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Biosynthetic Multitasking Facilitates Thalassospiramide Structural Diversity in Marine Bacteria

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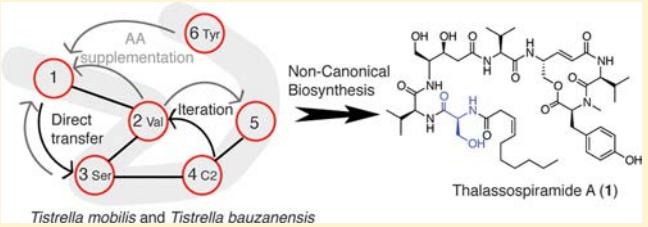
Supporting Information

ABSTRACT: Thalassospiramides A and B are immunosuppressant cyclic lipopeptides first reported from the marine α-proteobacterium *Thalassospira* sp. CNJ-328. We describe here the discovery and characterization of an extended family of 14 new analogues from four *Tistrella* and *Thalassospira* isolates. These potent calpain 1 protease inhibitors belong to six structure classes in which the length and composition of the acylpeptide side chain varies extensively. Genomic sequence analysis of the thalassospiramide-producing microbes revealed related, genus-specific biosynthetic loci encoding hybrid nonribosomal peptide synthetase/polyketide synthases consistent with thalassospiramide assembly. The bioinformatics analysis of the gene clusters suggests that structural diversity, which ranges from the 803.4 Da thalassospiramide C to the 1291.7 Da thalassospiramide F, results from a complex sequence of reactions involving amino acid substrate channeling and enzymatic multimodule skipping and iteration. Preliminary biochemical analysis of the N-terminal nonribosomal peptide synthetase module from the *Thalassospira* TtcA megasynthase supports a biosynthetic model in which *in cis* amino acid activation competes with *in trans* activation to increase the range of amino acid substrates incorporated at the N terminus.



INTRODUCTION

The multienzymatic thiotemplate mechanism of polyketide and nonribosomal peptide biosynthesis is a common strategy for the construction of complex natural products in microbes.[1,2] These biosynthetic assembly lines typically operate in a linear fashion whereby enzymatic substrates are sequentially incorporated into the growing product chain while it remains enzyme bound via thioester linkages throughout the entire synthetic process.[3,4] In many cases, such as for the prototypical polyketide synthase (PKS) 6-deoxyerythronolide B synthase[5] and the nonribosomal peptide synthetase (NRPS) surfactin synthetase,[6] product assembly displays a strict correlation between the enzymatic domain sequence of the megasynthase and the position of malonate/amino acid building blocks in the polyketide/peptide product. This colinear arrangement has facilitated the engineered biosynthesis of designer metabolites[7,8] and the bioinformatics-guided prediction and discovery of new chemical entities from genome-sequenced organisms.[9−12] However, the colinearity rule is not strictly enforced in nature.[13,14] Many polyketides, nonribosomal peptides, and hybrids thereof are products of pathways in which enzymatic functions are skipped, repeated, or complimented altogether.[15−20] Herein, we report an extreme departure from sequential assembly line biosynthesis, associated with the production of the thalassospiramide family of cyclic lipopeptides, which combines many noncanonical strategies for product diversity.

Thalassospiramides A (**1**) and B (**2**) were first described by Fenical and co-workers as immunosuppressant agents coproduced by the marine α-proteobacterium *Thalassospira* sp. CNJ-328.[21] These cyclic lipopeptides differ in structure at the N terminus in which the proteinogenic serine residue in **1** is replaced with the nonstandard phenylalanine-based statine residue 4-amino-3-hydroxy-5-phenylpentanoic acid (Ahppa). We report the characterization of an extended family of 14 new

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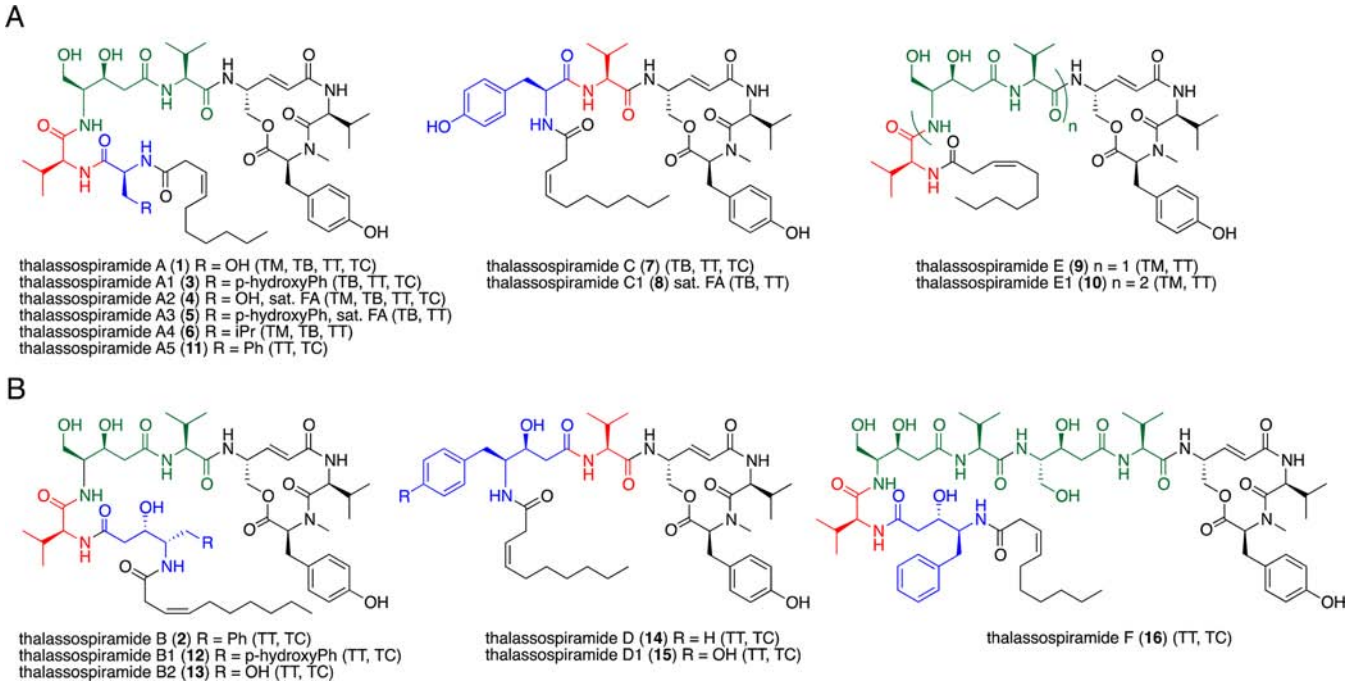

Figure 1. Structures of (A) thalassospiramide A-like and (B) thalassospiramide B-like natural products (blue portions indicate N-terminal amino acids used to distinguish the two groups, green portions highlight a repetitive amino acid motif, red portions indicate a common valine residue, and black portions are conserved throughout the family). Producing organism(s) shown in brackets include *Tistrella mobilis* (TM), *Tistrella bauzanensis* (TB), *Thalassospira* sp. TrichSKD10 (TT), and *Thalassospira* sp. CNJ-328 (TC). Several compounds (**4**, **5**, and **8**) have a saturated fatty acid at the N terminus (sat. FA).

thalassospiramide molecules from the original producer *Thalassospira* sp. CNJ-328 and three other marine α-proteobacteria *Thalassospira* sp. TrichSKD10, *Tistrella mobilis* KA081020-065, and *Tistrella bauzanensis* TIO7329. We further describe the genetic and biochemical basis for their biosynthetic diversity through the sequence analysis of their biosynthesis genes and the biochemical characterization of the thalassospiramide synthetase.

RESULTS AND DISCUSSION

Isolation and Characterization of Thalassospiramide Lipopeptides from Four Marine Bacteria. We recently reported that the marine α-proteobacteria *Tistrella mobilis* KA081020-065 and *Tistrella bauzanensis* TIO7329 from the Red Sea and the Pacific Ocean, respectively, produce the anticancer agent didemnin B.[22] Further chemical analysis of these microbes revealed that they produced a second group of unrelated lipopeptides. We first isolated and characterized thalassospiramide A (**1**) from both strains and noted its several-fold increased production upon iron supplementation of the growth media. However, we did not detect thalassospiramide B (**2**), which was previously reported along with **1** as a product of the marine α-proteobacterium *Thalassospira* sp. CNJ-328.[21] Instead, we isolated eight new thalassospiramide analogues (**3**–**10**). On the other hand, inspection of the original producer *Thalassospira* sp. CNJ-328 and the related strain *Thalassospira* sp. TrichSKD10 revealed their ability to synthesize not only **1** and **2** but all eight of the new *Tistrella* thalassospiramides as well as six additional derivatives (**11**–**16**) unique to the *Thalassospira* isolates.

We divided the variants into two structural classes, thalassospiramide A-like and thalassospiramide B-like, distinguished by the identity of the N terminal amino acid residue. Thalassospiramide A-like molecules (**3**–**11**) contain a standard amino acid residue at position one, whereas the thalassospiramide B-like molecules (**12**–**16**) have a statine-like amino acid at this position (Figure 1). We further grouped the 16 thalassospiramides into 6 subclasses based on distinguishing structural features: the A and B groups have a variety of amino acids in the N-terminal residue (shown in blue in Figure 1), the C and D groups are truncated (absence of green structure unit), and the E and F groups are elongated (repetition of the green structure unit). Structure diversity is also evident in the 3*Z*-decenyl lipid residue that is saturated in several variants (**4**, **5**, and **8**).

We extensively characterized at least one member of each subclass (A–F), including the previously reported **1** and **2**, by NMR, mass spectrometry (MS), and Marfey analyses (see the Supporting Information). The stereochemistry of the proteinogenic amino acid residues was assigned using the advanced Marfey method[23−25] in which the lipopeptides were first hydrogenated, hydrolyzed, and then reacted with either L- or D-1-fluoro-2,4-dinitrophenyl-5-leucine amide (FDLA, advanced Marfey's reagent). Analysis by liquid chromatography–mass spectrometry (LC-MS) revealed that all α-amino acids were in the L configuration and that the hydrogenated 4-amino-5-hydroxypenta-2-enoic acid (Ahpea) was assigned as R. These assignments are all in agreement with the results previously reported for **1** and **2**. The stereochemistry shown for the statine units 4-amino-3,5-dihydroxypentanoic acid (Adpa) and 4-amino-3-hydroxy-5-phenylpentanoic acid (Ahppa) is based upon prior assignments for A (**1**) and B (**2**) by Fenical and co-workers.[21]

Two of these compounds, thalassospiramides B1 (**12**) and D1 (**15**), were described previously in the patent literature from the α-proteobacterium *Oceanospirillum* sp. SANK 70992 and

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were shown to have nanomolar inhibitory action against the cysteine protease calpain 1.[26] We thus explored the activity of a selection of our thalassospiramide biosynthetic library against calpain 1 using a fluorescence-based assay (Table 1).[27] All

Table 1. Inhibitory Activity of Selected Thalassospiramides against Human Calpain 1 Protease[a]

compound	IC_{50} (nM)
thalassospiramide A (**1**)	56.5 ± 2.4
thalassospiramide A1 (**3**)	42.3 ± 3.0
thalassospiramide B (**2**)	29.0 ± 2.0
thalassospiramide C (**7**)	3.4 ± 1.2
thalassospiramide D1 (**15**)	34.7 ± 2.5
thalassospiramide E1 (**10**)	20.9 ± 1.6
positive control Z-Leu-Leu-Tyr-fluoromethylketone	16.2 ± 2.2

[a]Activity of **12** and **15** against human calpain protease 1 was previously reported, with IC_{50} values of 80 and 39 nM, respectively.[26]

compounds tested showed nanomolar inhibitory activity. However, the truncated thalassospiramide C (**7**) was the most potent at nearly an order of magnitude (3.4 ± 1.2 nM) more active than the others.

Identification and Analysis of the Noncanonical Thalassospiramide Biosynthetic Gene Clusters. To gain an understanding of the molecular basis for thalassospiramide chemical diversity, we characterized the associated biosynthetic gene clusters in all four strains. We had previously reported the complete genome sequence of *T. mobilis* KA081020-065 in the course of our work on didemnin B biosynthesis.[22] We compared the *T. mobilis* sequence with the publically available draft genome of *Thalassospira* sp. TrichSKD10 (available from https://moore.jcvi.org/moore/) and identified a common gene cluster encoding a hybrid nonribosomal peptide synthetase/polyketide synthase (NRPS/PKS). These were located on plasmid 1 (ORFs 1 and 603) of *T. mobilis* and contig 7 (nt 128 264−153 704) of *Thalassospira* sp. TrichSKD10. We also sequenced the draft genomes of *T. bauzanensis* TIO7329 and *Thalassospira* sp. CNJ-328 and identified related genus-specific thalassospiramide biosynthetic gene clusters (Figure 2). While the two thalassospiramide biosynthesis loci *ttm* and *ttb* from *Tistrella* share identical gene architecture, they differ in subtle yet distinct ways from the syntenic thalassospiramide biosynthesis loci *ttc* and *ttt* from *Thalassospira* (Figure 2). These key genetic differences correlate to the contrasts in thalassospiramide chemistry observed in each genus (Figure 1). Significantly, the *Thalassospira* thalassospiramide synthetase contains an additional PKS module (module 1b) that presumably allows for the assembly of the unusual N-terminal statine amino acid residue characteristic of the thalassospiramide B-like molecules particular to *Thalassospira*. A second striking difference between the two genetic systems is the absence of the vital catalytic adenylation (A) domains that are required for amino acid activation and loading in *Tistrella* modules 1 and 5. Protein alignment of the sequence bridging the condensation (C) and thiolation (T) domains in the five *Tistrella* NRPS modules and comparison with the crystal structure of the surfactin NRPS termination module[28] revealed that the N-terminal core A domain (~400 amino acid residues) is missing for modules 1 and 5, whereas the small C-terminal subdomain (~100 amino acids) persists (see Figures S18 and S19, Supporting Information). This loss of two critical enzymatic domains poses an interesting challenge regarding catalysis at these positions and suggests that *in trans* substrate activation must occur.

Similarly, the PKS module 4 in both systems is also lacking its substrate activating acyltransferase (AT) domain, while the *Thalassospira* module 1b PKS harbors an AT domain. We previously observed that the didemnin PKS in *T. mobilis* also lacks *cis* AT domains[22] and thus suggest that the fatty acid AT FabD may undertake this role *in trans* for the thalassospiramide and didemnin pathways. Alternatively, the *Thalassospira* system may uniquely employ the *in trans* application of the module 1b AT to complement module 4. Bioinformatics analysis of the ketosynthase (KS) domains in module 4 identifies them as *trans* KS domains and therefore likely capable of interaction with a *trans* AT.[17]

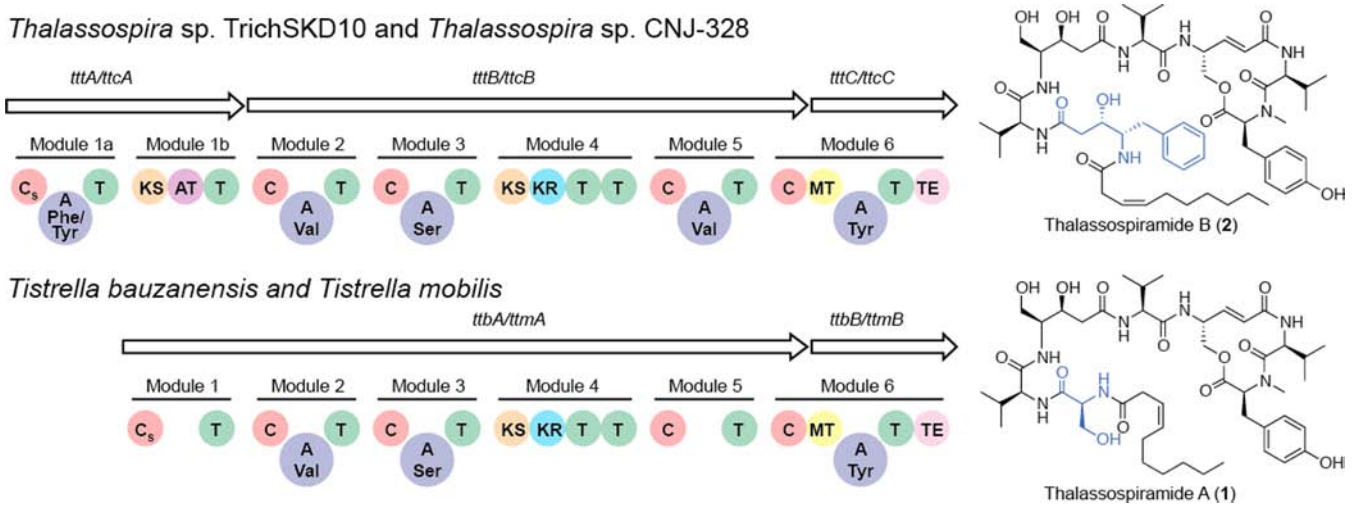

Figure 2. Homologous thalassospiramide gene clusters from *Tistrella* and *Thalassospira*. The *T. mobilis* KA081020-065 and *T. bauzanensis* TIO7329 thalassospiramide clusters *ttm* and *ttb*, respectively, encode the biosynthesis of thalassospiramide A-like molecules only, whereas the *Thalassospira* sp. CNJ-328 and *Thalassospira* sp. TrichSKD10 clusters *ttc* and *ttt*, respectively, encode the synthesis for both thalassospiramide A- and B-like molecules. Domain abbreviations: Starter condensation (C_S), condensation (C), adenylation (A), thiolation (T), ketosynthase (KS), acyltransferase (AT), ketoreductase (KR), methyltransferase (MT), and thioesterase (TE).

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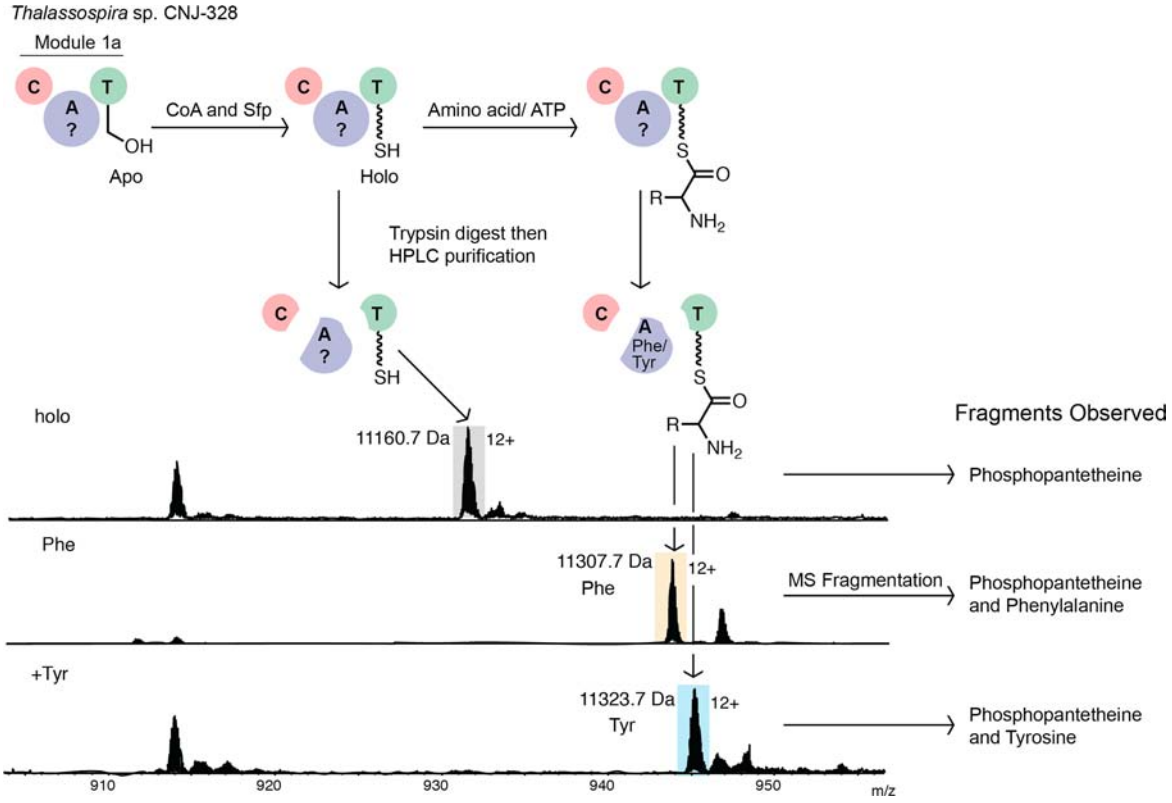

Figure 3. Determination of the amino acid selectivity of TtcA(CAT) via the phosphopantetheine ejection assay. Shown are the results for the holo enzyme without addition of amino acids and after treatment of the enzyme with L-phenylalanine or L-tyrosine. All other proteinogenic amino acids, including serine and valine, were not loaded as substrates (see Figures S1 and S2, Supporting Information).

Despite these differences, the *Thalassospira* and *Tistrella* thalassospiramide genotypes are clearly related and share many common biosynthetic features. For example, all clusters contain a primary condensation domain (C_S) consistent with the N-acylation of the N-terminal amino acid of lipopeptides such as in the coproduced didemnin X and Y from *T. mobilis*.[22] Similarly, all clusters feature a separately encoded terminal NRPS module responsible for incorporation of the C-terminal *N*-methyl tyrosine residue and macrocyclization to yield the 12-membered ring common to all thalassospiramide derivatives.

One of the most salient features of the thalassospiramide system is the apparent mismatch of biosynthetic modules necessary to accommodate all observed molecules. The thalassospiramide megasynthetase possesses six modules in *Tistrella* and seven modules in *Thalassospira*, which is consistent with the linear biosynthesis of thalassospiramide C and D groups, respectively. The other thalassospiramide groups require a previously unprecedented partially iterative usage of the synthetase involving modules 2−4. A further architectural anomaly that could support this hypothesis is the presence of two tandem thiolation domains at the end of module 4 (Figure 2), which may facilitate the shuttling of the peptide biosynthetic intermediate back to module 2 for further cycles of synthesis. Thus, in the case of thalassospiramides belonging to the A and B groups, we propose a single repeat of modules 2−4, while the biosynthesis of thalassospiramides E1 and F must undergo yet another repeat. To the best of our knowledge, this type of multimodular iteration has not been observed before in NRPS biochemistry. Previously reported examples of iteration include single module iteration such as for didemnins X and Y[22] and whole peptide oligomerization such as for gramicidin S[16] and enterobactin.[29,30] We propose that, in thalassospiramide biosynthesis, the growing peptide chain is actually translocated backward within the cluster from module 4 back to module 2 for a second (or third) round of synthesis.

Biochemical Evaluation of the TtcA CAT Tridomain. One of the main differences between the biosynthetic clusters for the two genera is the presence of the unique PKS module 1b within the *Thalassospira* cluster. This discrepancy is ascribed to the synthesis of the C2 portion of the statine-like amino acids at the N terminus of thalassospiramides in the B-like series. However, these organisms are still capable of making thalassospiramide A-like molecules, where in fact they dominate the thalassospiramide mixture. To investigate whether module 1a is responsible for incorporating the first amino acid residue observed in any or all of the isolated molecules, we probed the amino acid selectivity of its A domain. In silico predictions involving antiSMASH[31] and NRPSpredictor2[32] were unreliable and gave different results.

On the basis of the chemical diversity of amino acid analogues seen for residue 1 (Ser, Val, Phe, and Tyr), a promiscuous enzyme specific for four substrates of various polarity seemed unreasonable. Thus, we biochemically explored the amino acid selectivity of the module 1a CAT-tridomain of TtcA by the phosphopantetheine ejection assay (Figure 3).[33,34] We cloned and overexpressed the TtcA(CAT) as an *N*-terminal octahistidine-tagged fusion protein in *Escherichia coli* BL21-(DE3). The soluble apoprotein was purified by Ni-affinity chromatography and converted to the functional 124 kDa holo enzyme with the phosphopantetheinyl transferase enzyme Sfp. The active enzyme was then incubated with all proteinogenic amino acids in the presence of ATP, digested with trypsin, and

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fractionated by high-performance liquid chromatography (HPLC). The fragment containing the active-site serine residue post-translationally modified with CoA was first identified with bodipy (fluorescently) labeled coenzyme A.[35] Candidate fractions were then analyzed by Fourier transform ion cyclotron resonance (FT-ICR) MS, and fragmentation of candidate ions allowed identification of phosphopantetheinated peptides (Figure 3). MS^3 fragmentation of these ions yielded fragments consisting of phosphopantetheine and the covalently attached amino acid substrate. We found that the TtcA A domain activated L-phenylalanine (as in thalassospiramides B (**2**) and A5 (**11**)) and L-tyrosine (as in thalassospiramides A1 (**3**) and B1 (**12**)) but was unable to activate L-serine (as in thalassospiramide A (**1**) or B2 (**13**)), L-valine (as in thalassospiramide A4 (**6**)), or any other amino acids. This result confirms that the TtcA A domain has narrow amino acid specificity, limited to specific aromatic side chains.

Upon a second inspection, an intriguing parallel can be drawn between the two thalassospiramide synthetase systems. As with the *Tistrella* module 1 that lacks a cognate A domain and thus must be complemented *in trans*, the *Thalassospira* module 1a must also interact with a *trans* A domain to deliver serine and valine residues that are not activated by the *cis* Phe/Tyr-specific A domain (Figure 4). While a *trans* acting A domain encoded outside the gene cluster, which competes in the case of the *Thalassospira* or supplements in the case of the *Tistrella* thalassospiramide synthetase, is mechanistically plausible, a compelling biosynthetic model may involve internal supplementation from the other *cis* A domains in the thalassospiramide megasynthetase.

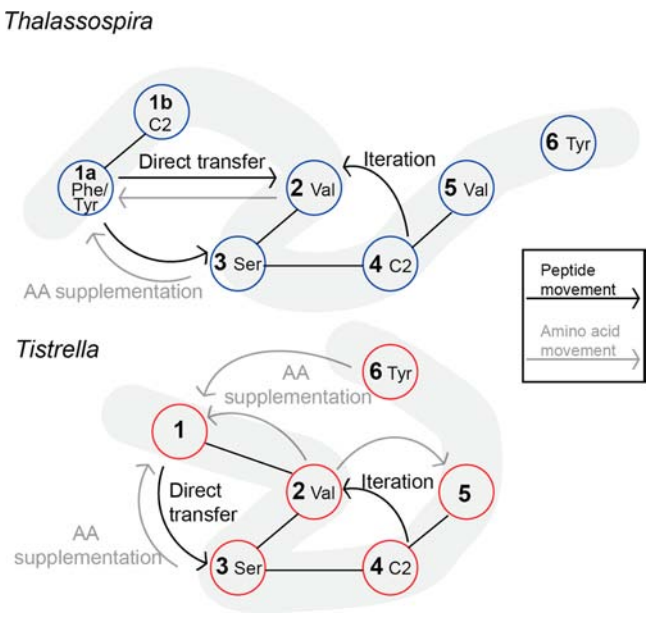

Figure 4. Representation of the proposed thalassospiramide biosynthetic pathways in *Thalassospira* and *Tistrella*. The pale gray ribbons represent the linear sequence of the gene products, black lines depict the modules located within the same protein, black arrows show the forward/backward movement of the growing peptide chain along the enzyme assembly line, and gray arrows show the intermodular movement of amino acid substrates from supplementing A domains to T domains.

All observed thalassospiramide analogues at position 1 carry amino acid residues that are also located elsewhere in the molecule, where specific A domains likely function dually to select and activate a specific amino acid residue as a substrate for its cognate T domain (*cis* acting) as well as for the module 1/1a/5 T domains (*trans* acting). This approach is reminiscent of the biosynthesis of yersiniabactin, where a single cysteine A domain loads three different T domains.[36−38] In the case of the thalassospiramides, multiple A domains would need to be involved. For instance, we propose that the valine-specific module 2 A domain in the *Tistrella* thalassospiramide synthetase services T domains in modules 1, 2, and 5 to produce thalassospiramide A4 (**6**). While an aminoacyltransferase could be involved in shuttling the amino acids from one module to another as seen in syringomycin biosynthesis,[20] it would require a highly promiscuous shuttle or a series of different transferases to account for all of the analogues observed. In Figure 4, we display a proposed two-dimensional representation of our biosynthetic proposal encompassing *in trans* amino acid supplementation, module skipping, and multimodule iteration to allow for the observed structural diversity represented in the natural thalassospiramide chemical library.

■ CONCLUSION

This study illuminates the molecular basis governing the noncanonical biosynthesis of the thalassospiramide family of cyclic lipopeptide calpain I protease inhibitors. While some of these atypical NRPS biochemical features of intrasynthetase *trans* A domain activation, module skipping, and multimodule iteration have been previously observed in other biosynthetic systems, the thalassospiramides represent an unprecedented amalgamation of these rare biosynthetic mechanisms. Efforts are ongoing to probe further this intriguing biosynthetic pathway and to learn what makes this multitasking synthetase so peculiar.

■ EXPERIMENTAL SECTION

Fermentation, Isolation, and Characterization of Thalassospiramides. To isolate thalassospiramides, *T. mobilis* and *T. bauzanensis* were grown in GYP media (glucose (10 g/L), yeast extract (4 g/L), peptone (2 g/L)) and $FeCl_3$ (0.5 mM at pH 7.4) at 28 °C for 4 days. For each species, 20 × 1 L cultures in 2.8 L Fernbach flasks were grown. *Thalassospira* sp. TrichSKD10 and *Thalassospira* sp. CNJ-328 were grown in a seawater-based GYP media at 28 °C for 2 days. For each species, 40 × 1 L cultures were grown for isolation purposes. The same extraction and crude purification was done for all strains. Briefly, an equal volume of EtOAc was added to the bacterial cultures and mixed. The aqueous layer was discarded, and the EtOAc was removed under reduced pressure. The crude extract was then subjected to flash C_{18} column chromatography, eluting with 30%, 70%, and 100% aqueous MeOH to yield three fractions. The fractions containing thalassospiramides were further purified using reversed-phase HPLC (Phenomenex Luna 5 μm C_{18}(2), 250 mm × 100 mm, 100 Å, 3.0 mL/min) with the following conditions. The 70% MeOH fraction from *T. mobilis* was separated with an isocratic method of 42% CH_3CN in water to yield **6** (3.0 mg) and **9** (3.0 mg) at 25.5−27 min and 19.5−21.0 min, respectively. From the same extract, **10** (1.0 mg) was obtained using an isocratic method of 38% CH_3CN in water and eluted at 74.0−84.0 min. The 70% MeOH fraction from *T. bauzanensis* was separated with an isocratic method of 51% CH_3CN in water to yield **1** (6.0 mg), **3** (3.0 mg), and **7** (3.0 mg) at 10.0−12.0, 14.5−16.0, and 26.7−28.2 min, respectively. The 100% MeOH fraction from *Thalassospira* sp. TrichSKD10 was separated with an isocratic method of 50% CH_3CN in water to yield **16** (1.2 mg), **2** (2.0 mg), **11** (3.0 mg), and **14** (1.5 mg) at 23.8−25.2, 29.2−32.0, 36.5−39.5, and 46.5−51.5 min, respectively.

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To determine the distribution of compounds produced by each strain, a 50 mL culture of each organism was extracted after 3 days using EtOAc (250 mL). Following concentration in vacuo, these samples were analyzed and compared by LC-MS (1.7 μm C_{18} column, 0.25 mL/min, gradient from 5% to 95% CH_3CN with 0.1% TFA, 25 min).

^{1}1D NMR and 2D NMR spectra were obtained with either a 500 or a 700 MHz Varian Inova 500 MHz spectrometer. All samples were dissolved in pyridine except thalassospiramides A4 and C, which were dissolved in DMSO-d_6. Electrospray ionization time-of-flight high-resolution mass spectrometry (ESI-TOF-HRMS) analysis was conducted with a Bruker micrOTOF instrument (Bruker Daltonics GmbH, Bremen, Germany). MS^n analysis was carried out on a LTQ Velos dual-pressure ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA).

Determination of the Absolute Amino Acid Configurations. To enhance the stability of the thalassospiramides in acid hydrolysis, the Ahpea unit of each compound was converted into 4-amino-5-hydroxy-pentanoic acid (Ahpa). Thalassospiramides A, A1, A4, A5, B, C, D, E, and F (0.5 mg each) were dissolved in MeOH (2 mL), and Pd/C was added to a final concentration of 10%. After stirring for 10 h under an atmosphere of H_2, the catalyst was removed by filtration, and the resultant product was examined by LC-MS. The hydrogenated product was then hydrolyzed with HCl (6 M, 0.5 mL) in a sealed glass tube at 110 °C for 24 h. The hydrolysate was evaporated to dryness to remove any residual acid, dissolved in $NaHCO_3$ (1 M, 100 μL), and split into two parts. L-FDLA (3 mg/mL, 50 μL) was added to one part, whereas D-FDLA (3 mg/mL, 50 μL) was added to the other. The two mixtures were incubated at 80 °C for 5 min and were then quenched by addition of HCl (2 M, 50 μL). Finally 50% aqueous CH_3CN (200 μL) was added to dissolve the reaction products. The derivative amino acids were analyzed by LC-MS (1.7 μm C_{18} column, 0.25 mL/min, gradient from 5% to 95% CH_3CN with 0.1% TFA, 25 min).

Calpain Activity Assay. Calpain 1 (active human calpain 1, BioVision) activity was measured according to the protocol for the calpain activity assay kit (BioVision). Z-Leu-Leu-Tyr-fluoromethylketone (Z-LLY-FMK, BioVision), a known calpain inhibitor, was used as a positive control. Thalassospiramides A, A1, C, D1, and E1 and Z-LLY-FMK were dissolved in DMSO to give 1 mM stock solutions and then serially diluted with MeOH or acetone, depending on solubility. The highest concentration tested was 0.1 mM. First, extraction buffer (85 μL) was added into each assay well of a 96-well plate, followed by addition of calpain 1 (0.1 U). Then, a test compound (1 μL) with desired concentration was added into each well. Reaction buffer (10 μL) and calpain substrate (5 μL) were added into each well, and the plate was incubated at 37 °C for 1 h in the dark. After incubation, the samples were recorded by a fluorometer equipped with a 400 nm excitation filter and a 505 nm emission filter. A blank assay was run without calpain or a test compound. A negative control was run without a test compound. There were three replicates for each tested concentration. The experiment was repeated for three times.

Gene Cluster Sequencing and Analysis. Sequence data was obtained for both *Tistrella bauzanensis* and *Thalassospira* sp. CNJ-328. Sequencing for *Tistrella bauzanensis* was performed on the Illumina Hiseq2000. A total of 17 million pairs of Illumina reads were obtained from a 170 bp and a 300 bp paired-end library. Genome assembly was performed using the program Velvet 1.0.15 with the following custom parameters: hash-length = 55 and coverage cutoff = 30. Ion Torrent technology was utilized for the *Thalassospira* sp. CNJ-328 draft genome. Following sequencing with the Ion PGM sequencer, the contigs were assembled to the putative cluster from *Thalassospira* sp. TrichSKD10 using the software Geneious (version 5.1.7 created by Biomatters). Sequencing of polymerase chain reaction (PCR) products was used to close gaps in the resultant sequence for the gene cluster from *Thalassospira* sp. CNJ-328. The sequence of the cluster in *Tistrella mobilis* was revised by sequencing PCR products.

The sequences of the thalassospiramide gene clusters can be accessed from the Genbank database with the following accession numbers: *T. bauzanensis* (KC181865), *Thalassospira* sp. CNJ-328 (KC181865), and plasmid 1 (cluster consists of ORF 1 and ORF 603) of *T. mobilis* (CP003237). Draft genome of *Thalassospira* sp. Trich SKD10 (cluster located at nt 128 264−153 704 on contig 7) can be obtained from the JCVI, Gordon and Betty Moore Foundation Marine Microbial Genome Sequencing Project (https://moore.jcvi.org/moore/).

In silico analysis of the gene clusters by protein−protein BLAST and Pfam analysis was performed.[39] This enabled assignment of putative roles for the proteins within the clusters. Prediction of adenylation domain specificity was done using the online software antiSMASH and NRPSpredictor2.[31,32] Analysis of condensation and ketosynthase domain functions was performed using the online software NaPDoS.[40]

Production of TtcA(CAT) by Heterologous Expression. The portion of the gene cluster coding for TtcA(CAT) was amplified by PCR using 5′-dGAC<u>CCATGG</u>**CTTG**CAGAAGCGCACAAACAC-3′ as the forward primer (*Nco*I site underlined and the translation start site is highlighted in bold) and 5′-dGAC<u>AAGCTT</u>**T-CA**GCGTTGGGTCTTTACCGGTT-3′ as the reverse primer (*Hin*dIII site underlined and the stop codon is highlighted in bold) from *Thalassospira* sp. CNJ-328 genomic DNA. The 3.3 kb PCR product was gel purified, digested with *Nco*I and *Hin*dIII followed by a second gel purification, and then, ligated (T4 DNA ligase) with *Nco*I/*Hin*dIII digested pHIS8[41] to generate the expression vector. The construct was transformed into *E. coli* TOP10 cells, and *ttcA*(CAT) insertion was checked by sequencing. The construct was then further transformed into *E. coli* BL21(DE3) for expression. Transformed *E. coli* was grown in Luria−Bertani media with kanamycin (50 μg/mL) at 28 °C until $A_{600\ nm}$ = 0.4−0.8. The culture was allowed to cool to 18 °C for 30 min and then induced with isopropyl-1-thio-β-D-galactopyranoside (0.5 M, 100 μL). The culture was grown for an additional 20 h, and then, the cells were pelleted by centrifugation. The cell pellet was resuspended in phosphate lysis buffer (50 mM sodium phosphate buffer pH 8, 300 mM NaCl, 10 mM imidazole) and incubated at 0 °C for 30 min in the presence of lysozyme (1 mg/mL). The mixture was then sonicated and incubated for a further 15 min at 0 °C with DNase 1 (1 U/μL). The cellular debris was removed via centrifugation, and the resultant supernatant was incubated with Ni−NTA resin (0.5 mL of a 50% slurry in lysis buffer) for 1 h at 0 °C. The mixture was loaded onto a column, and the resin was washed with the same phosphate buffer containing increasing concentrations of imidazole (20 mM, 60 mM). The desired proteins were eluted with phosphate buffer containing a high concentration of imidazole (250 mM imidazole). The elution fraction was dialyzed with Tris−HCl buffer (50 mM at pH 7.5 containing 10% (v/v) glycerol) and was then concentrated to 2.5 mg/mL. Protein samples were stored at −20 °C until use.

Synthesis of Bodipy−CoA (Adapted from La Clair et al.).[35] DMSO (600 μL) was added to 10 mM Tris−HCl buffer pH 7 (3.8 mL), followed by coenzyme A (10 mM, 58 μL). The mixture was allowed to cool to 0 °C in the dark, and then, bodipy (25 μg/μL in DMSO, 20 μL) was added. The reaction was allowed to stir at 0 °C for 30 min, and then after warming to room temperature, mercaptoethanol (12 μL) was added. The reaction mixture was washed with EtOAc (4 × 5 mL), and then, the resultant aqueous solution was stored at −80 °C.

In Vitro Formation of Aminoacylated TtcA(CAT), Digestion, and HPLC Purification. To generate holo-TtcA, 50 mM Tris−HCl buffer pH 7.5 with 10% (v/v) glycerol (2.5 μL), $MgCl_2$ (100 mM, 5 μL), tris(2-carboxyethyl)phosphine (2 mM, 1.5 μL), coenzyme A (10 mM, 3.13 μL), and Sfp (170 μM, 1.3 μL) were added to the apo form of TtcA(CAT) (20 μM, 50 μL), and the mixture was then incubated at 25 °C for 1 h. To test the amino acid loading capability of the holo enzyme, ATP (100 mM, 3.5 μL), an amino acid (50 mM, 1.4 μL), and 50 mM Tris−HCl buffer pH 7.5 with 10% (v/v) glycerol (1.6 μL) were added, and the mixture was incubated for 30 min at 25 °C. After adjusting the reaction mixture to pH 8 with 1 M Tris−HCl, it was incubated at 25 °C with trypsin (1 μg, Sigma−Aldrich proteomics grade) for 5 min. Formic acid (3.5 μL) was then added, and the sample was directly analyzed by HPLC. Using a Higgins Analytical PROTO 300 5 μm C_4 300 Å column, the samples were purified using

the following 66 min method with 0.1% TFA in both the water and CH_3CN solutions: 10% CH_3CN from 0 to 10 min, ramp to 30% by 15 min, ramp to 70% by 55 min, ramp to 90% by 60 min, hold at 90% until 60.1 min, ramp down to 5% by 60.2 min, hold at 5% until 62.6 min, ramp to 95% by 63 min, hold at 95% until 65 min, and ramp to 5% by 66 min. One minute fractions were collected, immediately frozen at −80 °C, and then, lyophilized. Candidate fractions for analysis were collected based upon retention time of bodipy−CoA holo-TtcA(CAT) fragments (HPLC monitored by UV/vis spectroscopy at 220, 254, and 504 nm). The assay was completed with a mixture of all 20 amino acids or with tyrosine/phenylalanine as substrates for the loading step.

Mass Spectrometric Analysis of Protein Samples. Lyophilized HPLC fractions were dissolved in ESI solution (30 μL, containing 50% LC-MS grade MeOH, 49% LC-MS grade water, and 1% formic acid), then gently vortexed for 30 s, and centrifuged for 2 min at 14 000 rpm. Analysis was performed using a hybrid 6.4T LTQ-FT (Thermo Electron, North America) mass spectrometer. The instrument was first tuned to m/z 816.3 using bovine cytochrome c (Sigma−Aldrich). Samples were introduced by electrospray ionization (1.4−1.5 kV, 0.4 psi) using a Biversa Nanomate 100 (Advion Biosciences) nanospray device in positive ion mode. Detection using the low resolution LTQ was performed using 1000 ms maximum ion accumulation time. An 8000 ms maximum ion accumulation time and a resolution of 100 000 were used for FT-ICR-MS detection. MS^2 and MS^3 analysis for PPant ejection and PPant fragmentation were performed as described previously.[42]

Active Site Mapping and Substrate Identification of TtcA-(CAT). Candidate active site peaks were identified based on bodipy retention time and PPant ejection.[34] Peak lists were generated from RAW files with XTRACT software (Thermo Finnigan, Bremen, Germany). The generated peak list was imported into the freeware PAWS to identify the active sites. A match to the active site of the T domain was confirmed by PPant ejection, that is, MS^2 fragmentation of the corresponding active site peak, and PPant fragmentation, that is, MS^3 fragmentation of the ejected PPant ion.[43] Loaded substrates on the T domain active site were identified by mass shift of the active site fragment (MS^1), the PPant ion (MS^2), and the PPant fragments (MS^3).

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra and characterization tables, annotated MS^2 spectra, high-resolution MS spectra, and protein sequence alignments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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