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Discovery and Assembly-Line Biosynthesis of the Lymphostin Pyrroloquinoline Alkaloid Family of mTOR Inhibitors in *Salinispora* Bacteria

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Supporting Information

ABSTRACT: The pyrroloquinoline alkaloid family of natural products, which includes the immunosuppressant lymphostin, has long been postulated to arise from tryptophan. We now report the molecular basis of lymphostin biosynthesis in three marine *Salinispora* species that maintain conserved biosynthetic gene clusters harboring a hybrid nonribosomal peptide synthetase−polyketide synthase that is central to lymphostin assembly. Through a series of experiments involving gene mutations, stable isotope profiling, and natural product discovery, we report the assembly-line biosynthesis of lymphostin and nine new analogues that exhibit potent mTOR inhibitory activity.

Since the discovery of discorhabdin C in 1986,[1] a group of cytotoxic natural products that contain a pyrroloquinoline core has emerged (Figure 1). Most members of this diverse family have been isolated from marine sponges[2] and include the batzellines, damirones, and makaluvamines.[3] More recently, compounds of this class have been obtained from terrestrial organisms, including makaluvamine A from a myxomycete[4] and the sanguinones and mycenarubins from mushrooms of the genus *Mycena*.[5] Variations in the structural complexity of these compounds arise from substitutions about the pyrrolo[4,3,2-*de*]quinoline core, which is thought to be derived from tryptophan. However, despite the large number of compounds currently known, biosynthetic studies have been hampered by the uncultivability and genetic intractability of the producer organisms.

To date, two natural products containing a pyrrolo[4,3,2-*de*]quinoline core have been isolated from bacteria: the myosin-targeting ammosamides from *Streptomyces* strain CNR-698[6] and the immunosuppressant lymphostin (**1**) that was first isolated from *Streptomyces* sp. KY11783.[7] Lymphostin has been shown to inhibit both lymphocyte kinase (IC_{50} 0.05 μM) and phosphatidylinositol 3-kinase (IC_{50} 0.001 μM).[8] This biological activity, along with lymphostin's tricyclic pyrroloquinoline skeleton, inspired a biomimetic total synthesis starting from tryptophan in 2004.[9] We report here the molecular basis for lymphostin (**1**) biosynthesis in *Salinispora* spp., which involves a uniquely

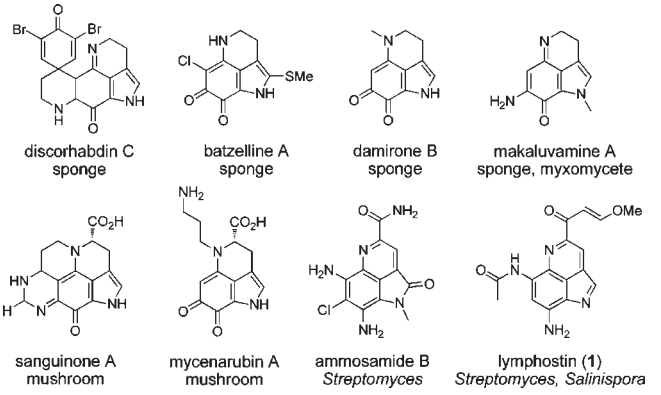

Figure 1. Structures of pyrrolo[4,3,2-*de*]quinoline natural products.

organized modular synthetase that through gene targeting provided the novel analogue lymphostinol (**2**). We also report eight new *N*-acyl derivatives, including the potent mTOR inhibitor neolymphostin A (**3**).

Genome sequencing of the actinomycetes *Salinispora tropica* strain CNB-440 and *Salinispora arenicola* strain CNS-205 revealed that these marine bacteria have the genetic capacity to produce a wide variety of structurally and biologically diverse natural products.[10] While the majority of their secondary metabolism genes are relatively unique, they share at least a dozen natural product gene clusters. Among these is the hydrid nonribosomal peptide synthetase−polyketide synthase (NRPS−PKS) locus *lym* (Stro3051−3057, Sare3277−3283), which has tentatively been assigned to encode the biosynthesis of **1** (Figure 2A).[10] The *lym* cluster is maintained in a third species, "*Salinispora pacifica*", including strain DPJ-0019, where its gene organization is conserved [Table S1 in the Supporting Information (SI)].

Bioinformatic analysis of the syntenic *lym* loci in *S. tropica*, *S. arenicola*, and "*S. pacifica*" established the approximate physical boundaries of the gene cluster, which includes seven genes

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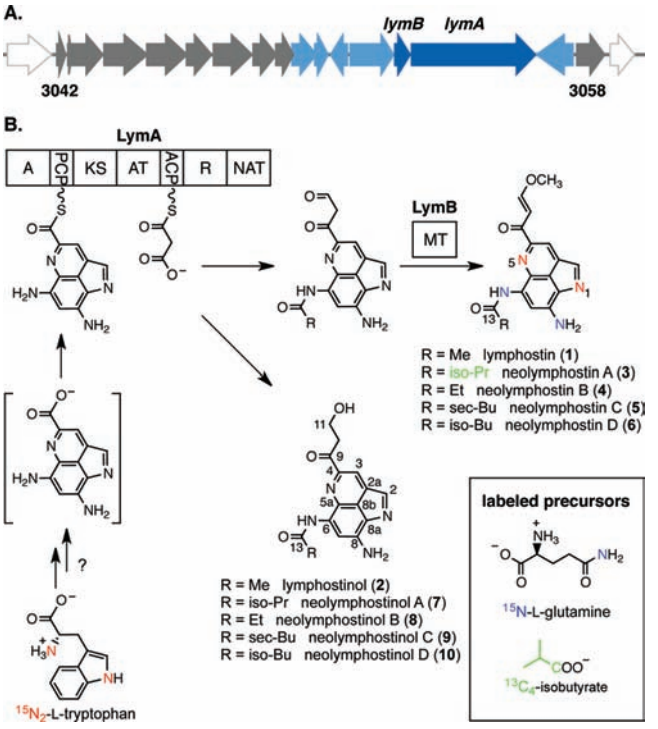

Figure 2. Organization of the *lym* gene cluster and proposed biosynthesis of lymphostin (**1**), lymphostinol (**2**), and related *N*-acyl derivatives **3**−**10**. (A) The putative *lym* gene cluster (including genes *lymA* and *lymB* that were inactivated in this study) is shown in blue and adjacent open reading frames (ORFs) associated with ribosomal peptide biosynthesis in gray. Strop gene numbers are shown flanking the cluster. Each arrow represents the direction of transcription of an ORF. See the SI for the deduced functions of the ORFs and a comparison of the *lym* gene products (Tables S2 and S3, respectively). (B) Neolymphostin A (**3**) biosynthesis was probed in "*S. pacifica*" strain DPJ-0019 by isotope labeling with $^{15}N_2$-L-tryptophan, ^{15}N-L-glutamine, and $^{13}C_4$-isobutyrate as shown. Abbreviations: A, adenylation; ACP, acyl carrier protein; AT, acyltransferase; KS, ketosynthase; MT, methyltransferase; NAT, *N*-acyltransferase; PCP, peptidyl carrier protein; R, reductase.

covering 14.8 kb (Figure 2A). The central *lymA* gene encodes a heptadomain synthetase that putatively functions to malonate-extend a tryptophan-derived peptidyl carrier protein (PCP)-bound residue and reductively release an *N*-acetylated diketide product as an aldehyde from an acyl carrier protein (ACP)-bound intermediate (Figure 2B). Trans methylation by the LymB methyltransferase (MT) would preserve the oxidation state of the released aldehyde as a methoxy enolate characteristic of **1** as a product of an atypical hybrid NRPS−PKS. Further sequence analysis of the LymA adenylation (A) domain revealed that the invariant Asp residue, which is required to stabilize the α-amino group of an amino acid substrate,[11] is replaced by Asn 226, which may activate an aryl acid substrate that may already contain the pyrrolo[4.3.2-*de*] core.

In order to explore this biosynthetic model of **1** assembly, we first established lymphostin production in *Salinispora* independently at UCSD and Pfizer. Standard growth conditions in A1 media that yielded products such as the salinosporamide proteasome inhibitors in *S. tropica*[12] and the cyclomarin peptides in *S. arenicola*[13] did not yield **1**. At UCSD, we explored different fermentation methods leading to a lymphostin production medium that ultimately gave **1** in *S. tropica* and *S. arenicola* at ∼0.4 and ∼3 mg/L,

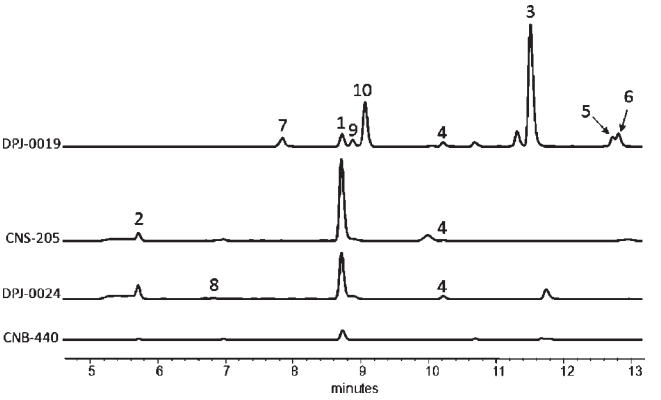

Figure 3. Comparative HPLC analysis (467 nm) of crude extracts from fermentations of "*S. pacifica*" DPJ-0019, *S. arenicola* CNS-205, "*S. pacifica*" DPJ-0024, and *S. tropica* CNB-440. Lymphostin (**1**); lymphostinol (**2**); neolymphostins A (**3**), B (**4**), C (**5**), and D (**6**); and neolymphostinols A (**7**), B (**8**), C (**9**), and D (**10**) are noted. See Table S6 for titers.

respectively. We additionally observed a new derivative in *S. arenicola* with the molecular composition $C_{15}H_{14}N_4O_3$, as established by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) ($[M + H]^+$, *m*/*z* 299.1141 obs, 0.7 ppm error). Analysis of the ^1H and ^{13}C NMR spectra, with the aid of gradient-enhanced heternuclear multiple-bond correlation (HMBC) data, clearly indicated that lymphostinol (**2**) was a novel lymphostin derivative with a modified C4 side chain in which the β-methoxy enone moiety of **1** was replaced with a β-hydroxy ketone residue. Key HMBC signals connected the C10/C11 ethyl alcohol residue to the C9 carbonyl at 200.2 ppm, which was supported by the MS data showing that **1** and **2** differ by a single carbon atom.

In the Pfizer laboratory, **1** production conditions were first established with *S. arenicola* strain MOSO-0003 in 12 different media that ultimately gave a titer of ∼20 mg/L in the medium M48-9. Subsequently, five of 11 different marine-invertebrate-derived strains of "*S. pacifica*" were found to produce **1**. Although most strains, such as DPJ-0024, produced **1** alone, strain DPJ-0019 was found to produce a mixture of lymphostin analogues. To improve the fermentation profile and facilitate downstream processing of material, a simpler production medium containing only soluble starch and yeast extract (YESS) was developed. For comparative HPLC analysis of **1**, strains from all three species (*S. tropica* CNB-440, *S. arenicola* CNS-205, and "*S. pacifica*" DPJ-0024 and DPJ-0019) were fermented in YESS medium at various scales, processed, and analyzed in parallel (Figure 3).

Analysis of the comparative *lym* chemistry of the three strains revealed that *S. tropica* CNB-440 produced **1** alone, whereas *S. arenicola* CNS-205 and "*S. pacifica*" DPJ-0024 produced **1**, **2**, **4**, and **8** and "*S. pacifica*" DPJ-0019 uniquely produced neolymphostins **3**, **5**, and **6** and the corresponding neolymphostinols **7**, **9**, and **10** (Figure 3 and Table S6). In the neolymphostins and neolymphostinols, the C13 *N*-acetyl found in **1** and **2** is replaced by larger acyl groups such as isobutyryl, propyl, *sec*-pentyryl, and isopentyryl (Figure 2B). All eight derivatives were isolated and fully characterized by HRMS and NMR analysis (Table S4). The most abundant member was neolymphostin A (**3**), whose ^1H and ^{13}C NMR spectra showed the presence of a 2-propylamide moiety [δ 3.06 (1H, m)/δ 34.7 and 1.22 (6H, d)/δ 18.9] in place of the acetamide in **1**. Lymphostin and the neolymphostins showed potent mTOR inhibition and cytoxicity (Table 1),

Table 1. mTor Kinase Inhibition and Cytotoxicity of Lymphostin and Neolymphostins

compound	mTOR (nM)	LNCap (nM)	MDA-468 (nM)
1	1.7	38	14
3	0.8	22	58
4	1.5	48	85
5	1.8	230	700
6	1.8	230	700

whereas the neolymphostinols as a whole were 1000-fold less active than their neolymphostin counterparts (Table S5).

Buoyed by the characterization of lymphostin chemistry, we next interrogated the *lym* clusters by PCR-targeted gene replacement recently optimized for *Salinispora* genetics.[13,14] Inactivation of the conserved *lymA* gene resulted in *S. tropica* and *S. arenicola* mutants devoid of **1** and **2** (Figure S1), thereby confirming the central role of the LymA synthetase. To establish further the biosynthetic relationship between **1** and **2**, we inactivated the *S. arenicola lymB* MT. On the basis of biosynthetic precedent,[15] we anticipated that the resulting mutant would accumulate an aldehyde intermediate that would lead to further carbonyl reduction by the LymA reductase in the absence of LymB (Figure 2B). Chemical analysis of the *lymB*::*apr*^{R} mutant revealed the complete loss of **1** and increased production of **2**, thereby confirming their biosynthetic linkage (Figure S1).

While the biosynthetic endgame is understood, we have yet to clarify the early stages of **1** biosynthesis that includes the formation of the pyrrolo[4,3,2-*de*]quinoline core structure. We established its biogenesis in "*S. pacifica*" strain DPJ-0019 from tryptophan through isotope experiments in which both nitrogen atoms were retained in the lymphostin products at N1 and N5 (Figure 2B). FTMS/MS analysis of $^{15}N_2$-L-tryptophan-derived **3** revealed both single (21%) and double (41%) ^{15}N labeling. The remaining nitrogen atoms in **3** were likewise shown to derive from glutamine, providing a possible role of the conserved amidotransferase encoded by Strop_3057 in adding the arylamine groups.

The molecular basis for the larger diversity of analogues produced by "*S. pacifica*" strain DPJ-0019 likely relies on the different substrate discrimination of the unusual LymA *N*-acetyltransferase (NAT) domain. As expected in the case of **3**, we confirmed through ^{13}C isotope labeling that the *N*-isobutyryl group is derived from isobutyrate (Figure 2B). While NATs are common biosynthetic enzymes, inclusion of a NAT in a modular synthetase is to the best of our knowledge unprecedented. We are presently studying the molecular basis of this difference in substrate specificity, which offers an exciting opportunity to explore acyl group discrimination as well as biosynthetic engineering of new lymphostin and other pyrroloquinoline alkaloids.

In summary, we have established the molecular basis of lymphostin biosynthesis in three *Salinispora* species by an unusual hybrid nonribosomal peptide−polyketide synthetase pathway that for the first time has shed light on the formation of alkaloids that contain a pyrrolo[4,3,2-*de*]quinoline core. Our work has also resulted in the discovery of nine new lymphostin derivatives and the preliminary description of their potent mTOR inhibitory activity.

■ ASSOCIATED CONTENT

Supporting Information. Figures S1−S4, Tables S1−S6, experimental methods, HRMS data, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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