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Synthetic Peptidoglycan Substrates for Penicillin-Binding Protein 5 of Gram-Negative Bacteria

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The major constituent of the bacterial cell wall, peptidoglycan, is comprised of repeating units of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) with an appended peptide. Penicillin-binding proteins (PBPs) are involved in the final stages of bacterial cell wall assembly. Two activities for PBPs are the cross-linking of the cell wall, carried out by DD-transpeptidases, and the DD-peptidase activity, that removes the terminal D-Ala residue from peptidoglycan. The DD-peptidase activity moderates the extent of the cell wall cross-linking. There exists a balance between the two activities that is critical for the well-being of bacterial cells. We have cloned and purified PBP5 of *Escherichia coli*. The membrane anchor of this protein was removed, and the enzyme was obtained as a soluble protein. Two fragments of the polymeric cell wall of Gram-negative bacteria (compounds **5** and **6**) were synthesized. These molecules served as substrates for PBP5. The products of the reactions of PBP5 and compounds **5** and **6** were isolated and were shown to be D-Ala and the fragments of the substrates minus the terminal D-Ala. The kinetic parameters for these enzymic reactions were evaluated. PBP5 would appear to have the potential for turnover of as many as 1.4 million peptidoglycan strands within a single doubling time (i.e., generation) of *E. coli*.

The cell wall is essential for survival of bacteria. The major constituent of the bacterial cell wall, peptidoglycan, is comprised of repeating units of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) with an appended peptide. Structure **1** depicts the type of peptidoglycan that is found in all Gram-negative and some Gram-positive bacteria. The last steps of cell wall assembly are carried out by penicillin-binding proteins (PBPs) on the outer surface of the bacterial cytoplasmic membrane (in the periplasmic space, in the case of Gram-negative organisms).[1,2] It is understood that the vast majority of these proteins are active-site-serine enzymes, but their biochemical properties have not been studied in detail.[2] Various PBPs undergo acylation by the polymeric peptidoglycan at the active site serine (**1** → **2**, Scheme 1). Depending on the nature of the given PBP, two fates await this intermediate. If another peptidoglycan undergoes a coupling with the acyl-enzyme species (i.e., **2**), one gets cross-linking of the cell wall (**2** → **3**), a reaction catalyzed by DD-transpeptidases. A glimpse of how the cross-linked entity looks was provided recently by a high-resolution X-ray structure.[3] Alternatively, the acyl-enzyme species may undergo hydrolysis to give rise to species **4**, a reaction that has been attributed to DD-peptidases. In essence, the reaction of DD-peptidases would moderate the degree of cross-linking of cell wall by DD-transpeptidases. The degree of cross-linking varies among bacterial species, but the balanced interplay between the two activities is critical for the well-being of these microorganisms.[4]

Reports on the chemical synthesis of small cell wall fragments have appeared in the literature.[5] The syntheses of lipids I and II and their analogues, which serve as precursors in biochemical transglycosylation, and hence the first step in preparation of the bacterial cell wall, have also been described.[6]

We report herein the syntheses of two fragments of the peptidoglycan of Gram-negative bacteria, compounds **5** and **6** (Scheme 2). These molecules served as substrates for PBP5, the turnover products for which were studied (**5** → **7** and **6** → **8**). Furthermore, catalytic activity of this

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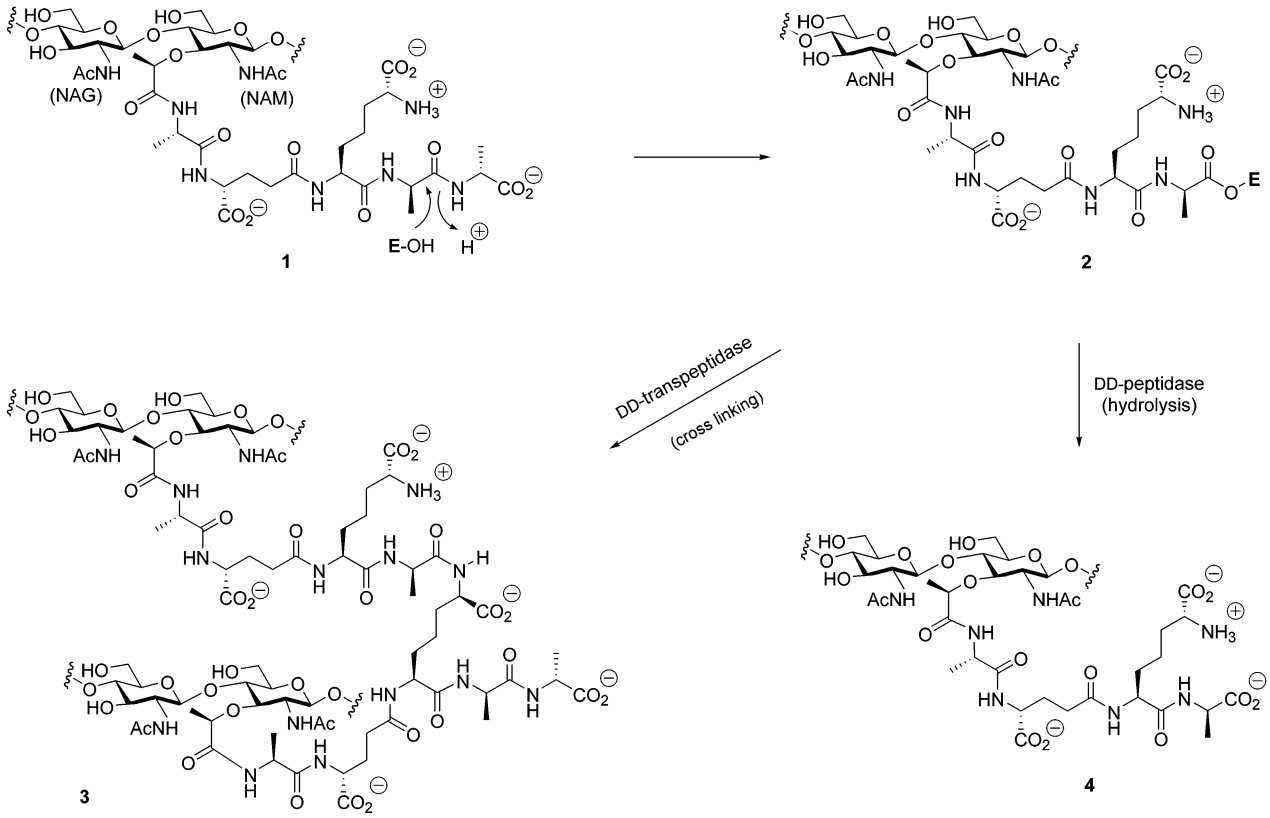
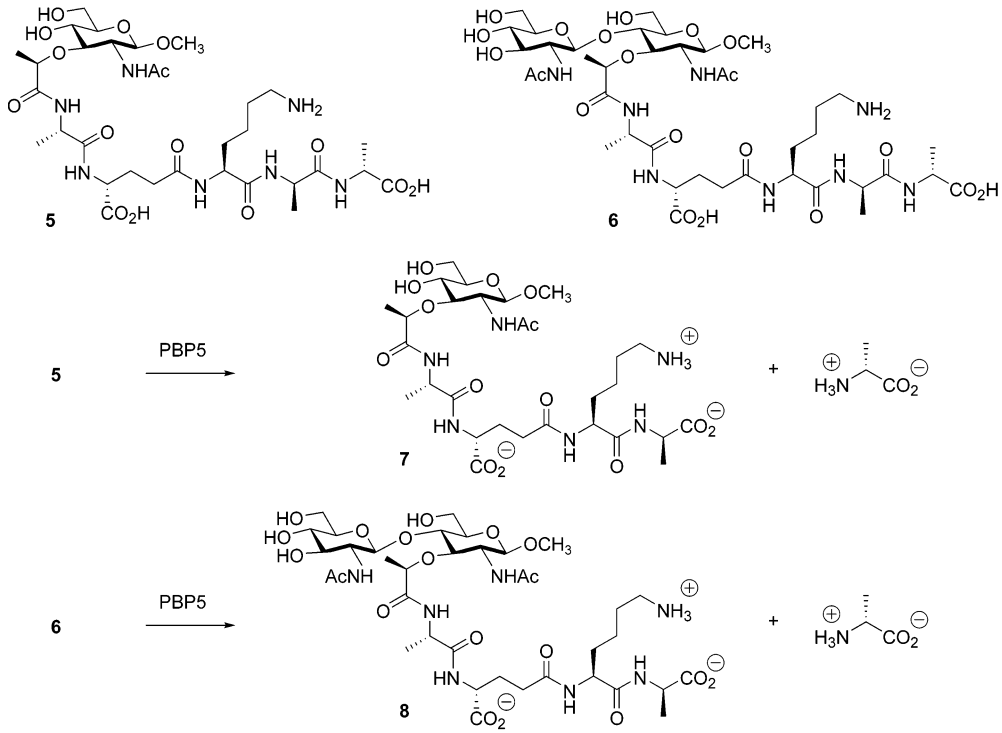
SCHEME 1



SCHEME 2



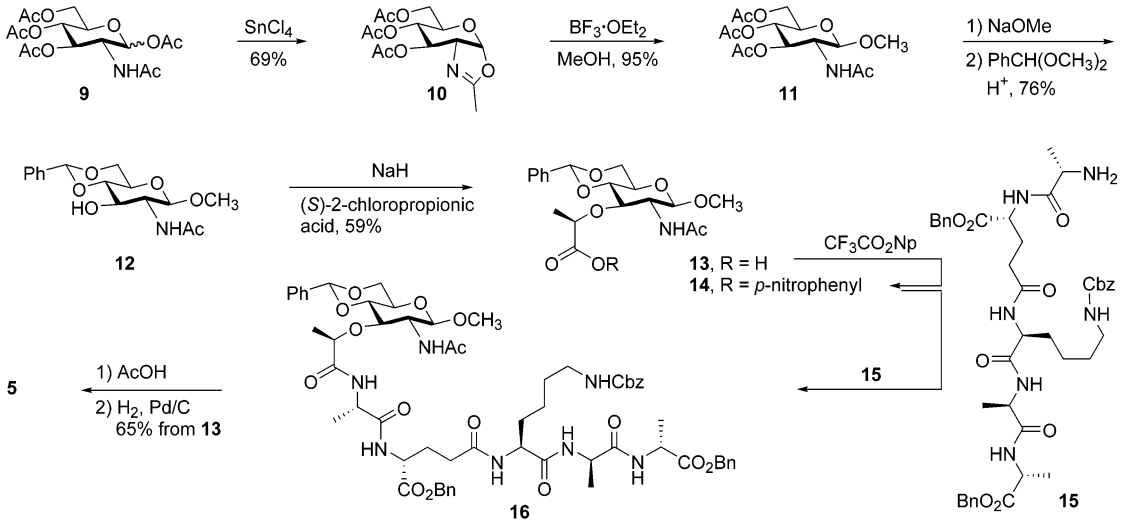
enzyme is discussed in the context of the final stages of the assembly of the bacterial cell wall.

Results and Discussion

We undertook the syntheses of compounds **5** and **6**, two fragments of the polymeric peptidoglycan of *E. coli* and other Gram-negative bacteria. Diaminopimelate of the peptidoglycan was substituted by lysine in the synthetic targets. These are the first "peptidoglycan-like" molecules that have been prepared for study with DD-peptidases.[7]

The synthetic approach incorporated a combination of the oxazoline method[8] in the NAM fragment and dimeth-

SCHEME 3



ylmaleoyl (DMM)[9] as an amine-protecting group in the NAG fragment. The general synthetic plan for the monosugar derivative **5** and bis-sugar derivative **6** called for the use of orthogonal protection-deprotection strategies. The first step for the synthesis of **5**, outlined in Scheme 3 was the formation of an oxazoline derivative **10** from peracetylated glucosamine **9**, using the standard protocol of Srivastava[8] to afford the desired derivative **10** in 69% yield. Upon treatment with $BF_3{\cdot}OEt_2$ in MeOH, this material was stereoselectively converted into *β*-OMe derivative **11** in 95% yield. Chemical manipulation (hydrolysis and benzylidene formation) was then carried out to mask and differentiate among the three hydroxyl groups for the regioselective introduction of a lactate moiety at the 3-*O*-position of **12**. The formation of muramyl fragment **13** by coupling **12** and an excess of (*S*)-2-chloropropionic acid in the presence of NaH proceeded in 59% yield. The coupling of the fully protected peptide residue **15** with **13** was accomplished by prior activation of the lactate carboxylate of **13** by *p*-nitrophenyl trifluoroacetate in the presence of pyridine to give **16**. After 4,6-*O*-benzylidene deprotection in 60% acetic acid, we carried out a global hydrogenolysis of the benzyl groups (esters, ethers and benzyloxycarbonyl functionalities) over 10% Pd/C in methanol to give compound **5** (65% from **13**).

The synthesis of the bis-sugar derivative **6** (Scheme 4) required a different protection strategy. An important consideration here was to distinguish between both the *β*-(1→4)-glycosidic bond formation and the hydroxyl groups (anomeric and peripheral) of the monosaccharide unit. For this purpose, we chose the commonly used sugar protective groups such as *O*-benzylidene-, *O*-benzyl-, *O*-acetyl-, and *O*-TBDMS. Another important consideration for the synthesis of the disaccharide was the stereoselective construction of the *β*-(1→4)-glycosidic bond. Trichloroacetimidate was used as the leaving group and trimethylsilyl trifluoromethanesulfonate (TMSOTf) served as the promoter during glycosyl bond formation with neighboring group participation of DMM.[9]

In a one-pot reaction, glucosamine (**17**) was treated with 2,3-dimethylmaleic anhydride in the presence of base. The resulting mixture was treated with acetic anhydride at room temperature for 5 h, followed by an aqueous workup and column chromatography to give **18** in 55% yield. Regioselective deacetylation at the C-1 anomeric position using hydrazine acetate in DMF, followed by silylation of the unmasked hydroxyl functionality, provided the desired orthogonally protected glucosyl building block **19**. Glucosamine triacetyl **19** was subsequently converted to the corresponding trihydroxy intermediate, which was converted to **20**. Due to the base-sensitive nature of the DMM group, loss of the DMM group was expected when the DMM-protected glucoside derivatives were subjected to a typical benzylation procedure, using benzyl bromide and sodium hydride. However, treatment with benzyl 2,2,2-trichloroacetimidate and TfOH (acting as a promoter) gave the derivative **21** in 67% yield, which was subsequently desilylated using fluoride anion to afford an anomeric mixture of 1-hydroxy derivative **22** in 72% yield. The glucosyl acceptor **24** was prepared by acetylation of **12** and the subsequent reductive benzylidene acetal ring opening with triethylsilane and trifluoroacetic acid.[10]

The glycosidation reaction between **23** and **24** was carried out using TMSOTf as the promoter to yield the dissacharide **25** in 47%. The DMM protective group in **25** was removed by the reaction with NaOH (4 h), followed by adjustment of pH to 3.0−3.5 by the addition of HCl. The mixture was kept at pH of 3.0−3.5 for 1 day, prior to the conversion to the corresponding acetylated derivative **26** by treatment with Ac_2O in pyridine.[9] The overall yield (49%) was modest. The 3-acetyl group in compound **26** was selectively unmasked by treatment with NaOMe to afford a free hydroxyl, which was readily coupled with (*S*)-2-chloropropionic acid. The incorporation of lactate and the subsequent condensation with the

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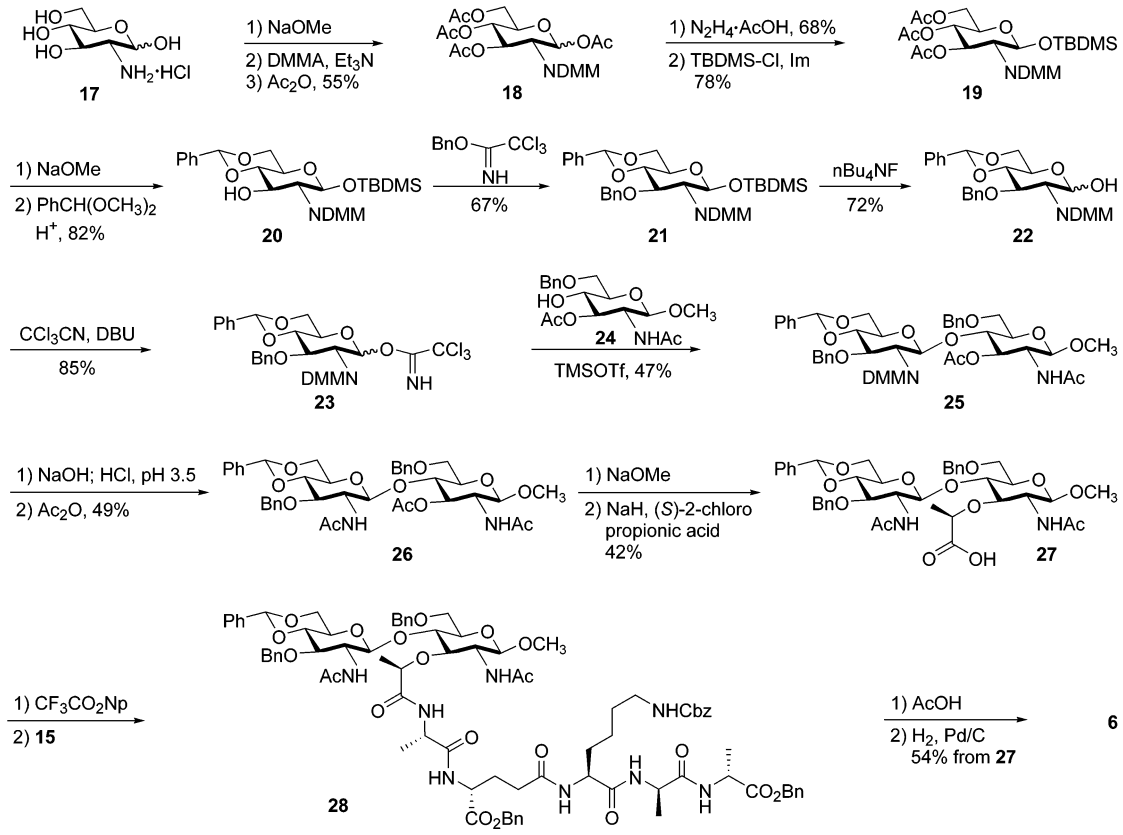
SCHEME 4



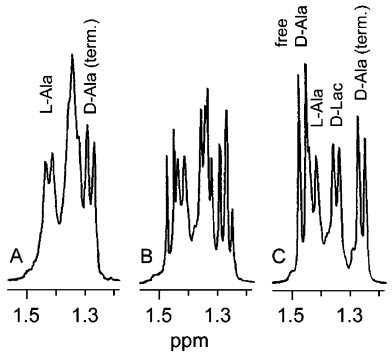


FIGURE 1. (A) The methyl NMR resonances of compound **6** before the addition of PBP5, (B) in the middle of turnover after the addition of PBP5, and (C) at the end of the PBP5 reaction. The terminal D-Ala in **6** (panel A) and in **8** (panel C) are designated as "D-Ala (term.)".

pentapeptide **15**, followed by global deprotection, as was accomplished in the preparation of **5**, resulted in **6**.

With the two desired synthetic peptidoglycan fragments in hand, we were ready to test them in biochemical experiments with purified PBP5. The DD-peptidase reaction, as shown for compound **6**, should give rise to D-Ala and compound **8** (**6** → **8**). Figure 1A shows the ^{1}H NMR signals for the four methyl groups in the structure of compound **6**. On addition of PBP5, a clean transition to new species was seen, ending with a mixture of products (Figure 1C) that showed four resolved methyl doublet signals.

The products were purified by preparative thin-layer chromatography, and they were shown to be D-Ala and compound **8** by analyses of their NMR and MS data. The same experiment was carried out with compound **5**, which also showed a clean hydrolysis of the terminal D-Ala and the formation of a fragment of **5** missing one D-Ala (**7**).

The formation of D-Ala in the course of the reaction of PBP5 can be monitored readily by a coupled assay with D-amino acid oxidase and lactate dehydrogenase. The D-Ala that is generated by the reaction of PBP5 is oxidized to lactate by D-amino acid oxidase, which in turn is reduced by the NADH-dependent lactate dehydrogenase. Consumption of NADH as a function of time was monitored at 340 nm. Hydrogen peroxide that forms during the reaction of D-amino acid oxidase was neutralized by catalase, which was added to the reaction mixture. The reaction of PBP5 was limiting, as all other reagents (including enzymes) were kept in large excess.

Compounds **5** and **6** were turned over by PBP5 with k_{cat} values of 0.9 ± 0.1 and 1.1 ± 0.1 s^{-1}, and K_m values of 3.5 ± 0.5 and 3.7 ± 0.5 mM, respectively. The millimolar K_m values are in the typical range for many important metabolic enzymes in living organisms. For example, of the 21 enzymes for glycolysis and the tricarboxylic acid cycle, 10 have millimolar K_m values for their respective substrates. The effective concentration of peptidoglycan near the surface of the plasma membrane is expected to be high, so for entropic reasons, saturation of the membrane-anchored PBP5 should not be a problem. Regardless, K_m might be somewhat lower and k_{cat} might be a bit higher, if the energetic contribution of the carboxylate of diaminopimelate—which is absent in the structures of **5** and **6** were to be taken into account. This effect need not be large, as solvation may

temper its electrostatic contribution to binding to PBP to as low as 0.5 kcal/mol.[11,12]

PBP5 is the most abundant of the PBPs in *E. coli*.[13,14] It has been estimated at 790[13] (32% of total PBPs) or 1800[14] (65% of total PBPs) copies per *E. coli* cell. The measured k_{cat} values for compounds **5** and **6** would mean that even at the lower estimation of the copy numbers for PBP5, there would be the potential for turnover of 1.4 million strands of peptidoglycan by PBP5 within a 30-min doubling time for a typical *E. coli*. This level of activity is further supplemented by the function of PBP6, which is also presumed to be a DD-peptidase.[2]

The assembly and regulation of the bacterial cell wall is a complex process involving multiple PBPs. Studies of PBPs have been held back by the fact that these enzymes are generally difficult to work with. Furthermore, the substrates for PBPs are polymeric and complex entities that have been difficult to prepare for biochemical studies. In the present report we have attempted a systematic approach to studies of PBP5 of *E. coli* that provides a glimpse of the event that regulates the degree of bacterial cell wall cross-linking.

Methods

PBP5 was cloned and purified in our laboratory according to a literature procedure.[15] Compounds **10**[8] and **18**−**20**[9] were prepared according to earlier reports.

Kinetics of Turnover of Compounds 5 and 6 by PBP5. A modified version of the method of Grassle was used for monitoring the formation of D-alanine.[16] All reactions were carried out in 40 mM HEPES (pH 7.5) containing 100 mM NaCl, 56 μM NADH, 0.75 units of D-amino acid oxidase, 3.3 units of lactate dehydrogenase, 640 units of catalase, and 48 nM PBP5. The total volume of the reaction mixture was 150 μL, and the temperature was maintained at 25 °C. Consumption of NADH as a function of time was monitored spectrophotometrically at 340 nm ($\Delta\epsilon = 6250\ cm^{-1}\ M^{-1}$). The steady-state kinetic parameters for the DD-carboxypeptidase reaction were determined at concentrations of the substrates in the range of 0.4−4.0 mM. The kinetic parameters were calculated from Lineweaver−Burk plots. Linear regression was performed using the GraFit 4 software (Erithacus Software Ltd.).

Methyl 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-D-glucopyranoside (11). Compound **10**[8] (6.6 g, 20 mmol) was treated with $BF_3{\cdot}OEt_2$ (1.12 mL, 5.4 mmol) in anhydrous MeOH (40 mL) and was stirred at rt overnight. The mixture was concentrated to dryness, and the residue was taken up in CH_2Cl_2. The resulting solution was passed through a layer of silica gel, which was subsequently washed with CH_2Cl_2/MeOH (3:0.5). Evaporation of the filtrate gave the title compound (6.9 g, 95%), which was used without further purification. The title compound had been prepared previously by a different method.[17] Spectral data of compound **11** was identical with those previously synthesized. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.92, 2.00, 2.05 (4s, 12H), 3.47 (s, 3H, OCH_3), 3.70 (2m, 1H, H-5), 3.86 (dd, J = 10.5, 8.1 Hz, 1H, H-2), 4.12 (dd, J = 12.2, 2.4 Hz, 1H, H-6), 4.25 (dd, J = 12.2, 4.1 Hz, 1H, H-6′), 4.57 (d, J = 8.1 Hz, 1H, H-1), 5.05 (t_{app}, J = 9.7 Hz, 1H, H-4), 5.26 (dd, J = 10.5, 9.7 Hz, 1H, H-3), 5.84 (d, J = 8.1 Hz, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.9, 21.0, 23.6, 54.7 (C-2), 57.0 (OCH_3), 62.3 (C-6), 68.8 (C-4), 71.9 (C-3), 72.7 (C-5), 101.8 (C-1), 169.7, 170.6, 171.0, 171.1.

Methyl 2-Acetamido-4,6-*O*-benzylidene-2-deoxy-*β*-D-glucopyranoside (12). Compound **11** (3.6 g, 10 mmol) was dissolved in anhydrous MeOH (40 mL), and the solution was treated with NaOMe (0.2 g, 3.7 mmol). After being stirred at rt for 0.5 h, the solution was neutralized with an excess of Amberlite IR-120 (H^+) and stirred for an additional 20 min. The resin was filtered, and the resulting solution was concentrated to dryness. The residue was dissolved in acetonitrile, re-evaporated to dryness, and kept under vacuum for 1 h. The residue was dissolved in anhydrous acetonitrile (40 mL). Benzaldehyde dimethyl acetal (2.2 mL, 14.7 mmol) and *p*-toluenesulfonic acid monohydrate (0.1 g) were added to the mixture, and the resultant solution was stirred at rt for 2 h, followed by neutralization by the addition of a few drops of Et_3N. After being stirred at rt for 0.5 h, the reaction mixture was concentrated to dryness and the residue was chromatographed on silica gel (hexane to hexane/EtOAc, 2:1) to give the title compound as a white solid (2.96 g, 76%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.81 (s, 3H, NHCOCH_3), 3.32 (s, 3H, OCH_3), 3.35 (m, 1H, H-5), 3.41 (t_{app}, J = 8.9 Hz, 1H, H-4), 3.53 (dd, 1H, J = 8.9, 7.3 Hz, H-2), 3.57 (m, 1H, H-3), 3.72 (dd, J = 10.5, 9.7 Hz, 1H, H-6), 4.19 (dd, J = 9.7, 4.9 Hz, 1H, H-6′), 4.37 (d, J = 8.1 Hz, 1H, H-1), 5.58 (s, 1H, CHPh), 7.35−7.44 (m, 5H), 7.83 (d, J = 8.9 Hz, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 23.8 (NHCOCH_3), 56.6 (C-2), 56.8 (OCH_3), 66.6 (C-5), 68.5 (C-6), 71.2 (C-3), 81.9 (C-4), 101.4 (CHPh), 103.1 (C-1), 127.1, 128.7, 129.6, 138.4, 169.9. MS (ES) m/z 324.19 $[M + H]^+$, 346.18 $[M + Na]^+$.

Methyl 2-Acetamido-4,6-*O*-benzylidene-3-*O*-((*R*)-1′-carboxyethyl)-2-deoxy-*β*-D-glucopyranoside (13). NaH (1.25 g, 31.2 mmol, 60% oil dispersion) was added to a solution of **12** (1.6 g, 5 mmol) in anhydrous DMF (50 mL), which was charged with activated 4-Å molecular sieves (2.5 g). After the mixture was stirred at rt for 0.5 h, (*S*)-2-chloropropionic acid (3 g, 27.8 mmol) in DMF (10 mL) was added to the reaction mixture and the resultant solution was stirred at rt for 0.5 h. Additional NaH (1.25 g, 31.2 mmol) in two portions was added to the reaction mixture, and the mixture was stirred at rt overnight. After the reaction, the DMF was removed to dryness under vacuum and then the crude material was redissolved in water, stirred with charcoal, and filtered through a small layer of Celite. The filtrate was acidified to pH 4 by the addition of 1 N HCl, was filtered, and then evaporated to dryness. The residue was taken up in a mixture of water and chloroform, the layers were separated, and the aqueous solution was washed with chloroform. The combined organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated to dryness to yield the title compound (1.17 g, 59%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.22 (d, J = 6.4 Hz, 3H, OCHCH_3), 1.79 (s, 3H, NHCOCH_3), 3.31 (s, 3H, OCH_3), 3.38 (m, 1H, H-5), 3.49 (q, J = 8.5 Hz, 1H, H-2), 3.59 (t_{app}, J = 9.3 Hz, 1H, H-4), 3.72 (t_{app}, J = 9.7 Hz, 1H, H-3), 3.74 (t_{app}, J = 9.7 Hz, 1H, H-6), 4.17 (q, J = 7.3 Hz, 1H, O−CH-CH_3), 4.22, (dd, J = 9.7, 4.9 Hz, 1H, H-6′), 4.47 (d, J = 8.1 Hz, 1H, H-1), 5.66 (s, 1H, CHPh), 7.36−7.42 (m, 5H), 7.74 (d, J = 8.4 Hz, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 19.5 (OCHCH_3), 23.8 (NHCOCH_3), 55.2 (C-2), 56.8 (OCH_3), 66.1 (C-5), 68.5 (C-6), 76.0 (O-CH−CH_3), 78.7 (C-3), 82.1 (C-4), 100.7 (CHPh), 102.8 (C-1), 126.5, 128.8, 129.5, 138.3, 170.0, 174.7. MS (ES) m/z 396.24 $[M + H]^+$, 418.24 $[M + Na]^+$.

Compound 5. *p*-Nitrophenyl trifluoroacetate (0.18 g, 0.75 mmol) was added to the solution of **13** (0.20 g, 0.5 mmol) in pyridine (15 mL), and the mixture was stirred at rt for 18 h. Subsequently, water was added to the reaction mixture and the precipitate was filtered and washed with water. The resulting white solid (**14**) was recrystalized from MeOH/CH_3-CN. Peptide **15**·TFA salt (0.64 g, 0.7 mmol) in dry DMF

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(25 mL) was neutralized with Et_3N (98 μL, 0.7 mmol) and was stirred at rt for 15 min. *p*-Nitrophenyl ester **14** was added into this amine solution, and the resultant mixture was stirred at rt for 18 h. The solvent was removed in vacuo, and the residue was suspended in water and was filtered. The residue was purified by short-path column chromatography ($CHCl_3$/MeOH, 5:1) to give **16**, which was dissolved in 60% acetic acid (5 mL), and the mixture was stirred at 50 °C for 1 h. After removal of acetic acid in vacuo, the residue was dissolved with MeOH (10 mL), and 10% Pd/C was added. The mixture was refluxed for 5 h under an atmosphere of hydrogen. The reaction mixture was treated with charcoal and was filtered through a layer of Celite, which was washed with methanol. The filtrate was concentrated prior to purification of the desired compound by short-path column chromatography (MeOH/AcOH, 4:1) to give the title compound (0.25 g, 65% from **13**). ^{1}H NMR (500 MHz, D_2O): δ 1.27 (d, J = 7.3 Hz, 3H), 1.31 (d, J = 7.3 Hz, 3H), 1.34 (d, J = 6.5 Hz, 3H), 1.39 (d, J = 7.3 Hz, 3H), 1.27−1.39 (m, 2H), 1.65 (m, 2H), 1.76 (m, 2H), 1.90 (m, 1H), 1.92 (s, 3H), 2.10 (m, 1H), 2.28 (t, J = 7.3 Hz, 2H), 2.95 (t, J = 8.1 Hz, 2H), 3.45 (s, 3H), 3.50 (m, 3H), 3.69−3.78 (overlapping dd, J = 11.35, 8.9, 5.7 Hz, 2H), 3.90 (d, J = 10.5 Hz, 1H), 4.10−4.30 (overlapping dd and m, J = 11.4, 7.3, 6.5, 5.7 Hz, 6H), 4.36 (d, J = 8.1 Hz, 1H, H-1). ^{13}C NMR (125 MHz, D_2O): δ 16.6, 17.0, 17.3, 18.8, 22.2, 22.3, 26.4, 28.1, 30.5, 31.8, 39.3, 49.7, 49.9, 54.2, 55.1, 57.3 (OCH_3), 60.8 (C-6), 68.9, 75.8, 78.3 (O-*C*H−CH_3), 82.7, 102.1 (C-1), 174−176 (a total of 8 carbonyl signals). HRMS (ESI) $[M + H]^+$ calcd for $C_{32}H_{56}N_7O_{15}^+$ 778.3829, found 778.3813.

***tert*-Butyldimethylsilyl 3-*O*-Benzyl-4,6-*O*-benzylidene-2-dimethylmaleimido-2-deoxy-β-D-glycopyranoside (21).** This procedure has to be carried under scrupulously dry condition, or else the reaction may fail entirely. To a solution of **20**[9] (4.6 g, 10 mmol) in anhydrous CH_2Cl_2 (20 mL) and anhydrous cyclohexane (20 mL) were added benzyl 2,2,2-trichloroacetimidate (3.7 mL, 20 mmol) and activated 4-Å molecular sieves (5 g), and the mixture was stirred at rt for 0.5 h. Then, the mixture was cooled in an ice−water bath, and a catalytic amount of trifluoromethanesulfonic acid was added dropwise (0.1 mL). 2,2,2-Trichloroacetamide started to crystallize from the reaction solution. The reaction was monitored by TLC. When the starting material was consumed completely (approximately 1 h), the reaction was quenched by a few drops of Et_3N and additional cyclohexane (20 mL) was added. After the mixture was stirred for 0.5 h at rt, 2,2,2-trichloroacetamide was filtered and washed with hexane/EtOAc (4:1). The filtrate was concentrated to dryness, and the residue was chromatographed (hexane to hexane/EtOAc, 8:1) to give **21** as a colorless oil (3.9 g, 67%). ^{1}H NMR (400 MHz, $CDCl_3$): δ −0.07, 0.03, 0.75 (3s, 15H), 1.94 (s, 6H, $2CH_3$), 3.58 (m, 1H, H-5), 3.74 (dd, J = 10.5, 8.1 Hz, 1H, H-4), 3.86 (t_{app}, J = 10.5 Hz, 1H, H-6), 3.94 (dd, J = 10.5, 8.1 Hz, 1H, H-2), 4.29 (dd, J = 10.5, 8.9 Hz, 1H, H-3), 4.33 (t_{app}, J = 10.5 Hz, 1H, H-6′), 4.50, 4.82 (2d, J = 12.2 Hz, 2H, CH_2Ph), 5.24 (d, J = 8.1 Hz, 1H, H-1), 5.59 (s, 1H, C*H*Ph), 7.12−7.52 (m, 10H). ^{13}C NMR (100 MHz, $CDCl_3$): δ −5.4, −4.0, 8.9, 17.8, 25.6, 58.0 (C-2), 66.4 (C-5), 69.0 (C-6), 74.2 (*C*H_2Ph), 74.9 (C-3), 83.3 (C-4), 94.3 (C-1), 101.5 (*C*HPh), 126.3, 127.6, 128.4, 128.5, 128.8, 129.2, 130.0, 134.7, 137.1, 137.6, 138.7, 172.1. MS (ES) m/z 580.26 $[M + H]^+$, 602.23 $[M + Na]^+$, 618.21 $[M + K]^+$.

3-*O*-Benzyl-4,6-*O*-benzylidene-2-dimethylmaleimido-2-deoxy-β-D-glycopyranose (22). Compound **21** (5.8 g, 10 mmol) in dry THF (80 mL) was treated by the dropwise addition of tetrabutylammonium fluoride (12.5 mL, 1.0 M in THF) at −20 °C. The mixture was stirred until starting material was consumed (4 h). Subsequently, acetic acid (0.8 mL) was added, and the mixture was warmed to rt over 10 min. After the addition of a few drops of satd $NaHCO_3$, the solvent was removed in vacuo, the residue was taken up in CH_2Cl_2 (50 mL) and washed with satd $NaHCO_3$, and the organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated. The crude product was chromatographed (hexane/EtOAc, 3:1−1:1) to give the title compound (3.3 g, 72%) as a single isomer. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.83 (brs, 6H, $2CH_3$), 3.63 (m, 1H, H-5), 3.77 (t_{app}, J = 8.9 Hz, 1H, H-4), 3.80 (t_{app}, J = 9.7 Hz, 1H, H-6), 3.92 (dd, J = 10.1, 8.5 Hz, 1H, H-2), 4.36 (dd, J = 10.5, 3.2 Hz, 1H, H-6′), 4.37 (dd, J = 10.5, 8.9 Hz, 1H, H-3), 4.50, 4.84 (2d, J = 12.2 Hz, 2H, CH_2Ph), 5.24 (t, J = 8.5 Hz, 1H, H-1), 5.59 (s, 1H, C*H*Ph), 7.11−7.52 (m, 10H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 8.9 (CH_3), 57.8 (C-2), 66.5 (C-5), 68.9 (C-6), 74.4 (*C*H_2Ph), 75.1 (C-3), 83.1 (C-4), 93.9 (C-1), 101.5 (*C*HPh), 126.3, 127.7, 128.5, 129.3, 137.3, 137.5, 138.5, 172.0. MS (ES) m/z 488.21 $[M + Na]^+$, 504.18 $[M + K]^+$.

Methyl 2-Acetamido-3-*O*-acetyl-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (24). Compound **12** (3.2 g, 10 mmol) in CH_2Cl_2 (10 mL) was stirred with pyridine (2.4 mL, 30 mmol) and acetic anhydride (3.9 mL, 30 mmol) at rt overnight. The reaction mixture was diluted with CH_2Cl_2 and was washed with water and brine. The organic layer was concentrated to dryness, and the sample was kept under vacuum for 1 h. The residue was subjected to the regioselective benzylidene ring opening reaction, which was reported by Deninno et al.[10]

Methyl 2-Acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3-*O*-acetyl-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (26). This procedure has to be carried out under scrupulously dry conditions or else the reaction may fail entirely. Compound **22** (4.7 g, 10 mmol) in anhydrous CH_2Cl_2 (50 mL) was treated with 2,2,2-trichloroacetonitrile (2.5 mL, 25 mmol) and a catalytic amount of DBU. When the starting material was consumed completely (2 h), the reaction mixture was filtered through a layer of silica gel, which was washed with CH_2Cl_2. The filtrate was concentrated under scrupulously dry conditions, and the residue was kept under high vacuum for 0.5 h. The residue (**23**) was dissolved in anhydrous CH_2Cl_2 (50 mL) and was treated with compound **24** (4.4 g, 12 mmol) and activated 4-Å molecular sieves (5 g). After being stirred for 0.5 h at rt, the mixture was cooled to −5 °C and was treated with a catalytic amount of trimethylsilyl trifluoromethanesulfonate (0.1 mL). When the starting material was consumed completely, a few drops of Et_3N were added and the solution was stirred at rt for 0.5 h. The reaction mixture was concentrated to dryness, and the resultant residue was dissolved in a small amount of ethyl acetate, which was filtered through a small layer of silica gel. Silica gel was washed with hexane/EtOAc (4:1), and the combined filtrate was concentrated to dryness. The residue was chromatographed (hexane to hexane/EtOAc, 4:1) to give compound **25** (3.3 g, 40% from **22**).

The mixture of **25** (3.3 g, 4 mmol) and sodium hydroxide (1.44 g, 36 mmol) in a dioxane/water mixture (5:1, 70 mL) was stirred at rt. After 4 h, the pH was adjusted to 3.5 by 1 N HCl, and the solution was stirred for 12 h while pH was kept between 3.0 and 3.5. The solution was concentrated to dryness, and the residue was dissolved in anhydrous MeOH. The precipitate was filtered, the filtrate was evaporated to dryness, and the residue was kept under high vacuum for a while. The residue in pyridine (2.4 mL, 30 mmol) was treated with acetic anhydride (3.9 mL, 30 mmol) at rt overnight. Evaporation of the volatiles gave the crude product (**26**), which was purified by column chromatography (1.5 g, 49%). Compound **23**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.81 (s, 6H, $2CH_3$), 3.78−3.90 (m, 3H, H-4, H-5, H-6), 4.29 (dd, J = 9.7, 8.9 Hz, 1H, H-2), 4.44 (dd, J = 10.5, 8.9 Hz, 1H, H-3), 4.46 (dd, J = 6.5, 4.1 Hz, 1H, H-6′), 4.53, 4.86 (2d, J = 12.2 Hz, 2H, CH_2Ph), 5.62 (s, 1H, C*H*Ph), 6.33 (d, J = 8.9 Hz, 1H, H-1), 7.14−7.54 (m, 10H), 8.61 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 8.9 (CH_3), 54.8 (C-2), 67.1 (C-5), 68.8 (C-6), 74.5 (*C*H_2Ph), 75.0 (C-3), 82.7 (C-4), 94.7 (C-1), 101.7 (*C*HPh), 126.3, 127.8, 128.5, 128.6, 129.3, 137.3, 137.4, 138.4, 161.0, 171.3. MS (ES): m/z 631.14 $[M + Na]^+$. Compound **26**. ^{1}H NMR (400 MHz, 5% CD_3OD in $CDCl_3$): δ 1.71, 1.82, 1.91 (3s, 9H), 3.20 (m, 1H, H-5^{II}), 3.36 (s, 3H, OCH_3), 3.38 (m, 1H, H-5^{I}), 3.48−3.62 (overlapping dd, J = 9.7, 8.9, 8.1 Hz, 6H, H-2^{II}, H-4^{II}, H-6^{I}, H-6′^{I}, H-6^{II}, H-3^{II}),

3.74 (t, J = 8.9 Hz, 1H, H-4^{I}), 3.85 (m, 1H, H-2^{I}), 4.19 (dd, 1H, J = 7.3, 4.9 Hz, H-6′^{II}), 4.22 (d, 1H, J = 8.1 Hz, H-1^{I}), 4.40, 4.51, 4.61, 4.73 (4d, 4H, J = 12.2 Hz, CH_2Ph), 4.42 (dd, 1H, J = 8.1 Hz, H-1^{II}), 4.88 (dd, 1H, J = 9.7, 8.7 Hz, H-3^{I}), 5.43 (s, 1H, C*H*Ph), 7.14−7.37 (m, 15H). ^{13}C NMR (100 MHz, 5% CD_3OD in $CDCl_3$): δ 21.5, 23.4, 23.6, 53.7 (C-2^{I}), 55.9 (C-2^{II}), 56.6 (OCH_3), 66.1 (C-5^{II}), 67.9 (C-6^{I}), 68.8 (C-6^{II}), 73.9 (*C*H_2-Ph), 74.0 (C-3^{I}), 74.2 (*C*H_2Ph′), 74.8 (C-4^{I}), 75.6 (C-5^{I}), 77.6 (C-3^{II}), 82.2 (C-4^{II}), 101.3 (C-1^{II}), 101.3 (*C*HPh), 101.8 (C-1^{I}), 126.6, 127.9, 128.0, 128.7, 128.8, 128.9, 129.1, 129.3, 129.5, 138.3, 139.4, 139.5, 169.7, 170.0. MS (ES): *m*/*z* 749.15 [M + H]$^+$, 771.13 [M + Na]$^+$, 787.11 [M + K]$^+$.

Methyl 2-Acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-6-*O*-benzyl-3-*O*-((*R*)-1′-carboxyethyl)-2-deoxy-β-D-glucopyranoside (27). A solution of compound **26** (3.7 g, 5 mmol) in anhydrous MeOH (20 mL) was treated with NaOMe (0.27 g, 5 mmol). After being stirred at rt for 0.5 h, the solution was evaporated in vacuo to dryness and the sample was keep under high vacuum for 1 h. The residue was coupled with (*S*)-2-chloropropionic acid, as described for the preparation of **13**, to give the title compound (1.6 g) in 42% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.26 (d, J = 6.5 Hz, 3H, OCHC*H*$_3$), 1.78, 1.82 (2s, 6H, NHCOC*H*$_3$), 3.18 (m, 1H, H-5^{II}), 3.27 (s, 3H, OCH_3), 3.34−3.50 (overlapping 3dd, J = 10.5, 8.9, 8.1 Hz, 3H, H-5^{I}, H-2^{I}, H-4^{I}), 3.62−3.80 (overlapping dd and m, J = 10.5, 8.9 Hz, 7H, H-2^{II}, H-4^{II}, H-6^{I}, H-6′^{I}, H-6^{II}, H-3^{I}, H-3^{II}), 4.25 (m, 1H, H-6′^{II}), 4.30 (d, J = 8.1 Hz, 1H, H-1^{II}), 4.39 (q, J = 6.4 Hz, 1H, O−C*H*-CH$_3$), 4.55−4.73 (overlapping 4 d, J = 12.2, 4H), 4.65 (d, J = 8.1 Hz, 1H, H-1^{II}), 5.71 (s, 1H, C*H*Ph), 7.25−7.39 (m, 15H), 7.58 (d, J = 7.3 Hz, 1H), 8.19 (d, J = 8.1 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 19.4, 23.6, 23.9, 54.7 (C-2^{II}), 56.3 (OCH_3, C-2^{I}), 66.3 (C-5^{II}), 68.6 (C-6^{II}), 68.8 (C-6^{I}), 72.3 (*C*H_2Ph), 74.1 (*C*H_2Ph), 74.6 (C-5^{I}), 75.8 (O-*C*H−CH$_3$), 76.6 (C-3^{I}, C-3^{II}), 79.4 (C-4^{I}), 81.8 (C-4^{II}), 100.7 (C-1^{II}, *C*HPh), 102.1 (C-1^{I}), 126.7, 127.6, 128.0, 128.8, 128.9, 129.5, 138.3, 139.4, 170.0, 170.1, 175.8. MS (ES): *m*/*z* 779.35 [M + H]$^+$, 801.11 [M + Na]$^+$.

Compound 6. *p*-Nitrophenyl trifluoroacetate (0.18 g, 0.75 mmol) was added to a solution of **27** (0.39 g, 0.5 mmol) in pyridine (15 mL), which was stirred at rt for 18 h. Water was added to the reaction mixture, resulting in a precipitate which was filtrated and washed with water. The resulting white solid, *p*-nitrophenyl ester of **27**, was recrystallized from MeOH/CH_3-CN. Peptide **15**·TFA salt (0.64 g, 0.7 mmol) in dry DMF (25 mL) was mixed with Et_3N (98 μL, 0.7 mmol) and was stirred at rt for 15 min. *p*-Nitrophenyl ester of **27** was added to the solution, which was stirred at rt for 18 h. The solvent was removed in vacuo, and the residue was suspended in water and was filtered. The residue was purified by short-path column chromatography ($CHCl_3$/MeOH, 5:1) to give **28**. Compound **28** was dissolved in 60% acetic acid (5 mL) and was stirred at 50 °C for 1 h. After removal of acetic acid in vacuo, the residue was dissolved in MeOH (10 mL) and 10% Pd/C was added. The mixture was refluxed for 5 h under a hydrogen atmosphere. The reaction mixture was treated with charcoal and was filtered through a layer of Celite, which was washed with methanol. The filtrate was concentrated and purified by short-path column chromatography (MeOH/AcOH, 4:1) to give the title compound (0.26 g, 54% from **27**). ^{1}H NMR (400 MHz, D_2O): δ 1.30−1.49 (overlapping 4d and m, J = 7.3, 6.4 Hz, 14H), 1.62 (m, 2H), 1.73 (m, 2H), 1.63 (s, 6H), 1.89 (m, 1H), 2.00 (m, 1H), 2.28 (m, 2H), 2.93 (t, J = 7.3 Hz, 2H), 3.36 (m, 1H), 3.41 (s, 3H), 3.42−3.60 (m, 4H), 3.63−3.72 (m, 4H), 3.77 (dd, J = 9.6, 9.1 Hz, 1H), 3.79 (m, 2H), 4.09−4.38 (overlapping dd and m, J = 10.6, 8.6, 8.1, 7.1, 6.7 Hz, 6H), 4.32, 4.76 (2d, J = 8.1 Hz, 2H, H-1^{I} and H-1^{II}). ^{13}C NMR (100 MHz, D_2O): δ 16.8, 17.2, 17.3, 18.2, 22.3, 22.5, 26.5, 28.3, 30.6, 32.0, 39.4, 49.8, 50.0, 54.3, 54.5, 56.2, 57.3 (OCH_3), 60.2 (C-6^{I}), 61.3 (C-6^{II}), 70.4, 73.8, 75.4, 75.6, 76.3, 78.3, 79.7, 100.7, 102.3 (C-1^{I} and C-1^{II}), 174.3, 174.9, 175.9, 176.3. HRMS (ESI): [M + H]$^+$ calcd for $C_{40}H_{69}N_8O_{20}^+$ 981.4623, found 981.4650.

NMR Experiment for Turnover of Compound 6. A solution of 10 mg of compound **6** and PBP5 (0.6 μg) in 1.0 mL of deuterated 50 mM phosphate, 0.5 M NaCl, 0.1% Triton X-100, pD 7.0 was incubated at 37 °C. The progress of the enzymic reaction was monitored by recording NMR spectra periodically.

Purification and Analyses of the PBP5 Turnover Products. A solution of PBP5 (0.28 mg) and 5 mg of compound **5** in 600 μL of 50 mM phosphate, 0.5 M NaCl, and 0.1% Triton X-100, pH 7.0 was incubated at 37 °C for 20 h. The solution was concentrated in vacuo, and the reaction products were isolated by preparative thin-layer chromatography (^{n}BuOH/AcOH/H_2O, 3:2:1) to give D-Ala (R_f = 0.48) and compound **7** (R_f = 0.1). D-Ala. ^{1}H NMR (400 MHz, D_2O): δ 1.48 (d, J = 7.3 Hz, 3H), 3.72 (q, J = 7.3 Hz, 1H). Compound **7**. ^{1}H NMR (400 MHz, D_2O): δ 1.29 (d, J = 6.5 Hz, 3H), 1.34 (d, J = 6.5 Hz, 3H), 1.41 (d, J = 6.5 Hz, 3H), 1.29−1.41 (m, 2H), 1.66 (m, 2H), 1.77 (m, 2H), 1.90 (m, 1H), 1.94 (s, 3H), 2.11 (m, 1H), 2.29 (m, 2H), 2.97 (t, J = 8.1 Hz, 2H), 3.47 (s, 3H), 3.50 (m, 3H), 3.70−3.79 (overlapping dd, J = 12.2, 8.1, 4.9 Hz, 2H), 3.89 (dd, J = 5.7, 4.9 Hz, 1H), 4.10−4.30 (overlapping q and m, 5H), 4.38 (d, J = 8.1 Hz, 1H, H-1). MS (ES): *m*/*z* 729.29 [M + Na]$^+$, 745.37 [M + K]$^+$.

An identical experiment was performed with compound **6**. The resulting mixture was purified by preparative thin-layer chromatography (^{n}BuOH/AcOH/H_2O, 3:2:1) to give give D-Ala (R_f = 0.48) and compound **8** (R_f = 0.08). Compound **8**. ^{1}H NMR (400 MHz, buffer A-which was used for the NMR experiment): δ 1.32 (d, J = 7.3 Hz, 3H), 1.39 (d, J = 6.5 Hz, 3H), 1.45 (d, J = 7.3 Hz, 3H), 1.30−1.49 (m, 2H), 1.70 (m, 2H), 1.82 (m, 2H), 1.92 (s, 6H), 1.96 (m, 2H), 2.35 (m, 2H), 2.97 (t, J = 8.1 Hz, 2H), 3.40 (m, 1H), 3.45 (s, 3H), 3.54−3.62 (m, 3H), 3.63−3.75 (m, 5H), 3.81 (dd, J = 9.6, 9.1 Hz, 1H), 3.90 (m, 2H), 4.10 (q, J = 7.3 Hz, 1H), 4.19−4.40 (overlapping q and m, 4H), 4.25, 4.52 (2d, J = 8.1 Hz, 1H, H-1^{I} and H-1^{II}). MS (ES): *m*/*z* 932.76 [M + Na]$^+$.

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Supporting Information Available: ^{1}H and ^{13}C NMR, H−H COSY, and HMQC spectra for major compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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