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Exploration and engineering of biosynthetic pathways in the marine actinomycete *Salinispora tropica**

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Abstract: In recent years, members of the marine actinomycete genus *Salinispora* have proven to be a precious source of structurally diverse secondary metabolites, including the potent anticancer agent salinosporamide A and the enediyne-derived sporolides. The tremendous potential of these marine-dwelling microbes for natural products biosynthesis, however, was not fully realized until sequencing of the *Salinispora tropica* genome revealed the presence of numerous orphan biosynthetic loci besides a plethora of rare metabolic pathways. This contribution summarizes the biochemical exploration of this prolific organism, highlighting studies in which genome-based information was exploited for the discovery of new enzymatic processes and the engineering of unnatural natural products. Inactivation of key genes within the salinosporamide pathway has expanded its inherent metabolic plasticity and enabled access to various salinosporamide derivatives by mutasynthesis. New insights into the biosynthesis of the sporolides allowed us to increase production titers of these structurally complex molecules, thereby providing the means to search for the DNA cleaving presporolide enediyne.

Keywords: biosynthesis; mutasynthesis; *Salinispora*; salinosporamides; sporolides.

INTRODUCTION: *SALINISPORA*—NATURAL PRODUCT PRODUCERS FROM THE SEA

The genus *Salinispora* constitutes a discrete group of actinomycete bacteria thriving in marine environments. Originally discovered in Bahamian sediments in 1991, these organisms were later found to have a cosmopolitan distribution [1,2]. Unlike many other spore-forming bacteria that can be retrieved from near-shore locations, *Salinispora* has an obligate requirement of seawater for growth. In phylogenetic analyses based on 16S rRNA gene sequence comparisons, the *Salinispora* strains form a distinct monophyletic clade within the Micromonosporaceae [2]. Subsequent morphological studies revealed a number of phenotypic peculiarities that distinguished the marine isolates from other genera classified in this family, strongly supporting the idea to combine these strains in a novel taxon. In 2005, the genus was formally described along with the two species that had been cultivated at that time: *Salinispora tropica* and *Salinispora arenicola* [3]. To date, several thousand isolates have been cultured from numerous tropical and subtropical locations around the world and clear biogeographical patterns have

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been recognized. These studies have also led to the recognition of a third species for which the name *Salinispora pacifica* has been proposed [4].

Since their first isolation in the early 1990s, actinomycetes belonging to the genus *Salinispora* have attracted a lot of interest due to their rich and diverse metabolome including one compound (salinosporamide A) that is currently in human clinical trials for the treatment of cancer [5,6]. A recent study on various *Salinispora* strains revealed that the majority of secondary metabolites are produced in species-specific patterns [7]. Coinciding with minor alterations in the 16S rRNA gene sequence, the metabolic profile represents a consistent phenotype, conforming to a model of selection-driven pathway fixation. It is tempting to speculate that secondary metabolism contributed to niche differentiation and speciation in *Salinispora*. The finding of different chemotypes in closely related species further suggests that the biosynthetic pathways for many natural products in *Salinispora* were not inherited vertically from a common ancestor, but rather have been acquired by means of lateral gene transfer [7].

THE *SALINISPORA* GENOME PROJECT

The opportunity to use *Salinispora* as a model organism to address questions about the interplay between speciation and ecological differentiation as well as its biotechnological value with regard to antibiotic production were primary motivations for the whole genome sequencing of *S. tropica* CNB-440 and *S. arenicola* CNS-205. The Salinispora Genome Project was initiated in collaboration with the Joint Genome Institute in 2006. Since then both chromosomes have been completely assembled, and the annotated sequences have been released to the public (accession nos. CP000667 and CP000850, respectively).

As expected for actinomycete bacteria, both *Salinispora* species exhibit a high G+C content around 70 % (Table 1). At 5–6 Mbp, their genomes are smaller than those of *Streptomyces* species, but they are similar in size to other actinomycetes that harbor circular chromosomes, such as *Frankia* sp. CcI3 and *Nocardia farcinica*. Bacteria within the order Actinomycetales are a well-known source of antibiotics, and the genus *Salinispora* is no exception to this rule. Altogether 19 biosynthetic gene clusters have been annotated from the *S. tropica* genome [8], while the *S. arenicola* genome harbors at least 30 biosynthetic loci (M. N., D. W. Udwary, P. R. Jensen, B. S. M., unpublished observations). Among the products of these pathways are siderophores, polyketides, nonribosomal peptides, ribosomal peptides, terpenoids, melanin pigments, and indolocarbazoles. While a total of nine compounds have been characterized from the sequenced organisms (the structure of the polyene salinilactam A was actually solved in part due to genome analysis) [8], it is clear that the vast majority of the biosynthetic potential in each of these strains has yet to be realized (e.g., by selecting fermentation conditions which induce the expression of dormant loci). The *Salinispora* Genome Project did not only reveal novel and therefore attractive targets for genome mining, but also provided a wealth of information about how secondary metabolites are produced. Exploiting this knowledge, it became possible to manipulate specific pathways with the rationale to generate new structural diversity. This approach was particularly appealing with regard to the drug candidate salinosporamide A.

Table 1 Actinomycete genome data and biosynthetic potential.

Organism	Size, Mb	Topology	%G+C content	Open reading frames (ORFs)	Secondary metabolite biosynthetic loci[a]
S. tropica CNB-440	5.18	Circular	69.8	4654	≥19 (5)
S. arenicola CNS-205	5.79	Circular	69.5	5172	≥30 (4)
S. coelicolor A3(2)	8.67	Linear	72.1	7912	≥22 (13)
S. avermitilis ATCC 31267	9.03	Linear	70.7	7669	≥30 (7)
S. griseus IFO 13350	8.55	Linear	72.2	7224	≥34 (8)
S. erythraea NRRL 23338	8.21	Circular	71.1	7264	≥25 (2)
Frankia sp. CcI3	5.43	Circular	70.1	4618	ND
Nocardia farcinia IFM 10152	6.02	Circular	70.8	5747	ND

[a]The number of clusters whose products have been characterized is given in parentheses. *S. tropica*: salinosporamide, sporolide, salinilactam, lymphostin, desferrioxamine; *S. arenicola*: cyclomarin-cyclomarazine, rifamycin-saliniketal, staurosporin, desferrioxamine; *S. coelicolor*: actinorhodin, calcium-dependent antibiotic, prodiginine, TW95a, albaflavenone, germicidin, desferrioxamine, coelichelin, geosmin, 2-methylisoborneol, isorenieratene, hopene, butyrolactone; *S. avermitilis*: avermectin, oligomycin, filipin, melanin, geosmin, pentalenolactone, squalene; *S. griseus*: streptomycin, grixazone, desferrioxamine, isorenieratene, HPQ melanin, alkylresorcinol, geosmin, 2-methylisoborneol; *S. erythraea*: erythromycin, 2,5,7-trihydroxy-1,4-naphthoquinone (flaviolin).

SALINOSPORAMIDES—GENETIC ENGINEERING OF UNNATURAL PROTEASOME INHIBITORS

Background

Salinosporamide A was originally discovered in 2003 by Fenical and coworkers at the Scripps Institution of Oceanography [9]. The rare γ-lactam-β-lactone ring system of this marine natural product was reported just once before in omuralide, a transformation product of lactacystin characterized by Omura and coworkers in 1991 (Fig. 1) [10]. Extensive studies on lactacystin and omuralide have since shown that these natural products inhibit the proteasome, a multisubunit protease complex responsible for ubiquitin-mediated protein degradation [11], through a covalent interaction with the active site N-terminal threonine residue in the 20S catalytic core [10]. Salinosporamide A has enhanced potency over omuralide (IC_{50} values of 1.3 vs. 49 nM, respectively, against purified 20S proteasome), which implicates the different functionalization of the pharmacophore as being mechanistically significant [9]. Recent crystal structures of the yeast 20S proteasome catalytic core in complex with omuralide and salinosporamides A and B have illuminated the mechanism of this family of β-lactone inhibitors in which the drug is linked through an ester bond to the side chain hydroxyl group of the N-terminal threonine residue [12,13]. In the case of salinosporamide A, further chemistry ensues in which the newly created C3 hydroxyl adds to the C2 chloroethyl group to give a cyclic ether (Fig. 2). This intramolecular nucleophilic addition is unique to salinosporamide A and was first observed with the natural product in aqueous solution [14]. The consequence of the formation of the tetrahydrofuran ring is that it renders the ligand irreversibly bound to the enzyme, as the C3 oxygen atom occupies the same position as that for the hydrolytic water molecule in the unligated enzyme, thereby hindering deacylation [13]. The unreactive C2 methyl and ethyl groups in omuralide and salinosporamide B, however, prevent the formation of a tetrahydrofuran ring, rendering the covalent ester bond between proteasome and inhibitor prone to hydrolysis. Without the chloro leaving group, the C3 hydroxyl group in the ligated omuralide and salinosporamide B complexes may either reform the β-lactone ring or alternatively allow a water molecule to bind for hydrolysis. Depending on the reactivity of the C2 substituent, nature has thus evolved both potent reversible and irreversible inhibitors of the proteasome around the γ-lactam-β-lactone pharmacophore.

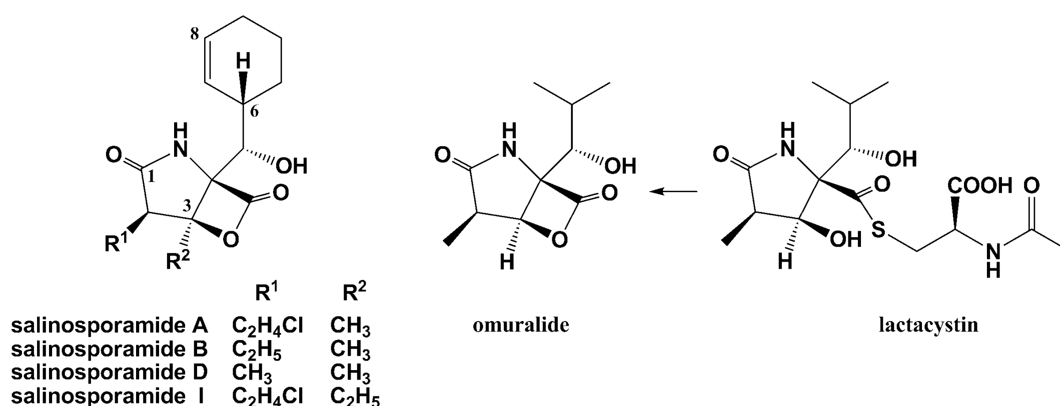

Fig. 1 Structures of the salinosporamides, omuralide, and lactacystin.

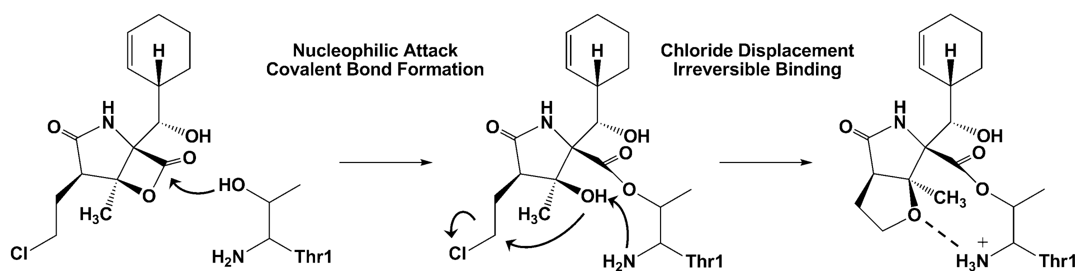

Fig. 2 Mode of action of salinosporamide A against the 20S proteasome and reactivity of the *N*-terminal nucleophilic threonine residue.

Biosynthesis and bioengineering of salinosporamides

The structures of the naturally occurring salinosporamides suggested that they originate from three biosynthetic building blocks, namely, two variable small-chain carboxylic acids and an amino acid. This hypothesis was largely based on the observation of different substitution patterns at C2 (methyl, ethyl, chloroethyl) and C3 (methyl, ethyl) (Fig. 1) [15]. Although the related lactacystin and its cyclization product omuralide were shown through feeding experiments to originate from valine-derived isobutyrate, leucine, and cysteine [10], we hypothesized that this pathway was not compatible for the salinosporamides due to the variable nature of the lower half of the molecule. To elucidate the identity of the salinosporamide building blocks, a series of labeling studies with stable isotopes was carried out. Initial feeding experiments with [U-$^{13}C_6$]glucose, [1,2-$^{13}C_2$]acetate, [1-^{13}C]butyrate, [1-^{13}C]phenylalanine, and [1,7-$^{13}C_2$]shikimate were particularly illuminating as they (1) confirmed that the salinosporamides are biosynthesized from three metabolic building blocks, (2) suggested an unprecedented polyketide synthase (PKS)–nonribosomal peptide synthetase (NRPS) hybrid pathway, (3) outlined the formation of the novel amino acid 3-cyclohex-2-enylalanine via a shunt in the phenylalanine pathway, and (4) showed that the chloroethyl and ethyl side chains in salinosporamides A and B, respectively, originate from two different precursors [16]. We originally expected salinosporamide B being a precursor of salinosporamide A and that chlorination of an aliphatic butyrate-derived methyl carbon was catalyzed by a non-heme iron halogenase. While the ethyl side chain in salinosporamide B is indeed derived from butyrate, the chloroethyl side chain in salinosporamide A is not. Instead, the observed labeling pattern of [U-$^{13}C_6$]glucose-enriched salinosporamide A was suggestive of a tetrose origin of the chlorinated fragment. Further feeding experiments with [1-^{13}C]propionate confirmed the modular nature of the C2 side chain in salinosporamide D and proved that propionate can replace the acetate-de-

rived starter unit in salinosporamide I (L. L. Beer, B. S. M., unpublished data). Together these incorporation experiments suggested a biosynthetic pathway in which acetyl-CoA (or propionyl-CoA) and a substituted malonyl-CoA molecule are condensed by a PKS to generate a β-keto thioester, which then reacts with the non-proteinogenic amino acid to give a linear hybrid PKS–NRPS product. This intermediate would ultimately undergo a novel series of reactions to yield the bicyclic γ-lactam-β-lactone (Fig. 3).

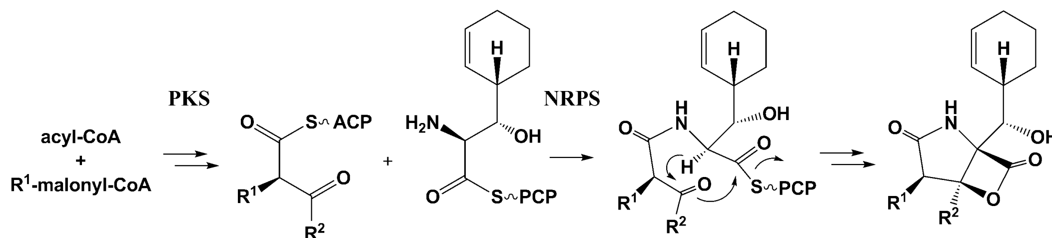

Fig. 3 Proposed biosynthesis of the salinosporamides via a hybrid PKS–NRPS pathway.

After gaining access to the genome sequence of *S. tropica*, the locus encoding salinosporamide biosynthesis was readily identified. A bioinformatic analysis revealed the pathway to be largely consistent with our previous proposal which was solely based on the isotope experiments. Moreover, the identification of the salinosporamide cluster (*sal*) provided valuable insights in an unprecedented pathway leading to the new PKS extender unit chloroethylmalonyl-CoA. To our surprise, none of the typical oxygen-based chlorinating enzymes, such as heme- and vanadium-dependent haloperoxidases that use H_2O_2 as a co-substrate or flavin- and non-heme iron-dependent halogenases that utilize dioxygen, were encoded by genes in the *sal* cluster [17]. Instead, we annotated the gene *salL*, the protein product of which is homologous (35 % amino acid identity) to the recently discovered fluorinase from *Streptomyces cattleya* [18]. Utilizing a nucleophilic substitution strategy, the latter displaces L-methionine from *S*-adenosyl-L-methionine (SAM) to generate 5'-fluoro-5'-deoxyadenosine. To test whether SalL might function in an orthogonal manner chlorinating SAM, we cloned the corresponding gene into the pET28a(+)-based expression vector pHIS8 and overexpressed the octahistidyl-tagged recombinant protein in *Escherichia coli* BL21(DE3). After purification via Ni^{2+}-affinity chromatography, the N-terminal His-tag of the recombinant protein was removed by thrombin cleavage. Incubation of the purified enzyme with SAM and sodium chloride established that SalL acts as a bona fide 5'-chloro-5'-deoxyadenosine synthase as confirmed by high-performance liquid chromatography (HPLC) analysis with authentic standards (Fig. 4) [19].

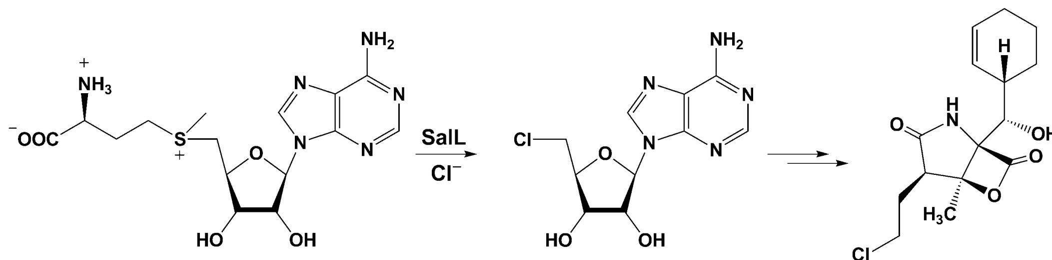

Fig. 4 Chloride is introduced into salinosporamide A via the SAM-derived intermediate 5'-chloro-5'-deoxyadenosine.

Interestingly, SalL accepted bromide and iodide as alternative substrates, but not fluoride. While kinetic data indicated that the brominase and iodinase reactions are not relevant in the natural environment of *S. tropica*, the plasticity of SalL allows for the in vivo production of bromosalinosporamide by precursor-directed biosynthesis [20,21]. When tested against the 20S proteasome the bromo and the (semisynthetic) iodo analog of salinosporamide A retained the activity of the chlorinated natural product [22]. Owing to the similar reactivity of these halogens, it can be assumed that the bromo and the iodo atom are also easily displaced by nucleophilic addition after the β-lactone has docked onto the catalytic threonine residue of the proteasome. The question about the bioactivity of a salinosporamide in which the chlorine is replaced with fluorine, however, remained unanswered. To study the effect of fluorine substitution in an engineered salinosporamide, we first had to bypass the halide specificity of the SAM-dependent chlorinase and to eliminate the background biosynthesis of chloroethylmalonyl-CoA. Therefore, we inactivated the *salL* gene by PCR-based gene replacement and supplemented the resulting mutant with synthetic 5'-fluoro-5'-deoxyadenosine. This approach resulted in the production of the desired fluorosalinosporamide (Fig. 6) [23], which was subsequently subjected to biological testing. Though the replacement of the chlorine atom in salinosporamide A by fluorine slightly reduced 20S proteasome inhibition, the engineered compound was still significantly more active than salinosporamide B. Based on the recovery of proteasome activity in a wash-out assay, the reversible binding mode of fluorosalinosporamide could be demonstrated [23]. Fluorosalinosporamide thus represents the most active reversible β-lactone proteasome inhibitor discovered so far.

We next aimed for a modification of the amino acid moiety of salinosporamide A which is known from previous structure–activity relationship (SAR) reports on omuralide to play a key role in proteasome binding [24]. The nonsymmetrical labeling pattern in salinosporamide A after feeding with [U-$^{13}C_6$]glucose suggested a pathway to the amino acid moiety that paralleled phenylalanine biosynthesis in which the cyclohexyl ring never becomes aromatized. We confirmed that while the amino acid was indeed derived from chorismate through feeding experiments with [1,7-$^{13}C_2$]shikimate and [U-$^{13}C_{10}$]chorismate, it did not originate from phenylalanine itself as [1-^{13}C]phenylalanine was not incorporated [16]. Based upon a bioinformatic analysis of the *sal* cluster, we then proposed a pathway in which prephenate is shunted into the cyclohexenylalanine pathway by the formal reduction of the pro-*R* C2'–C3' double bond. A prephenate dehydratase homolog putatively catalyzes the decarboxylative dehydration to the diene, which would be processed to the amino acid by further double bond reduction and transamination (Fig. 5).

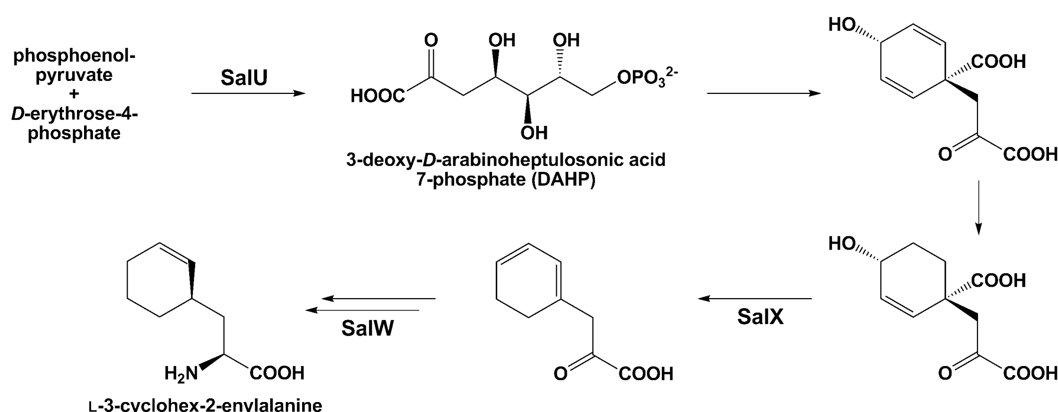

Fig. 5 Proposed biosynthesis of the novel non-proteinogenic amino acid L-3-cyclohex-2-enylalanine via a shunt in the phenylalanine pathway.

Many of the desired genes for such a pathway are encoded in the *sal* locus, including a copy of the initiating shikimic acid pathway enzyme DAHP synthase (SalU), a prephenate dehydratase homolog (SalX), and an aliphatic L-amino acid aminotransferase (SalW). Since SalU and SalW were expected to be complemented by housekeeping enzymes, we decided to target *salX* for gene disruption. The resulting mutant strain was no longer able to synthesize natural salinosporamide derivatives as confirmed by LC-MS analysis. Instead we observed the production of a new compound, which was absent in the wild-type organism. The structure of this compound was solved by NMR and unequivocally confirmed as antiprotealide, a low nanomolar proteasome inhibitor that was previously prepared by total synthesis (Fig. 6) [25]. It is evident that antiprotealide represents a biosynthetic hybrid between salinosporamide A and lactacystine/omuralide originating from chloroethylmalonate, acetate and L-leucine, respectively. Noteworthy, the compound has never been observed under any fermentation conditions before. To further probe the metabolic flexibility of the salinosporamide pathway enzymes, we chemically complemented the *salX* mutant with the nonproteinogenic amino acids L-3-cyclohexylalanine and DL-3-cyclopentylalanine. This strategy yielded the corresponding salinosporamide derivatives (Fig. 6) in increased biosynthetic yield with background production of antiprotealide. Having access to these analogs, we set out to investigate the effect of the C5 substituent toward proteasome inhibition. Antiprotealide with the smallest C5 side chain was the weakest inhibitor tested in this study, being about 40-fold less potent than salinosporamide A. The loss of the double bond in the cyclohexenyl moiety led to a 10-fold decrease in biological activity, whereas the impact of the ring contraction was less evident. In fact, the derivative containing the 5-membered saturated ring was slightly more potent than the cyclohexyl-substituted salinosporamide [26]. These results have now prompted further studies to generate salinosporamide derivatives with modifications in the amino acid-derived side chain.

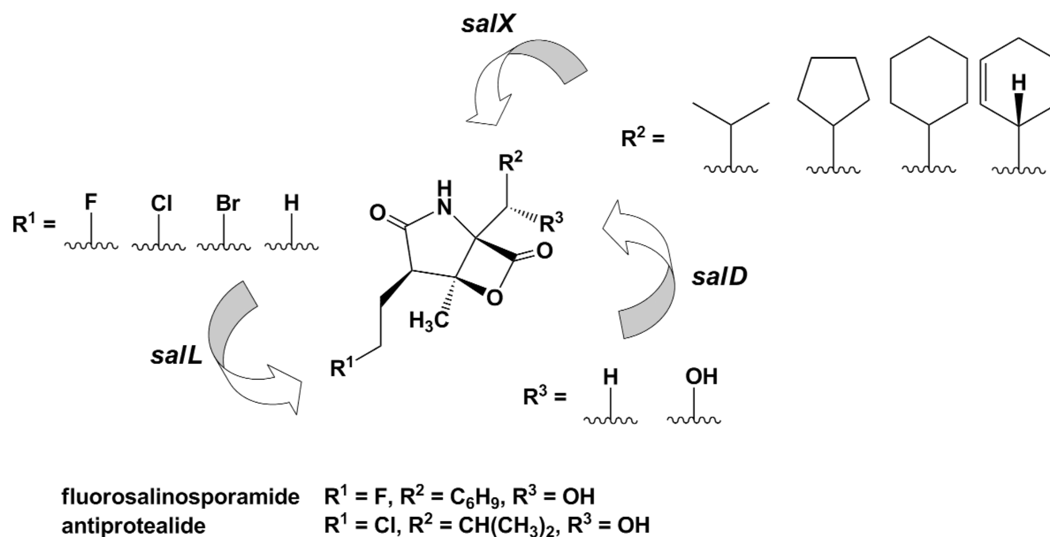

Fig. 6 β-lactone proteasome inhibitors engineered in *S. tropica*.

SPOROLIDE—OVERCOMING LIMITATIONS IN PRODUCTION

One of the rare features of the *S. tropica* genome is the presence of two independent iterative type-I polyketide gene clusters that are associated with the biosynthesis of distinct 9- and 10-membered enediyne natural products, respectively [8]. Although no enediynes have been directly characterized from *S. tropica* to date, sporolides A and B are putatively derived from a 9-membered enediyne pre-

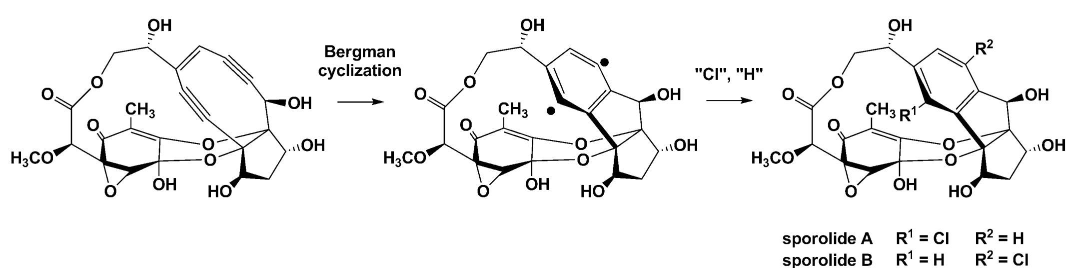

Fig. 7 Proposed cycloaromatization of a presporolide enediyne.

cursor upon cyclization to a *p*-benzyne biradical and subsequent nucleophilic addition of a chloride anion (Fig. 7) [27–29].

The candidate gene locus for sporolide biosynthesis (*spo*) comprises 47 open reading frames and encompasses two subclusters associated with the biosynthesis of the two structural fragments of the sporolide molecule, namely, the enediyne-derived cyclopenta[*a*]indene moiety and the cyclohexenone residue. To confirm the hypothesized origin of the sporolides, we inactivated the enediyne polyketide synthase gene *spoE*. A chemical characterization of the mutant confirmed the loss of sporolides (M. N., B. S. M., unpublished data). We are thus confident that the *spo* cluster indeed encodes sporolide biosynthetic enzymes. The subcluster that we postulate is involved in the synthesis, and attachment of the oxygenated cyclohexenone moiety is unique to the *spo* cluster, hence reflecting the novel chemistry of this structural residue. It comprises one gene (*spoT1*), homologs of which are known to play a key role in tyrosine metabolism. To test whether tyrosine is a building block of the sporolides, we conducted a series of feeding experiments with ^{13}C-labeled amino acids in *S. tropica*. These studies unequivocally showed the incorporation of L-tyrosine and two L-methionine-derived methyl groups into the sporolide carbon framework. Of particular interest was the finding that administration of L-tyrosine led to a 10-fold increase in sporolide production, thereby suggesting that this amino acid is limiting and providing a mechanism to ultimately search for the enediyne precursor because of increased titers. Bioinformatic analysis of *spoT1* suggested that its Fe(II)-dependent dioxygenase gene product functions as either the homologous 4-hydroxyphenylpyruvate dioxygenase or hydroxymandelate synthase, which convert 4-hydroxyphenylpyruvate to homogentisate and 4-hydroxymandelate, respectively [30]. In order to differentiate between these two enzymatic routes, we overexpressed SpoT1 in *E. coli* BL21(DE3) as an octahistidyl-tagged recombinant protein. Biochemical studies clearly revealed that SpoT1 functions as a hydroxymandelate synthase and thereby clarified the initial biochemical reaction in the pathway to the cyclohexenone unit (Fig. 8) [31]. The in vitro characterization of SpoT1 enabled us to postulate an entire biosynthetic pathway to the sporolides and has shown a further role for

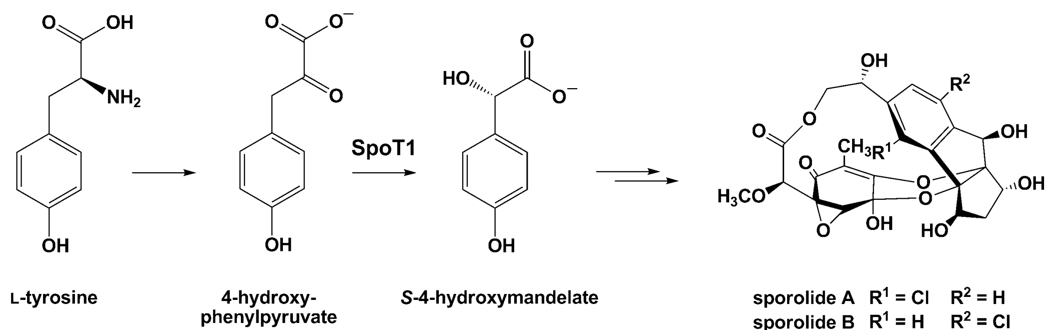

Fig. 8 Initial enzymatic reactions in the biosynthesis of the sporolides.

hydroxymandelate synthase, which until now has only been associated with the biosynthesis of hydroxyphenylglycine, a non-proteinogenic amino acid incorporated into several biologically active secondary metabolites [32].

SUMMARY

Genome sequencing has significantly impacted our understanding of microbial life and its adaptations to diverse environments. A recent trend that has emerged in the field of natural products chemistry is to exploit genomics for the discovery of orphan biosynthetic pathways and the subsequent isolation of their products, a process generally referred to as genome mining [33,34]. Endowed with unique enzymes which might reflect their environmental novelty and an above-average concentration of biosynthetic loci relative to the genome size of terrestrial actinomycetes, *S. tropica* and *S. arenicola* represent exquisite model organisms to further advance this technology. The successful adaptation of a PCR-targeted gene replacement approach has already benefited gene inactivation studies in both *Salinispora* species [8,35] and is expected to provide access to additional yet untapped chemistry in these prolific marine bacteria.

Beside the prediction of chemical structures, genome analysis has yielded valuable insights in the underlying biochemistry of natural products biosynthetic pathways. In the case of *Salinispora*, this knowledge was applied to the production of new chemical diversity as demonstrated in the case of the salinosporamides or to enhance production titers as has been shown with the sporolides. Furthermore, the discovery of an unprecedented SAM-dependent chlorination pathway associated with the novel salinosporamide A PKS extender unit chloroethylmalonyl-CoA offers intriguing opportunities for the combinatorial biosynthesis of halogenated polyketide compounds that may serve as vehicles in semisynthesis. Hence, the discovery and development of the *Salinispora* support the notion that marine actinobacteria harbor novel biosynthetic capabilities not present in their terrestrial counterparts and that these systems are biotechnologically accessible.

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