

Supporting Information

Effect of the Lipid II Sugar Moiety on Bacterial Transglycosylase: the 4-Hydroxy Epimer of Lipid II is a TGase Inhibitor

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Section A. Preparation and characterization of compounds

Abbreviations. (1) CC: column chromatography; (2) rt: room temperature; (3) HPLC: high performance liquid chromatography; (4) TLC: thin layer chromatography; (5) CH₂Cl₂: dichloromethane; (6) THF: tetrahydrofuran; (7) DMF: *N,N*-dimethylformamide; (8) TBAF: tetra-*n*-butylammonium fluoride (9) PyBOP: (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; (10) DIEA: *N,N*-diisopropylethylamine; (11) TMSOTf: trimethylsilyl trifluoromethanesulfonate; (12) *C. difficile* PBP: penicillin-binding proteins from *Clostridium difficile*; (13) TsCl: *p*-toluenesulfonyl chloride; (14) (*i*-Pr)₂NP(OBn)₂: dibenzyl *N,N*-diisopropylphosphoramidite; (15) PTSA: *p*-toluenesulfonic acid.

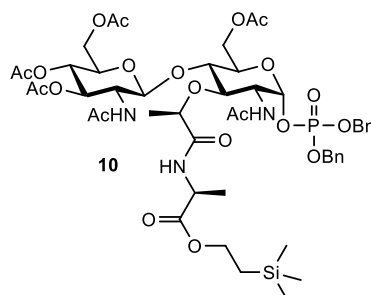
General information. All chemicals were obtained from commercial suppliers and used without further purification. All solvents were anhydrous grade unless indicated otherwise. All non-aqueous reactions were performed in oven-dried glassware under a slight positive pressure of argon unless otherwise noted. Reactions were magnetically stirred and monitored by thin-layer chromatography on silica gel. Flash chromatography was performed on silica gel of 40–63 µm particle size. Concentration refers to rotary evaporation. Yields are reported for spectroscopically pure compounds. NMR spectra were recorded on dilute solutions in D₂O, CDCl₃ and CD₃OD on Bruker AVANCE 600 at ambient temperature. Chemical shifts are given in δ values and coupling constants *J* are given in Hz. The splitting patterns are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and dd (double of doublets). High resolution mass spectra were obtained on a Bruker Daltonics BioTOF III spectrometer (ESI-MS). Optical rotations were measured with a JASCO P-2000 polarimeter.

Synthetic routes of Lipid II analogues.

Typical procedure (TP 1) for a sequence of debenzylolation and phosphoryl diester formation (the preparation of a dibenzyl phosphate; From 6, 7, 8, 9 to 10, 11, 12, 13, respectively.)

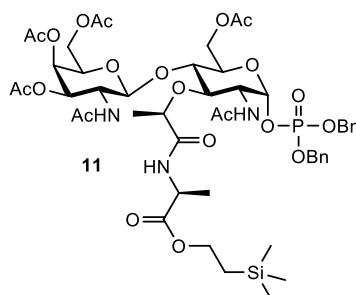
To a solution of a disaccharide (0.1 mmol) in MeOH (10 mL) was added 20% Pd(OH)₂/C (20 mg). The mixture was stirred vigorously under an atmosphere of hydrogen for 12 h, and then filtered through a pad of Celite. The filtrate was concentrated by rotary evaporation to give a lactol intermediate as a white solid. The lactol intermediate was used for next step without further purification. To a mixture of lactol intermediate (0.1 mmol) and 1*H*-tetrazole (21 mg, 3 equiv) in anhydrous CH₂Cl₂ (10 mL) was slowly added dibenzyl (*N,N*-diisopropyl) phosphoramidite (67 µL, 2 equiv) under an atmosphere of argon at –30 °C. The mixture was gradually warmed to room

temperature, and stirred for 2 h. The mixture was then cooled to $-40\text{ }^{\circ}\text{C}$, and *tetra*-butyl hydroperoxide (129 μL , 5 equiv) was added. The reaction mixture was allowed to warm to $0\text{ }^{\circ}\text{C}$ over a period of 1 h, diluted with CH_2Cl_2 , and washed with saturated $\text{Na}_2\text{S}_2\text{O}_3(\text{aq})$, $\text{NaHCO}_3(\text{aq})$, and brine. The organic layer was dried over MgSO_4 , filtered, concentrated by rotary evaporation, and purified by cc (silica gel, $\text{CH}_2\text{Cl}_2/\text{MeOH} = 50 : 1$) to afford the desired dibenzyl phosphate.



Compound 10.

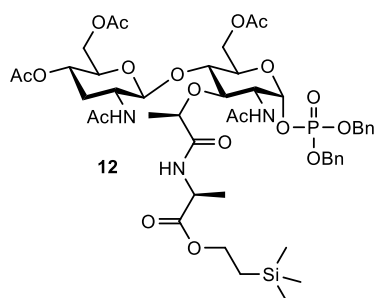
According to **TP 1**, disaccharide **6**^{S1} (500 mg, 0.540 mmol) was converted into **10** as a white solid (308 mg, 52% over three steps). $[\alpha]_{\text{D}}^{25} +32$ (*c* 1.1 in CHCl_3); ^1H NMR (600 MHz, CDCl_3): δ -0.03 (s, 9H), 0.84–0.94 (m, 2H), 1.37 (d, 3H, $J = 6.6$ Hz), 1.40 (d, 3H, $J = 7.2$ Hz), 1.81 (s, 3H), 1.94 (s, 3H), 2.00 (s, 3H), 2.01 (s, 3H), 2.03 (s, 3H), 2.04 (s, 3H), 3.50–3.58 (m, 2H), 3.81 (t, 1H, $J = 9.0$ Hz), 3.90–3.93 (m, 2H), 4.04–4.16 (m, 5H), 4.19 (dd, 1H, $J = 3.6, 12.6$ Hz), 4.30 (dd, 1H, $J = 4.2, 12.6$ Hz), 4.35 (d, 1H, $J = 8.4$ Hz), 4.47 (t, 1H, $J = 7.2$ Hz), 4.50 (q, 1H, $J = 6.6$ Hz), 4.99–5.08 (m, 5H), 5.10 (t, 1H, $J = 9.6$ Hz), 5.96 (dd, 1H, $J = 3.6, 4.8$ Hz), 6.11 (d, 1H, $J = 9.6$ Hz), 7.31–7.34 (m, 10H), 7.51 (d, 1H, $J = 5.4$ Hz). ^{13}C NMR (150 MHz, CDCl_3): δ -1.5 ($\times 3$), 14.1, 17.1, 17.8, 18.8, 20.6, 20.7, 22.8, 23.3, 48.1, 51.0, 53.5, 53.6, 60.4, 61.1, 61.9, 63.7, 66.4, 69.4, 69.5, 69.9, 70.9, 71.0, 73.6, 74.3, 95.8, 99.7, 127.8 ($\times 2$), 127.9 ($\times 2$), 128.5 ($\times 3$), 128.6 ($\times 3$), 135.5, 135.6, 170.0, 170.2, 170.6, 171.0, 171.1, 171.2, 172.5, 174.1; HRMS calcd for $[\text{C}_{49}\text{H}_{70}\text{N}_3\text{O}_{21}\text{PSi}+\text{H}]^+$ 1096.4009, found 1096.4001.



Compound 11.

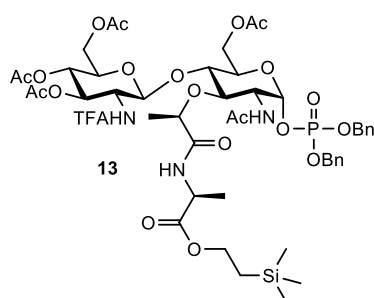
According to **TP 1**, disaccharide **7**^{S1} (500 mg, 0.45 mmol) was converted into **11** as a white solid (405 mg, 68% over three steps). $[\alpha]_{\text{D}}^{25} +39$ (*c* 1.3 in CHCl_3); ^1H NMR (600 MHz, CDCl_3): δ -0.04

(s, 9H), 0.84–0.93 (m, 2H), 1.40 (d, 3H, $J = 6.6$ Hz), 1.42 (d, 3H, $J = 7.2$ Hz), 1.81 (s, 6H), 1.97 (s, 3H), 2.02 (s, 3H), 2.03 (s, 3H), 2.12 (s, 3H), 3.53–3.55 (m, 1H), 3.80 (t, 1H, $J = 6.6$ Hz), 3.92–3.93 (m, 2H), 4.03–4.20 (m, 6H), 4.26 (q, 1H, $J = 9.6$ Hz), 4.38 (d, 1H, $J = 8.4$ Hz), 4.45–4.51 (m, 1H), 4.61 (td, 1H, $J = 6.6, 7.2$ Hz), 4.49 (d, 1H, $J = 12.6$ Hz), 4.96–5.05 (m, 5H), 5.31 (d, 1H, $J = 3.0$ Hz), 5.95 (d, 1H, $J = 9.6$ Hz), 5.97 (dd, 1H, $J = 3.0, 5.4$ Hz), 7.26–7.34 (m, 10H), 7.51 (d, 1H, $J = 5.4$ Hz); ^{13}C NMR (150 MHz, CDCl_3): δ -1.5 ($\times 3$), 17.1, 17.8, 18.8, 20.6 ($\times 2$), 20.8, 22.8, 23.3, 48.2, 51.0, 53.5, 53.6, 60.4, 61.1, 61.9, 63.7, 66.4, 69.3, 69.5, 69.9, 70.9, 71.0, 73.6, 74.3, 95.7, 99.7, 127.8 ($\times 3$), 127.9 ($\times 2$), 128.5 ($\times 4$), 128.6, 135.5, 135.6, 170.0, 170.2, 170.6, 171.0, 171.1, 171.2, 172.5, 174.7; HRMS calcd for $[\text{C}_{49}\text{H}_{70}\text{N}_3\text{O}_{21}\text{PSi}+\text{H}]^+$ 1096.4009, found 1096.4008.



Compound 12.

According to **TP 1**, disaccharide **8** (100 mg, 0.115 mmol) was converted into **12** as a white solid (93 mg, 78% over three steps). $[\alpha]_{\text{D}}^{25} +25$ (c 0.69 in CHCl_3); HRMS calcd for $[\text{C}_{47}\text{H}_{68}\text{N}_3\text{O}_{19}\text{PSi}+\text{H}]^+$ 1038.3954, found 1038.3951.



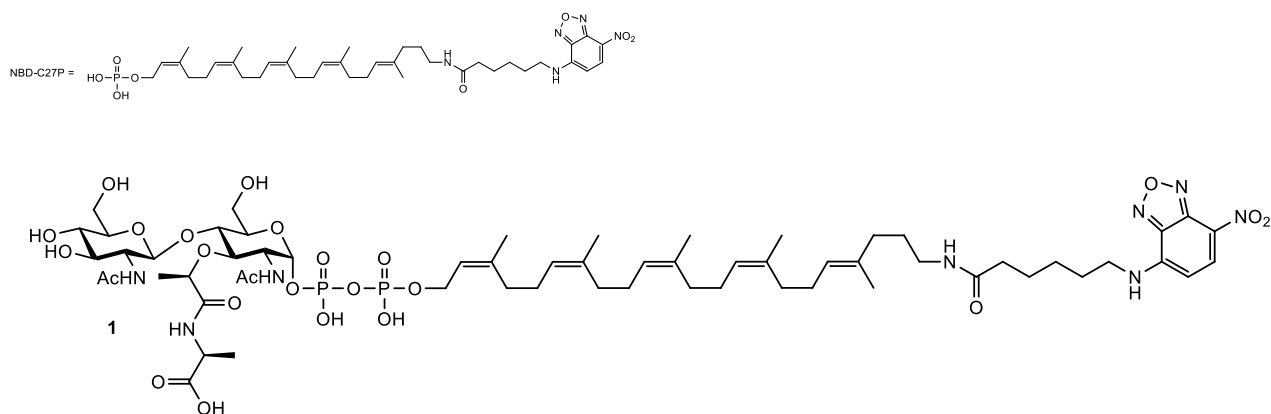
Compound 13.

According to **TP 1**, disaccharide **9** (200 mg, 0.204 mmol) was converted into **13** as a white solid (173 mg, 74% over three steps). $[\alpha]_{\text{D}}^{25} +43$ (c 0.78 in CHCl_3); ^1H NMR (600 MHz, CDCl_3): δ -0.02 (s, 9H), 0.89–0.93 (m, 2H), 1.37 (d, 3H, $J = 6.6$ Hz), 1.39 (d, 3H, $J = 7.2$ Hz), 1.78 (s, 3H), 1.99 (s, 6H), 2.02 (s, 3H), 2.03 (s, 3H), 3.49 (dd, 1H, $J = 9.0, 10.8$ Hz), 3.57–3.60 (m, 1H), 3.71 (t, 1H, $J = 9.6$ Hz), 3.79–3.83 (m, 1H), 3.89 (dd, 1H, $J = 1.2, 12.6$ Hz), 4.01–4.03 (m, 1H), 4.04 (dd, 1H, $J = 2.4, 12.6$ Hz), 4.10–4.16 (m, 3H), 4.23 (dd, 1H, $J = 3.6, 12.6$ Hz), 4.28–4.32 (m, 2H), 4.41–4.45 (m, 2H), 5.01–5.05 (m, 4H), 5.11–5.13 (m, 2H), 5.83 (dd, 1H, $J = 3.0, 6.0$ Hz), 6.87 (d, 1H, $J = 7.2$ Hz),

7.13 (d, 1H, $J = 6.6$ Hz), 7.29–7.35 (m, 10H), 7.81(d, 1H, $J = 9.6$ Hz); ^{13}C NMR (150 MHz, CDCl_3): δ -1.3 ($\times 3$), 17.4, 18.1, 18.7, 20.6, 20.7, 20.8, 21.1, 23.1, 48.3, 53.6, 54.9, 61.5, 61.9, 64.1, 68.0, 69.7, 69.8, 69.9, 69.9, 71.2, 72.1, 72.3, 74.8, 76.1, 96.2, 100.1, 128.1 ($\times 4$), 128.8 ($\times 2$), 128.9 ($\times 3$), 129.0, 135.6, 135.8, 167.1, 169.4, 170.7, 171.0, 171.2, 171.9, 172.8, 173.4; HRMS calcd for $[\text{C}_{49}\text{H}_{67}\text{F}_3\text{N}_3\text{O}_{21}\text{PSi}+\text{H}]^+$ 1150.3726, found 1150.3728.

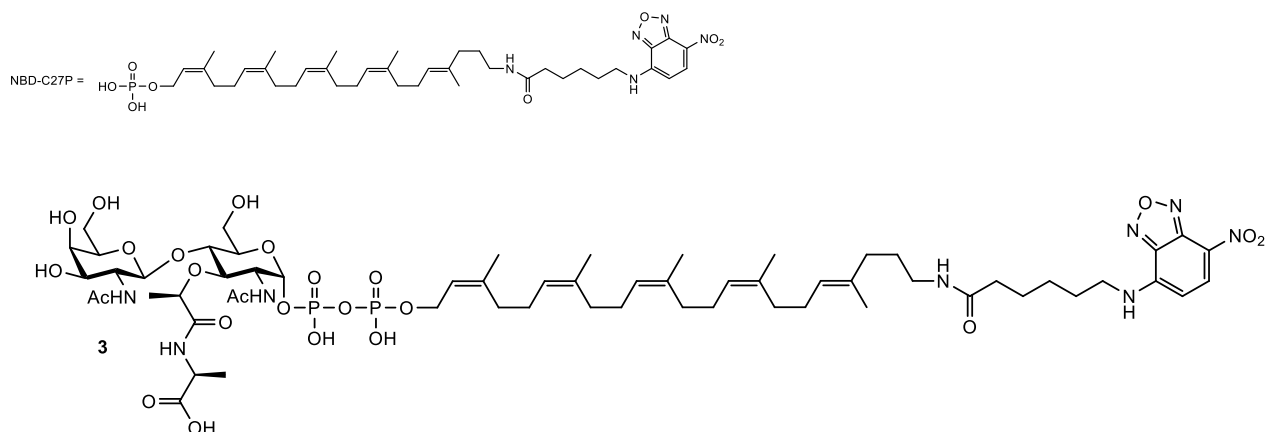
Typical procedure (TP 2) for a sequence of debenzylation, pyrophosphate formation, and global deprotection (the preparation of a Lipid II analogue; from 10, 11, 12, 13 to 1, 3, 4, 5, respectively).

A phosphoryldiester (0.1 mmol) was added to a suspension of 20% $\text{Pd}(\text{OH})_2/\text{C}$ (20 mg) in MeOH (20 mL) at rt. The mixture was stirred vigorously under an atmosphere of hydrogen for 4 h, and the catalyst was removed by filtration. The filtrate was concentrated by rotary evaporation to give phosphate intermediate as a white solid. The phosphate intermediate was used without further purification. The residual solid sample (0.1 mmol) was added to a solution of 1,1'-carbonyldiimidazole (81 mg, 5 equiv) in anhydrous DMF (3 mL) at rt. The mixture was stirred for 2 h and then anhydrous MeOH (140 μL) was added, and stirred for 40 min to destroy the excess 1,1'-carbonyldiimidazole. The reaction mixture was concentrated by rotary evaporation to give active phosphate intermediate as colorless oil. The active phosphate intermediate was mixed with lipid phosphate (76 mg, 1 equiv) in anhydrous THF (1 mL) and then 1*H*-tetrazole (88 mg, 12.5 equiv) was added into this reaction mixture at rt. The mixture was stirred under an atmosphere of argon for 24 h, and then concentrated by rotary evaporation under reduced pressure. The residue was dissolved in CH_2Cl_2 (20 mL), and extracted with water (3 mL \times 2). The organic layer was collected and concentrated to give a protected Lipid II intermediate. The protected Lipid II intermediate in MeOH (1 mL) was treated with $\text{LiOH}\cdot\text{H}_2\text{O}$ (42 mg, 10 equiv) and stirred at rt for 8 h. After the reaction was complete, as judged by TLC analysis, the mixture was directly purified by reverse-phase HPLC.



Compound 1.

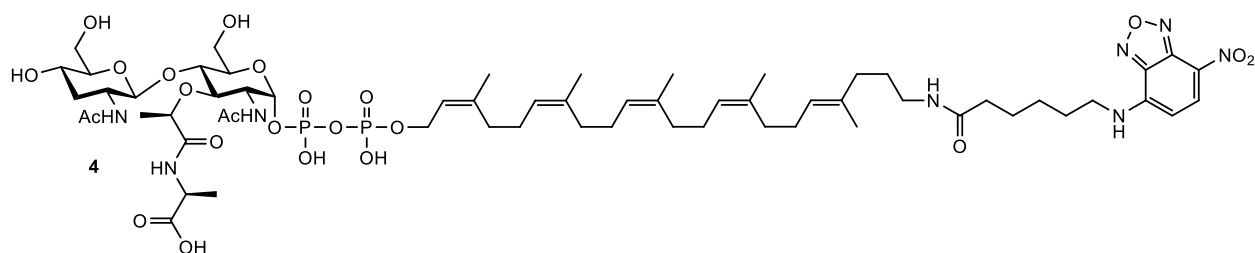
According to **TP 2**, **10** (312 mg, 0.285 mmol) and NBD-C27P (216 mg, 1 equiv) were utilized to prepare compound **1**. Purified by reverse-phase HPLC on a ZORBAX RX-C8 column (5 μ m, 9.4 \times 250 mm) using a gradient elution with A solution and MeOH (15:85 to 0:100 over 45 min) at a flow rate of 1 mL/min, where A solution is 0.05 M aqueous 50 mM NH₄OAc_(aq). The samples were monitored by fluorescent detector (E_{em} : 466 nm/ E_{ex} 535 nm) and the retention time of desired product **1** was 31.7 min. The eluting fractions of **1** were collected and concentrated as a red solid (153 mg, 39%). The purity of **1** was analyzed on an anion exchange column (SAX1, Supelco Co., 5 μ m, 4.6 \times 250 mm) by fluorescent-HPLC (E_{em} : 466 nm/ E_{ex} 535 nm) with a linear gradient elution of NH₄OAc (20 mM to 1 M in MeOH) at a flow rate of 1.0 mL/min over 30 min. Compound **1** had purity more than 90% by HPLC (retention time = 16.4 min). $[\alpha]_D^{25}$ -3.7 (c 0.21 in H₂O); ¹H NMR (600 MHz, MeOD): δ 0.90 (dd, 2H, J = 7.2, 15.6 Hz), 1.39–1.40 (m, 6H), 1.46–1.72 (m, 6H), 1.62 (s, 3H), 1.66 (s, 6H), 1.67 (s, 3H), 1.73 (s, 3H), 1.78–1.81 (m, 2H), 1.96 (s, 3H), 1.97 (s, 3H), 1.96–2.05 (m, 22H), 2.27 (t, 2H, J = 7.2 Hz), 3.13 (t, 2H, J = 7.2 Hz), 3.49–3.67 (m, 2H), 3.68–3.70 (m, 3H), 3.79–3.83 (m, 1H), 3.88–4.07 (m, 5H), 4.07 (m, 1H), 4.29 (m, 1H), 4.39 (m, 1H), 4.51–4.59 (m, 3H), 5.14–5.15 (m, 4H), 5.43–5.47 (m, 1H), 5.55 (m, 1H), 6.39 (d, 1H, J = 8.4 Hz), 8.55 (d, 1H, J = 8.4 Hz); HRMS calcd for [C₆₁H₉₆N₈O₂₄P₂-2H]²⁻ 692.2934, found 692.2931.



Compound 3.

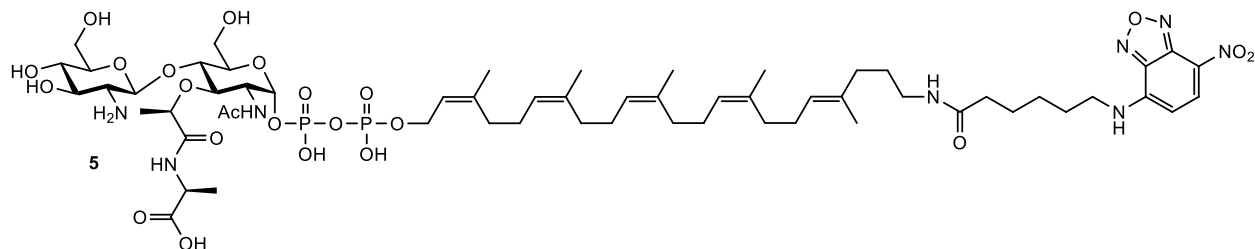
According to **TP2**, compound **11** (20 mg, 0.018 mmol) and NBD-C27P (14 mg, 1 equiv) were applied to prepare **3** as a red solid (11 mg, 44%). The purity of **3** was analyzed on an anion exchange column (SAX1, Supelco Co., 5 μ m, 4.6 \times 250 mm) by fluorescent-HPLC (E_{em} : 466 nm/ E_{ex} 535 nm) with a linear gradient elution of NH₄OAc (20 mM to 1 M in MeOH) at a flow rate of 1.0 mL/min over 30 min. Compound **3** had purity more than 90% by HPLC (retention time = 16.3 min). $[\alpha]_D^{25}$ -2.2 (c 0.17 in H₂O); ¹H NMR (600 MHz, MeOD): δ 0.87–0.90 (m, 2H), 1.38–1.41 (m, 6H), 1.49–1.72 (m, 6H), 1.60 (s, 3H), 1.66 (s, 9H), 1.72 (s, 3H), 1.79–1.82 (m, 2H), 1.96 (s, 3H), 1.97 (s, 3H), 1.96–2.11 (m, 22H), 2.22 (t, 2H, J = 7.2 Hz), 3.13 (m, 2H), 3.19–3.20 (m, 1H), 3.49–3.55 (m, 5H), 3.68–3.74 (m, 3H), 3.83–3.87 (m, 3H), 3.87–3.95 (m, 2H), 4.41–4.50 (m, 3H), 5.14–5.15 (m, 4H), 5.43–5.47 (m, 1H), 5.55 (m, 1H), 6.39 (d, 1H, J = 8.4 Hz), 8.55 (d, 1H, J =

8.4 Hz); HRMS calcd for $[C_{61}H_{96}N_8O_{24}P_2-2H]^{2-}$ 692.2934, found 692.2935.



Compound 4.

According to **TP 2**, compound **12** (10 mg, 0.010 mmol) and NBD-C27P (8 mg, 1 equiv) were applied to obtain **4** as a red solid (5.2 mg, 38%). The purity of **4** was analyzed on an anion exchange column (SAX1, Supelco Co., 5 μ m, 4.6 \times 250 mm) by fluorescent-HPLC (E_{em} : 466 nm/ E_{ex} 535 nm) with a linear gradient elution of NH_4OAc (20 mM to 1 M in MeOH) at a flow rate of 1.0 mL/min over 30 min. Compound **4** had purity more than 90% by HPLC (retention time = 16.0 min). $[\alpha]_D^{25}$ -2.7 (c 0.11 in H_2O); 1H NMR (600 MHz, MeOD): δ 0.90 (t, 2H, J = 6.6 Hz), 1.37–1.39 (m, 6H), 1.46–1.72 (m, 6H), 1.62 (s, 3H), 1.66 (s, 6H), 1.67 (s, 3H), 1.73 (s, 3H), 1.96 (s, 3H), 1.97 (s, 3H), 2.01–2.19 (m, 16H), 2.21 (t, 2H, J = 7.2 Hz), 3.10–3.11 (m, 2H), 3.39–3.47 (m, 2H), 3.51–3.70 (m, 4H), 3.79 (m, 1H), 3.82–4.01 (m, 5H), 4.07 (m, 1H), 4.30 (m, 1H), 4.46–4.50 (m, 3H), 4.58 (m, 1H), 5.11–5.14 (m, 4H), 5.40–5.43 (m, 1H), 5.51 (m, 1H), 6.35 (d, 1H, J = 8.4 Hz), 8.53 (d, 1H, J = 8.4 Hz); HRMS calcd for $[C_{61}H_{96}N_8O_{23}P_2-2H]^{2-}$ 684.2960, found 684.2962.

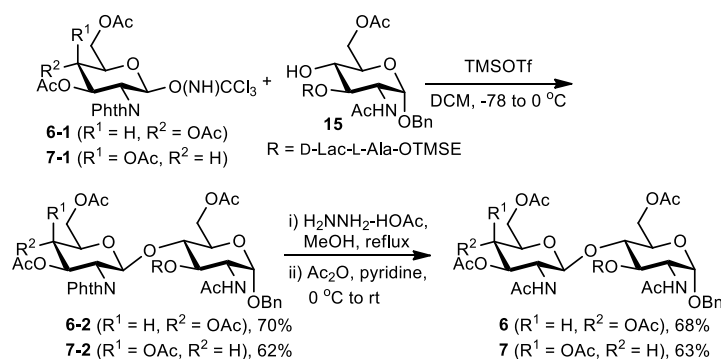


Compound 5.

According to **TP 2**, compound **13** (20 mg, 0.017 mmol) and NBD-C27P (13 mg, 1 equiv) were prepared to obtain **5** as a red solid (5.6 mg, 24%). The purity of **5** was analyzed on an anion exchange column (SAX1, Supelco Co., 5 μ m, 4.6 \times 250 mm) by fluorescent-HPLC (E_{em} : 466 nm/ E_{ex} 535 nm) with a linear gradient elution of NH_4OAc (20 mM to 1 M in MeOH) at a flow rate of 1.0 mL/min over 30 min. Compound **5** had purity more than 90% by HPLC (retention time = 16.3 min). $[\alpha]_D^{25}$ -4.3 (c 0.13 in H_2O); HRMS calcd for $[C_{59}H_{94}N_8O_{23}P_2-2H]^{2-}$ 671.2881, found 671.2879.

Typical procedure (TP 3) for a glycosylation (the preparation of a disaccharide). The donor

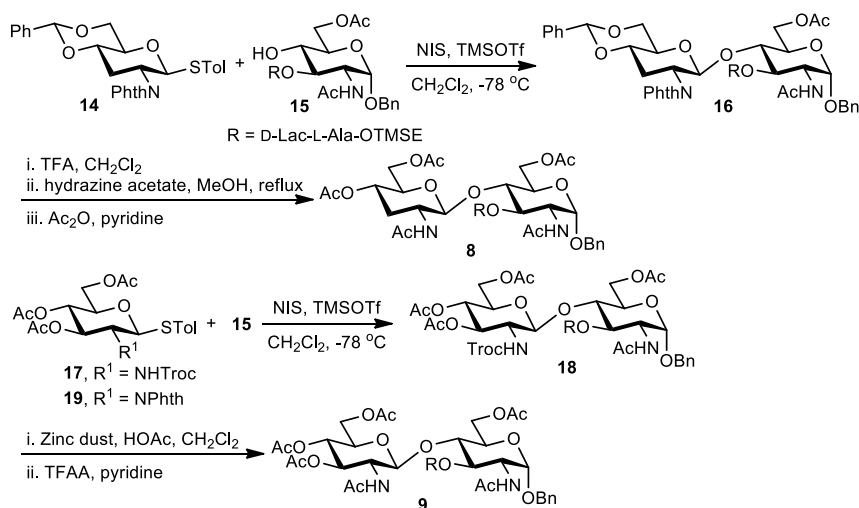
(1.3 equiv), NIS (226 mg, 0.67 equiv), and the acceptor **15** (895 mg, 1.5 mmol) was dissolved in CH₂Cl₂ (20 mL), followed by the addition of 4 Å molecular sieves. The solution was cooled to -78 °C, and treated with the slow addition of TMSOTf (182 µL, 0.67 equiv). The mixture was warmed to rt and stirred for 2 h. Triethylamine (209 µL, 1 equiv) was added to the mixture which was then filtered through a pad of Celite and concentrated.



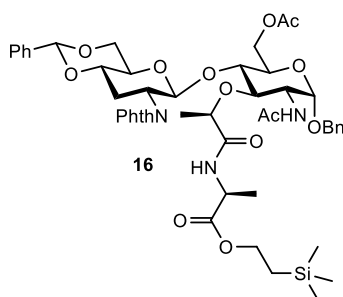
Preparation of disaccharides **6** and **7**.

The general and known procedure for the preparation of compounds **6 and **7**** (also shown in reference#S1).

Trimethylsilyl trifluoromethanesulfonate (54 µL, 0.2 equiv) was added to a mixture of the acceptor **15** (306 mg, 0.6 mmol), a trichloroacetimidate **6-1** or **7-1** (1.04 g, 3 equiv), and 4 Å molecular sieves (600 mg) in anhydrous dichloromethane (12 mL) at -78 °C. The reaction mixture was gradually warmed to rt and stirred for 14 h. *N,N*-Diisopropylethylamine (185 µL, 1 equiv) was added to the reaction mixture, which was then filtered through a pad of Celite, and concentrated to a syrup. The residue was purified by flash column chromatography [silica gel; EtOAc/toluene, 6:4 (v/v)] to afford the pure product (**6-2**: 390 mg, 70%; **7-2**: 345 mg) as a white foam. To a solution of **6-2** or **7-2** (928 mg, 1 mmol) in anhydrous MeOH (15 mL) was added hydrazine acetate (297 mg, 3.3 equiv). The solution was heated under refluxing for 3 h, and hydrazine acetate (297 mg, 3.3 equiv) was added again. After refluxing for another 15 h, the reaction mixture was concentrated by rotary evaporation. The residue was azeotroped twice with toluene, added DMAP (5 mg), anhydrous pyridine (10 mL), and Ac₂O (4 mL) at 0 °C. The mixture was then gradually warmed to room temperature and stirred for another 4 h. The solvent was removed by vacuum pump and the residue was dissolved in EtOAc (50 mL) and washed with 1 M HCl_(aq), sat. NaHCO_{3(aq)}, and brine. The organic layer was dried over MgSO₄, filtered, concentrated by rotary evaporation, and purified by flash column chromatography [silica gel; EtOAc/toluene, 6:4 (v/v)] to afford the desired disaccharide **6** (571 mg, 68%) and **7** (529 mg, 63%).

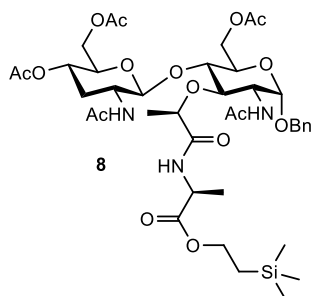


Preparation of disaccharides **8** and **9**.

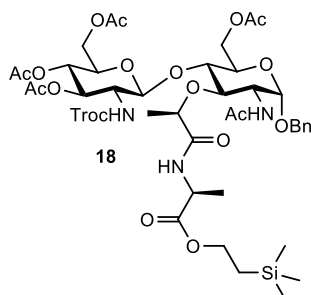


Compound **16**.

According to **TP 3**, **15** (895 mg, 1.5 mmol) and **14**^{S2} (951 mg, 1.3 equiv) were applied to give the desired **16** (1.240 g, 86%) after purification with CC (silica gel, EtOAc/CH₂Cl₂ = 1:4) [α]_D²⁵ +56 (*c* 0.87 in CHCl₃); ¹H NMR (600 MHz, CDCl₃): δ -0.00 (s, 9H), 0.95–0.98 (m, 2H), 1.43 (d, 3H, *J* = 6.6 Hz), 1.45 (d, 3H, *J* = 7.2 Hz), 1.89 (s, 3H), 2.09 (s, 3H), 3.44 (dd, 1H, *J* = 9.0, 10.8 Hz), 3.49–3.51 (m, 1H), 3.58–3.68 (m, 3H), 3.79 (t, 1H, *J* = 10.8 Hz), 3.95–4.00 (m, 2H), 4.14 (dd, 1H, *J* = 1.8, 12.0 Hz), 4.17 (d, 1H, *J* = 9.0 Hz), 4.18 (d, 1H, *J* = 9.0 Hz), 4.21–4.28 (m, 1H), 4.31 (q, 1H, *J* = 6.6 Hz), 4.36 (d, 1H, *J* = 12.0 Hz), 4.40 (dd, 1H, *J* = 4.8, 10.8 Hz), 4.49–4.52 (m, 2H), 4.98 (d, 1H, *J* = 3.6 Hz), 5.26 (d, 1H, *J* = 8.4 Hz), 5.54 (s, 1H), 6.82 (d, 1H, *J* = 7.2 Hz), 6.86 (d, 1H, *J* = 7.2 Hz), 7.17–7.23 (m, 5H), 7.32–7.36 (m, 3H), 7.45–7.46 (m, 2H), 7.72–7.74 (m, 2H), 7.84–7.85 (m, 2H); ¹³C NMR (150 MHz, CDCl₃): δ -1.3 (\times 3), 17.5, 18.2, 19.4, 21.0, 23.4, 29.8, 31.4, 48.3, 51.1, 53.7, 62.3, 63.9, 69.3 (\times 2), 70.1, 70.7, 74.7, 76.6, 77.6, 96.8, 98.7, 102.0, 124.0 (\times 2), 126.4 (\times 2), 127.8 (\times 2), 128.0, 128.5 (\times 2), 128.6 (\times 2), 129.4, 131.6 (\times 2), 134.5 (\times 2), 137.3 (\times 2), 168.0, 170.4, 170.7, 172.8, 173.7, 177.6; HRMS calcd for [C₄₉H₆₁N₃O₁₅Si+H]⁺ 960.3872, found 960.3867

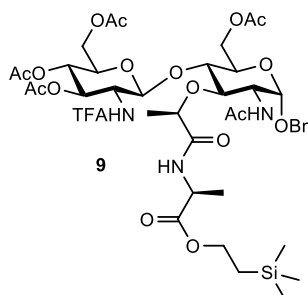


Compound 8. To a solution of **16** (300 mg, 0.312 mmol) in MeOH (20 mL) was added *p*-toluenesulfonic acid (6 mg, 0.1 equiv) at 0 °C. The reaction mixture was then heated to 60 °C and stirred for 30 min. After the reaction complete, the mixture was neutralized by adding Et₃N (43 μL, 1 equiv), concentrated and purified by CC (silica gel, CH₂Cl₂/MeOH= 25:1) to give the diol intermediate (0.29 mmol). The intermediate (0.29 mmol) in MeOH (10 mL) was added with hydrazine acetate (261 mg, 10 equiv) and the solution was heated to 80 °C for 15h. The solvent of the reaction mixture was removed. The resulting residues was re-dissolved in anhydrous pyridine (5 mL), followed by addition of Ac₂O (5.0 mL,) at 0 °C. The mixture was warmed to rt and stirred for 4h. The reaction mixture was diluted with CH₂Cl₂ (50 mL), and washed with brine (50 mL × 2) and 1 N HCl_(aq) (50 mL × 2). The organic layers were collected, dried over anhydrous MgSO₄, concentrated, and purified by CC (silica gel, CH₂Cl₂/MeOH = 50:1) to give **8** (156 mg, 62% over two steps). $[\alpha]_D^{25} +43$ (*c* 1.6 in CHCl₃); ¹H NMR (600 MHz, CDCl₃): δ 0.01 (s, 9H), 0.97 (dd, 2H, *J* = 7.7, 9.4 Hz), 1.34 (d, 3H, *J* = 6.6 Hz), 1.39 (d, 3H, *J* = 7.2 Hz), 1.92 (s, 3H), 1.96 (s, 3H), 2.00 (s, 3H), 2.01 (s, 3H), 2.13 (s, 3H), 2.39–2.42 (m, 1H), 3.51–3.53 (m, 1H), 3.62 (dd, 1H, *J* = 8.4, 10.2 Hz), 3.71 (dd, 1H, *J* = 8.4, 10.2 Hz), 3.74–3.75 (m, 1H), 3.85–3.92 (m, 1H), 3.96–3.99 (m, 1H), 4.05 (dd, 1H, *J* = 2.4, 12.0 Hz), 4.10 (dd, 1H, *J* = 1.2, 12.0 Hz), 4.15–4.18 (m, 2H), 4.25 (dd, 1H, *J* = 4.8, 12.0 Hz), 4.30 (d, 1H, *J* = 7.8 Hz), 4.35–4.47 (m, 2H), 4.50 (d, 1H, *J* = 12.6 Hz), 4.63 (d, 1H, *J* = 12.6 Hz), 4.74–4.80 (m, 1H), 5.10 (d, 1H, *J* = 3.6 Hz), 6.13 (d, 1H, *J* = 9.0 Hz), 7.10 (d, 1H, *J* = 6.6 Hz), 7.11 (d, 1H, *J* = 7.2 Hz), 7.26–7.33 (m, 5H); ¹³C NMR (150 MHz, CDCl₃): δ -1.3 (×3), 17.5, 18.1, 19.0, 20.9, 21.0, 21.3, 23.3, 23.5, 34.0, 48.3, 49.4, 53.5, 62.5, 62.7, 64.0, 66.1, 69.9, 70.3, 75.5, 75.8, 76.1, 76.9, 97.0, 102.3, 127.8 (×2), 128.0, 128.6 (×2), 137.6, 169.7, 170.4, 170.9, 171.0, 171.8, 173.1, 174.0; HRMS calcd for [C₄₀H₆₁N₃O₁₆Si+H]⁺ 868.3821, found 868.3890.

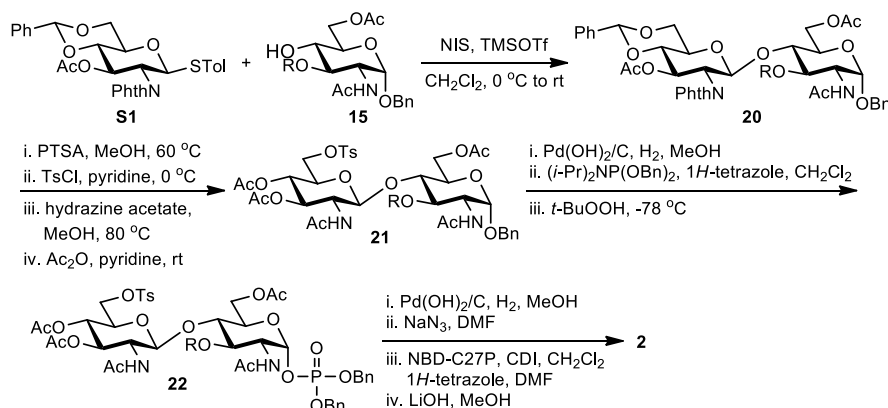


Compound 18.

According to **TP 3**, **15** (597 mg, 1 mmol) and **17**^{S3} (763 mg, 1.3 equiv) were applied to give the desired disaccharide **18** (742 mg, 70%) after Purification with CC (silica gel, EtOAc/CH₂Cl₂ = 1:4). $[\alpha]_D^{25} +38$ (*c* 0.96 in CHCl₃); HRMS calcd for [C₄₃H₆₂Cl₃N₃O₁₉Si+H]⁺ 1058.2812, found 1058.2818.



Compound 9. Compound **18** (60 mg, 0.057 mmole) in AcOH (5 mL) was added with zinc dust (186 mg, 50 equiv) at rt and stirred for 12 h. After the reaction was complete, the excess of zinc dust was removed by filtration through a pad of Celite. The filtrate was concentrated, and subsequently re-dissolved in anhydrous pyridine (2 mL), treated with trifluoroacetic anhydride (158 μ L, 20 equiv) at rt. After stirring for 4 h, the mixture was diluted with CH₂Cl₂ (15 mL), followed by washing with brine (15 mL \times 2), and 1.0 N HCl_(aq) (15 mL \times 2). The collected organic layers were dried over anhydrous MgSO₄, concentrated, and purified by CC (silica gel, CH₂Cl₂/MeOH = 50:1) to give **9** (46 mg, 82% over two steps). $[\alpha]_D^{25} +77$ (*c* 1.1 in CHCl₃); HRMS calcd for [C₄₂H₆₀F₃N₃O₁₈Si+H]⁺ 980.3593, found 980.3584.

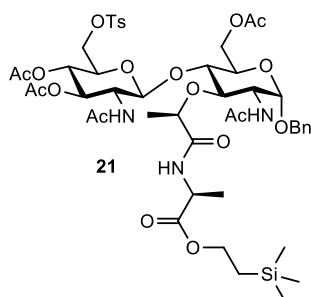


Preparation of Azido-Lipid II 2.

Compound 20.

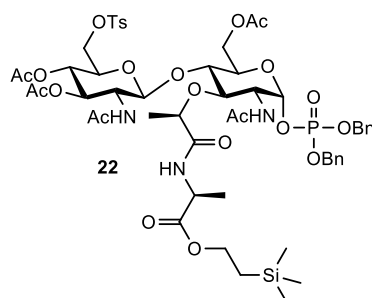
According to **TP 3**, **15** (597 mg, 1 mmol) and **S1**^{S4} (709 mg, 1.3 equiv) were applied to afford the desired disaccharide **20** (662 mg, 65%) after purification with CC (silica gel, EtOAc/CH₂Cl₂ = 1:4).

$[\alpha]_D^{25} +53$ (c 0.93 in CHCl_3); ^1H NMR (600 MHz, CDCl_3): δ -0.05 (s, 9H), 0.87–0.97 (m, 2H), 1.38 (d, 3H, J = 6.6 Hz), 1.48 (d, 3H, J = 7.2 Hz), 1.87 (s, 3H), 1.92 (s, 3H), 2.02 (s, 3H), 3.50 (dd, 2H, J = 9.0, 10.8 Hz), 3.64–3.67 (m, 2H), 3.79–3.82 (m, 1H), 3.99 (t, 1H, J = 9.6 Hz), 4.17–4.10 (m, 2H), 4.12 (t, 1H, J = 8.4 Hz), 4.36 (d, 1H, J = 12.6 Hz), 4.42 (d, 1H, J = 12.6 Hz), 4.47 (d, 1H, J = 12.6 Hz), 4.60 (dd, 1H, J = 8.4, 11.4 Hz), 4.63 (d, 1H, J = 6.6 Hz), 5.14 (d, 1H, J = 3.6 Hz), 5.28 (d, 1H, J = 8.4 Hz), 5.54 (s, 1H), 5.71 (dd, 1H, J = 3.6, 11.4 Hz), 7.08 (d, 1H, J = 7.2 Hz), 7.13–7.17 (m, 5H), 7.31–7.43 (m, 4H), 7.47–7.48 (m, 2H), 7.69–7.73 (m, 2H), 7.80–7.82 (m, 2H); ^{13}C NMR (150 MHz, CDCl_3): δ -1.4 ($\times 3$), 17.3, 18.2, 19.6, 20.8, 20.9, 23.1, 48.2, 51.7, 54.2, 62.4, 63.7, 66.8, 68.8, 68.9, 69.0, 70.0, 72.9, 75.4, 75.5, 77.8, 96.6, 97.1, 100.9, 123.9, 124.0, 126.4 ($\times 2$), 127.5 ($\times 2$), 127.6, 128.1 ($\times 2$), 128.3, 129.0, 131.3, 131.4, 134.4, 134.6, 137.6 ($\times 2$), 167.9, 168.3, 170.3, 170.4, 171.1, 172.7, 174.8, 178.2; HRMS calcd for $[\text{C}_{51}\text{H}_{63}\text{N}_3\text{O}_{17}\text{Si}+\text{H}]^+$ 1018.3927, found 1018.3924.



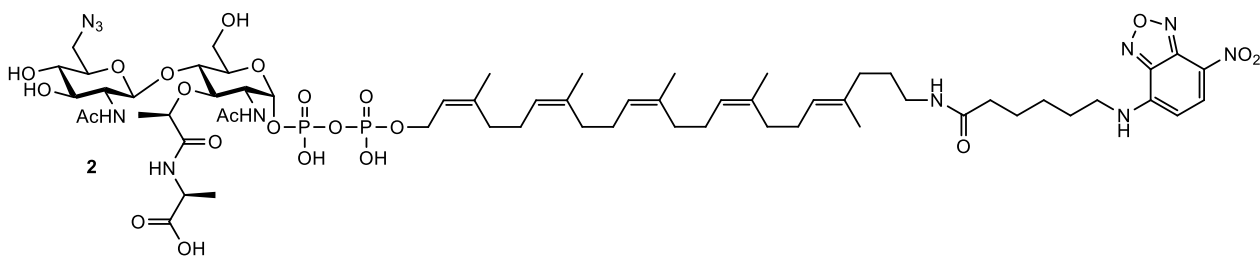
Compound 21. To a solution of **20** (600 mg, 0.589 mmol) in MeOH (20 mL) was added with *p*-toluenesulfonic acid (11 mg, 0.059 mmol) at 0 °C. The reaction mixture was then heated to 60 °C and stirred for 30 min. After the reaction was complete, the mixture was neutralized by adding Et_3N (82 μL , 1 equiv) and concentrated to give the diol. The resulting diol intermediate in pyridine (3 mL) was added with TsCl (114 mg, 1.02 equiv) at 0 °C under argon and stirred for 8 h. The reaction mixture was diluted with CH_2Cl_2 (30 mL) and washed with 0.5 N $\text{HCl}_{(\text{aq})}$ (30 mL \times 2). The organic layers were dried over anhydrous MgSO_4 , concentrated, and purified by CC (silica gel, $\text{CH}_2\text{Cl}_2/\text{MeOH}$ = 50:1) to give the tosylated intermediate (383 mg, 0.353 mmol). The tosylated intermediate (383 mg, 0.353 mmol) in MeOH (10 mL) was added with hydrazine acetate (318 mg, 10 equiv). The solution was heated to 80 °C for 15 h. The solvent of the reaction mixture was removed. The resulting residues was re-dissolved in anhydrous pyridine (5 mL), followed by addition of Ac_2O (5.0 mL) at 0 °C. The mixture was warmed up to rt and stirred for 4 h. The reaction mixture was diluted with CH_2Cl_2 (50 mL), and washed with brine (50 mL \times 2) and 1 N $\text{HCl}_{(\text{aq})}$ (50 mL \times 2). The organic layer was dried over anhydrous MgSO_4 , concentrated, and purified by CC (silica gel, $\text{CH}_2\text{Cl}_2/\text{MeOH}$ = 50:1) to give **21** (260 mg, 43% over four steps). $[\alpha]_D^{25} +27$ (c 0.85 in CHCl_3); ^1H NMR (600 MHz, CDCl_3): δ -0.01 (s, 9H), 0.96 (dd, 2H, J = 7.2, 9.6 Hz), 1.17 (d, 3H, J = 6.6 Hz), 1.39 (d, 3H, J = 7.2 Hz), 1.91 (s, 3H), 1.95 (s, 3H), 1.99 (s, 3H), 1.99 (s, 3H), 2.11

(s, 3H), 2.41 (s, 3H), 3.61 (dd, 1H, $J = 8.4, 10.8$ Hz), 3.64–3.70 (m, 1H), 3.71–3.76 (m, 2H), 3.87–3.90 (m, 1H), 3.96 (q, 1H, $J = 9.6$ Hz), 4.06–4.16 (m, 5H), 4.21 (dd, 1H, $J = 3.6, 12.0$ Hz), 4.40–4.47 (m, 2H), 4.49 (d, 1H, $J = 12.4$ Hz), 4.62 (d, 1H, $J = 12.4$ Hz), 4.94 (t, 1H, $J = 9.6$ Hz), 5.07 (dd, 1H, $J = 9.6, 10.2$ Hz), 5.13 (d, 1H, $J = 3.6$ Hz), 6.37 (d, 1H, $J = 9.6$ Hz), 7.23–7.34 (m, 8H, $J = 6.6$ Hz), 7.73 (d, 2H, $J = 8.4$ Hz); ^{13}C NMR (150 MHz, CDCl_3): δ -1.3 ($\times 3$), 17.4, 17.8, 19.3, 20.8, 20.8, 21.2, 21.9, 23.3, 23.4, 48.4, 53.6, 54.6, 62.6, 63.9, 67.2, 68.8, 69.4, 70.2, 72.1, 72.4, 75.1, 76.1, 76.6, 96.9, 100.0, 127.7 ($\times 2$), 127.9, 128.2 ($\times 2$), 128.6 ($\times 2$), 130.3 ($\times 2$), 132.3, 137.7, 145.6, 169.6, 171.0, 171.2, 171.2, 171.8, 173.2, 174.3; HRMS calcd for $[\text{C}_{47}\text{H}_{67}\text{N}_3\text{O}_{19}\text{SSi}+\text{H}]^+$ 1038.3859, found 1038.3864

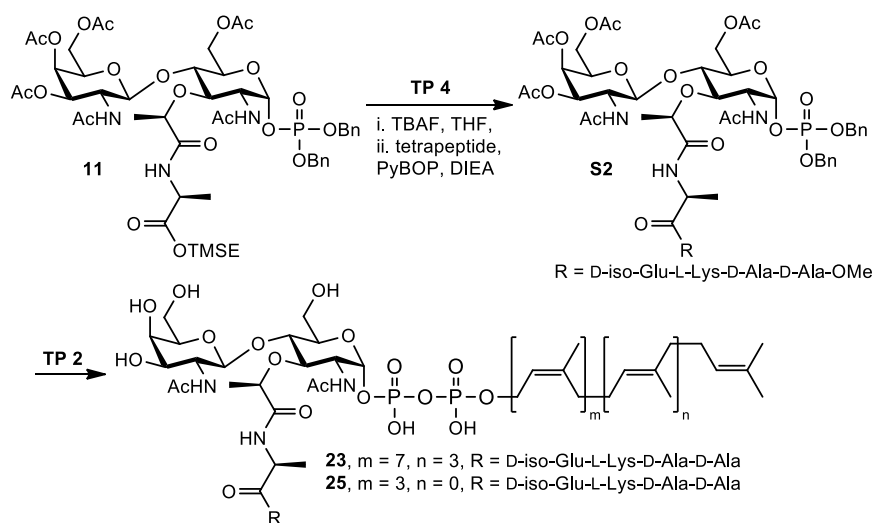


Compound 22.

According to **TP 1**, the disaccharide **21** (150 mg, 0.144 mmol) was converted into **22** as a white solid (113 mg, 65% over three steps). $[\alpha]_{\text{D}}^{25} +36$ (c 1.6 in CHCl_3); ^1H NMR (600 MHz, CDCl_3): δ -0.05 (s, 9H), 0.81–0.91 (m, 2H), 1.19 (d, 3H, $J = 6.6$ Hz), 1.39 (d, 3H, $J = 7.2$ Hz), 1.83 (s, 3H), 1.92 (s, 3H), 1.99 (s, 3H), 2.03 (m, 6H), 2.41 (s, 3H), 3.53 (dd, 1H, $J = 9.0, 10.8$ Hz), 3.68–3.69 (m, 1H), 3.81–3.87 (m, 3H), 4.01–4.16 (m, 7H), 4.41 (d, 1H, $J = 8.4$ Hz), 4.47 (t, 1H, $J = 7.2$ Hz), 4.51 (q, 1H, $J = 6.6$ Hz), 4.95 (t, d, 1H, $J = 9.6$ Hz), 4.98–5.06 (m, 6H), 5.98 (dd, 1H, $J = 3.6, 5.4$ Hz), 6.08 (d, 1H, $J = 9.6$ Hz), 7.30–7.34 (m, 11H), 7.64 (d, 1H, $J = 5.4$ Hz), 7.73 (d, 2H, $J = 8.4$ Hz); ^{13}C NMR (150 MHz, CDCl_3): δ -1.3 ($\times 3$), 17.3, 17.9, 19.3, 20.8, 20.9, 21.0, 21.9, 23.0, 23.4, 48.4, 53.9, 54.6, 62.1, 64.0, 67.3, 68.7, 69.6, 69.8, 71.2, 72.1, 72.2, 74.0, 74.8, 76.7, 95.8, 99.6, 128.1 ($\times 4$), 128.2 ($\times 3$), 128.7 ($\times 2$), 128.8 ($\times 4$), 130.3, 132.3, 135.8, 135.9, 145.6, 169.6, 171.2, 171.3, 171.4, 171.5, 172.8, 174.5; HRMS calcd for $[\text{C}_{54}\text{H}_{74}\text{N}_3\text{O}_{22}\text{PSSi}+\text{H}]^+$ 1208.3992, found 1208.3988.

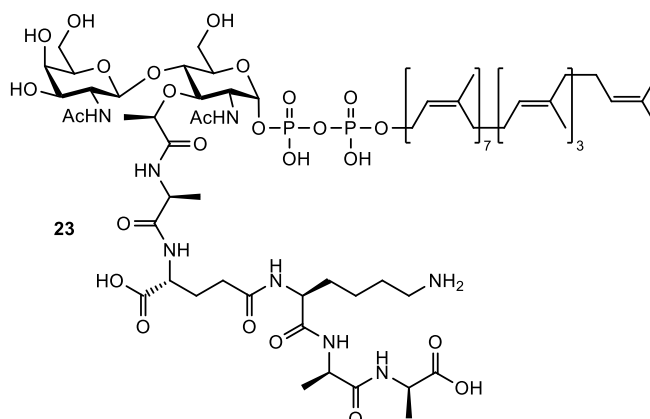


Compound 2. Compound **22** (25 mg, 0.021 mmol) was added to a suspension of 20% Pd(OH)₂/C (10 mg) in MeOH (3 mL) at rt. The mixture was stirred vigorously under an atmosphere of hydrogen for 4 h, and the catalyst was removed by filtration. The filtrate was concentrated by rotary evaporation and dried under high vacuum to give the phosphate intermediate as a colorless oil. The phosphate intermediate in DMF (5 mL) was added NaN₃ (94 mg, 69 equiv) in one portion, and the mixture was heated to 70 °C and stirred for 12 h. The crude mixture was diluted with CH₂Cl₂ (10 mL), 0.5 N HCl_(aq) (10 mL × 2). The organic layers were collected, dried over anhydrous MgSO₄, concentrated to the azido intermediate as white solid. Then, compound **2** as a red solid (5.3 mg, 18%) was prepared according to **TP 2**. The purity of **2** was analyzed on an anion exchange column (SAX1, Supelco Co., 5 μm, 4.6 × 250 mm) by fluorescent-HPLC (E_{em}: 466 nm/E_{ex} 535 nm) with a linear gradient elution of NH₄OAc (20 mM to 1 M in MeOH) at a flow rate of 1.0 mL/min over 30 min. Compound **2** had purity higher than 90% by HPLC (retention time = 16.5 min). [α]_D²⁵ −3.8 (*c* 0.25 in H₂O); HRMS calcd for [C₆₁H₉₅N₁₁O₂₃P₂-2H]²⁻ 704.7965, found 704.7965.



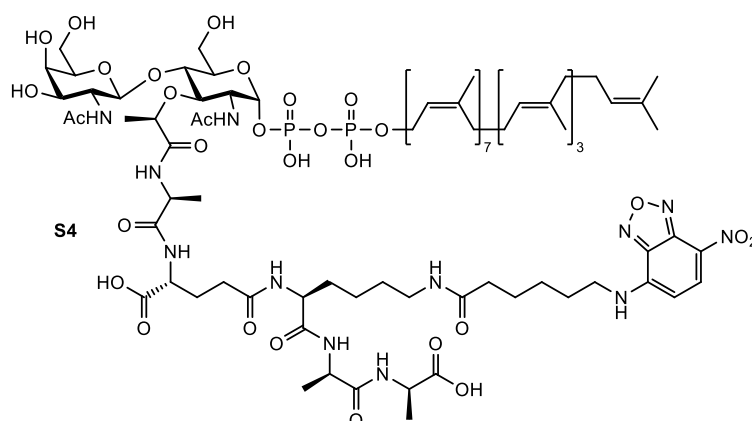
Typical procedure (TP 4) for a sequence of desilylation and peptide formation (the preparation of the pentapeptide). Compound **11** (0.11 mmol) dissolved in THF (10 mL) was treated with TBAF (1M in THF, 220 μL, 2 equiv). The mixture was stirred at rt. After stirring for 2 h, the mixture was diluted with EtOAc (15 mL), followed by washing with brine (15 mL × 2), and 1.0 N HCl_(aq) (15 mL × 2). The organic layer was dried over anhydrous MgSO₄ and concentrated by

rotary evaporation to afford the carboxylic acid. The acid and PyBOP (63 mg, 1.1 equiv) were stirred in CH₂Cl₂ (5 mL) for 5 min at 0 °C. The solution of tetrapeptide, D-Glu(OMe)-L-Lys(TFA)-D-Ala-D-Ala(OMe) (65 mg, 1.1 equiv) and DIEA (23 µL, 1.27 equiv) in THF (5 mL) were added to the solution above. The reaction was stirred for 30 min at rt, then CH₂Cl₂ (30 mL × 2) was added and the mixture was extracted with 1.0 N HCl_(aq) (30 mL × 2) and brine (30 mL × 2). The extract was dried over anhydrous MgSO₄ and concentrated to give **S2** as white solid (122 mg, 73%).



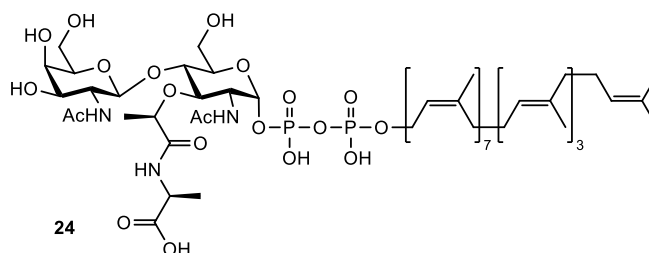
Compound 23.

According to **TP 2**, substrates **S2** and C55P were applied to obtain **23** as a white solid. Compound **23** had purity more than 95% by HPLC ([23.9 min, HPLC on a Eclipse XDB-C18 (4.6 mm × 250 mm, 5 µm) using a gradient eluent of 50 mM NH₄OAc/MeOH solution (15:85 to 0:100) over 30 min at a flow rate of 1.0 mL/min monitored by DAD detector with UV = 220 nm]). [α]_D²⁵ −2.1 (c 0.20 in H₂O); HRMS calcd for [C₉₄H₁₅₅N₈O₂₆P₂-H][−] 1874.0522, found 1874.0555.



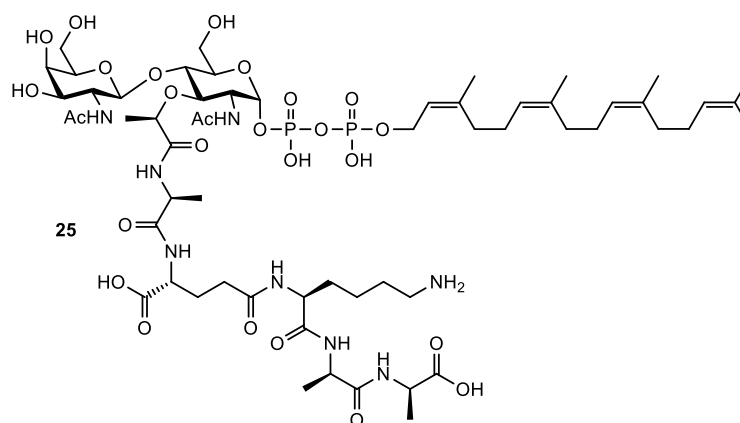
Compound S4. A mixture of **23** (2.5 mg, 1.3 µmol) and NBD-X-OSu (1 mg, 2.6 µmol) in a solution of DMF/sat. NaHCO_{3(aq)} (2 mL, v/v = 1:1) was stirred at rt for 2 h. The reaction mixture was purified by reverse-phase HPLC on a ZORBAX RX-C8 column to give compound **S4** as a reddish brown solid (2.3 mg, 82%). Compound **S4** had purity more than 90% by HPLC (retention time =

16.4 min). $[\alpha]_{\text{D}}^{25} -5.4$ (c 0.21 in H_2O); HRMS calcd for $[\text{C}_{106}\text{H}_{168}\text{N}_{12}\text{O}_{30}\text{P}_2-2\text{H}]^-$ 1074.5649, found 1074.5670.



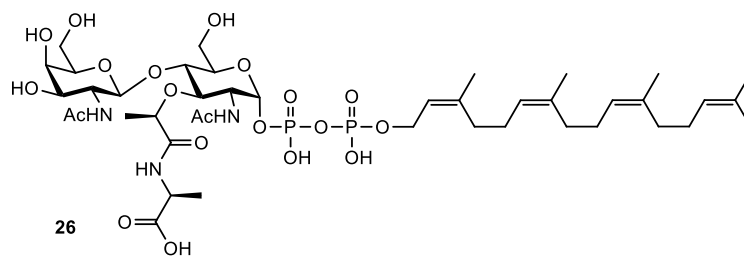
Compound 24.

According to **TP 2**, substrates **11** and C55P were applied to obtain **24** as a white solid. Compound **24** had purity more than 95% by HPLC ([23.5 min, HPLC on a Eclipse XDB-C18 (4.6 mm × 250 mm, 5 μm) using a gradient eluent of 50 mM NH₄OAc/MeOH solution (15:85 to 0:100) over 30 min at a flow rate of 1.0 mL/min monitored by DAD detector with UV = 220 nm)]. [α]_D²⁵ −3.7 (c 0.24 in H₂O); HRMS calcd for [C₇₇H₁₂₇N₃O₂₀P₂-2H]^{2−} 736.9166, found 736.9184.



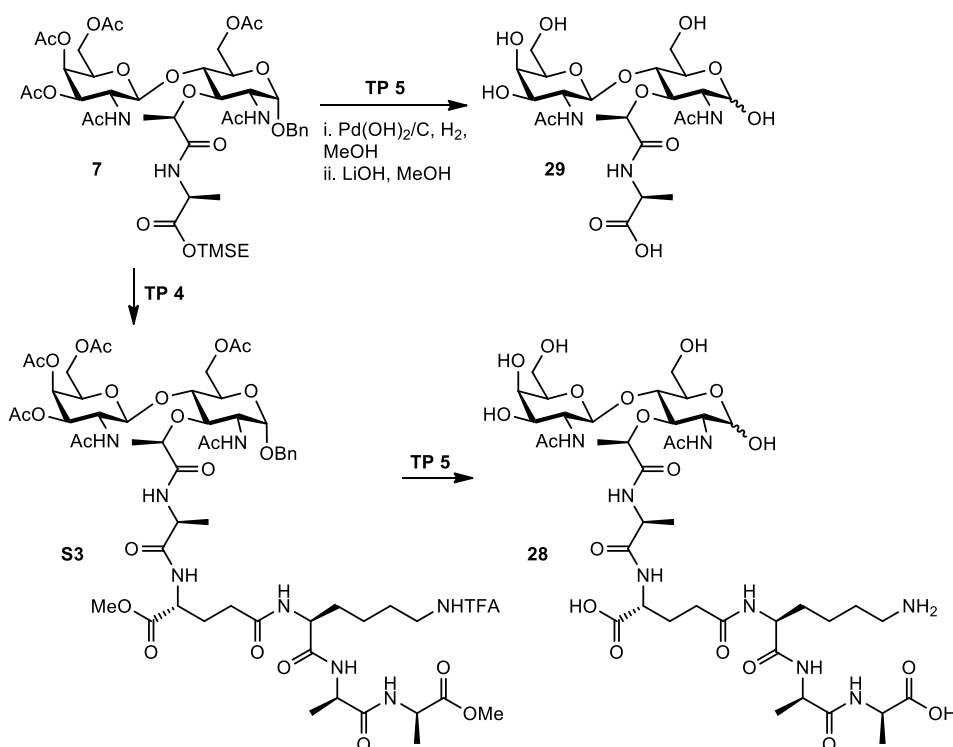
Compound 25.

According to **TP 2**, substrates **S2** and C20P were applied to obtain **25** as a white solid. Compound **25** had purity more than 95% by HPLC ([24 min, HPLC on a Eclipse XDB-C18 (4.6 mm × 250 mm, 5 μm) using a gradient eluent of 50 mM NH₄OAc/MeOH solution (15:85 to 0:100) over 30 min at a flow rate of 1.0 mL/min monitored by DAD detector with UV = 220 nm)]. $[\alpha]_{\text{D}}^{25} -2.2$ (c 0.22 in H₂O); HRMS calcd for [C₅₉H₁₀₀N₈O₂₆P₂+H]⁺ 1399.6297, found 1399.6318.



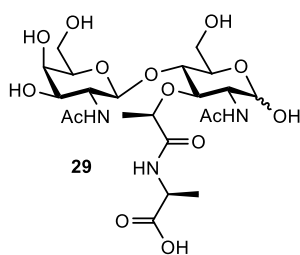
Compound 26.

According to **TP 2**, substrates **11** and C20P were applied to obtain **26** as a white solid. Compound **26** had purity more than 95% by HPLC ([24.2 min, HPLC on a Eclipse XDB-C18 (4.6 mm × 250 mm, 5 μm) using a gradient eluent of 50 mM NH₄OAc/MeOH solution (15:85 to 0:100) over 30 min at a flow rate of 1.0 mL/min monitored by DAD detector with UV = 220 nm]). $[\alpha]_D^{25} -3.5$ (*c* 0.21 in H₂O); ¹H NMR (600 MHz, MeOD): δ 1.35 (d, 3H, *J* = 6.0 Hz), 1.40 (d, 3H, *J* = 7.2 Hz), 1.61–1.73 (m, 15H), 1.91–2.10 (m, 18H), 3.42–4.03 (m, 13H), 4.34–4.52 (m, 3H), 5.12–5.25 (m, 4H), 5.41 (m, 1H), 5.57 (m, 1H); HRMS calcd for [C₄₂H₇₁N₃O₂₀P₂-2H]²⁻ 498.6980, found 498.6999.



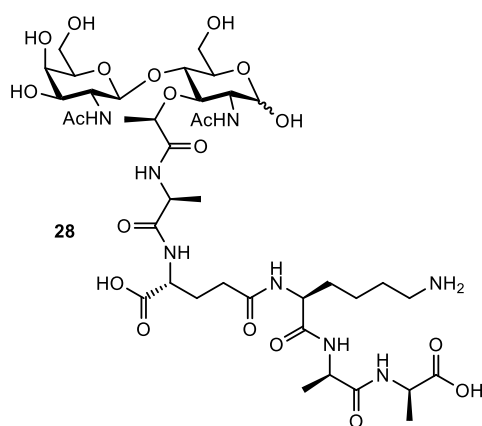
Typical procedure (TP 5) for a sequence of debenzylation and global deprotection. A benzyl glycoside **7** (0.1 mmol) was added to a suspension of 20% Pd(OH)₂/C (20 mg) in MeOH (20 mL) at rt. The mixture was stirred vigorously under an atmosphere of hydrogen for 4 h, and the catalyst was removed by filtration. The filtrate was concentrated by rotary evaporation to give a lactol. The lactol was used without further purification. The residual sample (0.1 mmol) in MeOH/H₂O (9:1, 5 mL) was treated with LiOH-H₂O (42 mg, 10 equiv) and stirred at rt for 2 h. The mixture was

neutralized by 1.0 N HCl_(aq), concentrated, and purified by CC (silica gel, *i*-PrOH/NH₄OH = 2/1).



Compound 29.

According to **TP 5**, compound **7** (100 mg, 0.108 mmol) was converted into **29** (45 mg, 73% over two steps) as a white solid. HRMS calcd for [C₂₂H₃₇N₃O₁₄+H]⁺ 568.2348, found 568.2318.



Compound 28.

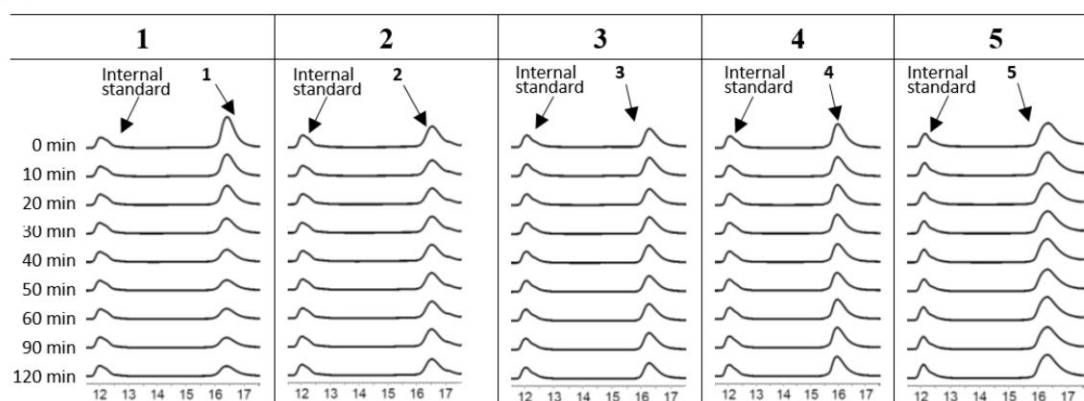
According to **TP 4** and **TP 5**, compound **7** (100 mg, 0.108 mmol) was converted into **28** (63 mg, 60% over four steps) as a white solid. HRMS calcd for [C₃₉H₆₆N₈O₂₀+H]⁺ 967.4466, found 967.4493.

Section B. Assay procedures and bio-results

Substrate activity analysis of Lipid II analogues

The activity assay was similar to the reaction as reported.^{S1} The assays were performed in 10 μ L buffer (50 mM Tris-HCl pH 8.0, 10 mM CaCl₂, 0.085% decyl PEG, 15% methanol and 10% DMSO) containing 2.5 μ g/mL *C. difficile* PBP in the presence or absence of Lipid IV (20 μ M) at 37 °C. Reactions were initiated with the addition of 30 μ M NBD-labeled Lipid II analogues. At various time points (0, 10, 20, 30, 40, 50, 60, 90, and 120 min), the mixture of 95 μ M moenomycin and 10 μ M internal standard were added to stop the transglycosylation reaction. The analysis of samples was performed on an anion exchange column (SAX1, Supelco Co.) with a linear gradient of NH₄OAc (20 mM to 1 M in MeOH) at a flow rate of 1.0 mL/min over 30 min. The fluorescence intensity was detected with λ_{ex} 466 nm and λ_{em} 535 nm.

(A)



(B)

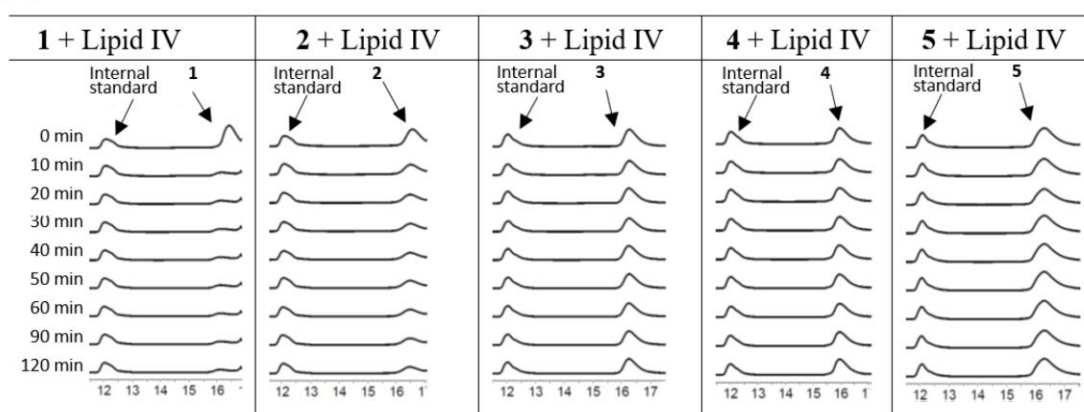
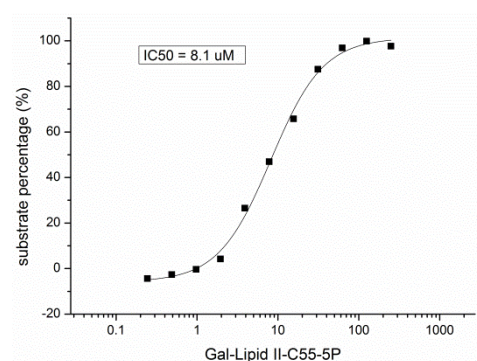


Figure S1. HPLC-based measurement of the substrate activity of NBD-Lipid II and 1–5 towards *C. difficile* PBP. The substrate activity of NBD-Lipid II and 1–5 was measured in the absence (A) or presence (B) of Lipid IV (20 μ M) as described above. The consumption of 1 and 2 in the reactions were significantly improved in the presence of Lipid IV.

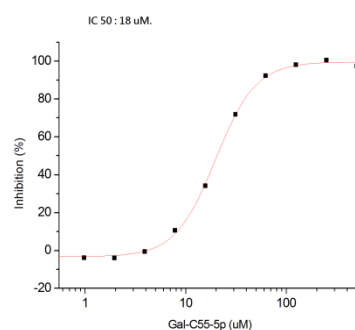
The inhibitory activity of Lipid II analogues

The inhibitory activity of Lipid II analogues was determined by HPLC-based TGase-functional assay. The inhibitory assay was performed in a solution of 50 mM Tris-HCl pH 8.0, 10 mM CaCl₂, 0.085% decyl PEG, 15% methanol, 10% DMSO, 8 μ M NBD-Lipid II and 6-(7-Nitro-2,1,3-benzoxadiazol-4-ylamino)hexanoic acid (5 μ M, internal standard). Reactions were initiated with the addition of 0.07 μ M active TGase. The reactions were incubated at 37 °C for 1 h, and then stopped by addition of 100 μ M moenomycin. Samples were analyzed by HPLC with an anion exchange column (SAX1, Supelco Co.). A linear gradient of NH₄OAc (20 mM to 1 M in MeOH) was used as eluent at a flow rate of 1.0 mL/min over 30 min. The fluorescent substrates were monitored with λ_{ex} 466 nm/ λ_{em} 535 nm by fluorescence detector. The inhibitory percentage was determined by the following formula: $(A_{\text{Rea}}/A_{\text{I}} - A_{\text{Int}}/A_{\text{I}})/(A_{\text{Int}}/A_{\text{I}}) \times 100$. A_{Rea} represents the peak area of NBD-Lipid II after 1 h reaction; A_{Int} represents the peak area of NBD-Lipid II without reaction; A_{I} represents the peak area of internal standard. The IC₅₀ value of Lipid II analogues against TGase was calculated by Origin 6[®].

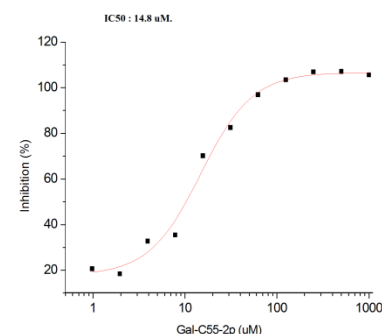
23 against *E. coli* TGase



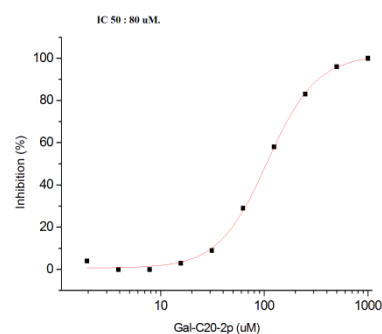
23 against *C.difficile* TGase



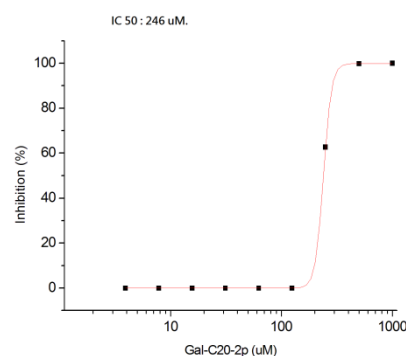
24 against *E. coli* TGase



26 against *E. coli* TGase

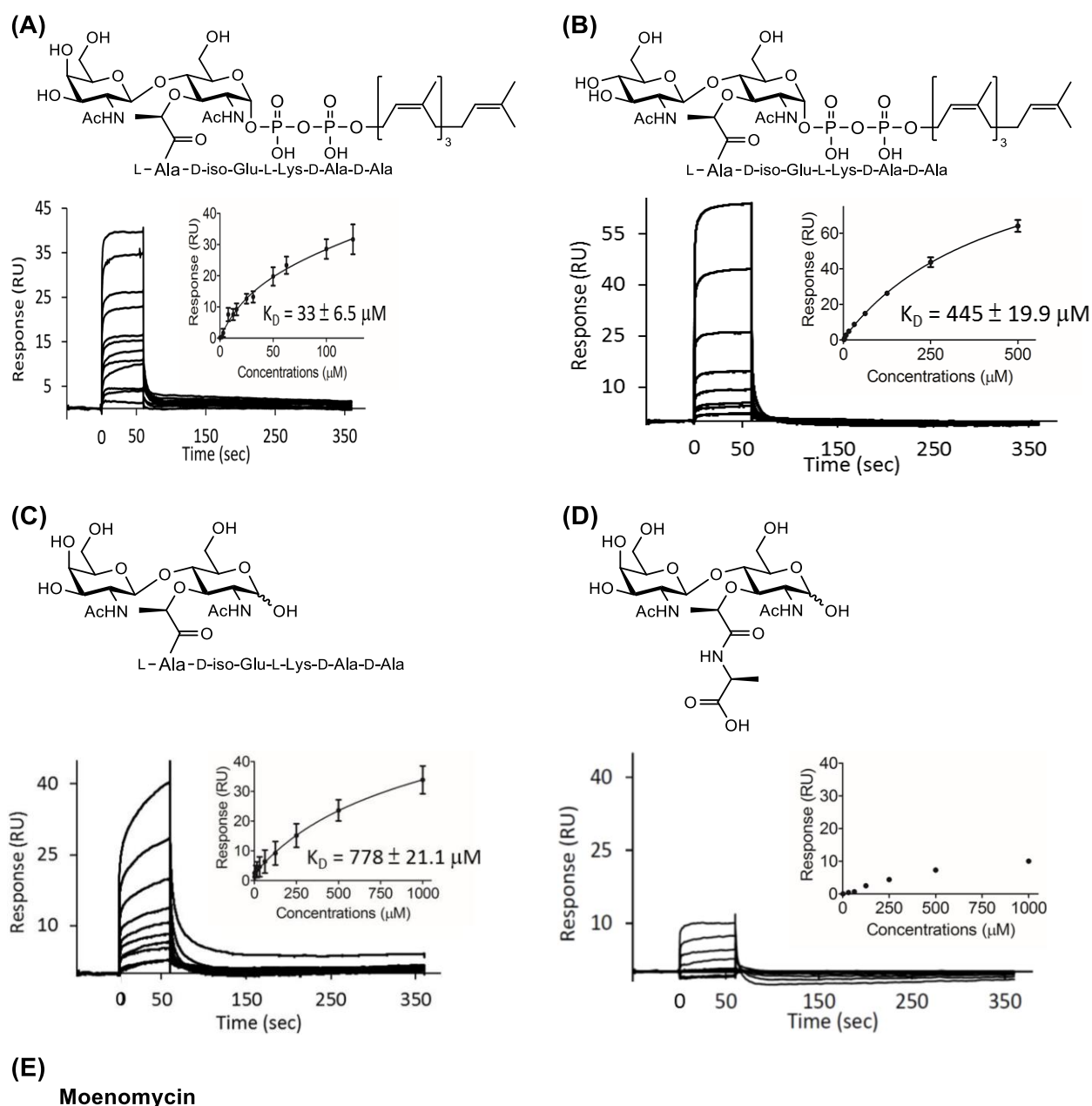


26 against *C.difficile* TGase



Surface plasma resonance (SPR) Analysis of Lipid II analogues

The assays were performed with BIAcore T200 (GE Healthcare). All experiments were performed in a HBS-EP+ buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% P20, GE Healthcare) at 25 °C. Purified *C. difficile* PBP was immobilized onto CM5 sensor chips (GE Healthcare) to the level of 4000 relative units (RU) via amine coupling. The chips were then passed over with sample mixtures which containing variable concentrations of Lipid II analogues (0 to 1000 μ M) and a constant concentration of moenomycin (1 μ M).



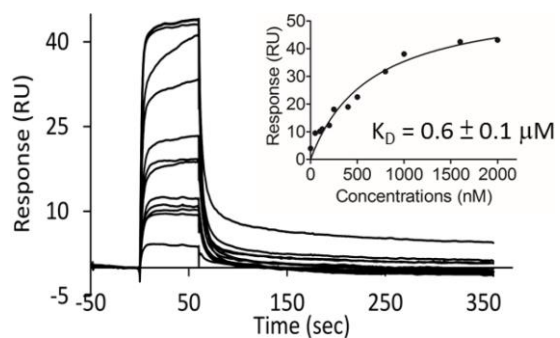


Figure S2. Results of SPR analysis of the binding activity of **25** (A), **27** (B), **28** (C), **29** (D) and moenomycin (E) in the presence of 1 μM MoeA to immobilized *C. difficile* PBP. The experiments were performed in triplicate, and the data were analyzed by using steady-state affinity and the K_D values were determined by BIAcore T100 evaluation software (GE Healthcare). The K_D values of **25**, **27**, **28** and moenomycin were 33 ± 6.5 , 445 ± 19.9 , 778 ± 21.1 and $0.6 \pm 0.1 \mu\text{M}$, respectively.

Determination of substrate activity of C4-epimer Lipid II (S4) in the presence of Lipid II.

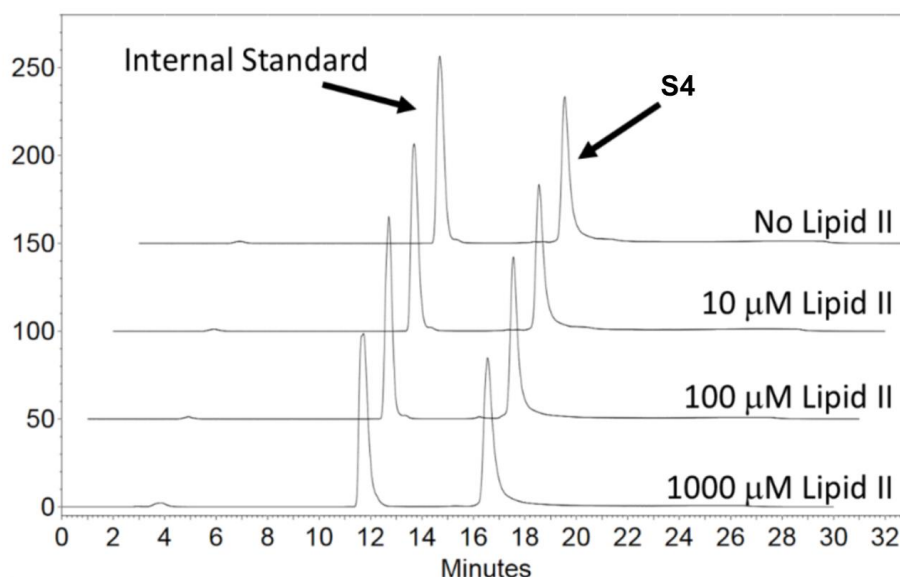
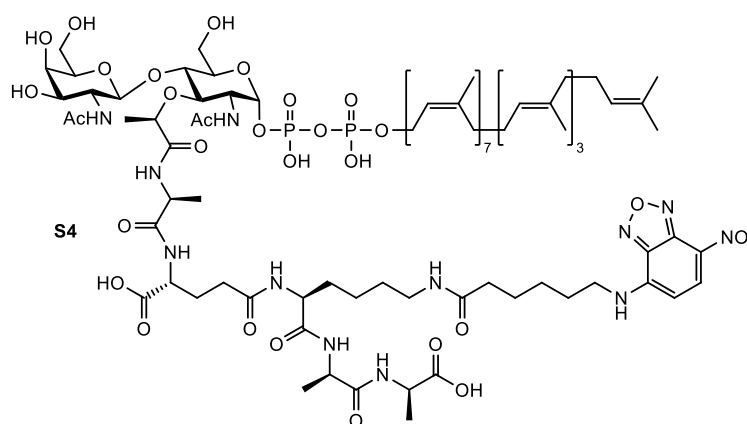


Figure S3. HPLC-based measurement of the substrate activity of **S4** without or with Lipid II.

The assays were performed in 10 μ L buffer (50 mM Tris-HCl pH 8.0, 10 mM CaCl_2 , 0.085% decyl PEG, 15% methanol and 10% DMSO) containing 10 μ M **S4**, 2.5 μ g/mL *C. difficile* PBP and various concentrations of Lipid II (0, 10, 100 and 1000 μ M) at 37 $^\circ\text{C}$ for 1 h. The samples were analyzed by an anion-exchange column (SAX1, Supelco Co., 5 μ m, 4.6 \times 250 mm) with a linear gradient elution of NH_4OAc (20 mM to 1 M in MeOH) at a flow rate of 1.0 mL/min over 30 min. The fluorescent substrates were monitored with λ_{ex} 466 nm/ λ_{em} 535 nm by fluorescence detector.

Based on our study, **S4** (the GalNAc-MurNAc type) is not a substrate of *C. difficile* PBP, even in the presence of 1 mM Lipid II.



Fluorescence anisotropy assay of **25** against F-MoeA

The fluorescence anisotropy (FA) assay was performed as described in our previous reports.^{S5} In general, FA measurements were carried out in triplicate in the wells of 384-well plates, using a laser fluorimeter equipped with a 488-nm laser (IsoCyte; Blueshift Biotech). The reaction was performed in a 10 μ L solution containing 100 nM F-Moe, 10 mM Tris, pH 8.0, 100 mM NaCl. The reaction was initial with addition of *C. difficile* PBP (1 μ M), and the anisotropy change was measured after a 5-min equilibration. The scanning focus was above the plate bottom to avoid detection interference. Data analysis was performed with the proprietary software BlueImage (Blueshift Biotech). Fluorescence anisotropy values (A) were calculated by using the equation: $A = (I_{\parallel} - G \cdot I_{\perp}) / (I_{\parallel} + 2G \cdot I_{\perp})$, where I_{\parallel} is the fluorescence intensity of emitted light parallel to excitation, I_{\perp} is the fluorescence intensity of emitted light perpendicular to excitation.

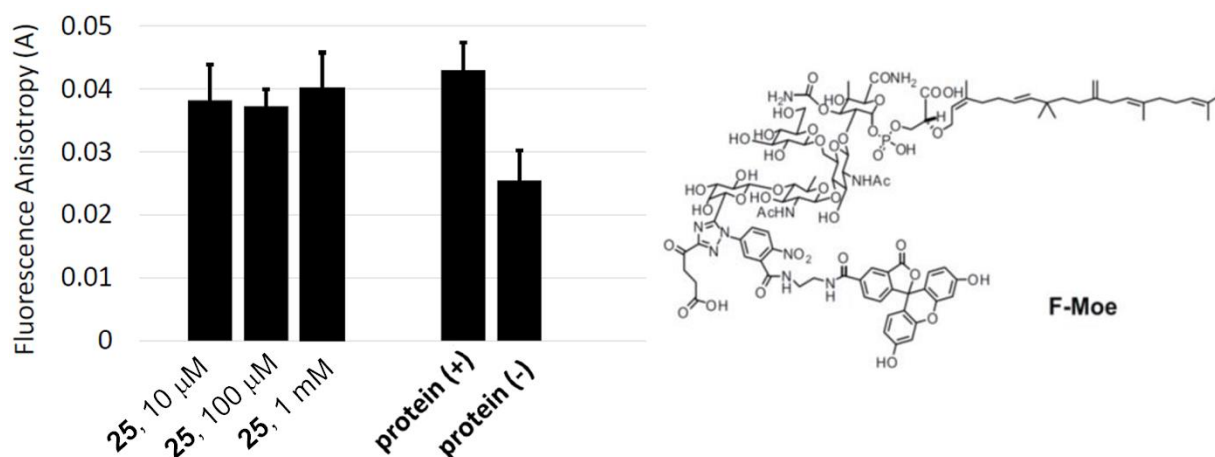
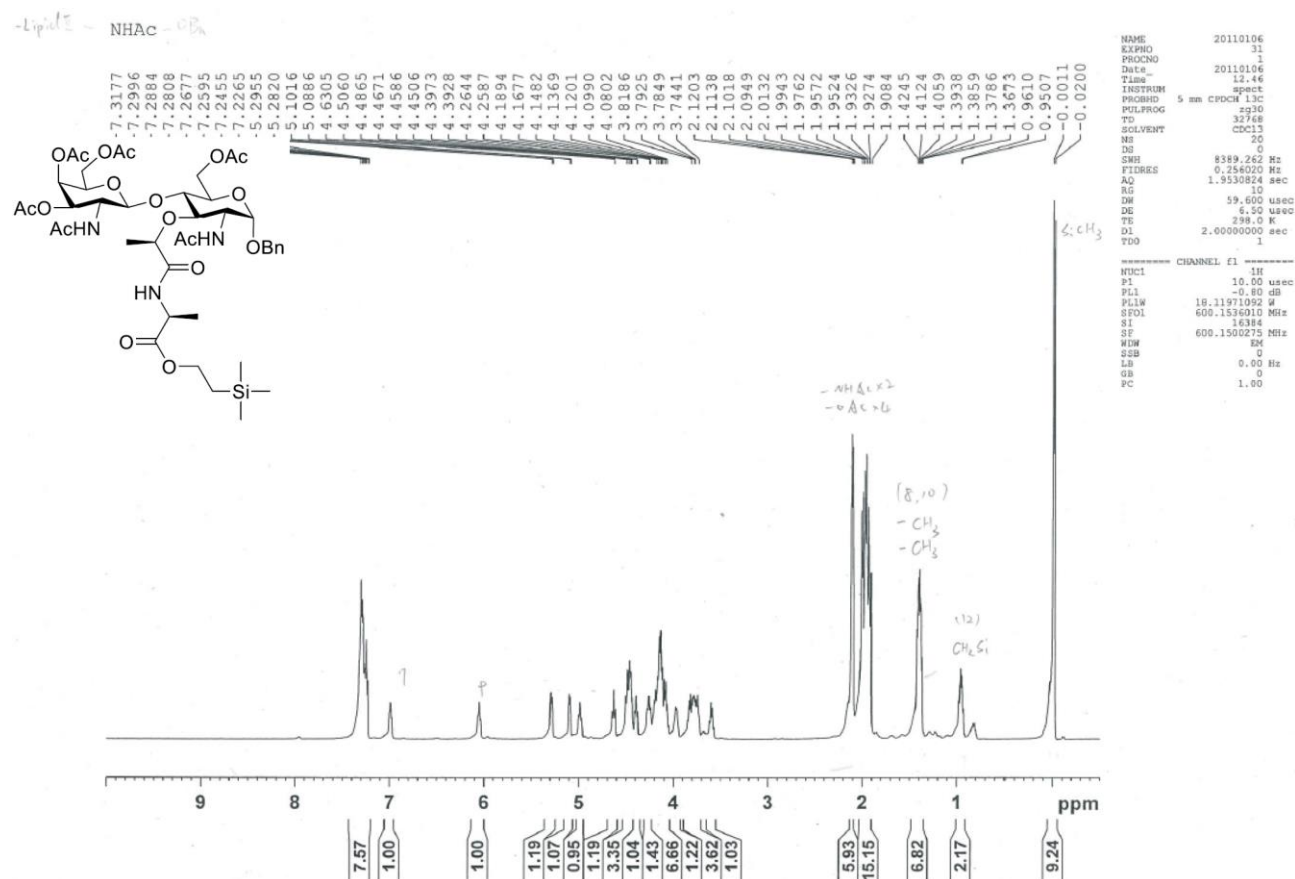
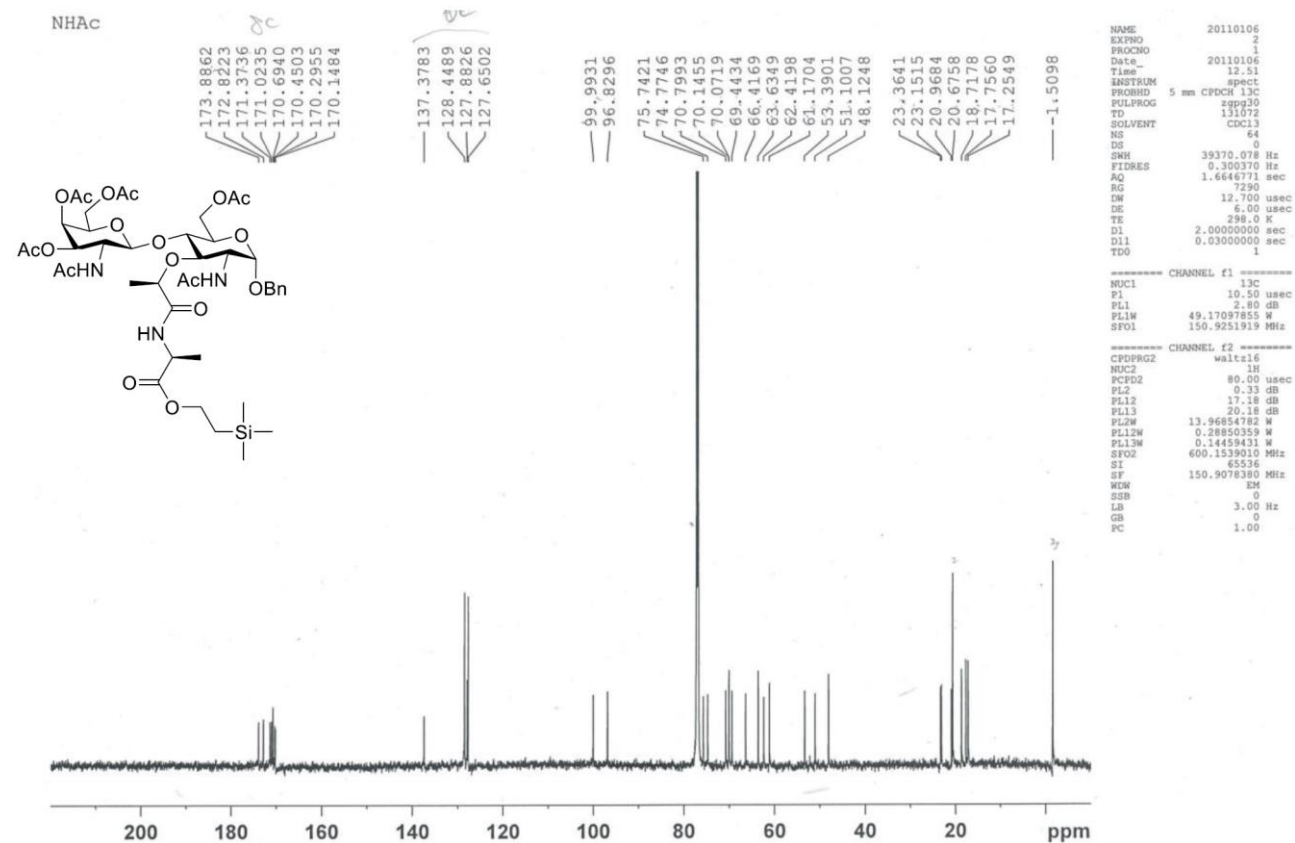


Figure S4. Competitive examination of **25** against F-MoeA by fluorescence anisotropy (FA) assay. The anisotropy of the *C. difficile* PBP-bound F-Moe complex incubation with **25** at various concentrations (10, 100, 1000 μ M) were showed. There are no significantly anisotropy changes were observed. The anisotropy of F-Moe incubation with (**protein** (+))/without (**protein** (-)) *C. difficile* PBP were illustrated as a control group.

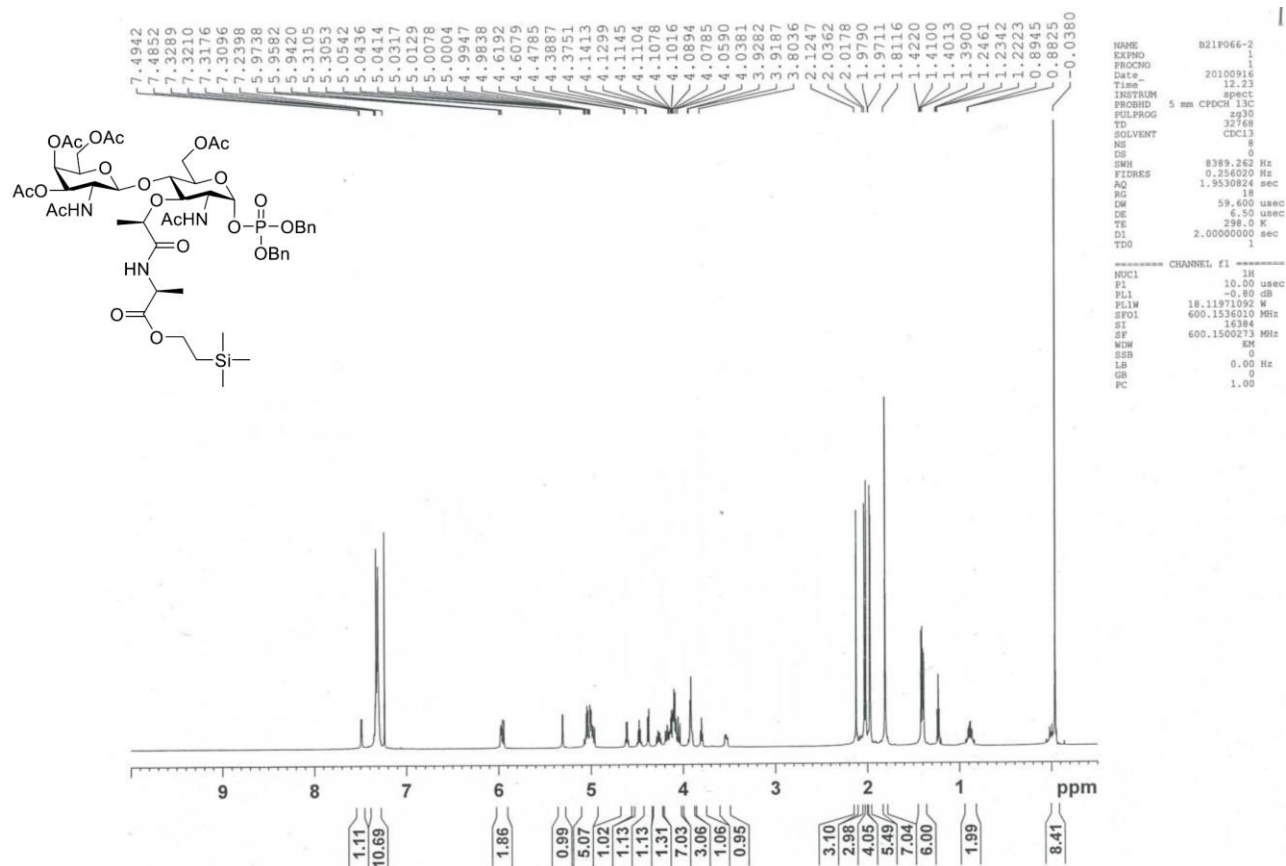
Section C. NMR spectra



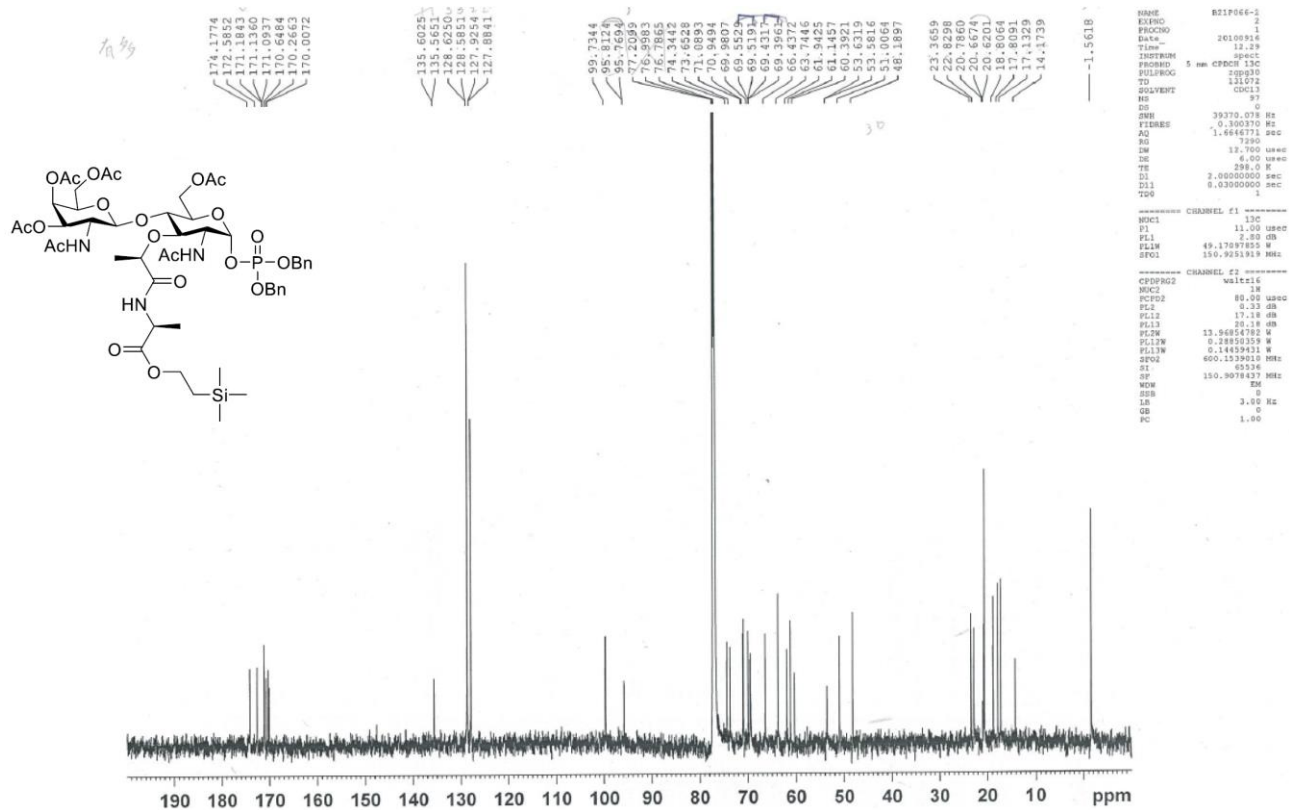
¹H NMR Spectra of compound **7** (600 MHz, CDCl₃)



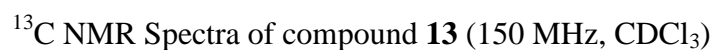
¹³C NMR Spectra of compound **7** (150 MHz, CDCl₃)

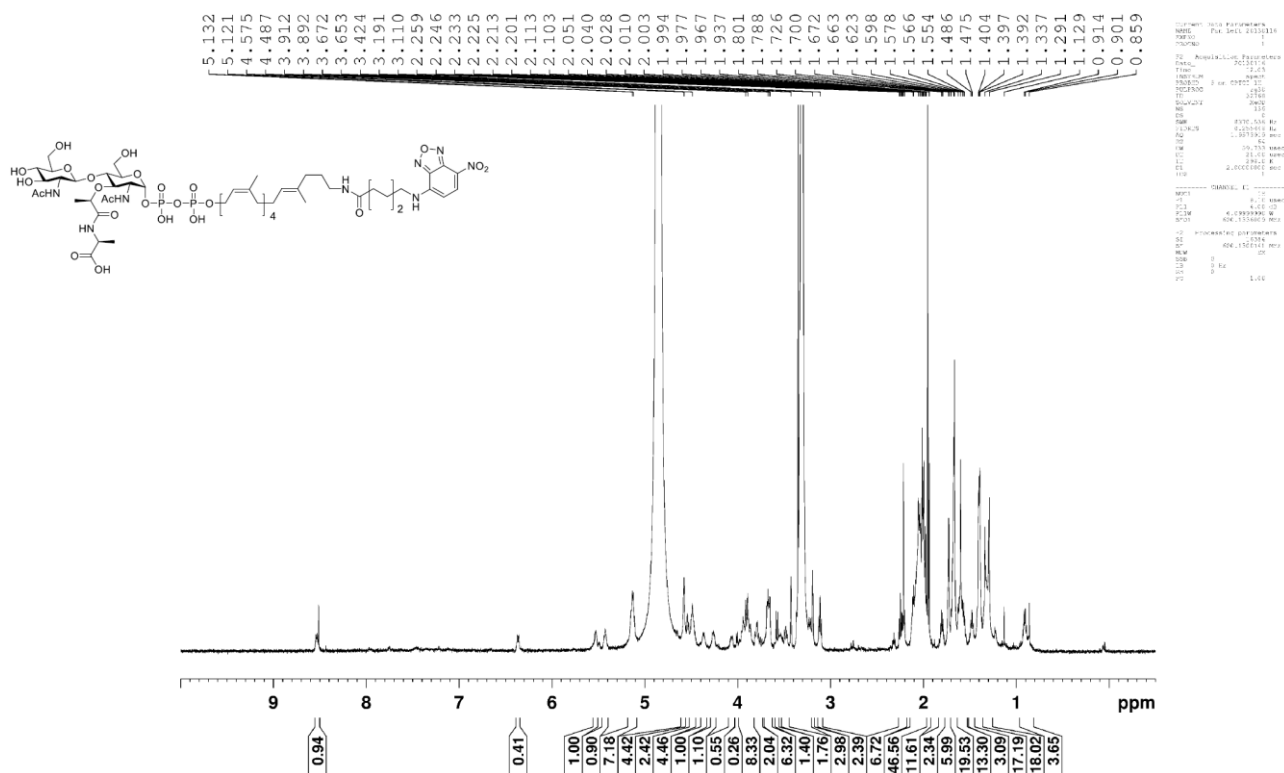


¹H NMR Spectra of compound **11** (600 MHz, CDCl₃)

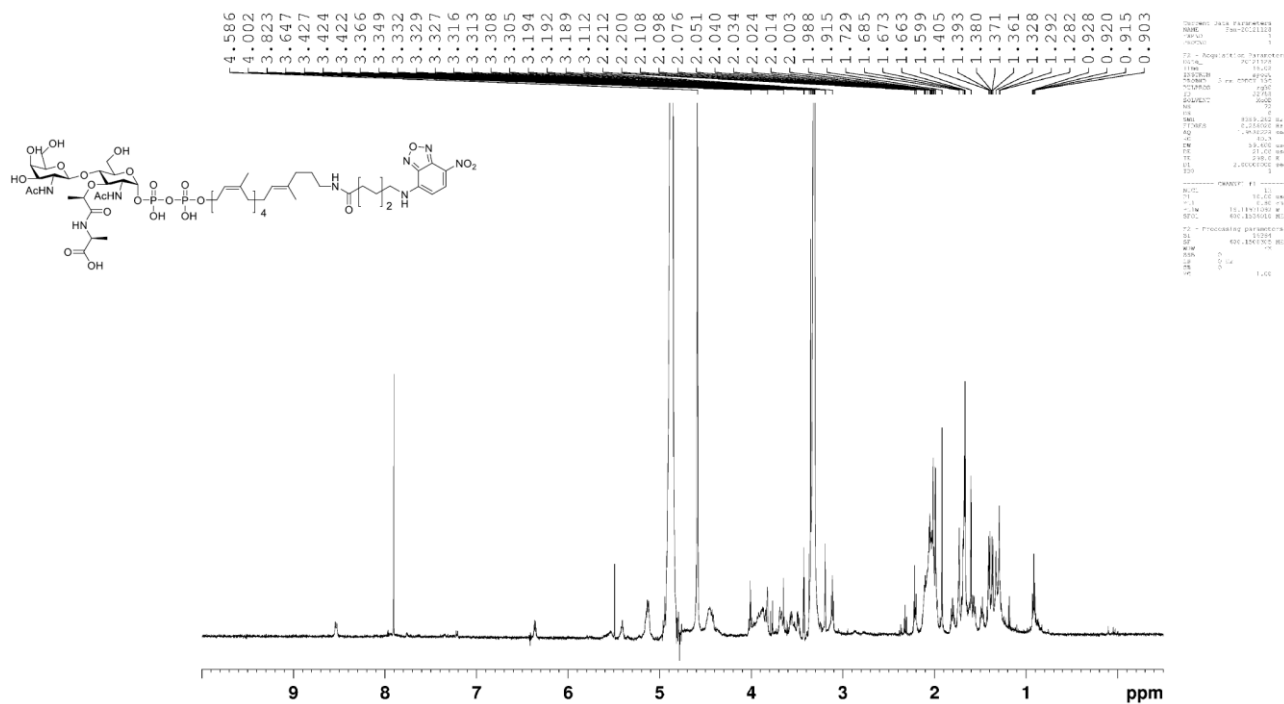


¹³C NMR Spectra of compound **11** (150 MHz, CDCl₃)

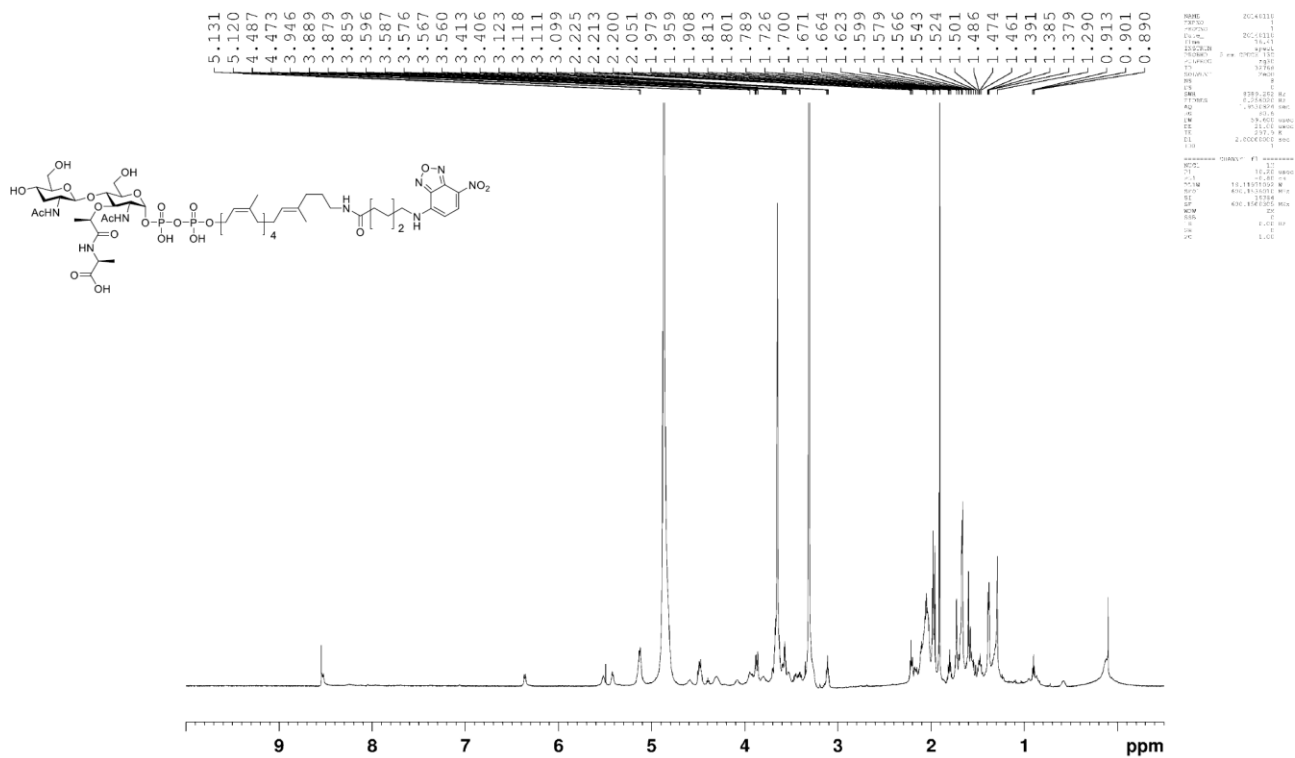




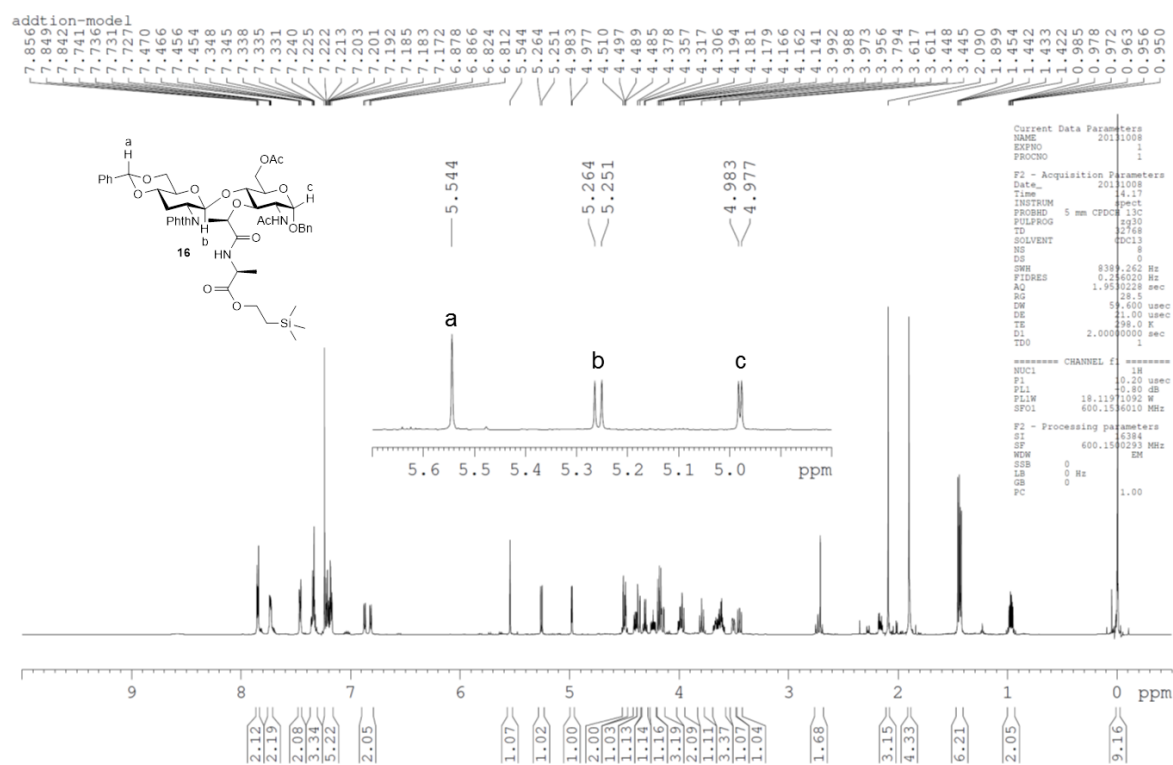
¹H NMR Spectra of compound **1** (600 MHz, MeOD)



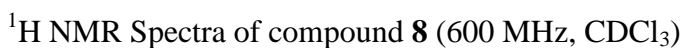
¹H NMR Spectra of compound **3** (600 MHz, MeOD)

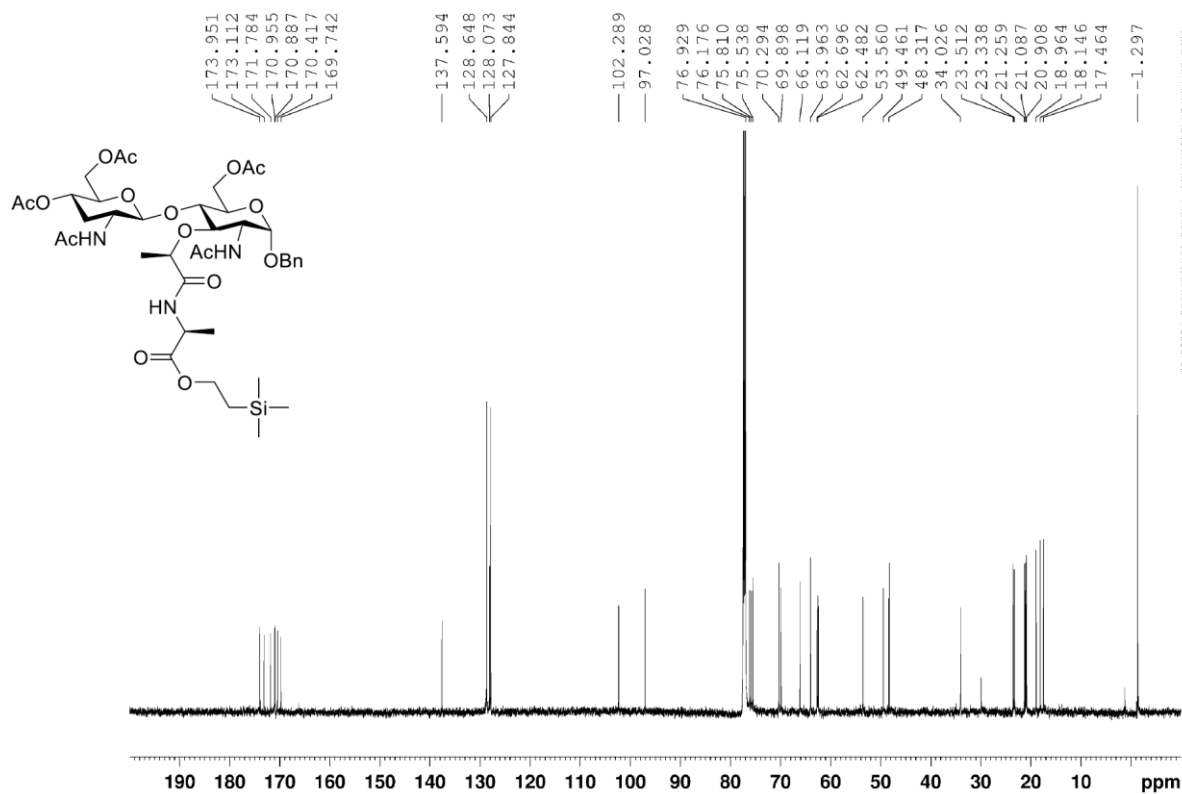


¹H NMR Spectra of compound **4** (600 MHz, MeOD)

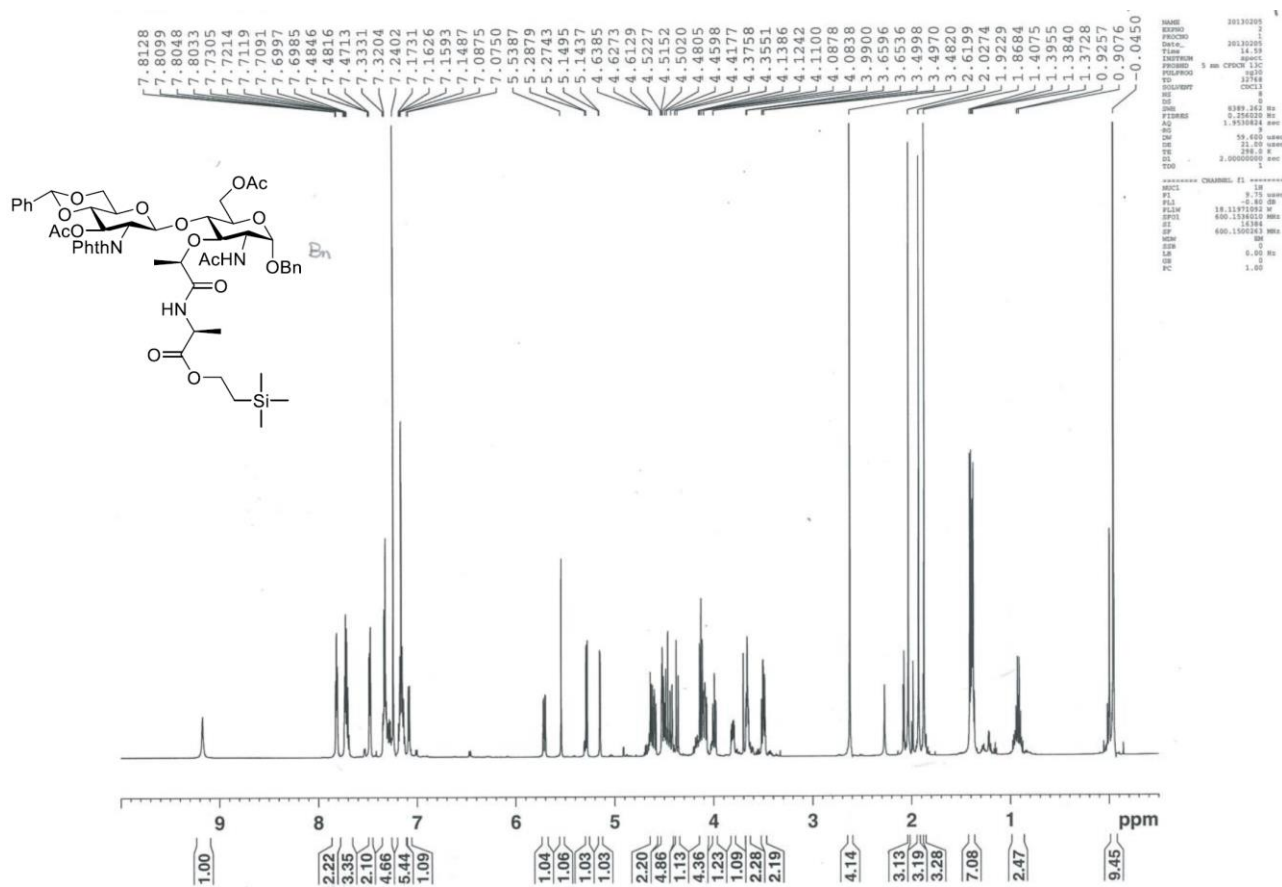


¹H NMR Spectra of compound **16** (600 MHz, CDCl₃)

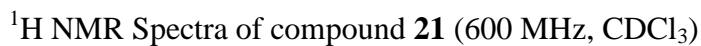




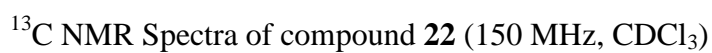
¹³C NMR Spectra of compound 8 (150 MHz, CDCl₃)



¹H NMR Spectra of compound 20 (600 MHz, CDCl₃)







References and Notes:

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- S5. T. J. Cheng, M. T. Sung, H. Y. Liao, Y. F. Chang, C. W. Chen, C. Y. Huang, L. Y. Chou, Y. D. Wu, Y. H. Chen, Y. S. Cheng, C. H. Wong, C. Ma and W. C. Cheng, *Proc. Natl. Acad. Sci.*, 2008, **105**, 431