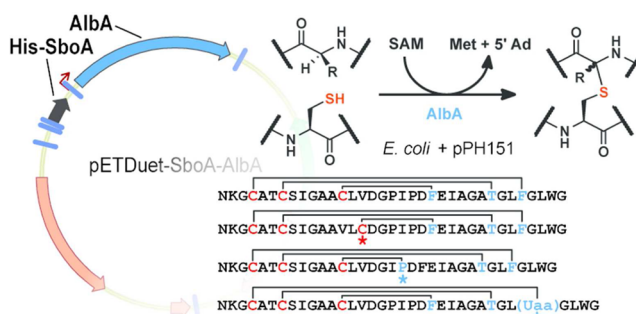


Production of Sactipeptides in *Escherichia coli*: Probing the Substrate Promiscuity of Subtilosin A Biosynthesis

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ABSTRACT: Sactipeptides are peptide-derived natural products that are processed by remarkable, radical-mediated cysteine sulfur to α -carbon coupling reactions. The resulting sactionine thioether linkages give rise to the unique defined structures and concomitant biological activities of sactipeptides. An *E. coli* heterologous expression system, based on the biosynthesis of one such sactipeptide, subtilosin A, is described and this expression system is exploited to probe the promiscuity of the subtilosin A sactionine bond-forming enzyme, AlbA. These efforts allowed the facile expression and isolation of a small library of mutant sactipeptides based on the subtilosin A precursor peptide, demonstrating broad substrate promiscuity where none was previously known. Importantly, we show that the positions of the sactionine linkages can be moved, giving rise to new, unnatural sactipeptide structures. *E. coli* heterologous expression also allowed incorporation of unnatural amino acids into sactipeptides by means of amber-suppression technology, potentially opening up new chemistry and new applications for unnatural sactipeptides.



Sactipeptides are an emerging class of peptide-derived natural products. The hallmarks of compounds in the class are intramolecular thioether bridges between cysteine sulfurs and the unreactive α -carbons of bridging partner amino acids (Figure 1a). To date, five sactipeptides have been isolated and structurally characterized, including subtilosin A, the sporulation killing factor (Skf), thurincin H, and the two-component sactipeptide thuricin CD (Figure 1b).^{1–8} Unlike the β -thioether bridges in the analogous lantipeptides, these sactionine linkages are quaternary due to the bridging partner side chain. Additionally, sactipeptide rings tend to be coaxial with the peptide backbone constrained in a U-like formation. The overall distribution of sactipeptide bridges combines with the stereochemistry of the α,α -disubstituted residues in the bridging partner residues to give sactipeptides regions of defined secondary structure, as in the 3_{10} -helical N-terminus of subtilosin A or the α -helical faces of thuricin CD (Figure 1c). The often amphipathic helicity of regions of the peptides is thought to grant subtilosin A and its congeners the ability to interact with and disrupt bacterial cell walls resulting in often bactericidal, typically narrow spectrum, activity.^{4,9,10} Still, the precise mechanisms of action of many sactipeptides are not yet fully understood.

In terms of biosynthesis, sactipeptides are ribosomally synthesized and post-translationally modified peptide natural products (RiPPs).¹¹ As such, they are produced from gene-encoded precursor peptides, which are post-translationally modified to install the distinctive sactionine linkages. Recently, Marahiel and co-workers characterized the key enzymes

responsible for installing thioethers in subtilosin A, Skf, and thurincin H, in particular, the radical SAM enzymes, AlbA, SkfB, and ThnB, respectively.^{12–14} These so-called sactionine synthetases are members of the newly defined SPASM-domain (Subtilosin A/PQQ/Anaerobic Sulfates Maturing) -containing family of enzymes and are predicted to have two [4Fe-4S] clusters, one each for activating the sulfhydryl and for coupling partner carbon center in what amounts to a radical–radical heterocoupling during cyclization (Figure 1a).¹⁵ Large gaps still exist in our understanding of the mechanism and promiscuity of the sactionine synthetases and the effect that changes in structure may have on their potential therapeutic properties. In particular, how the sactionine synthetases select multiple different bridging partner residues specifically at each bridge site and to what extent that can be manipulated or modified are still looming questions.

A homologous expression system has been reported for thurincin H, using a plasmid encoded copy of the precursor peptide in a precursor-knockout background of the native producer, *B. thuringiensis* SF361.¹⁶ Additionally, mutants of subtilosin A, genetically encoded on a pDG-148 plasmid and coexpressed with the native substrate, have been isolated in the native producer *B. subtilis* 168.¹³ While both of these approaches allowed some mutational sampling, the promiscuity

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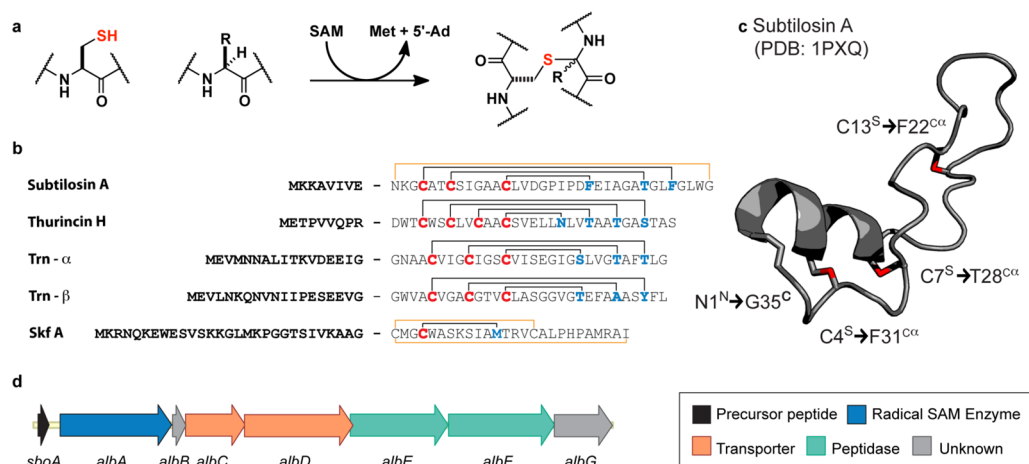


Figure 1. Sactipeptide biosynthesis. (a) Sactonine linkage formation from cysteines and cross-ring amino acid coupling partners. (b) Comparison of amino acid sequences of known sactipeptides with sactonine cysteines in red and bridging partners in blue. (c) NMR structure of subtilosin A, indicating sactonine linkages and head to tail cyclization. (d) The subtilosin A biosynthetic cluster from *Bacillus subtilis* sp. 168 including the structural gene *sboA* and radical SAM sactonine synthase-encoding gene *albA*.

of the respective radical SAM enzymes (rSAM) could be obscured by multiple factors: (1) poor downstream processing by other pathway enzymes potentially also contributing to (2) inefficient export by the dedicated natural product transporters in the clusters, (3) decreased transcriptional amplification by the known feedback regulation mechanisms or (4) rapid proteolytic degradation of partially modified peptides by the potent intracellular protease activity inherent to many strains of *Bacilli*.

We anticipated that development of a heterologous system for *in vivo* overexpression of modified sactipeptides would facilitate future investigations of their biosynthesis, as well as allow for rapid, large-scale production of modified variants.^{17–23} We sought a system that would allow robust overexpression of precursor peptides to allow isolation of sufficient materials for downstream characterization and/or potential application. We therefore set out to develop a system to allow heterologous expression of processed sactipeptides in *E. coli* under the control of the strong T7 promoter and purification from cell pellets. *E. coli* expression would further allow the adaptation of mature amber stop-codon suppression technology, as has been employed to varying extents with other RiPPs.^{20,24–28} We chose to focus these efforts first on the sactipeptide subtilosin A, its precursor peptide SboA, and radical SAM enzyme AlbA (Figure 1d). Subtilosin A contains three sactonine thioether bridges and therefore provides a number of opportunities to examine the interdependency of the different bridge formation sites.

■ HETEROLOGOUS PRODUCTION OF PRESUBTILOSIN A

In order to facilitate purification of heterologously expressed sactipeptides, we first tested enzyme compatibility with N-terminal or C-terminal hexahistidine tags. The subtilosin A precursor gene *sboA* was separately cloned into pMCSG7 (N-terminal-His tag) and pET28c (C-terminal-His tag) and expressed and purified from *E. coli* BL21(DE3) cells. Full-length peptides were purified from inclusion bodies using His-trap Ni-NTA columns according to the procedure of Li *et al.*²⁹ In parallel, N-terminal-His₆-tagged radical SAM AlbA was expressed, purified, and reconstituted as described by Flühe *et*

al. Upon incubation of peptides and enzyme, we could confirm complete formation of all three sactonine bridges by LC/MS and MS/MS. Therefore, AlbA appears indifferent to N- or C-terminal extensions. In the present application, this result suggested that tandem heterologous expression of AlbA and a His-tagged SboA could feasibly yield mature sactipeptides. We chose to move forward with the N-terminal tag as we anticipated that the fortuitous placement of several lysine residues in SboA would allow facile removal of the tag together with the leader peptide *via* trypsin digestion.

Precursor and enzyme were subsequently cloned into multiple cloning sites 1 (MCS1) and 2 (MCS2), respectively, of the pETDuet-1 vector to generate the bicistronic construct (Figure 2a). Unlike the native producer, this expression system would not be capable of producing true head-to-tail cyclized product because we did not include any candidate protease capable of forming this last linkage. It has been shown that this lack of head-to-tail cyclization does not affect the activity of the sactonine synthetase AlbA, and head-to-tail cyclization is not present in a number of other sactipeptides, such as Thurincin H and Thurincin CD.¹³ A variety of conditions were tested to affect sactonine formation *in vivo* (Figure 2b). Ultimately, we found that coexpression in the presence of pPH151 containing the *E. coli* suf ABCDSE genes and dropping the shake rate significantly improved expression of the modified sactipeptide.^{30,31} These two measures facilitate proper AlbA expression and activity: first by proper assembly and repair of the Fe–S cluster, assisted by suf ABCDSE, and second by limiting aeration and possible quenching of the enzyme. SDS-PAGE gels of the crude lysates showed substantially improved expression of AlbA under the optimized conditions (see Supporting Information). The high copy duet vector pRSFDuet-1 was also tested but showed negligible improvement over our pETDuet construct. We therefore employed the pETDuet-SboA-AlbA construct in our further examination of AlbA sactonine substrate promiscuity.

■ DESIGN AND EVALUATION OF SBOA MUTANTS

With a functional heterologous system in hand, we next sought to examine the ability of AlbA to accept alternate SboA substrates *in vivo*. To date, sactipeptide mutants have largely been limited to alanine scans or relatively conservative

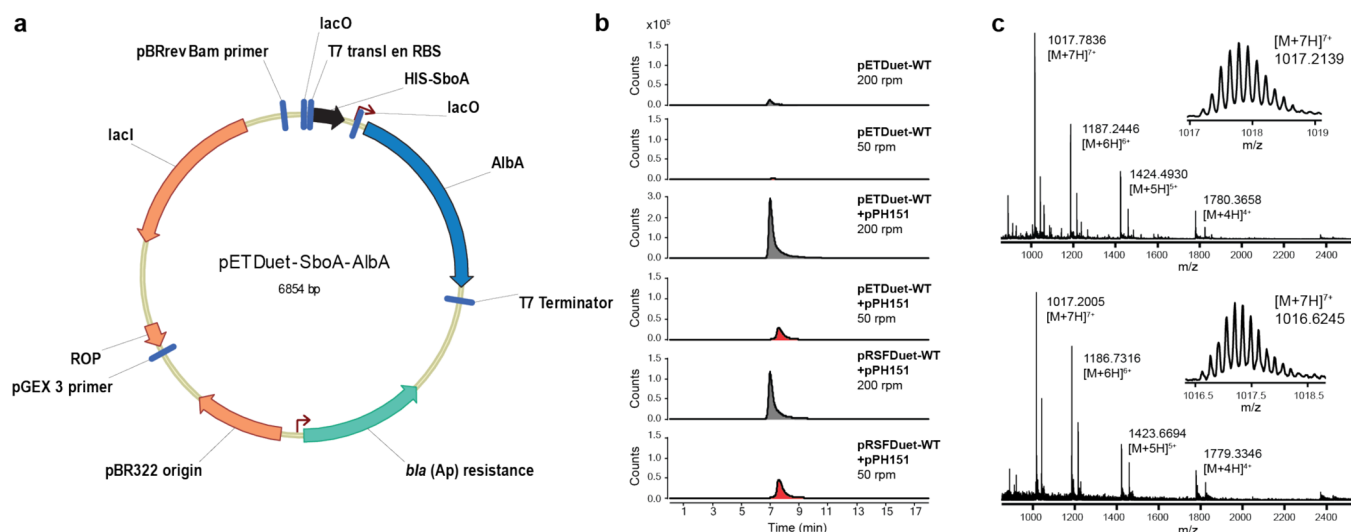


Figure 2. Expression of sactipeptides in *Escherichia coli*. (a) Vector map of pETDuet-SboA-AlbA, showing organization of genes in the two multiple cloning sites. (b) LC/MS comparison of *in vivo* production of precursor peptide (gray, unmodified) and sactipeptide (red, modified) from constructs under varied conditions. pPH151 containing the *E. coli* suf ABCDSE genes aids in expression and repair of Fe–S cluster sactinone synthetase (AlbA). (c) QTOF-MS data of unmodified (top) and modified (bottom) SboA peptide from *E. coli*.

mutations at bridging partner residues. Preliminary efforts by Marahiel and co-workers suggested that AlbA has somewhat strict substrate requirements, standing in contrast to the vast majority of RiPP enzymes characterized to date. We therefore designed our initial mutants with three primary goals in mind: (1) to test the promiscuity for bridging partner residues and to examine requirements for (2) substrate flexibility or “pre-organization” and (3) cysteine spacing relative to the brief leader sequence. Saturation mutagenesis would require a library size too large to allow specific probing of each of these areas; we instead pursued a rational design approach. In the case of the three bridging positions, Cys⁴-Phe³¹^{C α} , Cys⁷-Thr²⁸^{C α} , and Cys¹³-Phe²²^{C α} (Figure 1c), we incorporated a small subset of sterically and electronically distinct amino acids that are observed at bridging partner positions in other known sactipeptides, Ala, Gln, Met, Phe, Ser, and Thr (Table 1, entries 1–14). A second set of mutants targeted alanine scanning at flexible glycine residues adjacent to the sactinone bridging partners (Gly’s 26, 29, and 32), as well as potential turn-inducing prolines at residues 18 and 20. The latter two were also targeted for direct deletion in order to more extensively probe effects of the residues 14–21 loop on nucleating bridge formation. Finally, we moved the cysteine residues themselves around the “N-terminal side” of SboA. In order to minimize the number of potential changes to be tested, we chose to move cysteines by “swapping” them with residues at the respective position. The analogous Ala-swap could also be considered for further investigation.

In total, we designed and examined 40 mutants. Several of these mutants were accessed by QuikChange mutagenesis; however, the majority could be readily obtained by gene synthesis at equivalent or lesser cost (see Supporting Information). In each case, the mutant gene was incorporated into MCS1 of the pETDuet vector containing AlbA in MCS2 and expressed in the pPH151 background under the same optimized conditions as the wild-type SboA using small (100 mL culture size) grow-ups. Peptides were induced with IPTG, as with the wild type, and isolated in a medium throughput manner by using small spin Ni-NTA columns. To facilitate

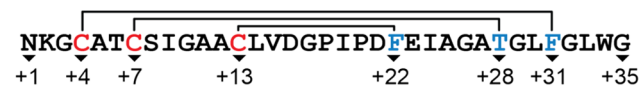
characterization, peptides were further trypsinized to remove the His-tag plus a portion of the leader peptide (cleaves at Lys, –6, Figure 1b).

The distinctive MS/MS fragmentation pattern of sactinone linkages (Figure 3a) was used to evaluate cross-bridge partnering of cysteines in the mutants.^{4,32} The thioamidals in sactinone bridges have been shown to undergo facile retro-elimination and tautomerization to the corresponding dehydroamino acid at low collision voltages as depicted in Figure 3a–d; the new amide is much more labile than a typical peptide bond, owing to the resonance-stabilized enamine leaving group. Thus, at low collision voltages, only the cleavage of these bonds is observed; other amide bonds stay intact, allowing the determination of the bridge location. This MS-generated formal dehydrogenation at bridging partners has previously been reported by Vederas *et al.*, who exploited it to correctly assign connectivity in the structure of sactipeptide thuricin CD. The alternative desulfurization method, employing nickel-borodeuteride, worked in our hands but often gave variable and incomplete results, even with subtilisin A itself.^{7,8} This was potentially due to metal contaminants carried over from Ni-NTA purification or other catalyst positions from the heterologous system. In the MS/MS method, sactinone linkages were easily the most labile at lower collision energies, and these provided strong qualitative confirmation of bridging partner residues with low parts-per-million error. In all cases, the presence of less than three bridges could be readily confirmed by reductive treatment with *N*-ethylmaleimide (NEM) and LC/MS to show masses of relevant adducts. Although sactinone linkages can be formed with differing stereochemistry at the bridge partner α -carbon, we did not examine effects of mutants on stereochemistry in this study. Outside of NMR, there are no good methods to measure sactinone stereochemistry that we could find reported in the literature to date.

We compared production of our modified peptides to native production of subtilisin A in *B. subtilis* 168. *B. subtilis* is reported to produce subtilisin A at roughly 5.5 mg/L.⁶ We obtained between 1 and 2 mg/L dry weight or ~20–40% of

Table 1. SboA Mutants Analyzed in This Study and Sites of Linkages Identified by MS/MS

Sequence and connectivity of wild-type SboA:



Entry	N-term changes	C-term changes	No. Bridges	Bridging Partners
1		F22A	3	A22, T28, F31
2		F22M	3	M22, T28, F31
3		F22S	no product	--
4		F22T	no product	--
5		T28A	3	F22, A28, F31
6		T28F	3	F22, F28, F31
7		T28N	3	F22, N28, F31
8		T28S	3	F22, S28, F31
9		T27/A28	3	F22, A28, F31
10		F31A	3	F22, T28, A31
11		F31M	no product	--
12		F31N	no product	--
13		F31S	3	F22, T28, S31
14		F31T	no product	--
15		G26A	3	F22, T28, F31
16		G29A	3	--
17		G32A	3	F22, T28, F31
18		G26A, G29A	3	--
19		G26A, G32A	3	F22, T28, F31
20		G29A, G32A	3	--
21		G26A, G29A, G32A	3	F22, T28, F31
22		P18A	3	F22, T28, F31
23		P20A	3	F22, T28, F31
24		P18A, P20A	3	F22, T28, F31
25		ΔP18	3	P19, T27, F30
26		ΔP20	3	F21, T27, F30
27		ΔP18, ΔP20	no product	--
28	C1/N4		no product	--
29	C2/K4		no product	--
30	C3/G4		1	F31, 1 disulfide
31	A4/C5		no product	--
32	C6/T7		no product	--
33	S7/C8		1	F31, 1 disulfide
34	I7/C9		no product	--
35	G7/C10		1	F31, 1 disulfide
36	C10/G13		3	F22, T28, F31
37	C11/A13		no product	--
38	C12/A13		3	F22, T28, F31
39	L13/C14		3	F22, T28, F31
40	V13/C15		3	F22, T28, F31

native production from the Duet system (see SI, Figure S29). Production levels of mutants are highly variable, and many of them could only be detected very faintly by UV/vis. Based on extracted ion chromatograms (EICs) of the products, yields of mutants varied from 10% to greater than 300% when compared to EIC of the modified wild type peptide (see SI, Figures S30–S59 and Table S2). In general, many of the cysteine swap mutants were produced at comparable or higher levels than the wild type SboA peptide in the Duet system (see SI, Table S2). In several instances (12 out of 40), we observed no product from induction of a given mutant.

The variable or complete lack of production may happen for a number reasons, such as intrinsic destabilization of the given mutant peptide or its transcript causing degradation or unanticipated metabolic processing, such as glutathionylation or other detoxifying post-translational modification that might cause the product to escape our isolation procedure. We expect that post-translation installation of sactonine linkages would improve peptide stability. Therefore, in the analysis, instances of “no product” were interpreted as a weak proclivity of the

enzyme to accept those substrates. Still, it should be noted that these are the results of expression under the current *in vivo* conditions. It also seems reasonable to speculate that while expression of some mutants may be lower than wild-type, even the production of these low levels has been buoyed by the presence of strong overexpression of the sactonine synthetase relative to levels in the native producer.

■ SUBSTRATE TOLERANCE AT BRIDGING PARTNERS

Several different amino acids appear at bridging partner residues in sactipeptides. Even within subtilisin A itself, phenylalanine and threonine appear at different positions, suggesting that the sactonine synthetases might have some level of tolerance for different bridging partners. A set of alanine mutants at the three bridging partners of subtilisin A was tested in our *E. coli* expression system (Table 1, entries 1, 5, and 10). In all three mutants, all three bridges were formed and MS/MS confirmed that the site of modification remained the same; that is, cross-linking occurred at the new Ala22, Ala28, or Ala31 residue. Marahiel and co-workers had reported a lack of production of F22A, T28A, and F31A bridging partner mutants expressed in parallel with the native cluster in *B. subtilis*. In our hands, however, in an *E. coli* heterologous expression system, these three mutants were well tolerated by the AlbA enzymatic machinery, demonstrating a potential benefit of this approach.

Beyond alanine, only the +28 position appeared particularly permissive to other amino acid residue substitutions. Thr28 could also be substituted with serine, phenylalanine, and asparagine and still be activated and incorporated into a sactonine bridge. Meanwhile, Phe22 could be substituted with methionine and still be processed to a sactonine linkage, but neither serine nor threonine substitutions at this position yielded peptide. Similarly, the F31S was processed to a three-bridge product, but no product could be observed with threonine, methionine, or asparagine substitutions at this position.

Given the promiscuity at the +28 residue, we wondered if there could be any proclivity for the native amino acid. We therefore exchanged Thr28 with the upstream Ala27 to see if this would drive bridge formation toward the newly positioned threonine. This was not the case, however, the sactonine bridge was instead formed at Ala28, suggesting minimal residue level control over steering modifications, at least at this position.

■ SUBSTRATE TOLERANCE AT UNMODIFIED POSITIONS

The precursor peptides for subtilisin and the two components of thuricin CD, Trn- α and Trn- β , all exhibit relatively high glycine content (22%, 26%, and 22% of noncysteine residues respectively). We therefore sought to probe whether substrate flexibility arising from these residues was necessary to allow enzymatic modification or if α -branching might drive a choice of alternative bridging partners. An alanine scan of the three C-terminal glycine residues, Gly26, Gly29, and Gly32, demonstrated that these positions can tolerate at least small, sterically unhindered amino acid side chains at these positions (Table 1, entries 15–21). Moreover, all three positions could be substituted with alanines at once, with no detriment to product formation. All products isolated from these grow-ups exhibited three complete sactonine bridges with the native residues

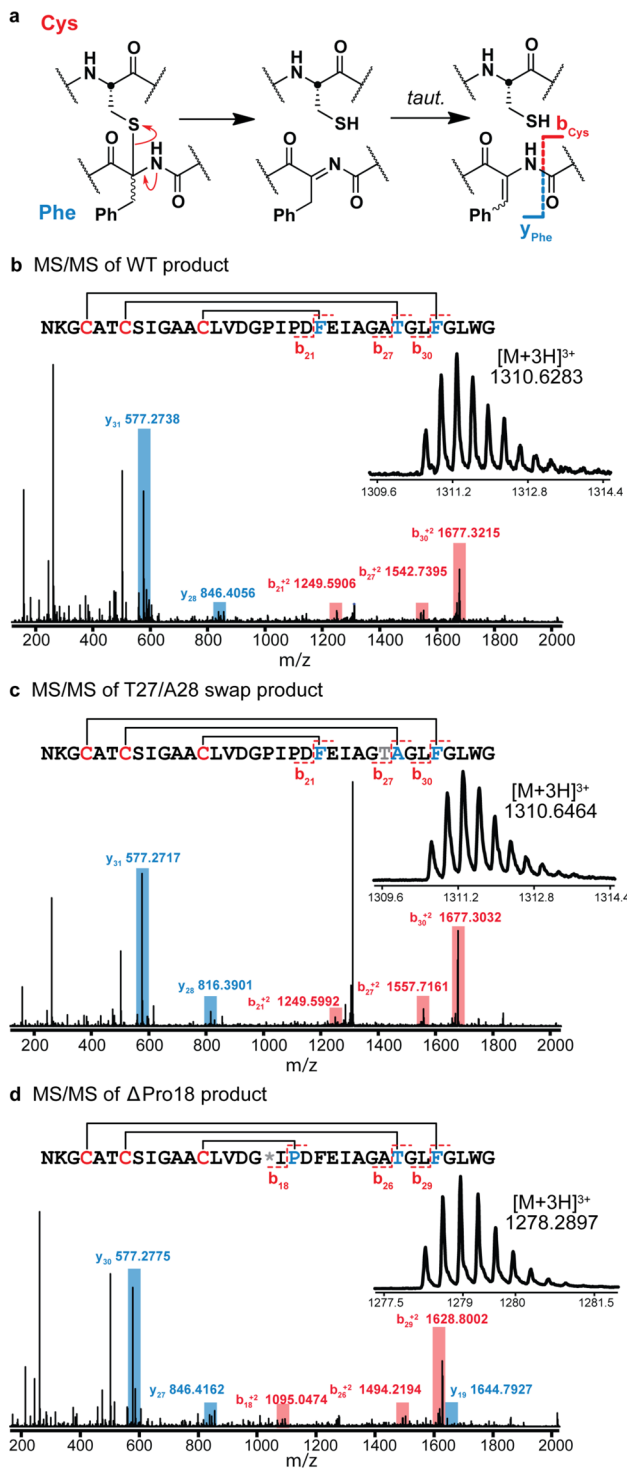


Figure 3. Confirming bridge formation in mutant sactipeptides. a) Mechanism of sactonine fragmentation. b) MS/MS on modified “wild type” SboA peptide from 50 rpm overnight culture. c) MS/MS on modified T27/A28 swap SboA mutant peptide from 50 rpm overnight culture. d) MS/MS on modified Δ Pro 18 SboA mutant peptide from 50 rpm overnight culture. Insets show $[M+3H]^{3+}$ ion targeted in MS/MS.

(Phe22, Thr28, and Phe31), suggesting that AlbA is indifferent to secondary structure or flexibility at these glycine residues.

Subtilisin A has the largest loop of the known sactipeptides (eight amino acids compared to seven in thuricin CD and four in thuricin H), and none of the other known sactipeptides

have prolines in their loop regions. We therefore sought to determine whether Pro18 and/or Pro20 were necessary for inducing a turn and allowing AlbA to thereby work on the sets of bridging residues (Table 1, entries 25–27). Again, alanine scans at these two positions demonstrated that AlbA was indifferent to the presence of prolines at these positions, at least with respect to the number and regioselectivity of sactonine bridge formation. However, complete deletion of Pro18 did appear to shift the bridging partner of Cys13 for the first time in our experiments (entry 22). Careful scrutiny of the MS/MS data indicates that the Cys13 sactonine thioether is formed at the α carbon of the new Pro19 in isolates from this mutant; the locations of the other two sactonine bridges are conserved. In contrast, deletion of Pro20 sustains formation of the now Phe21 linkage; again, locations of the Cys4 and Cys7 thioether bridges are conserved as well (entry 23). Overall, these mutants demonstrate substrate promiscuity on the part of AlbA and suggest that substrate–enzyme interactions may play a role in dictating the position of bridge formation. Alterations to the loop proline residues could also conceivably be expected to have an impact on stereochemistry, especially at the nearby Cys13–Phe21 sactonine linkage, a question for future investigations.

■ SUBSTRATE TOLERANCE FOR CYSTEINE PLACEMENT

Subtilisin A seemingly has relaxed specificity for the bridging partner residues and is able to accept Ala, Gln, Met, Phe, Ser, and Thr to varying degrees at the three different positions. Therefore, two major questions can be posed with respect to cysteine placement: (1) can cysteines be moved and still form bridges, and (2) will the bridging partner change depending on the placement of the cysteine? We examined these questions with a series of 14 cysteine swap mutants: four swap mutants each at Cys4 and Cys7, five mutants at Cys13, and a tandem mutant. Under our heterologous conditions, bridges at Cys4 and Cys7 seemed resistant to changes in placement (Table 1, entries 28–36). Cys4 did tolerate a move one residue toward the leader peptide (entry 30), but this move came at the expense of the remaining two bridges, which went unformed in this product. Similarly, Cys7 could be swapped with Ser8 or Gly10, while still allowing formation of the Cys4 bridge, but neither the newly placed middle bridge nor the anterior bridge were formed.

In stark contrast to the Cys4 and Cys7 bridges, Cys13 proved remarkably tractable. Of the five positional swaps tested (entries 36–40), only the Cys11/Ala13 swap (entry 37) did not yield a fully modified three-bridge product. Each new placement of Cys13, either at the +10, +12, +14, or +15 positions, resulted in a sactonine linkage to the same bridging partner as the original linkage (Phe22). Interestingly, the Gly10 position, which did not allow a swap with Cys7, is capable of tolerating a sactonine bridge when a +7 bridge is also formed in the final product (entry 36).

The relative success of the mutants tested may betray the order of AlbA-catalyzed bridge formation in this heterologous host. While the system does not appear to tolerate movement of Cys4 and Cys7, the third bridge at Cys13 seems to tolerate a wide array of changes. This could be due to the first two bridges being formed rapidly, allowing the rest of the peptide to reach a sort of conformational equilibrium before formation of the third bridge; further kinetic analysis of the bridge-forming reaction will be necessary to confirm this. The formation of a

bridge to F22 regardless of the position of the third cysteine indicates a degree of enzymatic control over regioselectivity. This control is overridden in the case of the deletion of Pro18, which suggests that this residue is crucial in spacing and in the conformation of the loop.

INCORPORATION OF UNNATURAL AMINO ACIDS

Unnatural amino acids (UAAs) have been incorporated in a number of heterologously expressed RiPP natural products by amber codon suppression technology, including lantipeptides and cyanobactins.^{20,24–28} In the case of the lantipeptides, UAAs allowed access to analogs with improved potency and solubility. We anticipate that similar effects can be obtained with UAA incorporation into bioactive sactipeptides. UAA incorporation may also allow access to new chemistry from sactione-forming radical SAM enzymes: since the enzyme putatively generates a reactive radical intermediate, specific placement of a radical trap or other reactive group on a bridge partner amino acid side chain could allow side-tracking into alternative reaction manifolds. This would only be possible if the radical SAMs prove permissive for UAAs at the bridging partner residue. We thus tested UAA incorporation *via* the orthogonal *O*-methyl tyrosine tRNA synthetase/tRNA (*O*-Me-Tyr) pair adapted from *M. janaschii*.

pUltra-*O*-Me-Tyr plasmid, replicated by an orthogonal pCDF origin of replication and bearing streptomycin resistance, was incorporated in BL21(DE3)s on top of pPH151 and a pETDuet-*sboA-AlbA* vector mutated to an amber stop codon (TAG) at F31. While we could observe unmodified peptide production/UAA incorporation at 200 rpm, this was accompanied by substantial amounts of truncate arising from disrupted translation at the newly incorporated TAG codon. No modified sactipeptide could be found at the lower, 50 rpm shake rate. We therefore turned to the recently reported “TAGless” *E. coli* strain, C321.Δ*A.exp*, developed by Isaacs *et al.*³³ In order to drive expression of our same pETDuet-*sboA-AlbA*, the λDE3 prophage was integrated into C321.Δ*A.exp*. Subsequent growth under our optimized conditions yielded cyclic presubtilosin A exhibiting fully modified cysteine thioether bridges at all three native positions (Figure 4). MS/MS confirmed the C4^S-(*O*-Me-Tyr)^{Cα} linkage at the newly installed UAA.

CONCLUSIONS

In summary, we have developed a system for the heterologous expression of sactipeptide derivatives of subtilosin A in *E. coli* and demonstrated unexpected biosynthetic promiscuity of the sactione synthetase AlbA. We anticipate that a similar strategy could be used to access other predicted sactipeptides or else chemistry from the ever-widening library of radical SAM enzymes in RiPP pathways.^{34–36} The pronounced promiscuity, especially in the loop region and at unmodified positions on the solvent-exposed exterior of the peptide macrocycle, could be exploited for the grafting of peptide epitopes as has been seen in lasso peptides and conotoxins. Moreover, we observed for the first time promiscuity with respect to sactione bridge placement and, in specific instances, bridging partner selection. An expanded understanding of control over sactione bridge placement could allow for the design of constrained sactipeptide conformations for inhibitory display of peptide epitopes; our *E. coli* expression system should facilitate such efforts. Results with sactione bridge movements suggest

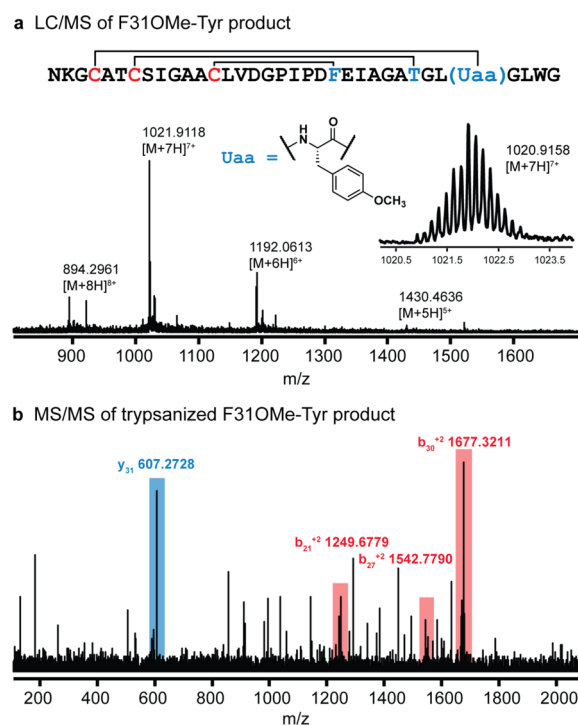


Figure 4. Incorporation of unnatural amino acids (UAAs) into sactipeptides in *E. coli*. (a) LC/MS of isolate from F31O-Me-Tyr mutant, structure of *O*-Me-Tyr, and blow-up of $[M+7H]^{7+}$ envelope. (b) MS/MS of trypanized F31O-Me-Tyr sactipeptide mutant.

interdependence between the positioning of these three bridges; this may further be compounded by the overall flexibility of the substrate. Therefore, many more modified sactipeptides may be possible from compound mutants that combine multiple mutations. Again, this is another place where the heterologous expression system may prove beneficial: based on the changes reported here, including the demonstrated mobility of the bridge partners, we estimate libraries far in excess of 10^{12} non-native subtilosin A analogs to be possible. Thus, this work enters the sactipeptides as another example of a secondary metabolic system capable of producing vast libraries with significantly high structural complexity. Finally, we have introduced nonproteinogenic amino acids (UAAs) into sactipeptides using stop-codon suppression technology, specifically at otherwise less permissive bridging partner position. This demonstrates the robustness of UAA incorporation in our system and may open up new chemistry and new applications for unnatural sactipeptides.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00042.

Experimental details, including molecular biology and isolation procedures, LC/MS, and MS/MS analysis techniques (PDF)

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Notes

The authors declare no competing financial interest.

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