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

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Supporting Information

Contents:

1. Materials and General Methods. All chemicals were purchased from Sigma-Aldrich unless noted otherwise. DNA sequencing was performed at the Molecular Biology Core Facilities of the Dana Farber Cancer Institute (Boston, MA). ^{1}H NMR spectra were recorded on a Varian 600 MHz spectrometer. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance resulting from incomplete deuteration as the internal standard ($CDCl_3$ δ 7.26, D_2O δ 4.79, CD_3OD δ 3.31). Data are reported as follows: chemical shift, multiplicity (d = doublet, dd = doublet of doublets, m = multiplet), coupling constants (Hz), and integration.

2. Cloning of SylC

Pseudomonas syringae pv. syringae B728a was kindly provided by Prof. Steven Lindow at UC-Berkeley. The strain was grown on 2xTY media overnight and the genomic DNA extracted using a QIAGEN DNeasy Kit following the manufacturer's protocol. The gene encoding SylC (GI: 66043271, 3969bp) was amplified from the purified genomic DNA using the following primers: 5'-GGTTGATGTCACCTGATC**CATATG**TTATCCGAGG -3' and 5'-CTGAA**GGATCC**CGCGGCCGTCAACGGCTCG -3' (Integrated DNA Technologies) which include a 5'-*Nde*I site and a 3'-*Bam*HI site, respectively. The PCR reaction (100 μL) contained 1 μL of 52 ng/μL template DNA, 1 μL of 2 U/μL Phusion DNA Polymerase (Finnzymes), 20 μL 5X GC Buffer, 3 μL DMSO, 2 μL of each 10 mM dNTP, 0.5 μL of each of 100 μM forward and reverse primer, and 72 μL ddH_2O. The PCR reaction was performed in a MyCycler Thermal Cycler (Biorad), with the following parameters: 98°C for 1 minute followed by 30 cycles of 98°C for 10 seconds, 65°C for 30 seconds, and 72°C for 1 minute; the final extension time was 10 minutes at 72°C. The PCR product was purified by gel extraction (QIAGEN). The amplified DNA was then restricted using *Nde*I and *Bam*HI restriction enzymes (New England Biolabs) and ligated into the pET22b expression vector (C-His_6 tag, Novagen) using T4 DNA ligase (New England Biolabs). Ligations were transformed into *E. coli* TOP10 cells (Invitrogen) for plasmid propagation and maintenance.

3. Expression and Purification of SylC

The protein was expressed in *E. coli* BL21-CodonPlus cells (Stratagene) in 4 L of LB media containing 100 μg/mL ampicillin and 35 μg/mL chloramphenicol. The cultures were shaken at 200 rpm for 3-4 hours at 37°C until the OD_{600} reached 0.8; the temperature was then reduced to 25°C to cool the cultures for 30 minutes before induction to a final concentration of 0.5 mM IPTG. The cultures were shaken for an additional 4 hours at 25°C before being harvested by centrifugation for 10 minutes at 5,000 g. The cells were resuspended in 60 mL of buffer containing 5 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9 (Buffer A). The suspension was passed through an EmulsiFlex-C5 (Avestin) cell disruptor twice and the lysate cleared by centrifugation at 35,000 g for 35 minutes. The supernatant was decanted into 3 mL of Ni-NTA resin (QIAGEN), and the solution was rotated 1 hour to bind protein. The solution was poured into a column, the flow-through discarded, and the resin rinsed with 20 mL of Buffer A. Weak-binding proteins were eluted with 12 mL Buffer A containing 25 mM imidazole, and SylC was eluted with 12 mL of Buffer A containing 200 mM imidizole. Fractions were analyzed by SDS-PAGE after which the purest were combined (lanes 4 and 5,

Figure S1) and dialyzed 3X into 20 mM Tris-HCl, pH 7.9, containing 250 mM NaCl. The protein was concentrated to 2-5 mg/mL using a 30K MWCO Amicon Centrafugation filter (Millipore). The protein concentration was determined at 280 nm using 143,740 M^{-1} cm^{-1} as the calculated extinction coefficent (ExPASY). Protein was frozen in $N_{2(l)}$ and stored at -80°C until use.

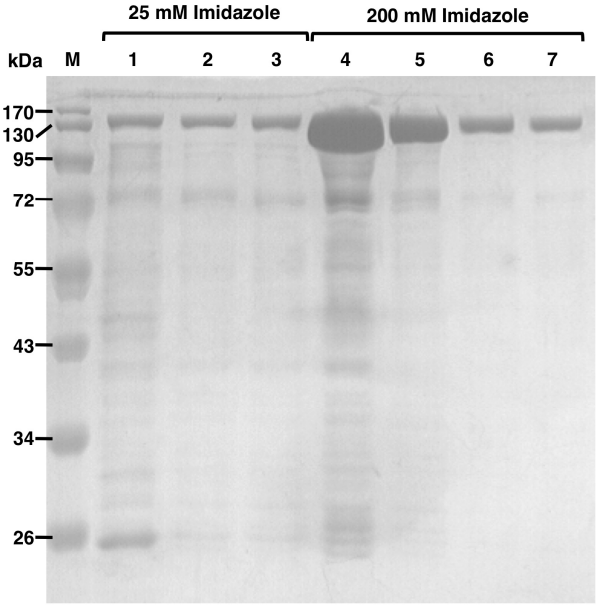

Figure S1. SDS-PAGE gel of of SylC purification

4. ATP-PP_i Exchange Assay

A typical experiment (300 µL total volume) was carried out as follows: $MgCl_2$ (10 mM), TCEP (0.5 mM), ATP (5 mM), tetrasodium pyrophosphate (1mM), tetrasodium [^{32}P]-pyrophosphate (1 µL/rxn, 1.2 mCi stock), $NaHCO_3$ (10 mM) and substrate amino acid (5 mM) were combined in reaction buffer (75 mM Tris, pH 7.5); reactions were then initiated by the addition of SylC to a final concentration of 1 µM. At time points of 0, 30, and 120 minutes 100 µL aliquots (90 µL for T = 0, before addition of enzyme) were quenched with a 500 µL solution of activated charcoal (1.6% w/v), 200 mM tetrasodium pyrophosphate, and 3.5% perchloric acid in water. The charcoal was pelleted by centrifugation and washed twice with a 500 µL solution of 200 mM tetrasodium pyrophosphate and 3.5% perchloric acid in water. The radioactivity of ATP bound to the charcoal was then measured by liquid scintillation counting. Turnover was calculated as (% incorporation of ^{32}P-PP_i)[total PP_i]/[Enz]. Relative turnover rates at the 120-minute time point are shown in SI Figure 2.

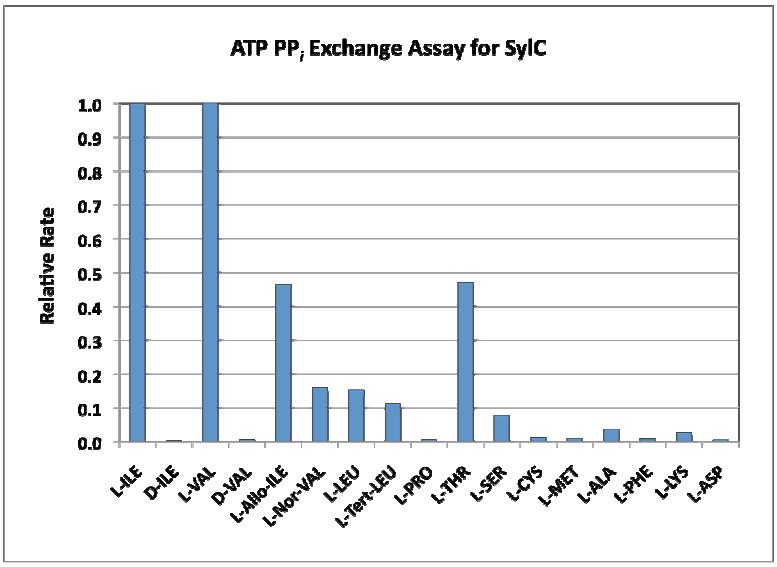

Figure S2. ATP-PP$_i$ exchange assay of the SylC A domain with various amino acids.

5. Apo-Holo Conversion Assay

The conversion of apo to phosphopantetheinylated holo SylC was assayed in a reaction containing 50 mM Tris, pH 7.9, 5 mM $MgCl_2$, 0.5 mM TCEP, 40 mM NaCl, 2.5 μM SylC and 94 μM [1-^{14}C]-acetyl coenzyme A (53.4 μCi/mmol; Moravek Biochemicals). The *B. subtilis* PPTase Sfp was added to a final concentration of 300 nM and time points were taken at 0.75, 1.5, 2.5, 5, 10, and 30 minutes by quenching 50 μL aliquots of the reaction mixture into 800 μL of 10% TCA containing 100 μL of 1 mg/mL BSA as a carrier protein. After all timed quenches were complete, the acidified solutions were centrifuged at 4°C at 10,000 g for 8 minutes to pellet the proteins. The supernatant was carefully aspirated, and the pellet was resuspended in 800 μL 10% TCA to remove unbound radioactive material. The centrifugation and wash was repeated again before the precipitate was finally dissolved in 250 μL of formic acid, vortexed, and pulsed to collect all liquid. The sample was added to 14 mL of Ultima Gold scintillation fluid (Perkin-Elmer) and the radioactivity quantified on a Beckman-Coulter LS 6500 Multi-Purpose Scintillation Counter. Disintegrations per minute (DPM) were converted to nmoles of [1-^{14}C]-acetyl coenzyme A counted using the specific radioactivity and divided by the nmoles of protein in the reaction mixture to obtain the fraction of protein labeled. Results are shown in Figure 2 of the main text.

6. T-Domain Loading Assay

Prior to amino acid loading, SylC was first converted from apo to holo *in situ* by incubation with coenzyme A and the *B. subtilis* PPTase Sfp for 15-30 minutes at room temperature. The reaction contained 50 mM Tris, pH 7.9, 5 mM $MgCl_2$, 0.5 mM TCEP, 40 mM NaCl, 2.5 μM SylC, 250 μM coenzyme A, and 300 nM Sfp. Radiolabeled amino acid (L-Val, 250 mCi/mol; L-Ile, 280 mCi/mmol; L-Thr, 155 mCi/mmol) was then added to a final concentration of 25 μM and the reaction initiated by addition of ATP to a final concentration of 5 mM. Time points were acquired and processed as above for the assay of apo to holo conversion. Results are shown in Figure 2 of main text.

7. Synthesis of Val-Ureido-Val Synthetic Standard

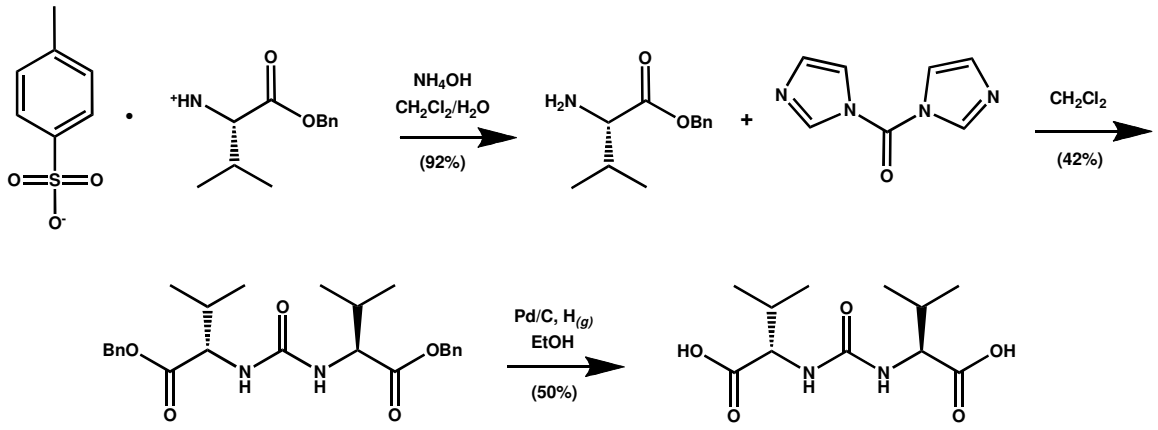

Figure S3. Synthesis of Val ureido dipeptide standard.

The L-valine benzyl ester *p*-toluenesulfonate salt (1 g, 2.6 mmol, Sigma; V2627) was dissolved in 50 mL of CH_2Cl_2/H_2O (4:1) and converted to the free base by extraction with 3 x 40 mL 0.25 M ammonium hydroxide. The organic layer was dried over $NaSO_4$, concentrated *in vacuo* to dryness, and used directly for coupling. In a 100 mL rounded bottom flask, the free base L-valine benzyl ester (500 mg, 2.4 mmol) was dissolved in 10 mL of CH_2Cl_2 and cooled on ice. To this solution was added 1,1′-carbonyldiimidazole (178 mg, 1.1 mmol, Sigma; 115533), and the solution was removed from the ice bath and warmed to room temperature. The reaction was stirred overnight before dilution with 10 mL CH_2Cl_2 and transfer to a 125 mL separatory funnel. The reaction was washed twice with 30 mL each of 1 M H_2SO_4, H_2O, and saturated NaCl. The organic layer was dried with $NaSO_4$ and concentrated *in vacuo* to give the benzyl-protected valine ureido dipeptide as a white solid (460 mg, 1.0 mmol). Deprotection was carried out in a 50 mL rounded bottom flask containing the protected valine ureido dipeptide (230 mg, 0.5 mmol), 10% wt. Pd/C (22 mg; Aldrich 205699), and 5 mL of ethanol. The flask was evacuated and purged with $H_{2(g)}$ twice and stirred overnight at room temperature. The reaction was filtered over celite, concentrated *in vacuo* to dryness, and taken up in minimal H_2O before being filtered through Millipore Type GV 0.22 mm paper. The clear, colorless solution was lyophilzed overnight to afford a white powder (65 mg, 0.25 mmol).

^{1}H NMR (600 MHz, D_2O) δ ppm 6.27 (d, *J*=9.0, 1H), 3.98 (dd, *J*=5.1, 6.3 1H), 1.98 (m, 1H), and 0.83 (dd, *J*=6.9, 19.2, 6H). MS–ESI$^+$ (*m/z*): expected for $C_{11}H_{20}N_2O_5$, 260.1450; observed, 261.1448 (Figure S7).

8. Enzymatic Formation of Ureido Peptides

Enzymatic formation of the ureido peptides was performed as follows (Figure S4). First, conversion of SylC from apo to holo was performed *in situ* by incubation with coenzyme A and the *B. subtilis* PPTase Sfp for 15-30 minutes at room temperature. The reaction contained 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.5 mM TCEP, 5 mM amino acid (L-Val, L-Ile, L-*allo*-Ile), 25 μM SylC, 200 μM coenzyme A, 25 mM $NaHCO_3$, and 300 nM Sfp. Next, 7.5 mM ATP was added to the reaction and incubated at room temperature for

two hours. The reaction was quenched with 750 μL cold methanol and cooled in an ice bath for five minutes. The solution was centrifuged at 4°C at 13,000 g for eight minutes to pellet the protein, the supernatant was removed and the wash was repeated three times (250 μL MeOH). The pellet was then redissolved in 100 μL of aqueous 0.1 M KOH, heated at 70°C for ten minutes, neutralized with 100 μL of aqueous 0.1 M HCl, and filtered through a 10K centrifugal filter at 4°C at 13,000 g for 15 minutes. The flow-through solution was analyzed by HRMS as described in Section 11 of the SI, and representative MS spectra are shown in Figure 3 of the main text and SI Figures S5-S9.

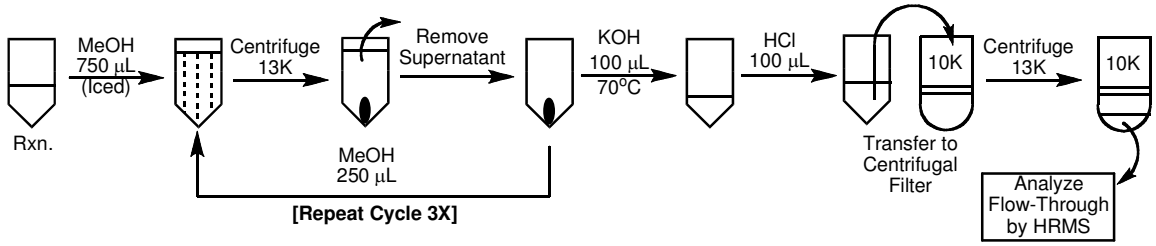

Figure S4. General procedure for hydrolysis of T-domain bound products.

9. ^{13}C-Labeling Assay

Enzymatic formation of the ureido peptides was performed as follows. First, conversion of SylC from apo to holo was performed *in situ* by incubation with coenzyme A and the *B. subtilis* PPTase Sfp for 15-30 minutes at room temperature. The reaction contained 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.5 mM TCEP, 5 mM amino acid (L-Val, L-Ile, L-*allo*-Ile), 25 μM SylC, 200 μM coenzyme A, 25 mM [^{13}C]-$NaHCO_3$, and 300 nM Sfp. Next, 7.5 mM ATP was added to the reaction and incubated at room temperature for two hours. The reaction was quenched with 750 μL cold methanol and cooled in an ice bath for five minutes. The solution was centrifuged at 4°C at 13,000 g for eight minutes to pellet the protein, the supernatant was removed and the wash was repeated three times (250 μL MeOH). The pellet was then redissolved in 100 μL of 0.1 M aqueous KOH, heated at 70°C for ten minutes, neutralized with 100 μL of 0.1 M aqueous HCl, and filtered through a 10K centrifugal filter at 4°C at 13,000 g for 15 minutes. The flow-through solution was analyzed by HRMS as described in Section 11 of the SI and the MS data is shown in Figure S7.

10. ^{18}O-Labeling Assay

Enzymatic formation of the ureido peptides was performed as follows. First, conversion of SylC from apo to holo was performed *in situ* by incubation with coenzyme A and the *B. subtilis* PPTase Sfp for 15-30 minutes at room temperature. The reaction contained 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.5 mM TCEP, 5 mM amino acid (L-Val, L-Ile, L-*allo*-Ile), 25 μM SylC, 200 μM coenzyme A, 25 mM [^{18}O]-$NaHCO_3$,[(1)] 300 nM Sfp, and

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[(1)] [^{18}O]-$NaHCO_3$ was synthesized via overnight incubation of 42.4 mg of $NaHCO_3$ in 500 μL of [^{18}O]-H_2O at 4°C.

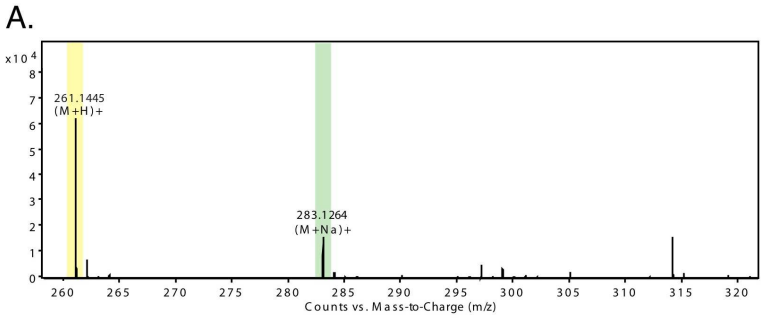
was performed in [^{18}O]-H_2O. Next, 7.5 mM ATP was added to the reaction and incubated at room temperature for two hours. The reaction was quenched with 750 μL cold methanol and cooled in an ice bath for five minutes. The solution was centrifuged at 4°C at 13,000 g for eight minutes to pellet protein, the supernatant was removed and the wash was repeated three times (250 μL in MeOH, Figure S3). The pellet was then redissolved in 100 μL of 0.1 M aqueous KOH, heated at 70°C for ten minutes, neutralized with 100 μL of 0.1 M aqueous HCl, and filtered through a 10K centrifugal filter at 4°C at 13,000 g for 15 minutes. The flow-through solution was analyzed by HRMS as described in Section 11 of the SI[(2)] and the MS data is shown in Figure S9.

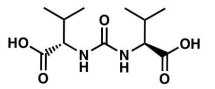
11. MS Analysis of Hydrolyzed Products

Hydrolyzed samples from all of the enzymatic experiments were analyzed on an Agilent 6520 QTOF-LCMS. The samples (40 μL) were separated over a Gemini-NX 5μm C18 110Å column (Phenomenex) at 2 mL/min with an elution of 0% A (0.1% formic acid in water) to 70% B (0.1% formic acid in acetonitrile) over 12 minutes. The eluant was subjected to Dual ESI-MS in positive ion mode with the following parameters: drying gas temperature, 225°C; gas flow, 12 L/min; fragmentor voltage, 180 V; capillary voltage, 3.5 kV; and nebulizer pressure, 30 psi. At this voltage, fragmentation to give the valine fragment was readily observed. The data was analyzed using Agilent MassHunter software with the search-by-formula function, which identifies compounds based on the calculated m/z generated from the empirical formula.

[(2)] Further experiments to distinguish between the two arms of the cyclization path of Figure 4 (main text) have not yet been conclusive. The commercially available Leuch anhydride of valine **IV** was hydrolytically unstable and yielded no diagnostic products with SylC, while the attempted labeling of SylC with a hydroxylamine fluorophore to trap **V** was unsuccessful.

12. HRMS Data of the Enzymatically Produced Ureido Peptides

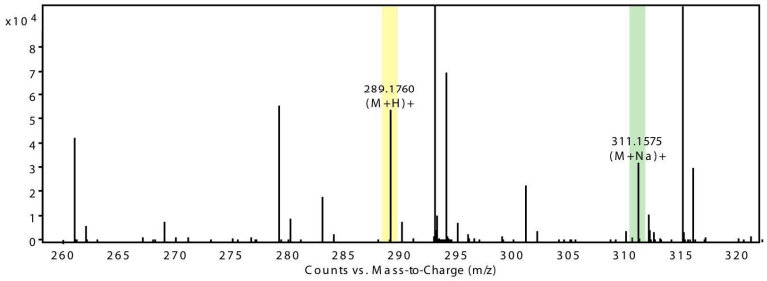


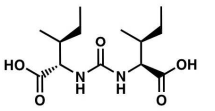


L-VAL-CO-L-VAL

Chemical Formula: $C_{11}H_{20}N_2O_5$

Ion	Retention time	Exp m/z	Obs m/z	Δ ppm
M+H	5.71	261.1445	261.1445	-0.12
M+Na	5.71	283.1264	283.1264	-0.33

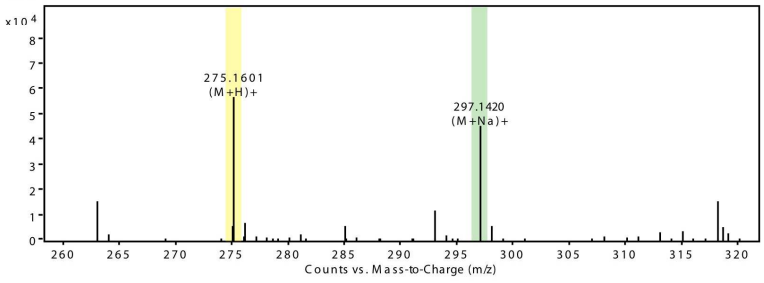


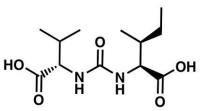


L-ILE-CO-L-ILE

Chemical Formula: $C_{13}H_{24}N_2O_5$

Ion	Retention time	Exp m/z	Obs m/z	Δ ppm
M+H	6.97	289.1758	289.1760	0.73
M+Na	6.97	311.1577	311.1575	-0.55



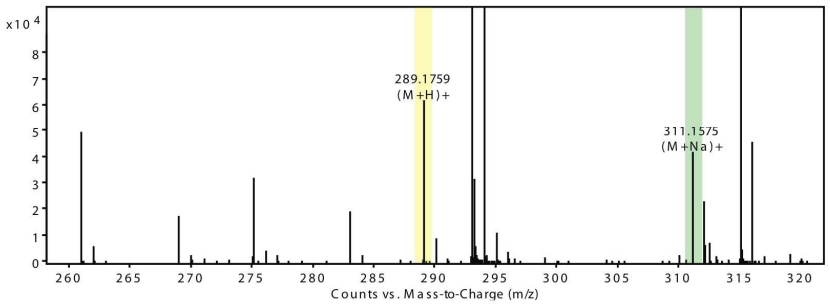


L-VAL-CO-L-ILE

Chemical Formula: $C_{12}H_{22}N_2O_5$

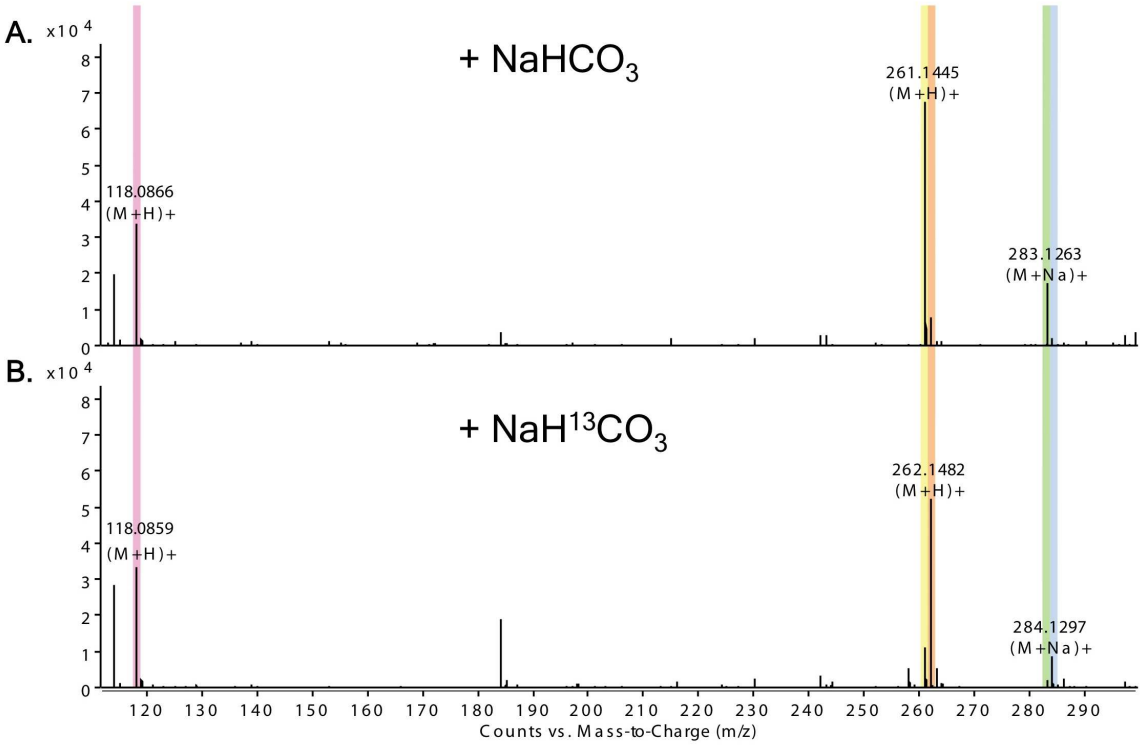
Ion	Retention time	Exp m/z	Obs m/z	Δ ppm
M+H	6.41	275.1601	275.1601	-0.12
M+Na	6.41	297.1421	297.1420	-0.45

Figure S5. HRMS of naturally occuring ureido dipeptide products hydrolyzed from the SylC T-domain (Section 8); Val ureido dipeptide (A), Ile ureido dipeptide (B), and the mixed Val/Ile ureido dipeptide (C).



Ion	Retention time	Exp m/z	Obs m/z	Δ ppm
M+H	6.87	289.1758	289.1759	0.89
M+Na	6.87	311.1577	311.1575	-0.32

Figure S6. HRMS of L-*allo*-Ile ureido dipeptide hydrolyzed from the SylC T-domain (Section 8).



Hydrolyzed Valine Ureido Dipeptide

Panel	$NaHCO_3$ Used	Formula Searched	Ion	Calc m/z	Obs m/z	Δ ppm	Abundance	Relative Abundance	Retention Time (min)
A	^{12}C	$C_{11}H_{20}N_2O_5$	M+H	261.1445	261.1445	-0.11	68553	1.0	5.71
A	^{12}C	$C_{11}H_{20}N_2O_5$	(M+1)+H	262.1476	262.1474	-0.50	7852	0.11	5.71
A	^{12}C	$C_{11}H_{20}N_2O_5$	M+Na	283.1264	283.1263	-0.33	17432	1.0	5.71
A	^{12}C	$C_{11}H_{20}N_2O_5$	(M+1)+Na	*Not found*					
B	^{13}C	$C_{11}H_{20}N_2O_5$	M+H	261.1445	261.1454	3.3	11980	1.0	5.69
B	^{13}C	$^{13}CC_{10}H_{20}N_2O_5$	M+H	262.1476	262.1482	1.3	52766	4.4	5.69
B	^{13}C	$C_{11}H_{20}N_2O_5$	M+Na	283.1264	283.1264	-0.06	2265	1.0	5.69
B	^{13}C	$^{13}CC_{10}H_{20}N_2O_5$	M+Na	284.1298	284.1297	-0.45	8810	3.9	5.69

Valine Fragment

Panel	$NaHCO_3$ Used	Formula Searched	Ion	Calc m/z	Obs m/z	Δ ppm	Abundance	Relative Abundance	Retention Time (min)
A	^{12}C	$C_5H_{11}NO_2$	M+H	118.0863	118.0866	2.6	33937	1.0	5.71
A	^{12}C	$C_5H_{11}NO_2$	(M+1)+H	119.0893	119.0893	-0.43	1863	0.05	5.71
B	^{13}C	$C_5H_{11}NO_2$	M+H	118.0863	118.0865	1.9	33848	1.0	5.69
B	^{13}C	$^{13}CC_4H_{11}NO_2$	M+H	*Not found*					

SI Figure S7. Comparison of HRMS spectra of unlabeled (A) and [^{13}C]-labeled (B) products hydrolyzed from the SylC-T domain (seetion 9) showing a M+1 mass shift in the Val ureido dipeptide from incubation with [^{13}C]-bicarbonate but no mass difference in the Val fragment.

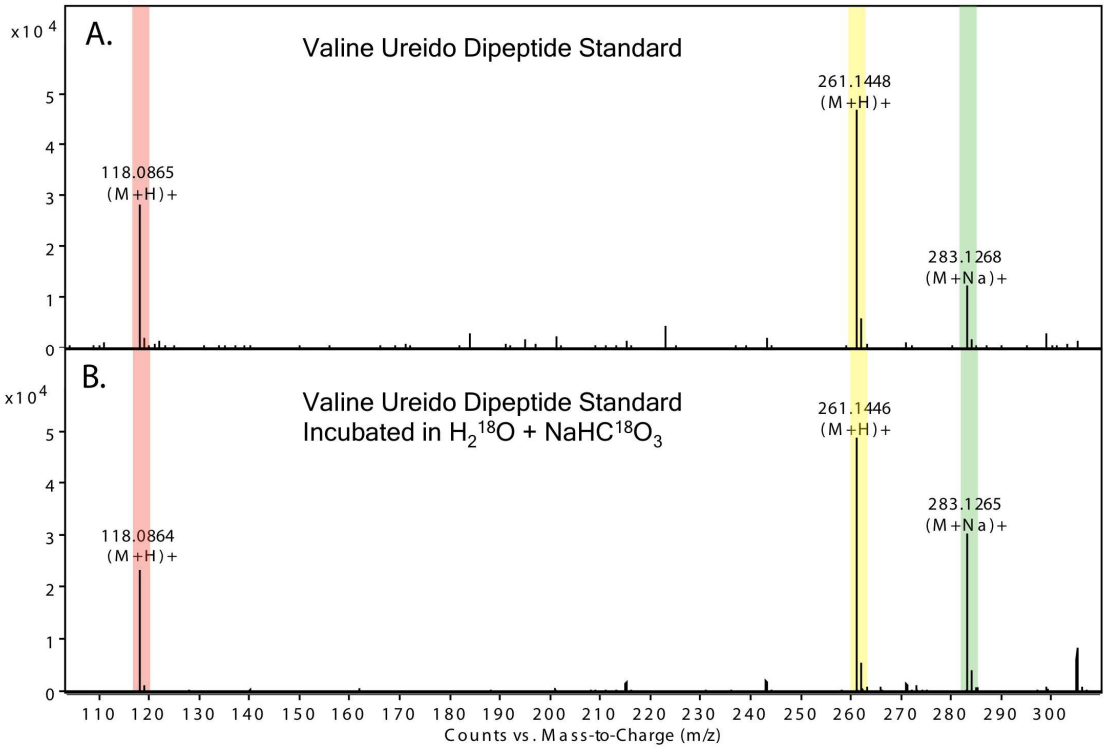
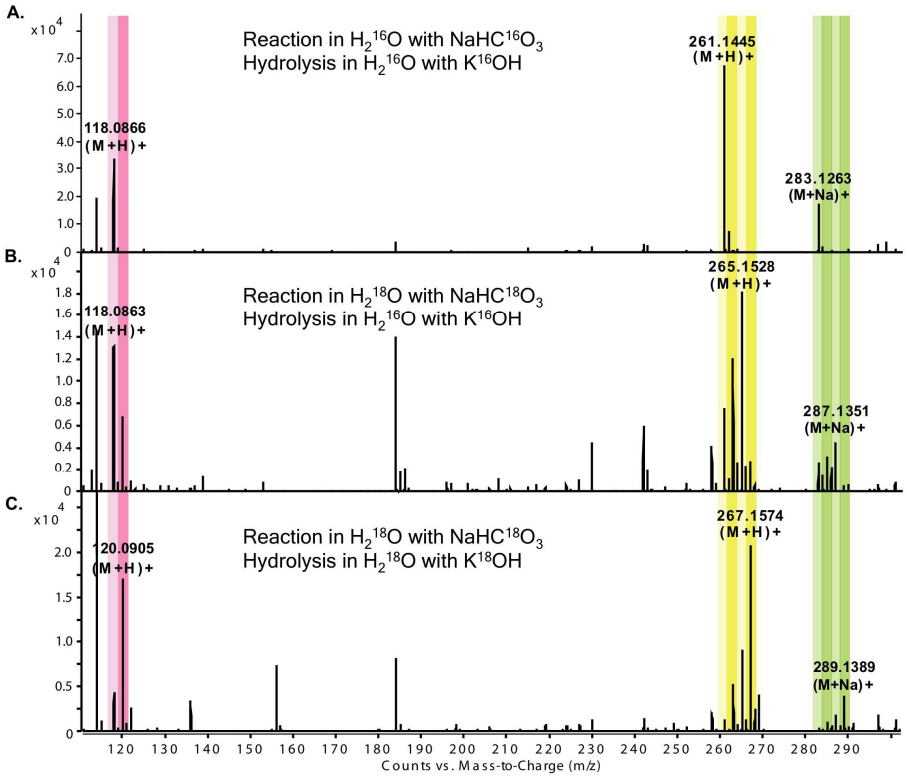


Figure S8. Comparison of HRMS spectra of the synthesized Val uredio dipeptide standard (A) and the result of incubation of that standard for 3 hours in [^{18}O]-H_2O and 25 mM [^{18}O]-$NaHCO_3$ showing no background incorporation of ^{18}O into either the ureido carbonyl or the carboxylate (B).



Hydrolyzed Valine Ureido Dipeptide

Panel	$NaHCO_3$ Used	KOH/H_2O Used	Formula Searched	Ion	Calc m/z	Obs m/z	Δ ppm	Abundance	Retention Time (min)
A	^{16}O	^{16}O	$C_{11}H_{20}N_2O_5$	M+H	261.1445	261.1445	-0.11	68553	5.71
A	^{16}O	^{16}O	$C_{11}H_{20}N_2O_5$	M+H	263.1496	263.1501	2.1	1038	5.71
A	^{16}O	^{16}O	$C_{11}H_{20}N_2O_5$	M+Na	283.1264	283.1263	-0.33	17432	5.71
A	^{16}O	^{16}O	$C_{11}H_{20}N_2O_5$	M+Na			*Not found*		
B	^{18}O	^{16}O	$C_{11}H_{20}N_2O_5$	M+H	261.1445	261.1453	3.1	7828	5.70
B	^{18}O	^{16}O	$^{18}OC_{11}H_{20}N_2O_4$	M+H	263.1487	263.1488	0.29	13351	5.70
B	^{18}O	^{16}O	$^{18}O_2C_{11}H_{20}N_2O_3$	M+H	265.1530	265.1528	-0.64	16692	5.70
B	^{18}O	^{16}O	$^{18}OC_{11}H_{20}N_2O_4$	M+Na	285.1307	285.1323	5.7	3627	5.70
B	^{18}O	^{16}O	$^{18}O_2C_{11}H_{20}N_2O_3$	M+Na	287.1349	287.1351	0.53	4208	5.70
B	^{18}O	^{16}O	$^{18}OC_{11}H_{20}N_2O_4$	M+Na			*Not found*		
C	^{18}O	^{16}O	$C_{11}H_{20}N_2O_5$	M+H			*Not found*		
C	^{18}O	^{18}O	$^{18}OC_{11}H_{20}N_2O_4$	M+H	263.1487	263.1483	-1.8	5806	5.56
C	^{18}O	^{18}O	$^{18}O_2C_{11}H_{20}N_2O_3$	M+H	265.1530	265.1528	-0.57	9054	5.56
C	^{18}O	^{18}O	$^{18}O_3C_{11}H_{20}N_2O_2$	M+H	267.1572	267.1574	0.65	22829	5.56
C	^{18}O	^{18}O	$C_{11}H_{20}N_2O_5$	M+Na			*Not found*		
C	^{18}O	^{18}O	$^{18}OC_{11}H_{20}N_2O_4$	M+Na	285.1307	285.1301	-1.9	1157	5.56
C	^{18}O	^{18}O	$^{18}O_2C_{11}H_{20}N_2O_3$	M+Na	287.1349	287.1341	-3.0	1916	5.56
C	^{18}O	^{18}O	$^{18}O_3C_{11}H_{20}N_2O_2$	M+Na	289.1392	289.1389	-0.96	4287	5.56

Valine Fragment

Panel	$NaHCO_3$ Used	KOH/H_2O Used	Formula Searched	Ion	Calc m/z	Obs m/z	Δ ppm	Abundance	Retention Time (min)
A	^{16}O	^{16}O	$C_5H_{11}NO_2$	M+H	118.0863	118.0866	2.6	33937	5.71
A	^{16}O	^{16}O	$C_5H_{11}NO_2$	M+H	120.0910	120.0915	4.3	182	5.71
B	^{18}O	^{16}O	$C_5H_{11}NO_2$	M+H	118.0863	118.0863	0.78	13653	5.70
B	^{18}O	^{16}O	$^{18}OC_5H_{11}NO_2$	M+H	120.0905	120.0906	0.84	7703	5.70
C	^{18}O	^{18}O	$C_5H_{11}NO_2$	M+H	118.0863	118.0865	1.7	5392	5.56
C	^{18}O	^{18}O	$^{18}OC_5H_{11}NO_2$	M+H	120.0905	120.0905	0.06	18785	5.56

Figure S9. Comparison of HRMS spectra of products hydrolyzed from the SylC T-domain after (A) unlabeled (^{16}O) reaction and hydrolysis, (B) labeled (^{18}O) reaction and unlabeled (^{16}O) hydrolysis and (C) both reaction and hydrolysis labeled (^{18}O).