Studying a Cell Division Amidase Using Defined Peptidoglycan Substrates

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Supporting information

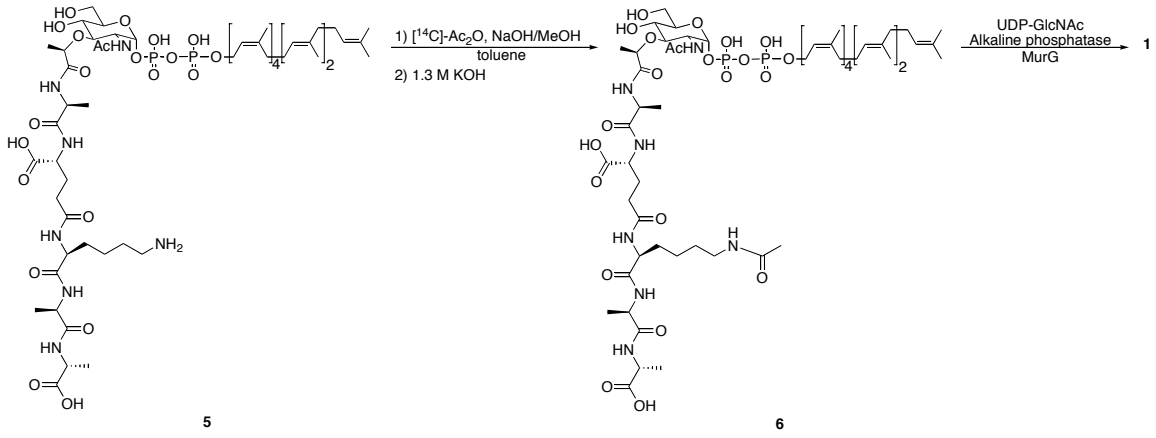
Materials: Heptaprenyl-[^{14}C]-Lipid IV (**2**, Specific Activity, SA = 110 mCi/mmol) was synthesized as described previously.[1] Heptaprenyl-[^{14}C]-Lipid II (**1**, SA = 55 mCi/mmol) and [^{14}C]-nascent PG (**3**) were prepared as detailed below. Unlabeled and [^{14}C]-pentapeptide (**4,** SA = 55 mCi/mmol) were synthesized as detailed below. [1-^{14}C] Acetic anhydride (SA = 110 mCi/mmol) was obtained from American Radiolabeled Chemicals, Inc. C18 SPE (solid phase extraction) columns were purchased from J.T. Baker. Non-stick PCR tubes and pipette tips used for enzymatic reactions were purchased from VWR and Axygen. Full-length *E. coli* PBP1A (with C-terminal His-tag) was purified as described previously.[1] Mature *E. coli* AmiA (with C-terminal His-tag) and mutants were cloned and purified as detailed below. Primers were purchased from Integrated DNA Technologies. Restriction endonucleases were purchased from New England Biolabs. Vectors and expression hosts were obtained from Novagen. Moenomycin A was isolated from flavomycin feedstock as previously reported.[2] EcoLite scintillation fluid was purchased from MP Biomedicals. All other chemicals were purchased from Sigma-Aldrich, unless otherwise indicated. Graphs were generated by GraphPad Prism 4.

General Methods: NMR spectra were recorded on a Varian Inova 500 MHz instrument. Low-resolution mass spectra (LRMS) were obtained on an Agilent Technologies 1100 series LC/MSD instrument using electrospray ionization (ESI), while high-resolution mass spectra (HRMS), ESI mode, were obtained at the Harvard University Mass Spectrometry Facility. MALDI-TOF spectra were obtained on an Applied Biosystems Voyager DE Pro at the Tufts University Core Facility. Preparative HPLC was performed on an Agilent Technologies 1200 Series using a Luna 5 μm C18 column (250 mm x 4.6 mm, Phenomenex). Reactions were monitored by either mass spectroscopy (MS) or thin

layer chromatography (TLC) on glass plates precoated with silica gel (250 μm, Sorbent Technologies). Commercially available reagents were used without further purification.

Radio-HPLC experiments were carried out on a Beckman Coulter System Gold instrument with attached β-Ram module 3 radioisotope detector (IN/US Systems). A Jupiter C12 small-pore column (250 x 4.60 mm, 4 μm, Proteo 90 Å, Phenomenex) equipped with a C12 security guard cartridge (4 x 2.0 mm, Phenomenex) was used for separations. Scintillation counting was performed with a Beckman LS 6000 Series. Radioactive gels were exposed to a phosphorimage screen (GE Healthcare). Image was captured with a Typhoon phosphorimager (GE Healthcare) and analyzed using ImageQuant.

Compound 1. Radiolabeled and unlabeled **1**[3] were made as follows:



Heptaprenyl-Lipid I (**5**) was synthesized as described previously.[4] The compound was then acetylated with radiolabeled or non-radiolabeled acetic anhydride as previously described for heptaprenyl-Lipid IV.[1] Non-radioactive and radioactive reactions were done in parallel to characterize the final product. Two aliquots of **5** (56.0 μg, 40.0 nmol each) were added to conical tubes and $(CH_3{}^{14}CO)_2O$ or $(CH_3CO)_2O$ (12.8 μL in toluene,

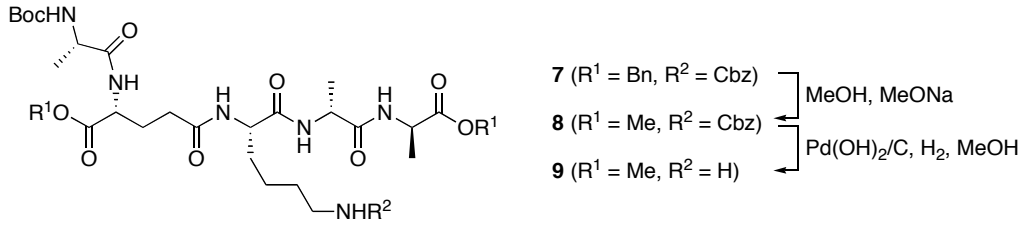
0.582 μmol) and NaOH (12.8 μL in MeOH, 0.192 μmol) were added. The tubes were sealed with parafilm and sonicated at 37 °C for 90 min. The reactions were concentrated by vacuum centrifugation, and then resuspended and incubated in KOH solution (1.3 M in H_2O, 12.8 μL) for 1 hr at RT. The mixtures were neutralized with 1.28 μL acetic acid and vortexed before being loaded onto reverse phase C18 SPE columns. A gradient from 100% 0.1% ammonium hydroxide in H_2O to 100% 0.1% ammonium hydroxide in methanol was employed. The desired product (**6**) was eluted between 50-70% 0.1% ammonium hydroxide in methanol, as detected by TLC phosphorimaging in comparison to an authentic standard. The fractions containing radiolabeled **6**, and analogous fractions of the non-radiolabeled compound, were combined and concentrated by vacuum centrifugation to afford **6** (27.2 μg, 18.9 nmol, 47% yield, based on liquid scintillation counting).

Compound **6** was converted to **1** using a previously described enzymatic method.[4] Separately, radiolabeled and non-radiolabeled **6** (26.7 μg, 18.5 nmol each) were placed in conical tubes. The compounds were dissolved in MeOH (9.2 μL) and 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH = 7.5), 10 mM $MgCl_2$ in H_2O and incubated with 0.9 mM UDP-*N*-acetyl glucosamine, 1 U/μL alkaline phosphatase and 0.5 mg/mL MurG in a final reaction volume of 63.8 μL for 2.5 hr at room temperature. The reactions were quenched with 63.8 μL ice cold H_2O, and purified on reverse phase C18 SPE columns. After washing with 0.1% ammonium hydroxide, the desired compound **1** was eluted with 0.1% ammonium hydroxide in methanol as detected by TLC phosphorimaging in comparison to an authentic standard. The fractions containing radiolabeled **1**, and analogous fractions of the non-radiolabeled compound, were

combined and concentrated by vacuum centrifugation to afford **1** (23.8 μg, 14.5 nmol, SA = 55 nCi/nmol, 78% yield, based on liquid scintillation counting). Purity of radiolabeled **1** was assessed by TLC and gel electrophoresis analysis, as detailed below. Non-radiolabeled **1** was detected by ESI-MS.

LRMS (ESI) calcd for $C_{76}H_{126}N_8O_{27}P_2^{1/2}$ $[M-2H]^{2-}$ 821.4, found 821.4.

Compound 4. Radiolabeled and unlabeled **4** were synthesized as follows:



To 44.0 mg NHBoc-L-Ala-γ-D-Glu(OBn)-L-Lys(NHCbz)-D-Ala-D-Ala-OBn (**7**)[5] (48.7 μmol) was added anhydrous MeOH (1 mL) and 25 wt. % sodium methoxide in methanol (0.05 mL), and the resulting solution was then stirred for 20 min. The reaction was quenched with acetic acid, and then the mixture was diluted with EtOAc and washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over sodium sulfate and concentrated *in vacuo* to afford **8**. Without further purification, MeOH and $Pd(OH)_2/C$ (Pearlman's catalyst, 10 mg) were added to the residue and the solution was stirred under hydrogen atmosphere for 30 min. The mixture was then filtered through celite and concentrated to afford **9**. Compound **9** was purified using reverse phase C18 column chromatography with 0.1% ammonium bicarbonate in water and ethanol. The product **9** was eluted with 40% ethanol (23.0 mg, 77% over 2 steps).

NHBoc-L-Ala-γ-D-Glu(OMe)-L-Lys(NHCbz)-D-Ala-D-Ala-OMe (8). ^{1}H NMR (CD_3OD) : δ 7.37 (m, ArH, 4H), 7.33 (m, ArH, 1H), 5.09 (s, 2H, CH_2Ph), 4.43 (q, CHα-Ala, *J* = 7.3 Hz, 1H), 4.40 (q, CHα-Ala, *J* = 7.3 Hz, 1H), 4.36 (dd, CHα-Glu, *J* = 5.4, 8.3

Hz, 1H), 4.22 (m, CHα-Lys, 1H), 4.07 (q, CHα-Ala, *J* = 7.3 Hz, 1H), 3.73 (s, $COOCH_3$, 3H), 3.69 (s, $COOCH_3$, 3H), 3.15 (t, $CH\varepsilon_2$-Lys, *J* = 6.8 Hz, 2H), 2.43 (dd, $CH\gamma_2$-Glu, *J* = 7.3, 7.3 Hz, 2H), 2.20 (m, CHβ-Glu, 1H), 1.98 (m, CHβ'-Glu, 1H), 1.88 (m, CHβ-Lys, 1H), 1.78 (m, CHβ'-Lys, 1H), 1.55 (m, $CH\delta_2$-Lys, 2H), 1.47 (s, $C(CH_3)_3$, 9H), 1.45 (d, CH_3-Ala, *J* = 7.3 Hz, 3H), 1.45-1.37 (m, $CH\gamma_2$-Lys, 2H), 1.41 (d, CH_3-Ala, *J* = 7.3 Hz, 3H), 1.34 (d, CH_3-Ala, *J* = 7.3 Hz, 3H).

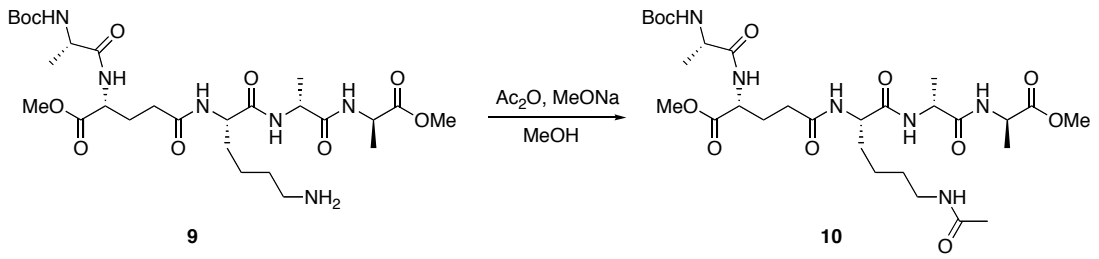
^{13}C NMR (CD_3OD) : δ 174.9, 173.5, 173.5, 173.3, 173.1, 172.4, 157.5, 156.3, 137.1, 128.1, 127.6, 127.4, 79.3, 65.9, 54.0, 52.6, 51.4, 50.8, 50.6, 48.8, 48.1, 40.1, 30.6, 29.5, 29.1, 27.4, 26.3, 22.7, 16.6, 16.5, 15.9.

LRMS (ESI) calcd for $C_{35}H_{54}N_6NaO_{12}$ $[M+Na]^+$ 773.4, found 773.3.

NHBoc-L-Ala-γ-D-Glu(OMe)-L-Lys-D-Ala-D-Ala-OMe (9). ^{1}H NMR (CD_3OD): δ 4.43 (q, CHα-Ala, *J* = 7.3 Hz, 1H), 4.40 (q, CHα-Ala, *J* = 6.8 Hz, 1H), 4.34 (m, CHα-Glu, 1H), 4.29 (m, CHα-Lys, 1H), 4.07 (q, CHα-Ala, *J* = 6.8 Hz, 1H), 3.74 (s, $COOCH_3$, 3H), 3.70 (s, $COOCH_3$, 3H), 2.84 (t, $CH\varepsilon_2$-Lys, *J* = 7.1 Hz, 2H), 2.46 (dd, $CH\gamma_2$-Glu, *J* = 7.3, 7.8 Hz, 2H), 2.21 (m, CHβ-Glu, 1H), 2.01 (m, CHβ'-Glu, 1H), 1.92 (m, CHβ-Lys, 1H), 1.79 (m, CHβ'-Lys, 1H), 1.63 (m, $CH\delta_2$-Lys, 2H), 1.52-1.37 (m, $CH\gamma_2$-Lys, 2H), 1.48 (s, $C(CH_3)_3$, 9H), 1.45 (d, CH_3-Ala, *J* = 7.3 Hz, 3H), 1.42 (d, CH_3-Ala, *J* = 7.3 Hz, 3H), 1.35 (d, CH_3-Ala, *J* = 7.3 Hz, 3H).

^{13}C NMR (CD_3OD) : δ 175.0, 173.5, 173.4, 173.3, 173.1, 172.5, 156.1 , 79.3, 53.6, 52.8, 51.4 , 50.8, 50.6, 48.9, 48.1, 39.8, 30.5, 29.5, 28.8, 27.4, 26.1, 22.5, 16.6, 16.6, 15.9.

LRMS (ESI) calcd for $C_{27}H_{49}N_6O_{10}$ $[M+H]^+$ 617.4, found 617.3.



9 **10**

To a solution of 13.9 mg **9** (22.5 μmol) in anhydrous MeOH (1 mL) was added 25 wt. % sodium methoxide in methanol (68 μL, 0.3 mmol) and acetic anhydride (55 μL, 0.6 mmol), which was stirred for 30 min at RT. The reaction was quenched with acetic acid, and the solvent was removed by evaporation. The acetylated compound was purified by silica gel column chromatography (EtOAc : MeOH / 20 : 1 to 10 : 1) to give **10** (6.8 mg, 10.3 μmol, 46%).

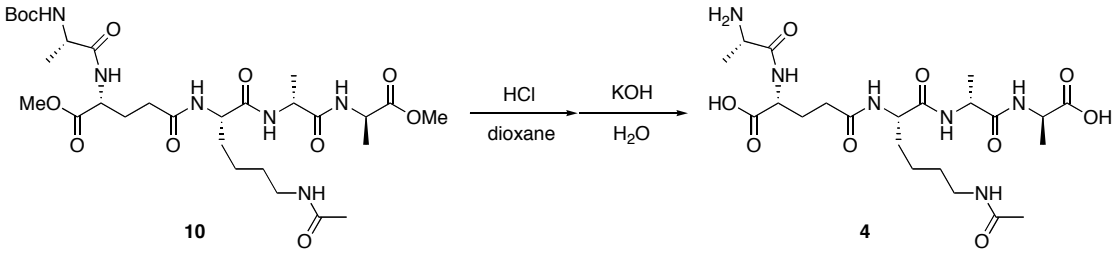
The radioactive reaction to make [^{14}C]-**10** was done in parallel to a non-radioactive reaction so that progress could be monitored by MS. The procedure above was repeated on 0.035 mg **9** (57.5 nmol) using $(CH_3{}^{14}CO)_2O$ or $(CH_3CO)_2O$ (45 mM in toluene) to give labeled **10** (27.7 μg, 42.1 nmol, 73.2%) after purification. The product was detected by TLC phosphorimaging (EtOAc : MeOH / 4 : 1) and quantified by liquid scintillation counting.

NHBoc-L-Ala-γ-D-Glu(OMe)-L-Lys(NHAc)-D-Ala-D-Ala-OMe (10). ^{1}H NMR (CD_3OD) : δ 4.44 (q, CHα-Ala, *J* = 7.3 Hz, 1H), 4.40 (q, CHα-Ala, *J* = 7.3 Hz, 1H), 4.36 (dd, CHα-Glu, *J* = 4.9, 8.3 Hz, 1H), 4.24 (m, CHα-Lys, 1H), 4.07 (q, CHα-Ala, *J* = 7.2 Hz, 1H), 3.73 (s, $COOCH_3$, 3H), 3.70 (s, $COOCH_3$, 3H), 3.19 (t, $CH\varepsilon_2$-Lys, *J* = 6.8 Hz, 2H), 2.45 (dd, $CH\gamma_2$-Glu, *J* = 7.3, 7.8 Hz, 2H), 2.20 (m, CHβ-Glu, 1H), 2.00 (m, CHβ'-Glu, 1H), 1.96 (s, Ac, 3H), 1.89 (m, CHβ-Lys, 1H), 1.78 (m, CHβ'-Lys, 1H), 1.55 (m, $CH\delta_2$-Lys, 2H), 1.47 (s, $C(CH_3)_3$, 9H), 1.46-1.34 (m, $CH\gamma_2$-Lys, 2H), 1.46 (d, CH_3-Ala, *J*

= 7.3 Hz, 3H), 1.42 (d, CH_3-Ala, *J* = 6.8 Hz, 3H), 1.35 (d, CH_3-Ala, *J* = 7.3 Hz, 3H).

^{13}C NMR (CD_3OD) : δ 174.9, 173.5, 173.4, 173.3, 173.1, 172.6, 171.8, 156.3, 79.3, 53.9, 52.6, 51.4, 50.8, 50.6, 48.8, 48.1, 38.8, 30.6, 29.5, 28.5, 27.4, 26.3, 22.9, 21.2, 16.5, 16.5, 15.9.

LRMS (ESI) calcd for $C_{29}H_{51}N_6O_{11}$ $[M+H]^+$ 659.4, found 659.3



Hydrochloric acid in dioxane (4 M, 1 mL) was added to **10** (4.9 mg, 7.4 μmol) and the resulting solution was vigorously stirred for 20 min. The mixture was concentrated *in vacuo*, and then the residue was dissolved in 0.67 M KOH aqueous solution (0.75 mL) and stirred for 30 min. The reaction was neutralized with DOWEX 100-H^+, filtered and concentrated. The residue was submitted to reverse-phase HPLC purification with a solvent system of 0.1% ammonium bicarbonate in water and methanol using a linear gradient of 0 % to 80 % methanol to afford **4** (0.9 mg, 1.7 μmol, 23% over two steps).

The reaction was repeated as described above for [^{14}C]-**10** (27.7 μg, 42.1 nmol) and unlabeled **10** (for reaction monitoring), except solutions were shaken, not stirred, and the reaction was neutralized with acetic acid in place of DOWEX. Reverse-phase radio-HPLC (without attached scintillation counter) was used for purification of labeled **4**. Fractions (1 mL) were analyzed by liquid scintillation counting, and labeled **4** (SA = 55 nCi/nmol, 4.47 μg, 8.40 nmol) was recovered in 20 % yield over 2 steps. All mention of

4 in the experiments below refers to labeled **4**, which was stored as a solution in H_2O at -20 °C.

For [^{14}C]-**4**: MALDI-TOF (linear) calcd for $^{14}CC_{21}H_{39}N_6O_9$ $[M+Na]^+$ 555.25, found 555.09

L-Ala-γ-D-Glu-L-Lys(NHAc)-D-Ala-D-Ala (4). ^{1}H NMR (CD_3OD) : δ 4.55 (q, CHα-D-Ala, *J* = 7.2 Hz, 1H), 4.40 (m, CHα-Lys, 1H), 4.38 (m, CHα-Glu, 1H), 4.19 (q, CHα-D-Ala, *J* = 7.1 Hz, 1H), 4.04 (q, CHα-L-Ala, *J* = 6.9 Hz, 1H), 3.20 (t, $CH\varepsilon_2$-Lys, *J* = 6.9 Hz, 2H), 2.45 (m, CHβ-Glu, 1H), 2.38 (m, $CH\gamma_2$-Glu, 2H), 1.97 (s, Ac, 3H), 1.91 (m, CHβ-Lys, 1H), 1.86 (m, CHβ'-Glu, 1H), 1.70 (m, CHβ'-Lys, 1H), 1.62 (d, CH_3-L-Ala, *J* = 6.9 Hz, 3H), 1.56 (m, $CH\delta_2$-Lys, 2H), 1.48-1.40 (m, $CH\gamma_2$-Lys, 2H), 1.43 (d, CH_3-D-Ala, *J* = 6.7 Hz, 3H), 1.40 (d, CH_3-D-Ala, *J* = 7.1 Hz, 3H).

^{13}C NMR (CD_3OD) : δ 177.4 (COα-D-Ala), 176.1 (COα-Glu), 174.0 (COγ-Glu), 172.9 (COα-Lys), 172.8 (COα-D-Ala), 171.9 ($\underline{C}OCH_3$), 169.6 (COα-L-Ala), 53.6 (Cα-Glu), 53.5 (Cα-Lys), 50.2 (Cα-D-Ala), 49.1 (Cα-L-Ala), 49.0 (Cα-D-Ala), 38.8 (Cε-Lys), 31.4 (Cγ-Glu), 31.1 (Cβ-Lys), 28.6 (CHδ-Lys), 28.2 (CHβ-Glu), 22.9 (Cγ-Lys), 21.3 ($CO\underline{C}H_3$), 17.3 (Cβ-L-Ala), 16.8 (Cβ-D-Ala), 16.5 (Cβ-D-Ala).

HRMS (ESI) calcd for $C_{22}H_{39}N_6O_9$ $[M+H]^+$ 531.2773, found 531.2777

Note that ^{1}H and ^{13}C NMR, and HRMS spectra for **4** are attached as Figures S6-S8.

*Enzymatic synthesis of nPG (**3**)*: Nascent PG (**3**) was made from polymerization of **1** by *E. coli* PBP1A by a previously described method.[1] Typically, PBP1A (0.4 μM) was incubated with 4 μM **1** in 50 mM HEPES (pH = 7.5), 10 mM $CaCl_2$, 20% DMSO, 1 kU/mL Penicillin G in a total reaction volume of 10-20 μL for 20 minutes at RT. The

reaction was terminated by boiling or addition of 8 μM Moenomycin A followed by vortexing. The product was visualized by gel electrophoresis, as reported previously.[3] Any changes to this procedure are noted below.

Cloning and expression of E. coli AmiA[K35-R289] and mutants: The *amiA (K35-R289)* gene encoding mature *E. coli* AmiA, lacking the predicted signal sequence, was PCR amplified from *E. coli* MC4100 purified genomic DNA using the following primer pair: 5′-TGTGCATATGAAAGACGAACTTTTAAAAACCAGC-3′ and 5′-TAGTAAGCTTTCGCTTTTTCGAATGTGCT-3′. After digestion with *NdeI* and *HindIII*, the PCR fragment was ligated into pET22b(+) to produce pAmiA[K35-R289] as a C-terminal His_6 fusion. The inserted *amiA (K35-R289)* gene was confirmed by sequencing (Genewiz or Dana-Farber/Harvard Cancer Center DNA Resource Core).

A point mutation was made in the parent plasmid pAmiA[K35-R289] using techniques described in the QuickChange site-directed mutagenesis kit (Stratagene) to make the mutant plasmid pAmiA[K35-R289] D191A with the forward primer 5′-CAACAAGTGCTGTTTGCTCTGGTGCAAACAGAT-3′ and the reverse primer 5′ATCTGTTTGCACCAGAGCAAACAGCACTTGTTG-3′ (mutagenized codon is underlined). Subsequent mutations were made on the parent mutant plasmid pAmiA[K35-R289] D191A using primer pairs given in Table S1.

Table S1. Primer pairs used for mutagenesis (mutagenized codon is underlined).

Mutations	Forward Primer	Reverse Primer
H65A	GTTCTCGATCCAGGTGCC GGCGGAATTGATACC	GGTATCAATTCCGCCGGC ACCTGGATCGAGAAC
D69V	GGTCACGGCGGAATTGTT ACCGGAGCGATCGGA	TCCGATCGCTCCGGTAAC AATTCCGCCGTGACC
E80A	CGCAACGGTTCGAAAGCA AAACATGTGGTGCTG	CAGCACCACATGTTTTGC TTTCGAACCGTTGCG
D109S	TTAACGCGTTCTGGCTCT ACGTTTATCCCACTT	AAGTGGGATAAACGTAGA GCCAGAACGCGTTAA
H133A	CTGTTTATGTCAATTGCT GCCGATGGCTTTACC	GGTAAAGCCATCGGCAGC AATTGACATAAACAG
E242A	CCTTCGGTGCTGGTGGCA ACCTCGTTTATCACC	GGTGATAAACGAGGTTGC CACCAGCACCGAAGG

The truncated and mutant plasmids were transformed into *E. coli* BL21(DE3) cells for overexpression. Cells were grown in 500 mL LB (Luria-Bertani) broth supplemented with 50 μg/mL carbenicillin at 37 °C until OD_{600} = 0.3 and were then cooled to 25 °C before induction at OD_{600} = 0.6 with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 5 hr. Cells were harvested by centrifugation (5,250 × g, 10 min, 4 °C) and the pellet was resuspended in 15 mL of 25 mM Tris-HCl (2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride, pH = 8.0), 400 mM NaCl, 10% glycerol (lysis buffer) supplemented with 10 μg/mL DNase and 1 mM PMSF (phenylmethanesulphonylfluoride) before being lysed with a French pressure cell twice at 16,000 lb/in^2. Lysozyme was not used in the lysis buffer because it was found to complicate later activity analysis. The resulting cell lysate was centrifuged (90,000 × g, 10 min, 4 °C) and the supernatant was incubated with 1.5 mL pre-washed Ni-NTA Superflow resin (Qiagen) supplemented with 2 mM imidazole for 1 hr at 4 °C with rocking. The resin was then washed and the His_6-tagged protein eluted with the following imidazole gradient: 5 CV (column volumes) of 30 mM imidazole in lysis

buffer; 4 CV of 60 mM imidazole in lysis buffer; 2.6 CV of 200 mM imidazole in lysis buffer and 2.6 CV of 500 mM imidazole in lysis buffer. The 200 mM imidazole fraction was shown to contain mainly pure protein by SDS-PAGE analysis and was concentrated using an Amicon Ultra Centrifugal Filter Device (10 kD MWCO, Millipore). The protein was further purified by gel filtration using a Superdex 200 10/300 GL column (GE Healthcare) with 25 mM HEPES (pH = 7.5), 400 mM NaCl, 10% glycerol buffer at 4 °C. The yields of AmiA[K35-R289] and AmiA[K35-R289] D191A were estimated by the *DC* protein assay (Bio-Rad) to be 10.7 mg/L and 13.3 mg/L, respectively. The mutant proteins of AmiA[K35-R289] D191A were overexpressed in yields ranging from 0.40 mg/L (D109S) to 4.40 mg/L (E242A).

Cleavage of PG fragments by AmiA (general procedure): AmiA was incubated with indicated amounts of PG fragments **1**, **2**, or **3** (in buffer from previous enzymatic step) in 50 mM HEPES (pH = 7.5), 10 mM $CaCl_2$, 20% DMSO, 60 μM $Zn(OAc)_2$ (Buffer A) in given volumes for specific time points. The reactions were terminated by boiling. Optimization of the amidase reaction was carried out in the rate analysis section, described below.

Paper chromatography assay for cleavage of compound **3**: To verify that AmiA could hydrolyze nPG (**3**), we used a paper chromatography assay previously reported for analyzing PG synthesis.[6,7] Compound **3** (40 pmol) was incubated with 4 μM AmiA or lysozyme (from chicken egg white) in Buffer A in a total reaction volume of 25 μL at RT for 10 min. AmiA storage buffer and BSA (bovine serum albumin, New England Biolabs) were used as negative controls. Reactions were terminated by boiling for 5 min and were spotted separately on 1 × 20 cm strips of cellulose chromatography paper (3

MM Whatman chromatography paper). Reactants and products were separated as detailed by Anderson *et al.*,[6] using isobutyric acid / 1M NH_4OH, 5:3 as the mobile phase and were quantified by scintillation counting (Figure S1a). Activity is demonstrated by a shift in radioactivity from the origin. Reactions were performed in duplicate.

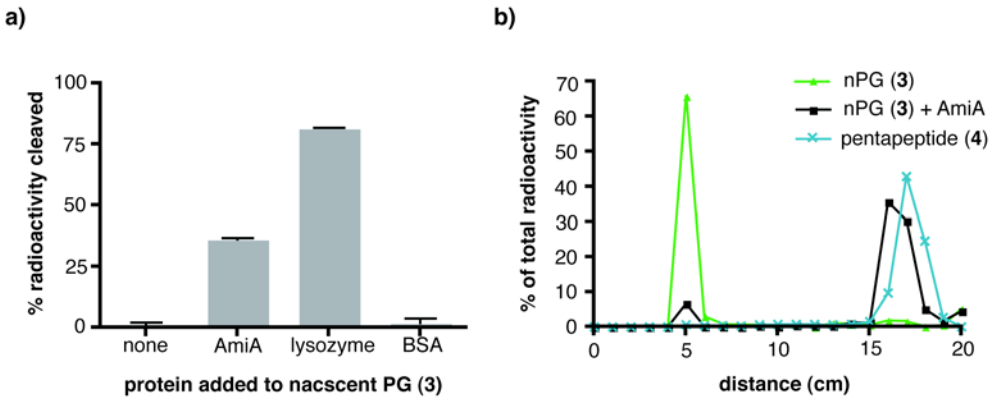


Figure S1. Validation of paper chromatography assay to analyze AmiA digestion of **3** to produce **4**: (a) Lysozyme and amidase treatment of **3** produce labeled fragments that migrate by paper chromatography, while added buffer and BSA do not. The percent radioactivity cleaved is found by dividing the amount of radioactivity that migrates away from the origin by the total amount of radioactivity loaded onto the paper. Unreacted **1** remaining from incomplete polymerization is subtracted from the migrated radioactivity; (b) AmiA digestion of nPG (**3**) produces a product that co-migrates with authentic **4**. Note that the origin is at 5 cm, and that nPG (**3**) does not migrate.

To confirm that the digestion of **3** by AmiA released a pentapeptide product (Figure 2b) that migrated by paper chromatography, we compared the R_f values of the radiolabeled standard **4** and the AmiA digestion product. Compound **3** was incubated with and without AmiA at 37 °C for 30 min in Buffer A. The reactions and compound **4** (15 pmol in triplicate) were analyzed by paper chromatography as detailed above. The paper strips were cut into 1 cm pieces and radioactivity was quantitated by scintillation counting (Figure S1b). Migration of the standard (**4**) and AmiA digestion product coincided, as expected.

*Zinc dependence of AmiA hydrolysis of **3***: In order to assess the predicted zinc-dependence of AmiA hydrolysis, we (a) varied the concentration of zinc (see section below and Figure 2a, right) and (b) added zinc chelators to the reaction. Compound **3** (80 pmol) was incubated with or without 4 μM AmiA in Buffer A in a total reaction volume of 20 μL at 37 °C for 2.5 hr. Reactions were carried out the absence or presence of indicated amounts (0 – 1 mM) of 1,10-phenanthroline (lanes 1 – 5) and EDTA (ethylenediamine tetra-acetic acid, lanes 6 - 9) with (lanes 5, 9) or without 5 mM $Zn(OAc)_2$ (Figure S2). Reactions were terminated by boiling and analyzed by gel electrophoresis on a 12% acrylamide gel (procedure described below). The appearance of a new low molecular weight band indicates activity, as described in detail below (Figure S4). AmiA is inhibited by 1 mM of 1,10-phenanthroline or EDTA (lanes 4 and 8, respectively), but activity can be rescued upon addition of 5 mM $Zn(OAc)_2$ (lanes 5 and 9, respectively).

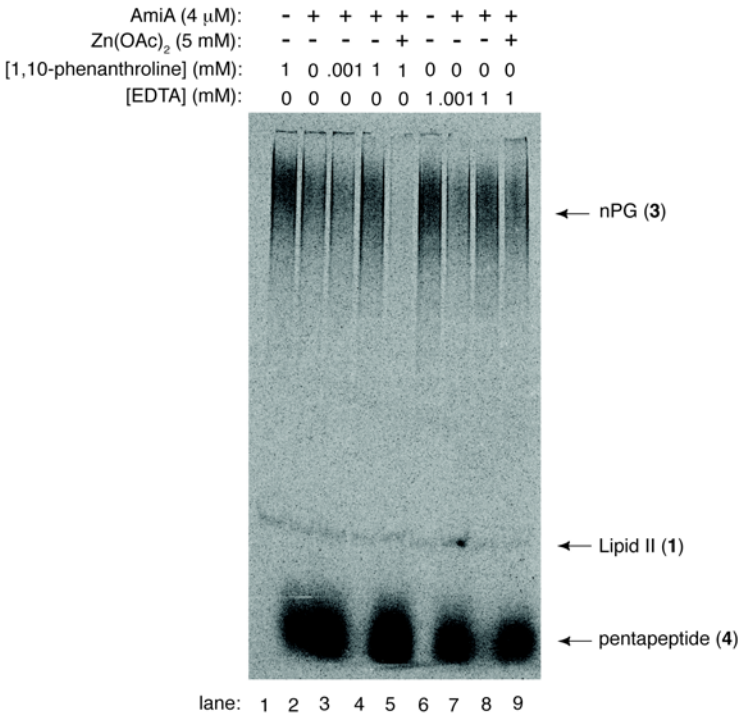


Figure S2. Gel electrophoresis analysis shows that high concentrations of zinc chelators inhibit AmiA cleavage of **3**, but added zinc restores activity. Cleavage is indicated by the presence of a new band that represents labeled product **4**, as confirmed in Figure S4. AmiA is active in the presence of 1 μM 1,10-phenanthroline and EDTA (lanes 3 and 7, respectively), but not 1 mM of each (lanes 4 and 8, respectively), unless 5 mM $Zn(OAc)_2$ is added (lanes 5 and 9, respectively). Lane 1 is a negative control that lacks AmiA. Lane 2 shows that AmiA is active over time without added zinc, which suggests that the purified enzyme contains zinc.

*Initial rate determination of AmiA/mutants hydrolysis of compound **3***: Rate determination experiments were performed as follows: PBP1A (4 μM) was incubated with 40 μM of **1** in 50 mM HEPES (pH = 7.5), 10 mM $CaCl_2$, 20% DMSO, 1 kU/mL Penicillin G in a total reaction volume of 12 μL for 30 minutes to produce **3**. The reaction was terminated by addition of 8 μM Moenomycin A followed by vortexing. The amount of **3** was determined by scintillation counting. After optimization of the reaction by varying substrate concentration (1.3 – 7.2 μM peptide concentration), enzyme concentration (0.4 – 40 μM), and zinc concentration (0 – 1.0 mM, Figure 2a, right), the following conditions

were used: amidase (4 μM) was incubated with 7.2 μM **3** in 50 mM HEPES (pH = 7.5), 10 mM $CaCl_2$, 10% DMSO, 50 μM $Zn(OAc)_2$ in a total reaction volume of 1.5 μL in nonstick conical tubes (0.2 mL, Axygen) for specific time points at RT. Reactions were quenched with 10 μL 10% SDS followed by vortexing. Reactions were done in at least triplicate and were analyzed by paper chromatography as described above, where percent product is equivalent to percent migrated minus percent migrated at time = 0 (found in triplicate for **3** without added amidase).

Note that AmiA[K35-R289] D191A was used as the "wt" in these experiments. This is because we identified a proteolytic cleavage site between Phe190 and Asp191 of AmiA[K35-R289] by N-terminal sequencing analysis (Tufts University Core Facility, data not shown). Mutation of Asp191 to Ala greatly reduced degradation, hence AmiA[K35-R289] D191A is more stable. The D191A mutant also has comparable activity to the parent protein (Figure S3a).

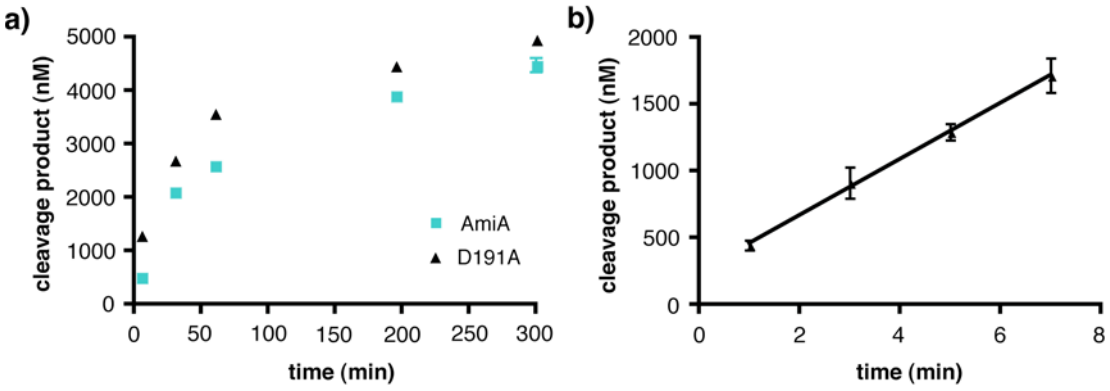


Figure S3. AmiA[K35-R289] D191A is used as the wild type protein for rate experiments: (a) time course of AmiA[K35-R289] D191A (black) and AmiA[K35-R289] (blue) demonstrates comparable activity; (b) D191A shows a linear time range from 1 to 7 min (slope = 209.9 ± 5.994 nM/min), R^2 = .9984). The turnover number given this rate is .05 min^{-1}. Amidase (4 μM) was incubated with **3** (7.2 μM) in 50 mM HEPES (pH = 7.5), 10 mM $CaCl_2$, 10% DMSO, 50 μM $Zn(OAc)_2$ in a total reaction volume of 1.5 μL for indicated time points in triplicate.

Using this assay, we compared the activity of predicted catalytic (H65A, E80A, H133A, and E242A, Figure 1a, left) and non-catalytic mutants (D109S and D69V) to wild type. All time courses were done in at least triplicate with at least three time points. The wild type protein exhibited a linear rate (209.9 ± 5.994 nM/min) from 1 to 7 min (Figure S3b). Mutant proteins H65A, E80A, H133A, and E242A were first screened for activity by collecting a time course of five time points between 1 and 90 min. E242A, H65A, and H133A showed no activity, even when reactions were repeated for time points of several hours. An initial rate was confirmed for E80A using a longer time course (60 – 180 min). D109S and D69V both exhibited activity (30 – 120 min). The rates were normalized with respect to wild type protein activity that was determined in parallel to each mutant in 50 μM $Zn(OAc)_2$ (Figure 1a, right).

Analysis of the substrate preferences of AmiA by gel electrophoresis: AmiA (4 μM) was incubated with 8 μM **1**, 8 μM **2**, or 4 μM **3** (about 4 nCi each) in Buffer A in a total reaction volume of 5 μL for **1**, 10 μL for **2**, and 20 μL for **3** for 2.5 hr at 37 °C (Figure 2c). For negative controls, substrates were incubated with an equal volume of storage buffer in place of AmiA. Reactions were terminated by boiling and were then dried by vacuum centrifugation, resuspended in sample buffer and analyzed by electrophoresis using a 12% acrylamide gel as detailed below. Results were confirmed by repeating reactions and monitoring for the appearance of labeled pentapeptide product using radio-HPLC (data not shown).

*Identification of amidase pentapeptide product by comparison to authentic standard **4** using gel electrophoresis and HPLC analysis*: Reactions were repeated exactly as described in the section above for AmiA digestion of **3** (4 nCi/rxn) with and without

enzyme for 2.5 hr at 37 °C. Reactions were terminated by boiling. For gel electrophoresis analysis, reaction samples and **4** (33 pmol, 1.8 nCi) were evaporated to dryness and analyzed by electrophoresis on a 12% acrylamide gel as detailed below, except the dye front was run only 12 cm down the gel to ensure that radioactivity did not run off the gel (Figure S4a).

Reaction samples that were analyzed by radio-HPLC were centrifuged (16,000 × g, 10 min) and the supernatant was diluted with 30 μL dH_2O before injection. Compound **4** (84 pmol, 4.6 nCi) was injected alone, and then co-injected (21 pmol, 1.2 nCi) with an AmiA + **3** reaction sample. Radio-HPLC analysis was carried out over a gradient of 1% to 80% 0.1% ammonium hydroxide in acetonitrile over 30 min after 5 min at 99% 0.1% ammonium hydroxide in H_2O (Figure S4b). Gel electrophoresis and radio-HPLC analysis demonstrate by co-migration/elution that the AmiA cleavage product is indeed **4**.

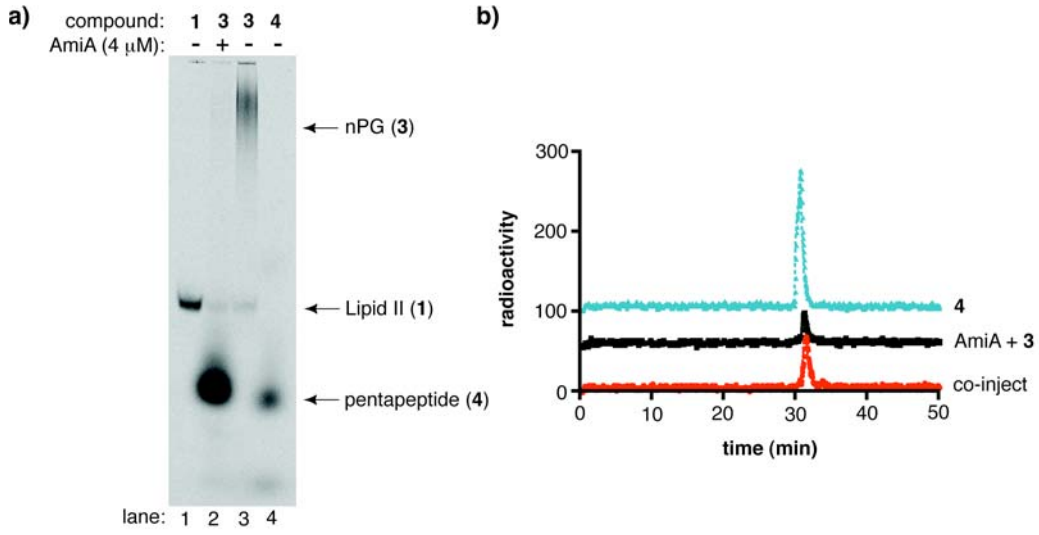


Figure S4. The product of AmiA cleavage of **3** co-migrates/elutes with authentic standard **4**: (a) gel electrophoresis analysis of **1** (lane 1), **3** with and without AmiA (lanes 2 and 3, respectively), and **4** (lane 4). The new low molecular weight band in lane 2 co-migrates with the authentic standard **4** (lane 4); (b) Radio-HPLC analysis of **4** (blue), AmiA + **3** (black) and co-injected **4** and AmiA + **3** (red) show that the product of AmiA cleavage of **3** co-elutes with **4** at t = 31 min (72% acetonitrile). A negative control of **3** without AmiA was injected and showed no peak formation (data not shown). Note that 4× the amount of **4** was injected alone than in the co-injection run.

Analysis of potential AmiA hydrolysis of disaccharide-pentapeptide PG fragments: Lytic transglycosylase *E. coli* Slt70 was cloned and purified in order to evaluate the potential cleavage of a disaccharide-pentapeptide PG fragment (**11**) by AmiA. The *slt (D28-Y645)* gene encoding mature *E. coli* Slt70, lacking the predicted signal sequence, was PCR amplified from *E. coli* MC4100 purified genomic DNA using the following primer pair: 5′-TAGTCATATGGACTCACTGGATGAGCAGCGTAGTCGT-3′ and 5′-TAGTAAGCTTGTAACGACGTCCCCATTCGTGGC-3′. After digestion with *NdeI* and *HindIII*, the PCR fragment was ligated into pET22b(+) to produce pSlt70[D28-Y645] as a C-terminal His_6 fusion. The inserted *slt (D28-Y645)* gene was confirmed by sequencing. The plasmid pΔSlt70[D28-Y645] was transformed into *E. coli* BL21(DE3)

cells and overexpressed and purified as described for AmiA, above. The final protein yield was an estimated 19.4 mg/L.

Slt70 is known to cleave glycosidic linkages in PG to produce 1,6-anhydromuramoyl disaccharide-pentapeptides (**11**) as the major product.[8] To examine if **11** could be accepted as a substrate by AmiA, PG that was pre-digested with Slt70[D28-Y645] was incubated with AmiA (Figure S5a). The experiment was performed as follows: **3** (in buffer from previous enzymatic step) was treated with 4 μM Slt70[D28-Y645] in a total reaction volume of 10 μL for 2 hr at 37 °C. The reactions were terminated by boiling for 5 min, and then cooled and centrifuged (16,000 × g, 10 min, 4 °C). To these reactions, 4 μM AmiA (lanes 4 and 5) or buffer (lane 3, negative control) were added for the indicated time points at 37 °C with 5 μL added Buffer A. As a positive control, AmiA was directly incubated with **3** under the same reaction conditions (lane 6). The reactions were terminated by boiling for 5 min, evaporated to dryness, and then analyzed by gel electrophoresis (Figure S5b), as described below. As indicated by lanes 4 and 5, AmiA does not accept the PG fragments produced by lytic transglycosylase cleavage of PG, even though AmiA is active on nPG (**3**) as shown by the appearance of **4** in lane 6. In a related experiment, Slt70 was replaced with lysozyme, which produces a lactol in place of the 1,6-anhydro moiety in **11**, and AmiA was still not active with the disaccharide-pentapeptide fragment (data not shown).

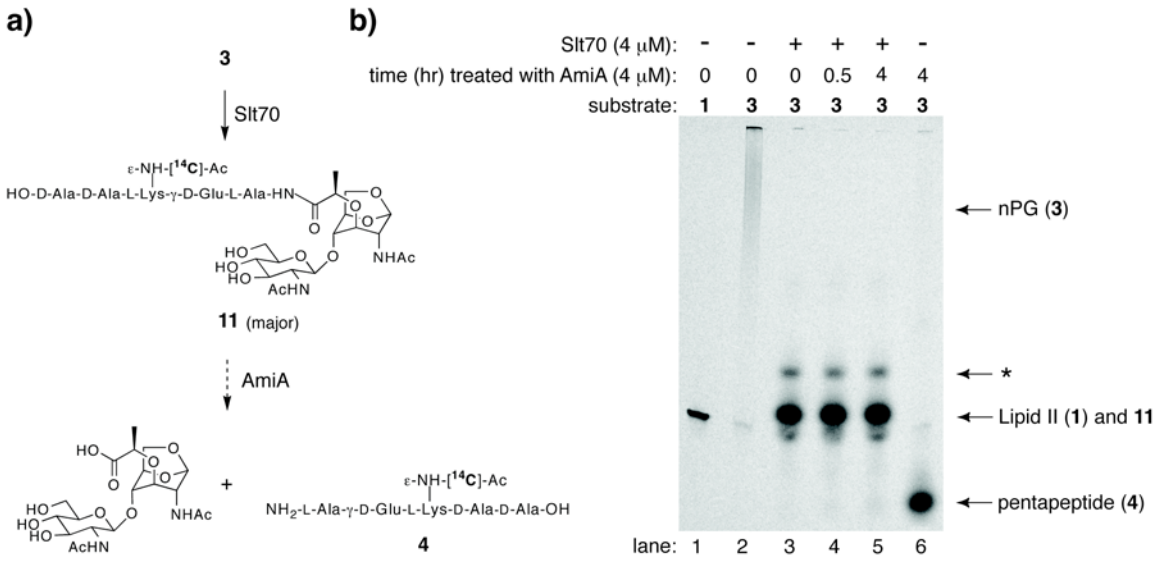


Figure S5. AmiA does not cleave disaccharide-pentapeptide PG fragment **11**: (a) reaction scheme depicting experimental design. Cleavage of **3** by Slt70 produces **11** as the major product, which is then incubated with AmiA to examine substrate requirements. Amidase activity is indicated by the appearance of **4**; (b) gel electrophoresis analysis of **3** (lane 2) treated with AmiA (lane 6) or with Slt70 alone (lane 3) or Slt70 followed by AmiA (lanes 4 and 5). AmiA does not cleave PG fragments produced by Slt70 (lanes 4 and 5), but does cleave **3** (lane 6), as expected. Note another new band (*) appears above **1**, which perhaps represents an unreacted fragment of **3** that cannot be broken down into disaccharide-pentapeptides. It is known that Slt70 does not digest tetrasaccharide fragments of PG,[9] and presumably this fragment cannot be cleaved by AmiA in the presence of **11**.

Gel electrophoresis analysis of PG fragments: Gel electrophoresis analysis was carried out as described in Barrett *et al.*[3] Briefly, samples were dried and resuspended in sample buffer (125 mM Tris-HCl (pH = 6.8), 40% glycerol, 9% SDS (sodium dodecyl sulfate), 0.004% bromophenol blue) before being loaded onto a 20 × 20 cm gel (1 mm thick) and separated at 30 mA until the dye front was 3.5 cm from the bottom. Gels were dried overnight, and exposed to a phosphorimage screen for >1 week.

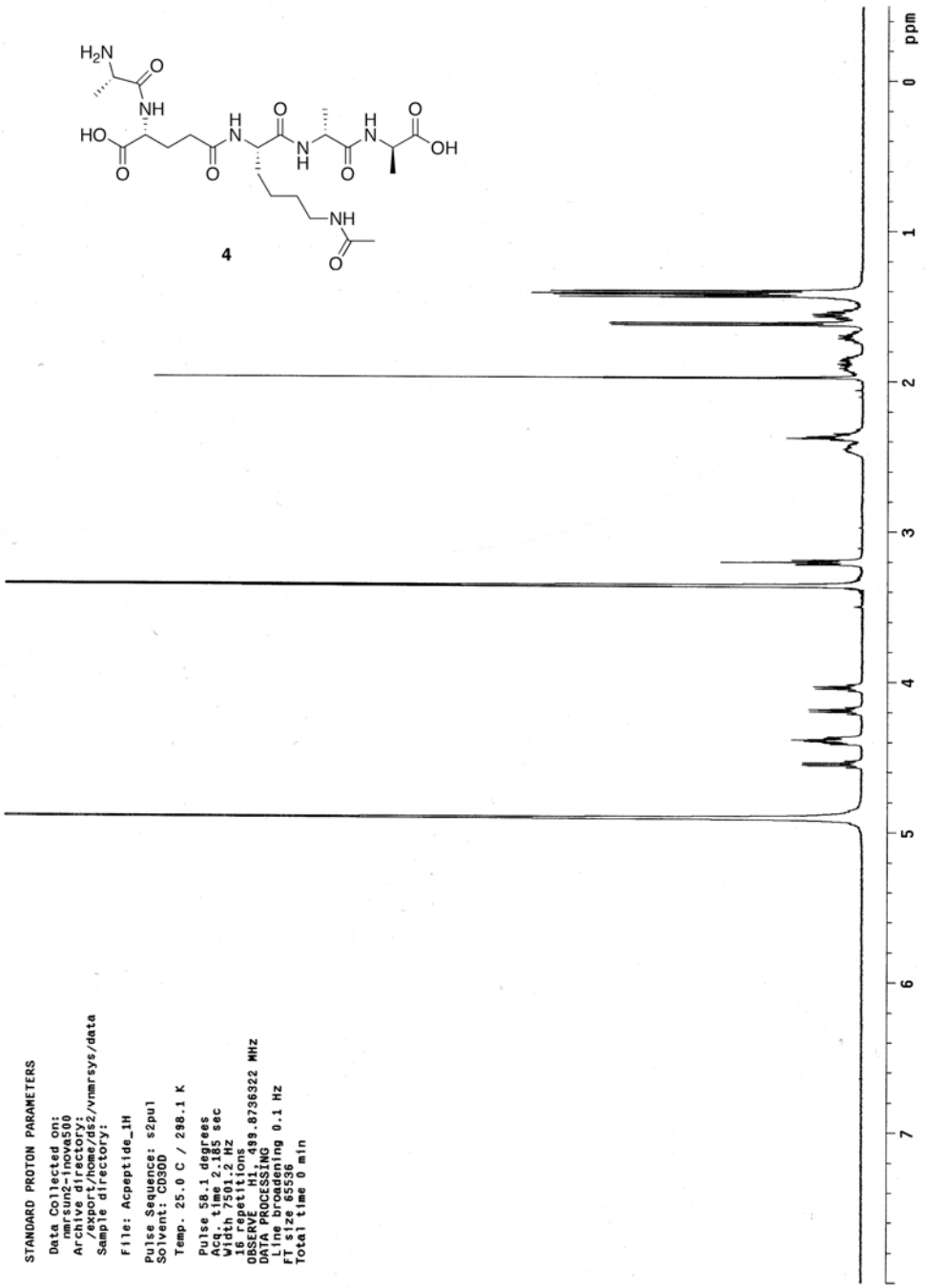


Figure S6. ^{1}H NMR spectra of **4**.

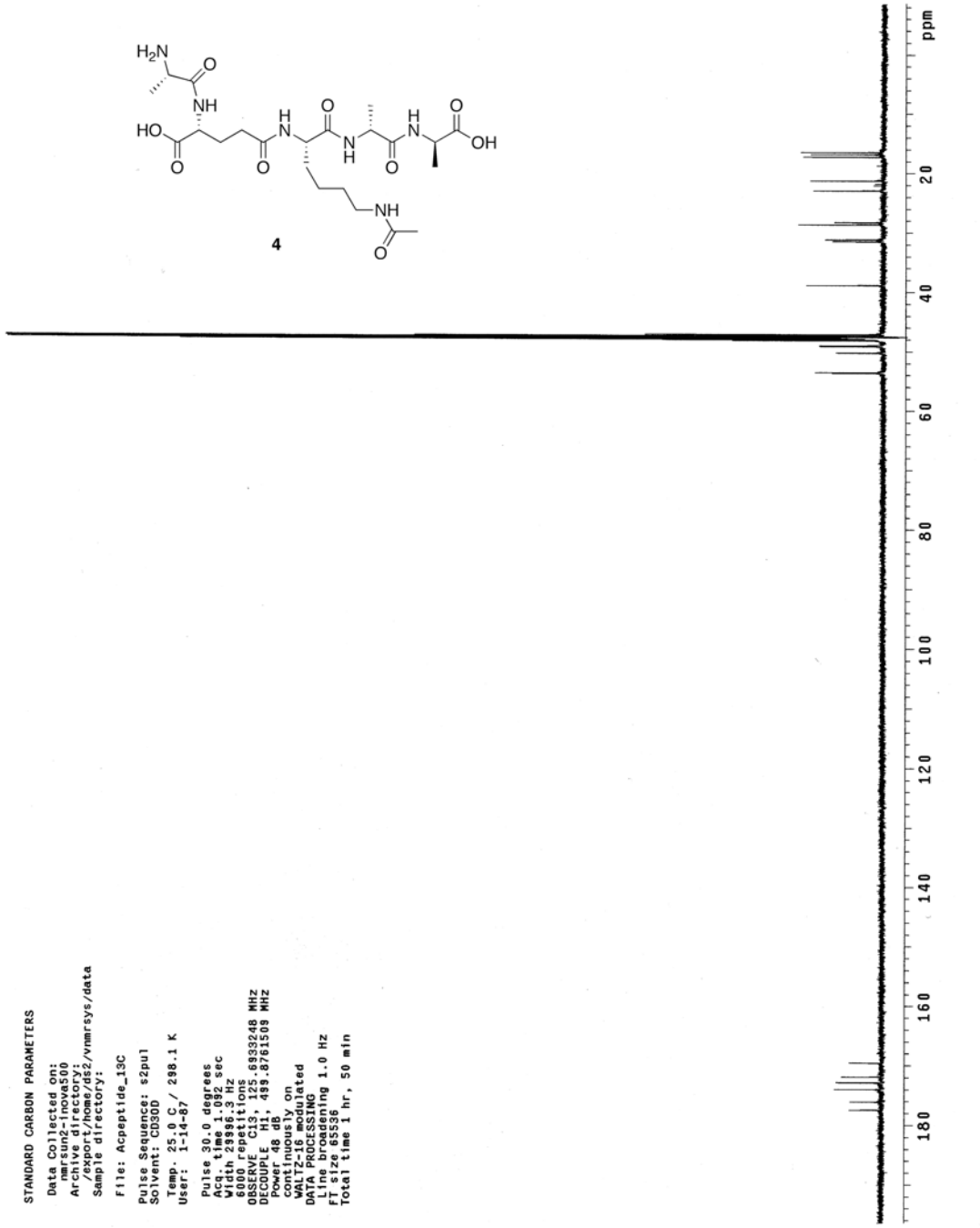
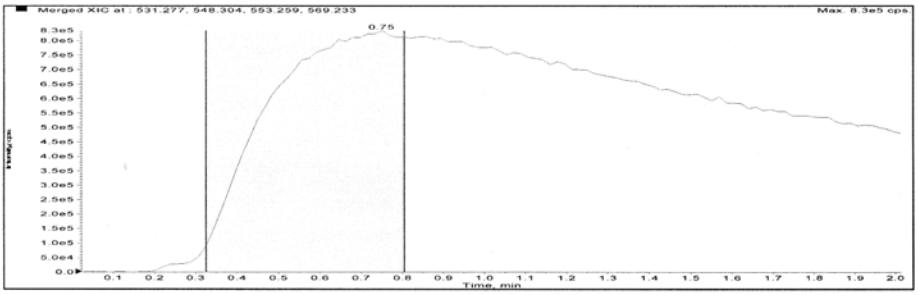
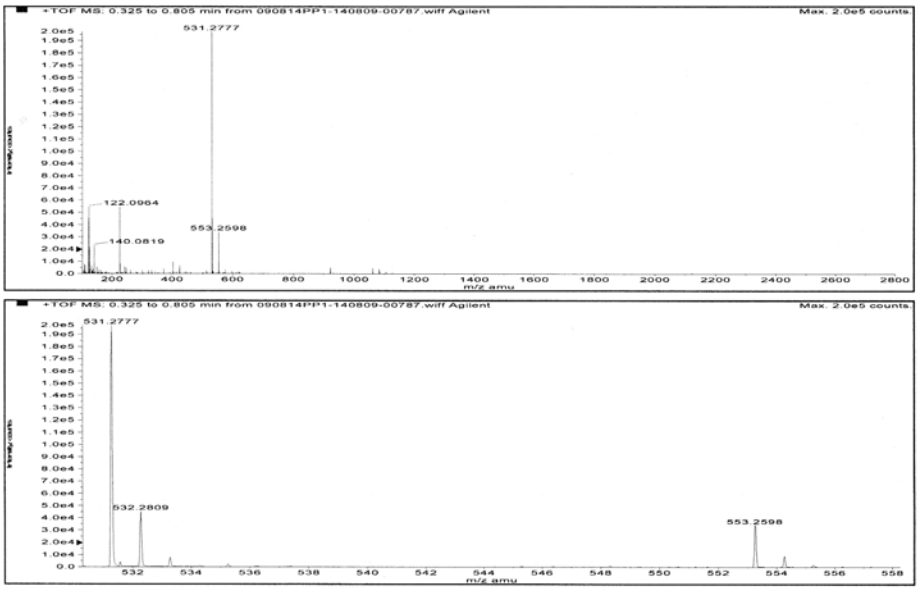


Figure S7. ^{13}C NMR spectra of **4**.



Merged XIC, Period# : 1 Experiment# : 1



Formula	Compound name	Mass	Peak RT (min)	Peak area	Description
C22H38N6O9	--	530.27003	0.75	5.88304 E6	--

Species	Abundance (counts)	Ion Mass	Measured Mass	Error (mDa)	Error (ppm)	Ret. Time Error (min)
[M+H]+	202143.05	531.27730	531.27773	0.42350	0.80	--
[M+Na]+	37067.19	553.25925	553.25983	0.58315	1.05	--

Figure S8. HRMS spectra of **4**.

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