JOC The Journal of Organic Chemistry

pubs.acs.org/joc Note

Synthesis of Bacterial-Derived Peptidoglycan Cross-Linked Fragments

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Cite This: https://dx.doi.org/10.1021/acs.joc.0c01852 Read Online

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ABSTRACT: Peptidoglycan (PG) is the core structural motif of the bacterial cell wall. Fragments released from the PG serve as fundamental recognition elements for the immune system. The structure of the PG, however, encompasses a variety of chemical modifications among different bacterial species. Here, the applicability of organic synthetic methods to address this chemical diversity is explored, and the synthesis of cross-linked PG fragments, carrying biologically relevant amino acid modifications and peptide cross-linkages, is presented using solution and solid phase approaches.

The bacterial cell wall is a unique structural component that surrounds the cell membrane of a bacterium, giving the organism strength and rigidity. The molecular structure of this layer contains as one of its core elements peptidoglycan (PG), a polymeric construct that is formed by repeating carbohydrate units of *N*-acetylglucosamine (NAG) and *N*-acetyl muramic acid (NAM), and short peptide bridges, extending from NAM units, are further cross-linked to complete this assembly (Figure 1).[1−3]

The bacterial cell wall is known to play an important role in bacterial cell biology as a protective layer against environmental stresses and to affect the integrity of the cell.[4] Moreover, the bacterial cell wall and several elements of its biosynthesis have been crucial targets for antibiotics, which has led to the development of major classes such as β-lactams (e.g., penicillins and cephalosporins) and glycopeptides (e.g., vancomycin and teicoplanin).[5] Furthermore, the PG structure is known to be a key factor in the immune response to bacteria. Specific molecular motifs derived from microorganisms, sometimes known as microbe-associated molecular patterns (MAMPs), have been shown to be sensed by the human immune system and consequently trigger the first immune responses. Different classes of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs)[6−8] and NOD-like receptors (NLRs)[9−15] have been shown to detect and bind to fragments of the PG structure and cause an immune response. Fragments of PG are released as the cell cycle progresses or as a result of cell lysis; at the molecular level, the chemical structure of these fragments plays an important role in their immunogenicity. As mentioned above, the chemical assembly of PG includes NAG and NAM units with a peptide extension off the lactoyl moiety of NAM. The peptide composition of the PG includes alternating L and D amino acids with a general order of L-alanine, D-isoglutamine, L-lysine, and two D-alanine residues in Gram-positive bacteria. However, in Gram-negative bacteria and some species of Gram-positive bacteria, the lysine residue is substituted with *meso*-diaminopimelic acid (*m*-DAP) (Figure 1).[16,17] Interestingly, these natural differences reflect themselves in their detection by the human immune system. For instance, the NOD2 protein, a member of the NLR family, binds to muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine or MDP) and triggers an immune response.[18,19] Conversely, NOD1, another member of this family, detects DAP-containing fragments such as D-isoglutamate-*m*-diaminopimelic acid (iE-DAP) or its tripeptide analogue with an L-alanine extension (Tri-DAP).[20] In addition to the specificity of human immune proteins for these predominantly accessible PG fragments, the PG structure also encompasses a variety of natural modifications.

Despite the commonality of the PG structure as discussed above, several chemical modifications are observed in the PG molecular structure, both pre- and postbiosynthesis, adding to the diversity of the bacterial cell wall. These changes can occur both on the polysaccharide chains and on the peptide residues

Special Issue: A New Era of Discovery in Carbohydrate Chemistry

Received: July 31, 2020

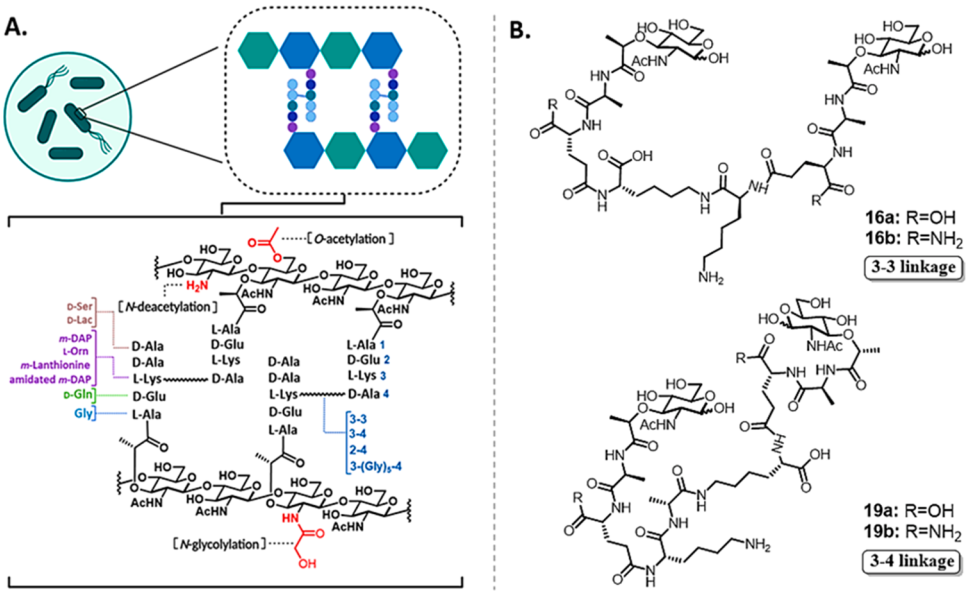

Figure 1. (A) General structure of the bacterial cell wall and examples of chemical modifications observed among different species. This diversity comes from changes in the carbohydrate backbone, amino acid constituents, and peptide linkages. (B) Synthesized cross-linked PG fragments mimicking 3−3 (top) and 3−4 (bottom) linkages containing D-isoglutamine or D-isoglutamate, as one of the common chemical modifications observed mostly in Gram-positive and Gram-negative bacteria, respectively.

(Figure 1).[17,21−23] O-Acetylation[24] and N-glycolylation[25,26] of NAM and N-deacetylation[27] of NAM and NAG in both Gram-positive and Gram-negative bacteria have been shown to increase lysozyme resistance and decrease the level of detection of corresponding fragments by NOD1 and NOD2.[28] Additionally, specific amino acid substitutions in different species are among the changes that can result in bacterial resistance. α-Amidation of D-isoglutmate and ε-amidation of *m*-DAP in several species of mycobacteria, Gram-positive and Gram-negative, are observed while reducing the level of immune activation.[29] Several spirochetes, such as the Gram-negative bacterium *Borrelia burgdorferi*, substitute the *m*-DAP with L-ornithine, thus enhancing the bacterium's ability to evade the host immune system.[30−32]

Another point for expanding the diversity of PG is amino acid cross-bridges, which link the peptide extensions (Figure 1). Various cross-linkages, including direct 3−3 and 3−4 bonds as well as connections through small peptide bridges such as pentaglycine, L-ornithine and D-aspartic acid, can be detected in different bacterial species (Figure 1). These are assembled through organism specific transpeptidases, which utilize different enzyme mechanisms, leaving some susceptible to β-lactams and others resistant.[21] While enzymes such as D,D-transpeptidases catalyze the formation of 3−4 cross-linkages in peptidoglycan, noncanonical transpeptidases like L,D-transpeptidases can form 3−3 bridges in some microorganisms such as *Enterococcus faecium* and *Mycobacterium tuberculosis*.[33−35] Despite appearing to be minor structural changes, these subtle chemical modifications can result in important functional outcomes in bacterial resistance, antimicrobial treatment for infectious diseases, and detection by the human immune system.

Various approaches have been pursued to study the biological implications of these changes for the PG structure, with regard to the biochemistry of the cell wall and their potential interactions with the human immune system. Metabolic labeling of the PG structure using bio-orthogonal probes and amino acid conjugates has been utilized to study PG cross-linking[34,36] and immunogenicity.[37] Moreover, by using noncanonical amino acids that are capable of forming covalent bonds, new unnatural cross-bridges are introduced.[38] In addition to *in vivo* modification of PG, chemical synthesis of diverse PG fragments has been shown to be a major step for gaining access to PG fragments. Several elegant syntheses have been designed and carried out to chemically produce fragments of PG for further biochemical and analytical evaluation. This includes some small fragments such as MDP and iE-DAP (and their derivatives) as well as larger disaccharides and PG cross-linked constructs.[39−53] For this purpose, different strategies, including solution and solid phase synthesis as well as chemoenzymatic approaches, have been utilized.[39−54] Despite the host of chemical syntheses for relatively small PG fragments (i.e., MDP), the chemical approaches for synthesizing larger fragments of PG have been underexplored. PG cross-linked fragments containing two immunostimulatory monomeric groups (such as MDP or iE-DAP monomers) mimicking the linkages found in the PG assembly would be useful tools for the biological community (Figure 1). These large PG subunits are shown to be among the fragments that are released from the cell wall. For instance, *Neisseria meningitidis* releases various cross-linked constructs during its normal growth.[55] Additionally, dimer-type PG fragments containing L-ornithine with glycine attachments are detected from *Borrelia burgdorferi*.[32] However, the potential immunogenicity of these fragments and their molecular interactions with innate immune receptors are not well understood, as synthesis of these PG fragments remains challenging. Furthermore, they contain structural information about the spatial orientations of PG constructs from different organisms.[56−59] Therefore, accessible chemical synthesis of these fragments will provide a method for producing cross-linked PG fragments for further biological studies.

PG-derived molecular probes have been shown to be essential tools for studying the complexities of the bacterial

https://dx.doi.org/10.1021/acs.joc.0c01852
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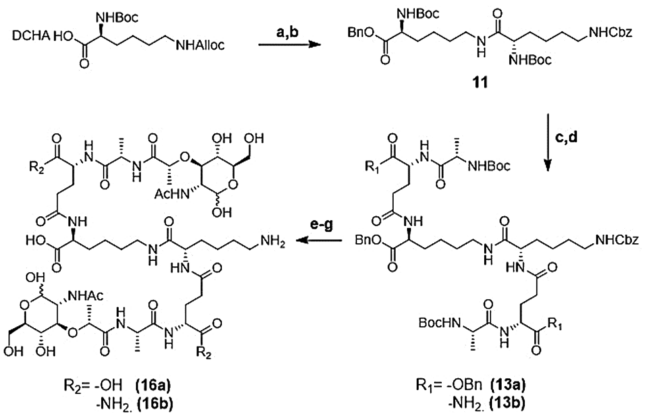
cell wall structure and its crucial role with regard to human immunity. The chemically synthesized PG probes decorated with fluorophores, cross-linkers, and modified chemical functionalities have been used to study the biology of the cell wall. Here, inspired by elegant syntheses of PG subunits,[45,53,60,61] we develop modified synthetic routes for synthesizing cross-linked fragments mimicking PG constructs and cross-bridges; specifically, the applicability of these methods to expand the library of these PG fragments based on the natural diversity of the PG structure is explored. For these purposes, the synthesis of a group of lysine-type PG fragments, derived from typical Gram-positive bacteria, such as *Staphylococcus aureus*, is used as a model. These synthesized substrates encompass biologically relevant amino acid substitutions, as well as various cross-linkages between the monomeric components, most notably, 3−3 and 3−4 bridges (Figure 1).

The first synthetic approach utilized solution chemistry, as we and others had been successful in synthesizing smaller PG fragments such as MDP and its derivatives. It was envisioned that a modular synthesis, relying on the orthogonality of the protecting groups for the peptide compartment as well as the carbohydrate moiety, could be applied. In this method, the core dipeptide linkage (L-Lys-L-Lys) was synthesized (Scheme S-4) followed by extending the peptide chains by adding L-Ala-D-Glu or L-Ala-D-Gln (Schemes S-3 and S-4). Finally, the two N-termini were capped by NAM moieties (Scheme 1). This approach would allow the synthesis of the peptide constructs first, before the incorporation of the carbohydrate rings into the structure, which can be considered for the synthesis of immunoactive PG fragments that do not contain the carbohydrate moiety, as it is known that in some biological settings the presence of the carbohydrate is not essential for the immunogenicity of the substrate. This approach was tested for the synthesis of compounds **16a** and **16b**, starting with formation of a 3−3 peptide bridge between two lysine groups; two 2:1 ratio coupling reactions were then followed to install the L-Ala-D-Glu/Gln dipeptide and the NAM group. The final substrates were achieved after a general catalytic hydrogenation to remove all of the protecting groups (Scheme 1). After this synthesis, it was noted that despite reasonable yields for the initial steps of this route, the solubility of the fully protected compounds in common organic solvents decreased significantly after installation of the NAM, which affected the purification and the yield for the final steps, resulting in a modest yield for the final deprotection (27−45%). The preliminary results from this approach showed a potential limitation for the applicability of this method, especially for larger derivatives.

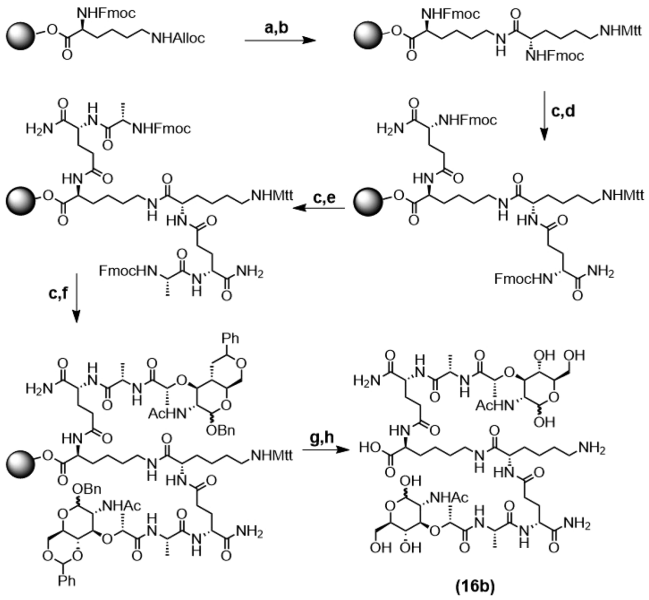
Scheme 1. Representative Solution Phase Synthesis of the Cross-Linked PG Fragments[a]



[a]Conditions: (a) BnBr, DMF (95%); (b) Boc-Lys(Z)-OH, HOBt, EDC, $Pd(PPh_3)_4$, DABCO, DCM (65%); (c) TFA/DCM [1:1 (v/v)]; (d) **5** or **9** (3 equiv), HATU, DIPEA, DMF (80−90% over two steps); (e) TFA/DCM [1:1 (v/v)] (90%); (f) **3** (3 equiv), HATU, DIPEA, DMF; (g) H_2, $Pd(OH)_2$/C, H_2O, TFA (27−45% over two steps). The full synthetic scheme is presented in Schemes S-3 and S-4.

To alleviate the solubility problems observed in the solution method, a synthesis on a solid base was used. This approach has previously yielded successful synthesis of a variety of PG-derived fragments.[13,46,60,62,63] Here, as an alternative approach, a solid phase synthesis of the 3−3- and 3−4-linked fragments was carried out (Scheme 2 and Schemes S-1 and S-2).

Scheme 2. Representative Solid Phase Synthesis of the Cross-Linked PG Fragments on 2-Chlorotrityl Chloride Resin (method A)[a]



[a]Conditions: (a) $Pd(PPh_3)_4$, $PhSiH_3$, DMF; (b) Fmoc-L-Lys(Mtt)-OH, HATU, DIPEA, DMF; (c) 20% piperidine in DMF; (d) Fmoc-D-Glu-NH_2, HATU, DIPEA, DMF; (e) Fmoc-L-Ala-OH, HATU, DIPEA, DMF; (f) **3**, HATU, DIPEA, DMF; (g) TFA/H_2O/TIPS (95:2.5:2.5); (h) Pd/C, H_2O, AcOH, H_2, 87%.

Following this method, partial removal of the protecting groups upon cleavage from the resin results in a better solubility of the released intermediate in aqueous solvent systems for the final hydrogenation. Using the multifunctional lysine amino acid as the starting point of peptide extension along with the orthogonal protecting strategy was shown to be efficient for this method. By changing the sequence of the peptide as well as incorporating unnatural amino acids or carbohydrate derivatives, one can expand this method to address the chemical diversity of the PG structure.

The relative symmetry of these substrates was translated to a modular synthesis on the solid support. This approach was used for compound **16b**, in which, after the formation of the dilysine linkage on the resin, a simultaneous growth of two peptide chains was followed by 2:1 coupling of D-Gln, L-Ala,

https://dx.doi.org/10.1021/acs.joc.0c01852
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and NAM (Scheme 2). This approach culminated in the successful assembly of partially protected **16b** on the resin that, following a catalytic hydrogenation, resulted in a fully deprotected substrate.

As an alternative approach, stepwise extension of each monomeric branch was applied to all of the substrates (**16a**, **16b**, **19a**, and **19b**) in which, following a complete synthesis of the muramyl tripeptide on the resin, a second muramyl tripeptide with the desired linkage was built subsequently, resulting in the same intermediate after being cleaved from the resin (Schemes S-1 and S-2). Furthermore, to show the ability of these methods to add chemical diversity, this route was applied to two forms of peptide cross-linking, resembling the natural PG cross-linking in the bacterial cell wall, to produce a 3−3 bridge (direct L-Lys-L-Lys bond, **16a** and **16b**) as well as a 3−4 bridge (L-Lys-D-Ala-L-Lys bridge, **19a** and **19b**). Additionally, two common forms of PG modifications on the second amino acid (D-Glx) in the form of D-isoglutamine (D-isoGln) and D-isoglutamate (D-isoGlu) were incorporated in the synthesis to produce two classes of biologically relevant PG fragments (Figure 1). The syntheses were performed on both Wang resin and 2-chlorotrityl chloride resin with a general Fmoc/*t*-Bu strategy to yield the acid peptide. These model substrates showcase the utility of the method to quickly access a range of functionality and cross-links found in PG.

■ CONCLUSION

Here, the applicability of various synthetic methods for the development of extended PG fragment analogues was analyzed, and an improved route was established. It was shown that using both solution-based and conventional resin-based syntheses, fragments with a higher level of structural complexity compared to short PG fragments can be produced, and the library of PG fragments was expanded while addressing the chemical diversity of the PG. These compounds can be further modified as molecular probes to investigate potential molecular interactions in the innate immune system as well as the structural properties of the bacterial cell wall. In recent years, PG conjugates decorated with cross-linkers, bioorthogonal handles, and fluorophores have been used to study the importance of these fragments. Additionally, these approaches can be used to incorporate other PG components to address the diversity in PG structure such as synthesis of DAP-containing PG fragments as well as biologically relevant modified carbohydrate derivatives, including N-deacetylated and N-glycolylated muramic acid. These compounds will be fundamental in the development of future immunotherapies.

■ EXPERIMENTAL SECTION

General Information. *Materials.* All amino acids and resins were purchased from Novabiochem, Bachem, or Chem-Impex. All other chemical reagents were purchased from Sigma-Aldrich, Fisher Scientific, Alfa Aesar, or Oakwood Chemical and used without further purification, unless otherwise noted. NMR solvents were purchased from Cambridge Isotope Laboratories, Inc.

General Procedures and Considerations. All of the reactions were performed in flame or oven-dried flasks or vials, equipped with rubber septa, a positive pressure of nitrogen, and magnetic stirring. The solid phase peptide synthesis was performed in Poly-Prep chromatography columns purchased from Bio-Rad. Unless otherwise noted, all solvents were anhydrous and transferred via a syringe and stainless steel needle. Reactions were monitored by thin layer chromatography (TLC) with glass plates coated with silica gel (silica HD TLC plates, w/UV254, 250 μm, Sorbent Technologies) and visualized with shortwave 254 nm UV light or developed upon heating with *p*-anisaldehyde or ninhydrin. Further monitoring was performed using electrospray ionization liquid chromatography mass spectrometry (ESI LC-MS). Analytical and semipreparative HPLC was performed on an Agilent Series 1100 instrument using a Phenomenex Luna 5 μm C18(2), 100 Å column (250 mm × 4.6 mm) and a Luna 5 μm C18(2), 100 Å column (250 mm × 10 mm), respectively. Preparative HPLC purification was performed on a Waters 2767 Sample Manager with HPLC and SQD2MS using a Sunfire Prep C18 OBD 5 μm 19 × 100 mm or 4.6 × 50 mm column.

All NMR spectra were recorded on a Bruker AV 400 MHz, AV III 600 MHz, or Neo 600 spectrometer at the NMR laboratory of the Department of Chemistry of the University of Delaware. The spectra were downfield from tetramethylsilane and referenced from an internal standard of residual protium in the NMR solvent. If necessary, the proton and carbon assignments were further confirmed by two-dimensional NMR analysis using COSY, HSQC, HMBC, and TOCSY experiments. Low-resolution mass spectra (LRMS) were recorded on an ACQUITY UPLC H-Class/SQD2 instrument using electrospray ionization (ESI), while high-resolution mass spectra (HR-MS), ESI mode, were recorded on a Thermo Q-Exactive Orbitrap, and Xevo G2-S QTof, at the Mass Spectroscopy Facility at the Department of Chemistry of the University of Delaware.

General Procedure for Solid Phase Synthesis of 16a, 16b, 19a, and 19b. *Loading the First Amino Acid.* 2-Chlorotrityl chloride resin (0.25 mmol) was transferred to a PolyPrep column and swollen by being mixed in 5 mL of anhydrous dichloromethane for 30−60 min. After the solvent was drained, a solution of the first amino acid (0.37 mmol, 1.5 equiv) [170 mg of Fmoc-L-Lys(Alloc)-OH in method A or 215 mg of Fmoc-L-Lys(ivDde)-OH in method B] and *N*,*N*-diisopropylethylamine (130 μL, 0.75 mmol, 3 equiv) in 3 mL of anhydrous dichloromethane was added to the resin and mixed for 6 h. Next, the resin was filtered and treated with 3 mL of a DCM/MeOH/*N*,*N*-diisopropylethylamine (DIPEA) (17:2:1) mixture (2 × 15 min). The resin was then washed with 5 mL of DCM (3 × 2 min) and 5 mL of DMF (3 × 2 min).

General Procedure for Fmoc Removal. The resin was washed with 5 mL of DMF (3 × 2 min). Fmoc removal was performed by treating the resin with a 5 mL solution of 20% piperidine in DMF (3 × 5 min), The resin was then washed with 5 mL of DMF (3 × 2 min) and used in the next coupling step.

General Procedure for Alloc Removal (method A). The resin was washed with 5 mL of DCM (3 × 2 min). Next, a 5 mL solution of tetrakis(triphenylphosphine) palladium(0) (29 mg, 0.025 mmol, 0.1 equiv) and phenylsilane (475 μL, 5 mmol, 20 equiv) in 5 mL of DCM was added and mixed (3 × 15 min). The resin was then washed with 5 mL of DCM (3 × 2 min) followed by 5 mL of DMF (3 × 2 min).

General Procedure for ivDde Removal (method B). The resin was washed with 5 mL of DMF (3 × 2 min). ivDde removal was performed by treating the resin with a 5 mL solution of 2% hydrazine in DMF (3 × 10 min), The resin was then washed with 5 mL of DMF (3 × 2 min) and used in the next coupling step.

General Procedure for Peptide Coupling. After deprotection of the amino group (Fmoc, Alloc, or ivDde), the next acid component in the peptide chain (1 mmol, 4 equiv),[a] 1-[bis(dimethylamino)-methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU) (361 mg, 0.95 mmol, 3.8 equiv), and *N*,*N*-diisopropylethylamine (380 μL, 2.2 mmol, 8.8 equiv) in 3 mL of DMF were added to the resin, and the reaction was carried out for 2−4 h,[b] after which the resin was washed with 5 mL of DMF (3 × 2 min) and used in the next step.

General Procedure for Peptide Cleavage. After the final coupling reaction, the resin was washed with 5 mL of DMF (3 × 2 min) and 5 mL of DCM (5 × 2 min), before a 6 mL solution of trifluoroacetic acid, triisopropylsilane, and water (95:2.5:2.5) was added to the resin and allowed to mix for 6 h. Next, the resin was filtered, and the filtrate was concentrated under a flow of N_2 gas to a volume of <1 mL, after which 12 mL of ice-cold diethyl ether was added and the peptide precipitated as a white solid. The white suspension was then centrifuged for 3 min at 2000 rpm. The diethyl ether supernatant was

https://dx.doi.org/10.1021/acs.joc.0c01852
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decanted, and this step was repeated with fresh ice-cold diethyl ether. Finally, the white crude product was dried, first under a flow of nitrogen for a short amount of time and then under vacuum.

The crude product was then purified by high-performance liquid chromatography using a semiprep C18 column [Luna 5 μm C18(2), 100 Å (250 mm × 10 mm)] and lyophilized to give **17a**, **17b**, **18a**, and **18b** as white solids (mixture of α and β anomeric isomers) (70−85 mg): buffer A, 98% water, 2% acetonitrile, and 0.06% trifluoroacetic acid; buffer B, 20% water, 80% acetonitrile, and 0.04% trifluoroacetic acid; flow rate of 3 mL/min; detection via dual absorbance detection at 214 and 254 nm; gradient of 100% A and 0% B from 0 to 4 min, from 100% A and 0% B to 80% A and 20% B from 4 to 10 min, from 80% A and 20% B to 30% A and 70% B from 10 to 40 min, from 30% A and 70% B to 0% A and 100% B from 40 to 42 min, of 0% A and 100% B from 42 to 47 min, from 0% A and 100% B to 100% A and 0% B from 47 to 50 min, and of 100% A and 0% B from 50 to 60 min.

17a: ^{1}H NMR (600 MHz, deuterium oxide) δ 7.45−7.38 (m, 10H, aromatic(OBn)), 4.91−4.90 (m, 2H, anomeric ***H***-1, anomeric ***H***-1′), 4.76−4.73 (m, 2H, -OC***H***HPh (×2)), 4.54 (d, *J* = 12.0 Hz, 2H, -OCH***H***Ph (×2)), 4.40−4.34 (m, 2H, $H_α$, $H_{α'}$ (D-Glu)), 4.31−4.25 (m, 5H, $H_α$, $H_{α'}$ (L-Ala), -COC***H***(Me)O- (×2), $H_α$ (L-Lys)), 4.17 (dd, *J* = 8.7, 5.8 Hz, 1H, $H_{α'}$ (L-Lys)), 3.96−3.93 (m, 2H, GlcNAc ring ***H***-2,2′), 3.85−3.76 (m, 6H, GlcNAc ring ***H***-5,5′,6,6′), 3.72−3.69 (m, 2H, GlcNAc ring ***H***-3,3′), 3.61−3.58 (m, 2H, GlcNAc ring ***H***-4,4′), 3.25−3.20 (m, 1H, $H_ε$ (L-Lys)), 3.16−3.11 (m, 1H, $H_ε$ (L-Lys)), 2.97 (t, *J* = 7.7 Hz, 2H, $2H_{ε'}$ (L-Lys)), 2.39−2.30 (m, 4H, $2H_γ$, $2H_{γ'}$ (D-Glu)), 2.24−2.16 (m, 2H, $H_β$, $H_{β'}$ (D-Glu)), 2.03−1.93 (m, 2H, $H_β$, $H_{β'}$ (D-Glu)), 1.89 (s, 3H, -COCH_3), 1.88 (s, 3H, -COCH_3), 1.85−1.75 (m, 2H, $H_β$, $H_{β'}$ (L-Lys)), 1.72−1.63 (m, 4H, $H_β$, $H_{β'}$ (L-Lys), $2H_{δ'}$ (L-Lys)), 1.53−1.46 (m, 2H, $2H_δ$ (L-Lys)), 1.43−1.32 (m, 16H, $2H_γ$, $2H_{γ'}$ (L-Lys), -CH_3, -C$H_{3'}$ (L-Ala), -COCH(CH_3)O- (×2)); ^{13}C{^{1}H} NMR (151 MHz, deuterium oxide) δ 175.7, 175.6, 175.5, 174.8, 174.77, 174.72, 174.70, 174.6, 174.5, 173.78, 173.74, 173.73, 136.9, 136.98, 136.96, 128.6, 128.5, 128.4, 128.33, 128.31, 95.7, 79.6, 79.5, 77.67, 77.63, 72.02, 72.00, 69.52, 69.50, 69.0, 68.9, 60.3, 54.0, 53.2, 53.1, 52.7, 51.8, 49.6, 49.5, 39.1, 38.8, 31.3, 31.2, 30.4, 30.0, 27.7, 26.7, 26.5, 26.2, 22.3, 22.1, 21.8, 18.5, 18.4, 16.73, 16.70; HRMS (ESI) *m*/*z* [M + H]$^+$ calcd for $C_{64}H_{97}N_{10}O_{25}$ 1405.6626, found 1405.6605.

17b: ^{1}H NMR (600 MHz, deuterium oxide) δ 7.45−7.38 (m, 10H, aromatic(OBn)), 4.90 (d, *J* = 3.6 Hz, 2H, anomeric ***H***-1, anomeric ***H***-1′), 4.75 (d, *J* = 12.0 Hz, 2H, -OC***H***HPh (×2)), 4.54 (d, *J* = 12.0 Hz, 2H, -OCH***H***Ph (×2)), 4.30−4.22 (m, 7H, $H_α$, $H_{α'}$ (D-Gln), $H_α$, $H_{α'}$ (L-Ala), -COC***H***(Me)O- (×2), $H_α$ (L-Lys)), 4.17 (dd, *J* = 8.6, 5.9 Hz, 1H, $H_{α'}$ (L-Lys)), 3.97−3.94 (m, 2H, GlcNAc ring ***H***-2,2′), 3.85−3.75 (m, 6H, GlcNAc ring ***H***-5,5′,6,6′), 3.72−3.68 (m, 2H, GlcNAc ring ***H***-3,3′), 3.61−3.58 (m, 2H, GlcNAc ring ***H***-4,4′), 3.24−3.20 (m, 1H, $H_ε$ (L-Lys)), 3.17−3.13 (m, 1H, $H_ε$ (L-Lys)), 2.97 (t, *J* = 7.7 Hz, 2H, $2H_{ε'}$ (L-Lys)), 2.40−2.31 (m, 4H, $2H_γ$, $2H_{γ'}$ (D-Gln)), 2.20−2.14 (m, 2H, $H_β$, $H_{β'}$ (D-Gln)), 2.00−1.88 (m, 8H, $H_β$, $H_{β'}$ (D-Gln), -COCH_3 (×2)), 1.87−1.75 (m, 2H, $H_β$, $H_{β'}$ (L-Lys)), 1.74−1.64 (m, 4H, $H_β$, $H_{β'}$ (L-Lys), $2H_{δ'}$ (L-Lys)), 1.53−1.48 (m, 2H, $2H_δ$ (L-Lys)), 1.45−1.30 (m, 16H, $2H_γ$, $2H_{γ'}$ (L-Lys), -CH_3, -C$H_{3'}$ (L-Ala), -COCH-(CH_3)O- (×2)); ^{13}C{^{1}H} NMR (151 MHz, deuterium oxide) δ 175.87, 175.83, 175.82, 175.78, 175.71, 174.9, 174.87, 174.82, 173.75, 173.73, 136.9, 128.7, 128.5, 128.4, 128.3, 95.8, 79.8, 79.7, 77.6, 71.9, 69.5, 68.9, 68.8, 60.3, 54.0, 53.24, 53.22, 52.79, 52.73, 52.5, 49.79, 49.72, 39.1, 38.9, 31.4, 31.3, 30.4, 29.9, 27.7, 26.8, 26.7, 26.2, 22.3, 22.1, 21.8, 18.6, 18.5, 16.54, 16.52; HRMS (ESI) *m*/*z* [M + H]$^+$ calcd for $C_{64}H_{99}N_{12}O_{23}$ 1403.6946, found 1403.6921.

18a: ^{1}H NMR (600 MHz, deuterium oxide) δ 7.44−7.37 (m, 10H, aromatic(OBn)), 4.91−4.89 (m, 2H, anomeric ***H***-1, anomeric ***H***-1′), 4.73 (d, *J* = 12.0 Hz, 2H, -OC***H***HPh (×2)), 4.53 (d, *J* = 12.0 Hz, 2H, -OCH***H***Ph (×2)), 4.39−4.34 (m, 2H, $H_α$, $H_{α'}$ (D-Glu)), 4.31−4.17 (m, 7H, $H_α$, $H_{α'}$ (L-Lys), $H_α$, $H_{α'}$ (L-Ala), $H_α$ (D-Ala), -COC***H***(Me)O- (×2)), 3.94−3.92 (m, 2H, GlcNAc ring ***H***-2,2′), 3.84−3.75 (m, 6H, GlcNAc ring ***H***-5,5′,6,6′), 3.72−3.68 (m, 2H, GlcNAc ring ***H***-3,3′), 3.59 (t, *J* = 9.3 Hz, 2H, GlcNAc ring ***H***-4,4′), 3.21−3.10 (m, 2H, $2H_ε$ (L-Lys)), 2.97 (t, *J* = 7.7 Hz, 2H, $2H_{ε'}$ (L-Lys)), 2.38−2.29 (m, 4H, $2H_γ$, $2H_{γ'}$ (D-Glu)), 2.21−2.15 (m, 2H, $H_β$, $H_{β'}$ (D-Glu)), 2.01−1.85 (m, 8H, $H_β$, $H_{β'}$ (D-Glu), -COCH_3 (×2)) 1.83−1.64 (m, 6H, $2H_β$, $2H_{β'}$ (L-Lys), $2H_{δ'}$ (L-Lys)), 1.51−1.31 (m, 21H, $2H_δ$ (L-Lys), $2H_γ$, $2H_{γ'}$ (L-Lys), -CH_3, -C$H_{3'}$ (L-Ala), -CH_3 (D-Ala), -COCH(CH_3)O- (×2)); ^{13}C{^{1}H} NMR (151 MHz, deuterium oxide) δ 175.7, 175.6, 175.5, 174.9, 174.8, 174.7, 174.6, 174.5, 174.49, 174.44, 174.0, 173.74, 173.72, 136.9, 128.6, 128.49, 128.45, 128.3, 95.7, 79.56, 79.55, 77.67, 77.63, 72.0, 69.5, 69.4, 69.09, 69.04, 60.3, 54.1, 53.2, 53.1, 52.7, 51.8, 51.7, 49.9, 49.59, 49.54, 39.1, 38.8, 31.3, 31.1, 30.1, 29.9, 27.7, 26.6, 26.5, 26.3, 22.2, 22.0, 21.8, 18.5, 18.4, 16.7, 16.5; HRMS (ESI) *m*/*z* [M + H]$^+$ calcd for $C_{67}H_{102}N_{11}O_{26}$ 1476.6997, found 1476.6973.

18b: ^{1}H NMR (600 MHz, deuterium oxide) δ 7.45−7.38 (m, 10H, aromatic(OBn)), 4.90 (d, *J* = 3.7 Hz, 2H, anomeric ***H***-1, anomeric ***H***-1′), 4.75 (d, *J* = 12.0, 2H, -OC***H***HPh (×2)), 4.54 (d, *J* = 12.0 Hz, 2H, -OCH***H***Ph (×2)), 4.30−4.19 (m, 9H, $H_α$, $H_{α'}$ (D-Gln), $H_α$, $H_{α'}$ (L-Ala), $H_α$, $H_{α'}$ (L-Lys), $H_α$ (D-Ala), -COC***H***(Me)O- (×2)), 3.97−3.95 (m, 2H, GlcNAc ring ***H***-2,2′), 3.85−3.75 (m, 6H, GlcNAc ring ***H***-5,5′,6,6′), 3.72−3.69 (m, 2H, GlcNAc ring ***H***-3,3′), 3.61−3.58 (m, 2H, GlcNAc ring ***H***-4,4′), 3.23−3.18 (m, 1H, $H_ε$ (L-Lys)), 3.16−3.11 (m, 1H, $H_ε$ (L-Lys)), 2.98 (t, *J* = 7.7 Hz, 2H, $2H_{ε'}$ (L-Lys)), 2.40−2.31 (m, 4H, $2H_γ$, $2H_{γ'}$ (D-Glu)), 2.20−2.12 (m, 2H, $H_β$, $H_{β'}$ (D-Glu)), 1.99−1.89 (m, 8H, $H_β$, $H_{β'}$ (D-Glu), -COCH_3 (×2)), 1.85−1.65 (m, 6H, $2H_β$, $2H_{β'}$ (L-Lys), $2H_{δ'}$ (L-Lys)), 1.52−1.32 (m, 21H, $2H_δ$ (L-Lys), $2H_γ$, $2H_{γ'}$ (L-Lys), -CH_3, -C$H_{3'}$ (L-Ala), CH_3 (D-Ala), -COCH(CH_3)O- (×2)); ^{13}C{^{1}H} NMR (151 MHz, deuterium oxide) δ 175.87, 175.83, 175.80, 175.7, 175.6, 174.99, 174.94, 174.92, 174.8, 174.5, 174.0, 173.75, 173.73, 136.9, 128.7, 128.5, 128.4, 128.3, 95.8, 79.79, 79.76, 77.7, 77.6, 71.9, 69.52, 69.50, 68.9, 68.8, 60.3, 54.0, 53.23, 53.22, 52.8, 52.7, 52.6, 49.9, 49.77, 49.73, 39.1, 38.8, 31.4, 31.2, 30.1, 29.9, 27.8, 26.9, 26.7, 26.3, 22.2, 22.0, 21.8, 18.6, 18.5, 16.6, 16.5; HRMS (ESI) *m*/*z* [M + H]$^+$ calcd for $C_{67}H_{104}N_{13}O_{24}$ 1474.7317, found 1474.7288.

16a. In a round-bottom flask, **17a** (15 mg, 0.01 mmol, 1.0 equiv) was dissolved in 2 mL of water and 100 μL of acetic acid was added followed by 10 mg of Pd/C (20 wt % loading). The flask was then purged with hydrogen and allowed to stir for 24 h under hydrogen (balloon). Removal of benzyl protecting groups was confirmed by LC-MS. Next, the Pd/C catalyst was filtered by being passed through a 0.22 μm filter, and the filtrate was lyophilized to give **16a** (12 mg, 94% yield) as a white solid [as a mixture of anomeric α and β isomers (1:0.25)] (protium counts for the major isomer are reported): ^{1}H NMR (600 MHz, deuterium oxide) δ 5.16 (d, *J* = 3.4 Hz, 2H, anomeric ***H***-1(α), anomeric ***H***-1′(α)), 4.67 (d, *J* = 8.5 Hz, 2H, anomeric ***H***-1(β), anomeric ***H***-1′(β)), 4.40−4.18 (m, 8H, $H_α$, $H_{α'}$ (D-Glu), $H_α$, $H_{α'}$ (L-Ala), -COC***H***(Me)O- (×2), $H_α$, $H_{α'}$ (L-Lys)), 3.98−3.95 (m, 2H, GlcNAc ring ***H***-2,2′(α)), 3.93−3.74 (m, 6H, GlcNAc ring ***H***-5,5′,6,6′(α), ***H***-2,2′,5,5′,6,6′(β)), 3.72−3.69 (m, 2H, GlcNAc ring ***H***-3,3′(α)), 3.60−3.47 (m, 2H, GlcNAc ring ***H***-4,4′(α), ***H***-3,3′,4,4′(β)), 3.28−3.23 (m, 1H, $H_ε$ (L-Lys)), 3.19−3.14 (m, 1H, $H_ε$ (L-Lys)), 2.99 (t, *J* = 7.4 Hz, 2H, $2H_{ε'}$ (L-Lys)), 2.40−2.34 (m, 4H, $2H_γ$, $2H_{γ'}$ (D-Glu)), 2.24−2.18 (m, 2H, $H_β$, $H_{β'}$ (D-Glu)), 2.05−1.95 (m, 8H, $H_β$, $H_{β'}$ (D-Glu), -COCH_3 (×2)), 1.88−1.68 (m, 6H, $2H_β$, $2H_{β'}$, $2H_{δ'}$ (L-Lys)), 1.55−1.50 (m, 2H, $2H_δ$ (L-Lys)), 1.46−1.35 (m, 16H, $2H_γ$, $2H_{γ'}$ (L-Lys), -CH_3, -C$H_{3'}$ (L-Ala), -COCH(CH_3)O- (×2)); ^{13}C{^{1}H} NMR (151 MHz, deuterium oxide) δ 177.2, 176.9, 176.5, 176.28, 176.20, 175.4, 175.2, 174.7, 174.4, 174.3, 95.4, 91.4, 83.0, 82.9, 80.1, 80.0, 78.5, 78.2, 76.2, 72.0, 69.48, 69.42, 69.27, 69.21, 61.2, 61.0, 59.8, 56.6, 54.5, 54.2, 53.4, 52.7, 50.2, 50.0, 39.6, 39.4, 31.98, 31.90, 31.0, 30.6, 28.3, 27.3, 27.1, 26.7, 22.8, 22.7, 22.6, 22.5, 20.9, 19.1, 17.3, 17.2; HRMS (ESI) *m*/*z* [M + H]$^+$ calcd for $C_{50}H_{85}N_{10}O_{25}$ 1225.5687, found 1225.5670.

16b. In a round-bottom flask, **17b** (13 mg, 0.009 mmol, 1.0 equiv) was dissolved in 2 mL of water and 100 μL of acetic acid was added followed by 10 mg of Pd/C (20 wt % loading). The flask was then purged with hydrogen and allowed to stir for 24 h under hydrogen (balloon). Removal of benzyl protecting groups was confirmed by LC-MS. Next, the Pd/C catalyst was filtered by being passed through a 0.22 μm filter, and the filtrate was lyophilized to give **16b** (10 mg, 87% yield) as a white solid [as a mixture of anomeric α and β isomers (1:0.23)] (protium counts for the major isomer are reported): ^{1}H

https://dx.doi.org/10.1021/acs.joc.0c01852
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NMR (600 MHz, deuterium oxide) δ 5.16−5.15 (m, 2H, anomeric *H*-1(α), anomeric *H*-1′(α)) (m, 2H, anomeric *H*-1(β), anomeric *H*-1′(β)), 4.33−4.19 (m, 8H, H_α, $H_{\alpha'}$ (D-Gln), H_α, $H_{\alpha'}$ (L-Ala), -COC*H*(Me)O- (×2), H_α, $H_{\alpha'}$ (L-Lys)), 3.98−3.95 (m, 2H, GlcNAc ring *H*-2,2′(α)), 3.92−3.75 (m, 6H, GlcNAc ring *H*-5,5′,6,6′(α), *H*-2,2′,5,5′,6,6′(β)), 3.72−3.69 (m, 2H, GlcNAc ring *H*-3,3′(α)), 3.59−3.46 (m, 2H, GlcNAc ring *H*-4,4′(α), *H*-3,3′,4,4′(β)), 3.27−3.22 (m, 1H, H_ε (L-Lys)), 3.20−3.14 (m, 1H, H_ε (L-Lys)), 3.00 (br, 2H, $2H_{\varepsilon'}$ (L-Lys)), 2.42−2.38 (m, 4H, $2H_\gamma$, $2H_{\gamma'}$ (D-Gln)), 2.22−2.15 (m, 2H, H_β, $H_{\beta'}$ (D-Gln)), 2.03−1.95 (m, 8H, H_β, $H_{\beta'}$ (D-Gln), -COCH_3 (×2)), 1.87−1.66 (m, 6H, $2H_\beta$, $2H_{\beta'}$, $2H_{\delta'}$ (L-Lys)), 1.55−1.49 (m, 2H, $2H_\delta$ (L-Lys)), 1.45−1.35 (m, 16H, $2H_\gamma$, $2H_{\gamma'}$ (L-Lys), -CH_3, -C$H_{3'}$ (L-Ala), -COCH(CH_3)O- (×2)); ^{13}C{^{1}H} NMR (151 MHz, deuterium oxide) δ 176.9, 176.7, 175.8, 175.0, 174.8, 174.7, 94.8, 90.9, 79.68, 79.63, 78.0, 77.7, 75.6, 71.4, 68.89, 68.84, 60.6, 60.4, 59.6, 56.1, 54.0, 53.6, 53.4, 52.8, 52.6, 49.8, 49.7, 39.1, 38.9, 31.5, 31.4, 30.5, 30.3, 27.8, 26.8, 26.2, 22.4, 22.2, 22.1, 21.9, 20.4, 18.6, 16.5; HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{50}H_{87}N_{12}O_{23}$ 1223.6007, found 1223.5978.

19a. In a round-bottom flask, **18a** (19 mg, 0.012 mmol, 1.0 equiv) was dissolved in 2 mL of water and 100 μL of acetic acid was added followed by 10 mg of Pd/C (20 wt % loading). The flask was then purged with hydrogen and allowed to stir for 24 h under hydrogen (balloon). Removal of benzyl protecting groups was confirmed by LC-MS. Next, the Pd/C catalyst was filtered by being passed through a 0.22 μm filter, and the filtrate was lyophilized to give **19a** (15 mg, 87% yield) as a white solid [as mixture of anomeric α and β isomers (1:0.23)] (protium counts for the major isomer are reported): ^{1}H NMR (600 MHz, deuterium oxide) δ 5.16 (d, J = 3.5 Hz, 2H, anomeric *H*-1(α), anomeric *H*-1′(α)), 4.68 (d, J = 8.4 Hz, 2H, anomeric *H*-1(β), anomeric *H*-1′(β)), 4.39−4.19 (m, 9H, H_α, $H_{\alpha'}$ (D-Glu), H_α, $H_{\alpha'}$ (L-Ala), H_α (D-Ala), -COC*H*(Me)O- (×2), H_α, $H_{\alpha'}$ (L-Lys)), 3.98−3.95 (m, 2H, GlcNAc ring *H*-2,2′(α)), 3.92−3.74 (m, 6H, GlcNAc ring *H*-5,5′,6,6′(α), *H*-2,2′,5,5′,6,6′(β)), 3.72−3.68 (m, 2H, GlcNAc ring *H*-3,3′(α)), 3.60−3.46 (m, 2H, GlcNAc ring *H*-4,4′(α), *H*-3,3′,4,4′(β)), 3.25−3.20 (m, 1H, H_ε (L-Lys)), 3.18−3.14 (m, 1H, H_ε (L-Lys)), 2.99 (t, J = 7.7 Hz, 2H, $2H_{\varepsilon'}$ (L-Lys)), 2.42−2.33 (m, 4H, $2H_\gamma$, $2H_{\gamma'}$ (D-Glu)), 2.23−2.17 (m, 2H, H_β, $H_{\beta'}$ (D-Glu)), 2.04−1.97 (m, 8H, H_β, $H_{\beta'}$ (D-Glu), -COCH_3 (×2)), 1.87−1.66 (m, 6H, $2H_\beta$, $2H_{\beta'}$, $2H_{\delta'}$ (L-Lys)), 1.54−1.34 (m, 21H, $2H_\gamma$, $2H_{\gamma'}$ (L-Lys), -CH_3, -C$H_{3'}$ (L-Ala), -CH_3 (D-Ala), -COCH(CH_3)O- (×2)); ^{13}C{^{1}H} NMR (151 MHz, deuterium oxide) δ 176.6, 176.2, 176.1, 175.9, 175.7, 175.5, 175.4, 175.2, 175.1, 175.0, 174.7, 174.6, 174.47, 174.46, 95.4, 91.4, 82.97, 82.94, 80.08, 80.06, 78.5, 78.25, 78.22, 76.2, 72.0, 69.48, 69.42, 69.27, 69.22, 61.2, 61.0, 56.65, 56.62, 54.6, 54.2, 54.1, 53.5, 52.8, 52.7, 50.5, 50.16, 50.12, 50.08, 50.05, 39.6, 39.4, 31.9, 31.8, 30.68, 30.60, 28.3, 27.4, 27.2, 26.8, 22.8, 22.7, 22.5, 22.4, 19.12, 19.10, 17.34, 17.32, 17.3, 17.0; HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{53}H_{90}N_{11}O_{26}$ 1296.6058, found 1296.6028.

19b. In a round-bottom flask, **18b** (11 mg, 0.007 mmol, 1.0 equiv) was dissolved in 2 mL of water and 100 μL of acetic acid was added followed by 10 mg of Pd/C (20 wt % loading). The flask was then purged with hydrogen and allowed to stir for 24 h under hydrogen (balloon). Removal of benzyl protecting groups was confirmed by LC-MS. Next, the Pd/C catalyst was filtered by being passed through a 0.22 μm filter, and the filtrate was lyophilized to give **19b** (9 mg, 92% yield) as a white solid [as mixture of anomeric α and β isomers (1:0.20)] (protium counts for the major isomer are reported): ^{1}H NMR (600 MHz, deuterium oxide) δ 5.16−5.15 (m, 2H, anomeric *H*-1(α), anomeric *H*-1′(α)), 4.68 (d, J = 8.5 Hz, 2H, anomeric *H*-1(β), anomeric *H*-1′(β)), 4.33−4.19 (m, 9H, H_α, $H_{\alpha'}$ (D-Gln), H_α, $H_{\alpha'}$ (L-Ala), H_α (D-Ala), -COC*H*(Me)O- (×2), H_α, $H_{\alpha'}$ (L-Lys)), 3.99−3.96 (m, 2H, GlcNAc ring *H*-2,2′(α)), 3.92−3.75 (m, 6H, GlcNAc ring *H*-5,5′,6,6′(α), *H*-2,2′,5,5′,6,6′(β)), 3.73−3.69 (m, 2H, GlcNAc ring *H*-3,3′(α)), 3.60−3.46 (m, 2H, GlcNAc ring *H*-4,4′(α), *H*-3,3′,4,4′(β)), 3.25−3.20 (m, 1H, H_ε (L-Lys)), 3.18−3.14 (m, 1H, H_ε (L-Lys)), 2.99 (t, J = 7.7 Hz, 2H, $2H_{\varepsilon'}$ (L-Lys)), 2.44−2.35 (m, 4H, $2H_\gamma$, $2H_{\gamma'}$ (D-Gln)), 2.22−2.14 (m, 2H, H_β, $H_{\beta'}$ (D-Gln)), 2.04−1.94 (m, 8H, H_β, $H_{\beta'}$ (D-Gln), -COCH_3 (×2)), 1.87−1.66 (m, 6H, $2H_\beta$, $2H_{\beta'}$, $2H_{\delta'}$ (L-Lys)), 1.54−1.33 (m, 21H, $2H_\gamma$, $2H_{\gamma'}$ (L-Lys), -CH_3, -C$H_{3'}$ (L-Ala), -CH_3 (D-Ala), -COCH(CH_3)O- (×2)); ^{13}C{^{1}H} NMR (151 MHz, deuterium oxide) δ 176.7, 176.2, 175.9, 175.8, 175.7, 175.0, 174.8, 174.5, 174.0, 173.9, 94.8, 90.9, 82.5, 79.68, 79.64, 78.0, 77.7, 75.6, 71.4, 68.9, 68.8, 68.6, 60.6, 60.4, 56.1, 54.0, 53.6, 53.2, 52.8, 52.6, 49.9, 49.79, 49.73, 39.1, 38.9, 31.4, 31.3, 30.19, 30.14, 27.8, 26.9, 26.8, 26.3, 22.3, 22.1, 22.0, 21.9, 20.3, 18.63, 18.61, 16.59, 16.55; HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{53}H_{92}N_{13}O_{24}$ 1294.6378, found 1294.6362.

(R)-2-{[(2S,4aR,6R,7R,8R,8aS)-7-Acetamido-6-(benzyloxy)-2-phenylhexahydropyrano[3,2-d][1,3]dioxin-8-yl]oxy}propanoic acid (**3**) was synthesized according to the previously published procedure.[64] Starting with commercially available *N*-acetylglucosamine (Scheme S-3), and over three steps (51% yield), **3** was obtained as an amorphous white solid.

(R)-Benzyl 5-Amino-4-{(S)-2-[(tert-butoxycarbonyl)amino]-propanamido}-5-oxopentanoate (**4**). D-Glu(OBn)-NH$_2$·HCl (1.25 g, 4.5 mmol, 1.0 equiv) was dissolved in THF (30 mL, 0.15 M) on ice. Boc-L-Ala-OSu (1.3 g, 4.5 mmol, 1.0 equiv) was added followed by DIPEA (1.0 mL, 1.27 equiv) dropwise. The reaction mixture was allowed to warm to room temperature and stirred overnight. LC-MS confirmed the production of the product. The reaction mixture was condensed and then diluted with 200 mL of ethyl acetate. The organic layer was washed with 125 mL of 1 N HCl, saturated sodium bicarbonate, and saturated brine. The washed organic layer was condensed and confirmed as being pure by TLC and LC-MS, yielding the product as a white solid (1.759 g, 95%): ^{1}H NMR (600 MHz, methanol-d_4) δ 7.37−7.30 (m, 5H), 5.14 (d, J = 12.4 Hz, 1H), 5.13 (d, J = 12.4 Hz, 1H), 4.39 (dd, J = 9.6, 4.5 Hz, 1H), 4.03 (q, J = 7.2 Hz, 1H), 2.48 (t, J = 7.6 Hz, 2H), 2.30−2.24 (m, 1H), 1.97−1.91 (m, 1H), 1.44 (s, 9H), 1.30 (d, J = 7.2 Hz, 3H); ^{13}C{^{1}H} NMR (151 MHz, methanol-d_4) δ 176.3, 176.2, 174.1, 157.9, 137.5, 129.5, 129.2, 129.1, 80.7, 67.3, 53.4, 52.0, 31.3, 28.6, 28.0, 17.6; HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{20}H_{30}N_3O_6$ 408.2135, found 408.2130.

(R)-5-Amino-4-{(S)-2-[(tert-butoxycarbonyl)amino]propanamido}-5-oxopentanoic Acid (**5**). To a solution of **4** (400 mg, 0.98 mmol, 1.0 equiv) in 10 mL of methanol was added 150 mg of Pd(OH)$_2$/C, and under H_2 (balloon), the reaction mixture was stirred overnight. The reaction progress was checked by TLC. After completion, the catalyst was filtered over a pad of Celite and the filtrate was concentrated to give a colorless oil, which upon treatment with chloroform gave 310 mg (quantitative) of the product as a white solid: ^{1}H NMR (600 MHz, methanol-d_4) δ 4.37 (dd, J = 9.6, 4.4 Hz, 1H), 4.03 (q, J = 7.1 Hz, 1H), 2.39 (t, J = 7.6 Hz, 2H), 2.25−2.19 (m, 1H), 1.93−1.87 (m, 1H), 1.45 (s, 9H), 1.32 (d, J = 7.1 Hz, 3H); ^{13}C{^{1}H} NMR (151 MHz, methanol-d_4) δ 176.4, 176.3, 157.9, 80.7, 53.6, 52.0, 31.2, 28.6, 28.1, 17.6; HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{13}H_{24}N_3O_6$ 318.1665, found 318.1651.

(R)-1-Benzyl 5-[2-(Trimethylsilyl)ethyl]-2-[(tert-butoxycarbonyl)-amino]pentanedioate (**6**). Boc-D-glutamic acid-1-benzyl ester (6.0 g, 17 mmol, 1.0 equiv) was dissolved in 60 mL of anhydrous *N*,*N*-dimethylformamide on ice. Dicyclohexylcarbodiimide (DCC) (4.0 g, 19 mmol, 1.1 equiv) was added followed by 2-(trimethylsilyl)ethanol (TMSE) (3.3 mL, 21 mmol, 1.3 equiv) and 4-(dimethylamino)-pyridine (DMAP) (1.3 g, 10.6 mmol, 0.6 equiv). The reaction mixture was warmed to room temperature and stirred overnight. The cloudy white mixture was filtered to remove the DCC urea. The filtrate was then diluted in DCM, washed with 1 N HCl (once), saturated bicarbonate (once), and brine (once), and then dried with sodium sulfate. The organic layer was condensed and purified via column chromatography with 0−25% ethyl acetate in hexane to yield an oil product (5.4 g, 70%): ^{1}H NMR (400 MHz, methanol-d_4) δ 7.36−7.27 (m, 5H, benzyl), 5.18−5.08 (m, 2H, -CH_2OBn), 4.18−4.06 (m, 3H, H_α (D-Glu), CH_2-OTMSE), 2.39−2.33 (m, 2H, $2H_\gamma$ (D-Glu)), 2.14−2.05 (m, 1H, H_β (D-Glu)), 1.90−1.81 (m, 1H, H_β (D-Glu)), 1.40 (s, 9H, Boc-(CH_3)$_3$), 0.97−0.88 (m, 2H, CH_2-TMSE), 0.01 (s, 9H, Si-(CH_3)$_3$); ^{13}C{^{1}H} NMR (101 MHz, methanol-d_4) δ 209.8 (carbonyl), 174.1 (carbonyl), 173.4 (carbonyl), 157.8 (aromatic), 137.0 (aromatic), 129.3 (aromatic), 129.07 (aromatic), 129.01 (aromatic), 80.4 (Boc-**C**(CH$_3$)$_3$), 67.6 (**C**H$_2$OBn), 63.5 (**C**H$_2$TMSE), 54.2 (**C**$_\alpha$ (D-Glu)), 31.1 (**C**$_\gamma$ (D-Glu)), 28.4 (Boc-(**C**H$_3$)$_3$), 27.3 (**C**$_\beta$ (D-Glu)),

https://dx.doi.org/10.1021/acs.joc.0c01852
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17.9 ($\mathbf{C}H_2$TMSE), −1.6 (Si-($\mathbf{C}H_3$)$_3$); HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{22}H_{36}NO_6Si$ 438.2312, found 438.2312.

(R)-1-Benzyl 5-[2-(Trimethylsilyl)ethyl] 2-Aminopentanedioate Hydrochloride (7). **6** (5.2 g, 11.8 mmol, 1 equiv) was dissolved in 60 mL of anhydrous DCM on ice; 4 M HCl in 1,4-dioxane (30 mL, 118 mmol, 10 equiv) was then added. The reaction mixture was stirred on ice for 1 h and then brought to room temperature for 2 h. The reaction mixture was then condensed (ensure proper ventilation for HCl gas on the evaporator) and placed under high vacuum overnight to yield a clear oil (4.4 g, quantitative): ^{1}H NMR (400 MHz, methanol-d_4) δ 7.40−7.32 (m, 5H, benzyl), 5.24 (s, 2H, -C$\mathbf{H}_2$OBn), 4.17−4.10 (m, 3H, $\boldsymbol{H}_\alpha$ (D-Glu), C$\mathbf{H}_2$-OTMSE), 2.50−2.37 (m, 2H, 2$\boldsymbol{H}_\gamma$ (D-Glu)), 2.18−2.07 (m, 2H, 2$\boldsymbol{H}_\beta$ (D-Glu)), 0.95−0.90 (m, 2H, C$\mathbf{H}_2$-TMSE), 0.00 (s, 9H, Si-($\mathbf{C}H_3$)$_3$); ^{13}C{^{1}H} NMR (101 MHz, methanol-d_4) δ 174.2 (carbonyl), 170.8 (carbonyl), 136.9 (aromatic), 130.5 (aromatic), 130.45 (aromatic), 130.40 (aromatic), 69.9 ($\mathbf{C}H_2$OBn), 64.7 ($\mathbf{C}H_2$TMSE), 53.5 ($\mathbf{C}_\alpha$ (D-Glu)), 30.9 ($\mathbf{C}_\gamma$ (D-Glu)), 27.2 ($\mathbf{C}_\beta$ (D-Glu)), 18.7 ($\mathbf{C}H_2$TMSE), −0.8 (Si-($\mathbf{C}H_3$)$_3$); HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{17}H_{28}NO_4Si$ 338.1788, found 338.1789.

(R)-1-Benzyl 5-[2-(Trimethylsilyl)ethyl] 2-{(S)-2-[(tert-Butoxycarbonyl)amino]propanamido}pentanedioate (8). **7** (4.3 g, 11.5 mmol, 1.2 equiv) and Boc-Ala-OSu (2.75 g, 9.6 mmol, 1.0 equiv) were dissolved in 64 mL of anhydrous THF on ice. DIPEA (2.43 mL, 14 mmol, 1.5 equiv) was added dropwise slowly. The reaction mixture was then allowed to warm room temperature and stirred overnight. The solution was condensed, dissolved in ethyl acetate, washed with 1 N HCl (twice), saturated bicarbonate (twice), and brine (once), dried with sodium sulfate, and condensed to yield a clear liquid oil as the pure product (4.5 g, 77%): ^{1}H NMR (400 MHz, chloroform-d) δ 7.41−7.33 (m, 5H, benzyl), 5.22−5.15 (m, 2H, -OC$\boldsymbol{H}_2$Ph), 4.98 (s, 1H, N$\boldsymbol{H}$), 4.68−4.63 (m, 1H, $\boldsymbol{H}_\alpha$ (D-Glu)), 4.22−4.11 (m, 3H, $\boldsymbol{H}_\alpha$ (L-Ala), -OC$\boldsymbol{H}_2$(TMSE)), 2.43−2.19 (m, 3H, 2$\boldsymbol{H}_\gamma$ (D-Glu), $\boldsymbol{H}_\beta$ (D-Glu)), 2.06−1.97 (m, 1H, $\boldsymbol{H}_\beta$ (D-Glu)), 1.46 (s, 9H, -($\mathbf{C}H_3$)$_3$, 1.37 (d, J = 7.1 Hz, 3H, C$\boldsymbol{H}_3$ (L-Ala)), 1.01−0.95 (m, 2H, -OCH$_2$C$\boldsymbol{H}_2$-TMSE), 0.05 (s, 9H, -Si-($\mathbf{C}H_3$)$_3$); ^{13}C{^{1}H} NMR (101 MHz, chloroform-d) δ 172.8 (carbonyl), 172.6 (carbonyl), 171.5 (carbonyl), 135.1 (aromatic), 128.6 (aromatic), 128.5 (aromatic), 128.2 (aromatic), 80.2 (Boc-$\mathbf{C}$(CH$_3$)$_3$), 67.3 ($\mathbf{C}H_2$OBn), 63.0 ($\mathbf{C}H_2$TMSE), 51.6 ($\mathbf{C}_\alpha$ (D-Glu)), 50.0 ($\mathbf{C}_\alpha$ (L-Ala)), 30.2 ($\mathbf{C}_\beta$ (D-Glu)), 28.3 (Boc-($\mathbf{C}H_3$)$_3$), 27.1 ($\mathbf{C}_\gamma$ (D-Glu)), 18.1 ($\mathbf{C}H_3$ (L-Ala)), 17.2 ($\mathbf{C}H_2$OTMSE), −1.4 (Si-($\mathbf{C}H_3$)$_3$); HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{25}H_{41}N_2O_7Si$ 509.2683, found 509.2687.

(R)-5-(Benzyloxy)-4-{(S)-2-[(tert-butoxycarbonyl)amino]propanamido}-5-oxopentanoic Acid (9). To a solution of **8** (408 mg, 0.8 mmol, 1.0 equiv) in THF (4.8 mL) was added tetrabutylammonium fluoride (TBAF) (3.2 mL, 1 M in THF, 4.0 equiv), and the reaction mixture was stirred at room temperature for 3 h. The reaction progress was checked with TLC, and upon completion, the reaction mixture was concentrated, diluted in ethyl acetate, and washed with 1 N HCl (twice). Combined aqueous fractions were back extracted with ethyl acetate. The combined organic fractions were then dried with sodium sulfate and concentrated. The crude product was purified by column chromatography (2−6% methanol in DCM) to give **9** (255 mg) as a white waxy solid (78% yield): ^{1}H NMR (600 MHz, methanol-d_4) δ 7.39−7.31 (m, 5H, benzyl), 5.20−5.15 (m, 2H, -C$\boldsymbol{H}_2$Ph), 4.51 (dd, J = 9.2, 5.0 Hz, 1H, $\boldsymbol{H}_\alpha$ (D-Glu)), 4.10−4.07 (m, 1H, $\boldsymbol{H}_\alpha$ (L-Ala)), 2.37 (t, J = 7.4 Hz, 2H, 2$\boldsymbol{H}_\gamma$ (D-Glu)), 2.23−2.17 (m, 1H, $\boldsymbol{H}_\beta$ (D-Glu)), 2.01−1.94 (m, 1H, $\boldsymbol{H}_\beta$ (D-Glu)), 1.45 (s, 9H, -($\mathbf{C}H_3$)$_3$), 1.30 (d, J = 7.2 Hz, 3H, C$\boldsymbol{H}_3$ (L-Ala)); ^{13}C{^{1}H} NMR (151 MHz, methanol-d_4) δ 176.1 (carbonyl), 175.1 (carbonyl), 172.7 (carbonyl), 157.5 (carbonyl), 137.1 (aromatic), 129.5 (aromatic), 129.3 (aromatic), 129.2 (aromatic), 80.6 (Boc-$\mathbf{C}$(CH$_3$)$_3$), 68.0 ($\mathbf{C}H_2$OBn), 53.1 ($\mathbf{C}_\alpha$ (D-Glu)), 51.7 ($\mathbf{C}_\alpha$ (L-Ala)), 30.9 ($\mathbf{C}_\beta$ (D-Glu)), 28.6 (Boc-($\mathbf{C}H_3$)$_3$), 27.6 ($\mathbf{C}_\gamma$ (D-Glu)), 18.3 ($\mathbf{C}H_3$ (L-Ala)); HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{20}H_{29}N_2O_7$ 409.1975, found 409.1964.

(S)-Benzyl 6-{[(Allyloxy)carbonyl]amino}-2-[(tert-butoxycarbonyl)amino]hexanoate (10). N^α-Boc-N^ε-allyloxycarbonyl-L-lysine dicyclohexyl ammonium salt (3 g, 5.86 mmol, 1.0 equiv) was dissolved in anhydrous DMF (40 mL). While the mixture was on ice and under nitrogen, benzyl bromide (1.53 mL, 12.89 mmol, 2.2 equiv) was added dropwise. The reaction mixture was warmed to room temperature and stirred overnight. Product formation was checked by thin layer chromatography. The reaction mixture was then diluted with water and extracted with ethyl acetate (thrice). The combined organic portions were then washed with 1 N HCl (thrice), saturated bicarbonate (thrice), and brine (once), dried over sodium sulfate, and concentrated. The crude product was then purified by column chromatography [gradient from ethyl acetate/hexane (5:95) to ethyl acetate/hexane (30:70)] to give **10** (2.51 g, 95%) as a colorless oil: ^{1}H NMR (600 MHz, methanol-d_4) δ 7.40−7.32 (m, 5H, benzyl), 5.94 (m, 1H, -COOCH$_2$C$\boldsymbol{H}$CH$_2$), 5.31 (dd, J = 17.2, 1.7 Hz, 1H, -COOCH$_2$CHC$\boldsymbol{H}$H), 5.24−5.17 (m, 3H, -COOCH$_2$CHCH$\boldsymbol{H}$, -C$\boldsymbol{H}_2$Ph), 5.13 (d, J = 12.4 Hz, 1H, -C$\boldsymbol{H}_2$Ph), 4.53 (appd, J = 5.4 Hz, 2H, COOC$\boldsymbol{H}_2$CHCH$_2$), 4.15−4.12 (m, 1H, $\boldsymbol{H}_\alpha$ (L-Lys)), 3.09 (t, J = 6.9 Hz, 2H, 2$\boldsymbol{H}_\varepsilon$ (L-Lys)), 1.83−1.77 (m, 1H, $\boldsymbol{H}_\beta$ (L-Lys)), 1.70−1.64 (m, 1H, $\boldsymbol{H}_\beta$ (L-Lys)), 1.53−1.37 (m, 13H, 2$\boldsymbol{H}_\gamma$, 2$\boldsymbol{H}_\delta$ (L-Lys) -C($\mathbf{C}H_3$)$_3$); ^{13}C{^{1}H} NMR (151 MHz, methanol-d_4) δ 174.2 (carbonyl), 158.8 (carbonyl), 158.1 (carbonyl), 137.3, 134.5, 129.5, 129.27, 129.24, 117.3, 80.5, 67.7, 66.2, 55.1, 41.3, 32.1, 30.3, 28.7, 24.0; HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{22}H_{33}N_2O_6$ 421.2339, found 421.2324.

(S)-Benzyl 6-((S)-6-{[(Benzyloxy)carbonyl]amino}-2-[(tert-butoxycarbonyl)amino]hexanamido)-2-[(tert-butoxycarbonyl)amino]-hexanoate (11). **11** was synthesized on the basis of the previously reported tandem deprotection/coupling of alloc group.[65] Boc-L-Lys(Z)-OH (1.53 g, 4.04 mmol, 2.0 equiv) was dissolved in 30 mL of anhydrous and degassed DCM followed by addition of *N*-[3-(dimethylamino)propyl]-*N*′-ethylcarbodiimide hydrochloride (EDC) (774 mg, 4.04 mmol, 2.0 equiv) and 1-hydroxybenzotriazole (HOBt) (545 mg anhydrous basis, 4.04 mmol, 2.0 equiv) on ice. The reaction mixture was allowed to stir for 30 min at 0 °C and 30 min at room temperature before **10** (850 mg, 2.02 mmol, 1.0 equiv), tetrakis-(triphenylphosphine)palladium(0) (231 mg, 0.2 mmol, 0.1 equiv), and 1,4-diazabicyclo[2.2.2]octane (DABCO) (1.13 g, 10.1 mmol, 5 equiv) were added. The reaction mixture was stirred for 3 h. Next, the reaction mixture was diluted with DCM, washed with 1 N HCl (thrice), saturated bicarbonate (thrice), and brine (once), dried over sodium sulfate, and concentrated. The crude product was then purified by column chromatography (gradient from 0.5 to 2% MeOH in DCM) to give 916 mg of **11** (65%) as an off-white foam:[c] ^{1}H NMR (600 MHz, methanol-d_4) δ 7.37−7.27 (m, 10H, aromatic), 5.18 (d, J = 12.3 Hz, 1H, -OC$\boldsymbol{H}_2$Ph), 5.10 (d, J = 12.3 Hz, 1H, -OC$\boldsymbol{H}_2$Ph), 5.06 (s, 2H, -NCOOC$\boldsymbol{H}_2$Ph), 4.12−4.08 (m, 1H, $\boldsymbol{H}_\alpha$ (L-Lys)), 3.95−3.93 (m, 1H, $\boldsymbol{H}_{\alpha'}$ (L-Lys)), 3.19−3.09 (m, 4H, 2$\boldsymbol{H}_\varepsilon$, 2$\boldsymbol{H}_{\varepsilon'}$ (L-Lys)), 1.80−1.74 (m, 1H, $\boldsymbol{H}_\beta$ (L-Lys)), 1.72−1.68 (m, 1H, $\boldsymbol{H}_{\beta'}$ (L-Lys)), 1.67−1.62 (m, 1H, $\boldsymbol{H}_\beta$ (L-Lys)), 1.60−1.56 (m, 1H, $\boldsymbol{H}_{\beta'}$ (L-Lys)), 1.52−1.45 (m, 4H, 2$\boldsymbol{H}_\delta$, 2$\boldsymbol{H}_{\delta'}$ (L-Lys)), 1.45−1.32 (m, 22H, 2$\boldsymbol{H}_\gamma$, 2$\boldsymbol{H}_{\gamma'}$ (L-Lys), -C($\mathbf{C}H_3$)$_3$ (Boc$_1$), -C($\mathbf{C}H_3$)$_3$ (Boc$_2$)); ^{13}C{^{1}H} NMR (151 MHz, methanol-d_4) δ 175.1, 174.2, 158.9, 158.2, 157.8, 138.4, 137.3, 129.5, 129.4, 129.29, 129.27, 128.9, 128.7, 80.5, 67.7, 67.3, 56.1, 55.2, 41.4, 39.9, 33.0, 32.1, 30.5, 29.8, 28.7, 24.1, 24.0; HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{37}H_{55}N_4O_9$ 699.3969, found 699.3959.

13a. 11 (595 mg, 0.85 mmol, 1.0 equiv) was dissolved in 10 mL of a trifluoroacetic acid (TFA)/DCM (1:1) mixture, and the reaction mixture was stirred for 4 h until the removal of Boc groups was confirmed my LC-MS. The reaction mixture was then concentrated and kept under high vacuum overnight to give **12** as a yellow syrup. The crude product was used in the next step without further purification. **9** (1.04 g, 2.55 mmol, 3.0 equiv) was dissolved in 45 mL of anhydrous DMF. HATU (936 mg, 2.46 mmol. 2.90 equiv) and DIPEA (1.34 mL, 7.66 mmol, 9.0 equiv) were added, and the reaction mixture was stirred on ice for 10 min before a solution of **12** in 5 mL of anhydrous DMF was added and the reaction mixture was stirred overnight at room temperature. The reaction mixture was then diluted with water. The product was extracted with ethyl acetate (thrice). Combined organic fractions were then washed with 1 N HCl (thrice), saturated bicarbonate (thrice), and brine (once). The organic fraction was then condensed, and the product was purified by column

https://dx.doi.org/10.1021/acs.joc.0c01852
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chromatography (gradient from 2 to 6% MeOH in DCM) to give **13a** (870 mg, 80% over two steps): ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.24−8.21 (m, 3H, -N*H*-, -N*H*′- (D-Glu), (α)N*H*- (L-Lys)), 7.86−7.80 (m, 2H, (α)N*H*′- (L-Lys), (ε)N*H*- (L-Lys)), 7.37−7.27 (m, 20H, aromatic), 7.20 (t, J = 5.8 Hz, 1H, -N*H*Cbz), 6.86−6.82 (m, 2H, -N*H*Boc (×2)), 5.11−5.02 (m, 6H, OCH_2Ph (×3)), 4.99 (s, 2H, OCH_2Ph), 4.30−4.24 (m, 2H, H_α, $H_{\alpha'}$ (D-Glu)), 4.22−4.18 (m, 1H, H_α (L-Lys)), 4.12−4.09 (m, 1H, $H_{\alpha'}$ (L-Lys)), 4.02−3.99 (m, 2H, H_α, $H_{\alpha'}$ (L-Ala)), 3.02−2.92 (m, 4H, 2H_ε, 2$H_{\varepsilon'}$ (L-Lys)), 2.20−2.15 (m, 4H, 2H_γ, 2$H_{\gamma'}$ (D-Glu)), 2.00−1.93 (m, 2H, H_β, $H_{\beta'}$ (D-Glu)), 1.86−1.79 (m, 2H, H_β, $H_{\beta'}$ (D-Glu)), 1.67−1.62 (m, 1H, H_β (L-Lys)), 1.60−1.54 (m, 2H, H_β, $H_{\beta'}$ (L-Lys)), 1.45−1.15 (m, 33H, $H_{\beta'}$ (L-Lys), 2H_δ, 2$H_{\delta'}$ (L-Lys), -C(CH_3)$_3$ (Boc$_1$), -C(CH_3)$_3$ (Boc$_2$), 2H_γ, 2$H_{\gamma'}$ (L-Lys), -CH_3, -C$H_{3'}$ (L-Ala)); ^{13}C{^{1}H} NMR (151 MHz, DMSO-d_6) δ 172.0, 171.5, 171.1, 156.0, 154.9, 137.2, 135.9, 135.8, 128.4, 128.3, 127.9, 127.75, 127.73, 127.71, 78.1, 65.9, 65.7, 65.0, 52.6, 52.0, 51.6, 51.4, 49.7, 49.6, 38.2, 31.8, 30.4, 29.1, 28.6, 28.1, 26.9, 22.7, 18.4, 18.3; HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{67}H_{91}N_8O_{17}$ 1279.6497, found 1279.6457.

13b. **11** (1.29 g, 1.84 mmol, 1.0 equiv) was dissolved in 12 mL of a TFA/DCM (1:1) mixture, and the reaction mixture was stirred for 4 h until the removal of Boc groups was confirmed my LC-MS. The reaction mixture was then concentrated and kept under high vacuum overnight to give **12** as a yellow syrup. The crude product was used in the next step without further purification. **5** (1.74 mg, 5.5 mmol, 3.0 equiv) was dissolved in 90 mL of anhydrous DMF. HATU (2.02 g, 5.33 mmol. 2.90 equiv) and DIPEA (2.8 mL, 16.5 mmol, 9.0 equiv) were added, and the reaction mixture was stirred at room temperature for 10 min on ice, before a solution of crude **12** in 10 mL of anhydrous DMF was added and the reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction mixture was then diluted with water, after which a precipitate was formed. The precipitate was then filtered and washed with 1 N HCl, water, saturated bicarbonate, and water. The filtered product was then washed with diethyl ether and dried under vacuum to give **13b** (1.85 g, 90% over two steps): ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.37−7.28 (m, 10H, aromatic), 5.09 (s, 2H, -OCH_2Ph), 4.98 (s, 2H, -NCOOCH_2Ph), 4.19 (dd, J = 9.0, 5.3 Hz, 1H, H_α (L-Lys)), 4.12−4.08 (m, 3H, H_α, $H_{\alpha'}$ (D-Gln), $H_{\alpha'}$ (L-Lys)), 3.96−3.92 (m, 2H, H_α, $H_{\alpha'}$ (L-Ala)), 3.01−2.92 (m, 4H, 2H_ε, 2$H_{\varepsilon'}$ (L-Lys)), 2.15−2.10 (m, 4H, 2H_γ, 2$H_{\gamma'}$ (D-Gln)), 1.98−1.90 (m, 2H, H_β, $H_{\beta'}$ (D-Gln)), 1.74−1.62 (m, 3H, H_β, $H_{\beta'}$ (D-Gln), H_β (L-Lys)), 1.60−1.54 (m, 2H, H_β, $H_{\beta'}$ (L-Lys)), 1.47−1.41 (m, 1H, $H_{\beta'}$ (L-Lys)), 1.38−1.31 (m, 22H, 2H_δ, 2$H_{\delta'}$ (L-Lys), -C(CH_3)$_3$ (Boc$_1$), -C(CH_3)$_3$ (Boc$_2$)), 1.25−1.15 (m, 10H, 2H_γ, 2$H_{\gamma'}$ (L-Lys), -CH_3, -C$H_{3'}$ (L-Ala)); ^{13}C{^{1}H} NMR (151 MHz, DMSO-d_6) δ 173.4, 173.0, 172.9, 172.2, 172.1, 171.78, 171.70, 156.2, 155.5, 137.3, 136.1, 128.6, 128.5, 128.2, 127.94, 127.90, 127.8, 78.56, 78.53, 65.9, 65.2, 65.1, 52.7, 52.1, 52.0, 50.0, 49.9, 39.9, 38.2, 31.8, 31.7, 31.5, 30.4, 29.2, 28.7, 28.3, 27.8, 22.9, 17.8, 17.7; HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{53}H_{81}N_{10}O_{15}$ 1097.5883, found 1097.5847.

Note that the amide protons are exchanged with deuterated solvent.

14a. **13a** (470 mg, 0.36 mmol, 1.0 equiv) was dissolved in 6 mL of a TFA/DCM (1:1) mixture, and the reaction mixture was stirred for 4 h until the removal of Boc groups was confirmed my LC-MS. The reaction mixture was then concentrated and treated with ice-cold diethyl ether to give **14a** as a white solid (432 mg, di TFA salt, 90%), which was used in the next step without further purification: ^{1}H NMR (600 MHz, methanol-d_4) δ 7.38−7.28 (m, 20H, aromatic), 5.22−5.12 (m, 6H), 5.06 (s, 2H), 4.52−4.49 (m, 2H), 4.40−4.38 (m, 1H), 4.22−4.19 (m, 1H), 3.99−3.95 (m, 2H), 3.20−3.15 (m, 1H), 3.12−3.07 (m, 3H), 2.37−2.32 (m, 4H), 2.27−2.17 (m, 2H), 2.03−1.93 (m, 2H), 1.84−1.78 (m, 1H), 1.76−1.71 (m, 1H), 1.70−1.60 (m, 2H), 1.53−1.44 (m, 10H), 1.39−1.33 (m, 4H); ^{13}C{^{1}H} NMR (151 MHz, methanol-d_4) δ 174.6, 174.4, 173.5, 172.68, 172.66, 171.2, 171.1, 162.6, 162.3, 158.9, 138.4, 137.2, 137.06, 137.04, 129.64, 129.61, 129.48, 129.46, 129.37, 129.36, 129.33, 129.2, 128.9, 128.7, 118.9, 68.2, 67.9, 67.3, 55.0, 53.9, 53.6, 53.4, 50.2, 49.5, 41.4, 39.9, 32.7, 32.6, 32.5, 31.9, 30.5, 29.7, 28.3, 24.1, 24.0, 17.74, 17.73; HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{57}H_{75}N_8O_{13}$ 1079.5454, found 1079.5404.

14b. **13b** (500 mg, 0.45 mmol, 1.0 equiv) was dissolved in 6 mL of a TFA/DCM (1:1) mixture, and the reaction mixture was stirred for 4 h until the removal of Boc groups was confirmed by LC-MS. The reaction mixture was then concentrated and treated with ice-cold diethyl ether to give **14b** as a white solid (461 mg, di TFA salt, 90%), which was used in the next step without further purification: ^{1}H NMR (600 MHz, deuterium oxide) δ 7.45−7.36 (m, 10H, aromatic), 5.21 (d, J = 12.2 Hz, 1H), 5.14 (d, J = 12.2 Hz, 1H), 5.06 (s, 2H), 4.33−4.28 (m, 3H), 4.14−4.09 (m, 3H), 3.21−3.15 (m, 1H), 3.09−3.02 (m, 3H), 2.40−2.32 (m, 4H), 2.12−2.02 (m, 2H), 2.00−1.89 (m, 2H), 1.81−1.75 (m, 1H), 1.72−1.63 (m, 3H), 1.54−1.52 (m, 6H), 1.47−1.42 (m, 4H), 1.32−1.22 (m, 4H); ^{13}C{^{1}H} NMR (151 MHz, deuterium oxide) δ 175.6, 175.5, 174.8, 174.6, 173.8, 173.7, 170.9, 170.8, 163.3, 163.0, 162.8, 162.6, 158.4, 135.2, 128.8, 128.74, 128.71, 128.3, 128.2, 127.5, 119.2, 117.3, 115.3, 67.5, 66.7, 54.1, 53.1, 53.0, 49.1, 40.0, 38.8, 31.3, 30.7, 29.8, 28.4, 27.7, 27.0, 26.9, 22.3, 22.1, 16.6, 16.5; HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{43}H_{65}N_{10}O_{11}$ 897.4834, found 897.4811.

15a. **3** (354 mg, 0.75 mmol, 3.0 equiv) was dissolved in 25 mL of anhydrous DMF. HATU (276 mg, 0.72 mmol, 2.90 equiv) and DIPEA (392 μL, 2.2 mmol, 9.0 equiv) were added, and the reaction mixture was stirred on ice for 10 min before **14a** (330 mg, 0.25 mmol. 1.0 equiv) was added and the reaction mixture was stirred overnight at room temperature. The reaction mixture was then diluted with water after which a precipitate was formed. The precipitate was the filtered and washed with 1 N HCl, water, saturated bicarbonate, and water. The filtered product was then washed with diethyl ether and dried under vacuum to give crude **15a** (460 mg) as a white solid. The crude was directly used in the next step.

15b. **3** (377 mg, 0.80 mmol, 3.0 equiv) was dissolved in 25 mL of anhydrous DMF. HATU (295 mg, 0.77 mmol, 2.90 equiv) and DIPEA (418 μL, 2.4 mmol, 9.0 equiv) were added, and the reaction mixture was stirred on ice for 10 min before **14b** (300 mg, 0.26 mmol. 1.0 equiv) was added and the reaction mixture was stirred overnight at room temperature. The reaction mixture was then diluted with water after which a precipitate was formed. The precipitate was then filtered and washed with 1 N HCl, water, saturated bicarbonate, and water. The filtered product was then washed with diethyl ether and dried under vacuum to give crude **15b** (367 mg) as a white solid. The crude was directly used in the next step.

16a. **15a** (50 mg) was dissolved in a mixture of water (0.24 mL) and TFA (2.4 mL) before Pd(OH)$_2$/C (50 mg, 20 wt % loading) was added and the reaction mixture was allowed to stir under H_2 (balloon) for 12 h. After that, the catalyst was filtered and the filtrate was condensed under a flow of N_2. The crude product was then precipitated out by adding ice-cold diethyl ether and isolated. The crude product was then purified by HPLC to give **16a** (15.0 mg, 45% yield over two steps) as a white solid.

16b. **15b** (50 mg) was dissolved in a mixture of water (0.24 mL) and TFA (2.4 mL) before Pd(OH)$_2$/C (50 mg, 20 wt % loading) was added and the reaction mixture was allowed to stir under H_2 (balloon) for 12 h. After that, the catalyst was filtered and the filtrate was condensed under a flow of N_2. The crude product was then precipitated out by adding ice-cold diethyl ether and isolated. The crude product was then purified by HPLC to give **16b** (12 mg, 27% yield over two steps) as a white solid.

Crude **16a** and **16b** were purified by high-performance liquid chromatography using a semiprep C18 column [Luna 5 μm C18(2), 100 Å column (250 mm × 10 mm)] and then lyophilized to give the products as white solids (mixture of α and β anomeric isomers): buffer A, 98% water, 2% acetonitrile, and 0.06% trifluoroacetic acid; buffer B, 20% water, 80% acetonitrile, and 0.04% trifluoroacetic acid; flow rate of 3 mL/min; dual absorbance detection at 214 and 254 nm; gradient of 100% A and 0% B from 0 to 5 min, from 100% A and 0% B to 90% A and 10% B from 5 to 40 min, from 90% A and 10% B to 0% A and 100% B from 40 to 50 min, of 0% A and 100% B from 50 to 52 min, from 0% A and 100% B to 100% A and 0% B from 52 to 55 min, and of 100% A and 0% B from 52 to 55 min.

https://dx.doi.org/10.1021/acs.joc.0c01852
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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.joc.0c01852.

Additional synthetic schemes, NMR spectra, and high-resolution MS data (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors are thankful for support from the Delaware COBRE program, supported by a grant from the National Institute of General Medical Sciences (NIGMS 1 P30 GM110758 and 1 P20 GM104316-01A1), as well as Grant GM138599 from the National Institutes of Health. C.L.G. is a Pew Scholar in the Biomedical Sciences, supported by the Pew Charitable Trusts. C.L.G. is a Camille Dreyfus Teacher Scholar and thanks the Camille and Henry Dreyfus Foundation. L.A.G., T.H., and J.R. thank the University of Delaware for their support through the University Summer Scholarship. K.L.B. thanks the University of Delaware for their support through the University Doctoral Fellowship Program. The authors gratefully acknowledge the National Science Foundation (CAREER CHE 1554967) for support of this research. The authors thank our colleagues at the University of Delaware: Dr. PapaNii Asare-Okai, the Director of the MS facility, and Dr. Shi Bai at the NMR facility of the University of Delaware.

■ ADDITIONAL NOTES

[a]Amounts of each acid component for a 0.25 mmol scale: Fmoc-L-Lys(Mtt)-OH (625 mg), Fmoc-D-Glu-OtBu (425 mg), Fmoc-D-Glu-NH_2 (368 mg), Fmoc-L-Ala-OH (311 mg), Fmoc-D-Ala-OH (311 mg), compound **3** (471 mg).

[b]In method A and for concurrent extension of two peptide branches, after the formation of the dilysine core, each coupling step was performed as a double-coupling reaction, in which after the first coupling reaction, following the general procedure discussed above, the resin was washed with 5 mL of DMF (3 × 2 min), and the coupling reaction was repeated under the same condition before proceeding to the next deprotection step or final cleavage.

[c]The residual leak (~5%) of a catalyst impurity was observed on occasions after purification.

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