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Structures and Comparative Characterization of Biosynthetic Gene Clusters for Cyanosporasides, Enediyne-Derived Natural Products from Marine Actinomycetes

Amy L. Lane,[†,‡] Sang-Jip Nam,[†,£] Takashi Fukuda,[†,⊥] Kazuya Yamanaka,[†] Christopher A. Kauffman,[†] Paul R. Jensen,[†] William Fenical,*[,†,§] and Bradley S. Moore*[,†,§]

[†]Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92093-0204, United States

[§]The Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California at San Diego, La Jolla, California 92093, United States

Supporting Information

ABSTRACT: Cyanosporasides are marine bacterial natural products containing a chlorinated cyclopenta[*a*]indene core of suspected enediyne polyketide biosynthetic origin. Herein, we report the isolation and characterization of novel cyanosporasides C−F (**3**−**6**) from the marine actinomycetes *Salinispora pacifica* CNS-143 and *Streptomyces* sp. CNT-179, highlighted by the unprecedented C-2′ *N*-acetylcysteamine functionalized hexose group of **6**. Cloning, sequencing, and mutagenesis of homologous ~50 kb cyanosporaside biosynthetic gene clusters from both bacteria afforded the first genetic evidence supporting cyanosporaside's enediyne, and thereby *p*-benzyne biradical, biosynthetic origin and revealed the molecular basis for nitrile and glycosyl functionalization. This study provides new opportunities for bioengineering of enediyne derivatives and expands the structural diversity afforded by enediyne gene clusters.

Enediyne natural products have drawn substantial interest among chemists and pharmacologists owing to their structurally fascinating molecular scaffolds, potent DNA damaging activity, and unique biosynthetic assembly.[1] The study of this fascinating class of bacterial compounds has been complicated by the lability of most 9-membered enediyne scaffolds in the absence of enediyne-binding apoproteins.[2] This is one likely reason why remarkably few enediyne chemical structures have been fully elucidated, despite observations that genes encoding modular, iteratively acting enediyne polyketide synthases (PKSEs) are relatively widespread among select genera of actinomycetes.[3] Several known natural products, including the sporolides, cyanosporasides and fijiolides, have been postulated to represent spontaneous enediyne degradation products.[4] Such products are of interest because they may offer insight into the divergence of biosynthetic pathways between those enediyne gene clusters that encode apoproteins and those that do not, and may afford access to unique biosynthetic genes that hold potential for engineering designer molecules with functional groups beyond the aromatic and glycosyl moieties common among most known enediynes. Herein, we report four new natural products of the cyanosporaside structure class from two phylogenetically distinct marine actinomycetes and suggest that their chlorinated cyclopenta[*a*]indene cores are derived from enediyne polyketide precursors based on the discovery and interrogation of their biosynthetic genes.

We previously reported that the marine bacterium *Salinispora pacifica* CNS-103 produces cyanosporasides A−B (**1**−**2**), novel chloro- and cyano-cyclopenta[*a*]indene glycosides, and hypothesized that their tricyclic aglycones were cyclized products of an enediyne polyketide precursor (Figure 1b).[4b] Support for this proposal was later provided by Perrin et al. who demonstrated facile addition of nucleophilic halides to model enediyne substrates to yield halogen-substituted aromatic products via a proposed *p*-benzyne biradical intermediate (Figure 1b).[5] This halogenation mechanism contrasts common synthetic and biosynthetic mechanisms for aromatic halogen substitution that rather invoke electrophilic halogens and represents a new cyclization outcome of an enediyne substrate.

Chemical analysis of *S. pacifica* CNS-143 revealed several unreported cyanosporaside analogues most closely related to previously described **2**. Through a combination of high-resolution mass spectrometry, detailed NMR analysis (Table S2, Figures S2−S17), CD measurements (Figure S1), and advanced Mosher's analysis (Figure S18), structures of cyanosporasides C−E (**3**−**5**) were elucidated. Cyanosporaside C (**3**) is the C-4′-acetate analogue of **2**, with the position of the acetate group of **3** established by NMR spectral differences at C-4′. The relative stereochemistry and absolute configuration of **3** were deduced by ROESY experiments and Mosher analysis, respectively (Figure S18). Cyanosporasides **4** and **5** featured the aglycone of **2** without the deoxysugar moiety and differed from one another in the presence or absence, respectively, of an acetyl group at C-3 as revealed by long-range HMBC correlations.

Fermentation of the distantly related marine actinomycete *Streptomyces* sp. CNT-179 coincidentally revealed that it too biosynthesizes the cyanosporaside aglycones **4** and **5** with the same absolute configuration as those isolated from *S. pacifica* CNS-143 (Figure S1). Further analysis of the *Streptomyces* sp.

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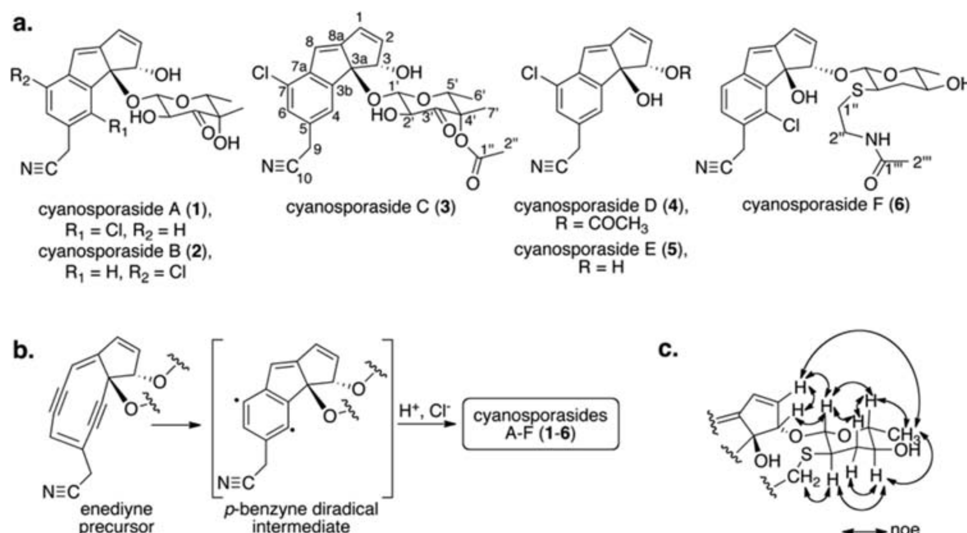

Figure 1. (a) Structures of cyanosporasides A–F (**1**–**6**); (b) proposed enediyne origin and abiotic cycloaromatization via a diradical intermediate to yield cyanosporasides;[4b,5] (c) Key NOESY correlations for determination of the relative stereochemistry of the pyranohexose unit in **6**.

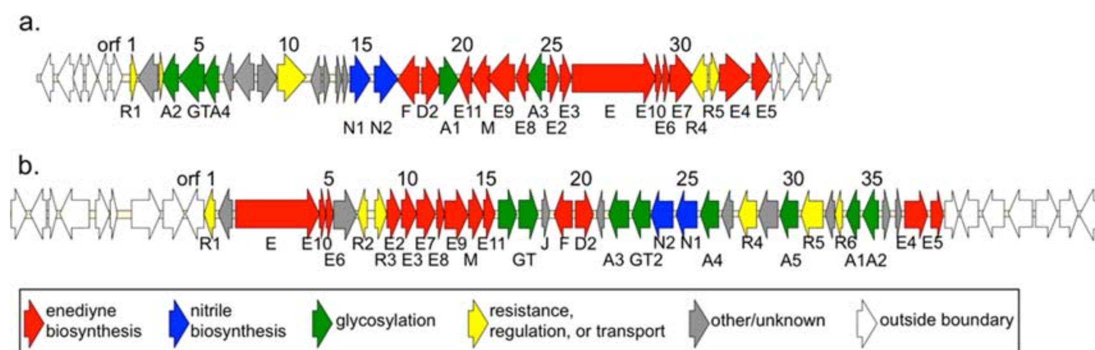

Figure 2. Organization and functional assignment of cyanosporaside biosynthetic gene clusters from (a) *S. pacifica* CNS-143 (*cya* cluster, ~49 kb) and (b) *Streptomyces* sp. CNT-179 (*cyn* cluster, ~52 kb).

organic extract yielded a novel thioether analogue, cyanosporaside F (**6**), with the molecular formula $C_{24}H_{27}{}^{35}ClN_2O_5S$ based on the HRESIMS pseudomolecular ion m/z 513.1219 $[M+Na]^+$. The main feature differentiating **6** from **1**[4b] was the attachment of an unusual pyranohexose sugar (C-1′ to C-6′) with an *N*-acetyl ethanamine unit (C-1″ to C-2″) at C-3 of **6**, as established by COSY and HMBC data. Analysis of the CD spectroscopic data of the aglycone of **6** and comparison to CD spectra for **3**–**5** (Figure S1) revealed their identical absolute configurations. The relative stereochemistry of pyranohexose ring was determined by $^1H-^1H$ coupling constants and NOE correlations (Figure 1c); attempts to determine the absolute configuration of the deoxysugar residue by Mosher ester derivatization were unsuccessful. From these data, the absolute and relative configurations of **6** were determined as 3*S*, 3a*R*, 1′*R**, 2′*S**, 4′*R** and 5′*S**. To the best of our knowledge, the hexose residue of **6** represents the first natural example of an *N*-acetylcysteamine functionalized sugar.

With the discovery of cyanosporasides from two taxonomically distinct actinomycetes, we had the opportunity to explore the molecular basis for the biosynthesis of these unique nitrile and sugar functionalized molecules of hypothesized enediyne origin (Figure 1b). From the draft genome of *S. pacifica* CNS-143,[6] we identified just one enediyne biosynthetic gene cluster (*cya*), sharing homology with the previously established sporolide (*spo*) pathway from *S. tropica*.[7] Sequence gaps within the putative cyanosporaside gene cluster (*cya*) were filled by sequencing portions of three overlapping cosmids encompassing the complete cluster. In total, a contiguous region of ~56 kb of DNA was established, with 34 putative open reading frames (orfs) spanning the ~49 kb proposed *cya* locus (Figure 2a, Table S3).

In the case of *Streptomyces* sp. CNT-179, PCR screening[3] similarly established a single enediyne-associated gene cluster in this strain. Construction and screening of a cosmid library for these enediyne-encoding genes yielded the putative *Streptomyces* sp. CNT-179 cyanosporaside gene cluster (*cyn*) of 39 proposed orfs spanning ~52 kb (Figure 2b, Table S4). Although the *cya* and *cyn* cyanosporaside clusters are rearranged and nonsyntenic, the majority of their shared gene products are >50% sequence identical (Table S4). Both the *Streptomyces* sp. and *S. pacifica* cyanosporaside clusters contain a predicted PKSE-encoding gene as well as other putative orfs highly conserved across previously reported biosynthetic pathways for 9-membered enediynes (Tables S3–S4).[3,7,8] Further analysis of the KS domains from these PKSE genes using the online tool NapDos (http://napdos.ucsd.edu/)[6] revealed a shared evolutionary history with other nine-membered enediynes.

To establish functional linkage of the *S. pacifica cya* locus with cyanosporaside production, we inactivated the conserved enediyne epoxide hydrolase *cyaF* by employing PCR-targeted

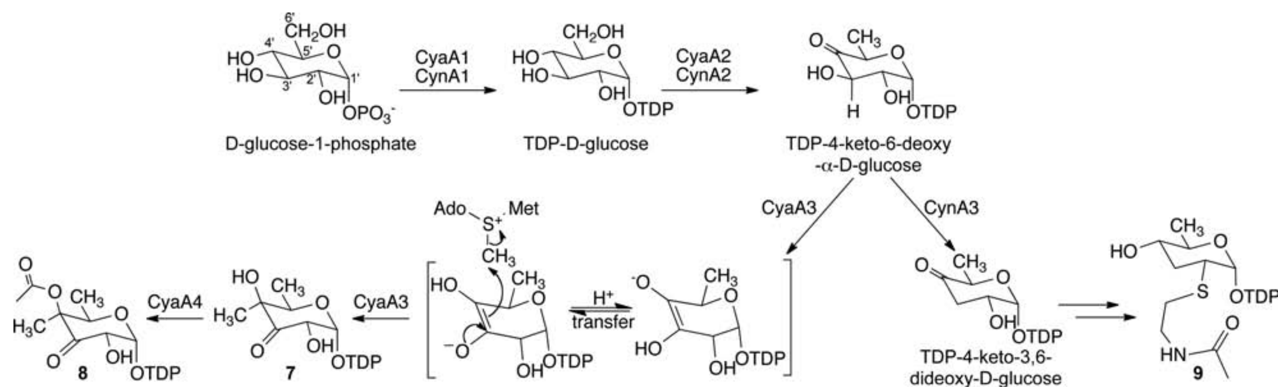

Figure 3. Proposed pathway for biosynthesis of unprecedented sugar groups **7**–**8** of cyanosporasides A–C (**1**–**3**) from *S. pacifica* and **9** of cyanosporaside F (**6**) from *Streptomyces* sp.

gene replacement methods that we previously established in *S. tropica*.[9] Chemical analysis of the resultant mutant bacterium revealed the abolished production of all known cyanosporasides, thereby providing the first direct support linking the production of these molecules to an enediyne biosynthetic pathway. We postulate that the enediyne polyketide precursor rapidly decomposes abiotically under the saline laboratory fermentation conditions to give the chlorinated cyanosporaside products, since we did not detect activity in the biochemical induction assay (BIA) for DNA-interfering compounds[10] or observe evidence for enediynes in LC-MS or NMR screens of crude chemical extracts (data not shown). This rapid decomposition may be explained by the lack of predicted proteins with homology to known enediyne-binding apoproteins that function to stabilize labile 9-membered enediyne groups.[1,2] While it is plausible that an apoprotein may be encoded but lacks homology with characterized enediyne-stabilizing proteins, as found with the maduropeptin-binding apoprotein,[11] this scenario appears unlikely as the cyanosporaside clusters do not share short orfs consistent with the length of characterized enediyne-binding apoproteins.

Of >150 000 known natural products, fewer than 0.1% feature a nitrile moiety,[12] making the cyanosporaside nitrile group particularly fascinating. As the construction of 9-membered enediyne skeletons proceeds from eight malonate units,[1,13] cyanosporaside nitrile functionalization does not require formation of a new C–C bond but rather a new C–N bond at C-10. Pursuing the hypothesis of a unified cyanosporaside nitrile functionalization mechanism, sequences of putative proteins from both cyanosporaside gene clusters were compared (Table S4). Proteins with strong homology to those for enediyne core biosynthesis, glycosylation, pathway regulation, and antibiotic resistance were eliminated from consideration, leaving three candidates for nitrile biosynthesis: the aminotransferases CyaN1 and CynN1 (82% similarity), the oxidoreductases CyaN2 and CynN2 (78% similarity), and the cytochrome P450 monooxygenases Orf9 and Orf29 (80% similarity) from *S. pacifica* and *Streptomyces* sp., respectively. To explore their role in cyanosporaside biosynthesis, *cyaN1*, *cyaN2*, and *orf9* were individually inactivated via PCR-targeted gene replacement. Chemical profiling of resulting *S. pacifica* mutants revealed the loss of cyanosporaside production in the *cyaN1::apr*^{R} aminotransferase and *cyaN2::apr*^{R} oxidoreductase mutants, while deletion of *orf9* did not abolish cyanosporaside production (Figure S19), suggesting its noninvolvement in nitrile functionalization.

Thus, we propose that nitrile functionalization minimally occurs via the highly conserved two gene *N1*–*N2* operon (Figure 2). CynN1 and CyaN1 are pyridoxal phosphate-dependent family III aminotransferases (Figure S21),[14] with limited homology to BorJ (~35% identity), an aminotransferase implicated in the nitrile functionalization of borrelidin.[15] BorJ and related aminotransferases typically act upon carbonyl groups, catalyzing conversion to amines.[15] Thus, a C-10 aldehyde intermediate is plausible in cyanosporaside assembly.

CynN2 and CyaN2 are homologous to flavin-dependent oxidoreductases and dehydrogenases and are candidates for formation of the aldehyde intermediate on which the aminotransferase may act. Another possibility is that CynN2 and CyaN2 catalyze oxidation of the amine group, afforded by aminotransferases CynN1 and CyaN1, to yield the nitrile moiety. Borrelidin provides a biosynthetic literature precedent for bacterial conversion of a primary amine to a nitrile group, and a multifunctional cytochrome P450 was proposed for the conversion via a putative aldoxime intermediate.[15] Neither CynN2 nor CyaN2 exhibited significant similarity to the oxidoreductase proposed in nitrile functionalization of borrelidin, suggesting that nitrile functionalization of cyanosporasides proceeds through a divergent route. With two candidate genes now identified for nitrile functionalization of cyanosporasides, the stage is set for biochemical investigations to unveil the unique functionality expected for the N1 and N2 enzymes.

Cyanosporasides A–C (**1**–**3**) represent the only known natural products featuring a 3′-oxo-4′-methyl-*β*-fucopyranose group. Methylation at C-4′ is particularly intriguing, as methyl branching at this position is rare in comparison to C-3′ and C-5′.[16] Bioinformatics analysis of the *cya* gene cluster revealed six *cyaA1-A4*, *cyaGT*, *orf24* proteins homologous to characterized glycosylation enzymes (Figure 2a, Table S3). CyaA1 and CyaA2 are most closely related to characterized glucose-1-phosphate thymidylyltransferases and 4,6-dehydratases, respectively, and are together proposed to catalyze conversion of D-glucose-1-phosphate to TDP-4-keto-6-deoxy-*α*-D-glucose, the committed pathway intermediate to 6-deoxy sugar units.[16] CyaA3 exhibits 47% similarity to a biochemically characterized SAM-dependent *C*-methyltransferase that carries out regioselective C-3′ methylation in mycarose biosynthesis.[17] We propose that CyaA3 deprotonates H-3′ of TDP-4-keto-6-deoxy-*α*-D-glucose to give an enolate intermediate poised for nucleophilic methylation at C-4′ and afford the unique regiochemistry observed for sugar moieties **7**–**8** (Figure 3).

The essential role of the CyaA3 methyltransferase was probed by deleting the corresponding gene to abolish production of glycosylated cyanosporasides. In cyanosporaside C (**3**), the deoxysugar residue is acylated via acyltransferase CyaA4 to yield **8**, as supported by PCR-directed gene elimination of *cyaA4*, in which production of **3** was selectively lost in the corresponding deletion mutant (Figure S20). Finally, *cyaGT* is proposed to act as a glycosyltransferase, based on its high similarity to known *O*-glycosyltransferases, as supported by the elimination of glycosylated cyanosporasides among *cyaGT::apr*^{R} mutants. Interestingly, elimination of the putative epimerase-encoding gene, *orf24*, did not alter cyanosporaside biosynthesis (Figure S20), refuting an essential role of this gene in cyanosporaside biosynthesis.

Cyanosporaside F (**6**) is structurally distinguished from the *S. pacifica* cyanosporasides A–C (**1**–**3**) by the glycosyl group, which to our knowledge represents the first example of an *N*-acetylcysteamine functionalized secondary metabolic sugar. On the basis of examination of the putative *cyn* sugar biosynthetic gene cluster that shares close homologues to *cyaA1* and *cyaA2* (Table S4), biosynthesis of the sugar unit in **6** is proposed to diverge from the *S. pacifica* sugar pathway following assembly of TDP-4-keto-6-deoxy-α-D-glucose (Figure 3). On the basis of the proposed functions of glycosylation-associated proteins deduced from the *cyn* gene cluster (Table S4), sulfur functionalization of C-2′ is anticipated to proceed through substitution of the C-2′ hydroxyl group (Figure 3), potentially through a nucleophilic substitution mechanism. However, the biosynthetic details of this functionalization remain enigmatic.

In summary, we discovered new members of the cyanosporaside natural product family and showed that these chloro- and cyano-cyclopenta[*a*]indene glycosides are indeed enediyne polyketide biosynthetic products as previously hypothesized.[4b] We moreover identified a two-gene operon implicated in nitrile functionalization that distinguishes the cyanosporasides from other enediyne products. The relatively simple structures and small gene clusters of the cyanosporasides (~50 kb) in comparison with other characterized enediynes (~70–100+ kb) may render the cyanosporaside clusters as promising model systems for exploring hypotheses regarding the biochemistry of enediyne biosynthesis.

■ ASSOCIATED CONTENT

Supporting Information

Experimental section, supporting tables and figures, and detailed compound characterization are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

bsmoore@ucsd.edu; wfenical@ucsd.edu

Present Addresses

‡A.L.L.: Chemistry Department, University of North Florida, Jacksonville, FL 32224.

£S.-J.N.: Department of Chemistry and Nano Science, Ewha Womans University, Seoul 120-750, Republic of Korea.

⊥T.F.: School of Pharmacy, Kitasato University, 5-9-1, Shirokane, Minato-ku, Tokyo 108-8641, Japan.

Notes

The authors declare no competing financial interest.

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