the possibility of horizontal gene transfer between fungi and bacteria. Given that this compound appears to be a common fungal metabolite,[134] the most parsimonious explanation is that the gene cluster was acquired by *S. arenicola*, a hypothesis that can be readily tested by determining its relationship to the iterative type I PKS responsible for mevinolin (lovastatin) biosynthesis in fungi.



72, desferrioxamine B, n = 1
74, desferrioxamine N, n = 2



73, desferrioxamine E



75, mevinolin

6 Conclusions

Since its discovery 23 years ago,[22] the genus *Salinispora* has become a robust model for natural product research. It speaks to the value of assigning formal taxonomic names, which have provided opportunities to address species level differences in secondary metabolism, and the associated deposition of type strains, which have been accessed by researchers worldwide. The acquisition of thousands of strains from global collection sites over a twenty-plus year research endeavor at both the Scripps Institution of Oceanography and other universities around the world has created a resource that may truly be

unparalleled in terms of creating opportunities to compare natural product biosynthesis among closely related environmental bacteria. These types of comparisons have begun to reveal the enormous complexities associated with natural product gene evolution[44] and will continue to provide insight into the mechanisms by which bacteria generate new structural diversity. Research on *Salinispora* spp. has helped clarify the concept that new microbial taxa, especially those inhabiting poorly studied environments such as the world's oceans, represent a promising resource for natural product discovery. Certainly the ratio of new to known compounds discovered from this genus (Table 1) supports this concept. Interestingly, the first two discoveries, lomaiviticins A and B and salinisporamide A, represent two of the most promising biomedical leads discovered to date from the genus. While this may be largely due to chance, it does suggest that the discovery of new taxa can bring an initial wealth of new chemical structures.

The development of new methodologies in genome sequencing, bioinformatics, molecular genetics, and a better understanding of the biosynthetic principles that govern natural product assembly have driven the ongoing resurgence in natural product research. Coupled with improved mass spectral-based analytical approaches such as peptidogenomics,[135] glycogenomics,[114] and molecular networking,[136] it has become possible to interrogate strains using highly informed approaches that eliminate some of the randomness traditionally associated with natural product discovery. While the initial *Salinispora* discoveries were largely based on traditional cultivation and screening approaches, the more recent discoveries have been driven by genomics and genetic manipulations. These advances speak to the value of interdisciplinary collaboration and the importance of developing new approaches to natural product discovery. Certainly one of the challenges that remains in the exploitation of this genus, and this is by no means limited to *Salinispora* spp., is translating the unrealized biosynthetic potential observed in genome sequence data into new chemical discoveries.

7 Acknowledgements

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