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Chemistry of a unique polyketide-like synthase

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Abstract

Like many complex natural products, the intricate architecture of saxitoxin (STX) has hindered full exploration of this scaffold's utility as a tool for studying voltage-gated sodium ion channels and as a pharmaceutical agent. Established chemical strategies can provide access to the natural product; however, a chemoenzymatic route to saxitoxin that could provide expedited access to related compounds has not been devised. The first step toward realizing a chemoenzymatic approach toward this class of molecules is the elucidation of the saxitoxin biosynthetic pathway. To date, a biochemical link between STX and its putative biosynthetic enzymes has not been demonstrated. Herein, we report the first biochemical characterization of any enzyme involved in STX biosynthesis. Specifically, the chemical functions of a polyketide-like synthase, SxtA, from the cyanobacteria *Cylindrospermopsis raciborskii* T3 are elucidated. This unique megasynthase is comprised of four domains: methyltransferase (MT), GCN5-related *N*-acetyl-transferase (GNAT), acyl carrier protein (ACP), and the first example of an 8-amino-7-oxononanoate synthase (AONS) associated with a multi-domain synthase. We have established that this single polypeptide carries out the formation of two carbon-carbon bonds, two decarboxylation events and a stereospecific protonation to afford the linear biosynthetic precursor to STX (**4**). The synthetic utility of the SxtA AONS is demonstrated by the synthesis of a suite of α -amino ketones from the corresponding α -amino acid in a single step.

Nature's approach to complex molecule synthesis often exploits orthogonal strategies to those employed by synthetic chemists. In the case of saxitoxin (STX, **6**),¹ the natural product's complex structure coupled with potent biological activity^{2–4} has challenged chemists for decades to design increasingly streamlined approaches toward this 5,5,6-tricycle, which contains a hydrated ketone moiety and seven total nitrogen atoms in the form of two cyclic guanidinium units and a carbamate group.^{1, 5} Despite the effort that has been dedicated to STX total synthesis, Nature's approach to STX biosynthesis has yet to be fully

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Notes

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ASSOCIATED CONTENT

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elucidated and exploited for the chemoenzymatic synthesis of STX, STX analogs or STX biosynthetic intermediates. Herein, we report the first biochemical characterization of an enzyme involved in STX biosynthesis and demonstrate the potential of this enzyme as a biocatalytic tool for the synthesis of α -amino ketones.

STX's unique structure has inspired several proposed biosynthetic pathways that draw on known biosynthetic mechanisms and a limited number of feeding studies.^{6–7} These proposals were further refined following Neilan and coworkers' identification and annotation of putative STX gene clusters (*sxt*) from cyanobacterial STX producers.^{8–11} STX biosynthesis is proposed to be initiated by the synthesis of a linear intermediate such as **4**,^{12–13} which could be further elaborated through oxidative cyclizations to tricycle **5**.^{14–15} From **5**, a series of late-stage functionalization events would afford STX as well as higher oxidation level analogs (Fig. 1A).

Although nearly a decade has elapsed since the first report of a STX gene cluster, concrete *in vivo* or *in vitro* experiments to identify the function of *sxt* gene products and establish a biochemical link between putative STX gene clusters and STX biosynthesis have not been reported. To begin unraveling the chemistry involved in STX biosynthesis, we have taken an *in vitro* approach to characterization of STX biosynthetic enzymes starting with the enzyme responsible for initiating STX biosynthesis, SxtA.

Conserved in all known cyanobacterial STX gene clusters,¹⁶ *sxtA* encodes for a unique four-domain enzyme with polyketide synthase (PKS)-like features, yet lacking the hallmark ketosynthase domain of PKSs. SxtA is comprised of an *S*-adenosyl methionine (SAM)-dependent methyltransferase domain at the N-terminus, followed by a GCN5-related *N*-acetyltransferase (GNAT) domain,¹⁷ an acyl carrier protein (ACP) and an 8-amino-7-oxononanoate synthase (AONS).¹⁸ It has been postulated that SxtA is responsible for the assembly of the linear STX precursor **4** from a coenzyme A (CoA) substrate, a molecule of arginine and a methyl group derived from SAM (Fig. 1A).⁸ A number of questions were outstanding at the onset of this study, including the CoA building block utilized by SxtA, the identity of the SxtA product, the mechanism for product formation, and the substrate promiscuity of this enzyme.

Shimizu conducted a series of feeding experiments in toxin-producing microorganisms that demonstrated the incorporation of labeled acetate into **6**, to support the hypothesis that acetyl-CoA is a substrate in STX biosynthesis.¹⁹ In contrast, the annotation of a GNAT domain in SxtA in place of a more common PKS acyltransferase domain suggests that malonyl-CoA could be the true starter unit for SxtA. The most studied GNAT within a PKS, CurA, has been shown to have the dual function of malonyl-CoA decarboxylation and transfer of the remaining acetyl unit onto the *holo*-ACP domain.¹⁷ Following loading, it was proposed that the SxtA methyltransferase (MT) domain would methylate the alpha position of acetyl-ACP to generate propionyl-ACP (**20**, Fig. 3).⁸ Finally, the pyr-idoxal phosphate (PLP)-dependent AONS domain was postulated to catalyze a C–C bond formation between the α -carbon of arginine and thioester carbon of the acyl-ACP substrate (**20**), decarboxylate, and then, stereospecifically protonate to afford ethyl ketone **4** (Fig. 1B).¹⁸

To begin elucidating the chemistry of SxtA, a number of CoAs were evaluated as substrates for SxtA from the cyanobacteria *Cylindrospermopsis raciborskii*. Reactions with SAM, arginine and various CoA substrates were conducted with *holo*-SxtA, which possesses a phosphopantetheinyl (Ppant) group covalently tethered to the ACP conserved serine. In these reactions, two SxtA-generated products were observed: (1) the expected ethyl ketone **4**, as well as (2) the analogous truncated methyl ketone **16**, which is anticipated to be a shunt product that was not methylated by the MT domain (Table 1). Reactions with acetyl-CoA led to the exclusive formation of the methyl ketone (**16**, Table 1, entry 1). Likewise, propionyl-CoA was converted to the ethyl ketone (**4**) without detectable formation of isopropyl or *t*-butyl ketone products, which would arise through α -methylation (entry 2). Together, these data indicate that methylation does not occur on acetyl or propionyl substrates under the *in vitro* conditions provided, but that both acetyl and propionyl substrates can be accepted by the AONS domain to undergo productive condensation with arginine. Using malonyl-CoA as a substrate resulted in the formation of both **4** and **16**, where the MT domain had productively methylated 45% of the material processed by SxtA (entry 3). Finally, reactions with methylmalonyl-CoA exclusively afforded the ethyl ketone product (**4**, entry 4), supporting methylmalonyl as a biosynthetic intermediate that can be efficiently converted to the SxtA product. In control experiments, only **16** was observed when SAM was omitted (entry 5), confirming that SAM is the methyl group donor. A small amount of **4** was observed when arginine was not added (entry 6), most likely resulting from small amounts of arginine co-purifying with SxtA. From this survey of CoA substrates, the methyltransferase domain was only active when SxtA was presented with malonyl-CoA. These experiments support a recent proposal from Keatinge-Clay that malonyl-ACP is the true substrate for the SxtA MT²⁰ and are also consistent with Shimizu's feeding studies, as malonyl-CoA can be generated from acetyl-CoA in cyanobacteria.²¹

With insight into the building blocks processed by SxtA, we began investigating the role of the SxtA GNAT. Studies on the CurA GNAT demonstrated distinct decarboxylation and loading activities with malonyl-CoA.¹⁷ In the case of SxtA, this type of dual function GNAT would lead to acetyl-ACP, which is not productively methylated by the SxtA MT. To probe the role of the GNAT domain in SxtA ACP loading, SxtA ACP was excised and expressed as a standalone domain. *Holo*-ACP was incubated with malonyl-CoA alone or in the presence of SxtA GNAT or SxtA MT-GNAT. Each reaction was assessed at multiple time points for relative ratios of *holo*-ACP, malonyl-ACP (**18**) and acetyl-ACP (Fig. 2A). In the absence of GNAT or MT-GNAT, >80% of the ACP was loaded in 2 h. Addition of GNAT or MT-GNAT consistently led to a modest increase in the percentage of loaded ACP. This provides evidence for parallel ACP loading mechanisms, which in addition to GNAT-mediated loading could be attributed to promiscuous acyltransferases,²² a *trans*-acting type II ACP, the hyper-reactive nature of malonyl-CoA²³ or ACP self-loading.^{24–27}

Next, the decarboxylation activity of the GNAT domain was evaluated. Methylmalonyl-ACP, which is proposed to be generated by SxtA MT methylation of malonyl-ACP,^{20,27} was combined with SxtA GNAT (5 or 15 μ M). In the presence of GNAT, decarboxylation of methylmalonyl-ACP to propionyl-ACP is observed over 3 h, with increasing amounts of decarboxylation observed with higher concentrations of GNAT (Fig. 2B). Replacing the

standalone GNAT with the MT-GNAT didomain at the same concentrations produced parallel results.

Together, these results support the proposed sequence of SxtA reactions outlined in Fig. 3. We anticipate that SxtA catalysis is initiated by malonyl-CoA (**17**) loading onto the SxtA ACP followed by methylation of malonyl-ACP (**18**) by the SAM-dependent MT domain. Next, SxtA GNAT can decarboxylate methylmalonyl-ACP (**19**) to propionyl-ACP (**20**). The final SxtA-mediated step is C–C bond formation between the α -carbon of arginine and the thioester carbon of the acyl-ACP substrate by a PLP-dependent AONS domain.¹⁸ Alternatively, malonyl-CoA can be processed by SxtA without methylation, leading to the formation of the truncated SxtA product (**16**).

With a refined proposal for the sequence of reactions mediated by SxtA, we interrogated the function of each domain in the full SxtA reaction. A series of SxtA variants was generated to further elucidate the role of each domain. The conserved ACP Ppant attachment site (S773) was substituted with alanine to create a system in which substrates cannot be covalently bound to the full SxtA module. *In vitro* reactions with SxtA(S773A) demonstrated that the MT, GNAT and AONS domains can also act on CoA substrates (Table 2, entry 2). Addition of *holo*-ACP *in trans* to reactions with SxtA(S773A) led to a nearly 4-fold increase in product formation, providing support for an accelerated reaction and increased methylation with acyl-ACP substrates over CoA substrates (entry 3). Although initial alignment of the SxtA MT showed low sequence identity with any characterized MTs, recent work by Skiba and coworkers²⁸ on a SxtA MT homolog suggests that H372 is necessary for catalysis in the SxtA MT. Reactions with SxtA(H372A) led to exclusive formation of the truncated product (**16**) in significantly lower yields (entry 4). To further confirm the GNAT decarboxylation activity, two residues implicated in decarboxylation activity were substituted.¹⁷ Reactions with the SxtA GNAT variants, SxtA(T637V) and SxtA(H671A), demonstrated a dramatic decrease in product formation, and interestingly, ethyl ketone **4** was the exclusive product, further supporting the hypothesis that methylation only occurs on malonyl substrates (entries 5 and 6). Finally, the AONS was mutagenized to remove the lysine residue proposed to be critical for catalysis (K1077).¹⁸ As anticipated, SxtA(K1077L) did not form any detectable ethyl or methyl ketone products (**4** or **16**, entry 7).

Reactions with the SxtA variant panel provided important insight on the chemistry performed by SxtA. We were particularly intrigued by the ability of all SxtA domains to act on free acyl-CoA substrates in addition to ACP-bound intermediates and sought to capitalize on the utility of this system.^{28–30} Based on the complexity-generating nature of the transformation, we began by investigating the promiscuity of the AONS domain on CoA substrates unnatural to SxtA. In a single enzymatic step, SxtA AONS successfully transformed arginine (**1**) into a variety of ketone derivatives (Fig. 4). CoA substrates bearing linear alkyl chains ranging from one to seven carbons in length (entries 1–5), branched alkyl groups (entries 6 and 7), as well as aromatic and saturated rings (entries 8 and 9) were converted to the corresponding ketone derivatives of arginine. In contrast to the direct conversion mediated by SxtA AONS, several synthetic steps are required to transform an α -amino acid into an α -amino ketone. For example, the synthesis of **21** required a linear six-

step sequence to access each ketone.¹³ Thus, we anticipate the SxtA AONS has potential as a useful biocatalyst.

The experiments described herein provide the first biochemical link between saxitoxin biosynthesis and putative saxitoxin gene clusters. The chemistry of the unique polyketide-like synthase, SxtA, has been elucidated to determine the discrete transformations catalyzed by each domain (Fig. 1B) as well as the sequence of reactions (Fig 3). The potential utility of SxtA domains as general bio-catalysts is supported by the discovery that each domain can act on thioester substrates free in solution and are not restricted to ACP-bound substrates. As a first-step toward demonstrating this utility, a variety of α -amino ketones were synthesized in a single step from arginine. Knowledge of the basic chemistry mediated by each SxtA domain will fuel more in-depth structure and mechanism studies as well as the development of biocatalytic methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Proposed STX biosynthetic pathway. (A) Three phases of STX biosynthesis. (B) Proposed SxtA reactions.

Figure 2. Reactions with excised SxtA ACP. (A) Loading of *holo*-ACP with malonyl-CoA (**17**). (B) Decarboxylation of methylmalonyl-ACP (**19**) to propionyl-ACP (**20**). See SI for reaction conditions.

Figure 3.
Proposed sequence of SxtA reactions to produce 4.

Figure 4.

Reactions with excised SxtA AONS to generate α -amino ketones.

^aProduct yields were calculated by comparison to an ¹⁵N arginine internal standard by LC-MS, and normalized to entry 2. ^bProduct formed via an acyl-ACP intermediate. See SI for complete reaction conditions.

Table 1

Reactions of SxtA with various CoA substrates

entry	substrate (R =)	SAM	Arg	normalized product, yield ^a	% ethyl ketone (4)	% methyl ketone (16)
1	Me	+	+	91 ± 7	ND	100
2	Et	+	+	656 ± 40	100	ND
3		+	+	100 ± 15	45	55
4		+	+	519 ± 64	100	ND
5		-	+	165 ± 6	ND	100
6		+	-	4 ± 1	100	ND

^aProduct yields were calculated by comparison to an ¹⁵N arginine internal standard by LC-MS, and normalized to entry 3. See SI for complete reaction conditions. ND: not detected.

Table 2

Reactions of malonyl-CoA (7) and SxtA variants

entry	SxtA variant (modified domain)	normalized product yield ^a	% ethyl ketone (4)	% methyl ketone (16)
1	wt	100 ± 18	45	55
2	S773A (ACP)	106 ± 22	42	58
3	S773A (ACP) + excised wt ACP	387 ± 75	63	27
4	H372A (MT)	46 ± 9	ND	100
5	T637V (GNAT)	27 ± 1	100	ND
6	H671A (GNAT)	9 ± 2	100	ND
7	K1077L (AONS)	0	ND	ND

^aProduct yields were calculated by comparison to an ¹⁵N arginine internal standard by LC-MS, and normalized to entry 1. See SI for complete reaction conditions. ND: not detected.