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Flora Ngadjewa, Emmanuelle Braud, Saidbakhrom Saidjalolov, Laura Iannazzo, Dirk Schnappinger, et al.. Critical Impact of Peptidoglycan Precursor Amidation on the Activity of l,d -Transpeptidases from Enterococcus faecium and Mycobacterium tuberculosis. Chemistry - A European Journal, 2018, 24 (22), pp.5743 - 5747. 10.1002/chem.201706082 . hal-01817697

HAL Id: hal-01817697

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Submitted on 18 Jun 2018

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Critical impact of peptidoglycan precursor amidation on the activity of L,D-transpeptidases from *Enterococcus faecium* and *Mycobacterium tuberculosis*

Flora Ngadjeua^{#, [a]}, Emmanuelle Braud^{#, [b]}, Saidbakhrom Saidjalolov^[b], Laura Iannazzo^[b], Dirk Schnappinger^[c], Sabine Ehrh^[c], Jean-Emmanuel Hugonnet^[a], Dominique Mengin-Lecreulx^[d], Delphine Patin^[d], Mélanie Ethève-Quellejeu^{*, [b]}, Matthieu Fonvielle^{*, [a]} and Michel Arthur^{*, [a]}

Abstract: The bacterial cell wall peptidoglycan contains unusual L and D amino acids assembled in branched peptides. Insight into the biosynthesis of the polymer has been hampered by limited access to substrates and to suitable polymerization assays. Here we report the full synthesis of the peptide stem of peptidoglycan precursors from two pathogenic bacteria, *Enterococcus faecium* and *Mycobacterium tuberculosis*, and the development of a sensitive post-derivatization assay for their cross-linking by L,D-transpeptidases. Access to series of stem peptides showed that amidation of free carboxyl groups is essential for optimal enzyme activity, in particular the amidation of diaminopimelate (DAP) residues for the cross-linking activity of the L,D-transpeptidase Ldt_{M12} from *M. tuberculosis*. Accordingly, construction of a conditional mutant established the essentiality of AsnB indicating that this DAP amidotransferase is an attractive target for the development of anti-mycobacterial drugs.

Peptidoglycan is an essential and specific component of the bacterial cell wall.^[1] The main role of this giant (cell-sized) macromolecule is to protect bacterial cells against the osmotic pressure of the cytoplasm. The peptidoglycan subunit consists of a disaccharide substituted by a pentapeptide stem (figure 1), which is polymerized by glycosyltransferases for the elongation of the glycan chains (all glycosidic bonds are β-1,4) and by D,D-transpeptidases for cross-linking the glycan chains to each other.^[2] The amide bond formed by the D,D-transpeptidases links the carbonyl of D-Ala at the 4th position of an acyl donor stem to the side-chain amino group at the 3rd position of an acyl acceptor stem (4→3 cross-link). These enzymes are the targets of β-lactam antibiotics such as penicillin.

The structure of peptidoglycan is generally conserved in bacteria belonging to the same species, but highly diverse between species, including members of the same genus.^[3] The polymorphisms include the *N*-deacetylation, *O*-acetylation, and *N*-glycolylation of either or both GlcNAc and MurNAc. These modifications are mostly, if not exclusively, due to maturation of

occur at the 3rd (e.g. L-Lys instead of diaminopimelic acid [DAP]) and at the 5th (e.g. D-Lac instead of D-Ala) positions (figure 1b). Modifications of the pentapeptide stem involve the addition of a side-chain to the 3rd residue (e.g. D-isoAsn or Gly₅) and the amidation of the carboxyl groups (e.g. the α-carboxyl of D-Glu and D-isoAsp or the ε-carboxyl of DAP). A last source of polymorphism originates from the presence of 3→3 instead of 4→3 cross-links in mycobacteria (e.g. *Mycobacterium tuberculosis*) and in β-lactam-resistant mutants generated *in vitro* (e.g. *Enterococcus faecium*) (figure 1a). The 3→3 cross-links are formed by transpeptidases of the L,D specificity, which cleave the L-Lys³-D-Ala⁴ or DAP³-D-Ala⁴ bond of an acyl donor containing a tetrapeptide stem and form L-Lys³→L-Lys³ or DAP³→DAP³ cross-links.

Variability in the peptidoglycan structure has been known for decades based on biochemical analyses of the cell wall.^[3a] The corresponding enzymes have been described more recently, mostly because the complexity of their substrates has hampered their characterization.^[4] Consequently, the biological significance of structural variability is poorly understood. It may involve various selective advantages.^[3b] Resistance to vancomycin is mediated by replacement of D-Ala by D-Lac or D-Ser at the 5th position of peptide stems since this prevents binding of the drug to the precursors. Specific links in mature peptidoglycan are cleaved by hydrolytic enzymes produced by eukaryote hosts, such as lysozyme, which cleaves the MurNAc-GlcNAc β-1,4 bond, or by competing bacteria, such as lysostaphin, which cleaves glycylglycine bonds in the D-Ala⁴→(Gly₅)-L-Lys³ cross-bridges of *Staphylococcus aureus*. Variations in peptidoglycan structure are therefore potential defense mechanisms against hydrolytic enzymes.

Diversification of the structure of peptidoglycan precursors associated with speciation is thought to lead to a parallel evolution of the substrate specificity of the transpeptidases.^[5] Genetic evidence in favor of this hypothesis is limited since impaired maturation of peptidoglycan precursors may have combined effects on numerous peptidoglycan biosynthetic steps in addition to transpeptidation. Scarce evidence has been provided by biochemical studies due to limited access to purified enzymes and substrates.^[6] In this study, we have developed the chemical synthesis of peptidoglycan precursor analogues and a post-derivatization assay to directly assess the impact of amidation of peptidoglycan precursors on the formation of cross-links by purified L,D-transpeptidases from *E. faecium* and *M. tuberculosis*. We show that defects in amidation strongly impair the efficacy of these enzymes indicating that the amidotransferases^[7] are attractive targets to develop alternatives to transpeptidase inhibition by β-lactam antibiotics in drug resistant bacteria.

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^[#] and ^[*] These authors contributed equally to this work.

subunits containing the canonic GlcNAc-MurNAc motif. The most frequent variations in the sequence of the pentapeptide stem

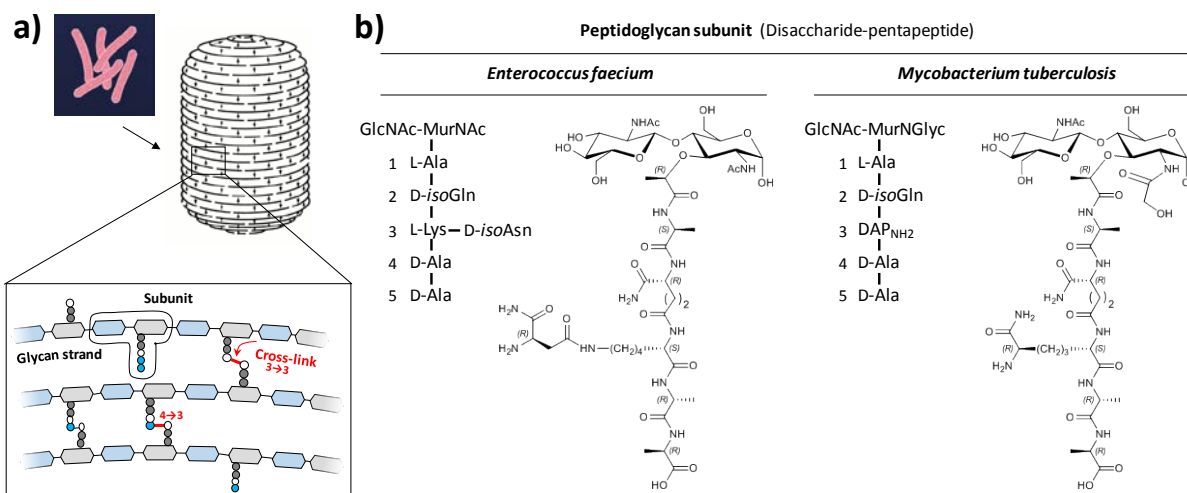
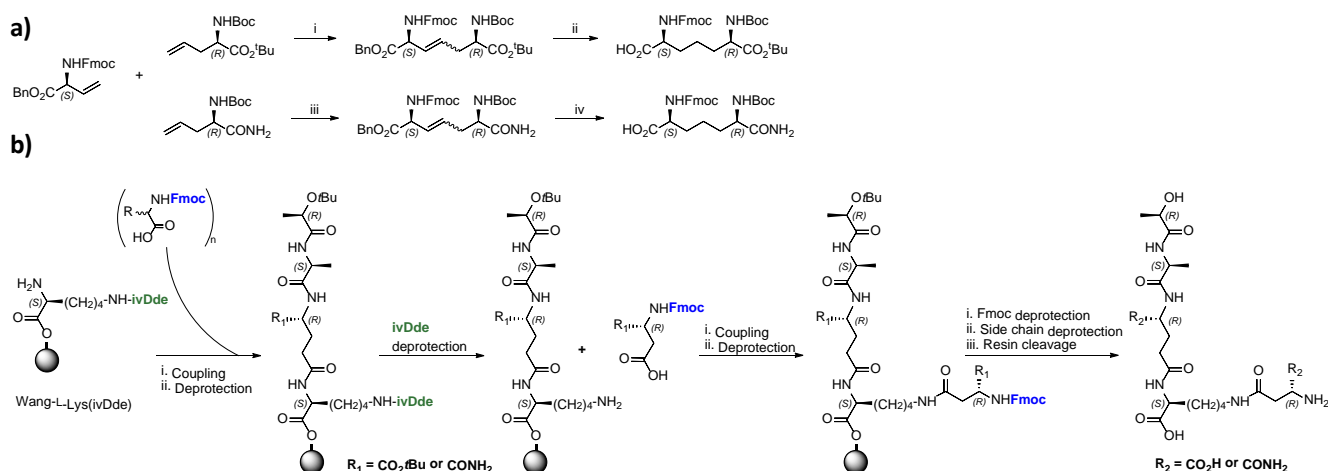


Figure 1. Peptidoglycan structure. a) Peptidoglycan is a giant mesh-like polymer that completely surrounds bacterial cells. b) Peptidoglycan is polymerized from disaccharide-pentapeptide subunits, which are assembled in the cytoplasm. MurNAC, *N*-acetyl muramic acid; MurNGlyc, *N*-glycolyl muramic acid; GlcNAc, *N*-acetyl glucosamine; D-isoGln, D-iso-glutamine; D-isoAsn, D-iso-asparagine; DAP_{NH2}, diaminopimelic acid amidated at the ϵ position.

Peptidoglycan precursors contain unusual amino acids (D-isoGln, D-Ala, D-isoAsn, amidated DAP), a non-peptide bond linking the γ -carboxyl of D-isoGln to the α -amino group of L-Lys or DAP, and a side-chain linked to the ϵ -amino group of L-Lys (figure 1b). Thus, solid-support peptide synthesis required access to non-commercial Fmoc-protected amino acids and the use of orthogonal protecting groups for the synthesis of branched peptides (Scheme 1). Synthesis of the Fmoc-protected DAP and amidated DAP has been performed using a cross-metathesis reaction as a key step (Scheme 1a).^[8] Synthesis of the other protected amino acids is described in the Supplementary Material. Orthogonal Fmoc and 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl (ivDde) protecting groups were used for sequential assembly of the peptide stem and of the side-chain residue branched at the 3rd position, respectively (Scheme 1b). Using this approach 9 peptides mimicking peptidoglycan precursors have been synthesized and fully characterized.

A post-functionalization assay was developed to quantify the substrates and the product of the enzymatic cross-linking reaction catalyzed by Ldt_{fm} from *E. faecium*, the prototypic enzyme of the

L,D-transpeptidase family (figure 2a). We took advantage of the presence of a single primary amine on these molecules to specifically introduce a 4-fluoro-7-nitrobenzofurazan group by nucleophilic aromatic substitution. This post-functionalization reaction applied to the crude enzymatic reaction converted the unreacted donor and acceptor substrates as well as the L,D-transpeptidation product (dimer) into fluorescent peptides. The post-functionalized peptides were separated by *rpHPLC* and detected by fluorescence (figure 2b). Since the fluorescence yield may vary with the acetonitrile concentration required for elution and with the molecular environment of the fluorophore within the peptides, calibration curves were obtained for every substrate and every product (figure 2c). For this purpose, each substrate and each product were individually post-functionalized with 4-fluoro-7-nitrobenzofurazan and known amounts of each fluorescent peptide were analyzed by *rpHPLC*. Access to sensitive determination of the peptides in the 10- to 200-pmol range provided a versatile method to determine the rate of the cross-linking reaction catalyzed by Ldt_{fm}.



Scheme 1. a) Synthetic scheme for the protected DAP and amidated DAP building blocks. i) 5% Grubbs' II catalyst, CH₂Cl₂, RT, 12 h; ii) 3% PtO₂, H₂, CH₂Cl₂/CH₃OH/H₂O (9/1/1), RT, 12 h; iii) 20% Grubbs' II catalyst, CH₂Cl₂, 70°C, 48 h; iv) PtO₂, H₂, CH₃OH, 4 atm, RT, 18 h. b) Solid-phase-synthesis of the peptidoglycan precursor analogues using orthogonal Fmoc and ivDde protecting groups.

The reaction catalyzed by Ldt_{fm} involves two peptidoglycan precursors that act as an acyl donor and as an acyl acceptor (figure 2a). For the donor, we used a linear tetrapeptide that cannot be used as an acyl acceptor since it does not harbor the D-isoAsn residue branched to L-Lys. Conversely, the peptides used as acyl acceptors cannot be used as acyl donors since they do not harbor the essential C-terminal D-Ala residue (D-Ala⁴). To specifically assess the impact of amidation in the acceptor substrate, we tested a tetrapeptide donor containing a D-isoGln residue (acyl donor 3A) and acceptors containing the four combinations of amidation of the D-isoGlu and D-isoAsp α -carboxyl groups (acyl acceptors 4A to 4D). The rate of formation of peptidoglycan dimers by Ldt_{fm} was the highest for the fully amidated acceptor, corresponding to the amidation status found in the peptidoglycan of the *E. faecium* host (figures 2d and 2e). The lack of amidation of D-isoGln had a moderate impact on the transpeptidase activity of Ldt_{fm} (36% residual activity). The impact of the lack of amidation was greater for the side-chain D-isoAsn residue (8.4% residual activity), whereas the combination of both modifications almost completely abolished the activity of Ldt_{fm} (0.83% residual activity). No transpeptidation product was

observed for substrates fully lacking amidation both in the acyl donor and acceptor substrates (data not shown). In conclusion, amidation of the D-isoGlu and D-isoAsp α -carboxyl groups of the acyl acceptor substrate was essential for optimal formation of peptidoglycan cross-links by Ldt_{fm} *in vitro*.

Our following objective was to evaluate the role of amidation of the α - and ϵ -carboxyl groups of D-isoGlu and DAP on the efficacy of formation of 3→3 cross-links by L,D-transpeptidases from *M. tuberculosis*. We based our analysis on Ldt_{M2} as a representative of the five L,D-transpeptidase paralogues produced by this species. Substrates containing the four combinations of amidation of the linear tetrapeptide stem of *M. tuberculosis* were synthesized and independently tested in the quantitative cross-linking assay (figure 3). No cross-linked dimer was observed with substrates lacking amidation of DAP, D-isoGlu, or both. To assess the impact of the lack of amidation of peptidoglycan precursors on the growth of *M. tuberculosis*, we constructed mutants of strain H37Rv conditionally producing the DAP amidotransferase AsnB (Rv2201). This analysis showed that the amidation of the ϵ -carboxyl of DAP is required for growth of *M. tuberculosis* H37Rv.

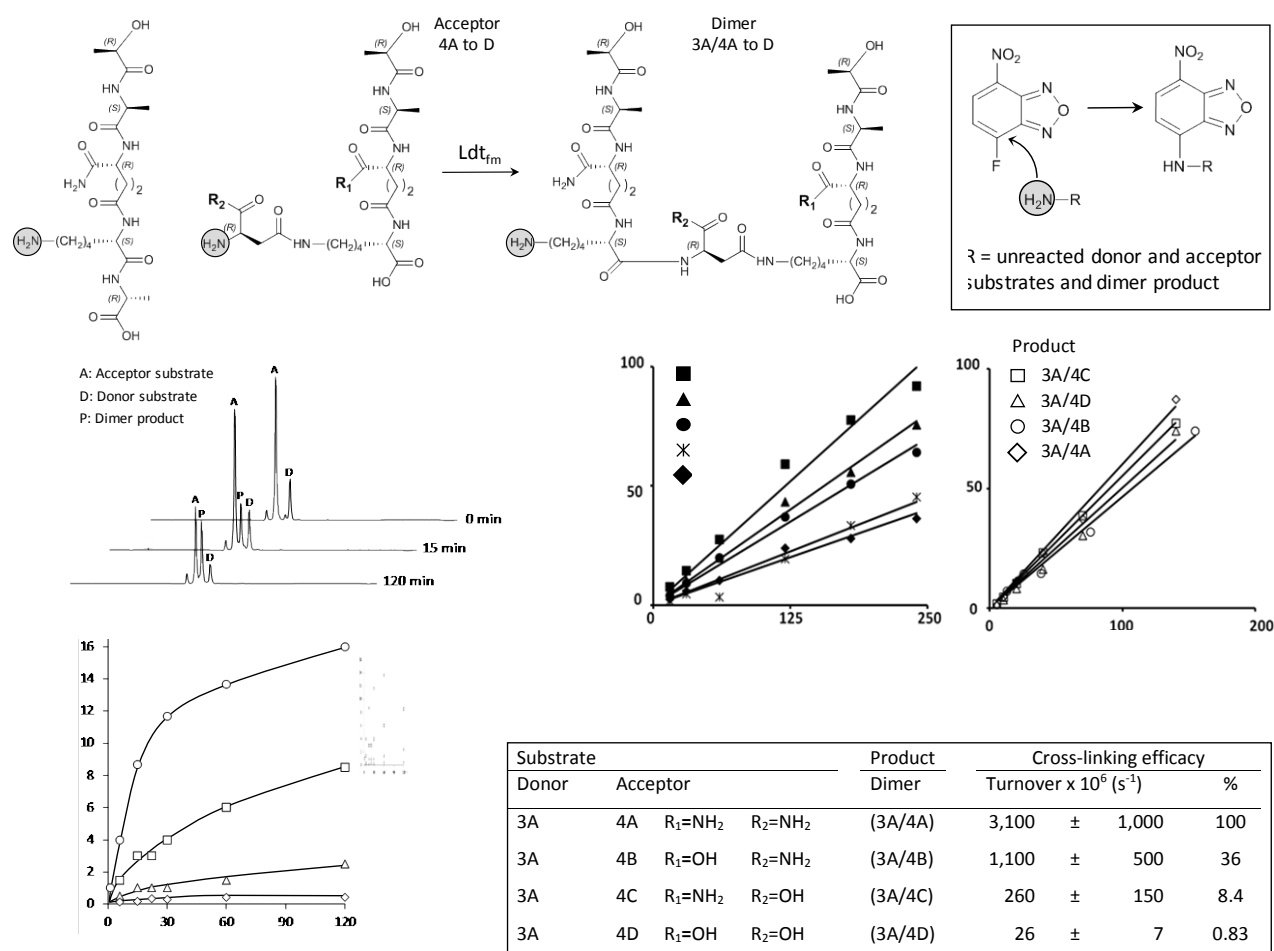


Figure 2. Post-functionalization assay for the cross-linking activity of peptidoglycan transpeptidases. a) Reaction catalyzed by the L,D-transpeptidase Ldt_{fm} . Inset, post-functionalization reaction. b) Separation by *r*PHPLC of the post-functionalized substrates 3A and 4A and of the reaction product (dimer 3A/4A), which were detected by fluorescence (λ_{ex} = 470 nm; λ_{em} = 530 nm). c) Calibration curves for quantitative determination of the substrates (left panel) and reaction products (right panel). d) Kinetics of dimer synthesis by Ldt_{fm} . e) Impact of amidation of the acyl acceptor on the cross-linking efficacy of Ldt_{fm} .

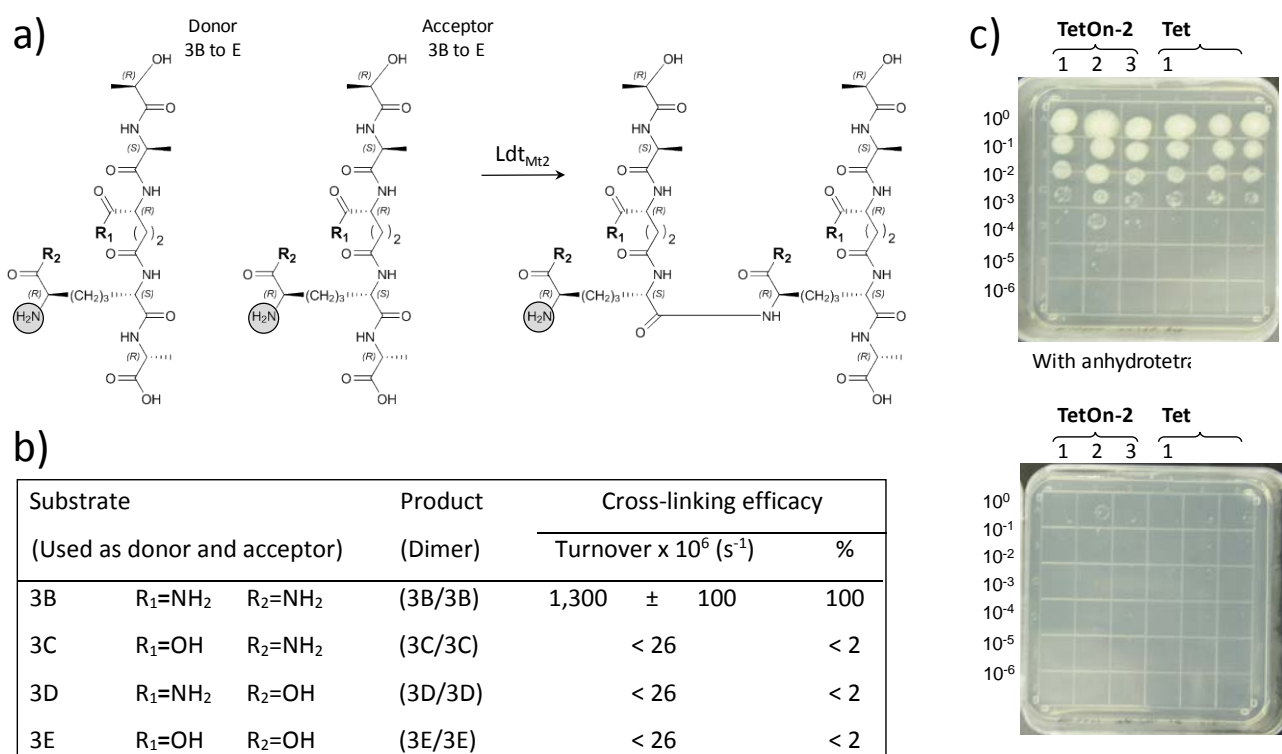


Figure 3. Impact of impaired peptidoglycan precursor amidation on the activity of *M. tuberculosis* L,D-transpeptidase Ldt_{MT2}. a) Transpeptidation reaction catalyzed by Ldt_{MT2}. b) *In vitro* cross-linking activity of Ldt_{MT2}. c) Impact of impaired amidation of DAP by AsnB (Rv2201) on the growth of *M. tuberculosis* H37Rv. Ten-fold dilutions of cultures of mutants Rv2201-TetON-2 and Rv2201-TetON-6 were spotted on the indicated media showing that depletion of AsnB in the absence of anhydrotetracycline prevents growth.

In conclusion, we have developed routes of synthesis of the peptide stems of peptidoglycan precursors that provide access to the full structural diversity found in bacteria, including the presence of DAP or L-Lys at the 3rd position, the presence or absence of a side-chain, and the amidation of carboxyl groups. We have also developed a sensitive assay for the detection of the products of the cross-linking reaction. Based on these new tools, functional analysis was focused on the impact of amidation of carboxyl groups in the D-isoGlu and DAP residues of peptidoglycan precursors on the activity of L,D-transpeptidases. For the first time, we directly establish that the diversification of the structure of peptidoglycan precursors is associated with a parallel evolution of the substrate specificity of cross-linking enzymes. Amidation of carboxyl groups was essential for the *in vitro* activity of L,D-transpeptidases from *E. faecium* and *M. tuberculosis*. Amidation of DAP was also essential for growth of *M. tuberculosis* indicating that the amidotransferase AsnB is a potential target for development of new drugs active on multi-drug resistant bacilli.

Acknowledgements

This work was supported by the project NAPCLI from the JPI AMR program to MA.

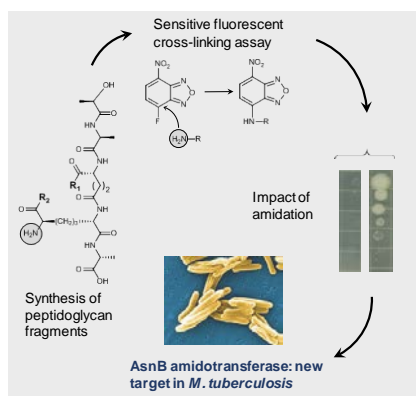
Keywords: Amidation • Amidotransferase • *Mycobacterium tuberculosis* • Peptidoglycan • Transpeptidase

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Layout 1:

COMMUNICATION

Synthetic routes to the peptide stem of peptidoglycan precursors and a sensitive fluorescent cross-linking assay were developed to assess the impact of structural variability on peptidoglycan polymerization. In the search for new targets for anti-mycobacterial drug development, this strategy was applied to the evaluation of diaminopimelate amidation in *Mycobacterium tuberculosis*, revealing the essential role of the AsnB amidotransferase for peptidoglycan transpeptidation both *in vitro* and *in vivo*.



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Supporting Information

Critical impact of peptidoglycan precursor amidation on the activity of L,D-transpeptidases from *Enterococcus faecium* and *Mycobacterium tuberculosis*

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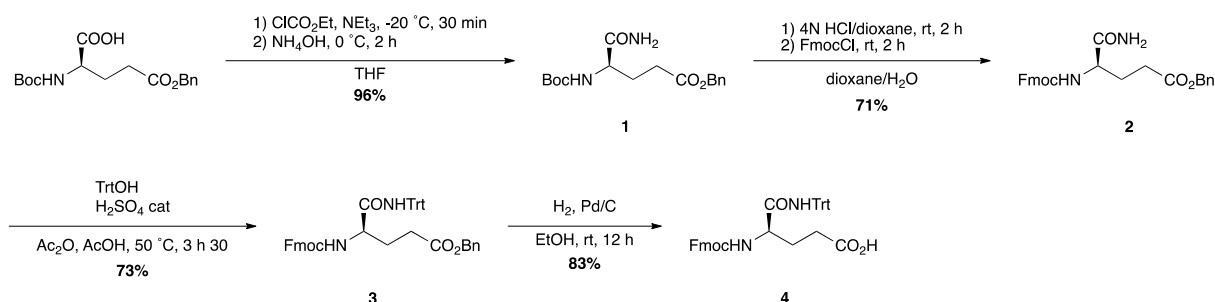
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1- General information

All reagents were obtained from commercial suppliers and used without further purification. Solvents were dried using standard methods and distilled before use. TLC: reconstituted silica gel thin layer sheets 60F₂₅₄ plates (Merck) were used for analytical thin layer chromatography. Flash column chromatography was performed on silica gel 60 (40-63 μm , Merck). Optical rotations were measured with a sodium lamp (589 nm) at 20°C on a Perkin Elmer polarimeter. NMR spectra were recorded at 300 K on Bruker AM250 or Bruker Advance II 500 spectrometers. Chemical shifts (δ) are expressed in ppm relative to the residual solvent resonance and coupling constants (J) are in Hertz (Hz). Multiplicities are reported as follows: singlet (s), doublet (d), triplet (t), multiplet (m), and broad resonance (br). Low resolution mass spectra (LRMS) were obtained on a LCQ Advantage mass spectrometer (ThermoElectron) and high resolution mass spectra (HRMS) were recorded on a TOF mass analyzer under electrospray ionisation.

2- Organic synthesis

2a- Synthesis of Fmoc-D-Glu-NHTrt (Compound 4)



Scheme S1. Synthesis of Fmoc-D-Glu-NHTrt (4).

(R)-Benzyl 5-amino-4-((tert-butoxycarbonyl)amino)-5-oxopentanoate (1)

Ethyl chloroformate (0.31 mL, 3.25 mmol) was added dropwise at -20 °C to a solution of Boc-D-Glu(OBn)-OH (1.00 g, 2.96 mmol) and triethylamine (0.45 mL, 3.25 mmol) in anhydrous THF (15 mL). The solution was stirred for 30 minutes before addition of NH₄OH (28%, 1 mL, 14.80 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 2 hours. The solvent was removed under reduced pressure and the residue dissolved in dichloromethane. The organic phase was washed with a saturated solution of NH₄Cl, dried over MgSO₄, filtered and concentrated to afford Boc-D-Glu(OBn)-NH₂ **1** as a white powder (0.96 g, 96%). ¹H NMR (250 MHz, CDCl₃): δ 7.39 (br s, 5H, Ar-H), 6.23 (br s, 1H, NH), 5.43 (br s, 1H, NH), 5.28 (d, *J* = 7.2 Hz, 1H, NH), 5.16 (s, 2H, CH₂), 4.26-4.19 (m, 1H, CH), 2.68-2.43 (m, 2H, CH₂), 2.27-2.13 (m, 1H, CH₂), 2.03-1.88 (m, 1H, CH₂), 1.46 (s, 9H, CH₃-Boc). ¹³C NMR (125 MHz, CDCl₃): δ 175.7 (C=O), 172.8 (C=O), 156.3 (C=O), 136.1, 128.1, 127.7, 79.3 (C-Boc), 65.9 (CH₂), 53.5 (CH), 30.0 (CH₂), 27.3 (CH₃), 27.2 (CH₂). LRMS: Calcd for C₁₇H₂₄N₂NaO₅ [M+Na]⁺ 359.3; Found: 359.3.

(R)-Benzyl 4-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-amino-5-oxopentanoate (2)

A solution of Boc-D-Glu(OBn)-NH₂ **1** (0.94 g, 2.85 mmol) in HCl (4 N) /dioxane (21 mL) was stirred at room temperature for 2 hours. The reaction mixture was concentrated and the residue was dissolved in H₂O/dioxane (80 mL, v/v). NaHCO₃ (1.43 g, 17.10 mmol) and FmocCl (0.96 g, 3.70 mmol) were then added. After 2 hours stirring at room temperature, EtOAc was added to the mixture. The organic phase was washed with a saturated solution of NaHCO₃, dried over MgSO₄, filtered, and concentrated under reduced pressure. A silica gel chromatography (cyclohexane/EtOAc, 8:2) was performed on the residue to obtain the Fmoc-D-Glu(OBn)-NH₂ **2** as a white powder (0.86 g, 71%). [α]_D - 2.2° (10 mg/mL, DMF). ¹H NMR (250 MHz, DMSO): δ 7.89 (d, *J* = 7.2 Hz, 2H, Ar-H), 7.73 (d, 2H, Ar-H), 7.47-7.29 (m, 9H, Ar-H), 7.07 (s, 1H, NH), 5.10 (s, 2H, CH₂), 4.30-4.21 (m, 3H, CH₂-Fmoc and CH-Fmoc), 4.06-3.92 (s, 1H, CH), 2.39 (t, *J* = 7.2 Hz, 2H, CH₂-CO), 2.00-1.76 (m, 2H, CH₂-CH). ¹³C NMR (125 MHz, CDCl₃): δ 173.2 (C=O), 172.1

(C=O), 155.9 (C=O), 143.8, 143.7, 140.7, 136.1, 128.4, 127.9, 127.8, 127.6, 127.0, 125.2, 120.0, 65.6 (CH₂), 65.4 (CH₂), 53.7 (CH), 46.7 (CH), 30.2 (CH₂), 27.1 (CH₂). LRMS: Calcd for C₂₇H₂₆N₂NaO₅ [M+Na]⁺ 481.4; Found: 481.3.

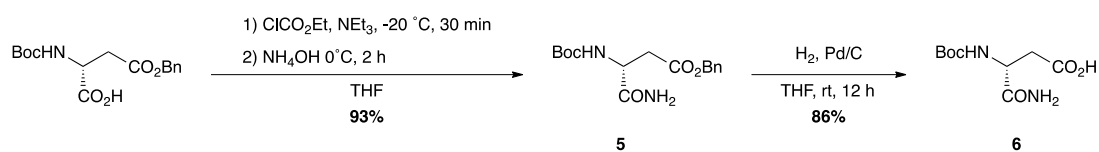
(R)-Benzyl 4-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-oxo-5-(tritylamino)pentanoate (3)

To a mixture of Fmoc-D-Glu(OBn)-NH₂ (**2**) (0.68 g, 1.60 mmol) and trityl alcohol (4.16 g, 16.00 mmol) in acetic acid (5.6 mL), sulfuric acid (51 μL, 0.96 mmol) and acetic anhydride (4.00 mmol) were added at 50 °C. The reaction mixture was stirred at 50 °C for 3.5 hours and diluted with EtOAc. The organic phase was washed with a saturated solution of NaHCO₃, dried over MgSO₄, filtered and concentrated under reduced pressure. Silica gel chromatography (cyclohexane/EtOAc, 7:3) afforded Fmoc-D-Glu(OBn)-NHTrt **3** as a white powder (0.82 g, 73%). [α]_D + 31.2° (10 mg/mL, MeOH). ¹H NMR (250 MHz, CDCl₃): δ 7.78 (d, 2H, *J* = 7.5 Hz, Ar-H), 7.60-7.57 (m, 2H, Ar-H), 7.46-7.28 (m, 24H, Ar-H), 5.64-5.62 (m, 1H, NH), 5.14 (br s, 2H, CH₂), 4.40 (m, 3H, CH₂-Fmoc and CH-Fmoc or CH), 4.22-4.19 (m, 1H, CH-Fmoc or CH), 2.61-1.87 (m, 4H, 2 CH₂). ¹³C NMR (125 MHz, CDCl₃): δ 172.8 (C=O), 171.6 (C=O), 157.2 (C=O), 144.2, 143.8, 143.2, 141.2, 141.1, 136.1, 128.4, 128.1, 127.8, 127.7, 127.3, 126.7, 126.5, 124.8, 124.7, 119.5, 70.1 (C_q), 66.6 (CH₂), 66.0 (CH₂), 54.6 (CH), 48.4 (CH), 29.8 (CH₂), 26.2 (CH₂). LRMS: Calcd for C₄₆H₄₀N₂NaO₅ [M+Na]⁺ 723.2; Found: 722.9.

(R)-4-(((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-5-oxo-5-(tritylamino)pentanoic acid (4)

10 wt. % Pd/C (0.04 g, 0.38 mmol) was added to a solution of Fmoc-D-Glu(OBn)-NHTrt **3** (0.40 g, 0.57 mmol) in anhydrous THF (6 mL). The solution was hydrogenated overnight under atmospheric pressure. The catalyst was removed by filtration through celite and the filtrate was evaporated. Silica gel chromatography (dichloromethane/MeOH, 95:5) afforded the Fmoc-D-Glu-NHTrt **4** as a white powder (0.29 g, 83%). [α]_D + 42.6° (10 mg/mL, MeOH). ¹H NMR (250 MHz, CDCl₃): δ 7.78 (d, *J* = 5.2 Hz, 2H, Ar-H), 7.60-7.57 (m, 2H, Ar-H), 7.55-7.42 (m, 2H, Ar-H), 7.39-7.23 (m, 17H, Ar-H), 5.66 (d, *J* = 6.5 Hz, 1H, NH), 4.49-4.39 (m, 3H, CH₂-Fmoc and CH), 4.22-4.16 (t, *J* = 6.8 Hz, 1H, CH-Fmoc), 2.53-2.07 (m, 3H, CH₂), 1.95-1.83 (m, 1H, CH₂). ¹³C NMR (125 MHz, CDCl₃): δ 176.4 (C=O), 170.4 (C=O), 156.8 (C=O), 144.1, 143.7, 143.6, 141.3, 128.6, 127.9, 127.8, 127.1, 127.0, 125.1, 120.0, 70.6 (C_q), 67.5 (CH₂), 54.2 (CH), 47.0 (CH), 29.7 (CH₂), 27.7 (CH₂). HRMS: Calcd for C₃₉H₃₄N₂NaO₅ [M+Na]⁺ 633.2360; Found: 633.2411.

2b- Synthesis of Boc-D-Asp-NH₂ (compound 6)



Scheme S2. Synthesis of Boc-D-Asp-NH₂ (6).

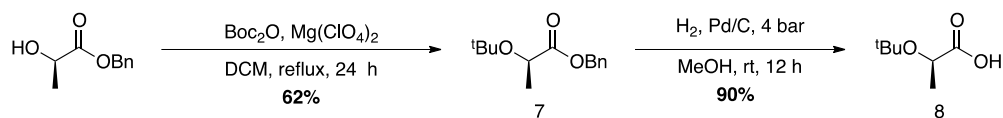
(*R*)-Benzyl 4-amino-3-((*tert*-butoxycarbonyl)amino)-4-oxobutanoate (5)

Ethyl chloroformate (0.15 mL, 1.70 mmol) was added dropwise at -20 °C to a solution of Boc-D-Asp(OBn)-OH (0.50 g, 1.55 mmol) and triethylamine (0.23 mL, 1.70 mmol) in anhydrous THF (15 mL). The solution was stirred for 30 minutes. NH₄OH (28%, 0.52 mL, 7.75 mmol) was added at 0 °C and the reaction mixture was stirred at 0 °C for 2 hours. The solvent was removed under reduced pressure and the residue was dissolved in dichloromethane. The organic phase was washed with water, dried over MgSO₄, filtered and concentrated affording Boc-D-Asp(OBn)-NH₂ **5** as a white powder (0.46 g, 93%). ¹H NMR (250 MHz, CDCl₃): δ 7.38 (br s, 5H, Ar-H), 6.45 (br s, 1H, NH), 5.70 (br s, 1H, NH), 5.43 (br s, 1H, NH), 5.17 (s, 2H, CH₂), 4.56 (br s, 1H, CH), 3.10 (dd, *J* = 17.0 Hz, *J* = 4.0 Hz, 1H, CH₂), 2.74 (dd, 1H, CH₂), 1.48 (s, 9H, CH₃-Boc). The spectroscopic data were consistent with the literature data for the *S* enantiomer.^[1]

(*R*)-4-Amino-3-((*tert*-butoxycarbonyl)amino)-4-oxobutanoic acid (6)

10 wt. % Pd/C (0.014 g, 0.13 mmol) was added to a solution of Boc-D-Asp(OBn)-NH₂ **5** (0.46 g, 1.43 mmol) in anhydrous THF (16 mL). The reaction mixture was hydrogenated overnight under atmospheric pressure. The solution was filtered through celite and evaporated under reduced pressure. Flash chromatography (Dichloromethane/MeOH, 9:1) afforded Boc-D-Asp-NH₂ **6** as a white powder (0.28 g, 86%). [α]_D + 29.4° (10 mg/mL, DMF). ¹H NMR (250 MHz, DMSO): δ 12.29 (br s, 1H, CO₂H), 7.18 (br s, 1H, NH), 7.03 (br s, 1H, NH), 6.93 (d, *J* = 8.2 Hz, 1H, NH), 4.25-4.16 (m, 1H, CH), 2.62 (dd, *J* = 16.2 Hz, *J* = 5.2 Hz, 1H, CH₂), 2.44 (dd, 1H, CH₂), 1.49 (s, 9H, CH₃-Boc). ¹³C NMR (125 MHz, CDCl₃): δ 173.0 (C=O), 171.9 (C=O), 155.1, (C=O), 78.1 (Cq), 50.8 (CH), 36.3 (CH₂), 28.1 (CH₃-Boc). LRMS: Calcd for C₉H₁₅N₂O₅ [2M-H]⁻ 463.1; Found: 463.1.

2c- Synthesis of D-lactic acid derivative (compound 8)



Scheme S3. Synthesis of D-lactic acid derivative (8).

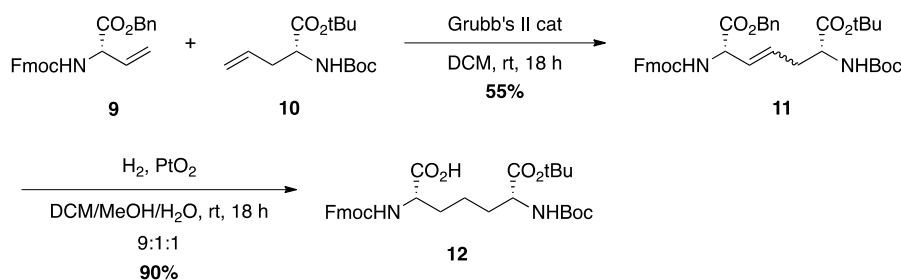
(*R*)-Benzyl 2-(*tert*-butoxy)propanoate (7)

A solution of D-lactic acid-benzyl ester (5.00 g, 27.74 mmol) in dry DCM (18 mL) was treated with di-*tert*-butyl dicarbonate (15.14 g, 69.40 mmol) and magnesium perchlorate (0.63 g, 2.80 mmol), and the mixture was refluxed for 12 hours. The solution was poured into a saturated aqueous NaHCO_3 solution and the mixture was extracted with EtOAc. The organic layer was washed with water, dried with brine and over MgSO_4 , filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (silica gel, cyclohexane/AcOEt 75/25) affording O-*tert*-Bu-D-lactic acid benzyl ester **7** as a colorless oil (4.00 g, 62%). $[\alpha]_{\text{D}} + 35.2^\circ$ (10 mg/mL, MeOH). ^1H NMR (500 MHz, CDCl_3): δ 7.34-7.29 (m, 5H, Ar-H), 5.14 (dd, $J = 12.5$ Hz, 2H, CH_2), 4.14 (q, $J = 7$ Hz, 1H, CH), 1.33 (d, $J = 7$ Hz, 3H, CH_3), 1.15 (s, 9H, CH_3 -*t*Bu), ^{13}C NMR (125 MHz, CDCl_3): δ 174.8 (C=O), 135.8 (C-Ar), 128.5 (CH-Ar), 128.2 (CH-Ar), 128.2 (CH-Ar), 74.9 (C_q), 67.5 (CH), 66.4 (CH_2), 27.7 (CH_3 -*t*Bu), 20.5 (CH_3). LRMS: Calcd for $\text{C}_{14}\text{H}_{20}\text{NaO}_3$ $[\text{M}+\text{Na}]^+$ 254.1; Found: 254.0.

(*R*)-2-(*tert*-Butoxy)propanoic acid (8)

A suspension of compound **7** (1.80 g, 7.62 mmol) in MeOH (75 mL) was treated with 10 wt. % Pd/C (0.26 g, 2.44 mmol) under 4 bar H_2 . The reaction mixture was stirred for 12 hours at room temperature. The residue was filtered through celite and concentrated under reduced pressure to afford D-lactic acid derivative **8** as a colorless oil (1.00 g, 90%). $[\alpha]_{\text{D}} + 42.3^\circ$ (10 mg/mL, EtOH). ^1H NMR (500 MHz, CDCl_3): δ 4.12 (q, $J = 7$ Hz, 1H, CH), 1.36 (d, $J = 7$ Hz, 3H, CH_3), 1.20 (s, 9H, *t*Bu). ^{13}C NMR (125 MHz, CDCl_3): δ 175.3 (C=O), 75.2 (C_q), 66.4 (CH), 26.8 (CH_3 -*t*Bu), 19.4 (CH_3). LRMS: Calcd for $\text{C}_7\text{H}_{15}\text{O}_3^+$ $[\text{M}+\text{H}]^+$ 147.1; Found: 146.9.

2d- Synthesis of DAP derivative (compound 12)



Scheme S4. Synthesis of DAP derivative (12).

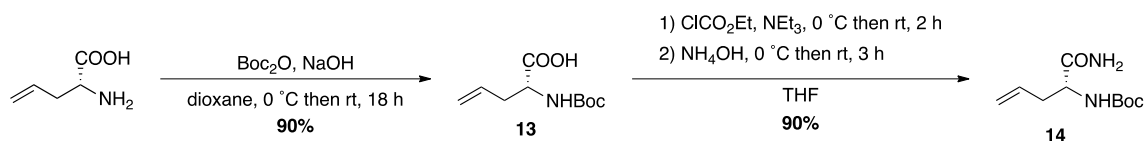
(2*S*,6*R*,*E*,*Z*)-1-Benzyl 7-*tert*-butyl 2-(((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-6-((*tert*-butoxycarbonyl)amino)hept-3-enedioate (11)^[2]

A solution of vinyl-glycine **9**² (0.42 g, 1.06 mmol) in dichloromethane (10 mL) was treated with allyl-glycine **10**^[3] (0.24 g, 0.88 mmol) and Grubb's second generation catalyst (0.04 g, 0.04 mmol). The solution was stirred for 18 hours at room temperature. Dichloromethane was removed under reduced pressure. The obtained residue was purified by flash chromatography (Cyclohexane/EtOAc, 7:3) affording olefin **11** as a colorless oil (0.32 g, 55%). ¹H NMR (500 MHz, CDCl₃): δ 7.80 (d, *J* = 7.5 Hz, 2H, Ar-H), 7.64 (d, *J* = 5 Hz, 2H, Ar-H), 7.46-7.34 (m, 9H, Ar-H), 5.72-5.68 (m, 2H, H_{allyl}), 5.53 (d, *J* = 7.5 Hz, 1H, NH), 5.28-5.10 (m, 3H, CH₂-Bn and NH), 4.99-4.94 (m, 1H, CH), 4.45-4.39 (m, 2H, CH₂-Fmoc), 4.30-4.22 (m, 2H, 2 CH), 2.62-2.43 (m, 2H, CH₂), 1.45 (s, 18H, CH₃-^tBu/Boc). HRMS: Calcd for C₃₈H₄₅N₂O₈ [M+H]⁺ 657.3170; Found: 657.3176.

(2*S*,6*R*)-2-(((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-7-(*tert*-butoxy)-6-((*tert*-butoxycarbonyl)amino)-7-oxoheptanoic acid (12)^[2]

The product **11** (0.32 g, 0.49 mmol) was dissolved in DCM/MeOH/H₂O (9:1:1 v/v/v) (33 mL) and PtO₂ (0.004 g, 0.02 mmol) was added under H₂ atmosphere. The reaction mixture was allowed to stir for 18 hours at room temperature. The solution was filtered through celite and concentrated under reduced pressure. The obtained residue was purified by flash chromatography (dichloromethane/MeOH, 9:1) to afford protected diaminopimelic acid (DAP) **12** as a white powder (0.29 g, 90%). ¹H NMR (500 MHz, MeOD): δ 7.75 (d, *J* = 7.8 Hz, 2H, Ar-H), 7.64 (d, *J* = 7.8 Hz, 2H, Ar-H), 7.39-7.26 (m, 2H, Ar-H), 7.00-6.79 (m, 2H, Ar-H), 4.36-4.31 (m, 2H, CH₂-Fmoc or 2 CH), 4.21-4.07 (m, 2H, CH₂-Fmoc or 2 CH), 3.97-3.93 (m, 1H, CH), 1.89-1.59 (m, 6H, CH₂), 1.44 (s, 9H, CH₃), 1.43 (s, 9H, CH₃). ¹³C NMR (125 MHz, MeOD): δ 173.8 (C=O), 158.5 (C=O), 158.1 (C=O), 145.4, 145.2, 142.6, 128.7, 128.2, 126.2, 120.9, 82.5 (C_q), 80.4 (C_q), 67.8 (CH₂-Fmoc), 55.9 (CH), 54.7 (CH), 49.7 (CH-Fmoc), 32.5 (CH₂), 30.6 (CH₂), 28.7 (CH₃), 28.3 (CH₃) 23.4 (CH₂). HRMS: Calcd for C₃₁H₄₁N₂O₈ [M+H]⁺ 569.2857; Found: 569.2861.

2e- Synthesis of D-Allyl-Glycine (**14**)



Scheme S5. Synthesis of D-Allyl-Glycine (**14**).

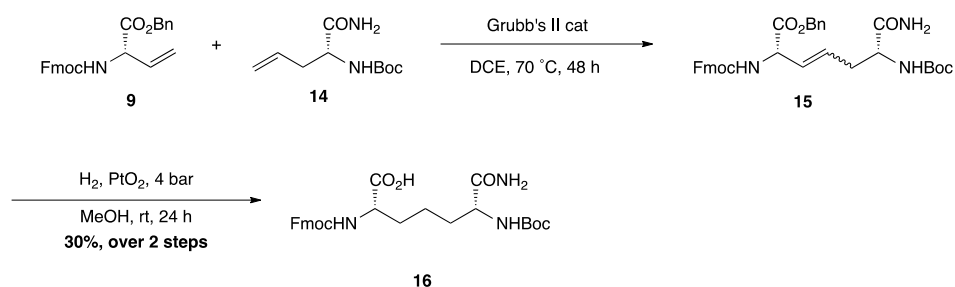
(*R*)-2-((*tert*-Butoxycarbonyl)amino)pent-4-enoic acid (**13**)^[4]

To a solution of commercially available (2S)-2-aminopent-4-enoic acid (1.00 g, 8.70 mmol) in 1 M NaOH (20 mL) and dioxane (10 mL) at $0\text{ }^\circ\text{C}$ was added di-*t*butyl dicarbonate (2.28 g, 10.5 mmol). The reaction mixture was allowed to warm to room temperature and stirred for an additional 18 hours. The pH was checked and adjusted to basic when necessary. The reaction mixture was concentrated under reduced pressure and the aqueous phase washed with Et_2O (2 x 10 mL). The aqueous phase was acidified to pH = 2 with 2 M H_2SO_4 and extracted with EtOAc (4 x 20 mL) while saturating the aqueous phase each time with NaCl. The combined organic layers were dried over MgSO_4 , filtered and concentrated under reduced pressure to afford the product **13** as a colorless oil (1.68 g, 90%). ^1H NMR (250 MHz, CDCl_3): δ 5.75-5.61 (d, $J = 15\text{ Hz}$, 1H, H_{allyl}), 5.14-5.03 (m, 2H, H_{allyl}), 4.47-4.40 (m, 1H, CH), 2.49-2.43 (m, 2H, CH_2), 1.33 (s, 9H, $\text{CH}_3\text{-Boc}$).

(*R*)-*tert*-Butyl (1-amino-1-oxopent-4-en-2-yl)carbamate (**14**)

To a solution of compound **13** (1.00 g, 4.67 mmol) in dry THF (40 mL), triethylamine (0.71 mL, 5.12 mmol) and ethyl chloroformate (0.43 mL, 5.6 mmol) were added at $0\text{ }^\circ\text{C}$. The reaction mixture was warmed to room temperature and stirred for 2 hours. The reaction mixture was then cooled to $0\text{ }^\circ\text{C}$ and treated dropwise with a solution of NH_4OH (28%, 1 mL, 14.80 mmol). The solution was stirred for extra 3 hours at room temperature. THF was removed under reduced pressure and the residue was dissolved in dichloromethane. The reaction mixture was washed with brine, dried over MgSO_4 , filtered and concentrated under reduced pressure. The obtained residue was purified by flash chromatography (Cyclohexane/ EtOAc , 7:3) to afford the allyl-glycine **14** as a white solid (0.90 g, 90%). $[\alpha]_{\text{D}} + 7^\circ$ (10 mg/mL, CHCl_3). ^1H NMR (500 MHz, CDCl_3): δ 6.08 (br s, 1H, NH), 5.84-5.68 (m, 1H, H_{allyl}), 5.44 (br, 1H, NH), 5.19-5.14 (m, 2H, H_{allyl}), 4.16-4.13 (m, 1H, CH), 2.52-2.47 (m, 2H, CH_2), 1.42 (s, 9H, $\text{CH}_3\text{-Boc}$). ^{13}C NMR (125 MHz, MeOD): δ 175.7 (C=O), 156.2 (C=O), 133.2 (C= CH_2), 117.1 ($\text{CH}_2=\text{CH}$), 79.0 (C_q), 54.0 (CH), 36.3 (CH_2), 26.9 (CH_3). HRMS: Calcd for $\text{C}_{10}\text{H}_{19}\text{N}_2\text{O}_3$ $[\text{M}+\text{H}]^+$ 215.1390; Found: 215.1387.

2f- Synthesis of amidated DAP (16)



Scheme S6. Synthesis of amidated DAP (**16**).

(2S,6R,E,Z)-Benzyl 2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-7-amino-6-((tert-butoxycarbonyl) amino)-7-oxohept-3-enoate (15**)^[5]**

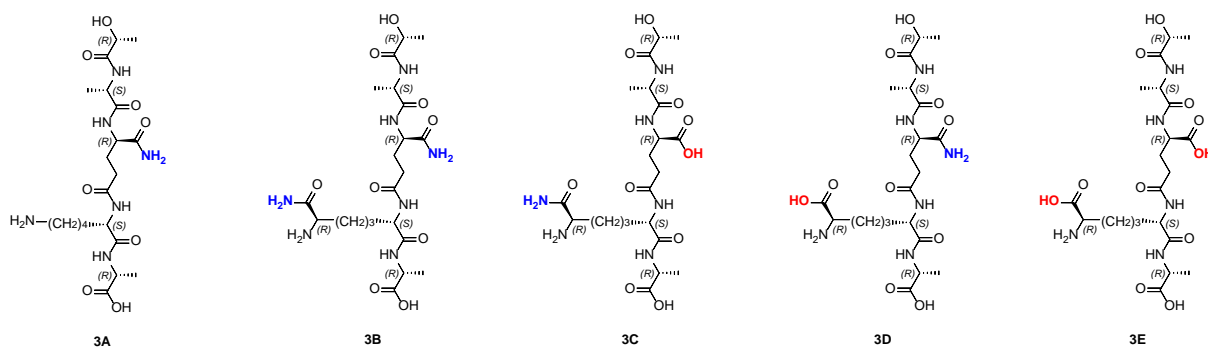
A solution of vinyl-glycine **9**^[2] (0.22 g, 0.53 mmol) in dichloroethane (10 mL) was treated with allyl-glycine **14** (0.27 g, 1.06 mmol) and Grubb's Second Generation Catalyst (0.09 g, 0.16 mmol). The reaction mixture was stirred for 48 hours at 70 °C. The solution was cooled to room temperature, treated with DMSO (0.5 mL), and stirred for additional 8 hours. The solution was diluted in dichloromethane (100 mL), washed with water (2 x 20 mL), dried over MgSO₄, filtered and concentrated under reduced pressure. The obtained residue was purified by flash chromatography (DCM/MeOH, 9:1) to afford a mixture (0.14 g) of **15** and unreacted **14** with a ratio of 1:2 (**15**:**14**) which was engaged in the next step. ¹H NMR (500 MHz, CDCl₃): δ 7.81 (d, *J* = 7.5 Hz, 2H, Ar-H), 7.63 (d, *J* = 7.5 Hz, 2H, Ar-H), 7.46-7.38 (m, 9H, Ar-H), 6.1 (br, 1H, NH), 5.79 (br, 1H, NH), 5.69-5.68 (m, 2H, H_{allyl}), 5.30 (br, 1H, NH), 5.18 (s, 2H, CH₂-Ar), 4.89 (m, 1H, CH-Fmoc or CH), 4.43-4.31 (m, 2H, CH₂-Fmoc), 4.26-4.10 (m, 1H, CH-Fmoc or CH), 2.63-2.37 (m, 2H, CH₂), 1.46 (s, 9H, CH₃-Boc). LRMS: Calcd for C₃₄H₃₈N₃O₇ [M+H]⁺ 600.2; Found: 600.1.

(2S,6R)-2-(((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-7-amino-6-((tert-butoxycarbonyl)amino)-7-oxoheptanoic acid (16**)**

The mixture **14**:**15** (0.22 g) was dissolved in MeOH (15 mL) and treated with PtO₂ (0.10 g, 0.44 mmol) under 4 bar H₂. The reaction mixture was stirred for 24 hours at room temperature. The mixture was filtrated through celite and concentrated under reduced pressure. The obtained residue was purified by flash chromatography (DCM/MeOH/H₂O, 8:2:1) to afford amidated DAP **16** as a white powder (0.06 g, 30% over 2 steps). [α]_D + 2° (5 mg/mL, MeOH). ¹H NMR (500 MHz, MeOD): δ 7.76 (d, *J* = 7.5 Hz, 2H, Ar-H), 7.65-7.62 (m, 2H, Ar-H), 7.37-7.27 (m, 4H, Ar-H), 4.33-4.31 (m, 2H, CH₂-Fmoc), 4.20-4.17 (m, 1H, CH-Fmoc), 4.05-3.99 (m, 2H, 2CH), 1.89-1.49 (m, 6H, CH₂) 1.40 (s, 9H, CH₃-Boc). ¹³C NMR (125 MHz, MeOD): δ 178.1 (C=O), 158.5 (C=O), 157.8 (C=O), 145.4 (C_q), 145.2 (C_q), 142.6 (C_q), 128.8 (CH-Fmoc), 128.2 (CH-Fmoc), 126.2 (CH-Fmoc), 120.9 (CH-Fmoc), 80.6 (C_q), 67.9 (CH₂-Fmoc), 56.6 (CH), 55.8 (CH),

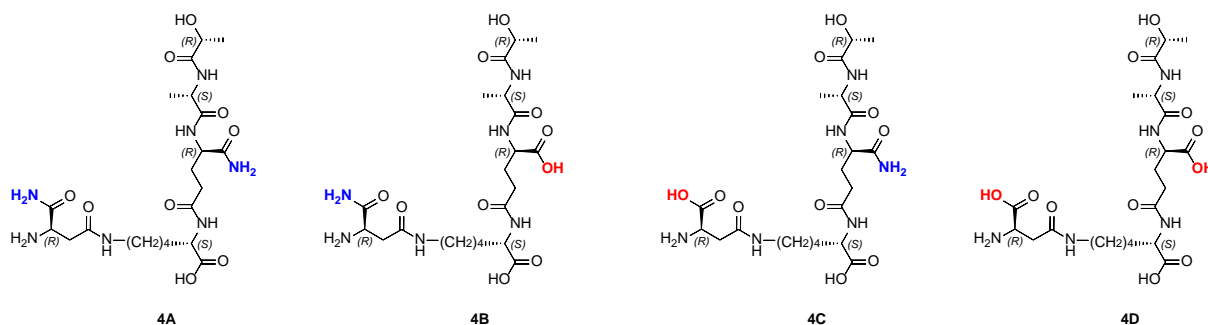
48.4 (CH-Fmoc), 33.2 (CH₂), 33.1 (CH₂), 28.7 (CH₃-Boc), 23.30 (CH₂). HMRS: Calcd for C₂₇H₃₂N₃O₇ [M - H]⁻ 510.2246; Found: 510.2245.

3- Solid-phase synthesis of linear peptides (3A to 3E)



Peptides were manually assembled on a Fmoc-D-Ala-Wang resin (0.80 mmol.g⁻¹, 75 mg). Coupling reactions were performed in 2 mL of anhydrous DMF containing 3 or 1.5 molar equivalent excess of commercial and non-commercial Fmoc-amino acids, respectively, 3 equivalents of K-Oxyma, and 6 equivalents of DIC and DIPEA. Each coupling was performed twice for 5 hours and 12 hours. Fmoc was removed twice (5 minutes and 7 minutes) with a 20% solution of piperidine in DMF. Final deprotection of acid labile protecting groups and cleavage from the resin were carried out under gentle agitation at room temperature for 2 hours with 2 mL of a TFA solution containing DCM, TIPS and water (80:20:5:5 v/v/v/v). The solution was filtered and the resin was washed with 1 mL of TFA. The TFA solutions were pooled and evaporated under reduced pressure. The crude product was dissolved in a 10% acetic acid solution (5 mL) and extracted with chloroform (3 x 10 mL). The aqueous solution was lyophilized and the peptides were purified by *rp*HPLC on a 5 μm Nucleosil preparative C-18 column (22 x 250 mm) using a linear gradient (0 to 100% buffer B) applied between 10 minutes and 40 minutes (Buffer A: 0.1 % TFA in H₂O; buffer B: 0.1 % TFA in CH₃CN; 10 mL.min⁻¹). Peptide-containing fractions were identified at 214 nm and analyzed by mass spectrometry (Supplementary figure S1). Peptides were lyophilized and dissolved in water at a final concentration of 10 mg.mL⁻¹. Purity was assessed by *rp*HPLC (analytical C18 Nucleosil column, 3 μm, 4.6 x 250 mm) using a linear gradient (0 to 50 buffer B) applied between 10.5 and 40.5 min (Buffer A: 0.1 % TFA in H₂O; buffer B: 0.1 % TFA in CH₃CN; 1 mL.min⁻¹) (Supplementary figure S2). Peptide concentration was determined in duplicate by acid hydrolysis and injection into a Hitachi L8800 amino acid analyzer equipped with a 2620 MSC-PS column.

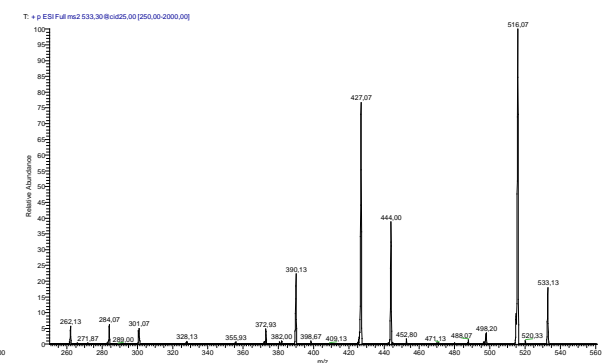
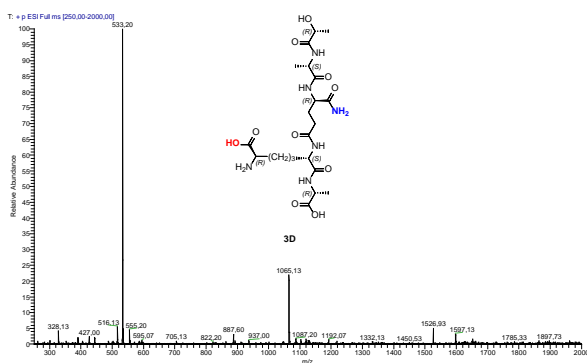
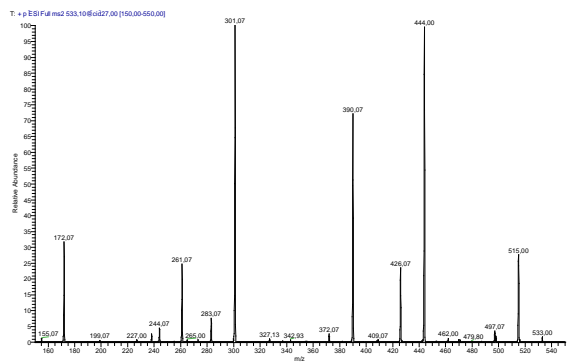
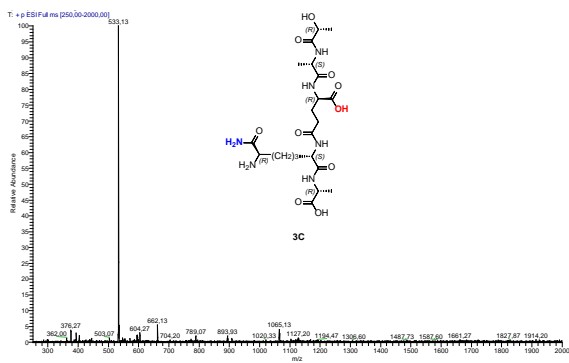
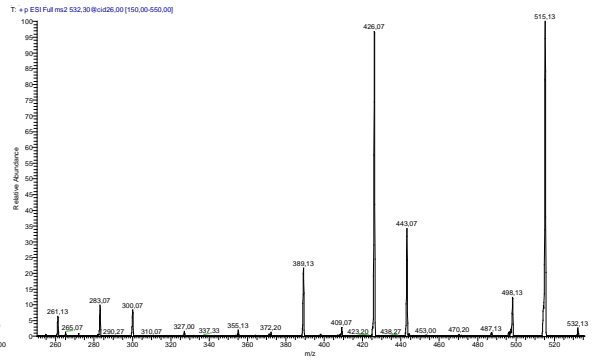
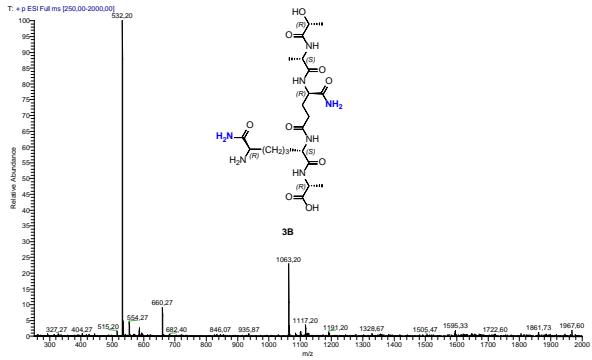
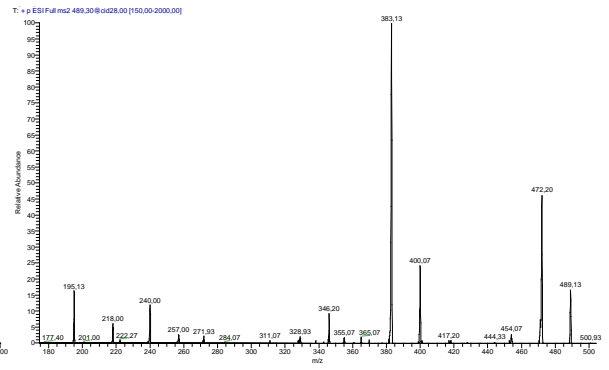
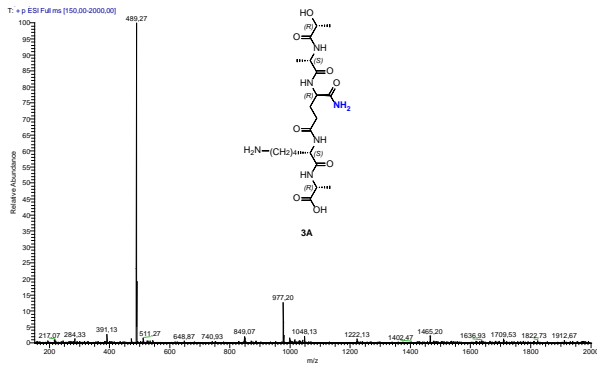
4- Solid-phase synthesis of branched peptides (4A to 4D)

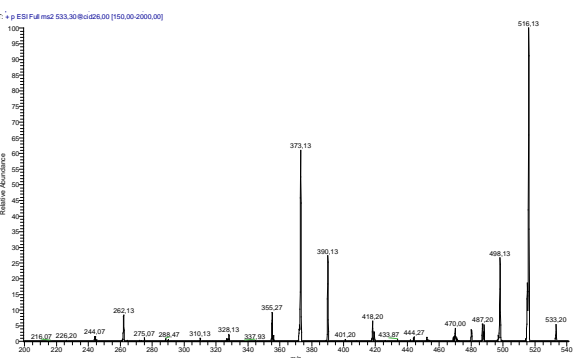
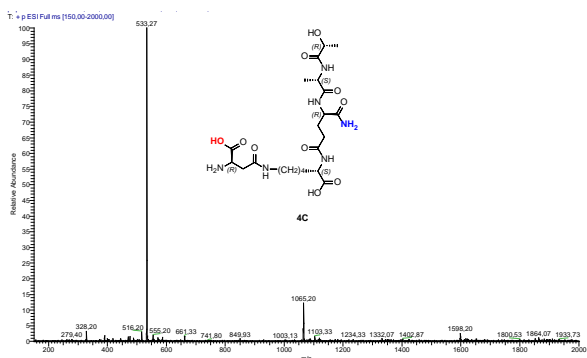
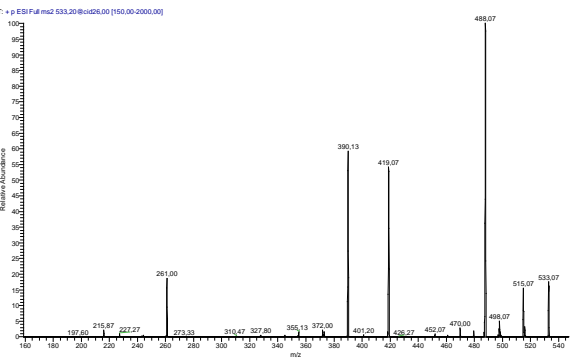
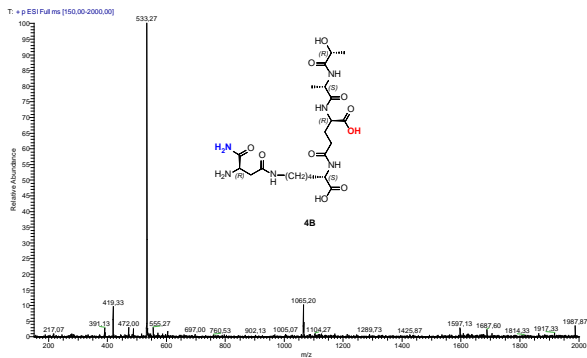
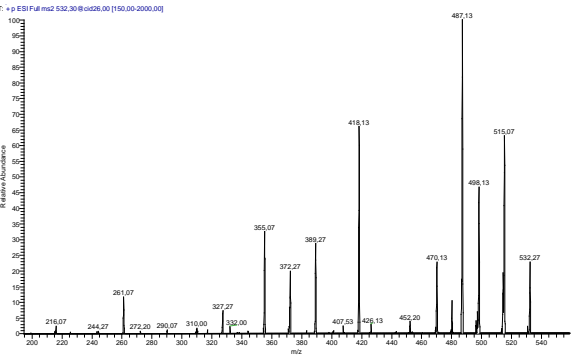
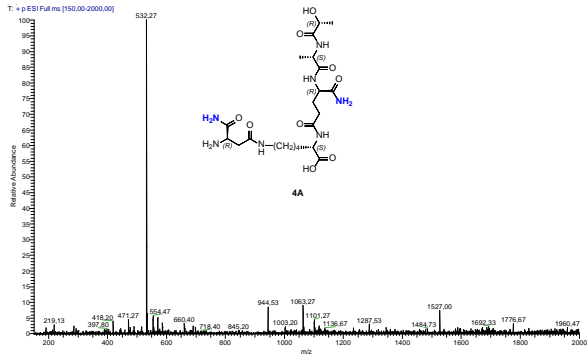
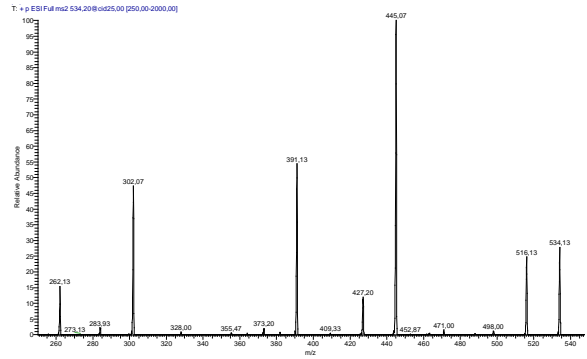
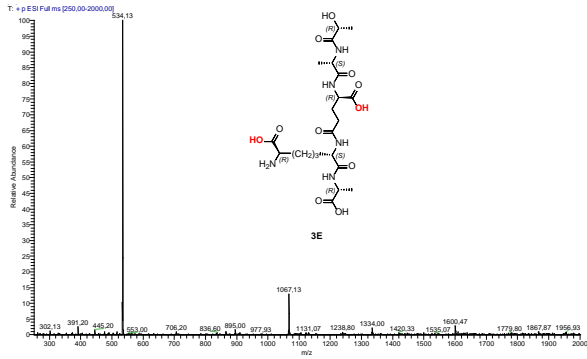


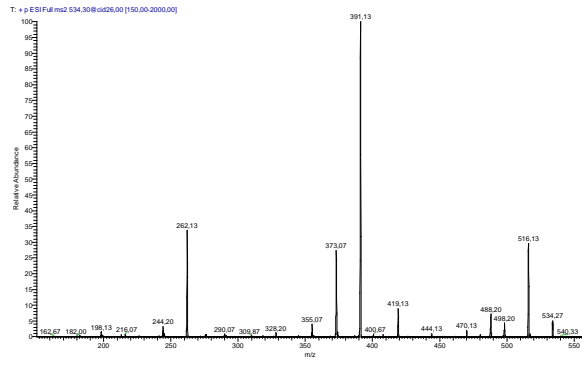
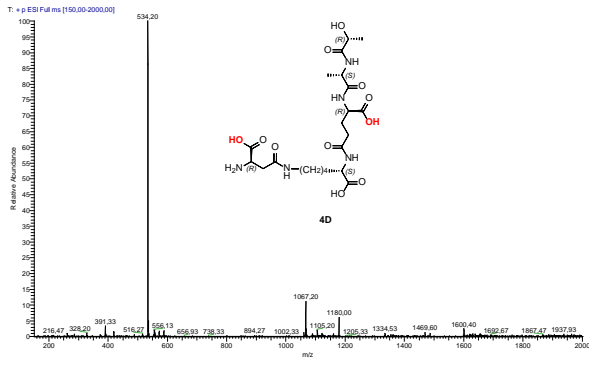
Branched peptides were synthesized via divergent Fmoc solid phase peptide synthesis using orthogonal protecting groups on the lysine residue. The starting material was a commercial Wang resin containing L-Lys with the α and ϵ NH_2 groups protected with Fmoc and ivDde groups, respectively. Peptides were manually assembled on the resin (0.69 mmol.g^{-1} , 75 mg), starting with the linear tripeptide to afford $\text{O}^t\text{Bu-D-Lac-L-Ala-D-iGlx-L-Lys(ivDde)-WANG}$ resin, as described above. Then, the ϵ NH_2 group of L-Lys was deprotected by hydrazinolysis of the ivDde protective group (5% of hydrazine mono-hydrate in DMF, 3 x 15 minutes). Fmoc-D-isoAsn or Fmoc-D-isoAsp was incorporated and the final deprotection steps were performed as described above.

5- Mass spectrometry analyses of the peptide

Peptide	Formula for $[\text{M}+\text{H}]^+$	Calc.	Found
3A	$\text{C}_{20}\text{H}_{37}\text{N}_6\text{O}_8^+$	489.27	489.27
3B	$\text{C}_{21}\text{H}_{38}\text{N}_7\text{O}_9^+$	532.27	532.20
3C	$\text{C}_{21}\text{H}_{37}\text{N}_6\text{O}_{10}^+$	533.26	533.13
3D	$\text{C}_{21}\text{H}_{37}\text{N}_6\text{O}_{10}^+$	533.26	533.20
3E	$\text{C}_{21}\text{H}_{36}\text{N}_5\text{O}_{11}^+$	534.24	534.13
4A	$\text{C}_{21}\text{H}_{38}\text{N}_7\text{O}_9^+$	532.27	532.27
4B	$\text{C}_{21}\text{H}_{37}\text{N}_6\text{O}_{10}^+$	533.26	533.27
4C	$\text{C}_{21}\text{H}_{37}\text{N}_6\text{O}_{10}^+$	533.26	533.27
4D	$\text{C}_{21}\text{H}_{36}\text{N}_5\text{O}_{11}^+$	534.24	534.20

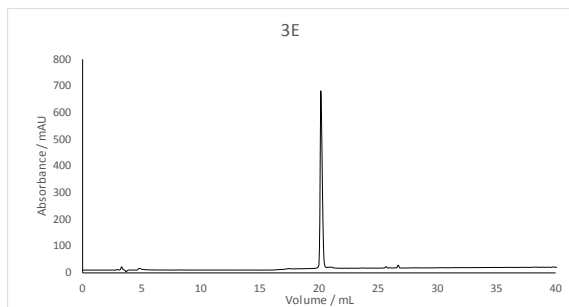
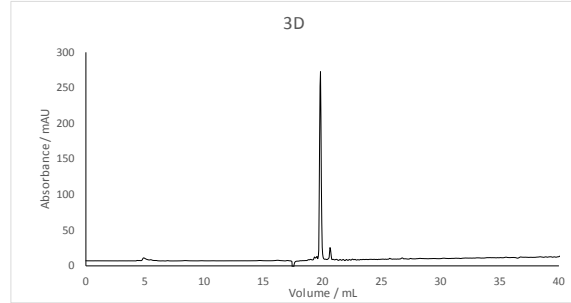
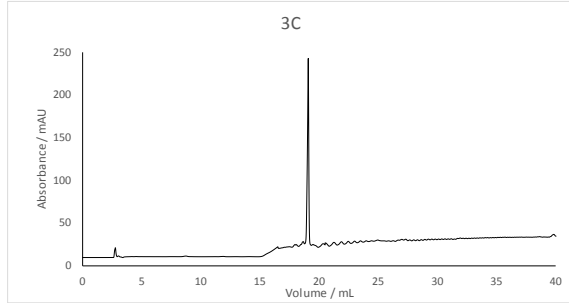
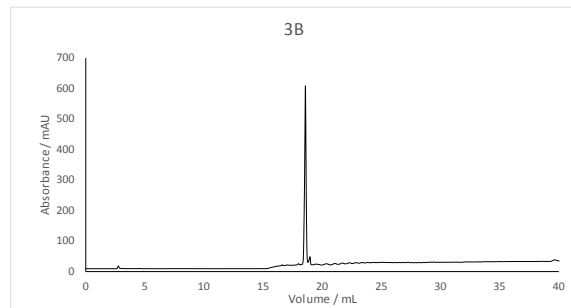
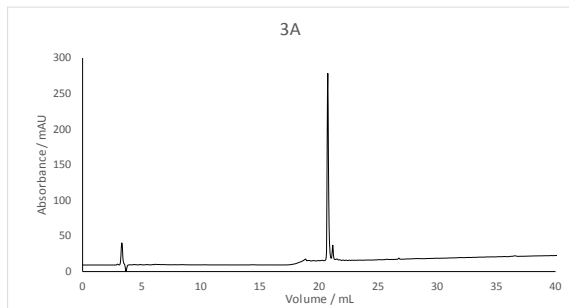


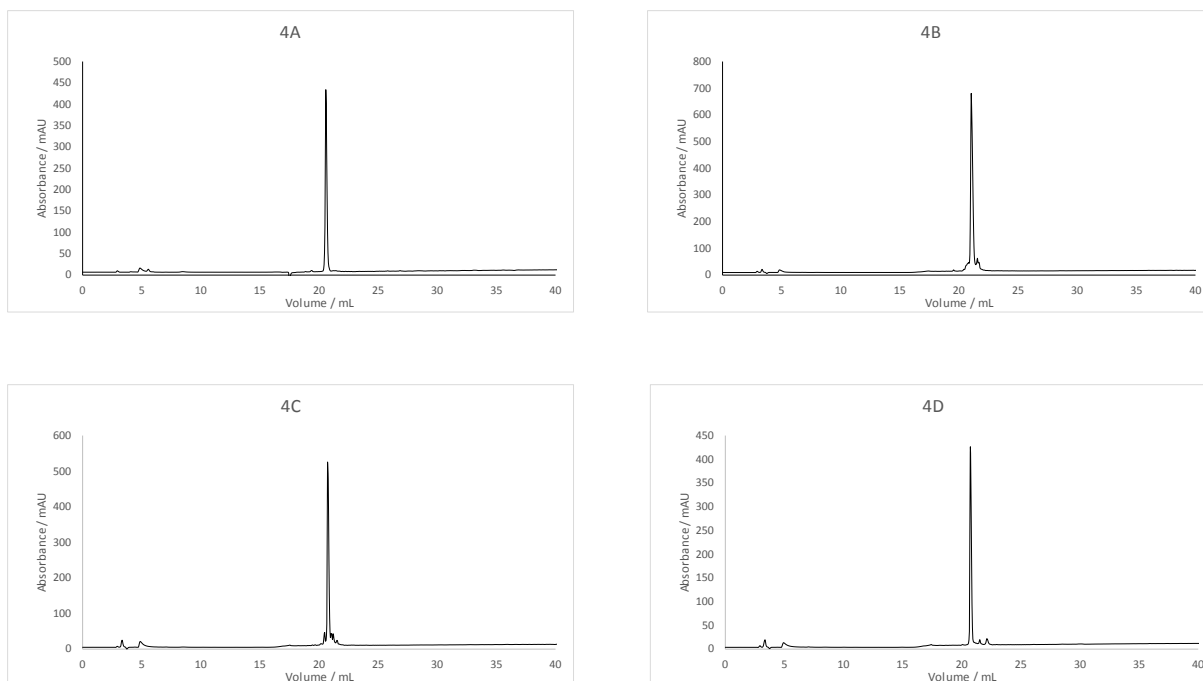




Supplementary figure S1. Mass spectrometry analysis of peptides. Left panels, mass spectrometry; right panels, tandem mass spectrometry.

6- *rp*HPLC analysis of peptides





Supplementary figure S2. Analysis of peptides by *rp*HPLC.

7- Protein purification

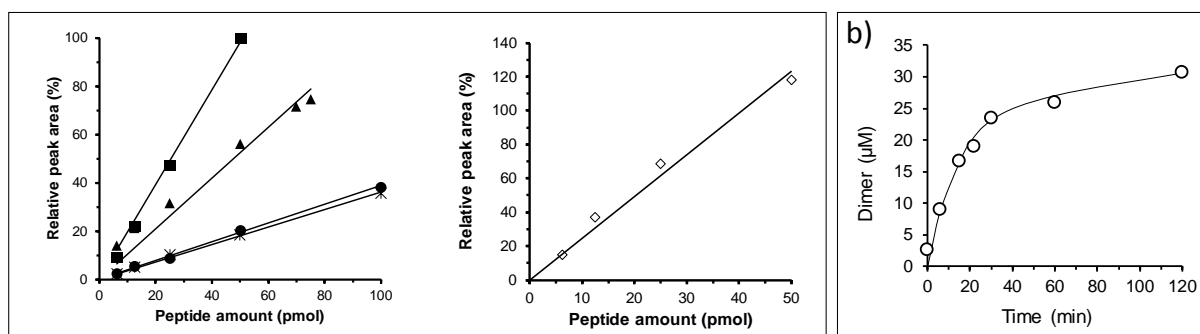
The catalytic domain of Ldt_{fm} (residues 341 to 466) and a soluble form of Ldt_{Mt2} (residues 55 to 408) were produced in *Escherichia coli* BL21(DE3) and purified by metal affinity and size-exclusion chromatography, as previously described.^[6] Protein concentration was determined by the Bradford method (Bio-Rad protein assay).

8- Cross-linking assay

Formation of 3→3 cross-links was determined in 140 μl of phosphate buffer (15 mM, pH 7.0) containing Ldt_{fm} (2.5 μM), the donor peptide **3A** (30 μM), and one of four branched acceptor peptides (**4A**, **4B**, **4C**, or **4D**; 30 μM). Ldt_{Mt2} (10 μM) was independently incubated with one of four DAP-containing tetrapeptides (**3B**, **3C**, **3D**, or **3E**; 100 μM), which were each used both as a donor and as an acceptor by the L_D-transpeptidase. The reaction was allowed to proceed at 37°C and aliquots of 4 μl were withdrawn at various times (0 to 120 min). For post-derivatization, each aliquot was incubated with the 4-fluoro-7-nitrobenzofurazan probe (12.5 mM; NBDF; SIGMA®F5883) for 8 min at 65°C in phosphate buffer (100 mM, pH 7.5) containing EDTA (1 mM) and β-mercaptoethanol (10 mM) in a total volume of 12.5 μl. The excess of unreacted NBDF was removed in a three-step extraction procedure comprising (i) acidification with citric acid (10% v/v) (total volume 162 μl), (ii) three extractions with 250 μl of isoamyl alcohol/chloroform (50/50 v/v), and (iii) five washings with 250 μl of chloroform. A portion of the aqueous phase containing the substrates and products (60 μl and 30 μl for the analyses of the reactions catalyzed by Ldt_{fm} and Ldt_{Mt2}, respectively) was mixed with an equal volume of the

rpHPLC buffer A (H₂O, TFA 0.05%) and injected into a C₁₈ *rpHPLC* column (Nucleosil[®]100-3, EC 250/4.6; Macherey-Nagel) at a flow rate of 1 mL.min⁻¹. For the substrates and products of Ldt_{fm}, a three-step gradient with buffer B (50% ACN, 0.035% TFA) was applied between 10 and 15 min (0 to 40%), 15 and 20 min (40 to 60%), and 20 and 40 min (60 to 100%). For the substrates and products of Ldt_{Mt2}, a three-step gradient with buffer B was applied between 10 and 12.5 min (0 to 20%), 12.5 and 32.5 min (20 to 60%), and 32.5 and 40 min (60 to 100%). Derivatized peptides were detected by fluorescence ($\lambda_{\text{ex}} = 470 \text{ nm}$; $\lambda_{\text{em}} = 530 \text{ nm}$).

To determine the relative fluorescence intensity of the substrates and products, authentic peptides (6 to 240 pmol) were derivatized and independently analyzed by *rpHPLC*, as described above. For this approach, dimers were obtained by incubation of Ldt_{fm} or Ldt_{Mt2} with their respective substrates and purified by *rpHPLC*. The concentration of the peptides was determined twice by acid hydrolysis and injection on a Hitachi L8800 amino acid analyzer equipped with a 2620 MSC-PS column.



Supplementary Figure S3. a) Calibration curves for quantitative determination of the substrates (left panel; 3B, square; 3C, triangle; 3D, circle; 3E, star) and the reaction products (right panel; 3B/3B, diamond) of Ldt_{Mt2} from *M. tuberculosis*. b) Kinetics of dimer synthesis by Ldt_{Mt2}. No dimer was detected with substrates 3C, 3D and 3E.

9- Conditional production of the DAP amidase AsnB in *M. tuberculosis*

To generate mutants that conditionally produce AsnB (Rv2201), *asnB* was tagged *in situ* with a DAS+4 tag as described.^[7] The resulting strain was transformed with plasmids that express the DAS+4-recognition protein SspB under the control of a tetracycline-controlled promoter and integrate into the chromosomal Giles phage attachment site. We used two regulated SspB-encoding plasmids (TetON-2 and TetON-6) that mediate SspB production only in the absence of anhydrotetracycline (atc). In the resulting mutants, designated AsnB-TetOn-2 and AsnB-TetOn-6, SspB-mediated AsnB degradation occurs in the absence of atc.

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