Tuning the Moenomycin Pharmacophore to Enable Discovery of Bacterial Cell Wall Synthesis Inhibitors

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Supporting Information

1. PBP/PGT Active Site Sequence Homology

The glycosyl transfer reaction can be carried out by monofunctional peptidoglycan glycosyltransferases (PGTs) and by penicillin binding proteins (PBPs). The latter are bifunctional enzymes with a PGT domain and a second active site that catalyzes transpeptidation (TP domain). Despite these differences between these two classes of enzymes, the catalytic amino acids in the PGT active sites are highly conserved across PGTs and PBPs of various bacterial species, Gram-positive and Gram-negative (Figure 1).

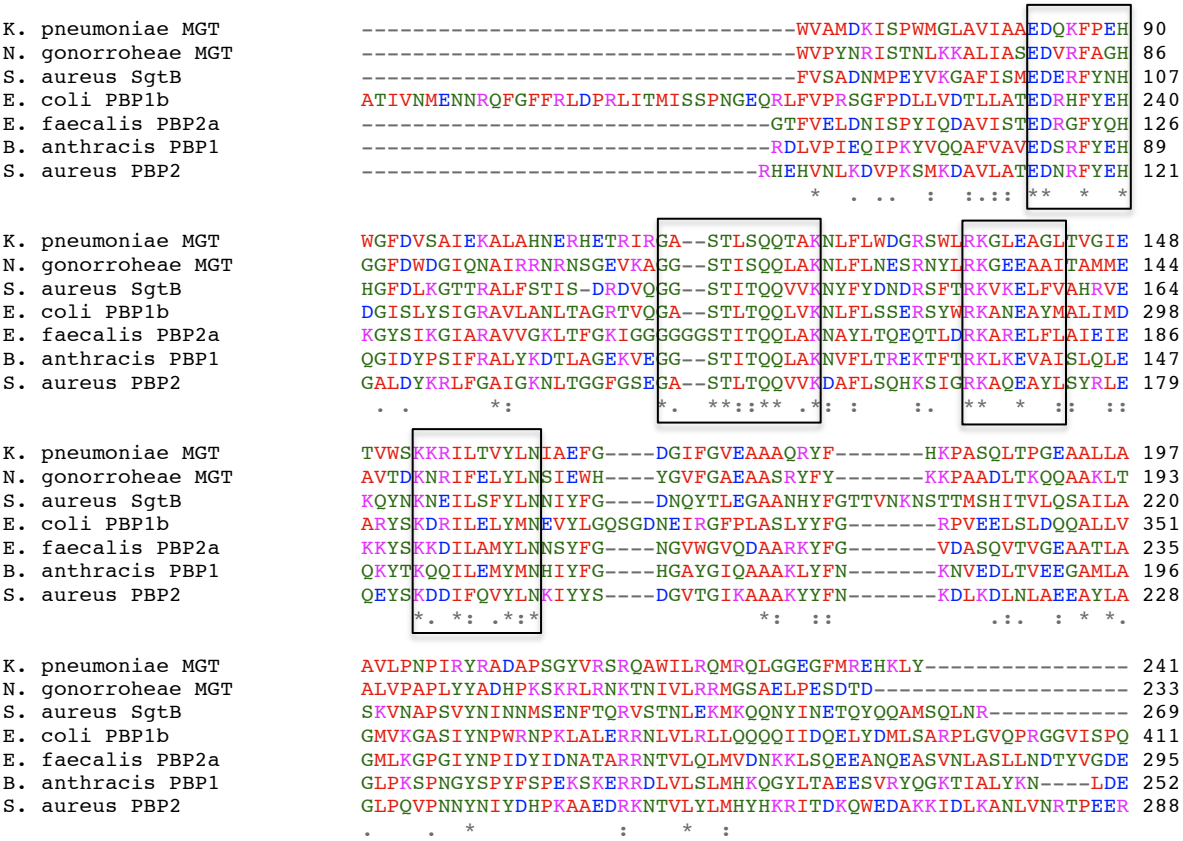


Figure 1. Protein sequence alignment of various PGTs and PBPs. Conserved sequences were identified by sequence alignment using ClustalW2. Invariant amino acids are marked "*", highly conserved are marked ":", similar residues are marked ".". The boxed sequences show the highly conserved glycosyl transferase motive. MGT: monofunctional peptidoglycan glycosyltransferase (PGT); PBP: penicillin binding protein (bifunctional PGT + TP); SgtB: *S. aureus* glycosyltransferase B (PGT)

2. Probe Design

The design of the saccharide part of the probe was based on various co-crystal structures of moenomycin A bound to PGTs. All structures show similar binding contacts of moenomycin (Figure 2). The E- and F-ring as well as the phosphoglycerate strongly participate in a hydrogen bonding network with the PGT active site. Thus, these features of moenomycin needed to be retained in the probe molecule to ensure specific binding to the PGT active site.

The C-ring *N*-acetyl group seemed well suited for attachment of the fluorophore (red arrow, Figure 2). The crystal structures available suggested that a fluorophore in this position could be accommodated, since it would point out of the enzyme binding pocket. Furthermore, we expected limited mobility of the C-ring, since it is held in place with one hydrogen bond to the *N*Ac group, thus restricting free rotation of the fluorophore.

The moenomycin A-, B-, and D-rings do not make significant contacts to the target protein and could thus be omitted in the probe molecule.

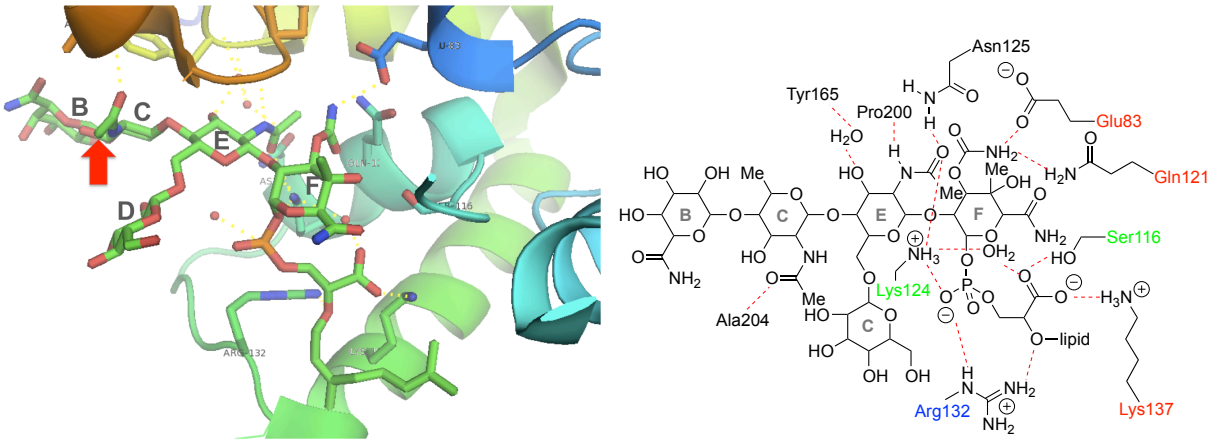


Figure 2. Contacts between moenomycin and PBP1a of *Aquifex aeolicus* (3D3H).[1] A-ring omitted for clarity; dotted lines indicate essential hydrogen bonds to amino acids in the PGT active site (red: invariant; green: highly conserved; blue: strong preference)

[1] Y. Yuan, S. Fuse, B. Ostash, P. Sliz, D. Kahne, S. Walker, *ACS Chem. Biol*, **2008**, *3*, 429-436.

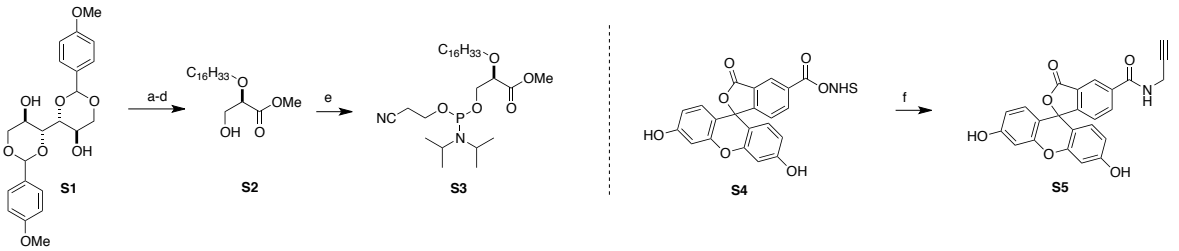
3. Methods and Materials

The following PGT enzymes were expressed and purified as previously described:
- PBP1b of *E. coli* , see ref. 2
- SgtB ΔTM of *S. aureus* (referred to as "SgtB" in the text), see ref. 3
- PBP2a (A68-N728) of *E. faecalis* (referred to as "PBP2a" in the text) see ref. 4
- PBP2 of *S. aureus*, see ref. 5

Moenomycin A (MmA) was isolated from flavomycin feedstock as described in ref. 6
Calf intestinal alkaline phosphatase (CIP, 1000 U, 20 U/μL) was purchased from Roche Diagnostics GmbH, Mannheim, Germany. GalT (Y289L) was generously provided (0.5 mg/mL in TRIS buffer) by Dr. Qasba and Dr. Ramakrishnan at the National Cancer Institute, Frederick, MD, USA.[7] UDP-*N*-azidoacetylgalactosamine was prepared according to a published procedure.[8] Commercial chemicals were used without prior purification. Solvents were dried by passage over columns filled with activated aluminum oxide (Glass Contour Solvent Systems, SG Water USA, Nashua, NH, USA).
MIC determinations were performed at the NERCE CORE A (Microbiology and Animal Resources Core Laboratory, Harvard Medical School) under BSL2+ conditions by Sean Fitzgerald and John Warner following *The Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically;* Approved Standard, 8th Edition; M07-A8; Volume 29, No.2. Clinical and Laboratory Standards Institute, January, 2009.

4. Chemical Syntheses

All reactions in non-aqueous reaction medium were carried out under an atmosphere of argon, unless otherwise noted.



(a) $C_{16}H_{33}Br$, DMF, NaH then: AcOH; (b) $NaIO_4$, THF, H_2O; (c) $NaClO_2$, NaH_2PO_4, H_2O, 2-methyl-2-butene; (d) $TMSCHN_2$, MeOH, THF; (e) $ClP(CEO)N^iPr_2$; (f) propargyl amine, NEt_3, DMF.

Synthesis of S2

Preparation of 2,5-di-*O*-*n*-$C_{16}H_{33}$-D-mannitol:
To a stirred suspension of 60% NaH (3 equiv.), washed twice with petroleum ether, in anhydrous DMF (8 mL/mmol starting material (SM)) was added 1,3:4,6-di-*O*,*O*-(4-methoxybenzylidene)-D-mannitol (1 equiv.) at room temperature. After being stirred for 30 min, the mixture was treated with a 1.2 M solution of n-hexadecanebromide (2.4 equiv.) in anhydrous DMF. The resulting mixture was stirred for 18 h at

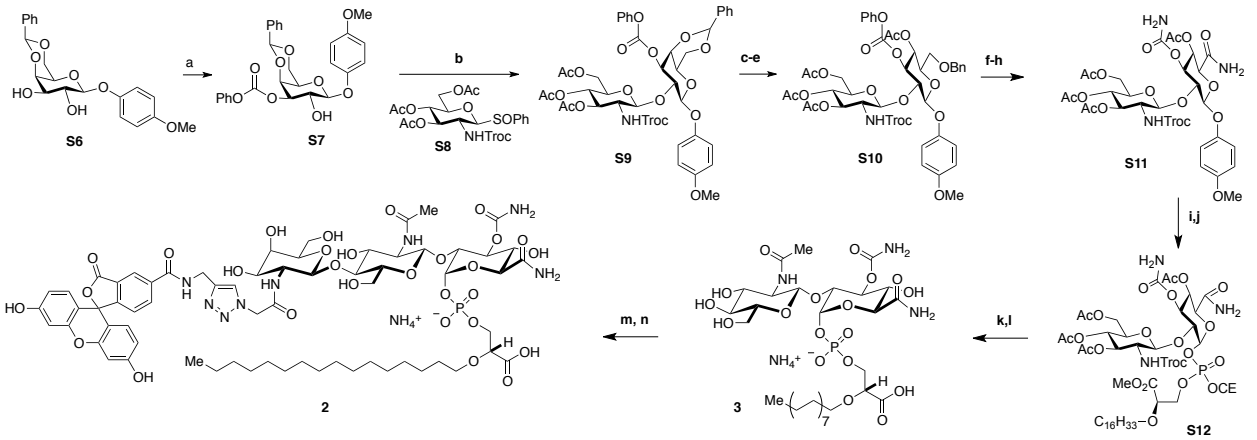
[2] C. Paradis-Bleau, M. Markovski, T. Uehara, T. J. Lupoli, S. Walker, D. E. Kahne, T. G. Bernhardt, *Cell* **2010**, *143*, 1110-1120.
[3] a) T.-S. A. Wang, T. J. Lupoli, Y. Sumida, H. Tsukamoto, Y. Wu, Y. Rebets, D. E. Kahne, S. Walker, *J. Am. Chem. Soc.* **2011**, *133*, 8528-8530; b) Heaslet, H.; Shaw, B.; Mistry, A.; Miller, A. A. *J. Struct. Biol*. **2009**, *167*, 129-135.
[4] T.-S. A. Wang, S. A. Manning, S. Walker, D. Kahne, *J. Am. Chem. Soc*. **2008**, *130*, 14068-14069.
[5] a) Zhang, Y.; Fechter, E. J.; Wang, T. S.; Barrett, D.; Walker, S.; Kahne, D. E. *J. Am. Chem. Soc.* **2007**, *129*, 3080; b) Barrett, D.; Leimkuhler, C.; Chen, L.; Walker, D.; Kahne, D.; Walker, S. *J. Bacteriol.* **2005**, *187*, 2215.
[6] Adachi, M.; Zhang, Y.; Leimkuhler, C.; Sun, B.; LaTour, J.V.; Kahne, D. *J. Am. Chem. Soc.* **2006**, *128*, 14012-14013.
[7] Ramakrishanan *et al. J. Biol Chem*. **2002**, *277*, 20833.
[8] Hang *et al. J. Am. Chem. Soc*. **2003**, *126*, 6.

room temperature and then poured into sat. aq. NH_4Cl (8 mL/mmol-SM). The immiscible mixture was extracted twice with Et_2O and the combined organic phases were washed with water, brine, dried over $MgSO_4$, and then concentrated *in vacuo*. The crude ether taken up in EtOH (12 mL/mmol-SM) and was treated with 3 M aq. HCl (12 equiv.) at room temperature. After being stirred at 70 °C for 3 h, the mixture was cooled to room temperature and basified with 4 M aq. K_2CO_3 (16 equiv.). The immiscible mixture was extracted twice with $CHCl_3$ and the combined organic phases were washed with brine, dried over $MgSO_4$, and then concentrated *in vacuo*. The residue was purified by recrystallization from Et_2O/EtOAc to give 2,5-di-*O*-*n*-$C_{16}H_{33}$-D-mannitol.

Preparation of Methyl 2-*O*-Alkyl-D-Glycerate (**S2**):
To a 5.5 M solution of 2,5-di-*O*-alkyl-D-mannitol (1 equiv., SM) in THF-H_2O (9:1) was added $NaIO_4$ (1.2 equiv.) at room temperature, and the mixture was stirred at 50 °C for 1 h. The resulting inorganic salt was removed by filtration through a pad of silica gel and washed with EtOAc. The filtrate was concentrated *in vacuo* and the crude aldehyde was used for the next reaction.
To a stirred solution of the residue in *t*-BuOH (20 mL/mmol–SM) were added 2-methyl-2-butene (100 equiv.) and a solution of 80% $NaClO_2$ (12 equiv.) and $NaH_2PO_4 \cdot H_2O$ (10 equiv.) in H_2O (8 mL/mmol - SM) at 0 °C successively. The resulting yellow mixture was allowed to warm to room temperature for 6 h, during which it turned into clear. Then, the mixture was cooled to 0 °C again and treated with 2.5 M aq. Na_2SO_3 (25 equiv.) to reduce an excess of $NaClO_2$. The mixture was acidified with 10% aq. citric acid (10 mL/mmol-SM) and extracted twice with $CHCl_3$ and the combined organic phases were washed with brine, dried over $MgSO_4$, and then concentrated *in vacuo*. The crude acid was used for the next reaction without further purification.
To a stirred solution of the residue in anhydrous THF-MeOH (1:1, 10 mL/mmol-SM) was treated with 2 M $TMSCHN_2$ solution in hexanes (3.2 equiv.) at 0 °C. After being stirred for 10 min, the resulting yellow mixture was decolorized by an addition of AcOH (3.2 equiv.) to consume an excess of $TMSCHN_2$. The mixture was concentrated *in vacuo* and the residue was purified by silica gel chromatography (petroleum ether : EtOAc = 4 : 1 to 3 : 2) to give methyl 2-*O*-alkyl-D-glycerate.
^{1}H NMR (500 MHz; $CDCl_3$): δ 3.99 (dd, *J* = 6.1, 3.8 Hz, 1H), 3.79 (d, *J* = 14.1 Hz, 4H), 3.73 (q, *J* = 7.9 Hz, 1H), 3.43 (t, *J* = 11.3 Hz, 1H), 1.65-1.62 (m, 2H), 1.35-1.26 (m, 28H), 0.89 (t, *J* = 7.0 Hz, 3H); **^{13}C NMR** (125 MHz, $CDCl_3$): δ 171.6, 79.8, 71.7, 63.7, 52.3, 32.2, 29.9 (multiple peaks), 29.8, 29.7, 29.6, 26.2, 22.9, 14.4; **HRMS** (ESI) calcd for $C_{20}H_{40}O_4Na^+$ $[M+Na]^+$ 367.2819, found 367.2823.

Preparation of S3 (0.2 M Solution)
To a 0.2 M solution of methyl 2-*O*-alkyl-D-glycerate (1 equiv.) in anhydrous CH_3CN were added *N*,*N*-diisopropylethylamine (1.5 equiv.) and ClP(OCE)N*i*-Pr_2 (1.2 equiv.) at room temperature successively. The reaction mixture was stirred for 1 h and directly used for the next coupling reaction.

Preparation of S5
To as solution of *N*-hydroxysuccinimide fluorescein (**S4**, 15 mg, 21 μmol) in DMF (300 μL) was added NEt_3 (20 μL) and propargyl amine (3.0 mg, 48 μmol). After stirring the solution for 24 h the solvent was removed *in vacuo* and the residue was purified by column chromatography (SiO_2, hexane/EtOAc = 2/8) to obtain **S5** (11 mg, 27 μmol, 84 %) as bright orange solid.
^{1}H NMR (500 MHz, CD_3OD) δ 8.43 (s, 1H), 8.20 (d, *J* = 9.50 Hz, 1H), 7.31 (d, *J* = 8.0 Hz, 1H), 6.69 (s, 1H), 6.61 (d, *J* = 8.5 Hz, 2H), 6.54 (d, *J* = 9.0 Hz, 2H), 4.59 (s, 1H), 4.21 (d, *J* = 2.0 Hz, 1H), 2.65 (d, *J* = 2.5 Hz, 1H); **^{13}C NMR** (500 MHz, CD_3OD) δ 169.4, 166.8, 152.9, 136.2, 134.4, 129.1, 129.0, 124.7, 123.9, 112.6, 112.2, 109.7, 102.5, 79.3, 78.1, 77.8, 71.2, 29.1.



(a) $ClCO_2Ph$, Py. (b) Tf_2O, DTBMP, ADMB, MS-4A, DCM; (c) Et_3SiH, TfOH, MS-4A, DCM; (d) Tf_2O, Py., DCM; (e) CsOAc, 18-Crown-6, PhMe; (f) H_2, 10% Pd-C, 1wt% Cl_3CCO_2H/MeOH; (g) TEMPO, $PhI(OAc)_2$, DCM-H_2O (2:1); (h) $ClCO_2$*i*Bu, NMM, THF then NH_3, *i*-PrOH; (i) CAN, ACN-H_2O (4:1); (j) **S3**, tetrazole, MS-3A, ACN, then *t*-BuO_2H; (k) Zn, Ac_2O, AcOH, THF; (l) LiOH, THF-H_2O_2 (8:1); (m) UDP-GalNAz, β1,4-GalT; (n) DMF, H_2O, $CuSO_4$, Na-ascorbate.

Synthesis of S7

Saccharide **S6** (18.7 g, 49.9 mmol, CAS: 176299-96-0) was dissolved in pyridine (160 mL) and cooled to −40 °C. Phenylchlorocarbonate (11 mL) was added drop wise to the stirred solution. After 2 h, methanol (11 mL) and toluene (100 mL) were added and the solvent was removed in vacuum. The residue was taken up in EtOAc and washed with HCl (1 M) and NaCl (sat.). The organic layer was dried over $MgSO_4$ and the solvent was removed in vacuum. Recrystallization from Et_2O yielded the title compound as colorless solid (18.5 g, 75 %).

^{1}H NMR (500 MHz; $CDCl_3$): δ 7.54 (d, *J* = 7.6 Hz, 2H), 7.37-3.35 (d, J = 1.9 Hz, 5H), 7.22-7.21 (m, 3H), 7.07 (d, J = 9.1 Hz, 2H), 6.83 (d, J = 9.1 Hz, 2H), 5.55 (s, 1H), 4.91 (dd, J = 10.2 Hz, 3.7 Hz, 1H), 4.85 (d, J = 7.8 Hz, 1H), 4.53 (d, J = 3.3 Hz, 1H), 4.34-4.31 (m, 2H), 4.05 (d, J=11.5 Hz, 1H), 3.76 (s, 3H), 3.51 (s, 1H), 2.89 (d, J = 2.8 Hz, 1H); **^{13}C NMR** (125 MHz, $CDCl_3$): δ 155.9, 153.6, 151.3, 137.8, 129.7, 129.4, 128.4, 126.6, 126.4, 122.0, 121.3, 119.5, 114.8, 102.8, 101.1, 73.2, 69.1, 68.6, 66.5, 55.9; **HRMS** (ESI) calcd for $C_{27}H_{26}O_9Na^+$ $[M+Na]^+$ 517.1469, found 517.1506.

Synthesis of S8

Sulfoxide **S8** was obtained by oxidation of peracyl-*N*-Troc-phenyl-(*S*,*O*)-glucosamine (CAS: 187022-49-7; 9.00 g, 15.1 mmol) with Selectfluor (6.00 g, 16.8 mmol) in MeCN (105 mL) and water (10.5 mL) at room temperature. The reaction was carried out in an open flask. After 1 h the solvent was removed in vacuum, and the residue was taken up in chloroform, washed with NaCl (sat.) and dried over $NaSO_4$. After concentration in vacuo the residue was recrystallized from EtOAc/hexane to yield sulfoxide **S8** as an off-white solid (9.10 g, 15.5 mmol, 98 %; 1/1 mixture of diastereomers).

Synthesis of S9

In a 100 mL round bottom flask, gylcosyl donor **S8** (1.50 g, 2.55 mmol), gylcosyl acceptor **S7** (840 mg, 1.70 mmol), 2,6-di-*tert*-butylpyridine (478 mg, 2.50 mmol), and a 4-allyl-1,2-dimethoxybenzene (2.73 g, 15.3 mmol) were combined and dried by azeotropic distillation with benzene. The residue was further dried in vaccum for 30 min before dichloromethane (17 mL) and molecular sieves 3 Å (ca. 500 mg) were added. The suspension was stirred at room temperature for 30 min and then cooled to −78 °C. Triflic anhydride (285 μL, 479 mg, 1.70 mmol) was slowly added and the resulting green solution was stirred for 1.5 h at −78 °C. $NaHCO_3$ (sat., 1 volume) was added, and the mixture was allowed to reach room temperature. The phases were separated, and the organic phase was washed with NaCl (sat.) and dried over Na_2SO_4. Removal of the solvent in vacuum was followed by column chromatography (SiO_2, toluene/EtOAc 8/2 → 7/3) to yield the title compound as colorless solid (910 mg, 0.951 mmol, 56 %).

^{1}H NMR (500 MHz; $CDCl_3$): δ 7.54 (d, *J* = 7.6 Hz, 2H), 7.39-3.37 (d, J = 1.9 Hz, 5H), 7.29-7.26 (m, 3H), 7.02 (d, J = 9.1 Hz, 2H), 6.80 (d, J = 9.1 Hz, 2H), 5.57 (s, 1H), 5.20-5.00 (m, 4H), 5.85-5.78 (m, 3H), 4.59

(br s, 1H), 4.40-4.22 (m, 3H), 4.21-4.19 (m, 1H), 4.16-4.12 (m, 1H), 3.86-3.75 (m, 2H), 3.77 (s, 3H), 3.59 (br s, 1H), 2.01 (s, 3H), 2.01 (s, 3H), 2.00 (s, 3H); **^{13}C NMR** (125 MHz, $CDCl_3$): δ 170.99, 170.88, 169.6, 155.63, 154.34, 152.75, 151.5, 151.2, 137.6, 128.9, 129.5, 128.5, 126.6, 121.3, 121.2, 118.8, 118. 7, 114.7, 101.9, 101.3, 100.9, 76.2, 74.6, 72.9, 72.4, 72.0, 69.1, 68.4, 66.1, 61.8, 55.9, 20.91, 20.86; **HRMS** (ESI) calcd for $C_{42}H_{44}Cl_3NO_{18}Na^+$ $[M+Na]^+$ 978.1516, found 978.1472.

Synthesis of S10

Disaccharide **S9** (1.06 g, 1.11 mmol) was dissolved in dichloromethane (22.2 mL) and $HSiEt_3$ (530 µL, 387 mg, 3.33) and molecular sieves 3 Å (ca. 500 mg) were added. The suspension was stirred for 30 min at room temperature and then cooled to −78 °C before triflic acid (333 µL, 566 mg, 3.77 mmol) was added dropwise. After 2.5 h at −78 °C, $NaHCO_3$ (sat.) was added and the mixture was allowed to reach room temperature. The phases were parted and the aqueous layer was extracted once with dichloromethane. The combined organic layers were washed with brine and dried over Na_2SO_4. Removal of the solvent in vacuum provided the corresponding C6-benzyl ether of **S9** in high purity, which was used in the next step without further purification.

The C6-benzyl ether previously obtained (1.15 g, 1.20 mmol) was dissolved in dichloromethane (12 mL) and pyiridine (290 µL, 284 mg, 3.59 mmol), and the solution was cooled to −40 °C. Triflic anhydride (242 µL, 406 mg, 1.44 mmol) was slowly added and the mixture was allowed to reach room temperature over 2 h. The reaction mixture was washed with 2 volumes of 0.5 M HCl, water, $NaHCO_3$ (sat.), and NaCl (sat), and then dried over Na_2SO_4. The solvent was removed in vacuum and the residue was dissolved in toluene (30 mL), and CsOAc (830 mg, 5.48 mmol) and 18-crown-6 (1.21 g, 4.58 mmol) were added. The resulting mixture was vigorously stirred for 14 h and then washed with $NaHCO_3$ (sat.) and NaCl (sat.). The residue obtained after drying of the solution over Na_2SO_4 and removal of the solvent in vacuum was purified by column chromatography (SiO_2, toluene/EtOAc 85/15) to obtain **S10** as colorless solid (668 mg, 667 µmol, 55 % over 3 steps).

^{1}H NMR (500 MHz; $CDCl_3$): δ 7.41-7.38 (m, 2H), 7.27 (s, 8H), 7.00 (d, J = 9.0 Hz, 2H), 6.80 (d, J = 8.50 Hz, 2H), 5.31 (t, J = 9.6, 1H), 5.20-5.18 (m, 2H), 5.14-5.05 (m, 4H), 5.01 (d, J = 8.1, 1H), 4.68 (d, J = 12.0, 1H), 4.56 (d, J = 11.8, 1H), 4.47 (d, J = 11.9, 1H), 4.27 (d, J = 12.2, 1H), 4.18 (dd, J = 11.9, 3.4, 1H), 4.07 (td, J = 8.2, 1.4, 1H), 3.77 (s, 3H), 3.67-3.60 (m, 3H), 3.59-3.57 (m, 1H), 2.01 (s, 3H), 2.01 (s, 3H), 2.00 (s, 3H), 1.95 (s, 3H); **^{13}C NMR** (125 MHz, $CDCl_3$): δ 170.93, 170.86, 170.11, 169.7, 155.69, 154.21, 153.32, 151.23, 151.12, 138.10, 137.91, 129.84, 129.27, 128.61, 128.46, 128.03, 127.98, 126.55, 125.52, 121.27, 118.12, 114.82, 114.79, 101.14, 100.44, 95.67, 79.60, 78.88, 74.53, 73.81, 73.22, 72.13, 72.00, 69.56, 68.81, 68.46, 61.72, 56.72, 55.91, 21.70, 20.91, 20.85, 20.81; **HRMS** (ESI) calcd for $C_{44}H_{48}Cl_3NO_{19}Na^+$ $[M+Na]^+$ 1022.1779, found 1022.1766.

Synthesis of S11

In a 100 ml round bottom flask, disaccharide **S10** (283 mg, 283 µmol) was dissolved in methanol (20 mL), and 10% Pd/C (100 mg) was added. The atmosphere above the solution was exchanged to H_2, and the solution was stirred vigorously. After 45 min the suspension was filtered through celite and the filtrate was concentrated *in vacuo*. The residue was dissolved in dichloromethane (3.4 mL) and water (1.7 mL), and $PhI(OAc)_2$ (237 mg, 756 µmol) and TEMPO (9.0 mg, 57 µmol) were added. After stirring the mixture for 2 h, the reaction was quenched by addition of $Na_2S_2O_3$ (sat.) and the solution was partitioned between dichloromethane and water. The organic layer was washed with NaCl (sat.) and dried over Na_2SO_4. The solvent was removed *in vacuo*, and the residue was taken up in THF (5.7 mL). This solution was cooled to −40 °C, *N*-methylmorpholine (78 µL, 72 mg, 0.71 mmol) was added and the mixture was treated with isobutyl chloroformate (93 µL, 97 mg, 0.71 mmol). After 5 min, NH_3 (2.0 M in ^{i}PrOH) was added and the mixture was stirred at room temperature for 24 h. Removal of the solvent in vacuum and column chromatography (SiO_2, $CHCl_3$/EtOH 99/1 → 95/5 → 9/1) yielded the title compound as colorless flakes (111 mg, 131 µmol, 46 % over 3 steps).

^{1}H NMR (500 MHz; $CDCl_3$/CD_3OD 9/1): δ 6.92 (d, J = 8.9, 2H), 6.76 (d, J = 9.0, 2H), 5.22 (t, J = 9.9, 1H), 5.16-5.12 (m, 2H), 4.98 (dd, J = 10.0, 2H), 4.90 (d, J = 8.4, 1H), 4.87 (d, J = 12.1, 1H), 4.49 (d, J = 12.1, 1H), 4.09 (dd, J = 12.3, 3.7, 1H), 3.99 (d, J = 8.9, 1H), 3.88 (t, J = 7.1, 1H), 3.78 (d, J = 11.6, 1H),

3.71 (s, 3H), 3.62 (d, J = 9.9, 1H), 3.55-3.48 (m, 5H), 2.00 (s, 3H), 1.94 (s, 3H), 1.93 (s, 3H), 1.92 (s, 3H); **^{13}C NMR** (125 MHz, $CDCl_3/CD_3OD$ 9/1): δ 171.20, 170.92, 170.6, 170.3, 170.0, 156.4, 155.8, 154.8, 150.7, 118.3, 114.9, 101.1, 100.1, 95.8, 79.4, 74.5, 73.9, 72.4, 71.8, 69.6, 68.7, 61.9, 56.5, 55.8, 49.7, 49.5, 49.4, 49.2, 48.9, 48.7, 20.7, 20.7; **HRMS** (ESI) calcd for $C_{31}H_{38}Cl_3N_3O_{18}Na^+$ $[M+Na]^+$ 868.1109, found 868.1067.

Synthesis of S12

Disaccharide **S11** (167 mg, 187 µmol) was dissolved in MeCN (8 mL) and water (2 mL), and cerium(IV) ammonium nitrate (542 mg, 989 µmol) was added. The mixture was stirred at room temperature for 1.5 h and then concentrated *in vacuo*. Purification of the residue by column chromatography (SiO_2, $CHCl_3$/EtOH 9/1 -> 4/1) gave the lactol of **S11** as colorless solid (115 mg, 79 %). This lactol (42.9 mg, 57.9 µmol) was further dried by azeotropic distillation with toluene (2 x), dissolved in tetrazole solution (0.34 M in MeCN, 1.0 mL), and stirred with molecular sieves 3 Å for 30 min at room temperature and 30 min at 0 °C. A solution of **S3** (0.2 M in MeCN, 0.58 mL) was added and the mixture was stirred at 0 °C for 2 h before ^{t}BuOOH (5.5 M in decane, 127 µL, 699 µmol) was added. After 1 h at 0 °C $P(OMe)_3$ (82 µL, 86 mg, 695 µmol) was added and the suspension was filtered through a pad of celite. Evaporation of the solvent *in vacuo* and column chromatographic purification (C18, gradient 30-100% MeCN/H_2O) of the residue gave phosphoglycerate **S12** (32.5 mg, 27.1 µmol, 47 %) as colorless solid as a 1/1 mixture of phosphate diastereomers.

analytical data for one diastereomer: **^{1}H NMR** (500 MHz; CD_3OD): δ 6.03 (dd, J = 6.3, 3.2, 1H), 5.27-5.20 (m, 3H), 5.07 (t, J = 9.7, 1H), 5.02 (d, J = 12.3, 1H), 4.78 (d, J = 8.5, 1H), 4.61 (d, J = 12.3, 1H), 4.46-4.42 (m, 4H), 4.45-4.41 (m, 4H), 4.22 (dd, J = 12.3, 2.2, 1H), 4.00 (dd, J = 6.9, 3.3, 1H), 3.87-3.83 (m, 2H), 3.82 (s, 3H), 3.72-3.65 (m, 2H), 3.59-3.55 (m, 1H), 3.01 (t, J = 6.0, 2H), 2.10 (s, 3H), 2.04 (s, 3H), 2.01 (s, 3H), 1.96 (s, 3H), 1.66 (t, J = 7.2, 2H), 1.30 (s, 28H), 0.91 (t, J = 7.0, 3H); **HSQC** (^{13}C signals, 125 MHz, CD_3OD): δ 102.7, 97.2; 77.9, 77.6, 74.2, 74.1, 72.2, 72.0, 71.5, 71.4, 70.4, 70.3, 69.5, 68.8, 68.2, 68.1, 63.6, 61.9, 61.8, 55.6, 51.8, 48.1, 31.6, 29.6 (multiple peaks), 25.8, 22. 5, 19.6, 19.3, 19.3, 19.3, 18.9, 13.2; **^{31}P NMR** (162 MHz; CD_3OD): δ –3.16; **HRMS** (ESI) calcd for $C_{47}H_{74}Cl_3N_4O_{23}PNa^+$ $[M+Na]^+$ 1223.3410, found 1223.3337.

Synthesis of 3

To a solution of phosphoglycerate **S12** (113 mg, 94.2 µmol) in THF (3 mL), Ac_2O (1 mL), and AcOH (2 mL) was added activated Zn (653 mg, 9.99 mmol), and the solution was stirred at room temperature for 10 h. The slurry was filtered through a pad of SiO_2 and the residue was thoroughly washed with $CHCl_3$/EtOH 2/1 (100 mL). The filtrate was concentrated, and the residue was dissolved in THF (19 mL), H_2O_2 (30 %, 4.8 mL) LiOH solution (1 M, 1.9 mL). After stirring the solution at 0 °C for 2 h, DOWEX50WX2-100 resin (HPy+ form, 0.8 g) was added, and the mixture was stirred for 2 h. The resin was filtered off by passing the reaction mixture through a cotton plug. Chromatographic purification (C18, gradient 20-50% MeCN in 0.2% NH_4HCO_3) of the residue obtained after concentration of the filtrate yielded disaccharide **3** (50.7 mg, 61.0 µmol, 63 %).

HPLC/MS retention time: 11.9 min (Phenomenex Luna, 3µ-C18 50 x 2 mm^2 3 micron, 0.3 mL/min, gradient 30-75% MeCN+0.1% HCO_2H in H_2O+0.1% HCO_2H over 6 min, then to 99% MeCN+0.1% HCO_2H over 5 min) **LRMS** (ESI) calcd for $C_{34}H_{61}N_3O_{18}P^-$ $[M–H]^-$ 830.4, found 830.3; **^{1}H NMR** (500 MHz; CD_3OD): δ 5.98 (dd, J = 7.1, 3.1, 1H), 5.05 (t, J = 9.7, 1H), 4.58 (d, J = 8.4, 1H), 4.36 (d, J = 10.0, 1H), 4.26-4.22 (m, 1H), 4.18-4.08 (m, 1H), 4.00-3.96 (m, 1H), 3.87-3.84 (m, 1H), 3.76-3.71 (m, 3H), 3.71 (s, 3H), 3.68-3.64 (m, 3H), 3.53-3.47 (m, 3H), 3.36-3.29 (m, 4H), 2.04 (s, 3H), 1.66-1.64 (m, 2H), 1.30 (s, 28H), 0.91 (t, J = 7.0, 3H); **^{13}C NMR** (125 MHz, D_6-DMSO): δ; 173.8, 171.3, 170.6, 157.3, 103.1, 94.9, 80.9, 80.2, 77.6, 74.6, 73.0, 73.0, 72.9, 72.8, 72.0, 72.0, 71.1, 66.4, 61.1, 56.1, 55.6, 32.0, 30.0, 29.8, 29.7, 26.4, 26.3, 23.7, 22.7, 14.6; **^{31}P NMR** (162 MHz; D_6-DMSO): δ –2.31; **HRMS** (ESI) calcd for $C_{34}H_{63}N_3O_{18}P^+$ $[M+H]^+$ 832.3839, found 832.7735.

Synthesis of 2

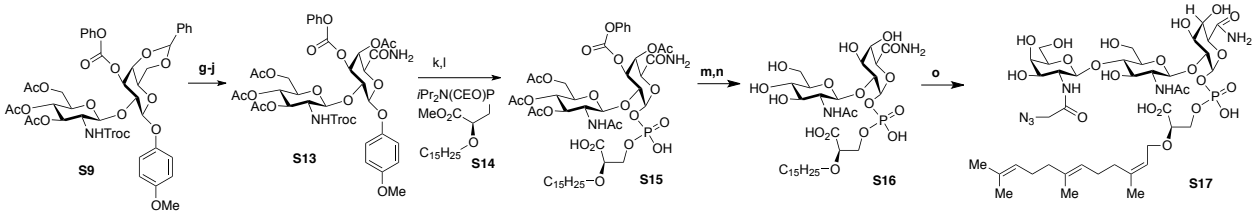
To a solution of TRIS (50 mM in H_2O, pH = 8.0, 2.5 mL) were sequentially added $MnCl_2$ (50 mM in H_2O, 2.5 mL), H_2O (5 mL), GalT Y289L (1 mg/mL in 50 mM TRIS buffer, pH = 8.0, 1.88 mL,), UDP-*N*-azidoacetylgalactosamine (UDP-Gal*N*Az, 20 mM in H_2O, 500 µL), disaccharide **3** (10 mM in H_2O, 125 µL), and calf intestinal alkaline phosphatase (CIP, 1000 U, 25 µL). The mixture was gently mixed and kept at 37 °C for 60 h. MeOH (7.5 mL) was added, and the mixture was vortexed and centrifuged (15 min at 5000 x g) to pellet precipitated proteins. The supernatant was concentrated *in vacuo*, and the residue obtained was loaded onto a Phenomenex Strata C-18 column, preequilibrated with H_2O. The column was eluted with H_2O to obtain unreacted UDP-Gal*N*Az. Elusion with H_2O/MeOH 1/9 provided the desired trisaccharide (**4**) in near quantitative yield.

HPLC retention time: 11.0 min (Phenomenex Luna, 3µ-C18 50 x 2 mm^2 3 micron, 0.3 mL/min, gradient 30-75% MeCN+0.1% HCO_2H in H_2O+0.1% HCO_2H over 6 min, then to 99% MeCN+0.1% HCO_2H over 5 min) **LRMS** (ESI) calcd for $C_{42}H_{73}N_7O_{23}P^-$ $[M–H]^-$ 1074.5, found 1074.3.

The Gal*N*Az-trisaccharide previously obtained (**4**, 4.0 mg, 3.8 µmol) was dissolved in DMF (400 µL) and $CuSO_4$ (0.9 M in H_2O, 4.0 µL, 3.6 µmol) and Na-ascorbate (1.8 M in H_2O, 4.0 µL, 7.2 µmol) were added. The mixture was stirred at room temperature, and after 24 h another portion of $CuSO_4$ (0.9 M in H_2O, 4.0 µL, 3.6 µmol) and Na-ascorbate (1.8 M in H_2O, 4.0 µL, 7.2 µmol) was added. After 48 the solution was concentrated *in vacuo* and the residue was purified by colum chromatography (C-18, gradient 30-90% MeOH in H_2O; then 10 % 2M NH_3 in MeOH to elute the product) to obtain the title compound (4.9 mg, 3.3 µmol, 87 %).

HPLC/MS retention time: 10.8 min (Phenomenex Luna, 3µ-C18 50 x 2 mm^2 3 micron, 0.3 mL/min, gradient 30-75% MeCN+0.1% HCO_2H in H_2O+0.1% HCO_2H over 6 min, then to 99% MeCN+0.1% HCO_2H over 5 min); **LRMS** (ESI) calcd for $C_{66}H_{88}N_8O_{29}P^-$ $[M–H]^-$ 1487.5, found 1487.3; **^{1}H NMR** (600 MHz; CD_3OD): δ 8.51 (s, 1H), 8.28-8.27 (m, 1H), 8.11 (s, 1H), 7.35 (d, J = 7.5, 1H), 6.72 (m, 2H), 6.65 (d, J = 8.5, 2H), 6.60-6.59 (m, 2H), 6.01 (br s, 1H), 5.29 (br s, 1H), 5.05 (t, J = 6.8, 1H), 4.77 (m, 2H), 4.61-4.56 (m, 2H), 4.38 (d, J = 7.5, 1H), 4.29-4.25 (m, 2H), 4.15-4.05 (m, 2H), 4.05-3.99 (m, 2H), 3.86-3.76 (m, 4H), 3.76-3.60 (m, 13H), 3.48 (br s, 1H), 2.02 (s, 3H), 1.65-1.64 (m, 6H), 1.48-1.42 (m, 12H), 1.31 (m, 38H), 0.92 (t, J = 5.4, 3H); **HSQC** (^{13}C signals, 125 MHz, CD_3OD): δ 129.1, 124.1, 112.9, 102.4, 102.3, 79.5, 75.8, 75.0, 73.3, 73.0, 71.5, 71.2, 70.5, 70.0, 68.3, 67.0, 61.2, 53.4, 52.0, 35.1, 31.8, 29.5, 26.0, 23.5, 22.6, 21.9, 13.1; **HRMS** (ESI) calcd for $C_{66}H_{90}N_8O_{29}P^+$ $[M+H]^+$ 1489.5546, found 1489.5420.

Synthesis of the model substrate for GalT-mediated Gal*N*Az transfer



(g) Et_3SiH, triflic acid, then: Ac_2O, Py., cat. DMAP, DCM; (h) H_2, 10% Pd-C, 1wt% Cl_3CCO_2H/MeOH; (i) TEMPO, $PhI(OAc)_2$, DCM-H_2O (2:1); (j) $ClCO_2iBu$, NMM, THF then NH_3, *i*-PrOH; (k) CAN, ACN-H_2O (4:1); (l) **S14**, tetrazole, MS-3A, ACN, then *t*-BuO_2H; (m) Zn, Ac_2O, AcOH, THF; (n) LiOH, THF-MeOH-H_2O (3:3:1); (o) UDP-GalNAz, β1,4-GalT;

Synthesis of S13

Disaccharide **S9** (650 mg, 679 µmol) was dissolved in dichloromethane (13.6 mL) and $HSiEt_3$ (325 µL, 237 mg, 2.04 mmol) and molecular sieves 3 Å (ca. 250 mg) were added. The suspension was stirred for 30 min at room temperature and then cooled to -78 °C before triflic acid (204 µL, 347 mg, 2.31 mmol) was added dropwise. After 2.5 h at -78 °C, $NaHCO_3$ (sat.) was added and the mixture was allowed to reach room temperature. The phases were parted and the aqueous layer was extracted once with dichloromethane. The combined organic layers were washed with brine and dried over Na_2SO_4. The solvent was removed *in vacuo* and the residue was dissolved in dichloromethane (3.4 mL), and pyridine (164 µL, 161 mg, 2.04 mmol), DMAP (8.3 mg, 0.07 mmol), and Ac_2O (94 µL, 104 mg, 1.02 mmol) were added. After 3 h at room temperature the reaction was diluted with dichloromethane and washed with HCl

(1 M), H_2O, $NaHCO_3$ (sat.), and NaCl (sat.). The organic layers were dried over Na_2SO_4 and then concentrated *in vacuo*. Purification of the residue yielded the C4-OAc, C6-OBn derivative of **S9** (539 mg, 538 µmol, 79 % over 2 steps) as colorless solid.
In a 10 mL roundbottom flask the product obtained in the previous step (56.4 mg, 56.3 µmol) was dissolved in a solution of 1% trichloroacetic acid in methanol (2.8 mL, 2.4 equiv. of TCA), 10% Pd/C (11.9 mg) was added and the atmosphere above the solution was exchanged to H_2. After stirring for 15 min the solution was filtered through a pad of Celite and poured into $NaHCO_3$ (sat.). The mixture was extracted with EtOAc (2 x), washed with NaCl (sat.), and dried over $MgSO_4$. Evaporation of the solvent in vacuum yielded the free C6-alcohol in quantitative yield.
The alcohol obtained (51.3 mg, 56.3 µmol) was dissolved in dichloromethane (0.2 mL) and water (0.1 mL). After addition of TEMPO (1.8 mg, 1.2 µmol) and diacetoxy iodobenzene (45.0 mg, 140 µmol) the mixture was stirred at room temperature for 1.5 h. $Na_2S_2O_3$ (sat.) solution was added, and the reaction mixture was extracted with EtOAc (2 x 3 mL). The combined organic layers were washed with NaCl (sat.) and dried over $MgSO_4$. The residue obtained after concentration of the solution in vacuum was purified by column chromatography (SiO_2, petrol ether/EtOAc/1% AcOH 2/1 → 1/4) to obtain pure C6-carboxylic acid (36.8 mg, 39.8 µmol, 71% over 2 steps).
The C6-carboxylic acid (22.0 mg, 23.8 µmol) was dissolved in THF (0.6 mL) and *N*-methyl-morpholine (5.2 µL, 47 µmol), and the solution was cooled to -30 °C before isobutylchloroformate (6.2 µL, 47 µmol) was added. After 30 min the turbid mixture was treated with 7M NH_3 solution in MeOH (14 µL) and stirred at 0 °C for another 30 min. The mixture was poured into NH_4Cl and extracted with EtOAc (2x). The organic layers were washed with NaCl (sat.) and dried over $MgSO_4$ before they were concentrated *in vacuo*. Column chromatographic purification of the residue (SiO_2, petroleum ether/EtOAc 2/1 → 1/4) gave **S13** as colorless solid (21.8 mg, 23.6 µmol, 99%).

Synthesis of S15
Deprotection of the PMP group was achieved by treatment of a solution of **S13** (21.8 mg, 23.8 µmol) in MeCN (1.2 mL) and water (0.3 mL) with cerium (IV) ammonium nitrate (40.2 mg, 73.3 µmol). After stirring at room temperature for 1 h the mixture was concentrated *in vacuo*, and the residue was purified by column chromatography (SiO_2, $CHCl_3$/MeOH 98/2 → 95/5) to give the free lactol as colorless solid (12.8 mg, 15.6 µmol, 66%). This material was dried by azeotropic distillation with toluene (2 x), dissolved in a solution of tetrazole in MeCN (0.34 M, 0.28 mL), and molecular sieves 3 Å (43 mg) were added. The mixture was stirred at room temperature for 15 min and then cooled to 0 °C before a solution of **S14** was added (0.2 M in MeCN, 0.15 mL). After 1 h at 0 °C, ^{t}BuOOH (5.5 M in decane, 23 µL) was added, and the suspension was stirred for another hour before $P(OMe)_3$ (22 µL, 186 µmol) was added. The mixture was filtered over Celite, and the residue was concentrated *in vacuo*. Purification of the residue by column chromatography (SiO_2, $CHCl_3$/MeOH 97/3 → 96/4) gave **S15** as colorless solid as a mixture of phosphate diastereomers (16.0 mg, 12.7 µmol, 81%).

Synthesis of S16
Phosphoglycerate **S15** (7.6 mg, 6.0 µmol) was dissolved in a mixture of THF (0.3 mL), Ac_2O (0.1 mL), and AcOH (0.2 mL) and activated zinc (70.1 mg) was added in portions over the course of 1.5 d. The suspension was filtered through a pad of Celite, concentrated *in vacuo*, and subjected to column chromatography (SiO_2, $CHCl_3$/MeOH 96/4). The product obtained was dissolved in a mixture of THF (0.66 mL), MeOH (0.22 mL), and H_2O (0.22 mL) and LiOH (1 M in H_2O, 66 µL, 66 µmol) was added. After stirring at room temperature for 1.5 h, AcOH (4 µL, 7 µmol) was added, and the solution was concentrated *in vacuo*. Purification of the residue by column chromatography (C18, gradient 25-75% MeOH in H_2O + 0.1 % AcOH) gave **S16** (3.3 mg, 4.3 µmol, 71 % over 2 steps) as colorless solid.

Synthesis of S17
To a solution (2 mL) containing HEPES (50 mM, pH = 7.5), NaCl (100 mM), $MnCl_2$ (1 mM), **S20** (1 mM), UDP-*N*-azidoacetylgalactosamine (UDP-Gal*N*Az,[8] 2 mM) were added calf intestinal alkaline phosphatase (1000 U, 5 µL) and GalT Y289L, and the mixture was incubated at 37 °C for 2 h. The

reaction was quenched by precipitation of the proteins by addition of MeOH (4 mL), was centrifuged, and the supernatant was passed over a 30 mg Strata-X C18 column (Phenomenex). The column was eluted with water (2 mL) to rinse off salts, UMP, and UDP-Gal*N*Az, and the desired trisaccharide **S17** was eluted with MeOH/H_2O 8/2.

5. FP-Assay Development and Screening Protocol

Enzyme titration with probe 2

Solution containing 10 mM TRIS (pH = 8.0), 100 mM NaCl, 0.3% DMSO, 75 nM **2**, and PGTs (2.2 µM *E. coli* PBP1b; 5.1 µM *E. faecalis* PBP2a; 2.1 µM *S. aureus* SgtB) were allowed to equilibrate for 30 min at 0 °C and were then serially diluted (1/1 dilutions) into buffer containing 10 mM TRIS (pH = 8.0), 100 mM NaCl, 0.3% DMSO, and 75 nM **2**. After equilibration at 0 °C for 30 min, 20 µL of the solutions were transferred to a black 384 well plate (Corning NBS Low Volume) and fluorescence polarization (FP, ex: 480 nm; em: 535 nm) was measured using a Perkin Elmer Envision microplate reader. Each series was performed in duplicate and the data was plotted FP vs. concentration of enzyme. For determination of the K_D, the average FP values were converted to fluorescence anisotropy and fitted to the standard equation describing an equilibrium L + E <-> LE (L = ligand; E = enzyme; LE = ligand enzyme complex) using GraphPad Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA, USA; Figure 1).

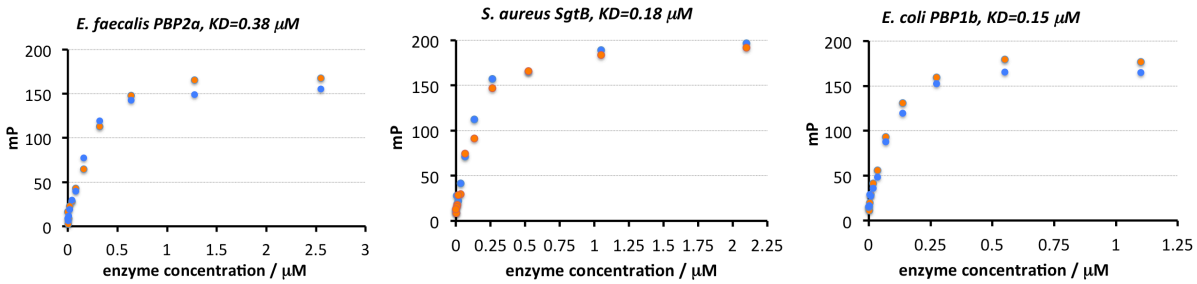


Figure 3: **Titration of the probe compound with PGTs in duplicate.** 75 nM **2**, 10 mM TRIS pH=8, 100 mM NaCl, 20 µl volume.

Validation of the assay

To equilibrated solutions containing 10 mM TRIS (pH = 8.0), 100 mM NaCl, 75 nM **2**, and PGTs (0.1-0.15 µM *E. coli* PBP1b; 0.38-0.46 µM *E. faecalis* PBP2a; 0.2-0.25 µM *S. aureus* SgtB) was added the test compound in DMSO or DMSO/H_2O solutions (stock solutions were typically 2 mM) to obtain a final concentration of test compound of ca. 200 µM. These solutions were serially diluted (1/1 dilutions) into buffer containing 10 mM TRIS (pH = 8.0), 100 mM NaCl, 75 nM **2**, and PGTs (same conc. as above). After equilibration at 0 °C for 30 min, 20 µL of the solutions were transferred to a black 384 well plate (Corning NBS Low Volume) and fluorescence polarization (FP, ex: 480 nm; em: 535 nm) was measured using a Perkin Elmer EnVision microplate reader. Each series was performed in duplicate and the data was plotted FP vs. concentration of test compound.

For determination of the K_i, the average FP values were first converted to fluorescence anisotropy. Using GraphPad Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA, USA), this data was fitted to the equation describing the competition for two ligands for a common binding site:

$$\frac{[RL]}{[R]} = \frac{1}{1 + \frac{K_D}{[L]}\left(1 + \frac{[A]}{K_i}\right)}$$

[RL]: conc. of receptor-ligand complex; [A]: conc. of test compound; [L]: conc. of probe = 75 nM; K_D: dissociation constant for the probe compound (determined above)

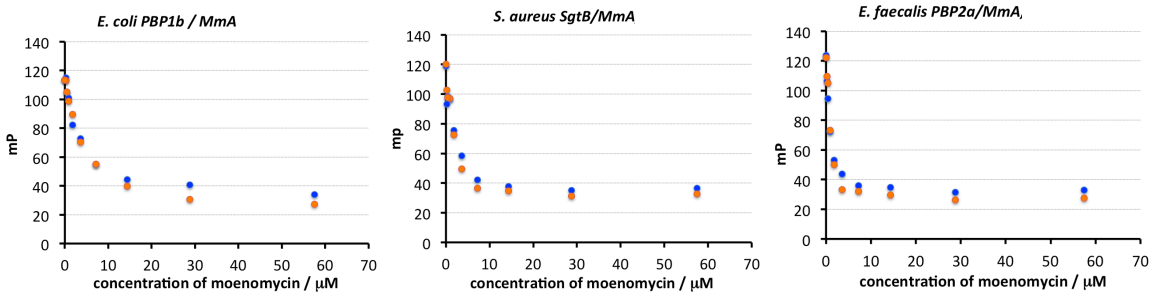


Figure 4: **Displacement of the probe from PGTs by addition of moenomycin A in duplicate.** 75 nM **2**, 10 mM TRIS pH=8, 100 mM NaCl, *E. coli* PBP1b: 0.1 µM, *E. faecalis* PBP2a: 0.4 µM, *S. aureus* ΔTM SgtB: 0.2 µM; K_i (Moenomycin/*E.coli* PBP1b) = 1.88 µM; K_i (Moenomycin/*S. aureus* SgtB) = 0.64 µM; K_i (Moenomycin/*E. faecalis* PBP2a) = 0.47 µM.

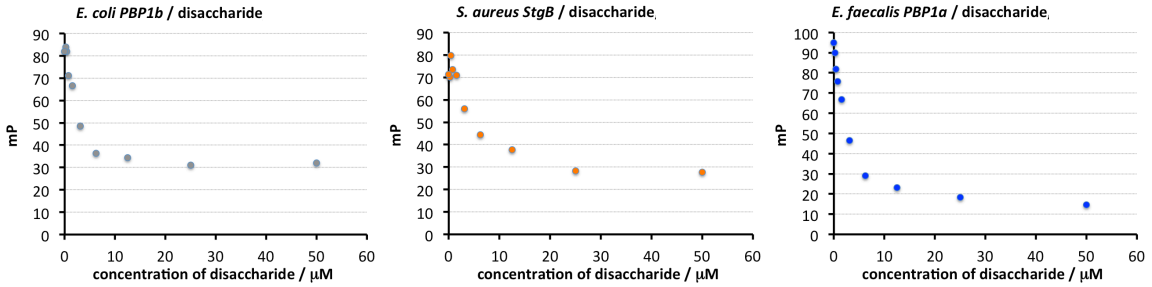


Figure 5: Displacement of the probe from PGTs by addition of disaccharide 3. 75 nM **2**, 10 mM TRIS pH=8, 100 mM NaCl, *E. coli* PBP1b: 0.11 µM, *E. faecalis* PBP2a: 0.38 µM, *S. aureus* ΔTM SgtB: 0.2 µM; K_i (disaccharide **3**/*E.coli* PBP1b) = 0.95 µM; K_i (disaccharide **3**/*S. aureus* SgtB) = 3.17 µM; K_i (disaccharide **3**/*E. faecalis* PBP2a) = 1.73 µM.

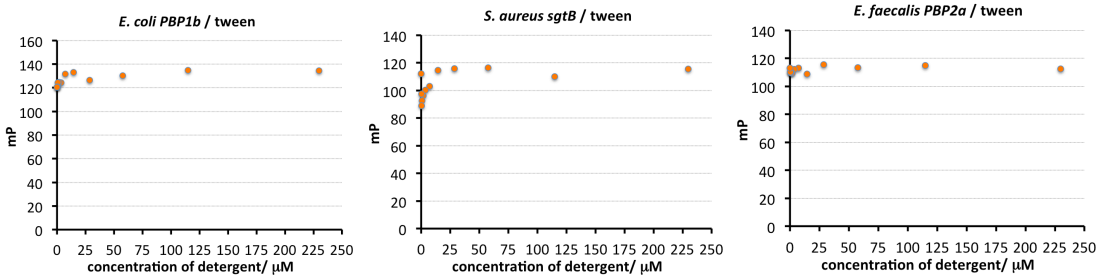


Figure 6: **Treatment of the probe-PGT complex with tween-20.** 75 nM **2**, 10 mM TRIS pH=8, 100 mM NaCl, *E. coli* PBP1b: 0.2 µM, *E. faecalis* PBP2a: 0.46 µM, *S. aureus* ΔTM SgtB: 0.25 µM.

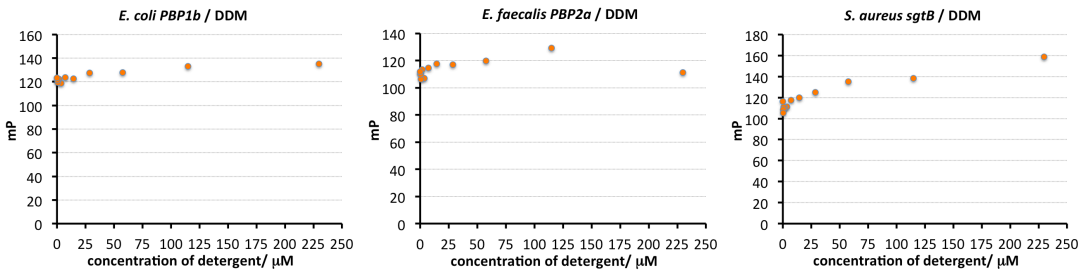


Figure 7: **Treatment of the probe-PGT complex with dodecylmaltoside.** 75 nM **2**, 10 mM TRIS pH=8, 100 mM NaCl, *E. coli* PBP1b: 0.2 µM, *E. faecalis* PBP2a: 0.46 µM, *S. aureus* ΔTM SgtB: 0.25 µM.

Determination of Z'-value[9]

384 well plates:

Using a Matrix WellMate, a black 384 well plate (Corning NBS Low Volume No. 3820) was filled (10 μL per well) with equilibrated solutions containing 10 mM TRIS (pH = 8.0), 100 mM NaCl, 75 nM **3**, and PGTs (0.2 μM *E. coli* PBP1b; 0.46 μM *E. faecalis* PBP2a; 0.25 μM *S. aureus* SgtB). From a second 384 well plate, filled half with DMSO and half with 10 mM disaccharide **3**, 100 nL were transferred to the test plate by pin transfer. After 10 min at room temperature fluorescence polarization (FP, ex: 480 nm; em: 535 nm) was measured using a Perkin Elmer EnVision microplate reader. The following Z'-values were obtained and were stable over a period of at least 30 min:

E. coli PBP1b: 0.70
E. faecalis PBP2a: 0.58
S. aureus SgtB: 0.64

1536 well plates:

Using a Thermo MultiDrop Combi nL, a black 1536 well plate (Greiner Low Volume, No. 782076) was filled (well volume indicated below) with equilibrated solutions containing 10 mM TRIS (pH = 8.0), 100 mM NaCl, 75 nM **3**, and 0.25 μM *S. aureus* ΔTM SgtB). From a 384 well plate, filled half with DMSO and half with 100 mM moenomycin A, 33 nL were transferred to the test plate by pin transfer. After 30 min at 4 °C fluorescence polarization (FP, ex: 480 nm; em: 535 nm) was measured using a Perkin Elmer EnVision microplate reader. The following Z'-values were obtained and were stable over a period of at least 2 h:

3 μL well volume: 0.72
5 μL well volume: 0.79

Protocol for high-throughput screening

Assay solutions consisted of: 1.0-1.5 μM *S. aureus* ΔTM SgtB (depending on protein batch), 75 nM probe **2**, 10 mM Tris pH 8.0, 100 mM NaCl. Note that, due to presence of 8 mM CHAPS detergent in the buffer of the *S. aureus* SgtB stock, the final assay solution contains 100-160 μM CHAPS. The assay was carried out in 384-well plates (Corning 3820) dispensing 10 μL of assay solution per well, followed by pin transfer of 100 nL of each experimental compound from library plates by a stainless steel pin array. The assay was subsequently adapted to 1536-well plates (Greiner 782076), which were filled with 3 μL assay solution per well, followed by a 33 nL pin transfer of experimental compounds. The final concentration of pertubator was ca. 100 μM. A solution containing 1.0-1.5 μM *S. aureus* ΔTM SgtB (depending on protein batch), 75 nM probe, 10 mM Tris pH 8.0, 100 mM NaCl and 20 μM moenomycin A was used as positive control. All wells in row 24 (384-well plate) or rows 47 and 48 (1536-well plate) were filled with 10 μL (384-well plate) or 3 μL (384-well plate) of this solution, respectively. Assay plates were incubated for 30 minutes at 4 °C after the addition of experimental compounds and then read on a PerkinElmer EnVision microplate reader (Excitation: 480 nm, Emission: 535 nm). Library plates were screened in duplicate, with both assay plates in a given set prepared on the same day.

For each plate, an adjusted FP threshold value was calculated using the formula: [plate average negative control FP - 0.9 * (average negative control FP - average positive control FP)]. Wells were considered positive if FP for both replicates was < threshold value (10% of the adjusted plate average negative control FP) and fluorescence intensity was below detector saturation.

Protocol for secondary assay

A black 384 well plate (Corning NBS Low Volume No. 3820) was filled (10 μL per well) with an equilibrated solution containing 10 mM TRIS (pH = 8.0), 100 mM NaCl, 75 nM **3**, and 1.0-1.5 μM *S. aureus* SgtB (depending on protein batch). Note that, due to presence of 8 mM CHAPS detergent in the

[9] Zhang et al. *J. Biomol. Screen* **1999**, *4*, 67-73.

buffer of the *S. aureus* SgtB stock, the final assay solution contains 100-160 µM CHAPS. Using an HP D300 Digital Dispenser, for each compound a 1/1 dilution series (12 wells) of the primary hit compound in DMSO (normalized to 1 µL with DMSO) was prepared and added to the aforementioned assay solution. The plate was incubated at 4 °C for 30 min and read with an Perkin Elmer EnVision microplate reader as described above. The dilution series was performed in duplicate.
For determination of the K_i, the average FP values were first converted to fluorescence anisotropy. Using GraphPad Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA, USA), this data was fitted to the equation describing the competition for two ligands for a common binding site:

$$\frac{[RL]}{[R]} = \frac{1}{1 + \frac{K_D}{[L]}\left(1 + \frac{[A]}{K_i}\right)}$$

[RL]: conc. of receptor-ligand complex; [A]: conc. of test compound; [L]: conc. of probe = 75 nM; K_D: dissociation constant for the probe compound (determined above)

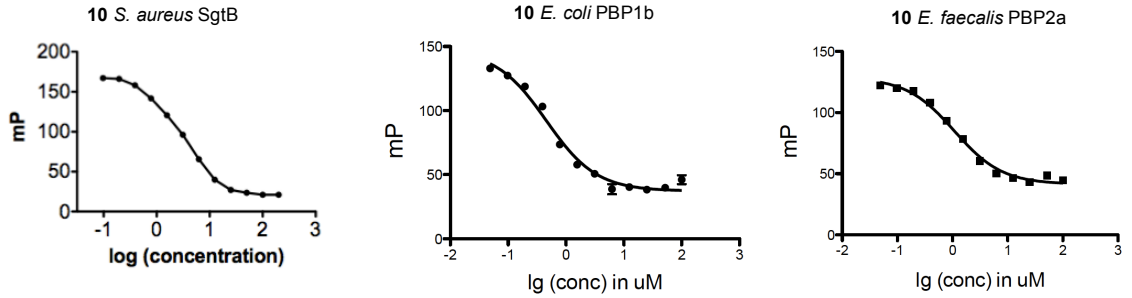


Figure 8: **Treatment of the probe-PGT complex with 10.** 75 nM **2**, 10 mM TRIS pH=8, 100 mM NaCl, *S. aureus* ΔTM SgtB: 1.5 µM; *E. coli* PBP1b: 0.05 µM; *E. faecalis* PBP2a: 0.5 µM; K_i (**10** *S. aureus* SgtB) = 2.6 µM, K_i (**10** *E. coli* PBP1b) = 94 nM, K_i (**10** *E. faecalis* PBP2a) = 0.90 µM.

<u>Aggregation assay with hit compound 10</u>

Compounds that aggregate in solution can non-specifically denature the target protein. This would result in displacement of the probe and the compound would be scored as false positive in the assay. In order to assess whether hit compound **10** is aggregating in the assay solution, we determined its IC_{50} for probe displacement in assay solutions containing detergents that would break up the aggregates. (Note that, due to presence of 8 mM CHAPS detergent in the buffer of the *S. aureus* SgtB stock, the primary assay solution already contains 100-160 µM CHAPS.) The assay solution was prepared as described above and 0.01 % triton X reduced, 2 mM CHAPS, and 0.2 mM CHAPS were added. The IC_{50} values measured for probe displacement did not vary significantly, indicating that **10** is aggregating in solution.

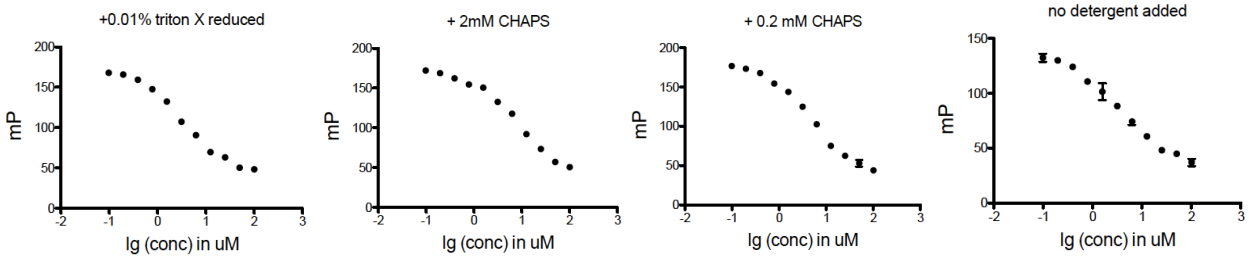


Figure 9: **Treatment of the probe-PGT complex with 10 in the presence of additional detergent.** 75 nM **2**, 10 mM TRIS pH=8, 100 mM NaCl, 1.5 µM *S. aureus* ΔTM SgtB; IC_{50} (**10** +0.01% triton X reduced) = 3.3 µM, IC_{50} (**10** + 2 mM CHAPS) = 8.5 µM, IC_{50} (**10** + 0.2 mM CHAPS) = 4.9 µM; IC_{50} (**10** + no detergent added) = 3.5 µM.

6. *In vitro* PGT Inhibition Assays

The PGT-inhibitor assay was carried out as described previously.[10]

In vitro inhibition of *S. aureus* SgtB:
A solutions of *S. aureus* SgtB (50 nM) in 12.5 mM HEPES (pH = 7.5), 2 mM $MnCl_2$, and 250 μM tween-80 (8 μL) were incubated with DMSO solutions containing the inhibitor of interest in different concentrations (1 μL) for 20 min. Then ^{14}C-labelled lipid II (1 μL, 40 μM, $^{14}C/^{12}C$ 1/3) was added and the polymerization reaction was allowed to proceed for 25 min at room temperature. The reaction was quenched with 10 μL of a solution of moenomycin (1 μM) in 10% triton-X reduced and the remaining lipid II was separated from peptidoglycan (PG) using paper strip chromatography (isobutyric acid / 1M NH_4OH 5/3). Using a scintillation counter the ratio of radioactivity in PG to total radioactivity was determined and plotted vs. inhibitor concentration. IC_{50}s were determined using the curve fitting program GraphPad Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA, USA).

In vitro inhibition of *S. aureus* PBP2:
Solutions of *S. aureus* PBP2 (1.2 μM) in 50 mM HEPES (pH = 5.0), 50 mM CHES, 50 mM AcOH, 10 mM $CaCl_2$, 50 mM MES, and 1000 U/min PenG (8 μL) were incubated with DMSO solutions containing the inhibitor of interest in different concentrations (1 μL) for 20 min. Then ^{14}C-labelled lipid II (1 μL, 40 μM, $^{14}C/^{12}C$ 1/3) was added and the polymerization reaction was allowed to proceed for 25 min at room temperature. The reaction was quenched and processed as described above.

In vitro inhibition of *E. coli* PBP1b and *E. faecalis* PBP2a
Solutions of the PGT (50 nM) in 50 mM HEPES (pH = 7.5), 10 mM $CaCl_2$, and 1000 U/min PenG (8 μL) were incubated with DMSO solutions containing the inhibitor of interest in different concentrations (1 μL) for 20 min. Then ^{14}C-labelled lipid II (1 μL, 40 μM, $^{14}C/^{12}C$ 1/3) was added and the polymerization reaction was allowed to proceed for 25 min at room temperature. The reaction was quenched and processed as described above.

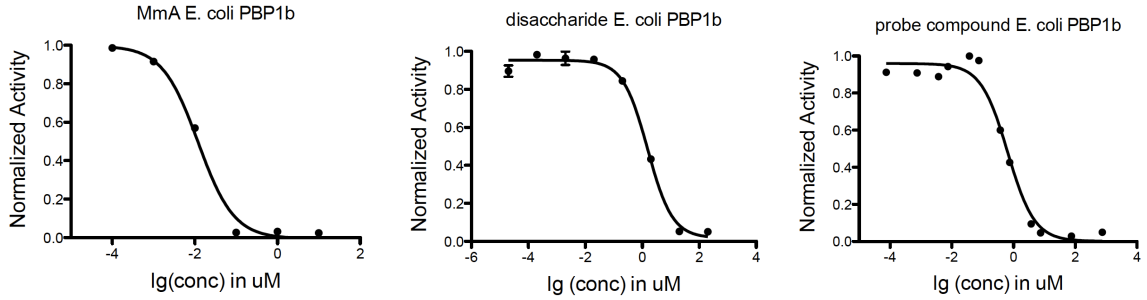


Figure 10: **Dose-response curve for inhibition of PG formation by *E. coli* PBP1b using various inhibitors.** 50 nM *E. coli* PBP1b, 4 μM lipid II. IC_{50} were determined as follows: Moenomycin: 12.0 nM (left); disaccharide **3**: 1.54 μM (middle); probe compound **2**: 650 nM (right).

[10] a) L. Chen, D. Walker, B. Sun, Y. Hu, S. Walker, D. Kahne, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 5658-5663; b) T.-S. A. Wang, T. J. Lupoli, Y. Sumida, H. Tsukamoto, Y. Wu, Y. Rebets, D. E. Kahne, S. Walker, *J. Am. Chem. Soc.* **2011**, *133*, 8528-8530.

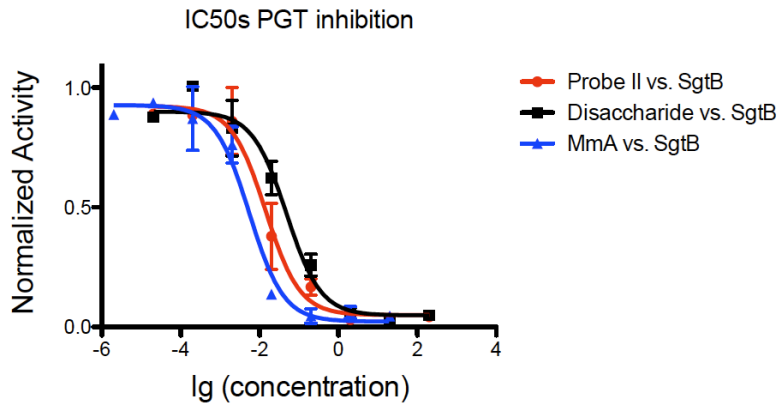


Figure 11: **Dose-response curve for inhibition of PG formation by *S. aureus* SgtB using various inhibitors.** 50 nM *S. aureus* SgtB, 4 μM lipid II. IC_{50} were determined as follows: Moenomycin: 6.0 nM (blue); disaccharide **3**: 48 nM (black); probe compound **2**: 14 nM (red).

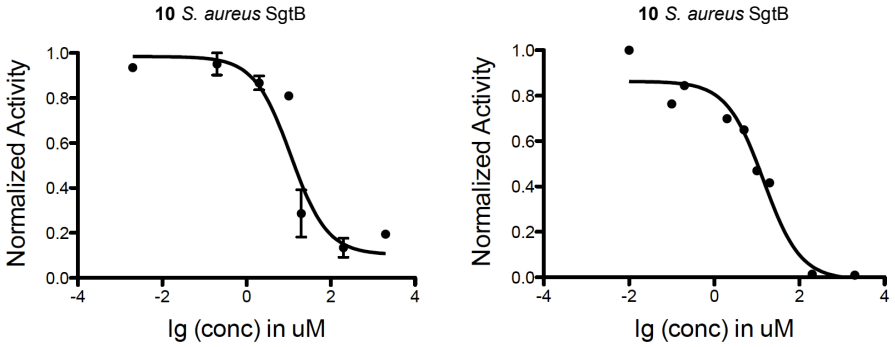


Figure 12: Dose-response curve for inhibition of PG formation by *S. aureus* SgtB using hit compound 10. 50 nM *S. aureus* SgtB, 4 μM lipid II; IC_{50} for compound **10** were determined in independent experiments as 11.3 μM (left) and 14.4 μM (right).

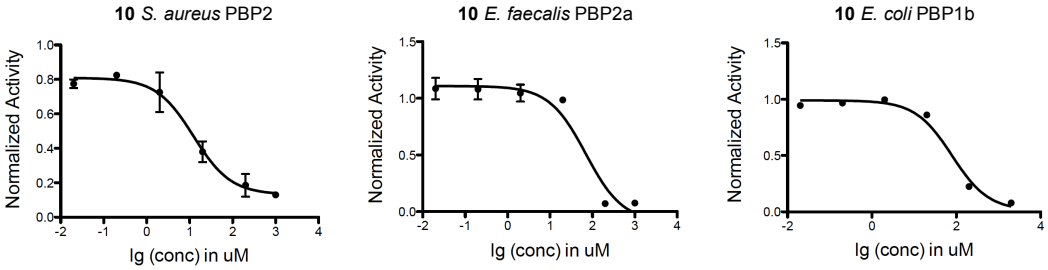
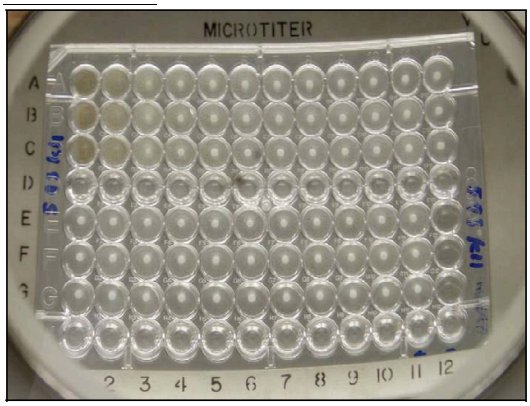


Figure 13: Dose-response curves for *in vitro* inhibition of various PGTs using hit compound 10. 1.2 μM *S. aureus* PBP2, 50 nM *E. faecalis* PBP2a, 50 nM *E. coli* PBP1b, 4 μM lipid II. IC_{50} (**10** *S. aureus* PBP2) = 12.0 μM; IC_{50} (**10** *E. faecalis* PBP2a) = 70 μM; IC_{50} (**10** *E. coli* PBP1b) = 79 μM.

6. MIC test



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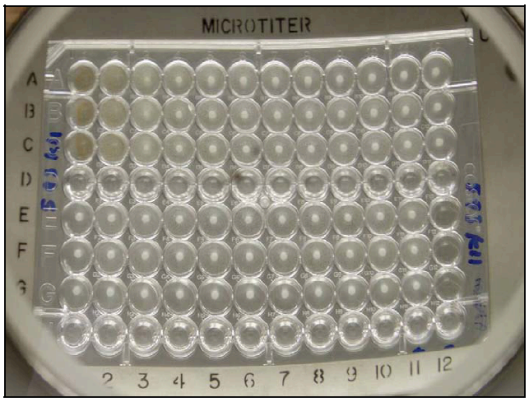
B	-	-	+	+	+	+	+	+	+	+	+	+
C	-	-	+	+	+	+	+	+	+	+	+	+
D	**32**	**16**	**8**	**4**	**2**	**1**	**0.5**	**0.25**	**0.125**	**0.0625**	**0.031**	**0.016**
E	+	+	+	+	+	+	+	+	+	+	+	-
F	+	+	+	+	+	+	+	+	+	+	+	-
G	+	+	+	+	+	+	+	+	+	+	+	-
H												
Conc. (µg/ ml):	**0.008**	**0.004**	**0.002**	**0.001**	**0.0005**	**0.00025**	**0.00013**	**0.00006**	**0.00003**	**0.000015**	**PC**	**NC**

Figure 14. MIC test of hit compound 10 against *S. aureus* ATCC 29213. Left: microtiter plate with cell cultures treated with various concentrations of **10**. The MIC is the concentration of compound that does not lead to cell growth as evidenced by clear wells. Right: plate map showing the concentrations in µg/mL. PC: positive control; NC: negative control.



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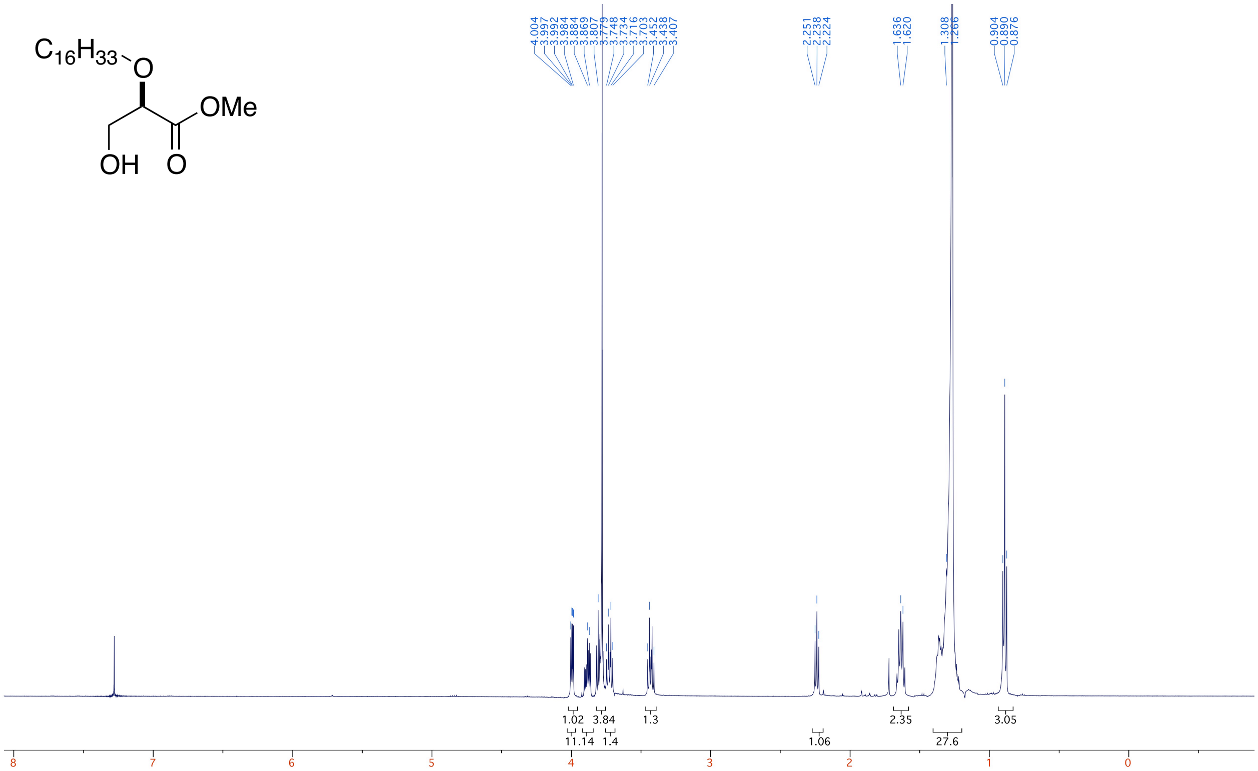
B	-	-	+	+	+	+	+	+	+	+	+	+
C	-	-	+	+	+	+	+	+	+	+	+	+
D	**32**	**16**	**8**	**4**	**2**	**1**	**0.5**	**0.25**	**0.125**	**0.0625**	**0.031**	**0.016**
E	+	+	+	+	+	+	+	+	+	+	+	-
F	+	+	+	+	+	+	+	+	+	+	+	-
G	+	+	+	+	+	+	+	+	+	+	+	-
H												
Conc. (µg/ ml):	**0.008**	**0.004**	**0.002**	**0.001**	**0.0005**	**0.00025**	**0.00013**	**0.00006**	**0.00003**	**0.000015**	**PC**	**NC**

Figure 15. MIC test of hit compound 10 against *S. aureus* USA 300. Left: microtiter plate with cell cultures treated with various concentrations of **10**. The MIC is the concentration of compound that does not lead to cell growth as evidenced by clear wells. Right: plate map showing the concentrations in µg/mL. PC: positive control; NC: negative control.

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	+	+	+	+	+	+	+	+
B	-	-	-	-	+	+	+	+	+	+	+	+
C	-	-	-	-	+	+	+	+	+	+	+	+
D	**32**	**16**	**8**	**4**	**2**	**1**	**0.5**	**0.25**	**0.125**	**0.0625**	**0.031**	**0.016**
E	+	+	+	+	+	+	+	+	+	+	+	-
F	+	+	+	+	+	+	+	+	+	+	+	-
G	+	+	+	+	+	+	+	+	+	+	+	-
H												
Conc. (µg/ ml):	**0.008**	**0.004**	**0.002**	**0.001**	**0.0005**	**0.00025**	**0.00013**	**0.00006**	**0.00003**	**0.000015**	**PC**	**NC**

Figure 16. MIC test of hit compound 10 against *B. anthracis* ANR-1. Left: microtiter plate with cell cultures treated with various concentrations of **10**. The MIC is the concentration of compound that does not lead to cell growth as evidenced by clear wells. Right: plate map showing the concentrations in µg/mL. PC: positive control; NC: negative control.

7. NMR Data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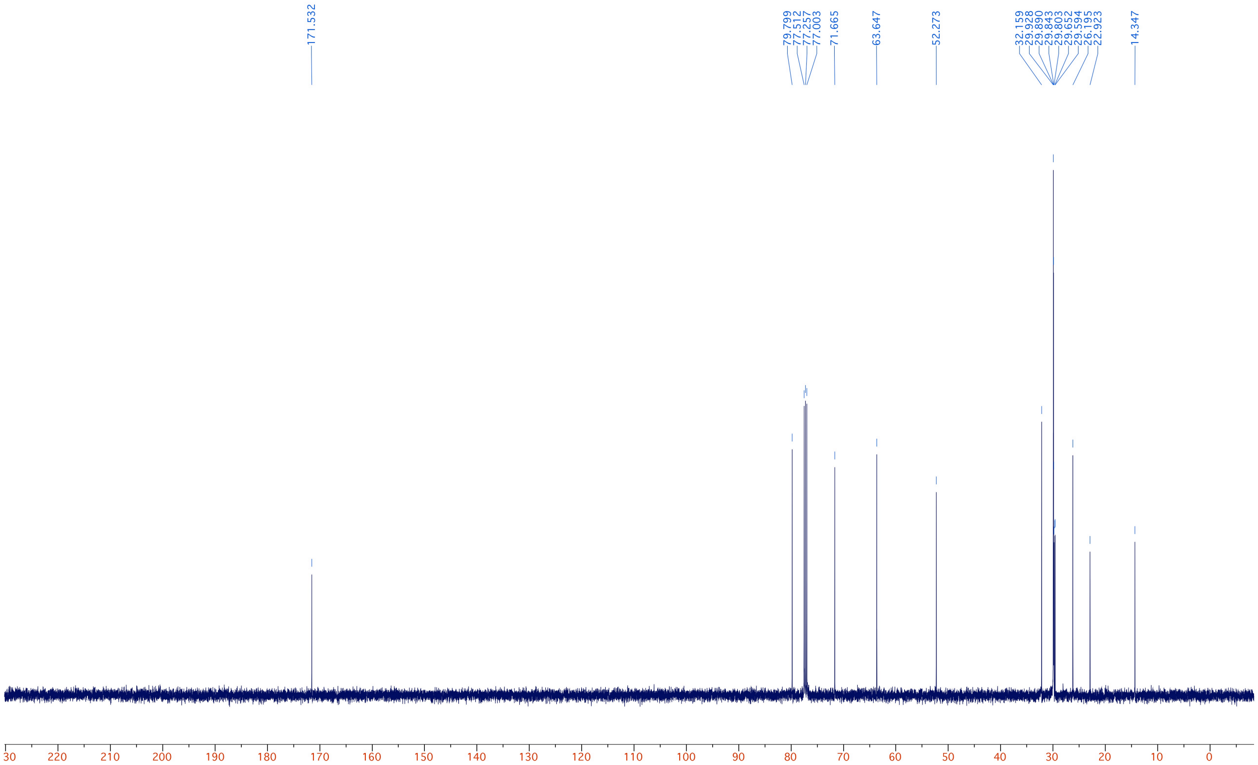
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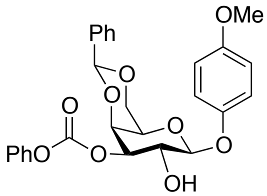
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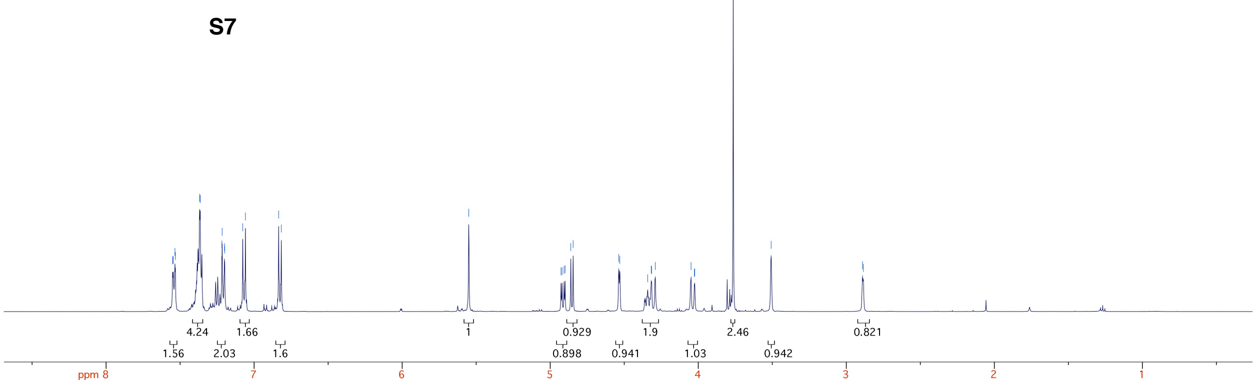
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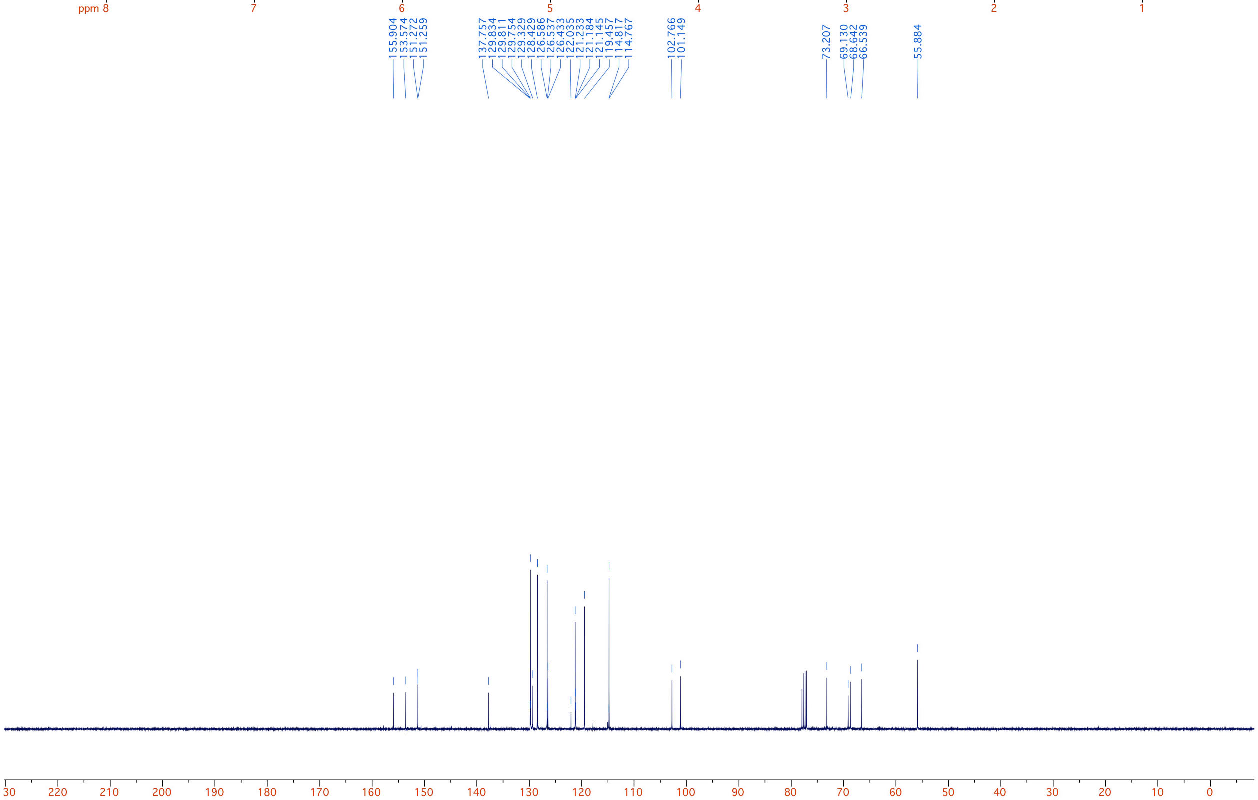
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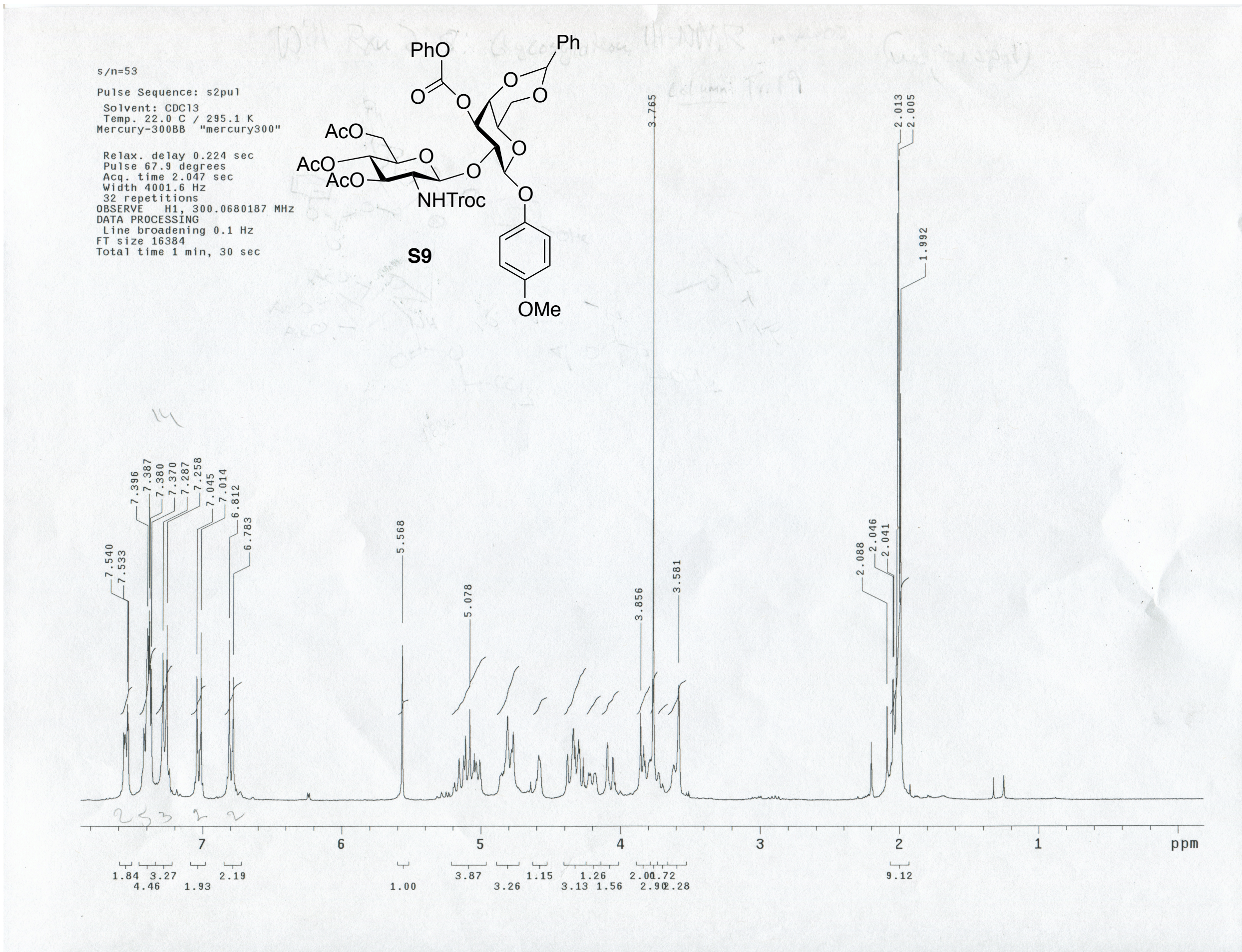
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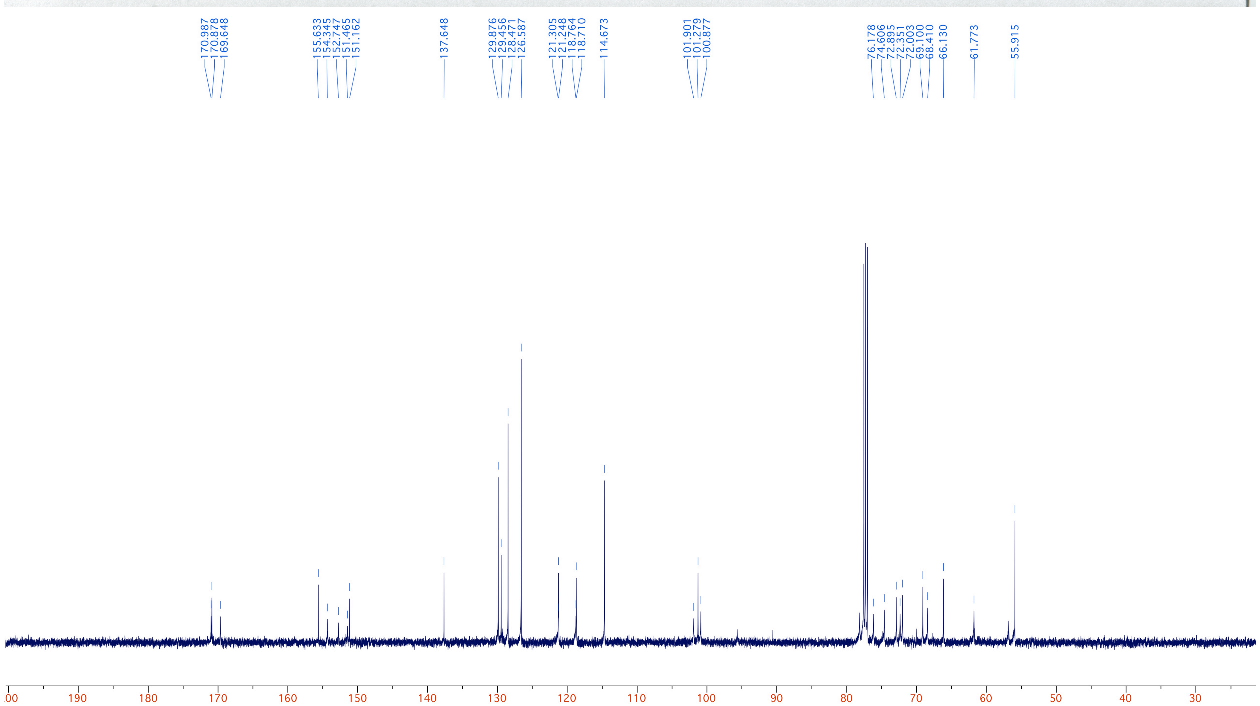

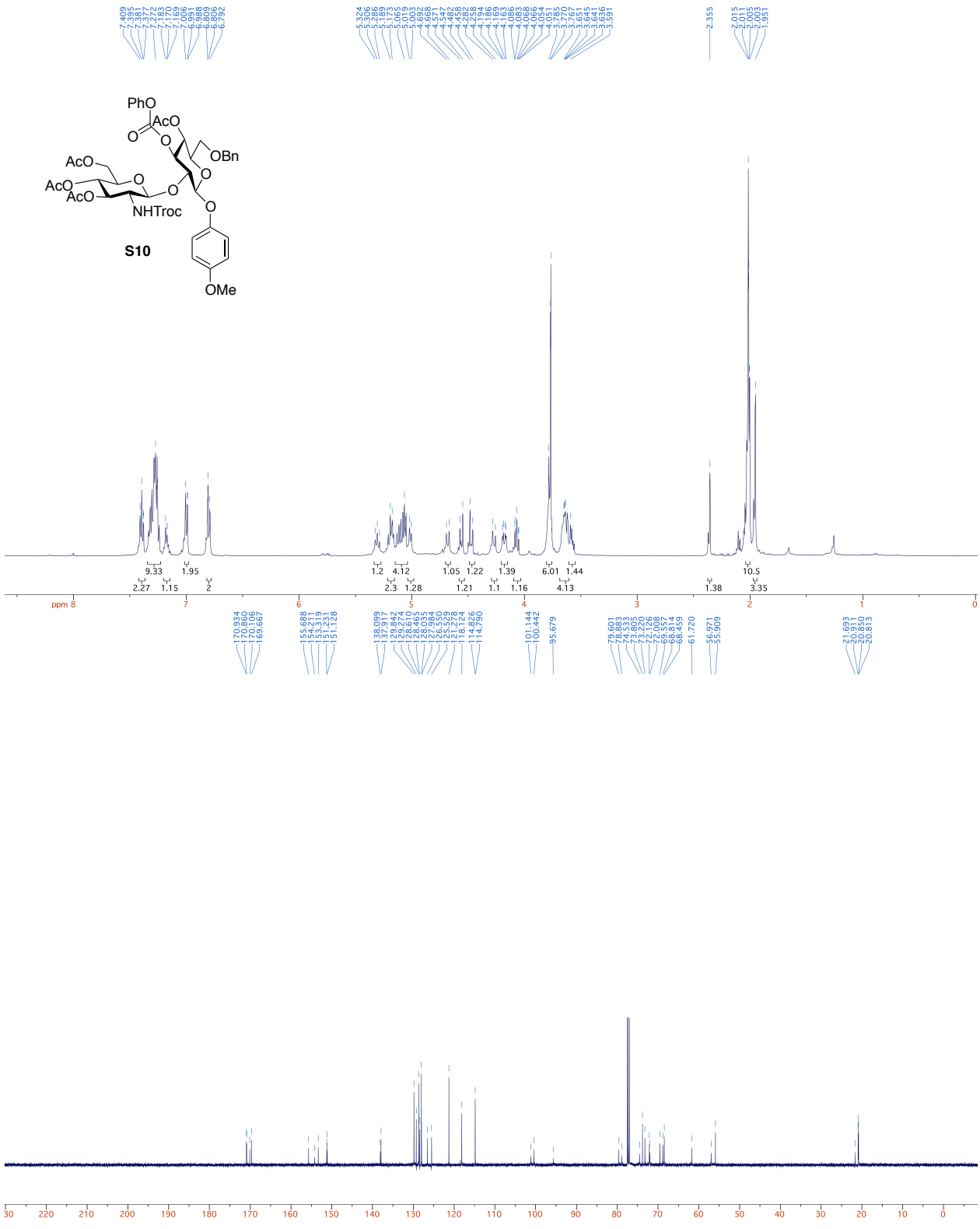
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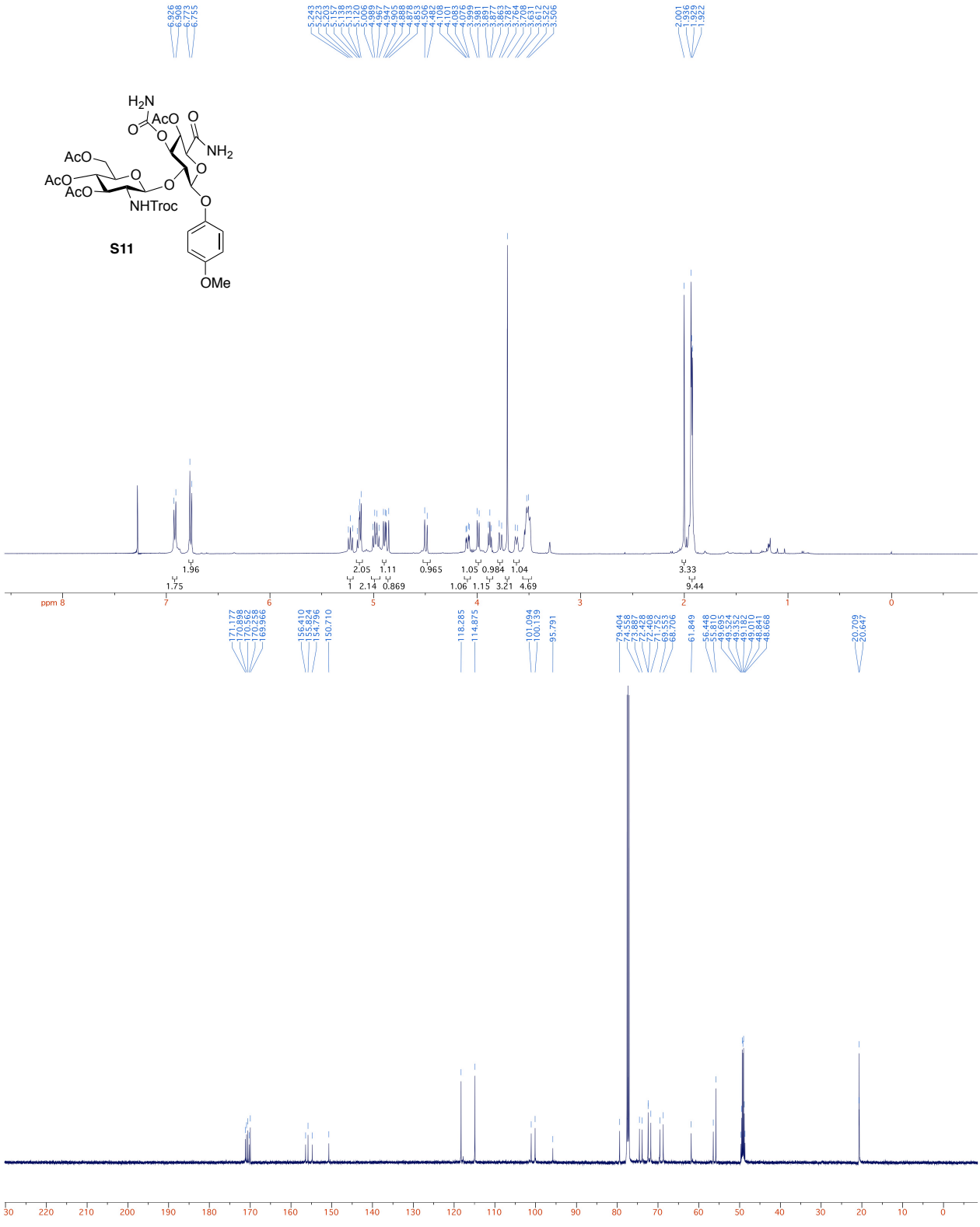
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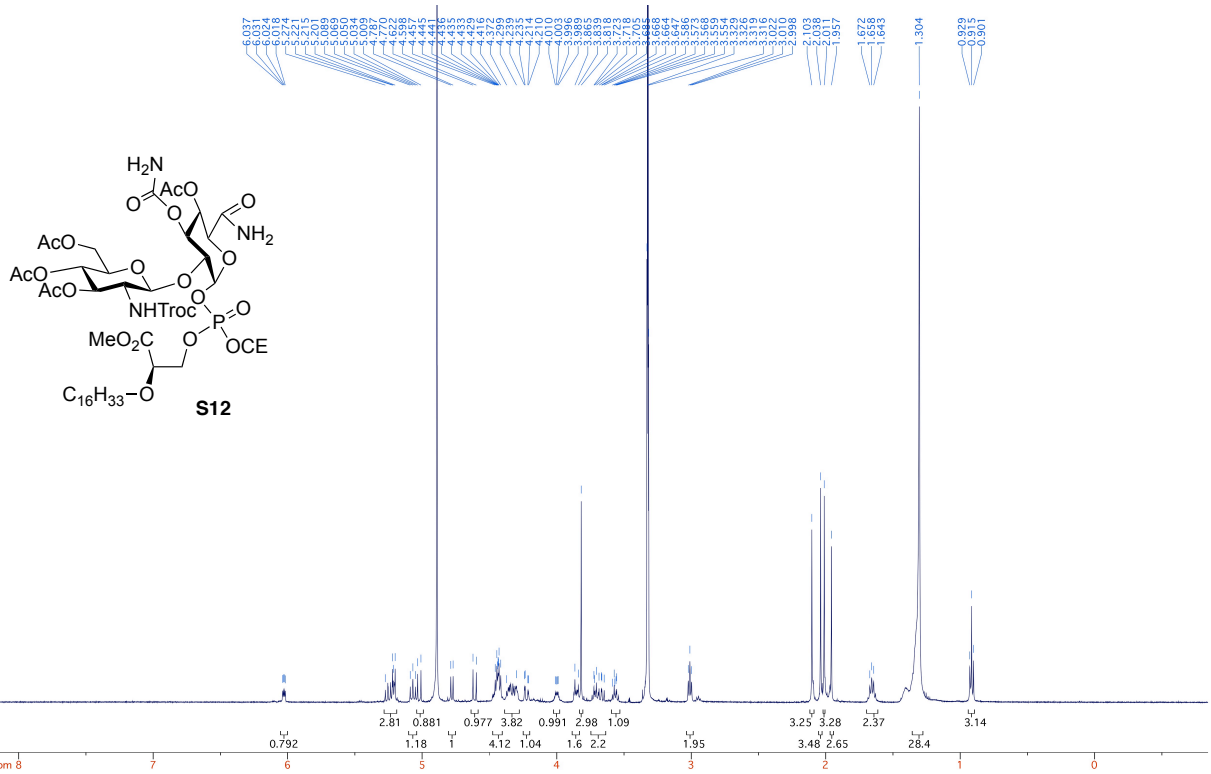
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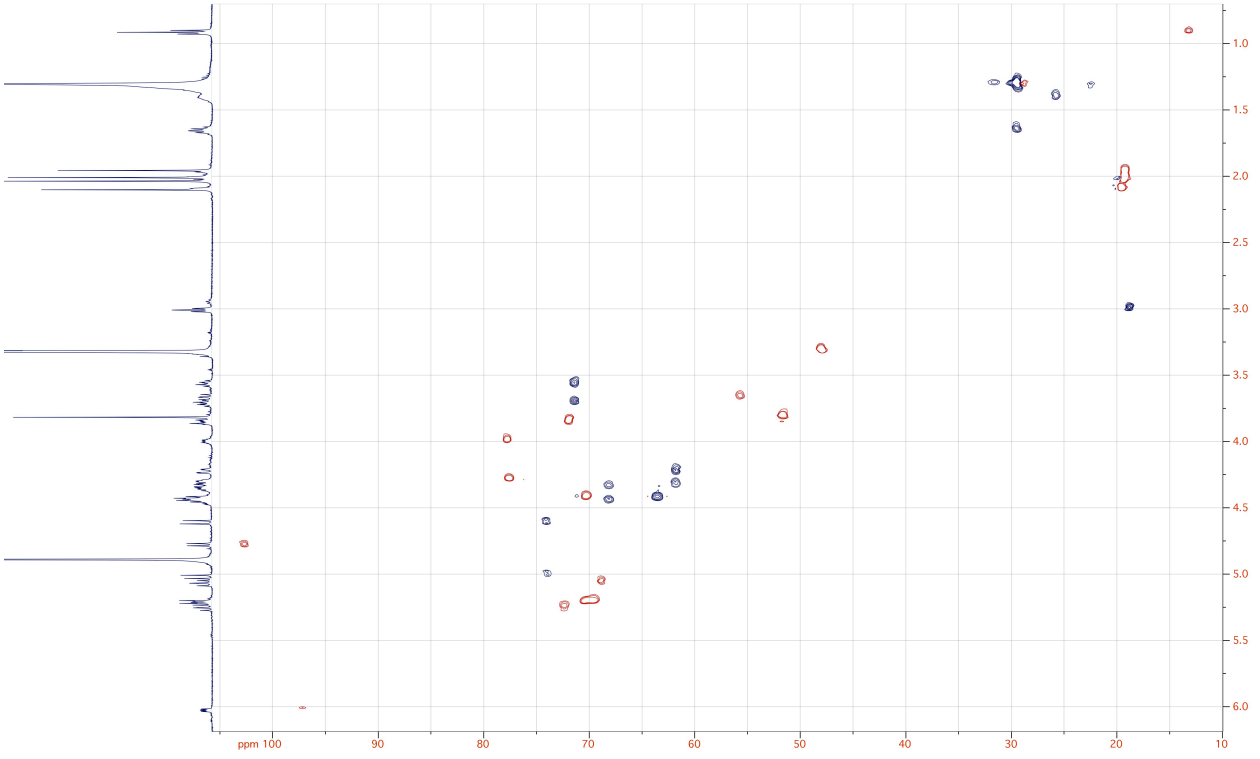

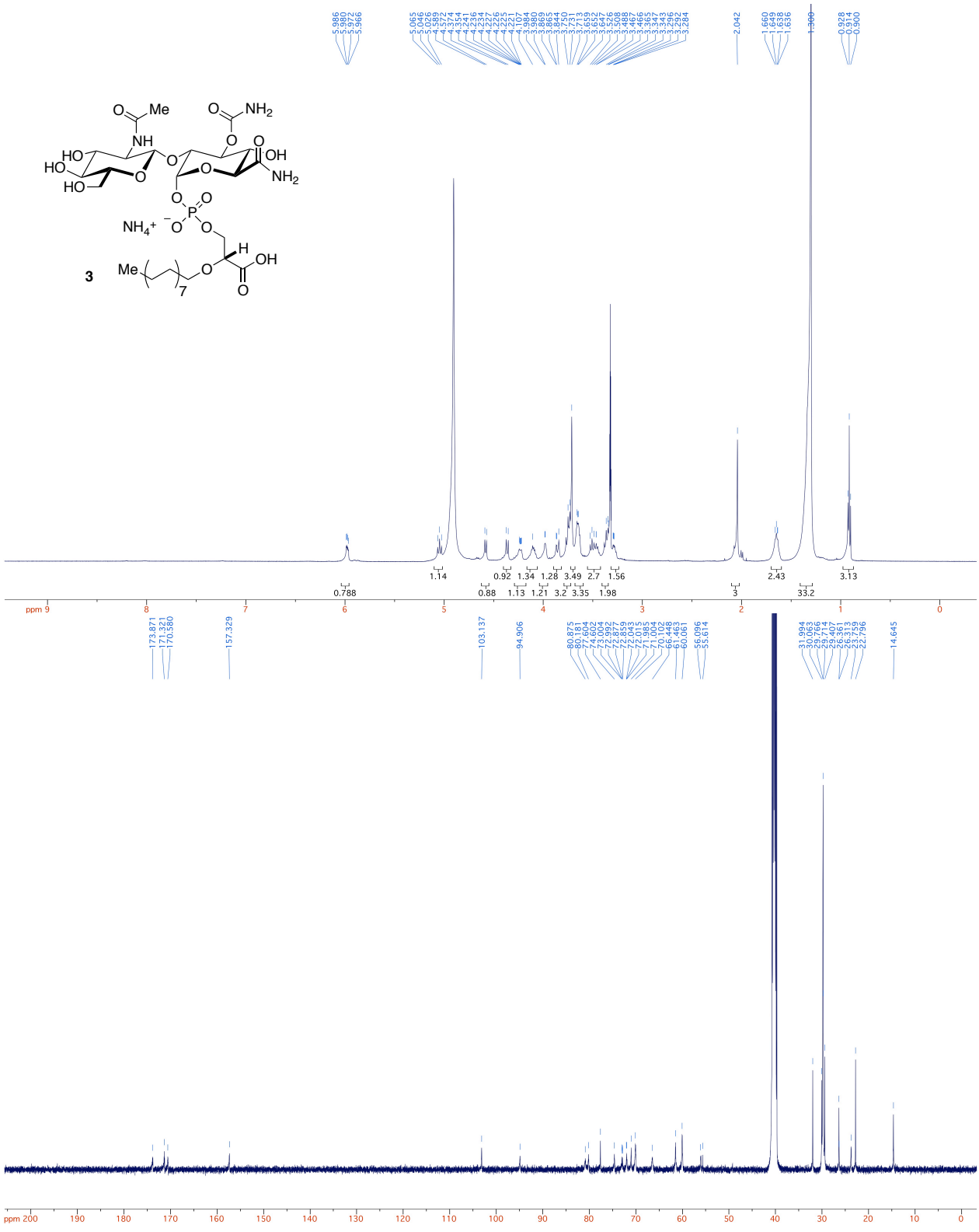
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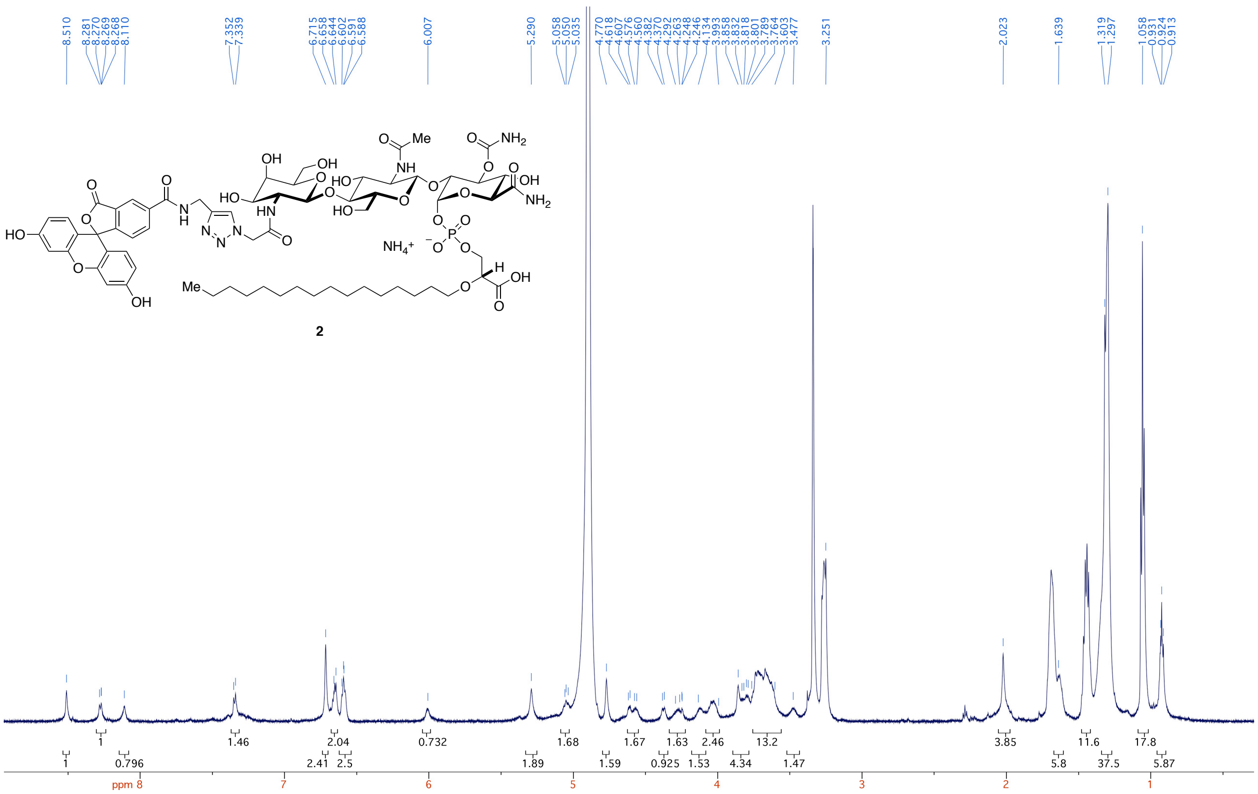
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