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SylC Catalyzes Ureido-Bond Formation During Biosynthesis of the Proteasome Inhibitor Syringolin A

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Abstract

Syringolins are a class of cyclic tripeptide natural products that are potent and irreversible inhibitors of the eukaryotic proteasome. In addition to being hybrid NRPS/PKS molecules, they also feature an unusual ureido-linkage (red) between two amino acid monomers. Here we report the first *in vitro* characterization of enzymatic ureido-linkage formation which is catalyzed by an NRPS, SylC. Using ^{13}C- and ^{18}O-labeling studies, we show that biosynthesis occurs via *N*-carboxylation to form an initial *N*-carboxy-aminoacyl-S-Ppant enzyme intermediate which undergoes intramolecular cyclization followed by condensation with a second amino acid to form the ureido-containing dipeptide product.

Nonribosomal peptide (NRPS) and polyketide synthetases (PKS) utilize numerous chemical reactions with precise control of regio- and stereochemistry on activated, tethered intermediates to construct diversely functionalized natural products with broad biological activity.[1] Typically these events occur through a controlled chain-elongation process where peptide and/or carbon-carbon bonds are constructed in an iterative manner. In NRPS the peptide scaffold is formed through successive assembly of amide bonds in a manner where chain polarity remains unidirectional. However, *N*-to-*N* terminal condensation via an ureido-linkage leads to a reversal of chain polarity and introduces a new point of diversification to modulate biological activity.

The syringolin family of proteasome inhibitors are NRPS-PKS hybrid molecules with notable structural features including a 12-membered ring and *N*-terminal acylation via a ureido-linkage.[2a] In syringolin A, Val_1 is *N*-acylated by an additional valine in a head to head condensation creating a ureido-linkage and affording a negative terminus rather than the usual positive terminus. Other natural products containing a chain reversal by way of a ureido-linkage include

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Supporting Information Available: Supplemental figures, experimental procedures, and HRMS characterization. This material is available free of charge via the internet at http://pubs.acs.org.

the anabaenopeptins,[2b] brunsvicamides,[2c] pacidomycins,[2d] mureidomycins,[2e] and napsamycins[2f] (Figure 1). Although the specific biological ramifications of chain reversal remain unknown at this time, *N*-acylation is known to dramatically influence the efficacy of syringolin A as a proteasome inhibitor.[3]

Syringolin A was first isolated from *P. syringae* and characterized in 1998 by Dudler and coworkers as an elicitor of stress response in rice, aiding in resistance toward the phytotoxic fungi, *P. oryzae*.[2a] Recent studies have shown that the active site Thr_1 of the 20S eukaryotic proteosome interacts covalently and irreversibly with the α,β-unsaturated amide of the syringolin macrocylic core via a Michael addition mechanism.[4] The resulting inhibition is consistent with studies that show syringolin-induced cell death of neuroblastoma, ovarian,[5a] and leukaemic[5b] cancer cells. The combined biomedical relevance and unusual ureido-functionality led us to examine the formation of this linkage in syringolin biosynthesis.

Recent *in vivo* investigations by Dudler and coworkers into the biosynthetic origin of the ureido-linkage of syringolin A revealed integration of either bicarbonate or carbon dioxide.[6] Feeding studies with [^{13}C]-bicarbonate followed by product characterization validated that incorporation was restricted to the carbonyl moiety. In this study we have focused on *in vitro* characterization of SylC, the NRPS enzyme presumed responsible for syringolin chain initiation, to evaluate its role, in generation of the ureido-linkage subsequent to amino acid monomer activation.

The full-length *syl*C gene was amplified from *P. syringae* B728a and cloned into *E. coli* expression vectors to generate a 147-kDa His_6-tag fusion. Overexpression and Ni-NTA purification provided soluble SylC, with the thiolation (T) domain in the apo-form as confirmed by subsequent phosphopantetheinylation with acetyl-coenzyme A (AcCoA) and the promiscuous phosphopantetheinyl transferase, Sfp.[7] The SylC adenylation (A) domain was first assayed using ATP-PP_i exchange, and both L-Val and L-Ile were preferentially reversibly adenylated.[8] Additionally, L-Thr and L-*allo*-Ile were also activated but at approximately 40% the level of the natural substrates (Figure S2). The covalent loading of amino acid monomers onto the SylC thiolation (T) domain was next investigated. Calibration experiments utilizing Sfp and radiolabeled [1-^{14}C]-AcCoA demonstrated that ~30% of SylC could be labeled during conversion from the apo-form to the holo-form by installation of the phosphopantetheinyl (Ppant) group on the T-domain (Figure 2, circles).[9] Intriguingly, when the HS-Ppant holo-form of SylC enzyme was formed by prior incubation with Sfp and unlabeled CoA and then subjected to amino acid loading via ATP and [1-^{14}C]-L-valine, approximately twice the level of radiolabeled protein was detected (Figure 2, squares). These results indicated that two equivalents of valine were incorporated into a single SylC enzyme and thus provided the first indication that SylC was in fact generating the ureido-Val-CO-Val-S-SylC as a covalently tethered thioester intermediate. [1-^{14}C]-L-Ile was also successfully loaded onto SylC at lower fractional stoichiometry, while the formation of [1-^{14}C]-Thr-S- SylC was not observed (data not shown).

To verify formation of the thioester bound Val-CO-Val moiety on the T-domain of SylC we turned to high-resolution mass spectrometry (HRMS). HRMS was utilized due to the accumulation of only picomolar quantities of the ureido-peptidyl-S-T intermediates as a result of the single turnover process. Quenching the assays with hydroxide, which hydrolyzed the T-domain thioester, allowed for the identification of the released ureido-dipeptides by their corresponding masses.

In vitro assays with ATP, bicarbonate, L-Val and/or L-Ile, and SylC, followed by hydrolytic release of the peptidyl-S-T domain intermediates, gave all three of the ureido-dipeptides (Val-CO-Val, Figure 3A; Val-CO-Ile, Ile-CO-Ile, Figure S5). Additionally, the enzyme accepted

and processed L-*allo*-Ile, presumably an unnatural substrate, producing the symmetric ureido-product (Figure S6). Next, [^{13}C]-bicarbonate was utilized as a chemical probe in SylC incubations to validate the carbon source of the carbonyl moiety of the ureido-linkage. The appearance of a [M+1] mass peak by HRMS corroborates the feeding studies of Dudler and coworkers and confirms that SylC alone forms the ureido-linkage from a bicarbonate source (Figure 3B).[6]

These studies establish that SylC, a free-standing NRPS module with a *single* C, A and T-domain, can activate L-Val or L-Ile *twice* and effect the chain reversal step to build the ureido-linkage on the HS-Ppant arm of its T-domain. This constitutes novel chemistry for an NRPS module, and the lack of precedent led us to question the mechanistic details of ureido-linkage formation. We hypothesized that the SylC A-domain conventionally activates one equivalent of amino acid as the aminoacyl-AMP, which is then captured by the thiol of the Ppant arm and loaded as the thioester onto the T-domain (Val-S-T SylC, Figure 4). It is likely that carboxylation of the amine of the tethered aminoacyl moiety by either bicarbonate or, more likely, carbon dioxide next forms a transient *N*-carboxy adduct, **I**. Examples of stabilized *N*-carboxy amines are known to participate in the catalytic mechanisms of RuBisCO[10a] and class D β-lactamases.[10b]

Formation of **I** would be followed by one of several possible transformations (Figure 4). The "adenylation" pathway involves *N*-carboxy-amino acid adenylation to form an activated mixed anhydride **II**, which would then react with a free amino acid forming the resulting ureido-dipeptide. Alternatively, in the "cyclization" pathway, the *N*-carboxy intermediate would cyclize intramolecularly, to generate a highly reactive covalently tethered thiohemiacetal intermediate, **III**. This transient thiohemiacetal would partition in one of at least two ways. In one scenario, formation of a "Leuch's anhydride" of type **IV**[11] would persist as a non-covalently bound species in the SylC active site during activation and loading of the second Val (Figure 4, pathway a). The nucleophilic amine of the second Val-S-T intermediate would capture **IV** regiospecifically and unravel it to the ureido-peptidyl-S- SylC.[12] Alternatively, the transient thiohemiacetal **III** would be intercepted first by a nucleophilic SylC residue (Nu) to form a covalent adduct **V** (Figure 4, pathway b).[13] This in turn would be captured by the nucleophilic amine of the second tethered Val yielding the ureido-dipeptide. Presumably, the ureido-peptidyl-S-SylC serves as the upstream intermediate that is transferred to the next NRPS module during chain elongation in syringolin biosynthesis.

To distinguish between adenylation or cyclization mechanisms we chose [^{18}O]-bicarbonate as a probe, since the adenylation pathway of Figure 4 would result in an [M+2] product while both arms of the cyclization pathway would furnish an ureido-dipeptide with an enrichment of [M+4].[14] SylC incubations were performed in [^{18}O]-bicarbonate/[^{18}O]- water (80% total ^{18}O enrichment) after which the protein was precipitated with MeOH and the pellet washed three times to remove [^{18}O]-water and [^{18}O]-bicarbonate. The thioester-bound intermediates were released by chemical hydrolysis in unlabeled water and analyzed by HRMS. Inspection of this data shows clear enrichment of the [M+4] mass peak (Figure 3C). Notably, one of the two [^{18}O] atoms is incorporated into the amino acid carboxylate (Val), while the other is presumably localized in the ureido-group (Figure S9); the separation of the two oxygen atoms is fully consistent with intramolecular cyclization of the *N*-carboxyaminoacyl-S-T species (**I**). Capture of a Leuch's anhydride **IV** or the covalent adduct **V** could equally account for the incorporation and placement of two [^{18}O] atoms in ureido-products.

In summary, we have completed the first *in vitro* characterization of enzymatic ureido-linkage formation. SylC, with a single C, A and T-domain, iteratively activates two amino acid monomers and constructs the ureido-linkage by incorporation of bicarbonate/CO_2 by cyclization of an initial *N*-carboxy-aminoacyl-S-Ppant enzyme intermediate.

Future studies will be directed at deducing evidence in favor of these or alternate mechanisms for formation of the ureido-group and evaluating parallel systems for the peptide chain reversal in pacidamycins and anabaenopeptins. The mode of action of syringolin A is similar to that of the anti-cancer drug Velcade[15] and presents an opportunity to expand the proteasome inhibitor class of cancer therapeutics. We anticipate that exploration of the promiscuity inherent to SylC, as evidenced by formation of the unnatural L-*allo*-Ile-containing ureido-dipeptide, should allow for the production of new syringolin analogues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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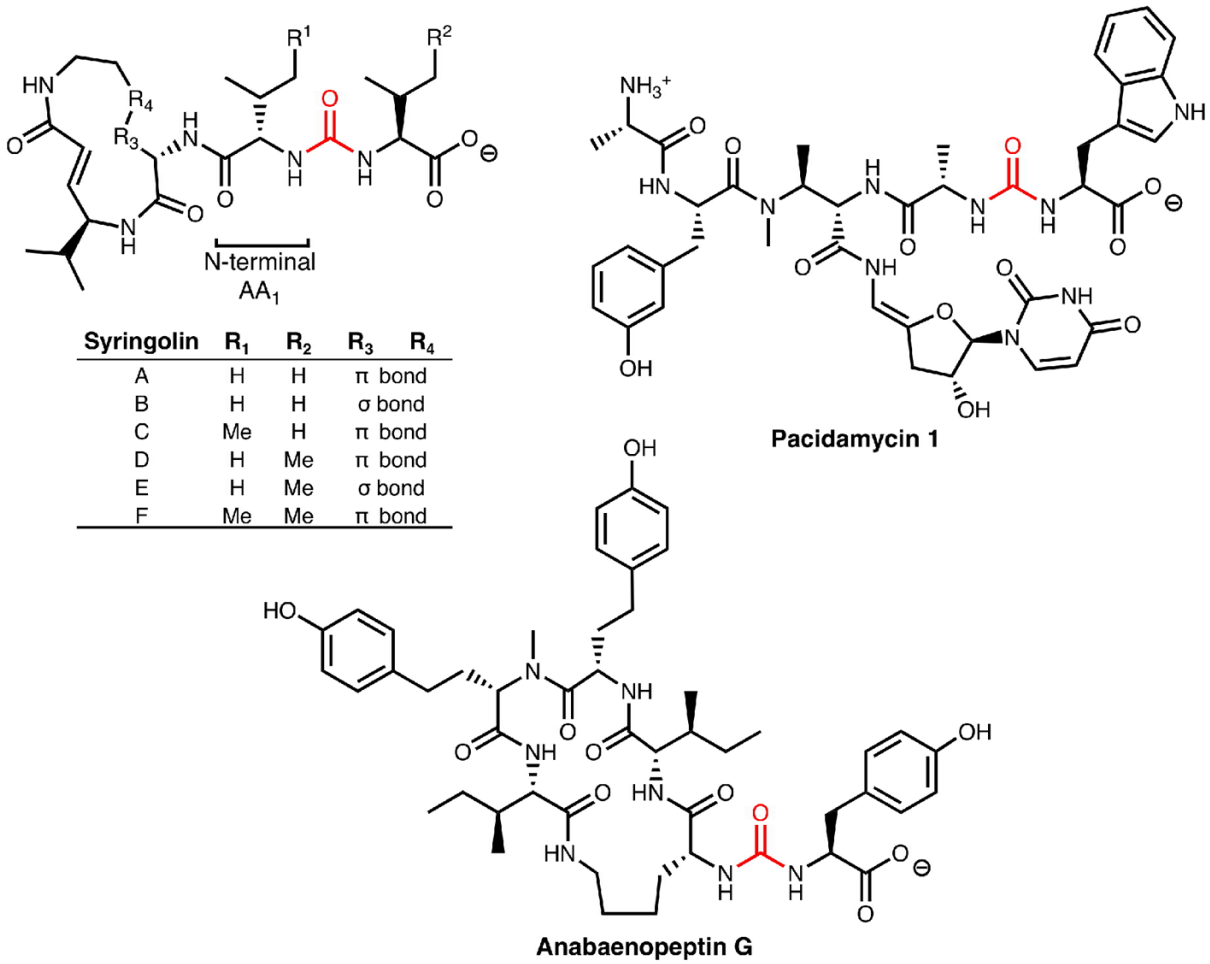


Syringolin	R_1	R_2	R_3	R_4
A	H	H	π bond	
B	H	H	σ bond	
C	Me	H	π bond	
D	H	Me	π bond	
E	H	Me	σ bond	
F	Me	Me	π bond	

Figure 1.
Ureido-containing natural products.

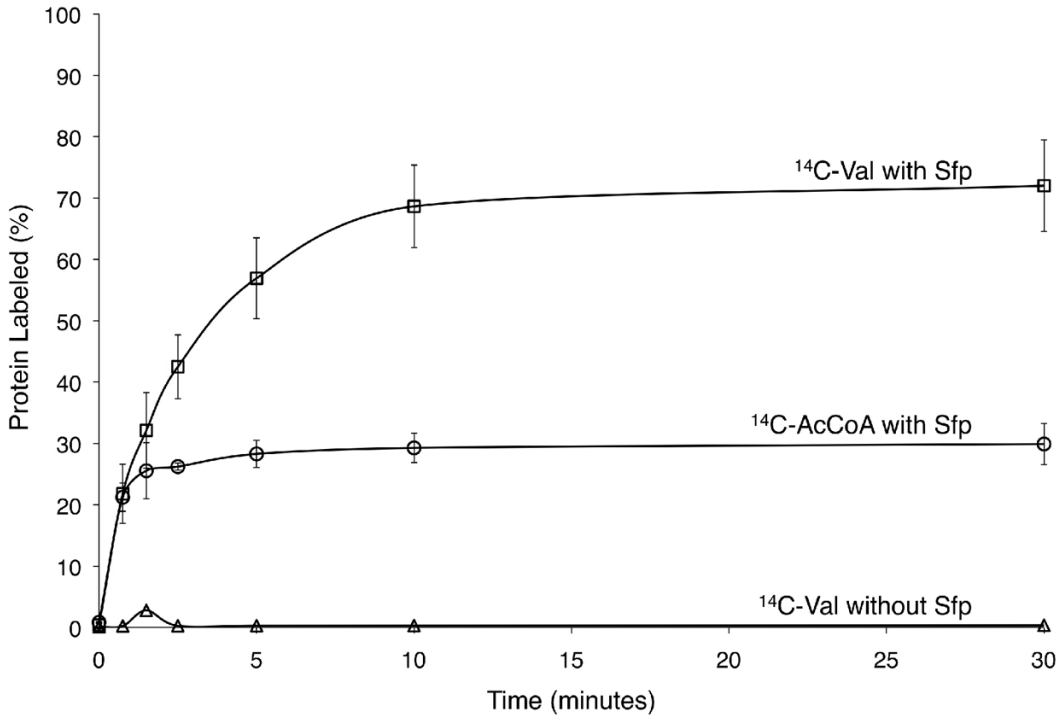
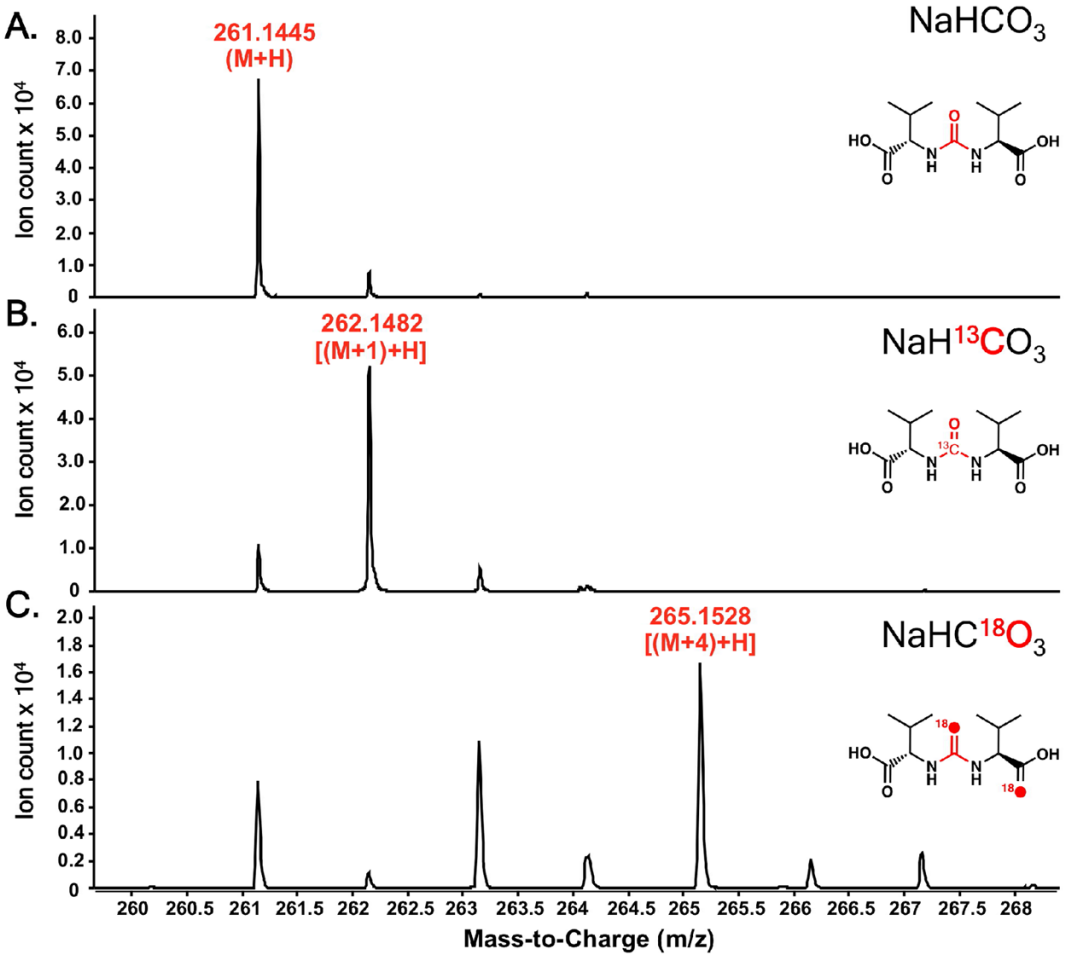


Figure 2.
Comparison of extent of conversion from apo-SylC to holo-SylC by Sfp (circles) to extent of [^{14}C]-L-Val loading onto the holo-SylC T-domain (squares). SylC is isolated entirely in the apo-form as indicated by no [^{14}C]-L-Val loading if Sfp is omitted from the assay (triangles).



Panel	Bicarbonate	Relative Ion Abundance to (M+H)				Retention time (min)
		(M+H)	[(M+1)+H]	[(M+2)+H]	[(M+4)+H]	
A	$NaHCO_3$	1.0	0.11	0.015	--	5.73
B	$NaH^{13}CO_3$	1.0	4.4	0.45	--	5.57
C	$NaHC^{18}O_3$	1.0	0.15	1.6	2.1	5.69

Figure 3.
HRMS of the **A)** unlabeled valine ureido-dipeptide, **B**) [^{13}C]-labeled valine ureido-dipeptide, and **C**) [^{18}O]-labeled valine ureido-dipeptide with normalization of ion abundance shown below.

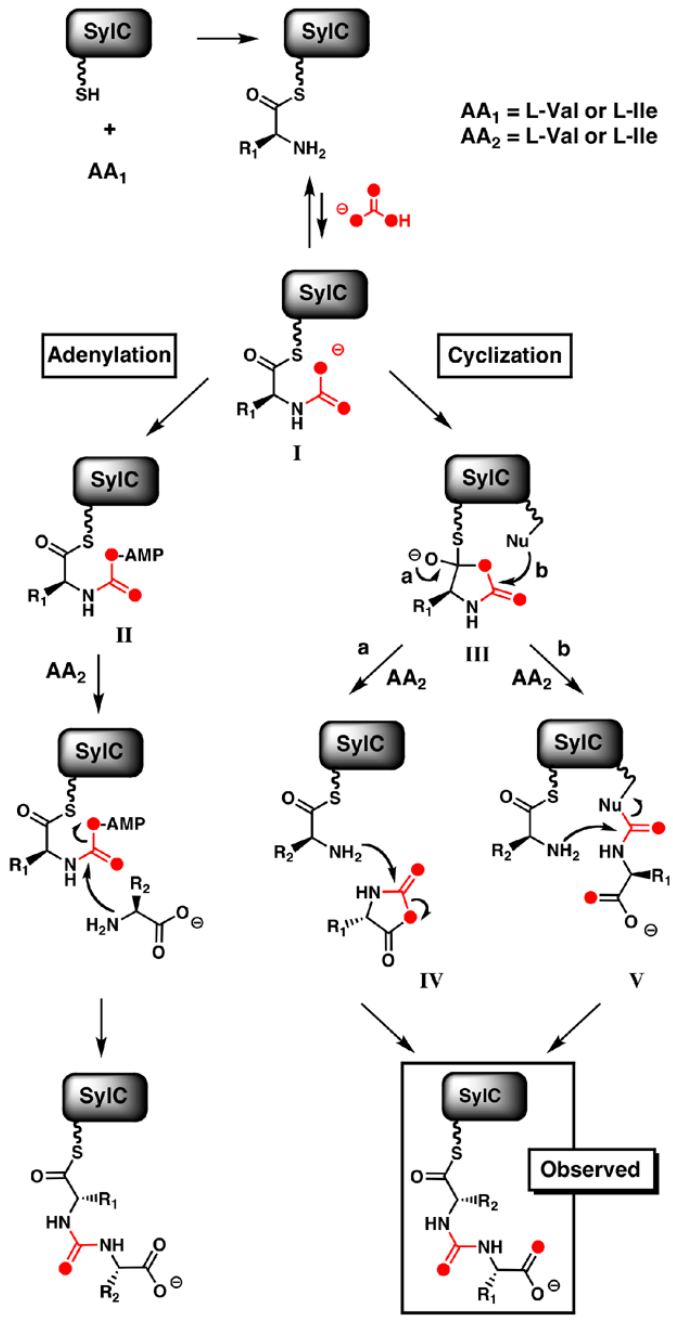


Figure 4.

Proposed mechanisms of SylC-catalyzed formation of the ureido-dipeptide showing two distinct pathways (adenylation and cyclization) which are distinguished by label incorporation when the reaction is carried out with [^{18}O]-bicarbonate.