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Biocatalytic synthesis of α-amino ketones

Stephanie W. Chun[a,b] **Alison R. H. Narayan**[a,b]

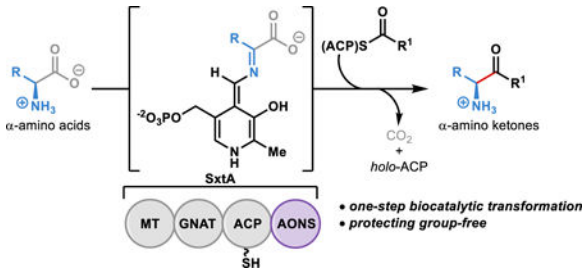
[a]Department of Chemistry, University of Michigan, 930 North University Ave, Ann Arbor, MI 48109-1055, USA

[b]Life Sciences Institute, University of Michigan, 210 Washtenaw Ave, Ann Arbor, MI 48109-2216, USA

Abstract

Stereospecific generation of α-amino ketones from common α-amino acids is difficult to achieve, often employing superstoichiometric alkylating reagents and requiring multiple protecting group manipulations. In contrast, the α-oxoamine synthase protein family performs this transformation stereospecifically in a single step without the need for protecting groups. Herein, we detail the characterization of the 8-amino-7-oxononanoate synthase (AONS) domain of the four-domain polyketide-like synthase SxtA, which natively mediates the formation of the ethyl ketone derivative of arginine. The function of each of the four domains is elucidated, leading to a revised proposal for the initiation of saxitoxin biosynthesis, a potent neurotoxin. We also demonstrate the synthetic potential of SxtA AONS, which is applied to the synthesis of a panel of novel α-amino ketones.

Graphical Abstract



Keywords

biocatalysis; biosynthesis; α-oxoamine synthases; neurotoxins; α-amino ketones; pyridoxal-5'-phosphate

1 Introduction

Pyridoxal-5'-phosphate (PLP) is a versatile enzyme cofactor that typically functions by stabilizing negative charge as a temporary sink for two electrons, while the subsequent

arhardin@umich.edu.

chemistry is controlled by the environment of the enzyme active site.[1] PLP-mediated enzymatic transformations include, but are not limited to, transamination, decarboxylation, epimerization and elimination reactions, many of which are difficult to perform with precise chemo- and stereoselectivity using traditional chemical methods (Scheme 1).[2,3]

The α-oxoamine synthase (AOS) family is a small subset of PLP-dependent enzymes.[4,5] Characterized members of this family catalyze the stereospecific carbon-carbon bond formation between the α-carbon of an α-amino acid and the carbonyl carbon of specific acyl carrier protein (ACP)-bound substrates or coenzyme A (CoA)-activated thioesters. This initial C–C bond formation is followed by decarboxylation to form α-amino ketone products (Scheme 2A). We recently characterized the function of the 8-amino-7-oxononanoate synthase (AONS) domain contained within the polyketide synthase-related protein SxtA.[6] This protein from the cyanobacterium *Cylindrospermopsis raciborskii* T3 was first annotated as an AOS by Neilan and coworkers in 2008.[7] SxtA contains four domains, three that are typical polyketide synthase (PKS) domains, but lacks a ketosynthase domain requisite for true PKSs.[7,8] Like type I PKSs, SxtA catalysis is initiated by loading of an acyl group from a CoA substrate onto the ACP domain. Acyl-ACP intermediates are then shuttled to various partner enzyme domains before release of the final small molecule product. Neilan and coworkers proposed that SxtA is the first protein in the biosynthetic pathway of the paralytic shellfish toxin saxitoxin (STX, **9**, Scheme 2).[7,9,10] Based on feeding studies with labeled precursors carried out by Shimizu and others, Neilan proposed the order of events outlined in Scheme 2B: (1) GCN5-related *N*-acetyltransferase (GNAT) loading of the ACP domain with the starter unit acetyl-CoA to form acetyl-ACP (**5**); (2) methylation at the alpha position by the *S*-adenosylmethionine-dependent methyltransferase (MT) domain to generate propionyl-ACP (**6**); (3) AONS-mediated condensation of propionyl-ACP and L-arginine (L-Arg) to afford ketone **8**, CO_2 and the regeneration of *holo*-ACP.[11] Later reports by Yotsu-Yamashita and others demonstrated that **8** is elaborated to the natural product, saxitoxin (**9**).[12]

While additional *sxt* gene clusters have been found in other saxitoxin-producing cyanobacteria since the Neilan group's initial disclosure, no homologs of SxtA or any other Sxt proteins had been biochemically characterized prior to our study.[6] Thus, in order to employ SxtA AONS as a biocatalytic tool, it was necessary to verify the amino acid substrate as well as the activity of the upstream domains leading to the formation of the correct acyl-ACP substrate. This knowledge provides a platform for exploring the synthetic utility of SxtA AONS as a biocatalyst.

2 Native SxtA module activity

Full length SxtA from *C. raciborskii* was expressed in *E. coli* strain BAP1[19] and purified to afford an approximately 144 kDa pale yellow protein for *in vitro* experiments. However, in the presence of acetyl-CoA, *S*-adenosylmethionine (SAM) and L-Arg, the expected product **8** was not observed; only the truncated methyl ketone **10** was detected, which is anticipated to be produced by AONS-mediated condensation of L-Arg and acetyl-ACP/CoA without methylation by the MT domain (Scheme 3). These experiments confirmed L-Arg as the AONS amino acid substrate, but no amount of optimization of reaction conditions

employing acetyl-CoA as a substrate afforded **8**. As no saxitoxin derivatives lacking C13 have been isolated to date, we concluded that acetyl-CoA is not the correct starter unit for SxtA.

The second domain of SxtA was annotated as a GNAT rather than a more common PKS acyltransferase loading domain. Gu *et al.* have shown that the closely related CurA GNAT from the curacin A biosynthetic pathway decarboxylates its starter unit malonyl-CoA to acetyl-CoA, suggesting that malonyl-CoA is also the true starter unit for SxtA.[20] Replacing acetyl-CoA with malonyl-CoA (**11**, Scheme 3) in reactions with SxtA yielded a mixture of the truncated methyl ketone **10** and the expected ethyl ketone **8**, where 45% of the ketone product produced had been methylated by the MT domain. Using related substrates, methylmalonyl-CoA (**12**) and propionyl-CoA (**13**), gave only the full-length ketone **8**, without additional α-methylation, which would afford the isopropyl or *tert*-butyl ketones. Of the four potential starter units, the MT was only active on the malonyl substrate. Based on the superior yield obtained when starting with propionyl-CoA, an on-pathway methylmalonyl intermediate would likely precede a propionyl species. Reactions with SxtA variant S773A, which removes the serine residue where the phosphopantetheine cofactor is attached to the ACP domain, with **11** demonstrated that all partner domains are active on acyl-CoA substrates in addition to acyl-ACP intermediates. These results and recent findings from Skiba *et al.* on related MTs in AprA and the GphF loading module support Keatinge-Clay's proposal that this panel of MTs methylate malonyl-ACP instead of acetyl-ACP, with adjacent intact GNAT domains responsible for subsequent decarboxylation.[21,22]

To investigate the reactions on ACP-bound intermediates, the MT-GNAT and GNAT were cloned as a didomain and single domain, respectively, to afford standalone proteins to probe the reactions of these domains in *trans* with the discretely expressed ACP. The ACP-bound products could then be analyzed more easily using the Ppant ejection mass spectrometry assay.[23] Malonyl-ACP and methylmalonyl-ACP were prepared enzymatically from *apo*-ACP and the corresponding CoA thioester using the promiscuous phosphopantetheinyl-transferase Sfp,[24] although acyl-ACP used at lower concentration due to its preparation method. Incubation of malonyl-ACP (**14**) with MT-GNAT and SAM produced mixtures of acetyl-ACP, methylmalonyl-ACP, and propionyl-ACP products (Scheme 4A). Some acetyl-ACP (**5**) was present before the addition of MT-GNAT due to spontaneous decarboxylation of **14**, and the relative concentration of acyl-ACP was constant over the three-hour reaction. Methylmalonyl-ACP (**15**) was only observed at early timepoints as it formed from malonyl-ACP, but then was decarboxylated to deliver propionyl-ACP (**6**). Malonyl-ACP was not methylated the absence of MT-GNAT. Consumption of malonyl-ACP and methylmalonyl-ACP was complete after two hours. In these experiments, none of the remaining acetyl-ACP was methylated to generate propionyl-ACP, and neither dimethylmalonyl-ACP nor isobutyryl-ACP resulting from a second methylation event were observed. Decarboxylation was probed separately in reactions with ACP loaded with carboxylated substrates and the excised GNAT. Both the putative GNAT native substrate, methylmalonyl-ACP (**15**), and malonyl-ACP (**14**) were decarboxylated in a concentration-dependent manner (Scheme 4B).

Given these data, we proposed the refined catalytic cycle outlined in Scheme 5. After loading of *holo*-ACP (**16**) with the starter unit malonyl-CoA (**11**, by an as-yet undetermined

mechanism), the SAM-dependent MT methylates malonyl-ACP (**14**) at the alpha position to form methylmalonyl-ACP (**15**). Subsequent decarboxylation by the GNAT domain affords the propionyl-ACP (**6**) that serves as the electrophile in the AONS-mediated condensation with arginine to provide the expected ketone product **8**. Spontaneous and GNAT-mediated premature decarboxylation of malonyl-ACP to acetyl-ACP (**5**) would account for the formation of the methyl ketone **10** observed with the full SxtA module. Malonyl-CoA is also carboxylated *in vivo* from acetyl-CoA, accounting for the incorporation of labeled acetate at STX carbons 5 and 6 observed by Shimizu in feeding studies (see **9**, Scheme 2).[25] We also anticipate that **10** is an *in vitro* shunt product only, as this ketone has never been isolated from STX-producing microorganisms.

3 New reactions with SxtA AONS

α-oxoamine synthases are involved in the biosynthetic pathways of critical metabolites such as tetrapyrroles and threonine.[4,26,27] Select examples of carrier protein-associated synthases similar to SxtA have also been reported.[28,29] The AONS domain of SxtA takes its name from the well-studied 8-amino-7-oxononanoate synthase protein (BioF) of biotin biosynthesis.[7] All members of the AOS family follow a common mechanism (Scheme 6A): from the internal aldimine resting state of PLP (**17**), the key lysine residue (Lys1077 in SxtA) is displaced by the amino acid substrate, forming the external aldimine (**18**). The alpha proton of the amino acid is deprotonated by a base, possibly the same catalytic lysine residue, to give the common quinonoid intermediate **19**. A new C–C bond is then formed between the α-carbon and the acyl-thioester substrate to afford a tetrahedral intermediate (see **20**) that then releases *holo*-ACP **16** or CoASH. The resulting 1,3-dicarbonyl (**21**) is readily decarboxylated except in the case of ketobutyric acid ligases. Finally, stereospecific protonation (see **22** to **23**) and displacement of product by the lysine residue or another molecule of the amino acid substrate closes the catalytic cycle.

The analogous chemical transformation of an unprotected amino acid to an alkyl ketone requires significantly more synthetic steps. First, the amine group and reactive side chains must be protected before conversion to a Weinreb amide (**25**, Scheme 6B).[30] Unlike the biocatalytic reaction in which the original carbonyl is lost as CO_2, the carbonyl carbon of the synthetic standard is retained and the new carbon-carbon bond is formed directly to the carbonyl carbon through a Grignard reaction. Deprotection of the amine and side chain groups affords the final product in a 4–6 step linear sequence, compared to a one-step enzymatic reaction without any protecting groups. The synthetic route to the SxtA native product **8** required 6 steps from L-ornithine due to protecting group manipulation on the side chain. Other chemical methods for synthesizing α-amino ketones from α-amino acids also involve the use of excess reactive organolithium or organocuprate reagents, or generate racemic products (the Dakin-West reaction).[31,32]

Reactions with the SxtA wild-type and S773A modules have demonstrated that the SxtA AONS domain can process at least one non-native substrate, acetyl-ACP, in addition to propionyl thioesters. Furthermore, the AONS domain was also compatible with free CoA thioesters. To probe the reactivity of the standalone domain, the AONS was cloned and expressed separately and incubated with a selection of commercially available acyl-CoA

thioesters (Scheme 7). As expected, **10** and **8** were produced from acetyl- and propionyl-CoA, respectively (entries 1 and 2). Longer straight alkyl chains from three to seven carbons (entries 3–5) underwent condensation with arginine to generate the corresponding α-amino ketone products. Interestingly, a chain of three carbons (entry 3) was produced in higher amounts than the native product that possesses a two-carbon chain. Branched substrates (entries 6–7) were also processed, although generally in lower yields. Finally, the AONS mediated the formation of two ketones bearing a cyclic substituent, one aromatic (entry 8) and one saturated (entry 9). For the cyclopentyl ketone (entry 9), the corresponding acyl-ACP (**29**) was made by *in situ* transthioesterification[33] with thioester **28** as cyclopentanoyl-CoA is not commercially available. This reaction also demonstrates that the AONS reacts with standalone acyl-ACP *in trans*. All products were verified by LC-MS comparison to synthetic standards, which were prepared by a 6-step route analogous to the preparation of **8** (Scheme 6B).

4 Conclusions and outlook

The chemical steps that initiate saxitoxin biosynthesis have been elucidated. The three building blocks are assembled by SxtA to generate the earliest known intermediate in the biosynthesis of this paralytic shellfish toxin. Malonyl-CoA, the true starter unit of SxtA, was found to be methylated at the α-carbon after loading onto SxtA ACP, subsequently decarboxylated, and condensed with L-Arg to form the expected ketone **8**. The potential of the final C–C bond-forming domain, AONS, as a new biocatalyst was then tested, with the AONS catalyzing the formation of several new arginine-derived ketones. Further development of SxtA AONS and related synthases will provide more direct access to chiral α-amino ketones.

Acknowledgment

All authentic standards were synthesized by Dr. Meagan Hinze. Dr. Meredith Skiba provided cloning assistance. A plasmid containing *sxtA* was provided by Prof. Brett Neilan, Dr. Paul D'Agostino and Dr. Rabia Mazmouz. Dr. Priyanka Bajaj is thanked for cloning and protein purification assistance. We are grateful to Prof. Janet Smith and Prof. Anna Mapp for helpful discussions.

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Biosketches

Alison Narayan holds a Ph.D. in organic chemistry from the University of California, Berkeley, where she worked under the direction of Prof. Richmond Sarpong. She completed her undergraduate studies in chemistry at the University of Michigan, where she later returned as a postdoctoral research fellow in the lab of Prof. David Sherman. She joined the Department of Chemistry and the Life Sciences Institute as an assistant professor in 2015.

Stephanie Chun graduated with a B.S. in chemistry and biology from Brandeis University, where she performed research under Prof. Isaac Krauss. She is currently a PhD candidate in the Department of Chemistry at the University of Michigan working in the Narayan lab.

References

(1). Eliot AC; Kirsch JF Annu. Rev. Biochem 2004, 73, 383–415. [PubMed: 15189147]

(2). Boville CE; Scheele RA; Koch P; Brinkmann-Chen S; Buller AR; Arnold FH Angew. Chem. Int. Ed 2018, 9, 14764–14768.

(3). Steffen-Munsberg F; Vickers C; Kohls H; Land H; Mallin H; Nobili A; Skalden L; van den Bergh T; Joosten H-J; Berglund P; Höhne M; Bornscheuer UT Biotechnol. Adv 2015, 33, 566–604. [PubMed: 25575689]

(4). Webster SP; Alexeev D; Campopiano DJ; Watt RM; Alexeeva M; Sawyer L; Baxter RL Biochemistry 2000, 39, 516–528. [PubMed: 10642176]

(5). Yard BA; Carter LG; Johnson KA; Overton IM; Dorward M; Liu H; McMahon SA; Oke M; Puech D; Barton GJ; Naismith JH; Campopiano DJ J. Mol. Biol 2007, 370, 870–886. [PubMed: 17559874]

(6). Chun SW; Hinze ME; Skiba MA; Narayan AR H. J. Am. Chem. Soc 2018, 140, 2430–2433. [PubMed: 29390180]

(7). Kellmann R; Mihali TK; Young JJ; Pickford R; Pomati F; Neilan BA Appl. Environ. Microbiol 2008, 74, 4044–4053. [PubMed: 18487408]

(8). Staunton J; Weissman KJ Nat. Prod. Rep 2001, 18, 380–416. [PubMed: 11548049]

(9). Mihali TK; Kellmann R; Neilan BA BMC Biochem 2009, 10, 8. [PubMed: 19331657]

(10). Mihali TK; Carmichael WW; Neilan BA PLoS One 2011, 6, e14657. [PubMed: 21347365]

(11). Shimizu Y Chem. Rev 1993, 93, 1685–1698.

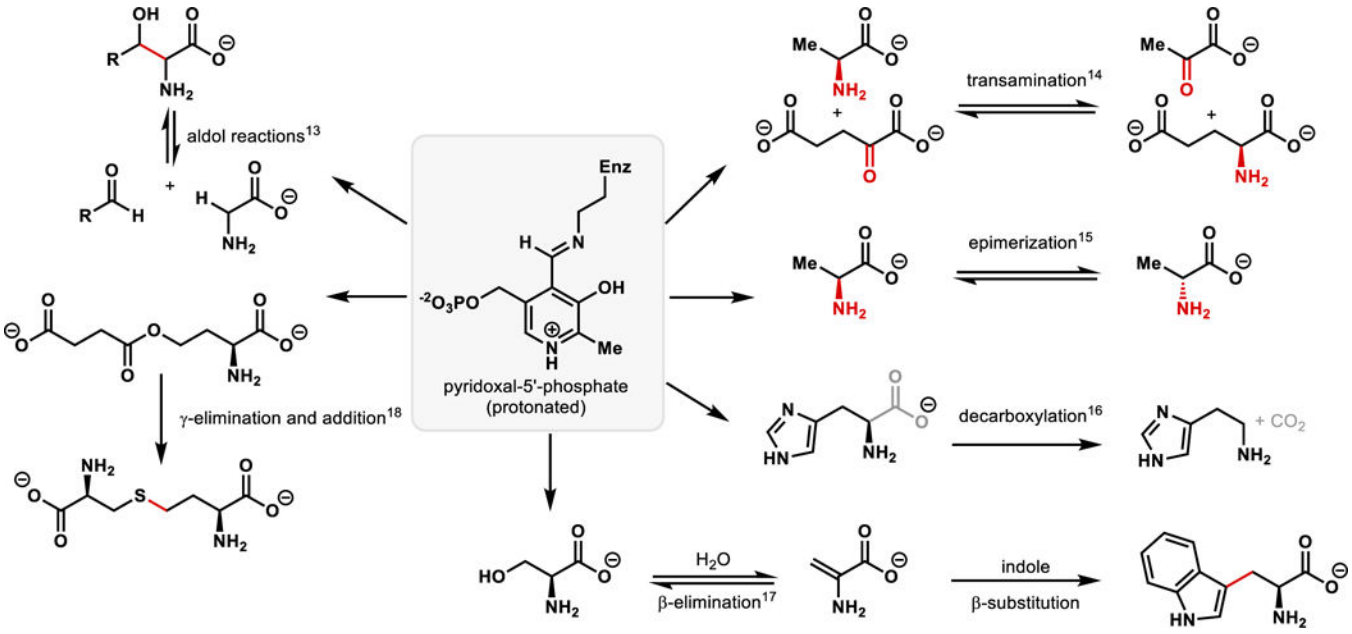
(12). Tsuchiya S; Cho Y; Konoki K; Nagasawa K; Oshima Y; Yotsu-Yamashita M Sci. Rep 2016, 6, 20340. [PubMed: 26842222]

(13). Fesko K Appl. Genet. Mol. Biotechnol 2016, 100, 2579–2590.

(14). Duff SMG; Rydel TJ; Mcclerren AL; Zhang W; Li JY; Sturman EJ; Halls C; Chen S; Zeng J; Peng J; Kretzler CN; Evdokimov A Arch. Biochem. Biophys 2012, 528, 90–101. [PubMed: 22750542]

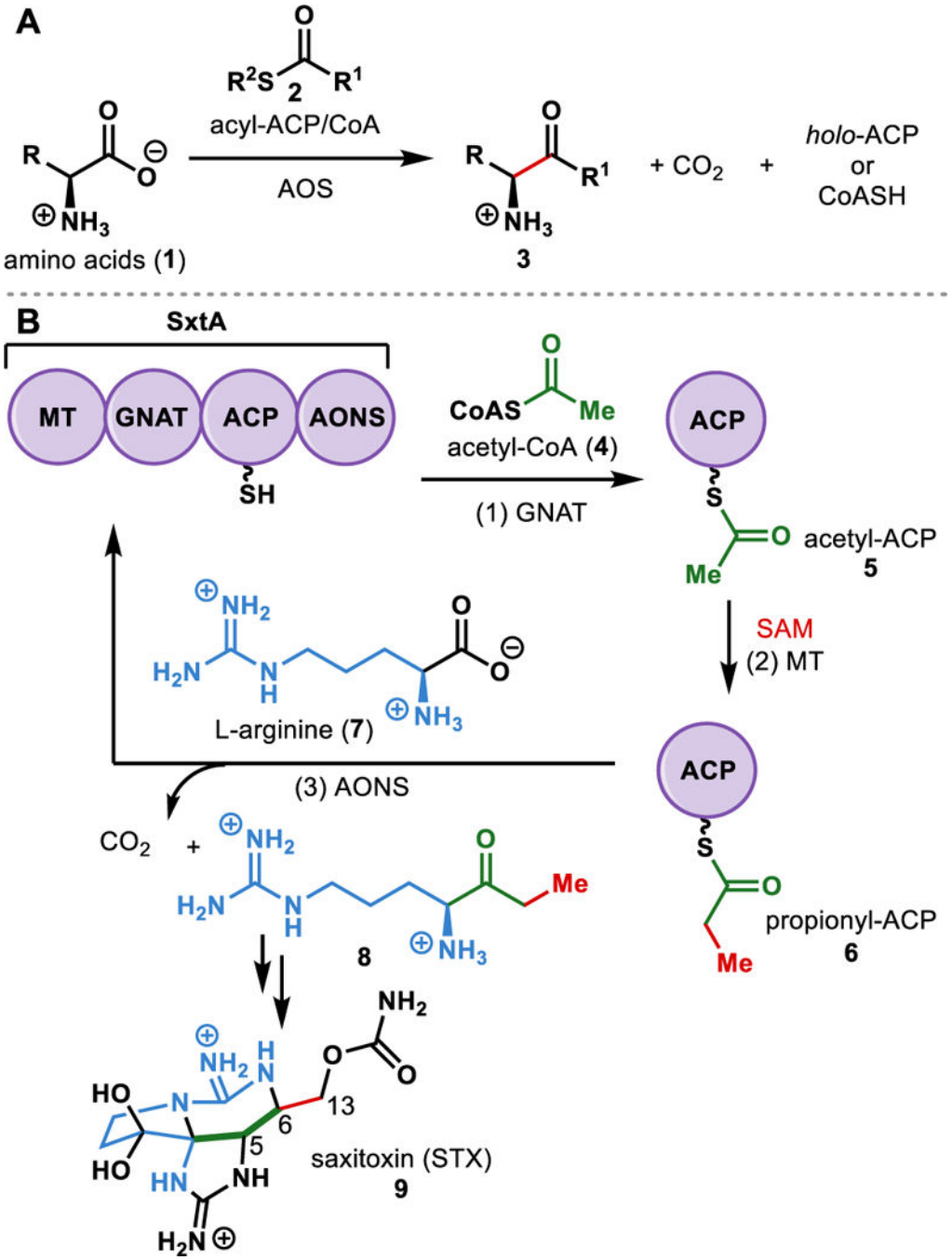
(15). Lemagueres P; Im H; Dvorak A; Strych U; Benedik M; Krause KL Biochemistry 2003, 42, 14752–14761. [PubMed: 14674749]

(16). Komori H; Nitta Y; Ueno H; Higuchi YJ Biol. Chem 2012, 287, 29175–29183.
(17). Schneider TR; Gerhardt E; Lee M; Liang P; Anderson KS; Schlichting I Biochemistry 1998, 37, 5394–5406. [PubMed: 9548921]
(18). Clifton MC; Abendroth J; Edwards TE; Leibly DJ; Gillespie AK; Ferrell M; Dieterich SH; Exley I; Staker BL; Myler PJ; Van Voorhis WC; Stewart LJ Acta Crystallogr. F 2011, 67, 1154–1158.
(19). Pfeifer BA; Admiraal SJ; Gramajo H; Cane DE; Khosla C Science 2001, 291, 1790–1792. [PubMed: 11230695]
(20). Gu L; Geders TW; Wang B; Gerwick WH; Hakansson K; Smith JL; Sherman DH Science 2007, 318, 970–975. [PubMed: 17991863]
(21). Skiba MA; Sikkema AP; Moss NA; Tran CL; Sturgis RM; Gerwick L; Gerwick WH; Sherman DH; Smith JL ACS Chem. Biol 2017, 12, 3039–3048. [PubMed: 29096064]
(22). Keatinge-Clay AT Chem. Rev 2017, 117, 5334–5366. [PubMed: 28394118]
(23). Dorrestein PC; Bumpus SB; Calderone CT; Garneau-Tsodikova S; Aron ZD; Straight PD; Kolter R; Walsh CT; Kelleher NL Biochemistry 2006, 45, 12756–12766. [PubMed: 17042494]
(24). Quadri LE; Weinreb PH; Lei M; Nakano MM; Zuber P; Walsh CT Biochemistry 1998, 37, 1585–1595. [PubMed: 9484229]
(25). Liu X; Sheng J; Curtiss III R Proc. Natl. Acad. Sci 2011, 108, 6899–6904. [PubMed: 21482809]
(26). Astner I; Schulze JO; Van Den Heuvel J; Jahn D; Schubert WD; Heinz DW EMBO J 2005, 24, 3166–3177. [PubMed: 16121195]
(27). Schmidt A; Sivaraman J; Li Y; Larocque R; Barbosa JARG; Smith C; Matte A; Schrag JD; Cygler M Biochemistry 2001, 40, 5151–5160. [PubMed: 11318637]
(28). Garneau-Tsodikova S; Dorrestein PC; Kelleher NL; Walsh CT J. Am. Chem. Soc 2006, 128, 12600–12601. [PubMed: 17002325]
(29). Manandhar M; Cronan JE Appl. Environ. Microbiol 2018, 84, e02084–17. [PubMed: 29054876]
(30). Fisher LE; Muchowski JM Org. Prep. Proced. Int 1990, 22, 399–484.
(31). Sheppard GS; Florjancic AS Synthesis 2003, 11, 1653–1656.
(32). Liebeskind LS; Yang H; Li H Angew. Chem 2009, 121, 1445–1449.
(33). Hansen DA; Koch AA; Sherman DH J. Am. Chem. Soc 2015, 137, 3735–3738. [PubMed: 25730816]



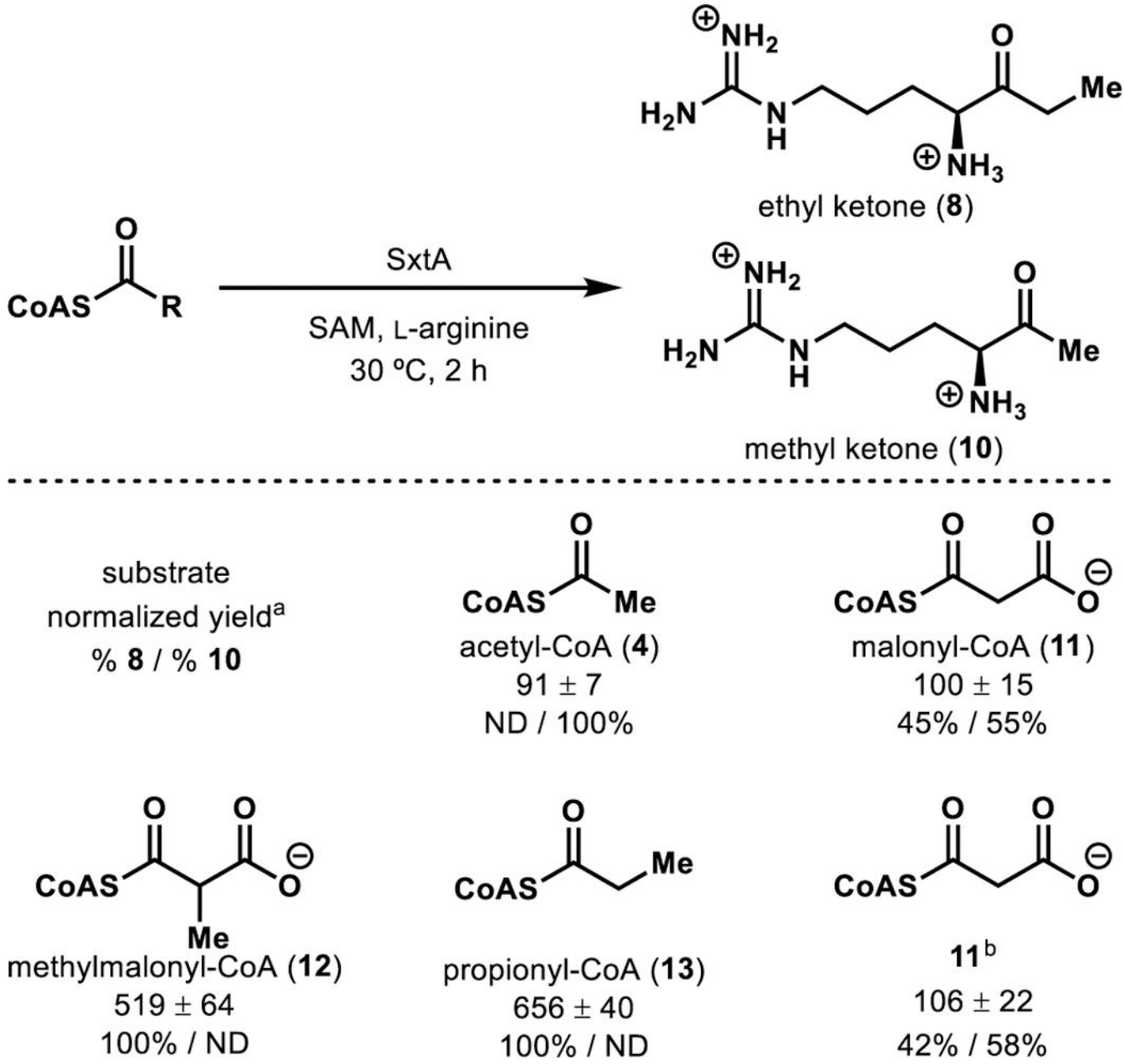
Scheme 1.

Select reactions mediated by PLP-dependent proteins: threonine aldolase[13], Ala aminotransferase[14], Ala racemase[15], His decarboxylase[16], Trp synthase[17], cystathionine gamma synthase.[18]



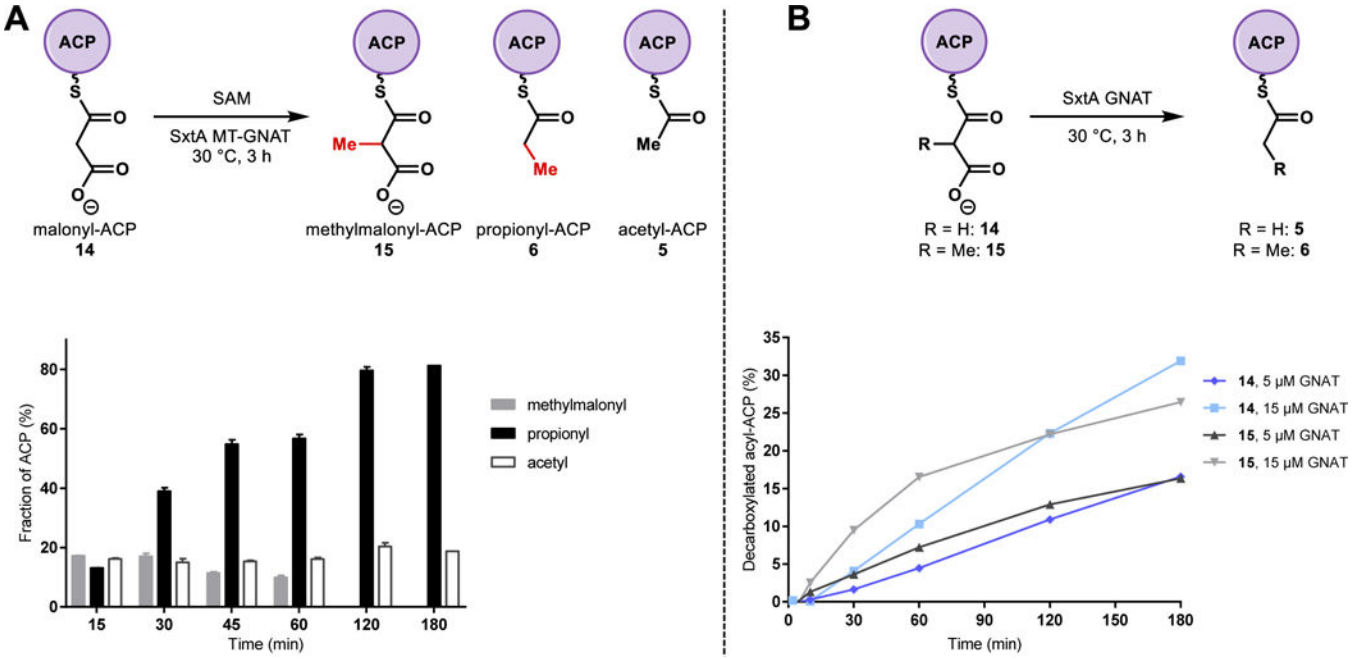
Scheme 2.

(A) Reactions of α-oxoamine synthases (AOS). (B) Initial proposed reactions of SxtA by Neilan and coworkers.[7] SAM: *S*-adenosylmethionine.



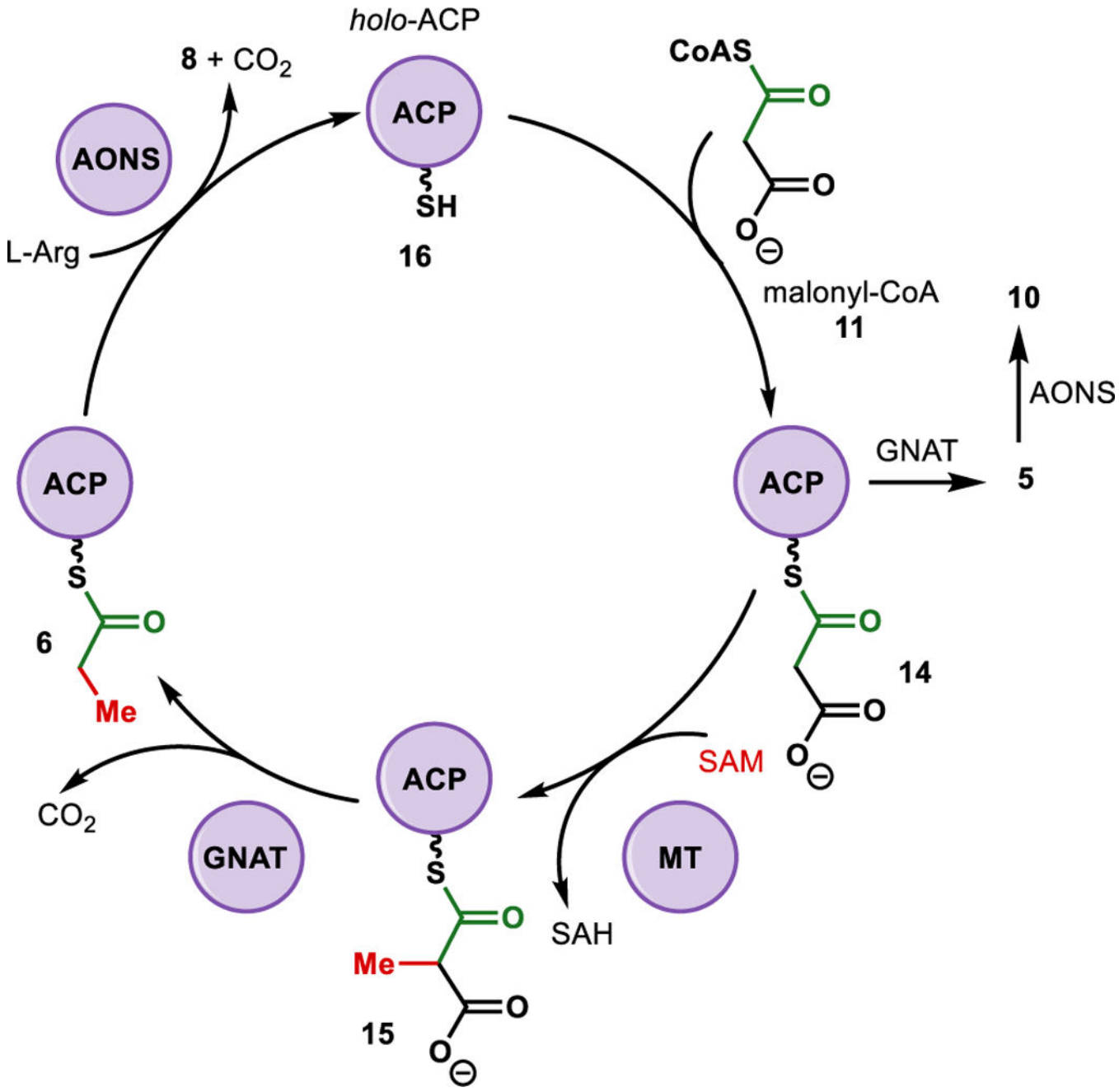
Scheme 3.

Reactions of the SxtA module. [a]Product yields were calculated by comparison to an ^{15}N arginine internal standard by LC-MS and normalized to the reactions of wild-type SxtA with malonyl-CoA. [b]Used SxtA(S773A). Reaction conditions: 50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.5 mM $MnCl_2$, 250 μM malonyl-CoA, 2 mM l-Arg, 1 mM SAM, 10 μM SxtA, 30 °C, 2 h. ND: not detected.

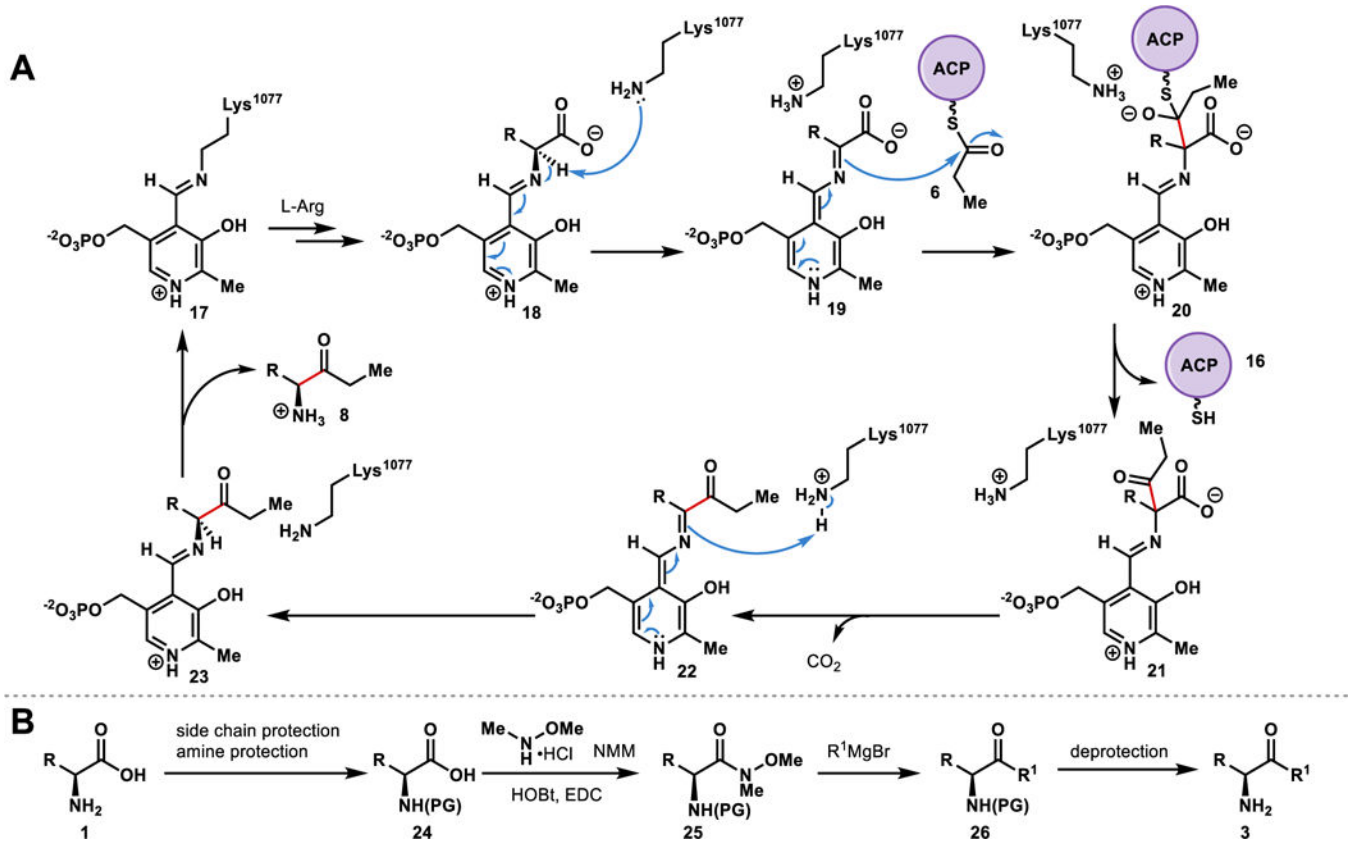


Scheme 4.

(A) Methylation and decarboxylation of malonyl-ACP. (B) GNAT-mediated decarboxylation of malonyl- and methylmalonyl-ACP. Reaction conditions: 50 mM HEPES pH 7.4, 150 mM NaCl, 1 mm $MgCl_2$, 0.5 mM $MnCl_2$, 100 μM acyl-ACP, 5–15 μM MT-GNAT or GNAT, 30 °C, 3 h. Methylation reactions only: 1 mM SAM.

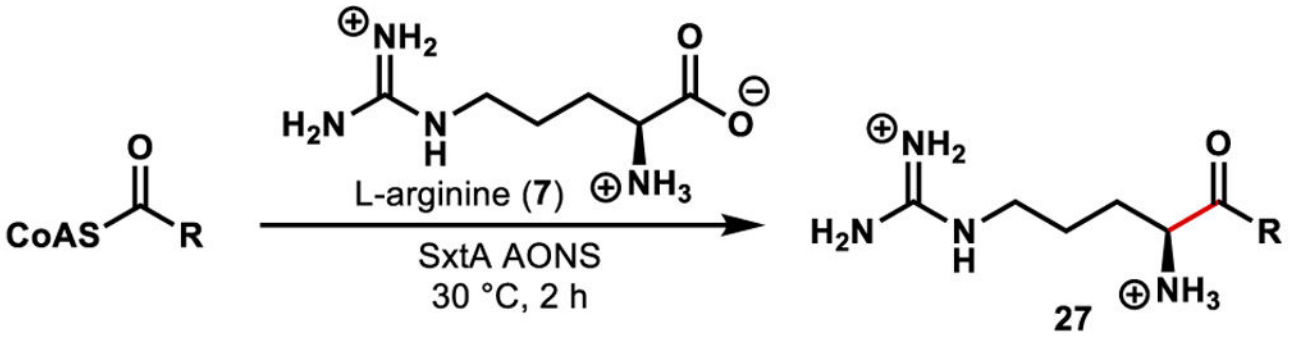


Scheme 5.
Our revised SxtA catalytic cycle. SAH: *S*-adenosylhomocysteine.

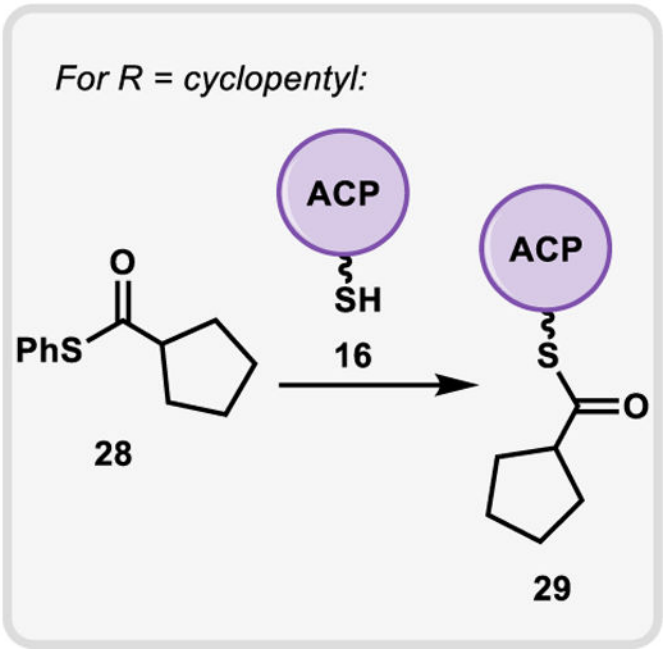


Scheme 6.

(A) Representative α-oxoamine synthase (AOS) catalytic cycle with SxtA AONS. (B). Synthesis of α-amino ketone standards.



entry	R =	normalized yield
1	Me	11 ± 2
2	Et	100 ± 17
3	*n*-Pr	190 ± 10
4	*n*-pentyl	9.2 ± 0.6
5	*n*-heptyl	67 ± 5
6	iPr	2.3 ± 0.5
7	iBu	39 ± 2
8	Ph	2.9 ± 0.2
9[a]	cyclopentyl	12 ± 2



Scheme 7.

Reactions of the excised AONS domain. [a]via *in situ* acyl-ACP. Reaction conditions: 50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.5 mM $MnCl_2$, 500 μM acyl-CoA, 2 mM L-Arg, 8 μM AONS, 30 °C, 2 h.