Reconstitution of peptidoglycan cross-linking leads to improved fluorescent probes of cell wall synthesis

Matthew D. Lebar,[x,‡] Janine M. May,[x,‡] Alexander J. Meeske,[y] Sara A. Leiman,[z] Tania J. Lupoli,[x,y] Hirokazu Tsukamoto,[x] Richard Losick,[z] David Z. Rudner,[y] Suzanne Walker,[y]* and Daniel Kahne [x]*

[‡] contributed equally

[x] Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138, United States

[y] Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts 02115, United States

[z] Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138, United States

Supporting Information

Materials

Heptaprenyl MurNAc pyrophosphate[1-2] and Gram-negative Lipid II (**1**)[3] were prepared as previously described. Fluorescein isothiocyanate (FITC)-derivatized D-Lysine (FDL, **3**) was prepared as previously described.[4] Full-length *E. coli* PBP1A was purified as reported previously, with modifications noted below.[3, 5-6] The *B. subtilis dacA* deletion mutant was generated as part of the ARRA-funded knock out collection and its construction will be described elsewhere. Non-stick conical vials and pipet tips used for enzymatic reactions were from VWR. Primers were purchased from Integrated DNA Technologies. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. All PCR amplification was performed with KOD Hot Start DNA polymerase from Novagen, and all plasmid preparation was conducted with Qiagen's spin miniprep kit. All other chemicals were purchased from Sigma Aldrich unless otherwise noted.

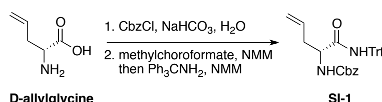
General Methods

NMR spectra were recorded on a Varian Inova 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) instrument in CD_3OD or $CDCl_3$. Chemical shifts are reported in units of parts per million (ppm). All reactions were conducted under nitrogen or argon atmosphere using anhydrous solvents passed through an activated alumina column, unless otherwise noted. Commercially available reagents were used without further purification. Low-resolution mass spectra (LRMS) were obtained on an Agilent Technologies 1100 series LC-MSD instrument using electrospray ionization (ESI). High-resolution mass spectra (HRMS) and LC/HRMS chromatograms were obtained on an Agilent Technologies 6520 LC/Q-TOF mass spectrometer. Reactions were monitored by thin layer chromatography on glass plates precoated with silica gel (250 µm, Merck) with detection by UV and cerium ammonium molybdate as the developing agent. Reactions were also monitored by LC/MS (Agilent Series 1100 instrument, using a Phenomenex Luna C18 3 mm column and ESI ionization.). Normal phase flash chromatography was carried out with silica gel from Sorbent Technologies (60 Å, 40-63 µm). Reverse phase flash chromatography was conducted on Phenomenex Strata C-18 SPE columns and Fluka C-18 reversed-phase silica. High-performance lipid chromatography (HPLC) purification was carried out on an Agilent Technologies 1260 Quanternary LC system using Agilent Zorbax 300SB C18 and Phenomenex Luna C18 columns. Scintillation counting of radioactive reactions was performed with a Beckman LS 6000 Series.

Fluorescence microscopy

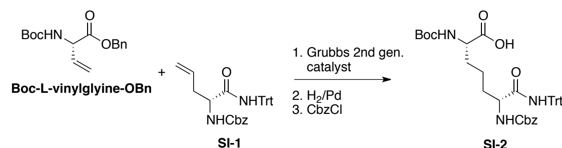
Fluorescence microscopy experiments were conducted with a Nikon Eclipse T*i* microscope equipped with a Plan Apochromat 100x/1.4NA oil phase contrast objective, a Chroma 49002 ET-EGFP filter cube, and a CoolSnapHQ^2 digital camera (Photometrics). The light source was a Lumencor SpectraX-6 LCR SA light engine. Images were captured using Nikon Elements software and processed using Fiji. All fluorescence images were scaled equally for comparison. For conditions that produced a weaker fluorescent signal, an autoscaled image was generated to highlight the staining pattern.

B. subtilis strains (168: **Figures 4**, **S2**, **S5**; 168 Δ*dacA*: **Figure S6**; 3610: Figure **S7**) were grown in the presence and absence of probe **3** or **4** at designated concentrations (10 - 500 μM) in CH medium. Exponentially growing cells were centrifuged at 8,000 RPM for 1 min, washed 3 times in CH medium, spotted onto agarose pads, and imaged immediately. *E. coli* MC4100 cells were grown to exponential phase in the presence and absence of probe **3** or **4** (500 μM) in M9 glucose media and washed with PBS. Prior to imaging, cells were immobilized on pads composed of 2% agarose (**Figure S4**).



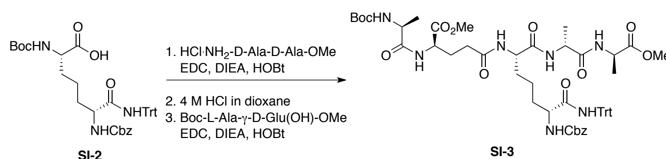
(*R*)-benzyl (1-oxo-1-(tritylamino)pent-4-en-2-yl)carbamate (Cbz-D-allylglycine-NHTrt, SI-1)

To a stirring mixture of (*R*)-2-aminopent-4-enoic acid (D-allylglycine, Parkway Scientific, 1.15 g, 10 mmol) and sodium bicarbonate ($NaHCO_3$, 2.35 g, 28 mmol) at rt in water (30 mL) was added dropwise benzyl chloroformate (CbzCl, 2.5 mL, 2.9 g, 17 mmol).[7] After 3.5 h, an additional 10 mL water was added and the mixture was partitioned with Et_2O (40 mL). The aqueous layer was again extracted with 40 mL Et_2O. The organic layers were discarded and the aqueous layer was acidified with 10% citric acid (30 mL). The aqueous layer was extracted 3X with 60 mL aliquots of Et_2O. The combined organic phases was dried over anhy. Na_2SO_4, and concentrated, then placed under high vacuum to afford (*R*)-2-(((benzyloxy)carbonyl)amino)pent-4-enoic acid (Cbz-D-allylglycine, 2.3 g, 9.1 mmol, 91%) as a viscous oil, which was used without further purification. To a stirring solution of Cbz-D-allylglycine (600 mg, 2.4 mmol) in dry THF (44 mL) at 0 °C was added N-methylmorpholine (NMM, 291 uL, 268 mg, 2.64 mmol).[8] This solution stirred for 5 min. then methylchloroformate (202 uL, 250 mg, 2.64 mmol) was added dropwise over 30 sec. This solution stirred for 2 min. then triphenylmethylamine (680 mg, 2.64 mmol) and NMM (291 uL, 268 mg, 2.64 mmol) dissolved in 5 mL dry THF was added. The reaction mixture stirred for 2 h at 0 °C then 16 h overnight at rt. The reaction mixture was then concentrated and partition in Et_2O (50 mL) and 0.1 M HCl (50 mL). The organic layer was washed with water (50 mL), 5% $NaHCO_3$ (50 mL), and brine (50 mL), and dried over anhy. Na_2SO_4. The dried organic layer was concentrated on Celite and purified via silica gel column chromatography (15% EtOAc/hexanes) to afford Cbz-D-allylglycine-NHTrt (**SI-1**, 220 mg, 0.45 mmol, 19%) as a white solid; δ_H (500 MHz, $CDCl_3$) 7.33-7.14 (20H, m), 5.68 (1H, m), 5.13, (4H, m), 4.28 (1H, m), 2.49 (2H, m) (**Figure S14**); δ_C (125 MHz, $CDCl_3$) 169.7, 156.4, 144.4 (3C), 136.2, 133.0, 128.7 (6C), 128.5, 128.4 (2C), 128.2 (2C), 128.0 (6C), 127.2 (3C), 119.5, 70.6, 67.2, 54.9, 36.4 (**Figure S15**); LRMS (*m/z*): 513.2 $[M+Na]^+$; HRMS (*m/z*): calcd for $C_{32}H_{30}N_2O_3Na$ 513.2149 $[M+Na]^+$, found 513.2153.



(2*S*,6*R*)-6-(((benzyloxy)carbonyl)amino)-2-((*tert*-butoxycarbonyl)amino)-7-oxo-7-(tritylamino)heptanoic acid (Boc-L-OH-DAP-Cbz-D-NHTrt, SI-2)

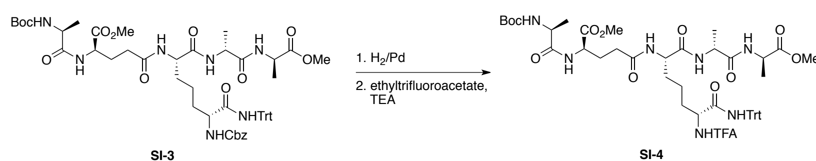
A solution of (*S*)-benzyl 2-((*tert*-butoxycarbonyl)amino)but-3-enoate[3] (Boc-L-vinylglycine-OBn, 175 mg, 0.60 mmol), Cbz-D-allylglycine-NHTrt (**SI-1**, 150 mg, 0.30 mmol], and Grubbs 2nd gen. catalyst (38 mg, 0.045 mmol) in dry DCM was refluxed under stirring and Ar for 12 h. The mixture was concentrated on Celite and purified by silica gel column chromatography (20%-30% EtOAc/hexanes) to afford the alkene intermediate (150 mg, 0.199 mmol, 66% from **SI-1**) as a white solid. A mixture of the alkene (150 mg, 0.20 mmol) and 20% $Pd(OH)_2$/C (38 mg) in MeOH (15 mL) was stirred under H_2 (1 atm) for 1 h. The mixture was filtered through Celite and concentrated to afford (2*S*,6*R*)-6-amino-2-((*tert*-butoxycarbonyl)amino)-7-oxo-7-(tritylamino) heptanoic acid (Boc-L-OH-DAP-NH_2-D-NHTrt, 100 mg, 0.19 mmol, 95%) as a white solid and used without further purification. A mixture of Boc-L-OH-DAP-NH_2-D-NHTrt (140 mg, 0.263 mmol), N-(Benzyloxy-carbonyloxy) succinimide (Cbz-OSu, 65 mg, 0.263 mmol), $NaHCO_3$ (22 mg, 0.263), water (3 mL), and acetone (3 mL) stirred for 12h at rt. The acetone was removed in vacuo and the mixtue was partitioned between EtOAc (25 mL) and 100 mM NaOAc, pH=4.0 (20 mL). The organic layer was washed 2X with 20 mL aliquots of 100 mM NaOAc, pH=4.0, dried over anhy. Na_2SO_4, and concentrated to afford Boc-L-OH-DAP-Cbz-D-NHTrt (**SI-2**, 177 mg, 0.260 mmol, ~95%) as a white solid, which was used without further purification; δ_H (500 MHz, CD_3OD) 7.40-7.15 (20H, m), 5.20 (1H, d, *J*=12.4 Hz), 5.02 (1H, d, *J*=12.4 Hz), 4.23 (1H, m), 4.05 (1H, m), 1.72 (2H, m), 1.54-1.40 (2H, m), 1.42 (9H, s), 1.31 (1H, m) (**Figure S16**); δ_C (125 MHz, CD_3OD) 176.0, 173.8, 171.2, 158.0, 145.6 (3C), 138.1, 130.1, 129.9 (6C), 129.5 (2C), 129.0, 128.9 (2C), 128.7 (6C), 127.8 (3C), 80.5, 73.7, 71.5, 54.7, 32.4, 28.7 (3C), 26.3, 23.3 (**Figure S17**); LRMS (*m/z*): 664.2 $[M-H]^-$; HRMS (*m/z*): calcd for $C_{39}H_{42}N_3O_7$ 664.3028 $[M-H]^-$, found 664.3057.



Boc-L-Ala-γ-D-Glu(OMe)-*meso*-2(L),2'(D)-DAP(Cbz)(NHTrt)-D-Ala-D-Ala-OMe (SI-3)

To a stirring mixture of **SI-2** (120 mg, 0.18 mmol), HCl·D-Ala-D-Ala-OMe[1] (40 mg, 0.18 mmol), HOBt·H_2O (34 mg, 0.22 mmol), and EDC (42 mg, 0.22 mmol) in dry DCM (2 mL) was added DIEA (38 μL, 28 mg, 0.22 mmol) at room temperature under Ar. After 45 min., the mixture was concentrated and partitioned between EtOAc (20 mL) and 10% citric acid (10 mL). The organic phase was subsequently washed with H_2O (10 mL), 5% $NaHCO_3$ (10 mL), brine (10 mL), dried over anhy. Na_2SO_4, and concentrated to afford Boc-*meso*-2(L),2'(D)-DAP(Cbz)(NHTrt)-D-Ala-D-Ala-OMe (130 mg, 0.158 mmol,

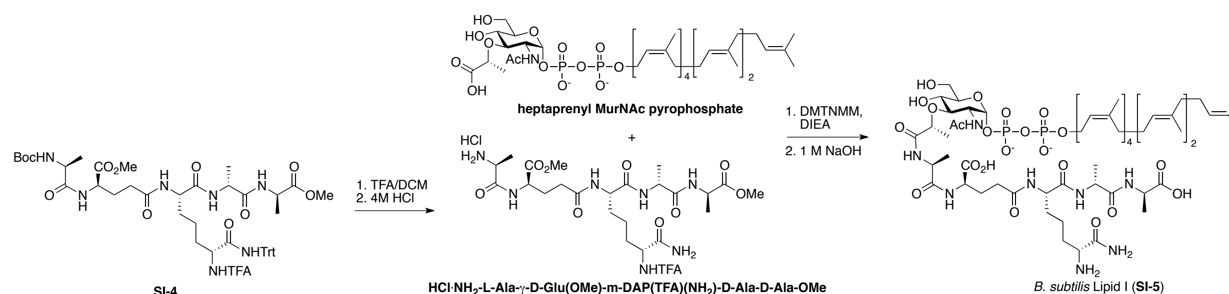
88%) as a white solid. Boc-*meso*-2(L),2'(D)-DAP(Cbz)(NHTrt)-D-Ala-D-Ala-OMe (113 mg, 0.140 mmol) was stirred in 4 M HCl (3 mL) under Ar for 20 min. The solution was concentrated to dryness then concentrated twice more from toluene to remove trace HCl yielding HCl·NH_2-*meso*-2(L),2'(D)-DAP(Cbz)(NHTrt)-D-Ala-D-Ala-OMe which was used without further purification. To HCl·NH_2-*meso*-2(L),2'(D)-DAP(Cbz)(NHTrt)-D-Ala-D-Ala-OMe (~0.14 mmol) was added Boc-L-Ala-γ-D-Glu(OMe)-OH[1] (51 mg, 0.154 mmol) dissolved in dry DCM (5 mL) under Ar at room temperature. To the stirring mixture were added HOBt·H_2O (26 mg, 0.168 mmol), EDC (32 mg, 0.168 mmol), and DIEA (30 µL, 22 mg, 0.168 mmol). After 2 h, the mixture was concentrated and partitioned with EtOAc (20 mL), and 10% citric acid (10 mL). The organic phase was subsequently washed with H_2O (10 mL), 5% $NaHCO_3$ (10 mL), brine (10 mL), dried over anhy. Na_2SO_4, and concentrated. The residue was purified via silica gel column chromatography (step gradient: $CHCl_3$, 1% MeOH in $CHCl_3$, 5% MeOH in $CHCl_3$) to afford Boc-L-Ala-γ-D-Glu(OMe)-*meso*-2(L),2'(D)-DAP(Cbz)(NHTrt)-D-Ala-D-Ala-OMe (78 mg, 0.075 mmol, 54%) as a white solid. δ_H (500 MHz, $CDCl_3$) 7.40-6.95 (20H + 7NH, m) 5.50 (1H, m), 5.17 (1H, d, *J*=12.2 Hz), 5.11 (1H, m), 5.04 (1H, d, *J*=12.2 Hz), 4.48 (2H, m), 4.22 (1H, m), 4.12 (1H, m), 3.71 (3H, s), 3.67 (3H, s), 2.34 (1H, m), 2.18 (2H, m), 1.90 (1H, m), 1.81 (2H, m), 1.66 (2H, m), 1.42 (9H, s), 1.38 (3H, d, *J*=7.26 Hz), 1.36 (3H, d, *J*=7.16 Hz), 1.33 (2H, m), 1.30 (3H, d, *J*=7.12 Hz) (**Figure S18**); δ_C (125 MHz, CD_3OD) 176.1, 175.0, 174.7, 174.5, 174.4, 173.8, 173.3, 158.6, 157.8, 145.7 (3C), 138.2, 129.9 (6C), 129.6 (2C), 129.1, 129.0 (2C), 128.8 (6C), 127.9 (3C), 80.8, 71.5, 67.7, 57.0, 55.5, 52.8, 52.7, 52.5, 52.0, 50.3, 49.5, 32.3, 32.1, 32.0, 28.8 (3C), 28.1, 23.3, 18.2, 17.8, 17.4 (**Figure S19**); LRMS (*m/z*): 1058.4 $[M+Na]^+$; HRMS (*m/z*): calcd for $C_{55}H_{69}N_7O_{13}Na$ 1058.4846 $[M+Na]^+$, found 1058.4891.



Boc-L-Ala-γ-D-Glu(OMe)-*meso*-2(L),2'(D)-DAP(TFA)(NHTrt)-D-Ala-D-Ala-OMe (SI-4)

A mixture of **SI-3** (10 mg, 0.010 mmol) and 20% $Pd(OH)_2/C$ (4 mg) in MeOH (0.5 mL) was stirred under H_2 (1 atm) for 35 min. The mixture was filtered through Celite and concentrated to afford the free base Boc-L-Ala-γ-D-Glu(OMe)-*meso*-2(L),2'(D)-DAP(NH_2)(NHTrt)-D-Ala-D-Ala-OMe (7.5 mg, 0.008 mmol, 75%) as a white solid. To a stirring soloution of Boc-L-Ala-γ-D-Glu(OMe)-*meso*-2(L),2'(D)-DAP(NH_2)(NHTrt)-D-Ala-D-Ala-OMe (7.5 mg, 0.008 mmol) in dry MeOH (0.5 mL) under Ar was added ethyl trifluoroacetate (10 µL of 12% stock solution in dry MeOH, 1.4 mg, 0.01 mmol) and triethylamine (TEA, 10 µL of 11% stock solution in dry MeOH, 0.8 mg, 0.008 mmol).[9] The solution stirred for 22 h. Some starting material was present via LC/MS so aliquots of ethyl trifluoroacetate (20 uL stock solution, 0.02 mmol) and TEA (20 µL of 11% stock solution, 0.016 mmol) were added. After stirring an additional 48 h, the mixture was concentrated and purified on reverse phase HPLC using a C18 column (linear gradient: 30% acetonitrile (0.1% formic acid)/H_2O (0.1% formic acid) to 99% acetonitrile

(0.1% formic acid)/H_2O (0.1% formic acid) affording starting material (2.5 mg, 0.0028 mmol, 33%) and product Boc-L-Ala-γ-D-Glu(OMe)-*meso*-2(L),2'(D)-DAP(TFA)(NHTrt)-D-Ala-D-Ala-OMe (**SI-4**, 5 mg, 0.005 mmol, 63%) as a white solid. δ_H (500 MHz, CD_3OD) 7.30-7.15 (15H m), 4.60 (2H, m), 4.37 (2H, m), 4.16 (1H, m), 4.04 (1H, m), 3.70 (3H, s), 3.67 (3H, s), 2.27 (2H, m), 1.94-1.75 (4H, m) 1.65 (2H, m), 1.44 (9H, s), 1.39 (3H, d, *J*=7.3 Hz), 1.37-1.26 (2H, m), 1.34 (3H, d, *J*=7.4 Hz), 1.31 (3H, d, *J*=7.2 Hz) (**Figure S20**); LRMS (*m/z*): 1020.3 $[M+Na]^+$; HRMS (*m/z*): calcd for $C_{49}H_{62}F_3N_7O_{12}Na$ 1020.4301 $[M+Na]^+$, found 1020.4229.



B. subtilis Lipid I (SI-5)

SI-4 (5 mg, 5 μmol) was stirred in trifluoroacetic acid (TFA, 500 μL) and DCM (150 μL) under Ar for 2.5 h. The solution gradually turned yellow. Triisopropylsilane (*i*-Pr_3SiH, 10 uL) was added and the solution became colorless. The solution was concentrated then triturated with 2X Et_2O (500 μL). The remaining solid was dried in vacuo then dissolved in 4 M HCl in dioxane (0.5 mL). The solution was concentrated to dryness, concentrated twice more from toluene to remove trace HCl, then dried under high vacuum affording HCl·NH_2-L-Ala-γ-D-Glu(OMe)-*meso*-2(L),2'(D)-DAP(TFA)(NH_2)-D-Ala-D-Ala-OMe as a colorless film, which was used without further purification. Heptaprenyl MurNAc pyrophosphate[1-2] (2 mg, 2 μmol) was evaporated twice with 0.5 M DIEA in MeOH (0.4 mL, 0.2 mmol) for ammonium salt exchange and dried under high vacuum for 1 h. To this residue was added HCl·NH_2-L-Ala-γ-D-Glu(OMe)-*meso*-2(L),2'(D)-DAP(Tfa)(NH_2)-D-Ala-D-Ala-OMe (3 mg, ~4 μmol) dissolved in MeOH (0.5 mL). The mixture was treated with DIEA (0.5 M in MeOH, 6.5 μL, 3.2 μmol) followed by dimethoxytriazine-*N*-methylmorpholinium chloride (DMTMM, 1.5 mg, 5.4 μmol). After being stirred at room temperature for 2 h ESI-MS confirmed product had formed. The reaction was concentrated *in vacuo*, and purified on reverse phase C18 silica using a step gradient: 0%, 20%, 40%, 60%, 80%, and 100% acetonitrile (ACN)/ 25 mM NH_4CO_3H. The 60% fraction was concentrated *in vacuo* to afford protected lipid I product. The protected compound (1.5 mg) was dissolved in 500 μL 1:1 dioxane/H_2O and 40 μL 1 M NaOH in H_2O was added under stirring. Global protecting group hydrolysis was observed after 7 h by ESI-MS. The NaOH was neutralized with addition of NH_4Cl (~2 mg), and then concentrated *in vacuo*. The residue was purified on reverse phase C18 silica using a step gradient: 0%, 10%, 20%, 30%, 40%, 50%, 60%, 80%, and 100% ACN/ 25 mM NH_4CO_3H. The 40% and 50% fractions were pooled then concentrated *in vacuo* to give *B. subtilis* Lipid I (**SI-5**, 0.7 mg, 0.5 μmol, 25%

from heptaprenyl MurNAc pyrophosphate) as a white solid; LRMS (*m/z*): 1441.7 [M-H]$^-$, 720.4 [M-2H]$^{2-}$; HRMS (*m/z*): calcd for $C_{67}H_{111}N_8O_{23}P_2$ 1441.7294 [M-H]$^-$, found 1441.7351, calcd for $C_{67}H_{110}N_8O_{23}P_2$ 720.3610 [M-2H]$^{2-}$, found 720.3650 (**Figure S21**).

UDP-GlcNAc, MurG, buffer

B. subtilis Lipid I (**SI-5**) → *B. subtilis* Lipid II (**2**)

B. subtilis Lipid II (2)

B. subtilis Lipid I (**SI-5**) was converted to **2** using a previously described enzymatic method.[10] A radiolabeled reaction was conducted in parallel to measure reaction yield. Non-radiolabeled UDP-*N*-acetyl glucosamine (UDP-GlcNAc, 50 nmol) was placed in a conical tube. A mixture of radiolabeled UDP-*N*-acetyl glucosamine (American Radiolabeled Chemicals 0152, 12.5 nmol) and non radiolabeled UDP-GlcNAc (37.5 nmol) was placed in a separate conical tube (1:3 labeled:unlabeled). To each tube was added **8** (75 µg, 50 nmol) dissolved in MeOH (24 µL), 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, pH = 7.4), 10 mM $MgCl_2$ in H_2O and incubated with 1 U/µL alkaline phosphatase and 0.5 mg/mL *E. coli* MurG in a final reaction volume of 160 µL for 1 h at room temperature. An additional aliquot of MurG (8 µL of 5 mg/mL stock) was added. After 1 h, the reactions were quenched with 160 µL ice cold H_2O, and purified on a reverse phase C18 SPE column. After washing with 0.1% ammonium hydroxide in water, the desired compound **2** was eluted with 0.1% ammonium hydroxide in methanol. Fractions from non-radiolabeled experiments were analyzed by ESI-MS. Vacuum centrifugation afforded non-radiolabeled **2**; LRMS (*m/z*): 822.1 [M-2H]$^{2-}$; HRMS (*m/z*): calcd for $C_{75}H_{124}N_9O_{27}P_2$ 1644.8087 [M-H]$^-$, found 1644.8089 (**Figure S22**); calcd for $C_{75}H_{123}N_9O_{27}P_2$ 821.9007 [M-2H]$^{2-}$, found 821.9029. No Lipid I starting material was observed via ESI-MS. Fractions containing radiolabeled **2**, were combined and concentrated by vacuum centrifugation to afford **2** (7.55 nmol, SA = 300 nCi/nmol, 60% yield, based on liquid scintillation counting).

1. CDI, DMF then 28% NH_3 (aq.)
2. $H_2/Pd(OH)_2$
3. FITC, DMF
4. TFA/DCM

Boc-D-Lys(Cbz)-OH → FDL-NH_2 (**4**)

FITC-derivatized D-Lysine carboxamide (FDL-NH_2, 4)

To a stirring solution of Boc-D-Lys(Cbz)-OH (Bachem A-1470, 381 mg, 1.0 mmol) in dry DMF (5 mL) was added carbonyldiimidazole (CDI, 203 mg, 1.25 mmol) in one portion. The mixture stirred for 1.5 h

at rt then aqueous ammonia (14.8 M, 0.1 mL, 1.48 mmol) was added dropwise. After stirring an additional 6 h, no starting material remained (via ESI-MS). The mixture was concentrated. The resulting residue was partitioned between DCM (30 mL) and sat. $NaHCO_3$ (10 mL). The organic layer was washed with sat. $NaHCO_3$ (2X 10 mL), water (10 mL), 5% citric acid (2X 10 mL), then brine (10 mL). The organic layer was then dried over anhy. Na_2SO_4, and concentrated to afford Boc-D-Lys(Cbz)-NH_2 (380 mg, >95%, crude), which was used without further purification. A mixture of Boc-D-Lys(Cbz)-NH_2 (380 mg, 1.0 mmol) and 20% $Pd(OH)_2/C$ (75 mg) in MeOH (1 mL) was stirred under H_2 (1 atm) for 1.5 h. The mixture was filtered through Celite and concentrated to afford Boc-D-Lys-NH_2 (190 mg, 0.77 mmol, 77%), which was used without further purification. To a stirring solution of Boc-D-Lys-NH_2 (100 mg, 0.4 mmol) in dry DMF (4 mL) under N_2 was added fluorescein isothiocyanate (FITC, 100 mg, 0.26 mmol) in one portion.[4] The solution stirred for 4.5 h and was then concentrated. The residue partitioned between EtOAc (30 mL) and 1M HCl (10 mL). The organic layer was washed with 1 M HCl (10 mL) then brine (10 mL). The organic layer was dried over anhy. Na_2SO_4 then concentrated to afford Boc-FDL-NH_2 (112 mg, 0.18 mmol, 69%) as an orange solid. Boc-FDL-NH_2 (35 mg, 0.055 mmol) was stirred under N_2 in TFA (0.5 mL) and DCM (0.5 mL) for 45 min. The solution was then concentrated to afford crude (**4**, ~28 mg), which was purified on reverse phase HPLC using a C18 column (linear gradient: 40-80% MeOH in water) to afford FDL-NH_2 as an orange solid (**4**, 21.9 mg, 0.04 mmol, 73%). (δ_H (500 MHz, CD_3OD) 7.95 (1H, d, *J*=2.1 Hz), 7.56 (1H, br d *J*=7.2 Hz), 7.22 (1H, d, *J*=8.2 Hz), 7.16 (2H, d, *J*=9.0 Hz), 6.64-6.61 (4H, m), 3.67 (3H, m), 1.86 (1H, m), 1.81-1.70 (3H, m), 1.63-1.50 (2H, m); LRMS (*m/z*): 535.2 $[M+H]^+$; HRMS (*m/z*): calcd for $C_{27}H_{27}N_4O_6$ 535.1646 $[M+H]^+$, found 535.1642.

Cloning of wild-type *B. subtilis* PBP1

The full-length *B. subtilis ponA (M1-N914)* gene encoding penicillin-binding protein 1A/1B was amplified from *B. subtilis* 168 purified genomic DNA using forward primer 5'- GA AGA GCA GAGCTC ATG TCA GAT CAA TTT AAC AGC CGT GAA G -3' and reverse primer 5'- GT AAT TAA CTCGAG TTA ATT TGT TTT TTC AAT GGA TGA TGA GTT TGT TTG T -3'. The parent vector, pCB22 (*kan^R $lacI^q$ PT_7::His_6-SUMO-E.coli ponB*), was obtained from the Bernhardt lab (Harvard Medical School).[5] To remove the *E. coli* gene, the vector was digested with *SacI* and *XhoI* and gel purified (QIAquick gel extraction kit from Qiagen). The *B. subtilis ponA* PCR fragment was digested with *SacI* and *XhoI,* ligated into the digested pCB22, and transformed into *E. coli* NovaBlue competent cells (Novagen) to produce pJM1.

As *B. subtilis* PBP1 did not express well with the N-terminal His_6-SUMO tag, site-directed mutagenesis (Dpn I from New England Biolabs) was employed to install a C-terminal His_6 tag with forward primer 5'- CAT TGA AAA AAC AAA TTA TCT CGA GCA CCA CCA C -3' and reverse primer 5'- GTG GTG GTG CTC GAG ATA ATT TGT TTT TTC AAT G -3'. The N-terminal His_6-SUMO tag was removed with forward primer 5'- AGA AGG AGA TAT ACC ATG TCA GAT CAA TTT -3' and reverse

primer 5'- AAA TTG ATC TGA CAT GGT ATA TCT CCT TCT -3'. The final construct (pJM25) of *B. subtilils ponA*-His$_6$ was transformed into NovaBlue cells and confirmed by DNA sequencing (Beckman Coulter and Genewiz).

Cloning of transpeptidase-inactive *B. subtilis* PBP1

The catalytic serine residue (Ser390) of the transpeptidase domain of *B. subtilis* PBP1 has been identified by sequence alignment with other PBPs.[11] A Ser390Ala mutation was installed into pJM25 by site-directed mutagenesis with forward primer 5'- CAC AGC CTG GTG CGA CCA TAA AAC -3' and reverse primer 5'- GTT TTA TGG TCG CAC CAG GCT GTG -3'. The final construct (pJM34) was transformed into NovaBlue cells and confirmed by DNA sequencing.

Purification of wild-type and TP-inactive *B. subtilis* PBP1

Plasmids pJM25 (wild-type PBP1) and pJM34 (TP-inactive PBP1) were transformed into *E. coli* BL21(DE3) competent cells (Novagen) for overexpression. Cells were grown in 1.5-L LB Miller medium with 50 μg/mL kanamycin at 37°C with shaking to OD_{600} of 0.35. Cultures were cooled to 16°C and grown to OD_{600} 0.8 before induction with 0.1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) for 20 h with shaking. Cells were harvested by centrifugation (5,250 xg, 20 min), resuspended in 40 mL lysis buffer (20 mM Tris-HCl [tris(hydroxymethyl)aminomethane], pH 8.0 with 10 mM $MgCl_2$, 10 μg/mL DNAse I, 1 mM PMSF [phenylmethanesulfonyl fluoride]), and lysed by three passages through the cell disruptor (15,000 psi, 4°C). The lysates were pelleted by ultracentrifugation (100,000 xg, 30 min, 4°C), and PBP1-His$_6$ was found in the pellets.

Each pellet was extracted in 20 mL extraction buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 7.6 with 2% reduced Triton X-100) and stirred gently for 20 h at 4°C. The extract was ultracentrifuged (100,000 xg, 30 min, 4°C), and the supernatant was supplemented with 20 mM imidazole (from a 4 M, pH 8.0 stock). To prepare the nickel column, 3 mL of Ni-NTA Superflow resin (Qiagen) slurry were washed and equilibrated with buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 7.6) supplemented with 20 mM imidazole. The supernatants were incubated with the resin for 1 h at 4°C, rocking. The resins were collected and washed with 9 mL of column buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 7.6 with 0.1% reduced Triton X-100) plus 20 mM imidazole and then 9 mL of column buffer plus 30 mM imidazole. The proteins were eluted from the resin with 3 mL column buffer with 200 mM imidazole.

The elution fractions were concentrated to 0.5 mL in Amicon Ultra 30 kDa MWCO centrifugal filter units and further purified by FPLC on a Superdex 200 column (buffer: 20 mM Tris-HCl, 0.5 M NaCl, pH 7.5, 0.1% reduced Triton X-100, 10% glycerol; flow-rate: 0.5 mL/min). Fractions were analyzed by SDS-PAGE, and those containing PBP1 were pooled and concentrated to 0.5 mL in 30 kDa MWCO centrifugal filter units. Protein concentrations were determined with the Thermo NanoDrop A280 function, and both the wild-type and the TP-inactive preparations were found to be approximately 36 μM. Proteins were stored at -80°C.

Cloning of transpeptidase-inactive *E. coli* PBP1A

Overexpression plasmid pCB21 (*kan*^{R} *lacI*^{q} *PT*$_7$*::His*$_6$*-SUMO-E. coli ponA*) was obtained from the Bernhardt lab (Harvard Medical School).[5] The catalytic serine residue (Ser465) of the transpeptidase domain of *E. coli* PBP1A has been identified.[11] A Ser465Ala mutation was installed into pCB21 by site-directed mutagenesis with forward primer 5'- AGG CAC TGC GTC AGG TGG GTG CCA ACA TCA AAC CGT TCC TCT A -3' and reverse primer 5'- TAG AGG AAC GGT TTG ATG TTG GCA CCC ACC TGA CGC AGT GCC T -3'. The final construct (pJM77) was transformed into NovaBlue cells and confirmed by DNA sequencing.

Purification of wild-type and TP-inactive *E. coli* PBP1A

Full-length *E. coli* PBP1A was purified as reported previously.[3, 5-6] Plasmids pCB21 (wild-type PBP1A) and pJM77 (TP-inactive PBP1a) were transformed into *E. coli* Rosetta2(DE3) competent cells (Novagen) for overexpression. Overnight starter cultures were grown in LB Miller supplemented with 25 µg/mL kanamycin, 30 µg/mL chloramphenicol, and 0.2% glucose. Expression cultures were grown in 1.5-L LB Miller medium with 25 µg/mL kanamycin and 30 µg/mL chloramphenicol at 37°C with shaking to OD_{600} of 0.35. Cultures were cooled to 25°C and grown to OD_{600} 0.6 before induction with 1 mM IPTG for 20 h with shaking. Cells were harvested by centrifugation (5,250 xg, 20 min), resuspended in 35 mL lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl with 1 mM PMSF, 1 mM EDTA, and 1% CHAPS [3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate]), and lysed by four passages through the cell disruptor (15,000 psi, 4°C). The lysates were pelleted by ultracentrifugation (100,000 xg, 30 min, 4°C), and His_6-SUMO-PBP1A was found in the supernatant.

To prepare the nickel columns, 3 mL of Ni-NTA Superflow resin (Qiagen) slurry were washed and equilibrated with column buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1% reduced Triton X-100). The supernatants were filtered (0.45 µM filter, VWR), supplemented with 2 mM imidazole, and incubated with the resins for 1.5 h at 4°C, rocking. The resins were collected and washed with 30 mL of column buffer plus 50 mM imidazole. The proteins were eluted from the resins with 10 mL column buffer with 300 mM imidazole. As described previously, His_6-SUMO protease (25 µL of a 10 mg/mL stock) was added to the elution fractions to cleave off the His_6-SUMO tag from PBP1A.[12] The samples were loaded into 10 KDa MWCO Slide-A-Lyzer dialysis cassettes and each dialyzed against 1.8 L of dialysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1% reduced Triton-X-100) overnight at 4°C. To separate the cleaved PBP1A from the His_6-SUMO tag and protease, the dialyzed samples were again incubated with 3 mL equilibrated Ni-NTA resins for 1 h at 4°C.

The flow-through fractions were collected and concentrated to 0.5 mL in Amicon Ultra 10 kDa MWCO centrifugal filter units and further purified by FPLC on a Superdex 200 column (buffer: 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1% reduced Triton X-100, 10% glycerol; flow-rate: 0.5 mL/min). Fractions were analyzed by SDS-PAGE, and those containing PBP1A were pooled and concentrated

to 0.5 mL in 10 kDa MWCO centrifugal filter units. Protein concentrations were determined with the Thermo NanoDrop A280 function, and both the wild-type and the TP-inactive preparations were found to be approximately 18 μM. Proteins were stored at -80°C.

LC/HRMS assay to evaluate *in vitro* TP and PGT activity

Lipid II (**1** or **2**, 40 μM) was incubated with *B. subtilis* PBP1 or *E. coli* PBP1A (400 nM) in buffer A (50 mM HEPES, 10mM $CaCl_2$, 20% DMSO, pH = 7.5, 10 μL total reaction volume) for 1 h at 25 °C in the presence and absence of D-Phe or D-Phe-NH_2 (1 mM final concentration). Control reactions were set up in parallel with TP-inactive *B. subtilis* PBP1 (S390A) and TP-inactive *E.* coli PBP1A (S465A) (**Figure S8**). The reactions were quenched at 95 °C for 5 min, then treated with mutanolysin (*Streptomyces globisporus*, Sigma, 1U for 1.5 h at 37 ^{o}C followed by another 1U aliquot for 1.5 h) to cleave polymeric products into disaccharide units and sodium borohydride (10 mg/mL, 30 min) to reduce the MurNAc termini. Phosphoric acid (20%, 1.2 μL) was then added to adjust the pH to ~4. The reaction mixture was then lyophilized, redissolved in 10 μL H_2O and subjected to LC/HRMS analysis.

LC/HRMS analysis was conducted with ESI-MS operating in positive mode. The instrument was equipped with a Waters Symmetry Shield RP18 column (5 μm, 3.9X150 mm) with matching column guard. The fragments were separated using the following method: 0.5 mL/min H_2O (0.1% formic acid) for 5 min followed by a gradient of 0% ACN (0.1% formic acid)/H_2O (0.1% formic acid) to 20% ACN (0.1% formic acid)/H_2O (0.1% formic acid) over 40 min. Molecular ions corresponding to expected disaccharide fragments were extracted from the chromatogram. For **Figure 2** (**i**, **iii**): ions corresponding to fragments **A, B, C**, and **D** were extracted; for **Figure 2** (**ii**, **iv**): **A'**, **B'**, **C'**, and **D'** were extracted. For **Figure 3** (**i**, **ii**): ions corresponding to fragments **A**-**D**, **E** and **F** were extracted; for **Figure 3** (**iii**, **iv**): **A'**, **B'**, **C'**, **D'**, **E'**, and **F'** were extracted. For **Figure S8** (**i**): ions corresponding to fragments **A-D** were extracted; for **Figure S8** (**ii**): **A'**, **B'**, **C'**, and **D'** were extracted. See **Table S1** for ion masses and **Figures S9** and **S10** for HRMS spectra.

Isolation and analysis of *B. subtilis* cell wall from culture

Bacillus subtilis 3610 was grown in 1-L LB Miller medium in the presence and absence of D-Phe or D-Phe-NH_2 (0.5 mM final concentration) with shaking at 37 °C to mid log phase (3.5 h). The cells were frozen at -80°C, and cell wall was isolated from the culture as described by Glauner et al.[13] and Uehera et al.[14] with modifications described below. The cells were resuspended in 10 mL Tris-buffered-saline and boiled for 30 min in 50 mL 5% SDS (sodium dodecyl sulfate). After the samples cooled, they were pelleted in the ultracentrifuge (100,000 xg, 25°C, 30-90 min) and washed six times by pelleting (100,000 xg, 25°C, 30-90 min) from 20 mL water aliquots to remove the SDS. The samples were resuspended in 600 μL digestion buffer (100 mM Tris-HCl, 20 mM $MgSO_4$, pH 7.5), treated with 2 μL DNAse I, 4 μL RNAse A, and 8 μL alpha-amylase (all stocks 10 mg/mL in 50% glycerol), and incubated at 37°C with shaking for 2 h. To cleave proteins attached to the cell wall, $CaCl_2$ (10 mM final

concentration) and trypsin (8 μL of a 10 mg/mL stock in 50% glycerol) were added, and the samples were incubated at 37°C with shaking overnight. An additional aliquot of trypsin was added, and the samples were digested for an additional night. To remove the trypsin, SDS was added to a final concentration of 1%, and the samples were incubated at 95°C for one h. The samples were again pelleted in the ultracentrifuge (100,000 xg, 25°C, 30-90 min) and washed with water to remove the SDS. The final peptidoglycan (PG) samples were resuspended in 400 μL 0.02% azide and stored at 4°C.

To analyze PG composition via LC/HRMS, 50 μL of each cell wall sample were incubated with mutanolysin (10 U, Sigma, stored at -20°C in 50 mM TES, pH 7.0, 1 mM $MgCl_2$, 10% glycerol) in 50 mM sodium phosphate buffer (pH 6.0, 100 μL total volume) at 37°C with shaking overnight. Another aliquot of mutanolysin (10 U) was added, and the mixture was incubated at 37°C with shaking for 3 h. Insoluble particles were separated by centrifugation (16,000 x g). The supernatant, containing soluble fragments, was treated with sodium borohydride (10 mg/mL in water, 100 μL) at room temperature for 30 min. Phosphoric acid was then added to adjust pH to ~4 (20%, 12.4 μL). When bubbling ceased, the samples were lyophilized and re-dissolved in 25 μL water, which was analyzed on LC/HRMS using the method described above (see **Figures S1**, **S11**, **S12**).

Isolation and analysis of *E. coli* cell wall from culture

E. coli MC4100 was grown in 1.5-L LB Miller medium in the presence and absence of D-Phe or D-Phe-NH_2 (0.5 mM final concentration) shaking at 37 °C to mid log phase (3 h). The cell wall was isolated almost exactly as described for the *B. subtilis* wall above. The cells were resuspended in 10 mL Tris-buffered-saline and boiled for 30 min in 50 mL 5% SDS. After the samples cooled, they were pelleted in the ultracentrifuge (100,000 xg, 25°C, 30-90 min) and washed five times by pelleting (100,000 xg, 25°C, 30-90 min) from 50 mL water aliquots to remove the SDS. The samples were resuspended in 1.8 mL digestion buffer (100 mM Tris-HCl, 20 mM $MgSO_4$, pH 7.5), treated with 2 μL DNAse I, 4 μL RNAse A, and 8 μL alpha-amylase (all stocks 10 mg/mL in 50% glycerol), and incubated at 37°C with shaking for 3 h. To cleave proteins attached to the cell wall, $CaCl_2$ (10 mM final concentration) and trypsin (8 μL of a 10 mg/mL stock in 50% glycerol) were added, and the samples were incubated at 37°C with shaking overnight. An additional aliquot of trypsin was added, and the samples were digested again for 8 h. To remove the trypsin, SDS was added to a final concentration of 1%, and the samples were incubated at 95°C for one h. The samples were again pelleted in the ultracentrifuge (100,000 xg, 25°C, 30-90 min) and washed with water to remove the SDS. The final peptidoglycan (PG) samples were resuspended in 300 μL 0.02% azide and stored at 4°C.

The *E. coli* cell wall samples (see **Figures S3**, **S13**) were digested with mutanolysin and analyzed by LC/HRMS exactly as described above for the *B. subtilis* samples.

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fragment	*formula*	*(M+3H)/3 ions*	*(M+2H)/2 ions*	*(M+H) ions*
A	**C40 H68 N8 O22**		507.2297 507.7314 508.2331	1013.4521 1014.4555 1015.4589
B	**C37 H63 N7 O21**		471.7112 472.2128 472.7145	942.4150 943.4184 944.4217
C	**C77 H129 N15 O42**	646.2879 646.6224 646.9568	968.9283 969.4300 969.9316	
D	**C74 H124 N14 O41**	622.6089 622.9434 623.2778	933.4097 933.9114 934.4131	
E	**C46 H72 N8 O22**		545.2454 545.7470 546.2487	1089.4834 1090.4868 1091.4902
F	**C46 H73 N9 O21**		544.7534 545.2550 545.7567	1088.4994 1089.5028 1090.5061

fragment	*formula*	*(M+3H)/3 ions*	*(M+2H)/2 ions*	*(M+H) ions*
A’	**C40 H69 N9 O21**		506.7377 507.2394 507.7411	1012.4681 1013.4715 1014.4748
B’	**C37 H64 N8 O20**		471.2191 471.7208 472.2225	941.4310 942.4344 943.4377
C’	**C77 H131 N17 O40**	645.6319 645.9664 646.3008	967.9443 968.4459 968.9476	
D’	**C74 H126 N16 O39**	621.9529 622.2873 622.6218	932.4257 932.9274 933.4291	
E’	**C46 H73 N9 O21**		544.7534 545.2550 545.7567	1088.4994 1089.5028 1090.5061
F’	**C46 H74 N10 O20**		544.2613 544.7630 545.2647	1087.5154 1088.5188 1089.5221

Table S1. Ions extracted in Figures 2, 3, and S8.

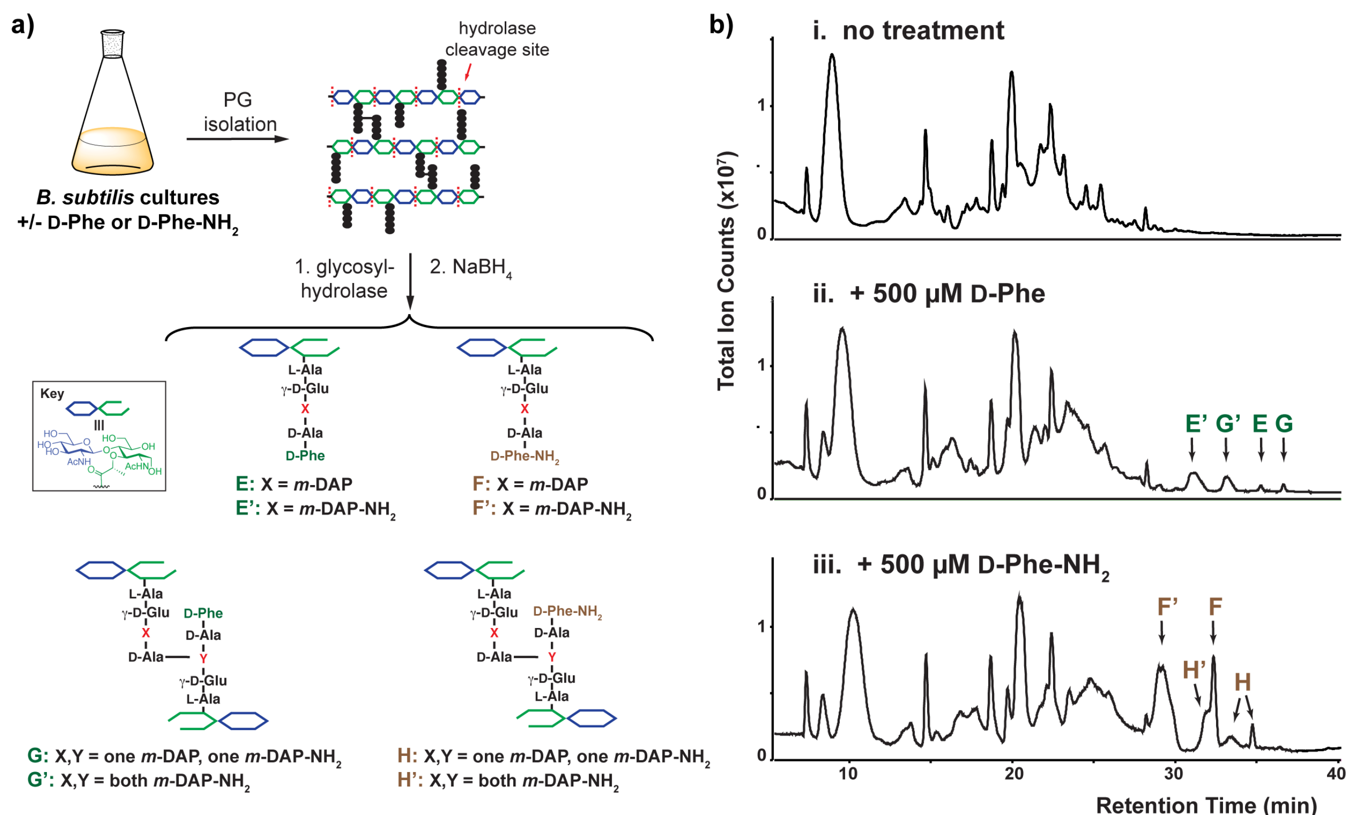


Figure S1. *B. subtilis* cells incorporate D-amino carboxamides into PG more than D-amino acids. (a) *B. subtilis* (strain 3610) was grown in LB supplemented with either 500 μM D-Phe or D-Phe-NH_2 and harvested at early log phase. Peptidoglycan isolated from the cells was digested with a glycosyl-hydrolase, and the resultant disaccharide-peptide fragments were analyzed by high-resolution LC/MS. Although the *m*-DAP residue in *B. subtilis* is normally amidated, the carboxylate form is also present. (b) Total ion chromatograms of the PG fragments. Fragment identities of the new peaks arising in the treatment samples were assigned by mass (see Figures S11 and S12).

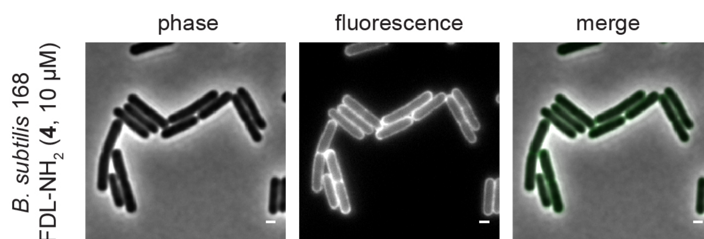


Figure S2. Amidated probe FDL-NH_2 (**4**) efficiently labels *B. subtilis* 168 at 10 μM. Scale bars: 1 μm.

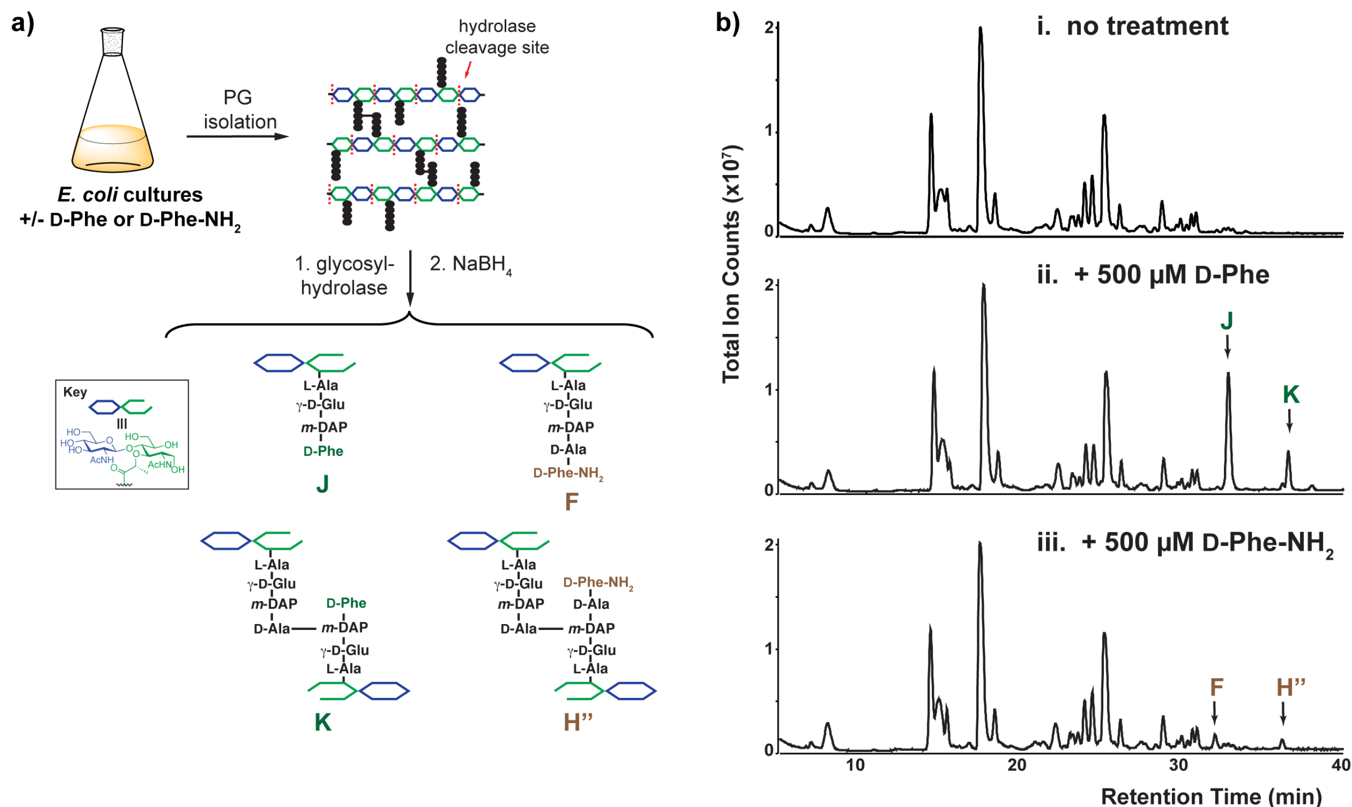


Figure S3. *E. coli* cells incorporate D-amino acids into PG much more than D-amino carboxamides. (a) *E. coli* (strain MC4100) was grown in LB supplemented with either 500 μM D-Phe or D-Phe-NH_2 and harvested at early log phase. Peptidoglycan isolated from the cells was digested with a glycosyl-hydrolase, and the resultant disaccharide-peptide fragments were analyzed by high-resolution LC/MS. (b) Total ion chromatograms of the PG fragments. Fragment identities of the new peaks arising in the treatment samples were assigned by mass (see Figure S13). No fifth-position incorporation into the peptide stem was detected for D-Phe, and no fourth-position incorporation was detected for D-Phe-NH_2.

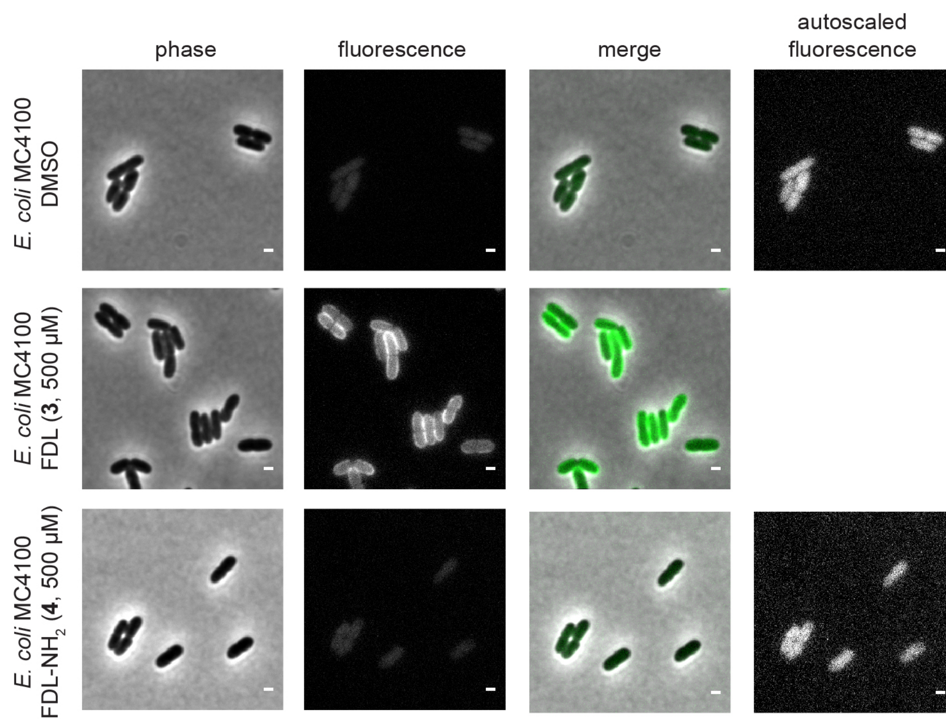


Figure S4. *E. coli* can incorporate FDL (**3**) but not FDL-NH_2 (**4**) at 500 μM. *E. coli* cells autofluoresce in the absence of probe. Scale bars: 1 μm.

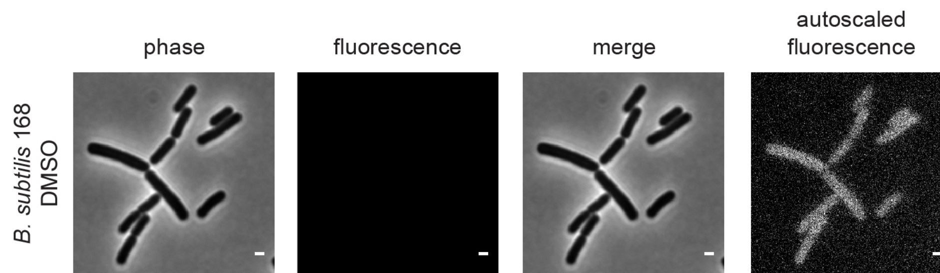


Figure S5. *B. subtilis* 168 displays no fluorescence at the same intensity scale as Figure 4 and S6. Scale bars: 1 μm.

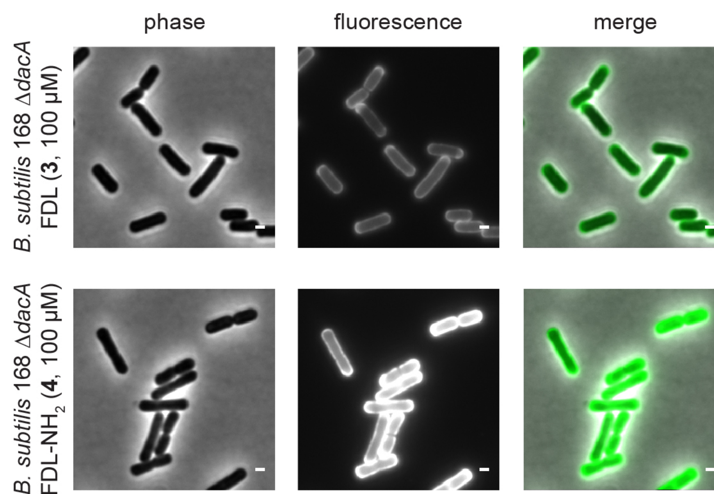


Figure S6. FDL-NH_2 (**4**) labels *B. subtilis* 168 Δ*dacA* intensely. Analogous to wild-type cells, FDL-NH_2 (**4)** labels *B. subtilis* 168 Δ*dacA* more efficiently than FDL (**3**). *B. subtilis* 168 Δ*dacA* incorporated more fluorescent label than wild-type when treated with **3**, as reported previously.[4] *B. subtilis* 168 Δ*dacA* is also able to incorporate **4** better than wild-type. The fluorescence intensity is at the same scale as Figure 4 and S5. Scale bars: 1 μm.

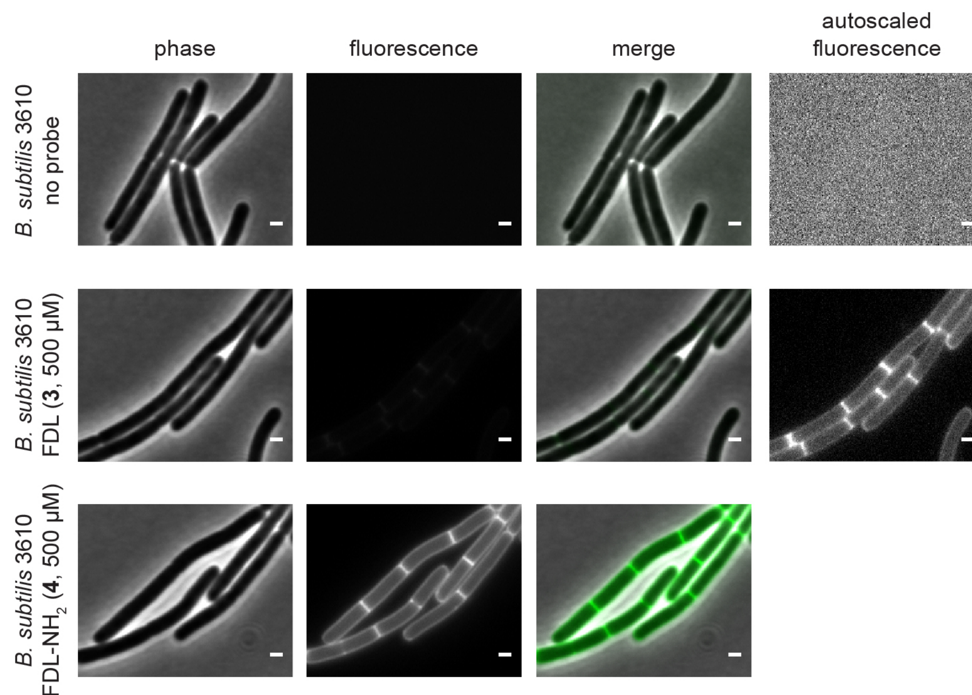


Figure S7. FDL-NH_2 (**4**) labels *B. subtilis* 3610 much more than FDL (**3**). Scale bars: 1 µm.

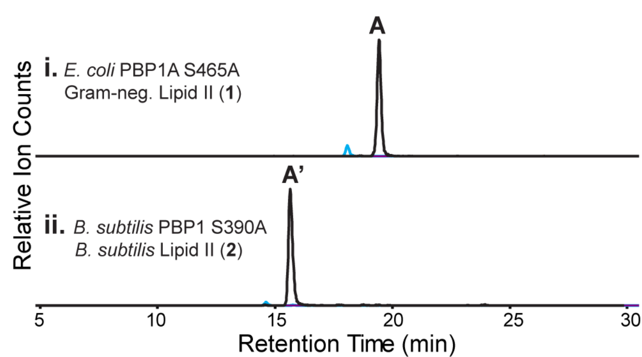


Figure S8. Purifications of *B. subtilis* PBP1 and *E. coli* PBP1A did not contain TP contaminants, as active-site mutants did not display cross-linking activity. Reactions were set up and analyzed as described for the wild-type enzymes in Figure 2.

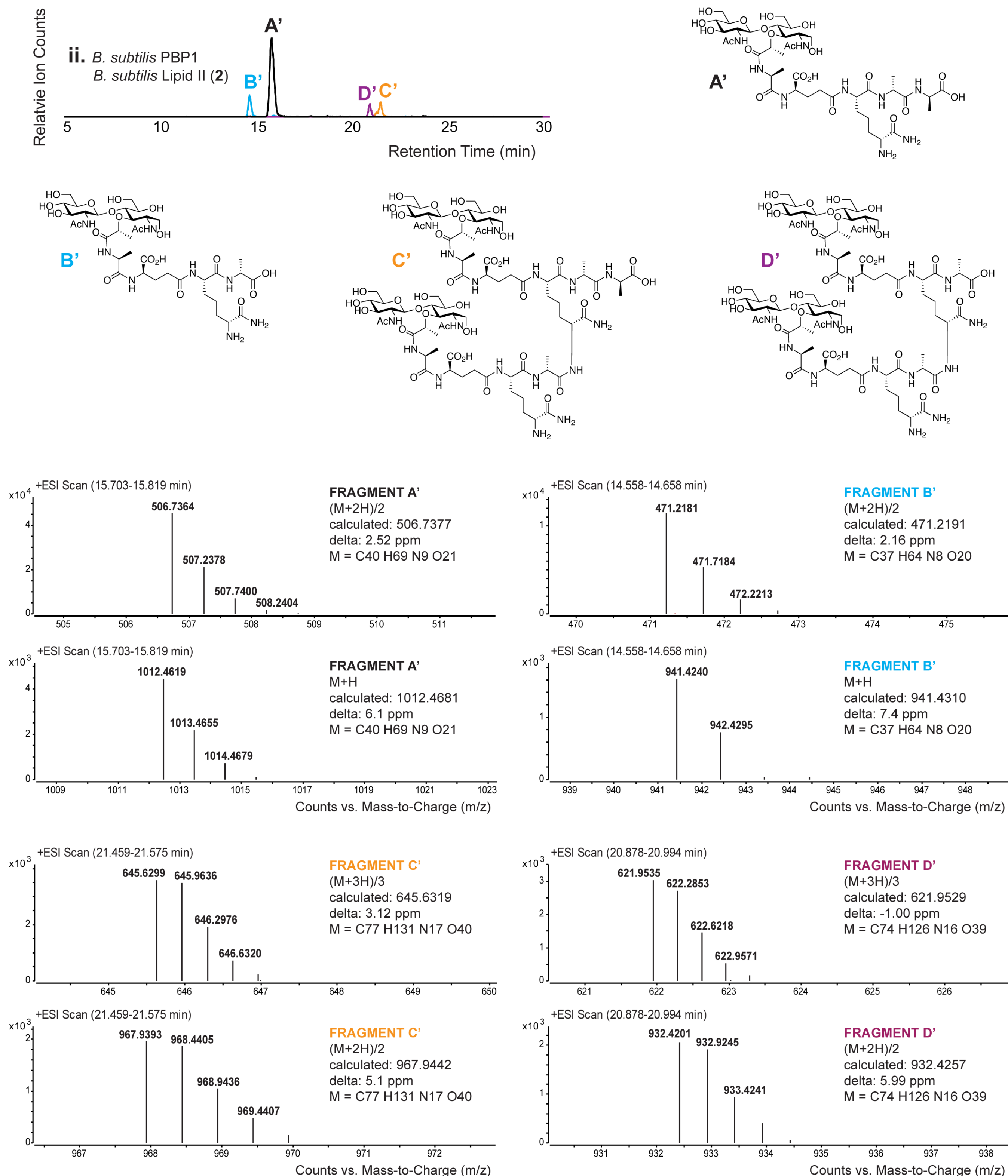
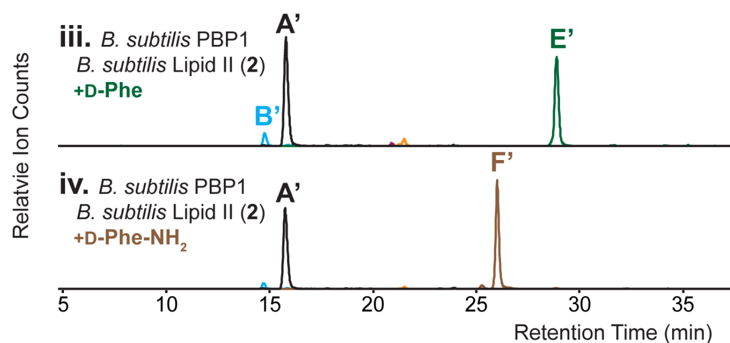
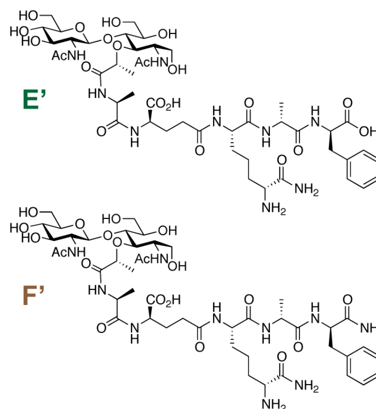
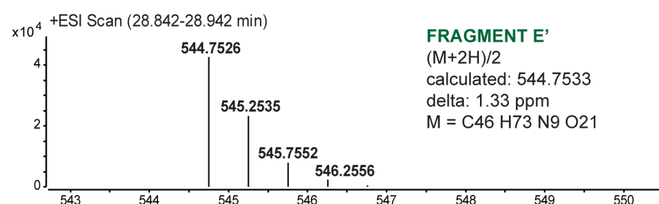
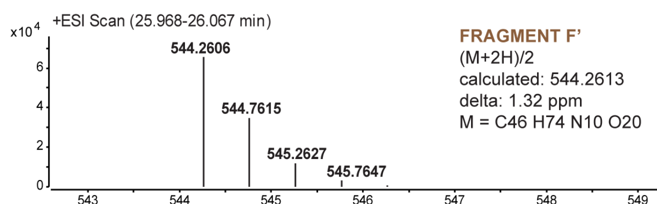


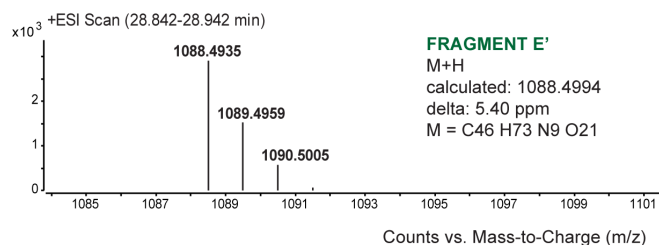
Figure S9. High-resolution LC/Q-TOF mass spectra of PG fragments generated in vitro from *B. subtilis* PBP1 and *B. subtilis* Lipid II (see Figure 2).











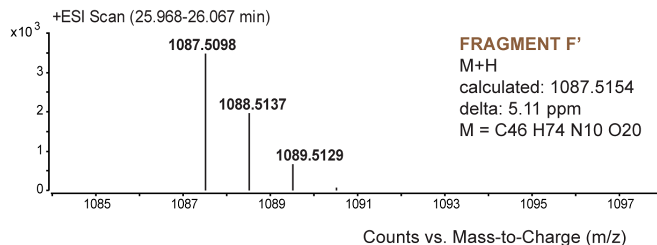


Figure S10. High-resolution LC/Q-TOF mass spectra of PG fragments generated in vitro from *B. subtilis* PBP1 and *B. subtilis* Lipid II treated with 1 mM D-Phe or D-Phe-NH_2 (see Figure 3).

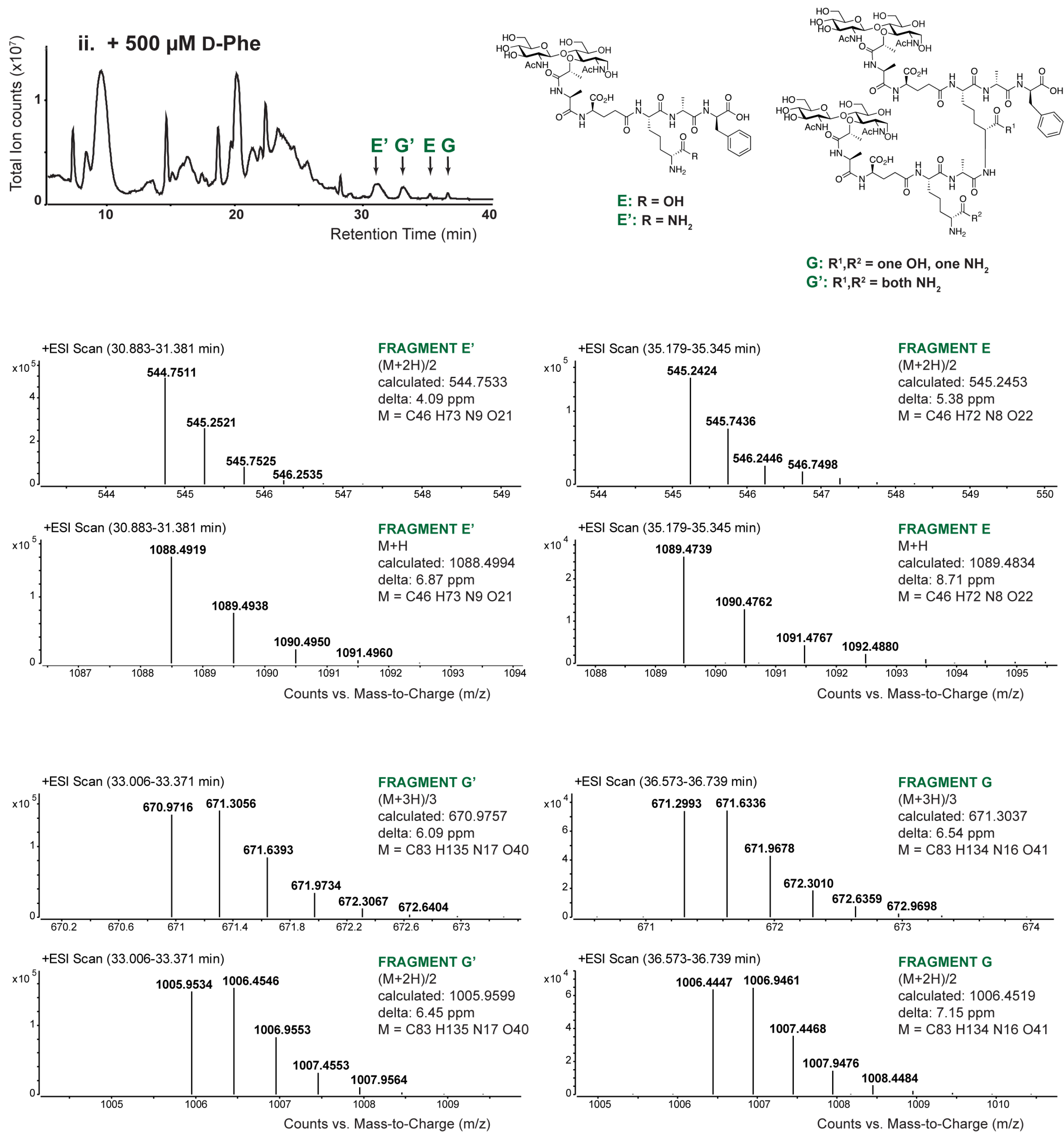


Figure S11. High-resolution LC/Q-TOF mass spectra of PG fragments isolated from *B. subtilis* cells treated with 500 µM D-Phe (see Figure S1).

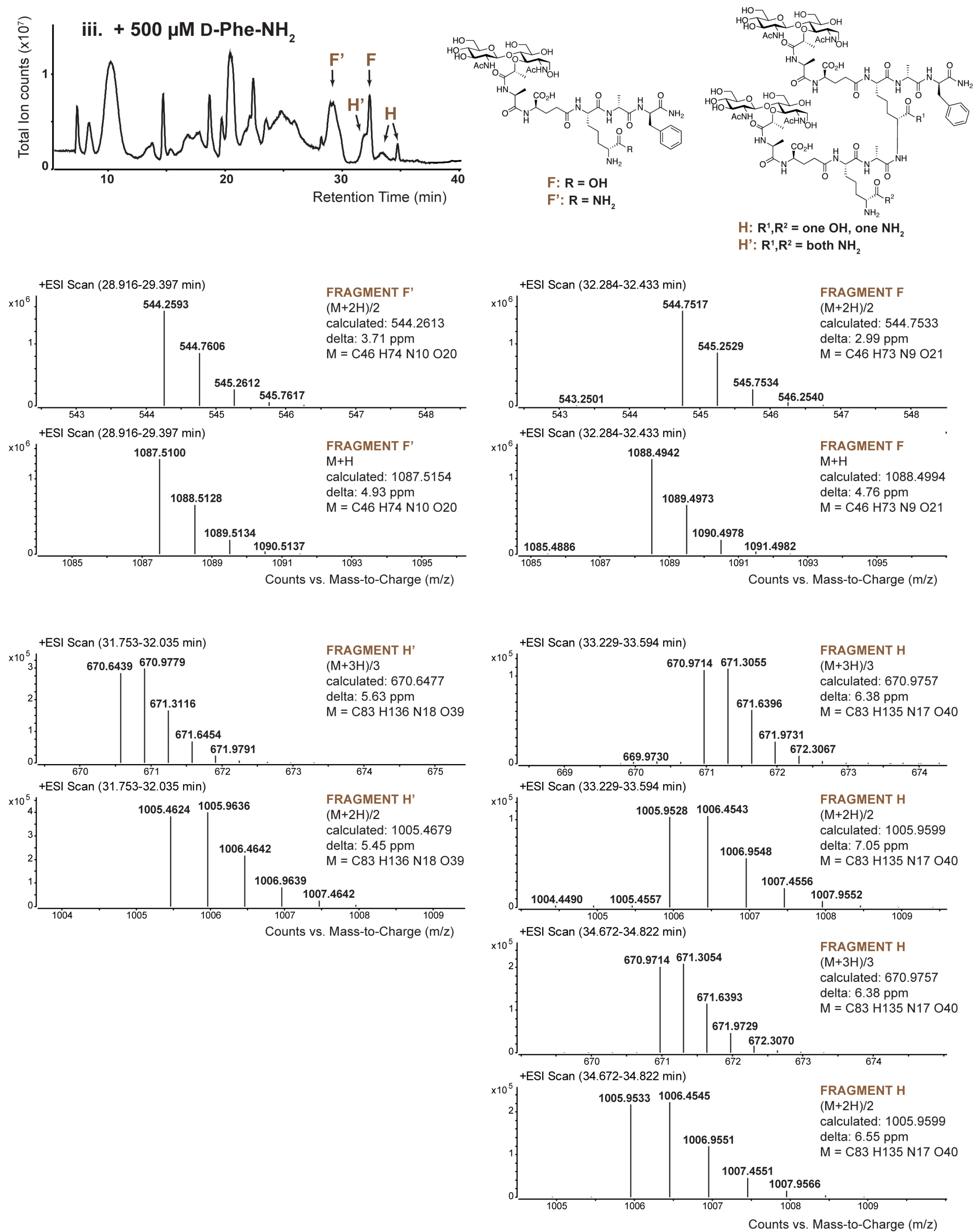
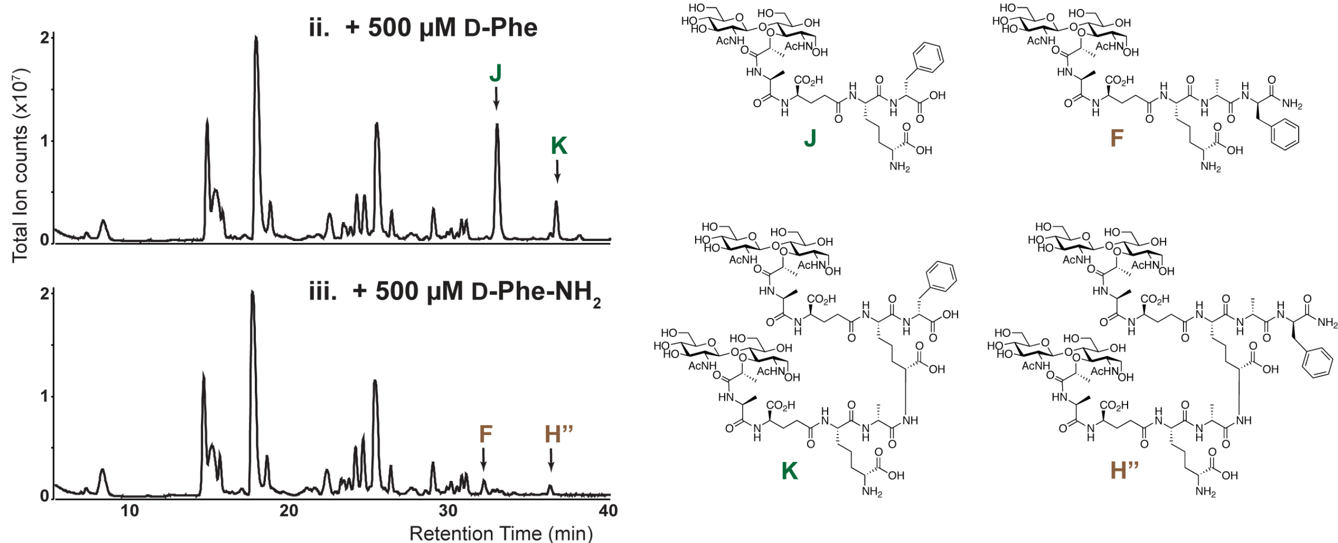
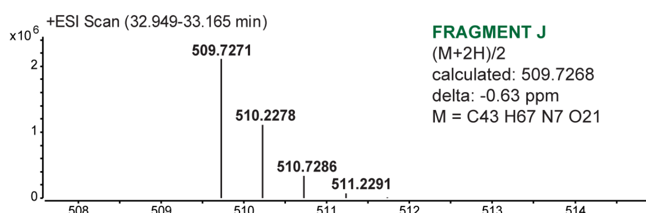
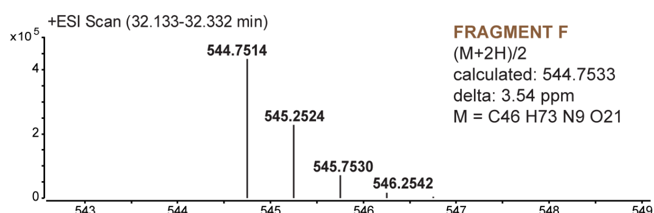
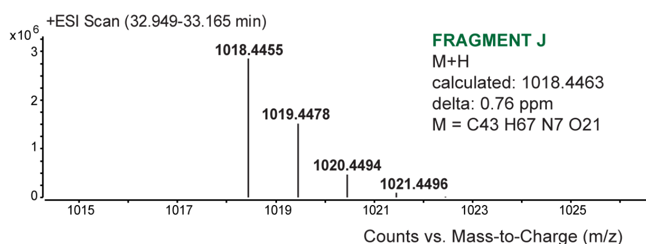


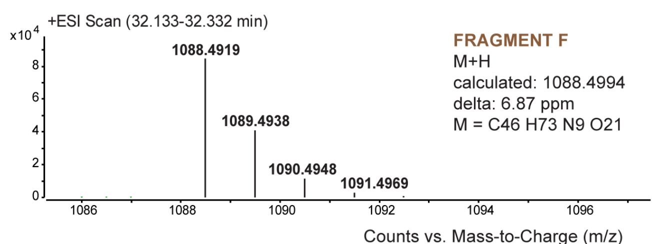
Figure S12. High-resolution LC/Q-TOF mass spectra of PG fragments isolated from *B. subtilis* cells treated with 500 µM D-Phe-NH_2 (see Figure S1).

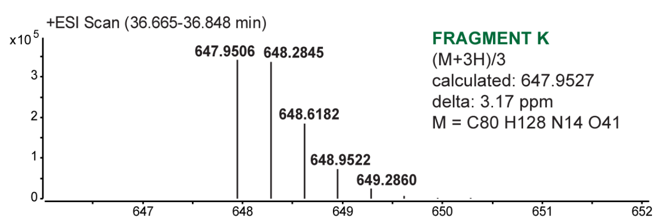


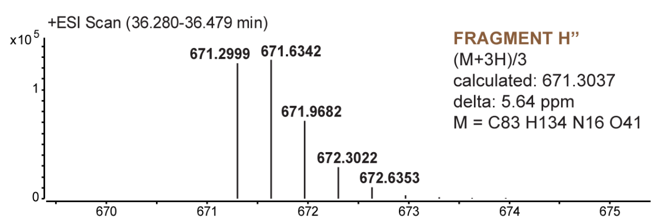


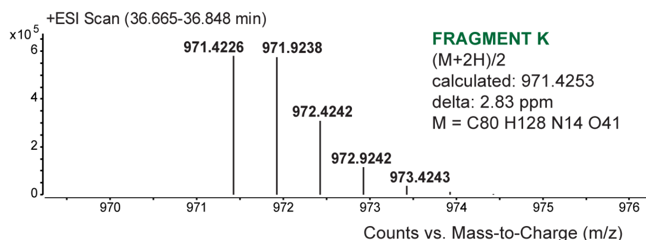












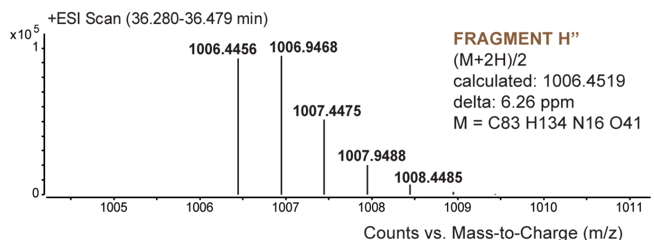


Figure S13. High-resolution LC/Q-TOF mass spectra of PG fragments isolated from *E. coli* cells treated with 500 μM D-Phe or D-Phe-NH_2 (see Figure S3).

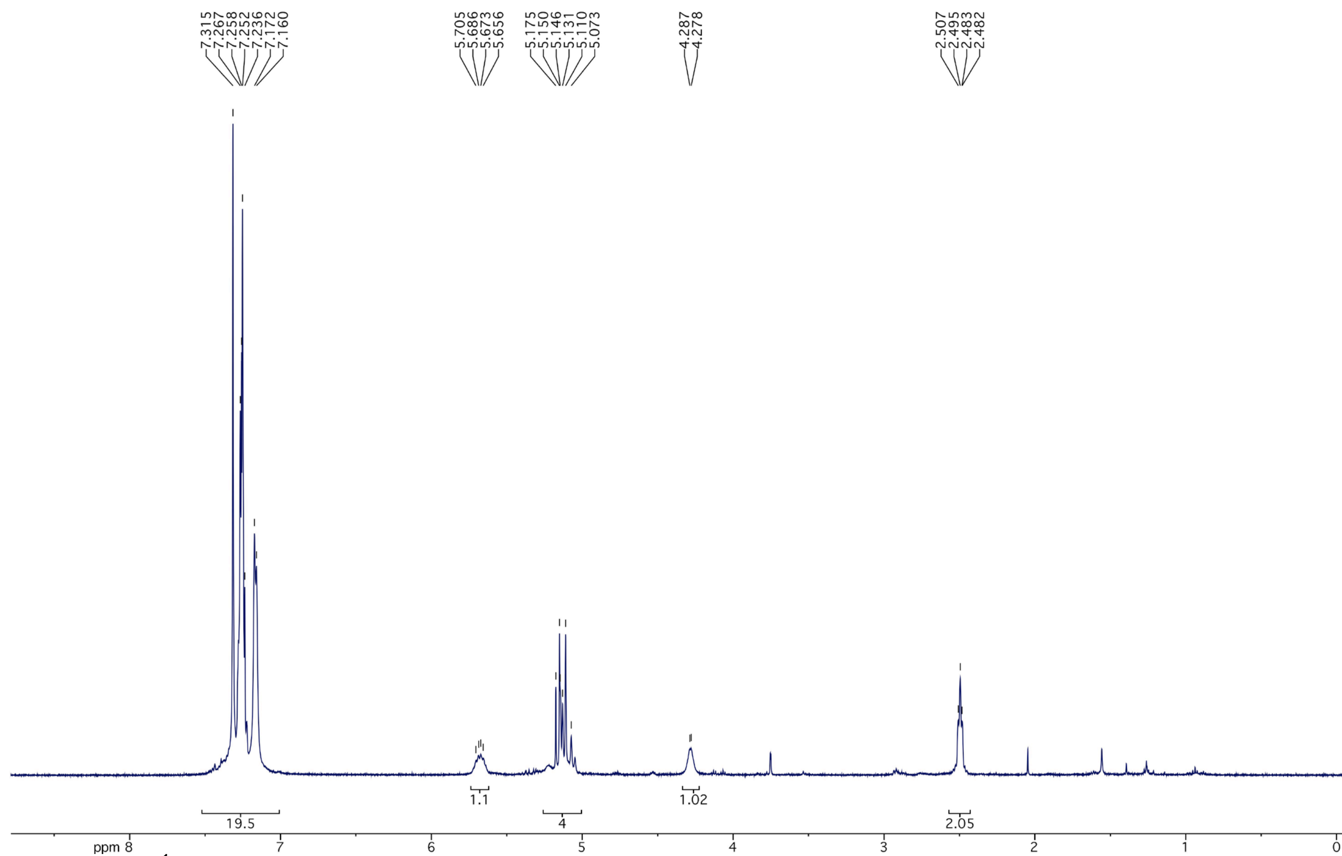


Figure S14. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of Cbz-D-allylglycine-NHTrt (**SI-1**)

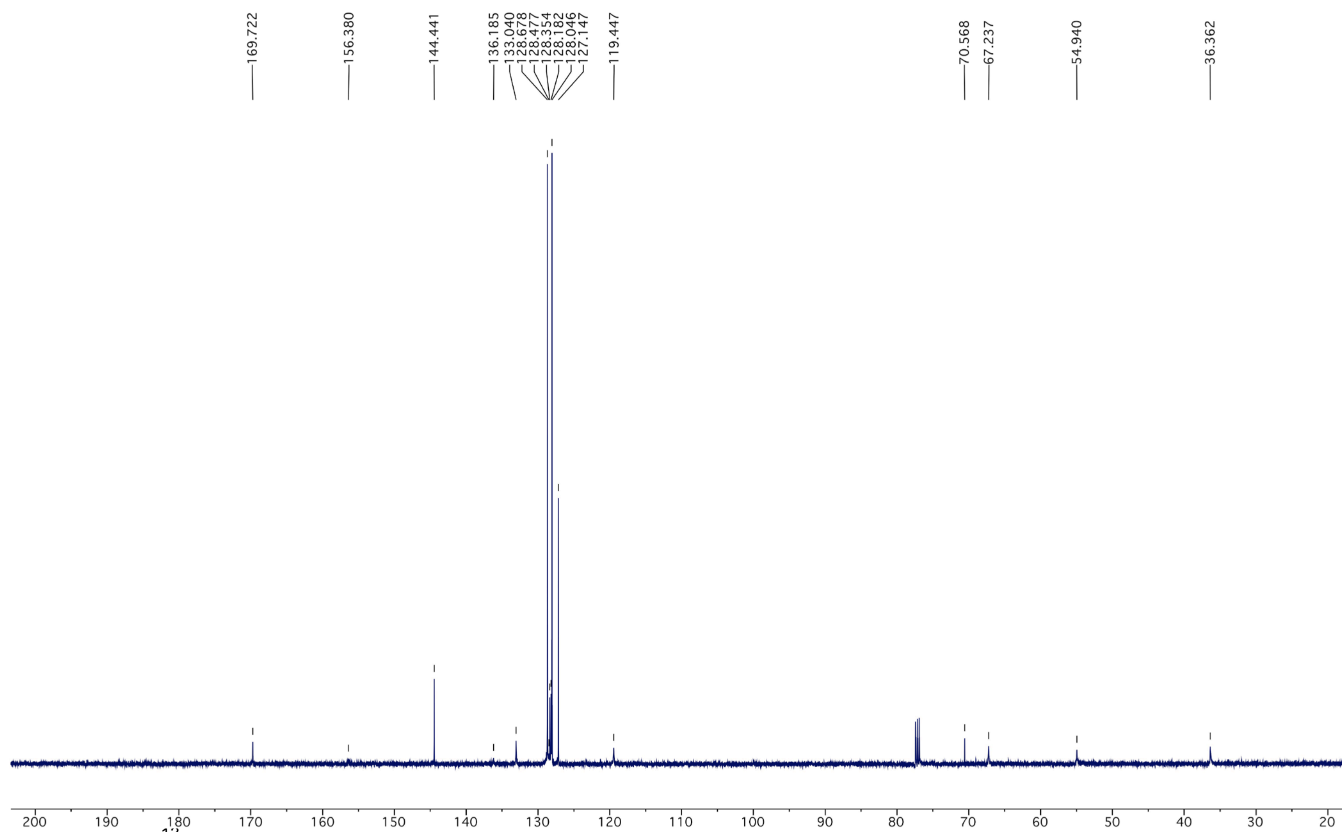


Figure S15. ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of Cbz-D-allylglycine-NHTrt (**SI-1**)

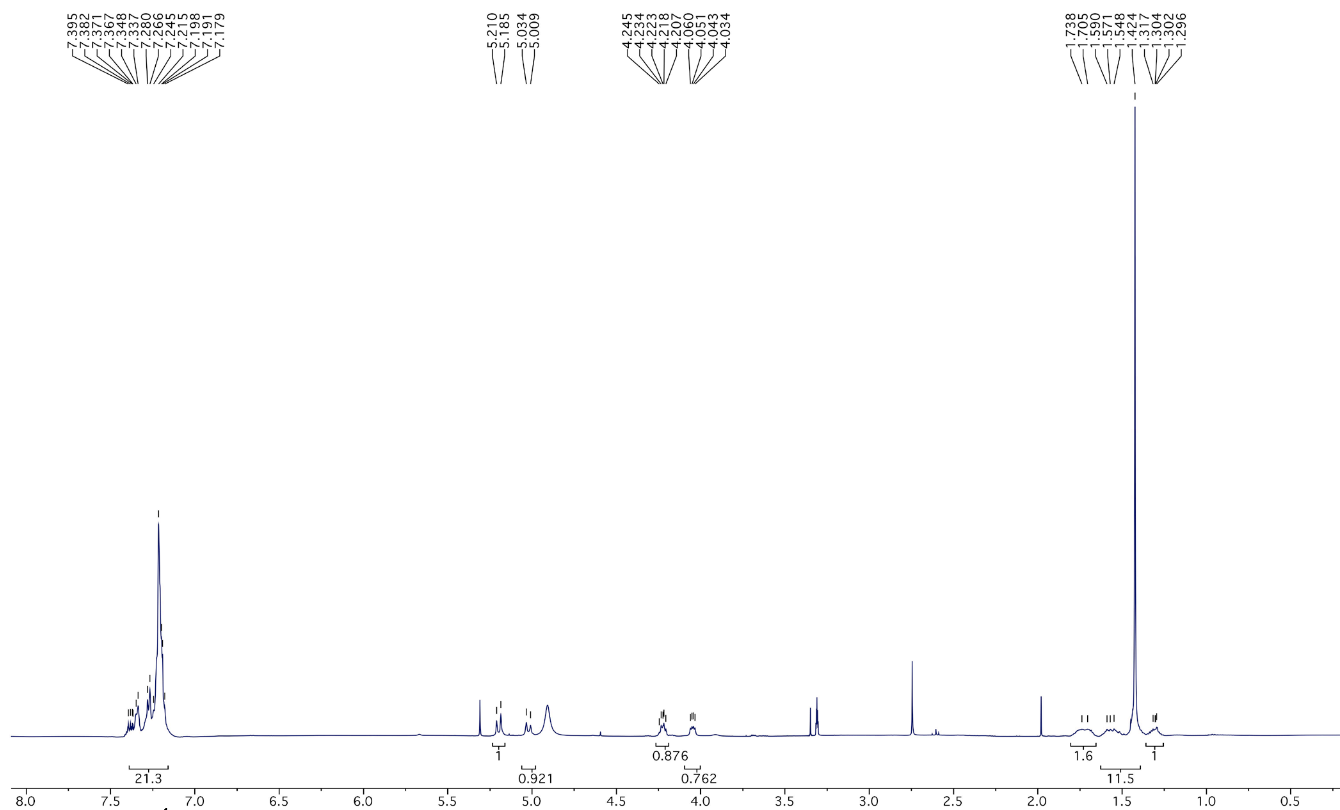


Figure S16. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of Boc-L-OH-DAP-Cbz-D-NHTrt (**SI-2**)

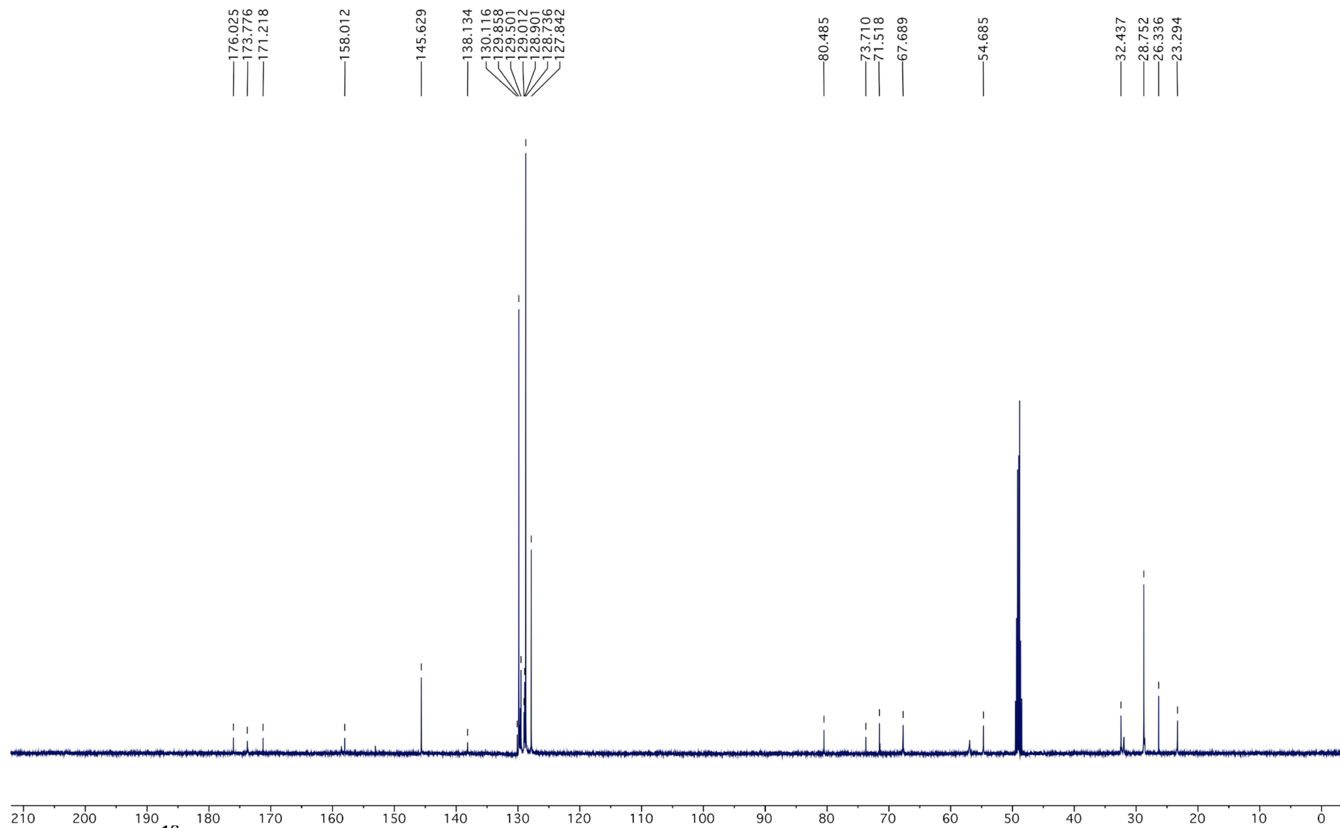


Figure S17. ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of Boc-L-OH-DAP-Cbz-D-NHTrt (**SI-2**)

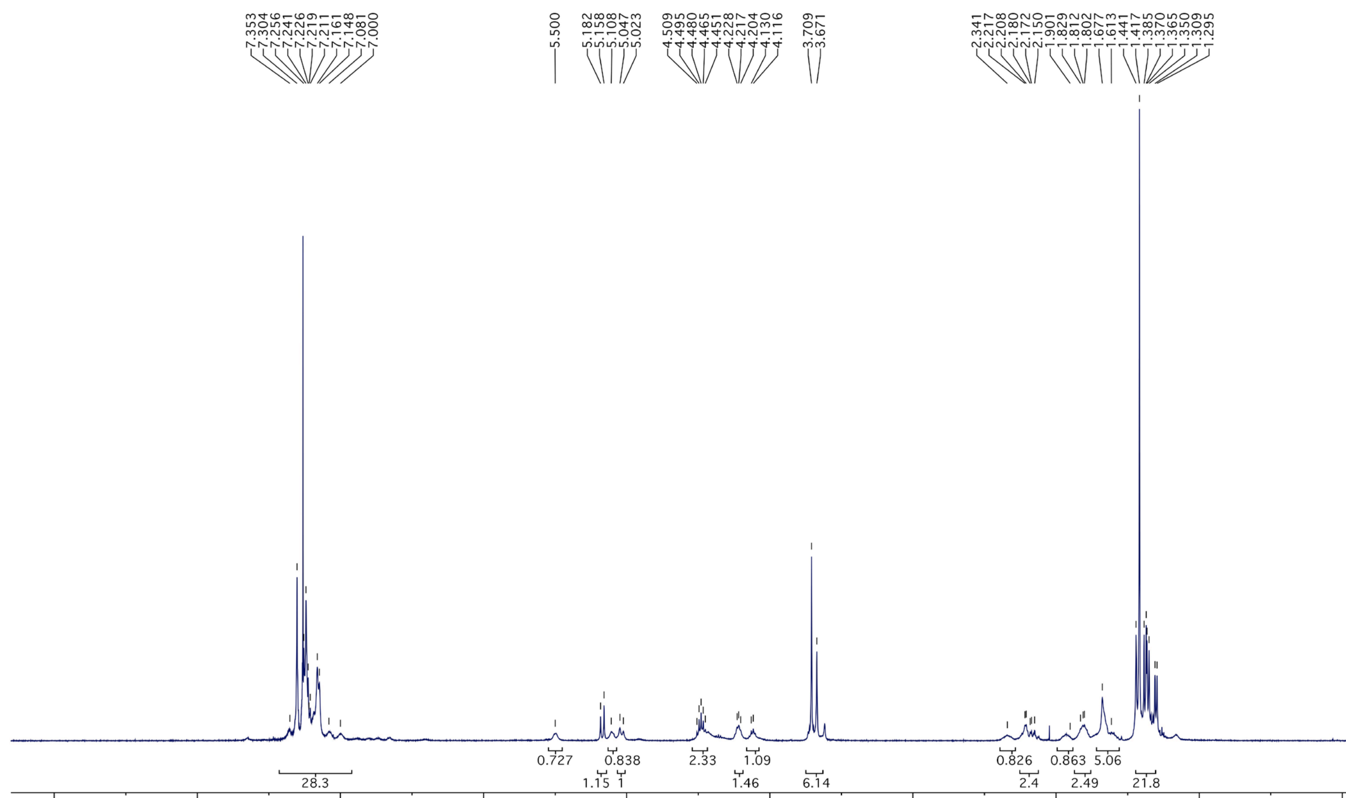


Figure S18. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of Boc-L-Ala-γ-D-Glu(OMe)-*meso*-2(L),2'(D)-DAP(Cbz)(NHTrt)-D-Ala-D-Ala-OMe (**SI-3**)

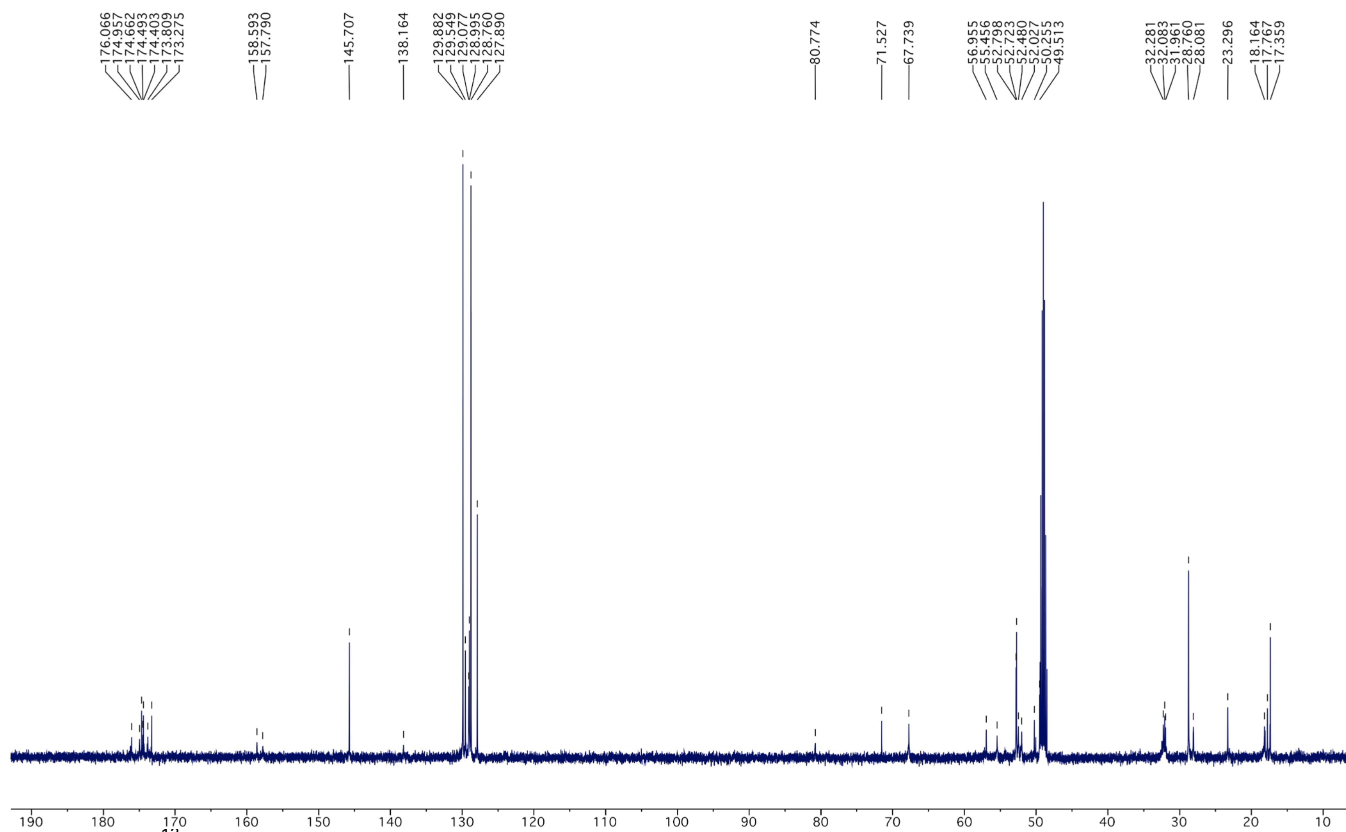


Figure S19. ^{13}C NMR spectrum (100 MHz, CD_3OD) of Boc-L-Ala-γ-D-Glu(OMe)-*meso*-2(L),2'(D)-DAP(Cbz)(NHTrt)-D-Ala-D-Ala-OMe (**SI-3**)

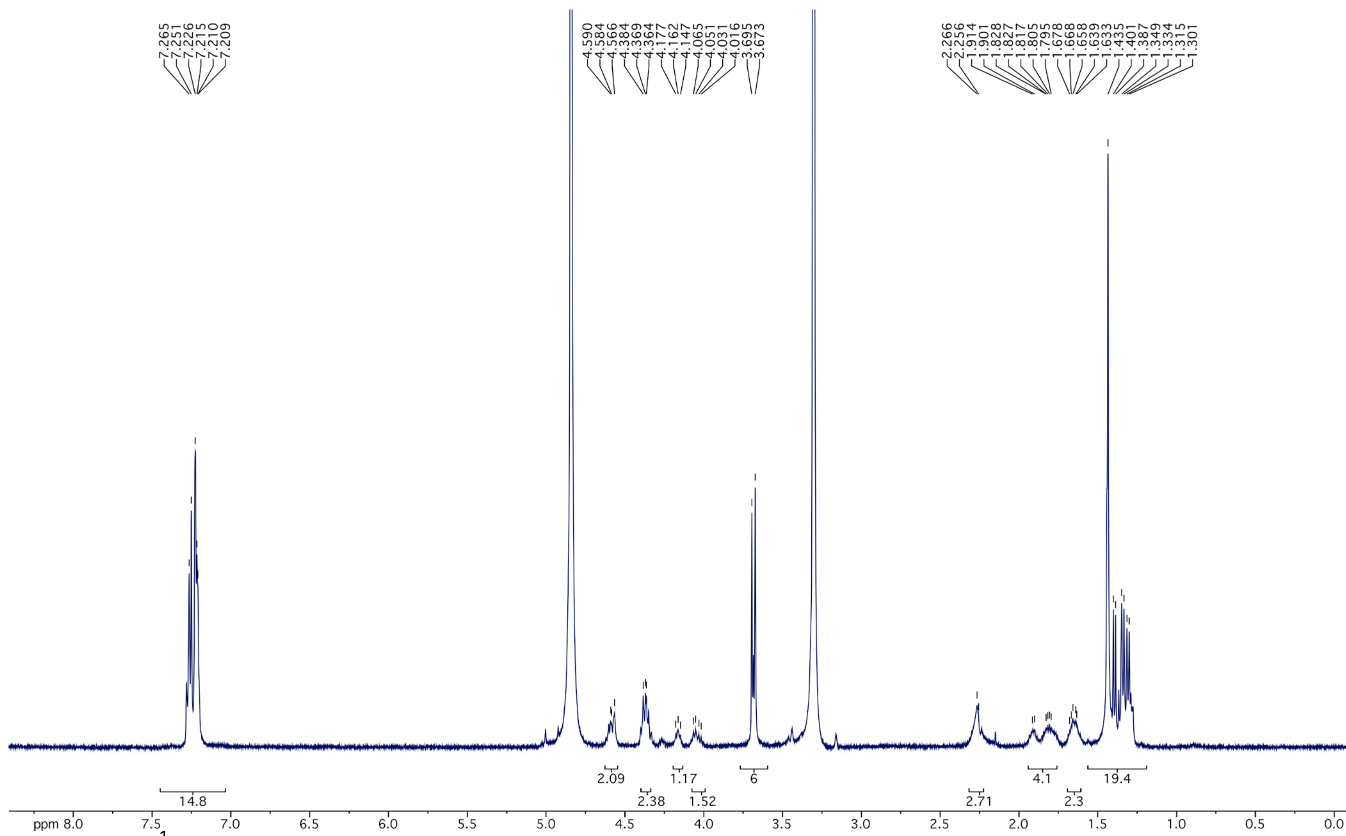
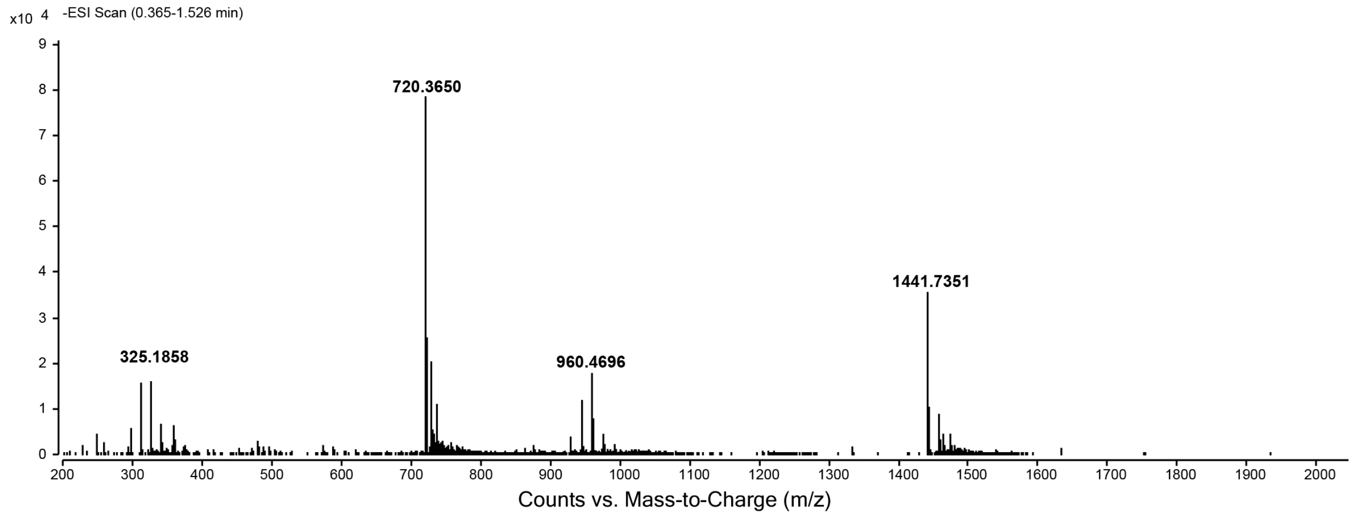
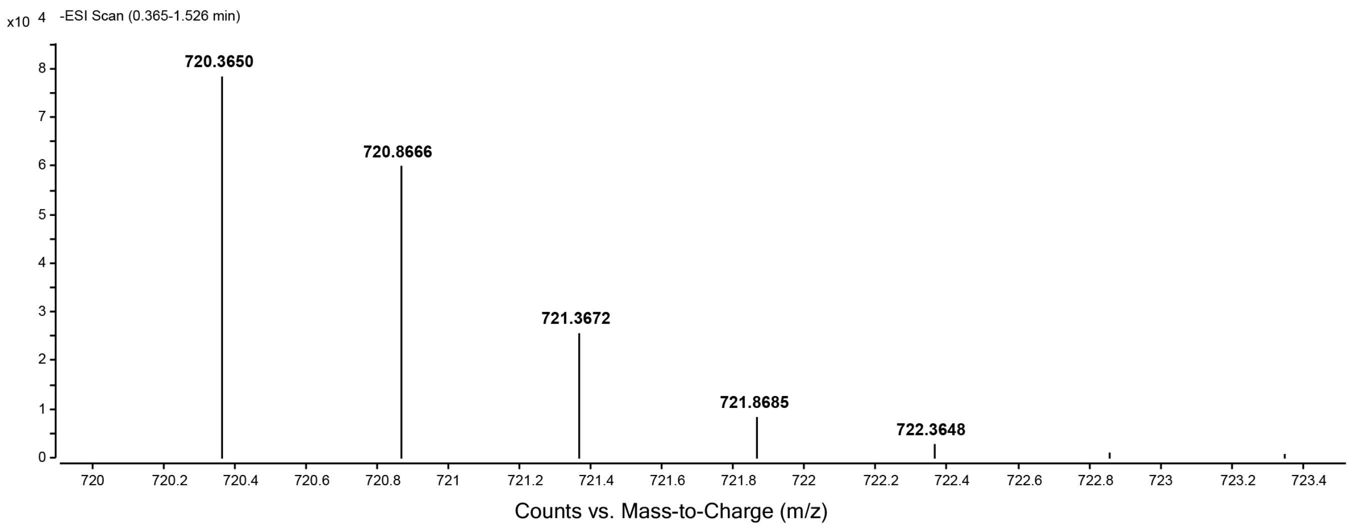


Figure S20. ^{1}H NMR spectrum (500 MHz, CD_3OD) of Boc-L-Ala-γ-D-Glu(OMe)-*meso*-2(L),2'(D)-DAP(TFA)(NHTrt)-D-Ala-D-Ala-OMe (**SI-4**)





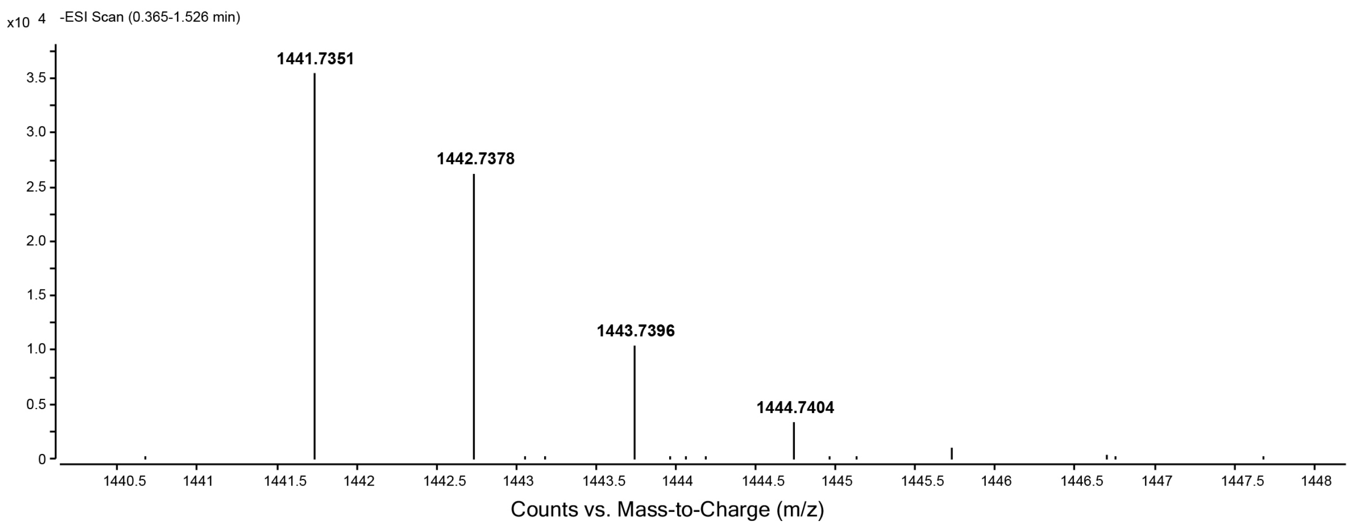
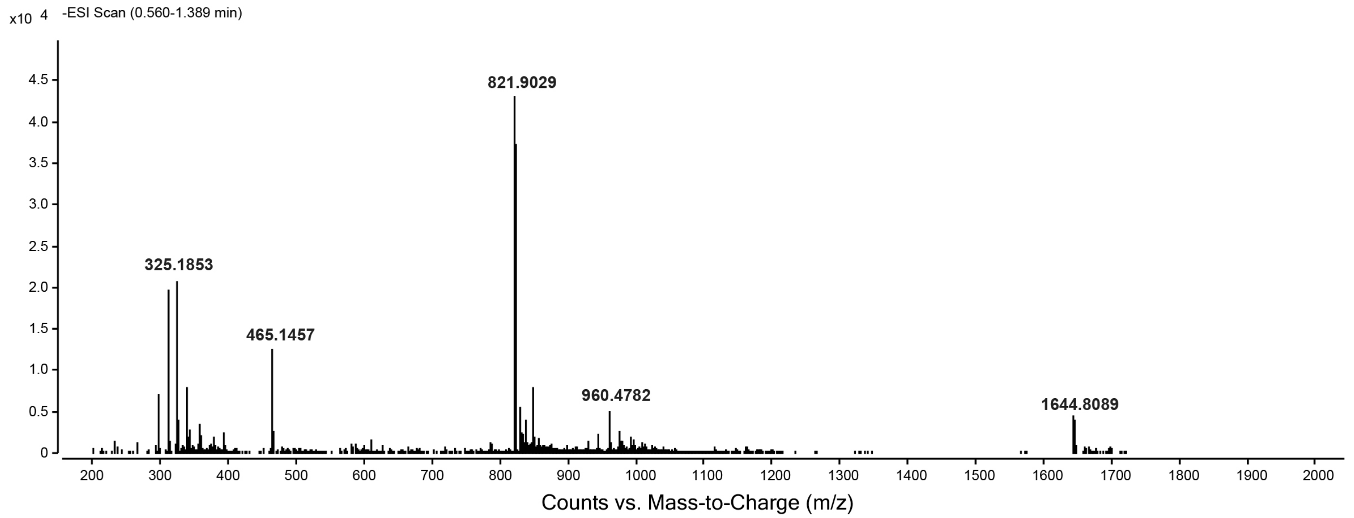
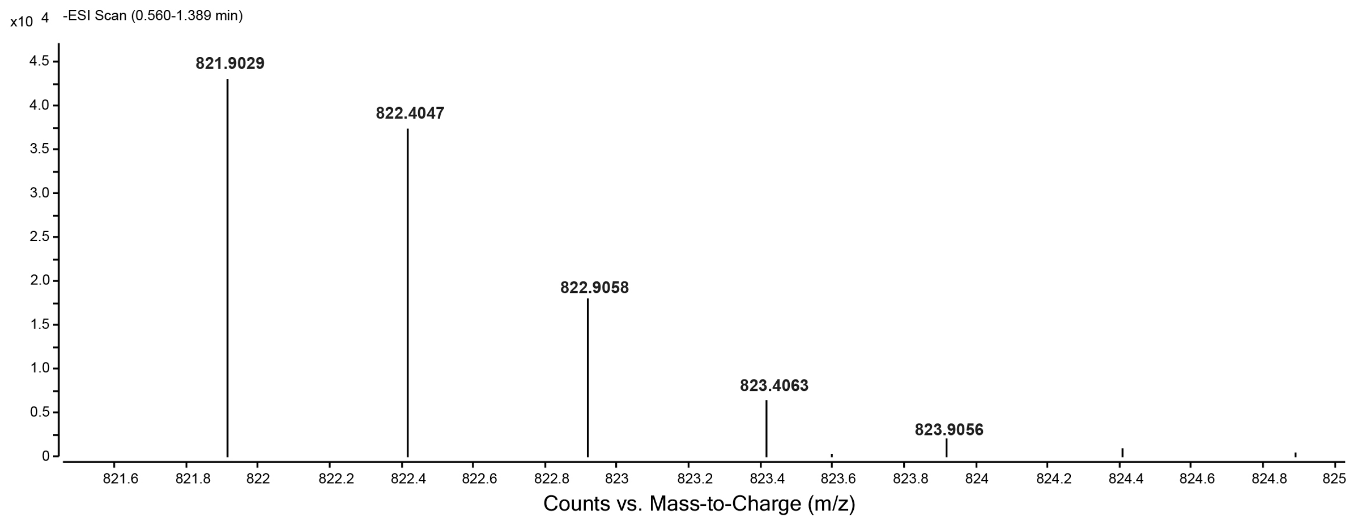


Figure S21. QTOF-MS (neg. mode) of *B. subtilis* Lipid I (**SI-5**)





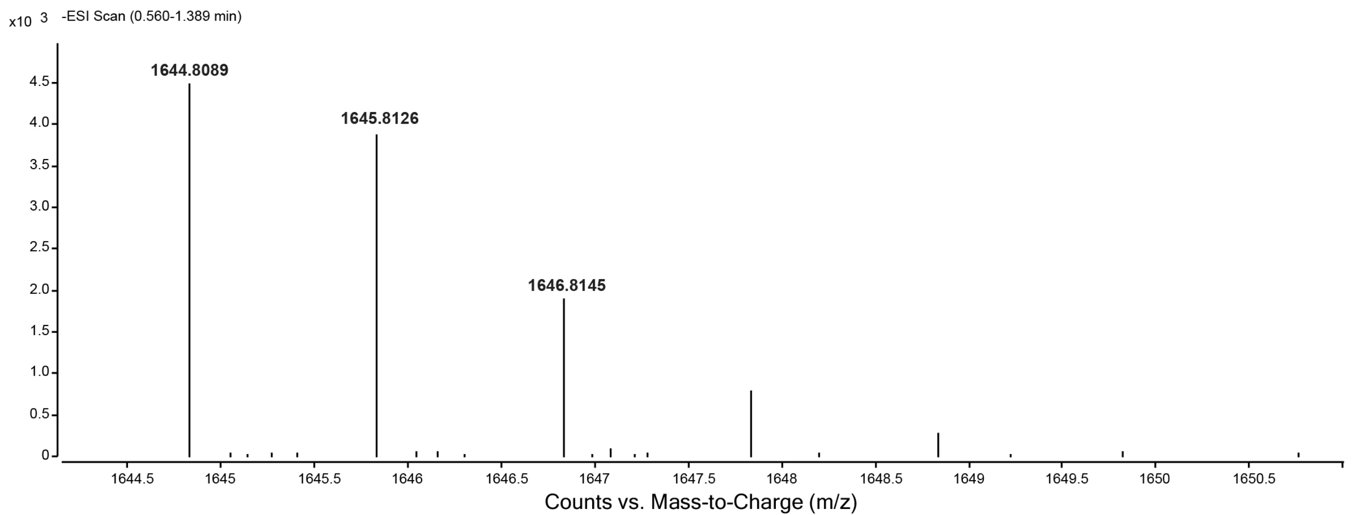


Figure S22. QTOF-MS (neg. mode) of *B. subtilis* Lipid II (**2**)