A Set of Phosphatase-Inert "Molecular Rulers" to Probe for Bivalent Mannose 6-Phosphate Ligand-Receptor Interactions

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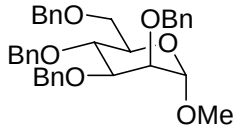
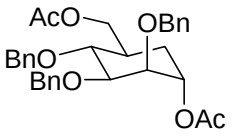
Organic Synthesis

General Experiment: Reactions were conducted under nitrogen atmosphere using flame-dried glassware. Methylene chloride and acetonitrile were distilled from CaH_2. THF was distilled from sodium-benzophenone ketyl. Methanol was distilled from magnesium and iodine. All other reagents were used as purchased without further purification. Flash chromatography was performed using Merck silica gel 60 (230-400 mesh). ^{1}H NMR, Proton-decoupled ^{13}C NMR and ^{31}P NMR spectra were recorded on a Bruker-DRXAvance-300 MHz and 400 MHz instrument with chemical shifts reported relative to residual $CDCl_3$ (7.25 ppm).

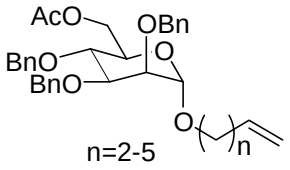


Methyl-2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranoside (22)[1]: Benzyl bromide (9.19 g, 77 mmol) was added to a stirred solution of methyl α-D-mannopyranoside (3.0 g, 15 mmol) in DMF (15 mL). The reaction mixture was cooled to 0°C and sodium hydride (2.23 g, 93 mmol) was then added portion-wise. The mixture was kept at that temperature for another 1 h, and then allowed to warm to room temperature. After 17 h, TLC (hexanes: ethyl acetate, 3:1) indicated the completion of reaction. The reaction was quenched with methanol, diluted with diethyl ether (200 mL) and washed with water (150

mL). The aqueous phase was extracted with diethyl ether (200 mL). The combined organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash chromatography (20%, EtOAc/hexanes) to provide **22** (7.52 g, 90%) as a colorless oil. The spectral data were identical to those reported.



1, 6-Di-*O*-acetyl-2,3,4-tri-*O*-benzyl-α-D-mannopyranose (1):[1] Concentrated sulfuric acid (0.15 mL) was added dropwise to a stirred solution of methyl-2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranoside (798 mg, 1.44 mmol) in a mixture of acetic acid and acetic anhydride (5mL:5mL) at 0°C. After 30min, TLC (hexanes: ethyl acetate, 3:1) indicated the complete conversion of starting material to a major product. The reaction mixture was diluted with CH_2Cl_2 (40 mL), washed with saturated $NaHCO_3$ solution (7×10 mL), ice cold water (10 mL). The aqueous layer was extracted with CH_2Cl_2 (3 ×20 mL). Combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash chromatography (25%, EtOAc/hexanes) to give **1** (632 mg, 82%) as a white solid. The spectral data were identical to those reported.



General method for glycosidation (2-5):

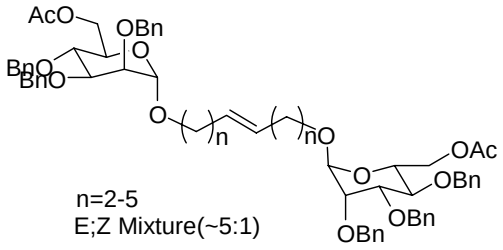
But-3-enyl 2,3,4-tri-*O*-benzyl-6-*O*-acetyl-α-D-mannopyranose (2): (160 µL, 1.9 mmol) 3-buten-1-ol and (59 µL, 0.34 mmol) trimethylsilyl trifluoromethanesulfonate were added to 0.4 mL acetonitrile at room temperature. After being stirred for 15 min, to the mixture was added 1,6-di-*O*-acetyl-2,3,4-tri-*O*-benzyl-α-D-mannopyranose (100 mg, 0.19 mmol) in 0.3 mL acetonitrile. The reaction mixture was stirred for 30 min and quenched by saturated $NaHCO_3$; the mixture was extracted with ethyl acetate (3 × 5 mL) and dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash chromatography (16%, EtOAc/hexanes) to give **2 (**88 mg, 85%) as colorless oil:

2: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.04 (s, 3H), 2.22-2.31 (m, 1H), 2.36-2.45 (m, 1H), 3.38-3.53 (m, 2H), 3.75-3.83 (m, 1H), 3.87-3.99 (m, 2H), 4.29-4.36 (m, 2H), 4.37-4.41 (m, 1H), 4.45 (dd, 1H), 4.53 (d, 1H), 4.58 (dd, 1H), 4.63 (s, 1H), 4.72 (q, 1H), 4.81-4.99 (m, 2H), 5.06 (m, 1H), 5.12 (dd, 1H), 5.67-5.88 (m, 1H), 7.21-7.46 (m, 15 H)

3: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.51-1.67 (m, 2H), 1.67-1.81 (m, 1H), 2.03 (s, 3H), 2.09-2.19 (m, 1H), 3.32-3.58 (m, 2H), 3.74-3.85 (m, 1H), 3.88-3.98 (m, 2H), 4.27-4.41 (m, 3H), 4.48 (q, 1H), 4.57 (dd, 1H), 4.63 (s, 1H), 4.72 (q, 1H), 4.81-4.85 (m, 1H), 4.89 (dd, 1H), 4.93-4.98 (m, 2H), 4.98-5.02 (m, 1H), 5.71-5.91 (m, 1H), 7.25-7.52 (m, 15H)

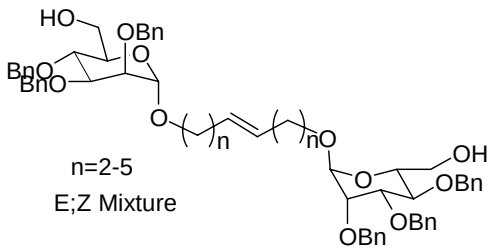
4: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25-1.35 (m, 1H), 1.38-1.49 (m, 2H), 1.49-1.61 (m, 3H), 2.05 (s, 3H), 3.31-3.42 (m, 1H), 3.58-3.71 (m, 1H), 3.74-3.86 (m, 2H), 3.92-3.99 (m, 2H), 4.32-4.38 (m, 2H), 4.61 (dd, 2H), 4.64 (s, 1H), 4.76 (q, 2H), 4.85 (d, 1H), 4.97 (dd, 1H), 4.98-5.03 (m, 1H), 5.03-5.06 (m, 1H), 5.72-5.93 (m, 1H), 7.29-7.43 (m, 15H)

5: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.20-1.44 (m, 4H), 1.45-1.58 (m, 3H), 2.05 (s, 3H), 3.28-3.38 (m, 1H), 3.56-3.68 (m, 1H), 3.72-3.84 (m, 2H), 3.87-3.96 (m, 2H), 4.12 (q, 1H), 4.28-4.37 (m, 2H), 4.58 (dd, 2H), 4.62 (s, 1H), 4.72 (t, 2H), 4.82 (d, 1H), 4.88-4.92 (m, 1H), 4.92-4.98 (m, 1H), 5.01-5.03 (m, 1H), 5.70-5.88 (m, 1H), 7.25-7.41 (m, 15H)



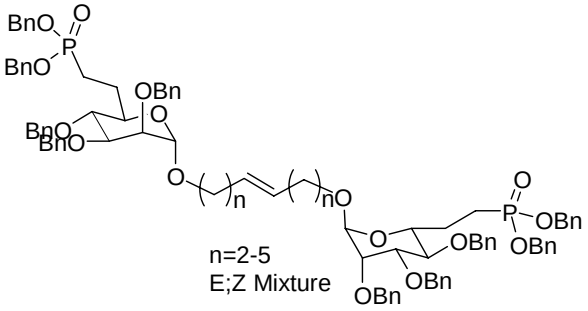
General method for cross-metathesis (6-9):

metathesis product 9E & 9Z: To a solution of **5** (125 mg, 0.21 mmol) in dry CH_2Cl_2 (2 mL) was added Grubbs I catalyst (17 mg, 0.021 mmol), and the resulting purple-colored solution allowed to reflux for 24 h. Then the solvent was evaporated and the residue was purified by flash chromatography (50→67%, EtOAc/hexanes), affording **9 (**84 mg, 70%), as a mixture of geometric isomers. (E/Z~5/1)



General method for deacetylation (10-13):

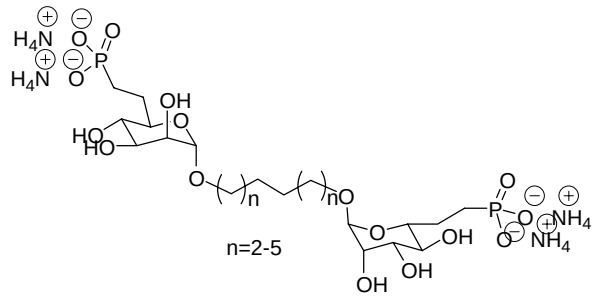
10: To a solution of **6** (E/Z mixture, 330 mg, 0.31 mmol) in methanol (10 mL) was added a solution of NaOMe in methanol (30%, 5 mL). The reaction mixture was stirred for 6 h until the TLC indicated all the starting material was converted to the product. Dowex 50 was added to quench the reaction. The mixture was filtered and concentrated *in vacuo* to afford **10 (**295 mg, 98%), as a mixture of geometric isomers.



General triflate displacement method (14-17):

Bis-triflate displacement product 14: To a solution of **10 (**E/Z mixture, 210 mg, 0.21 mmol) and 2,6-di-*tert*-butyl-4-methylpyridine (175 mg, 0.86 mmol) in CH_2Cl_2 (4 mL), was added dropwise trifluoromethanesulfonic anhydride (107 µL, 0.64 mmol) at -40 °C. The reaction was held at that temperature for 2.5 h, and then diluted with Et_2O. The organic layer was sequentially washed with saturated aqueous NH_4Cl and saturated aqueous $NaHCO_3$, then dried over $NaSO_4$, filtered and concentrated *in vacuo* to afford 362 mg crude product of bis-triflate as a white solid. To a magnetically stirred solution of dibenzyl methylphosphonate (265 mg, 0.96 mmol) in THF (4 mL) at -78 °C was added slowly a solution of *n*-BuLi (0.64 mL, of 1.5 M in hexane, 0.96 mmol), followed by 30

min stirring at that temperature. A solution of the bis-triflate in THF (2 mL) was then added, dropwise, via cannula, and the resulting reaction mixture was stirred for 1 h at -78 °C. The reaction was then quenched with saturated aqueous NH_4Cl. Following dilution with EtOAc (50 mL), the organic layer was washed sequentially with NH_4Cl (aq), water and brine. Drying ($MgSO_4$), filtration, evaporation and flash chromatography (100%, EtOAc) provided **14 (**211 mg, 67%) as a mixture of geometric isomers.



General deprotection method (18-21):

Bivalent 6-deoxy-6-phosphonomethyl-mannopyranose mimic 18: A solution of protected bis-phosphonate **14 (**E/Z mixture, 50 mg, 0.033 mmol) in anhydrous methanol (15 mL) containing 20% $Pd(OH)_2/C$ (10 mg) was stirred under a balloon pressure of hydrogen, at room temperature, overnight. Following filtration through Celite™ and washing of the filter cake with methanol, a solution of 50 mM NH_4HCO_3 (2 mL) was added to the filtrate. Removal of the volatiles *in vacuo* and further drying on the high vacuum gave **18 (**33 mg, 66%) as syrup:

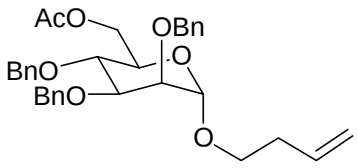
18: ^{1}H NMR (400 MHz, D_2O) δ 1.13-1.72 (m, 14H), 1.81-1.97 (m, 2H), 3.33-3.42 (m, 6H), 3.51-3.68 (m, 4H), 3.78 (dd, 2H), 4.68 (d, 2H); ^{13}C NMR (100 MHz, D_2O) δ 25.27 (2C), 28.31, 67.64, 69.90, 69.98, 70.39, 72.53, 72.70, 99.45; ^{31}P NMR (162 MHz, D_2O) δ 24.25

19: ^{1}H NMR (400 MHz, D_2O) δ 1.27-1.48 (m, 8H), 1.58-1.81 (m, 8H), 1.85-1.98 (m, 2H), 2.03-2.19 (m, 2H), 3.50-3.61 (m, 6H), 3.68-3.81 (m, 4H), 3.95 (dd, 2H), 4.82 (d, 2H); ^{13}C NMR (100 MHz, D_2O) δ 24.27, 24.83, 25.37, 28.41, 67.86, 70.08, 70.51, 71.24, 72.29, 72.46, 99.56; ^{31}P NMR (162 MHz, D_2O) δ 25.18

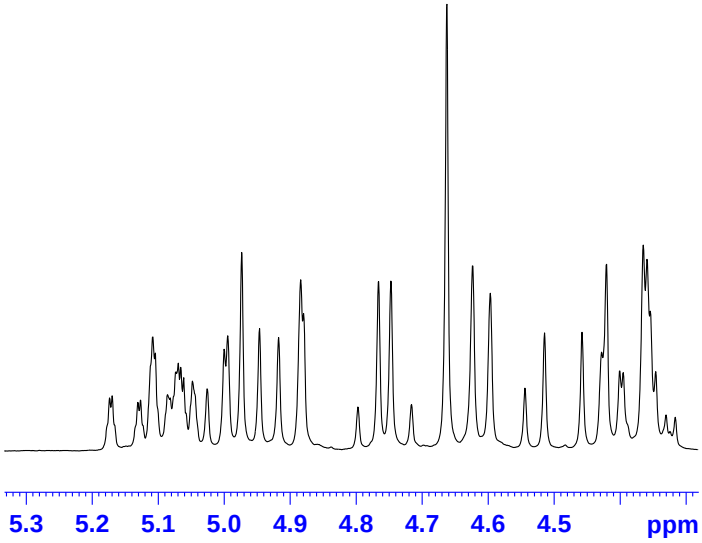
20: ^{1}H NMR (400 MHz, D_2O) δ 1.27-1.51 (m, 12H), 1.52-1.78 (m, 8H), 1.79-1.95 (m, 2H), 2.03-2.18 (m, 2H), 3.52-3.61 (m, 6H), 3.64-3.88 (m, 4H), 3.94 (dd, 2H), 4.82 (d, 2H); ^{13}C NMR (100 MHz, D_2O) δ 23.26, 25.08, 25.43, 25.58, 28.52, 67.86, 70.04, 70.50, 71.21, 72.42, 99.51; ^{31}P NMR (162 MHz, D_2O) δ 25.48

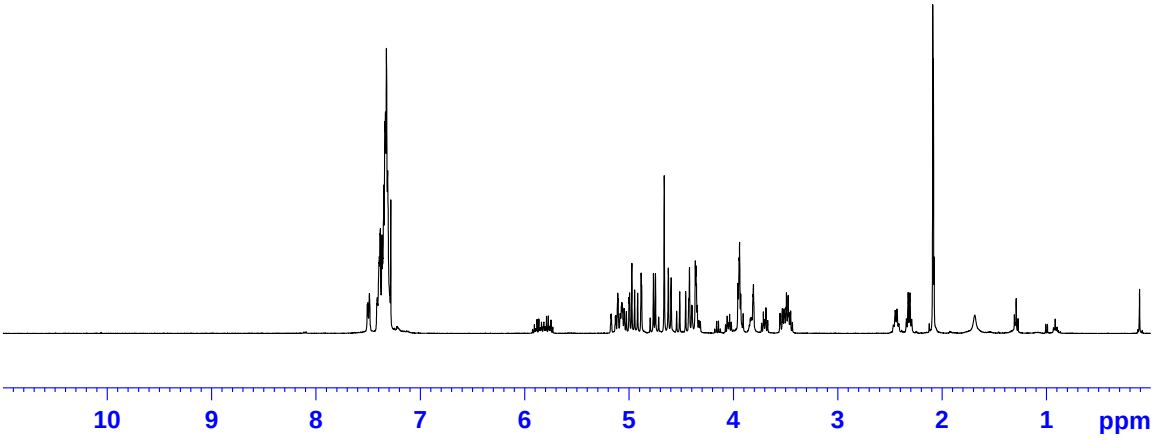
21: ^{1}H NMR (400 MHz, D_2O) δ 1.25-1.49 (m, 16H), 1.56-1.81 (m, 8H), 1.82-1.99 (m, 2H), 2.01-2.19 (m, 2H), 3.46-3.62 (m, 6H), 3.64-3.83 (m, 4H), 3.93 (brds, 2H), 4.82 (2H); ^{13}C NMR (100 MHz, D_2O) δ 23.10, 24.41, 25.04, 25.92, 29.05, 29.31, 67.70, 70.12, 70.28, 71.65, 72.26, 72.44, 99.76; ^{31}P NMR (162 MHz, D_2O) δ 26.41

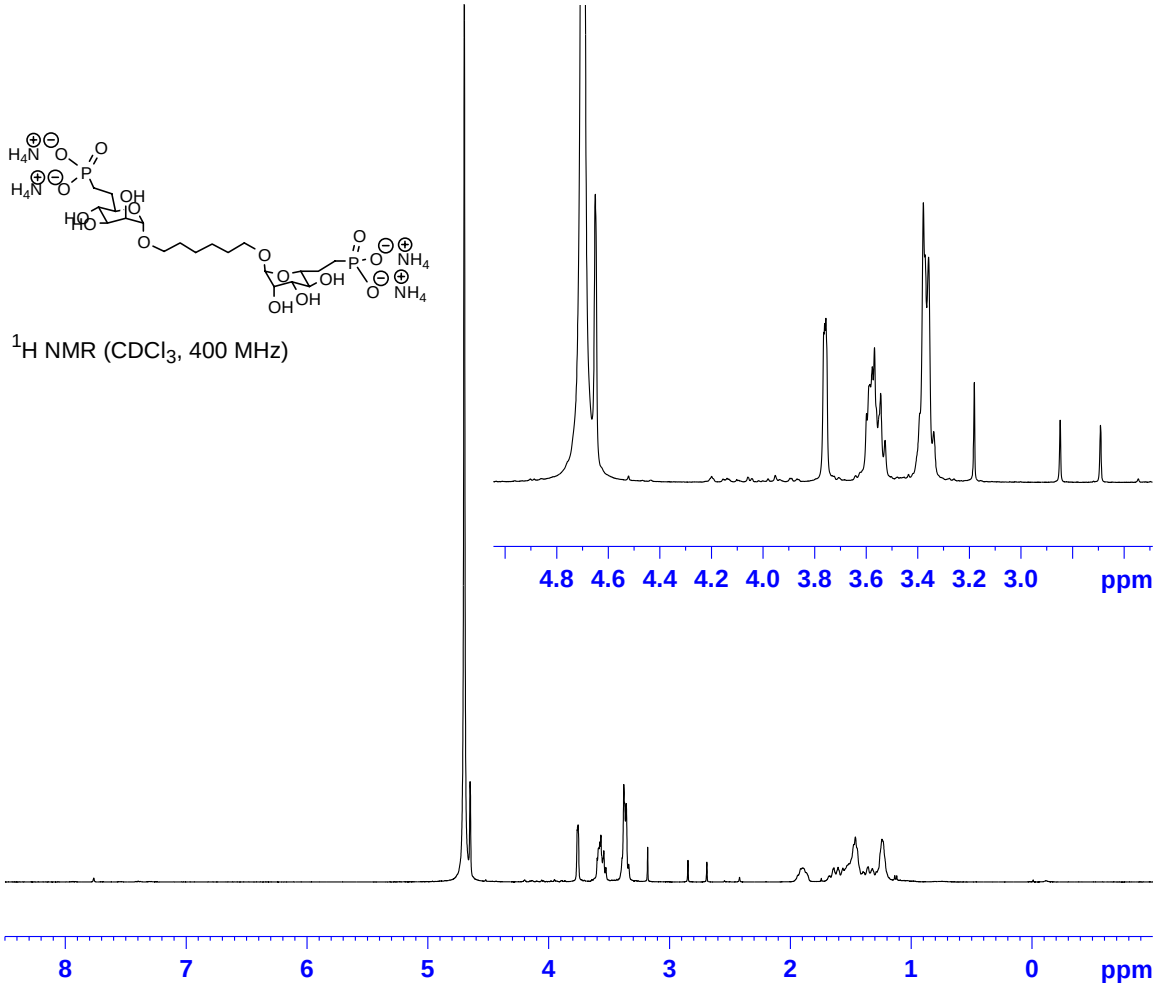
Spectral Data

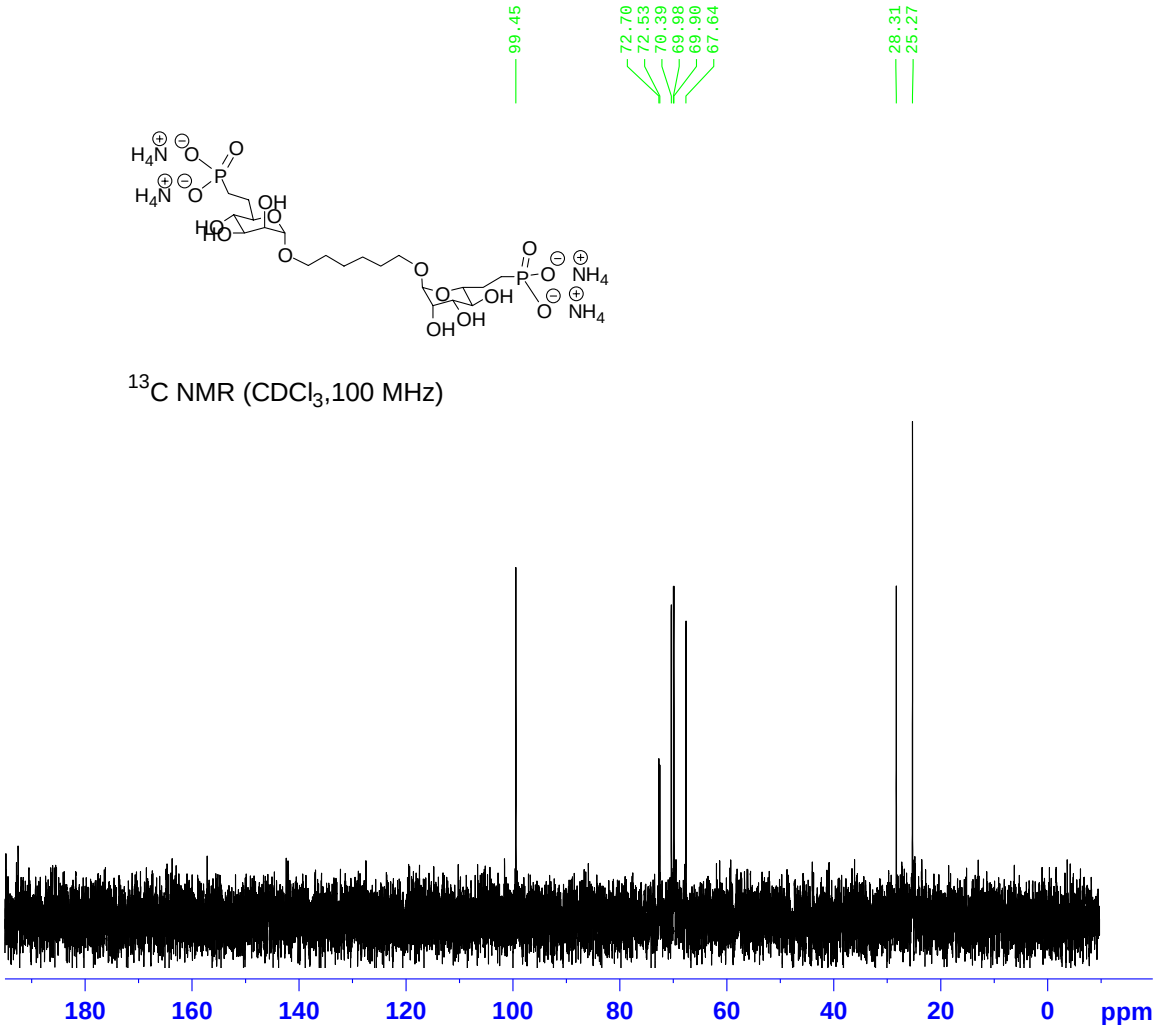


^{1}H NMR ($CDCl_3$, 400 MHz)

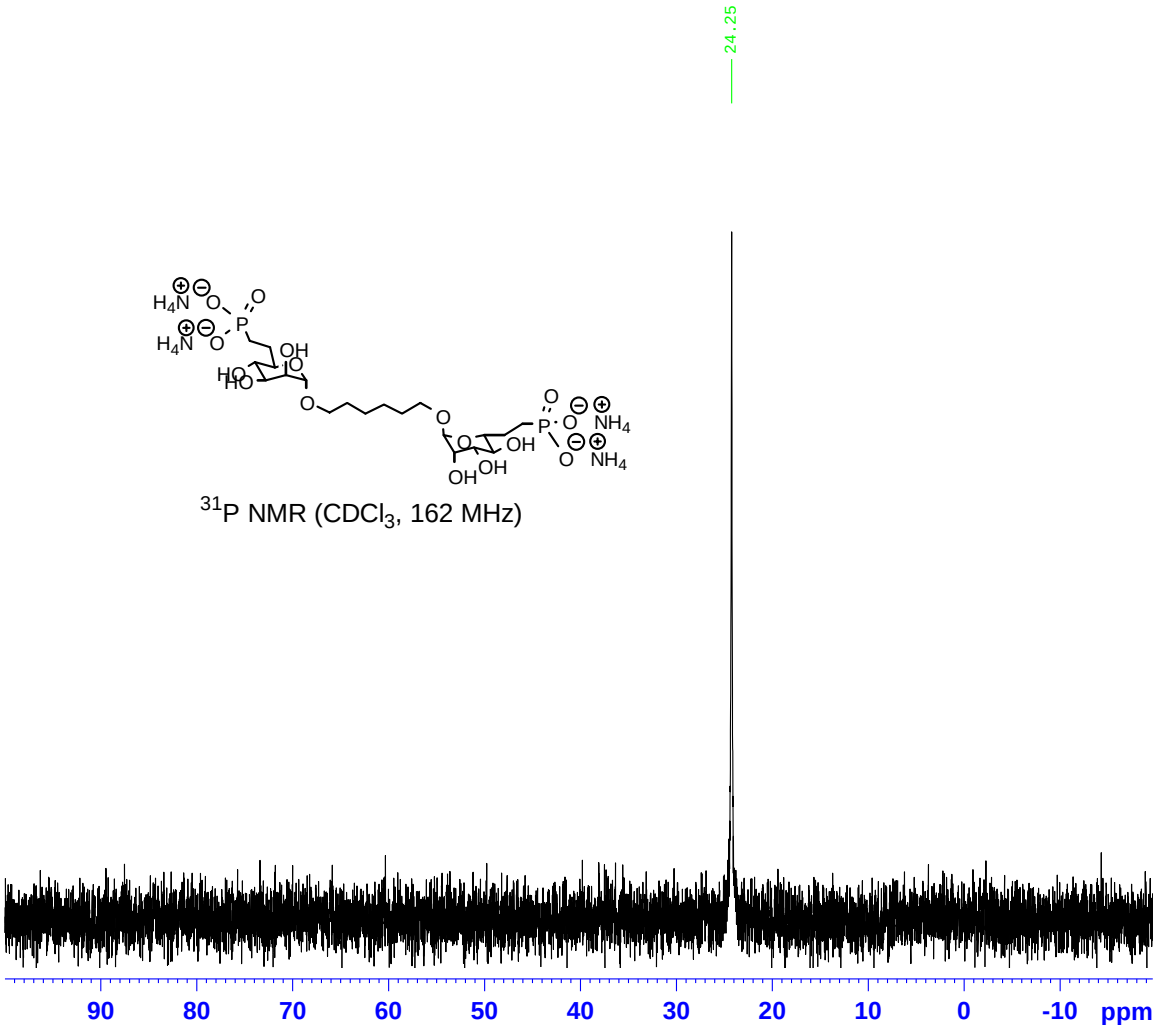




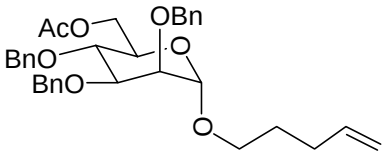

^{1}H NMR ($CDCl_3$, 400 MHz)
4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 ppm
8 7 6 5 4 3 2 1 0 ppm



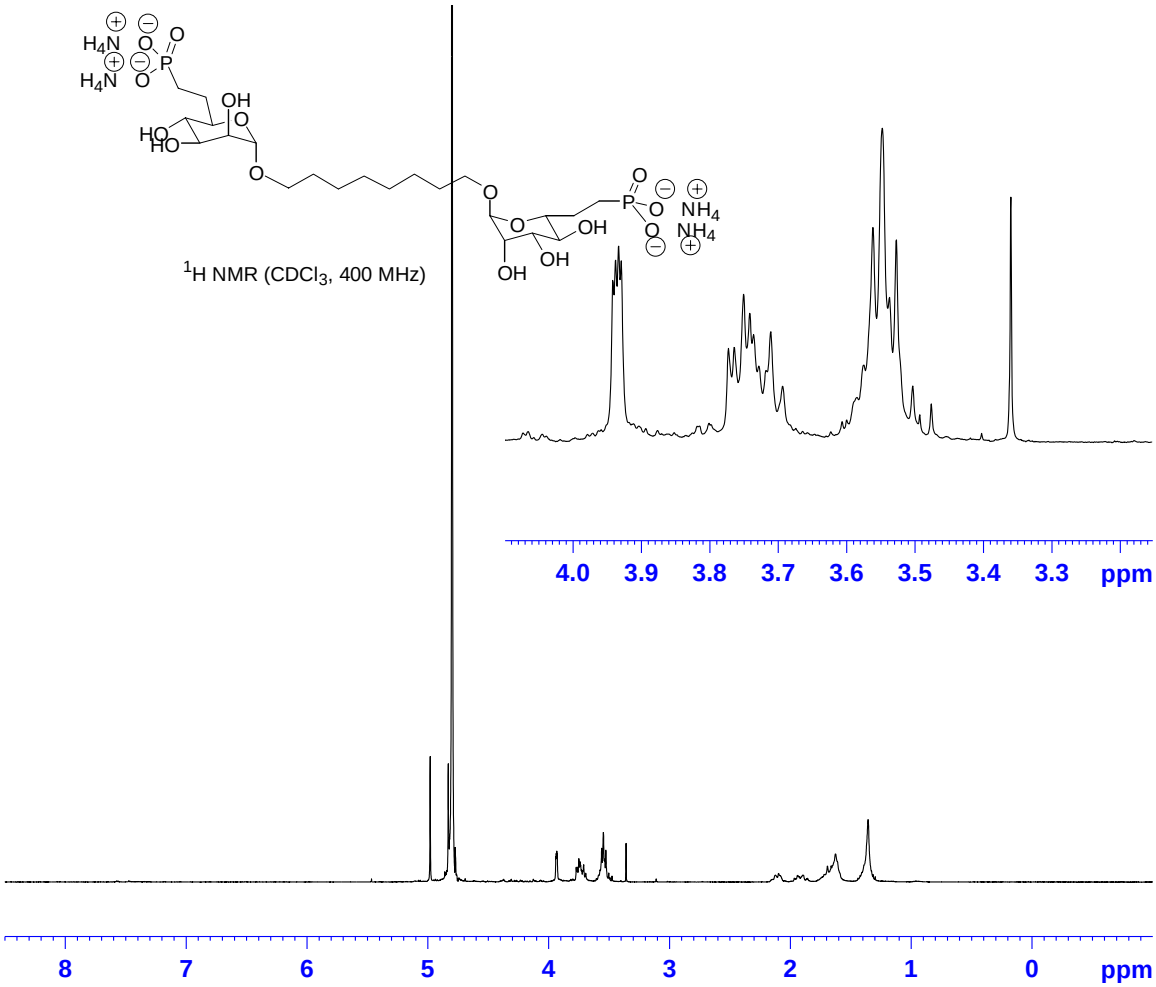
^{13}C NMR ($CDCl_3$,100 MHz)

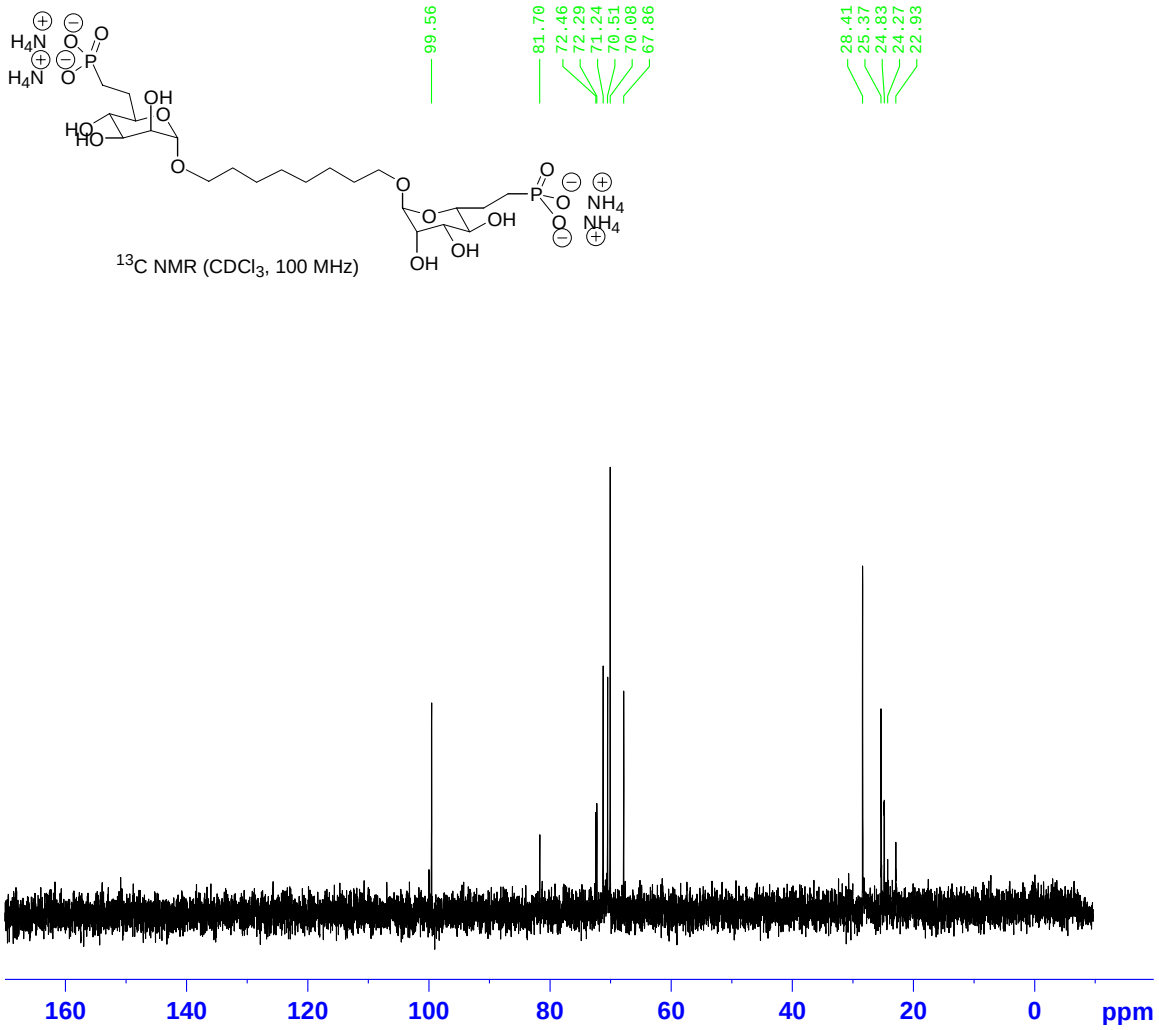


^{31}P NMR ($CDCl_3$, 162 MHz)

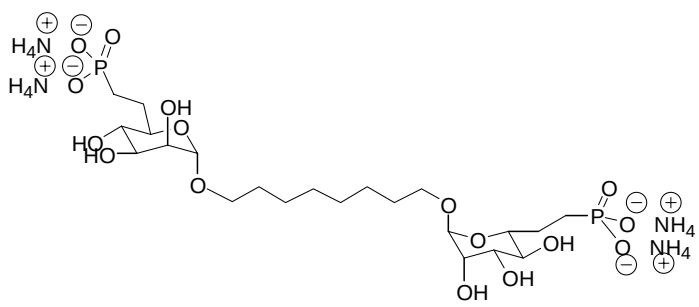


^{1}H NMR ($CDCl_3$, 400 MHz)

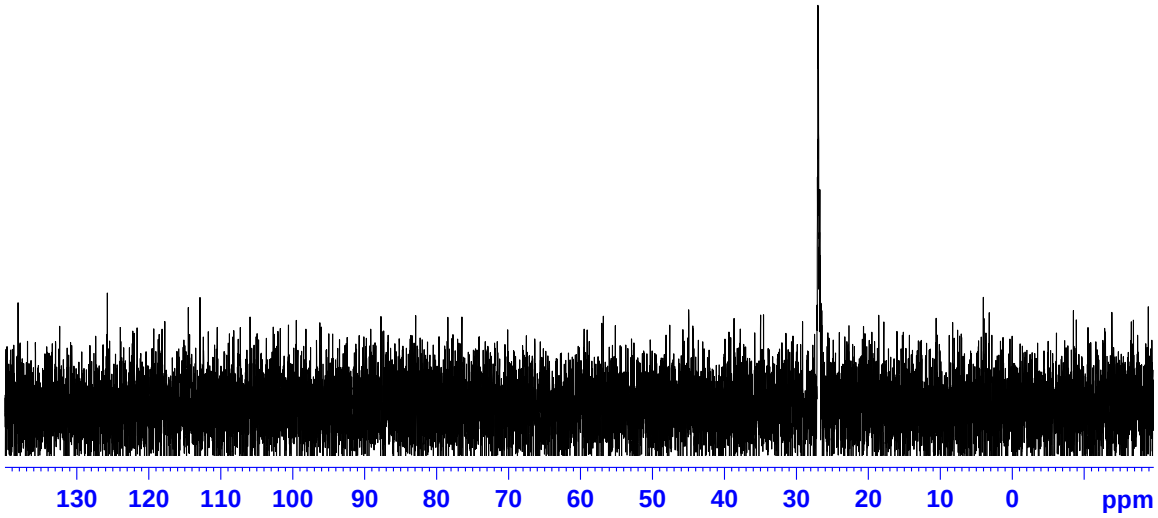

^{1}H NMR ($CDCl_3$, 400 MHz)
4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 ppm
8 7 6 5 4 3 2 1 0 ppm

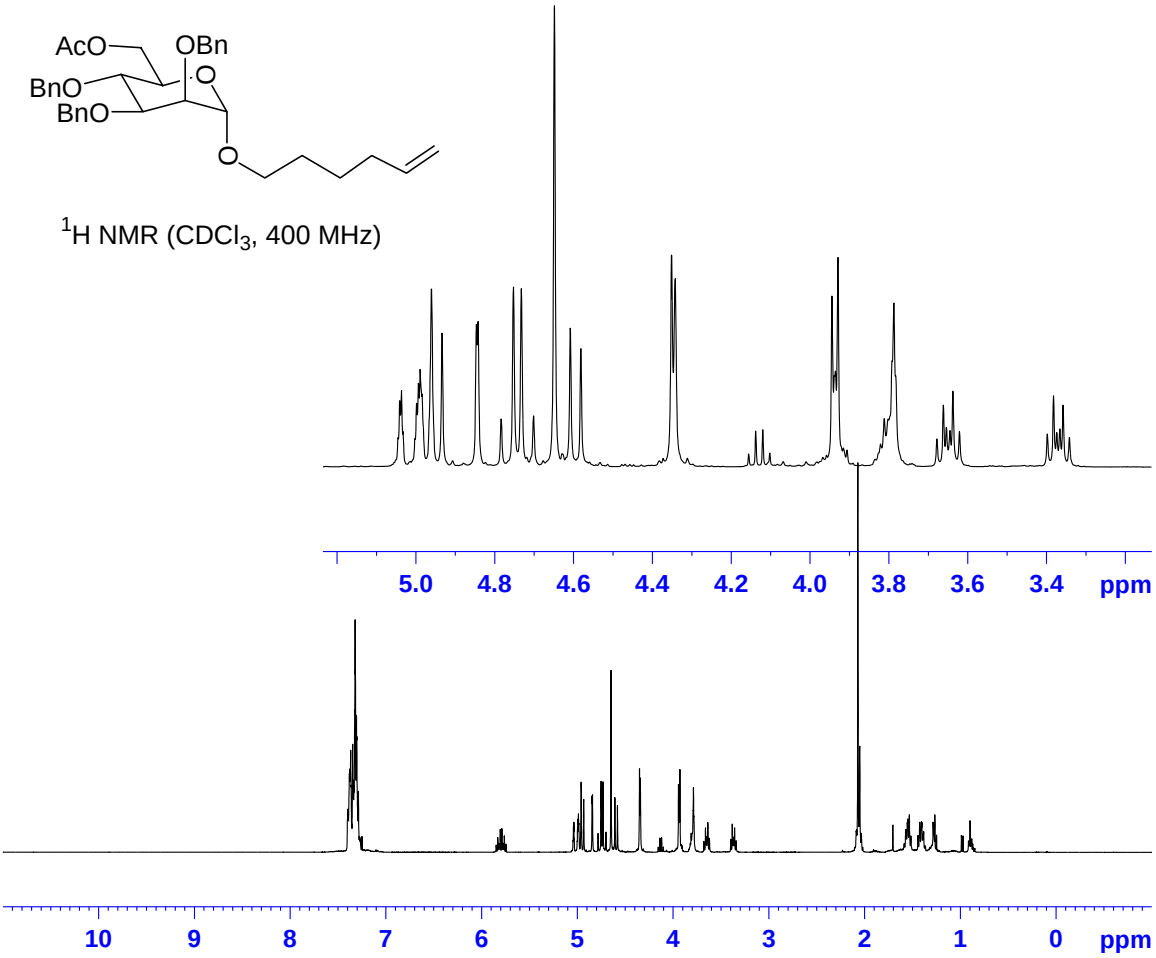


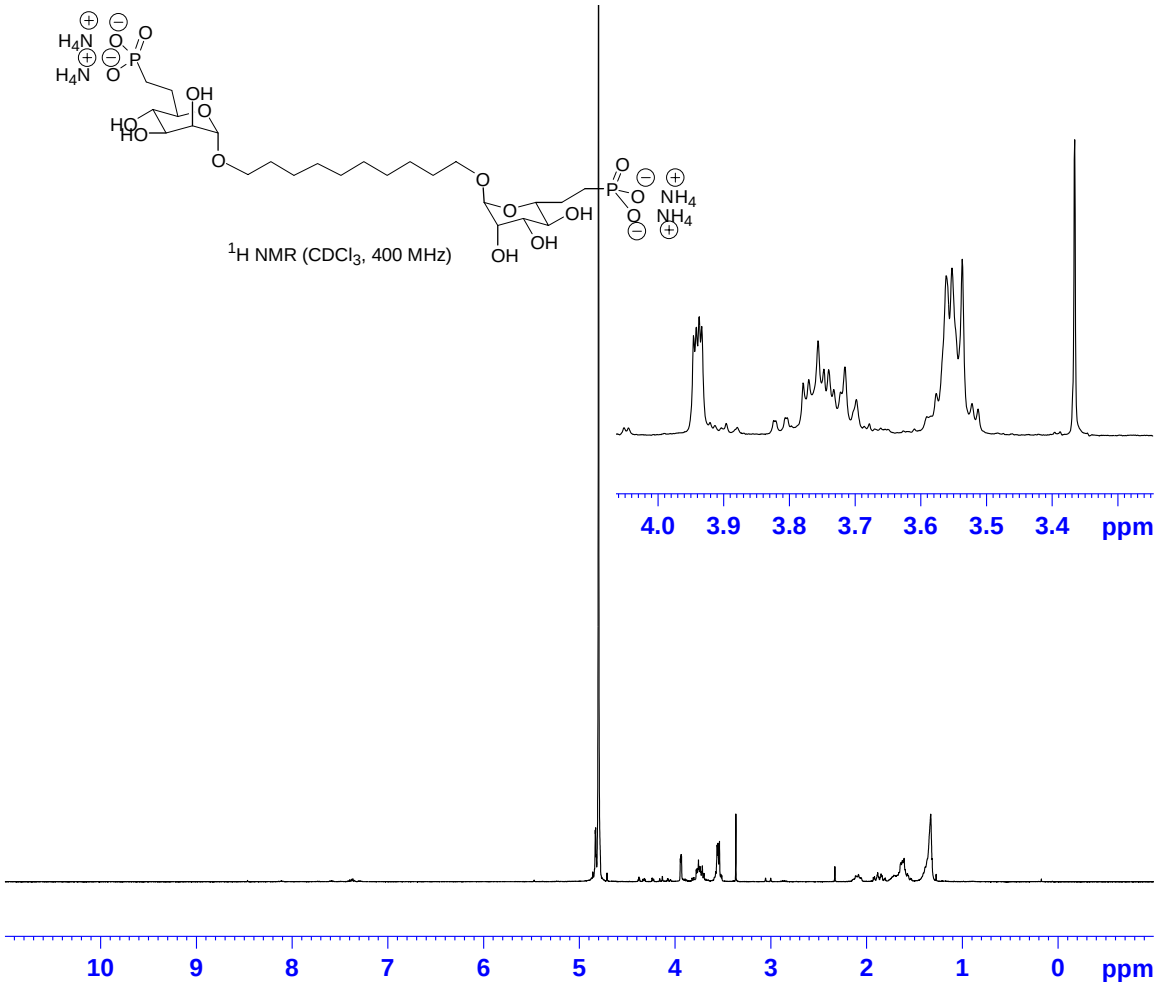
^{13}C NMR ($CDCl_3$, 100 MHz)

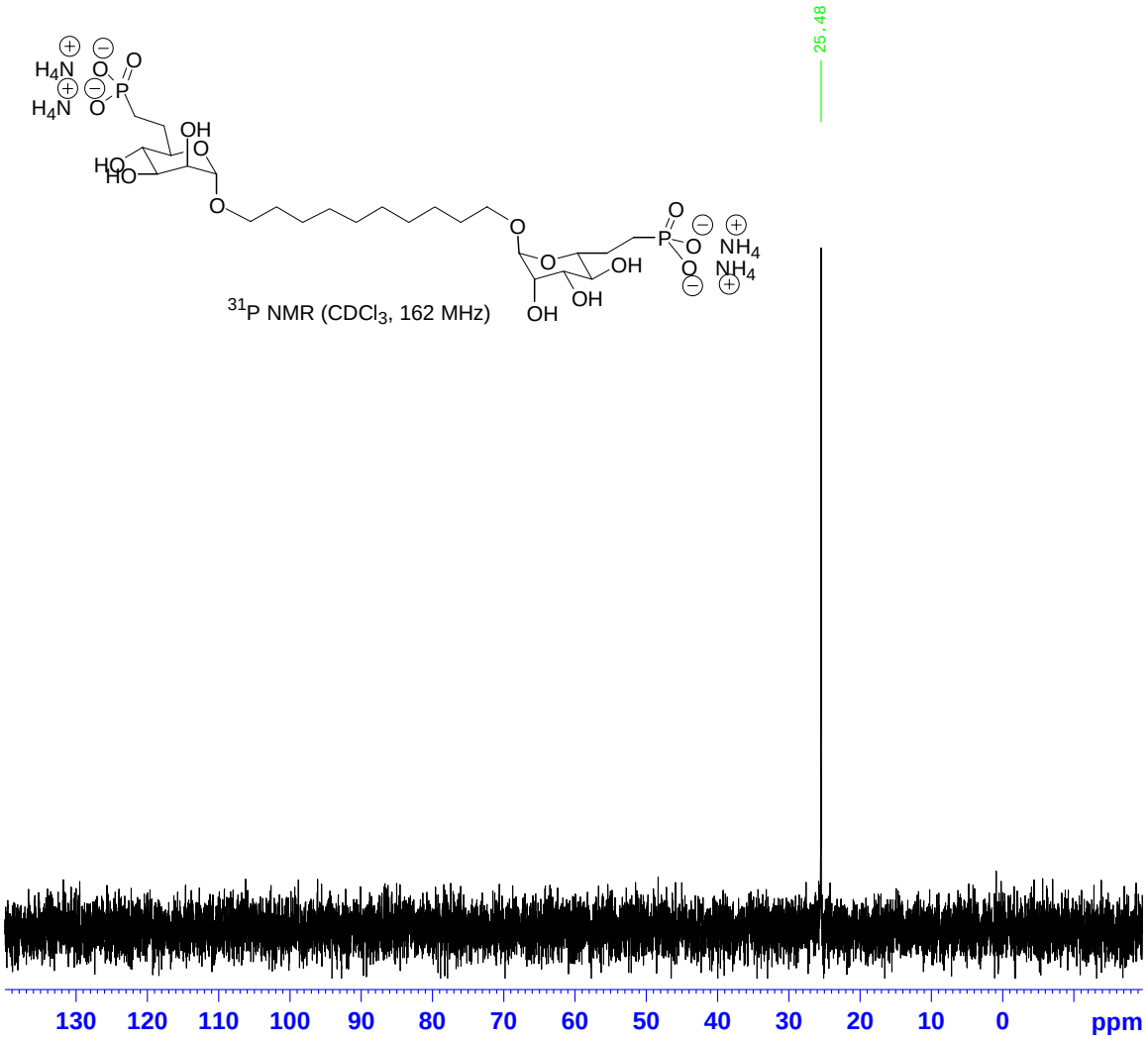


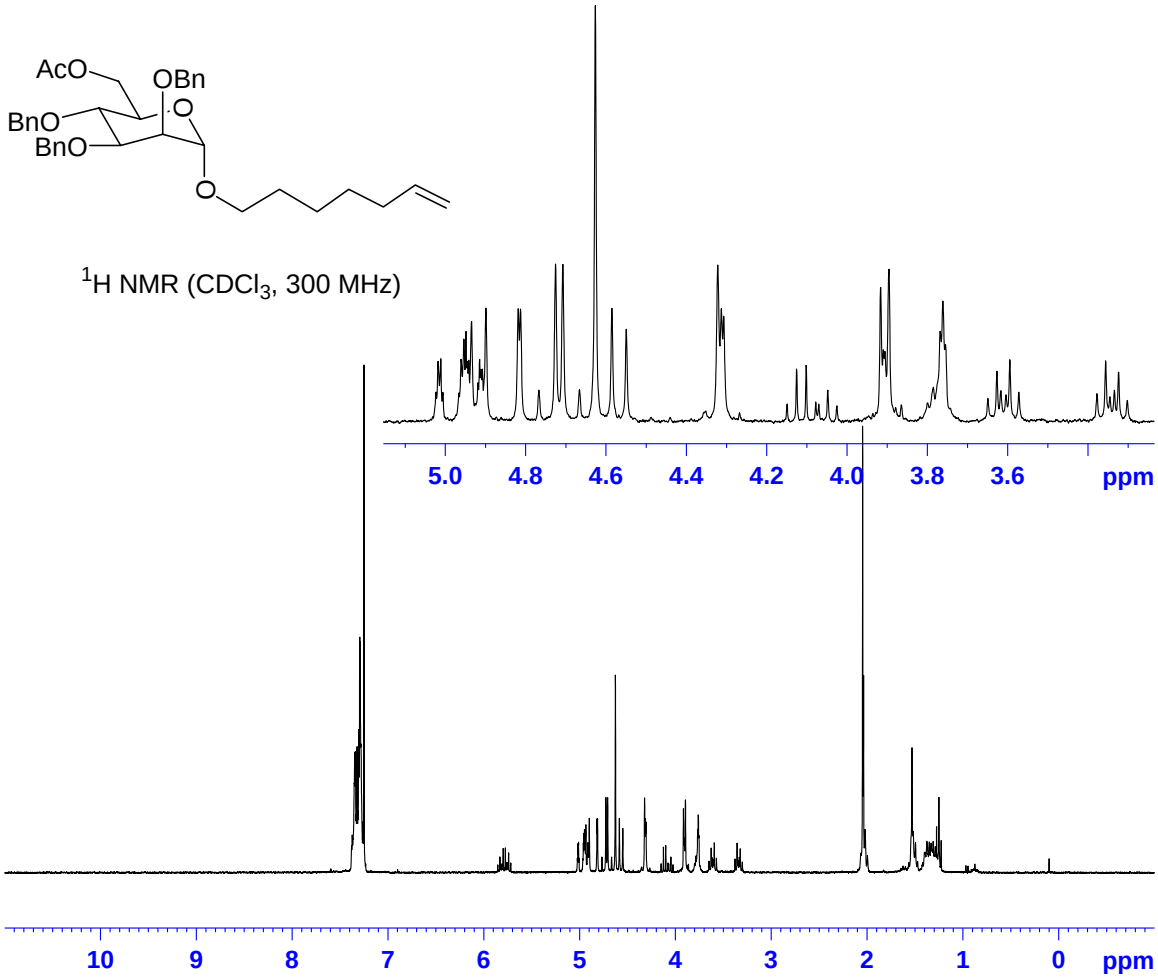
^{31}P NMR ($CDCl_3$, 162 MHz)

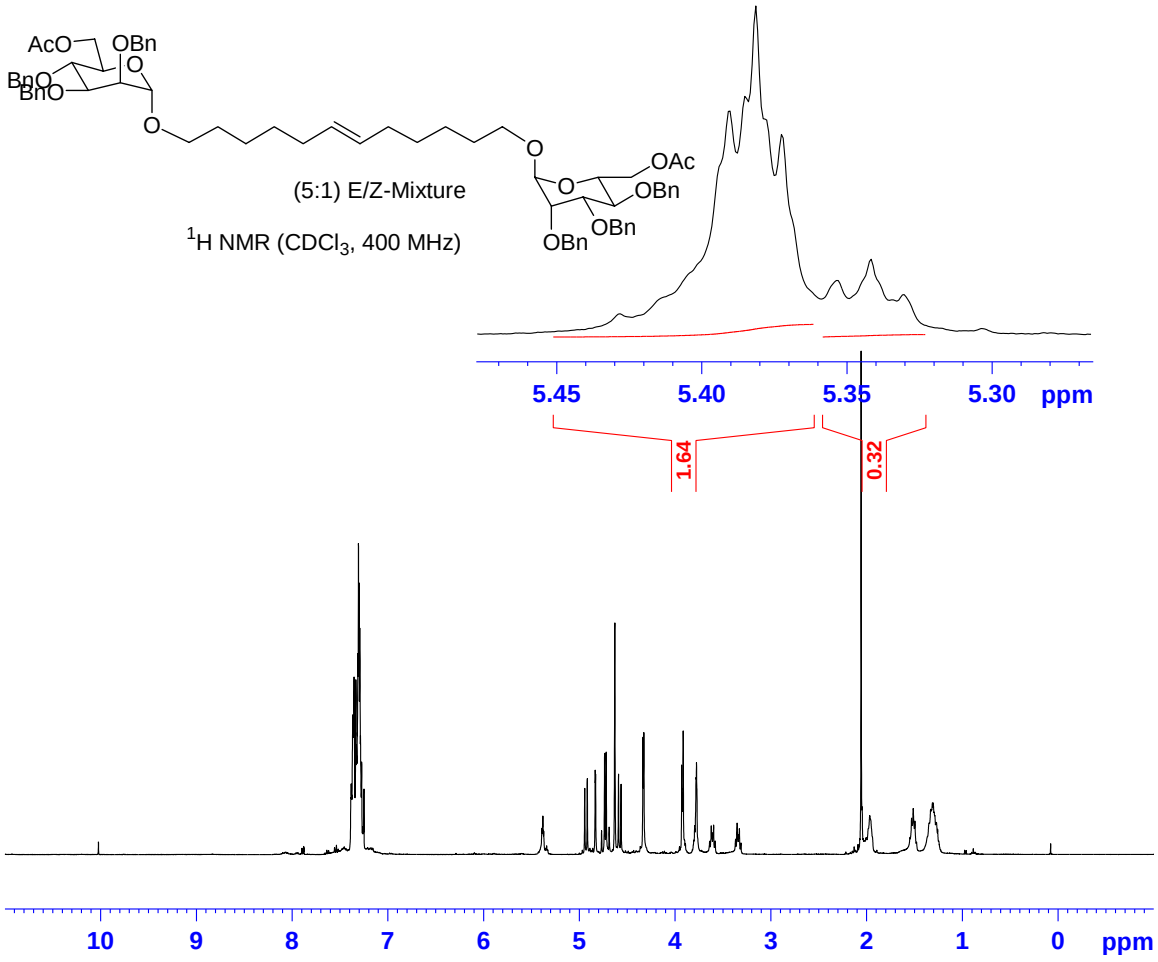


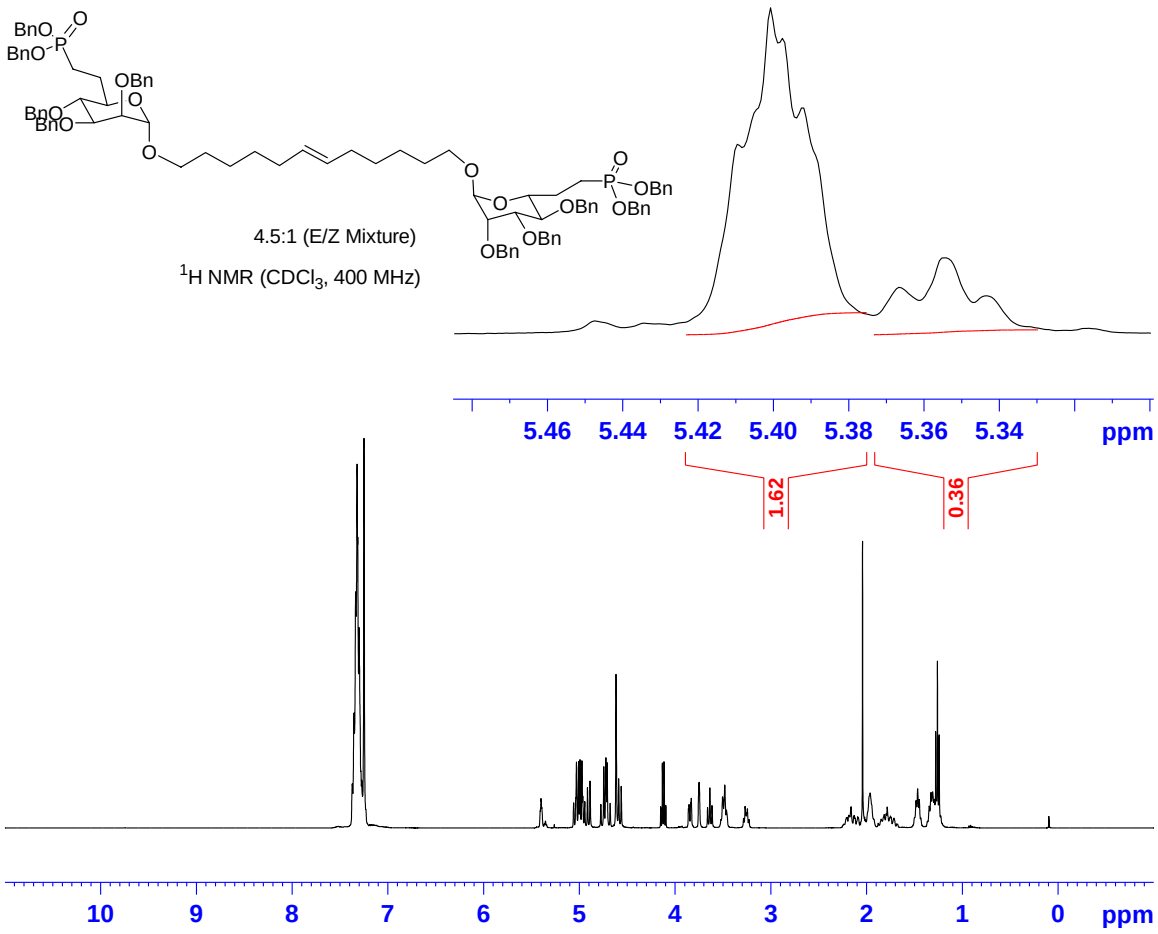

AcO
OBn
BnO
O
BnO
O
^{1}H NMR ($CDCl_3$, 400 MHz)
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
ppm
10
9
8
7
6
5
4
3
2
1
0
ppm


^{1}H NMR ($CDCl_3$, 400 MHz)
4.0 3.9 3.8 3.7 3.6 3.5 3.4 ppm
10 9 8 7 6 5 4 3 2 1 0 ppm


25.48
^{31}P NMR ($CDCl_3$, 162 MHz)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm


AcO
OBn
O
BnO
BnO
O
^{1}H NMR ($CDCl_3$, 300 MHz)
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
ppm
10
9
8
7
6
5
4
3
2
1
0
ppm


AcO
OBn
O
BnO
BnO
O
O
O
OAc
O
OBn
OBn
OBn
(5:1) E/Z-Mixture
1H NMR (CDCl3, 400 MHz)
5.45
5.40
5.35
5.30
ppm
1.64
0.32
10
9
8
7
6
5
4
3
2
1
0
ppm


BnO
BnO
O
P
OBn
BnO
BnO
O
O
O
O
O
P
OBn
OBn
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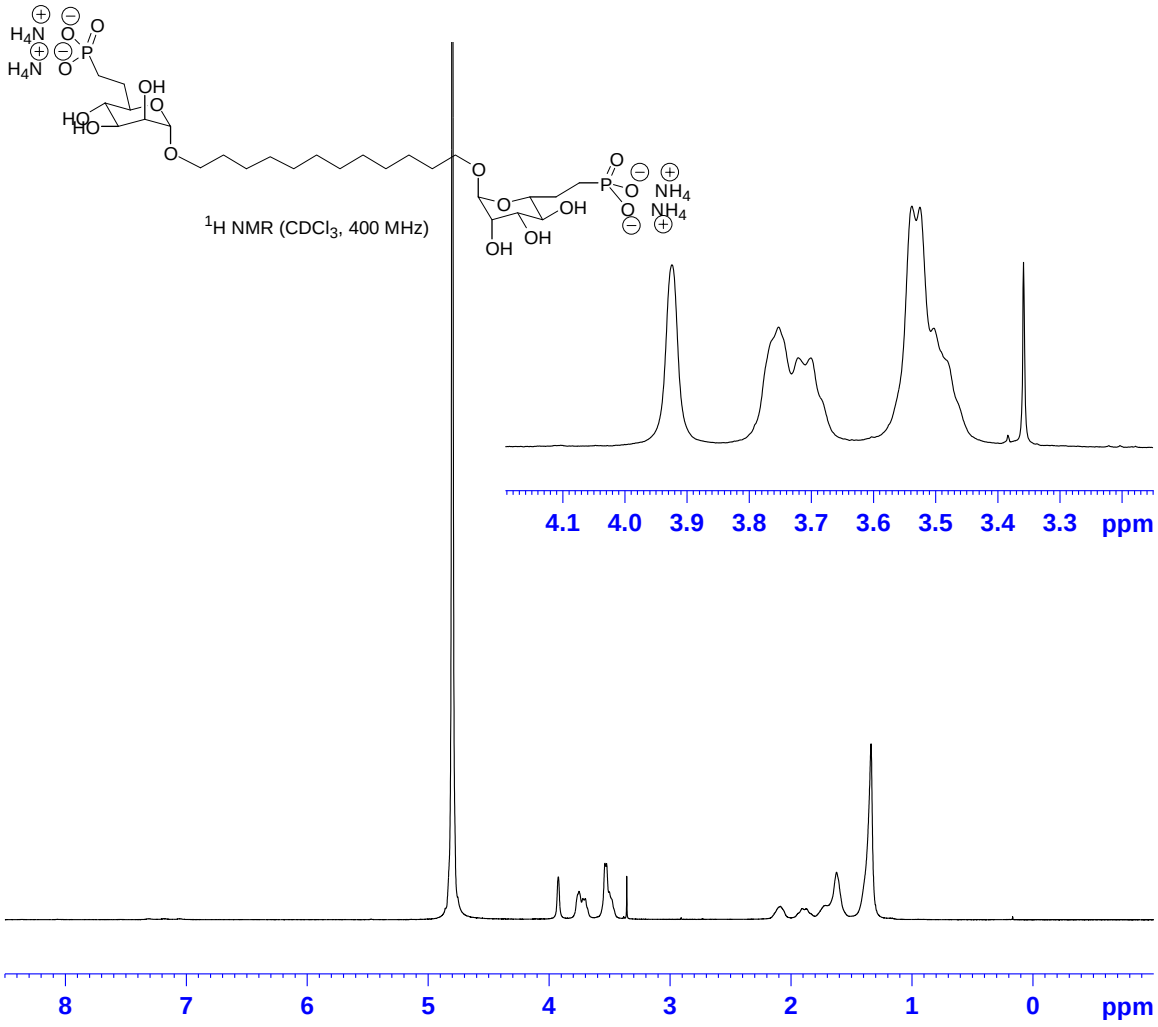
OBn
OBn
4.5:1 (E/Z Mixture)
1H NMR (CDCl3, 400 MHz)
5.46
5.44
5.42
5.40
5.38
5.36
5.34
ppm
1.62
0.36
10
9
8
7
6
5
4
3
2
1
0
ppm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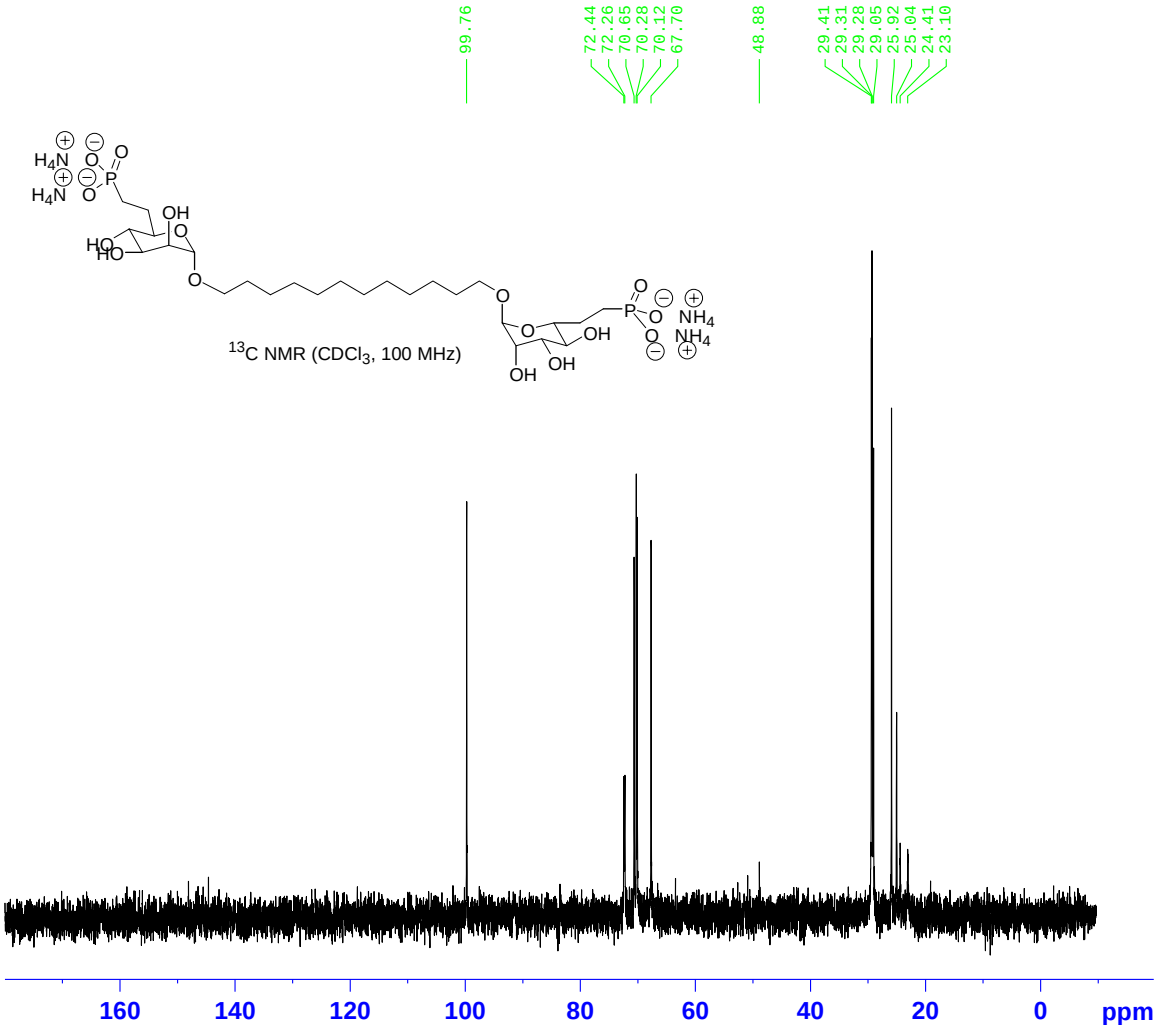
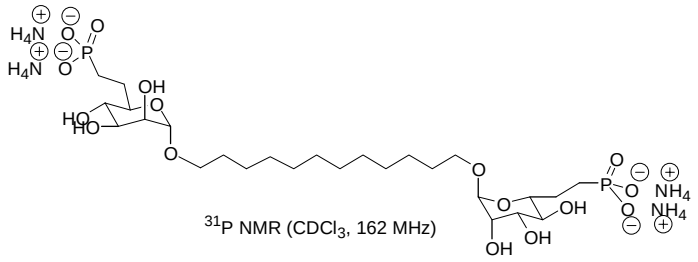
8 7 6 5 4 3 2 1 0 ppm


99.76
72.44
72.26
70.65
70.28
70.12
67.70
48.88
29.41
29.31
29.28
29.05
25.92
25.04
24.41
23.10
^{13}C NMR ($CDCl_3$, 100 MHz)
160
140
120
100
80
60
40
20
0
ppm


26.41
^{31}P NMR (CDCl$_3$, 162 MHz)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm

Radioligand Displacement Assays

Materials: D-Mannose 6-phosphate (Man-6-P), disodium salt, hydrate (M-6876), β-D-glucose 6-phosphate (Glc-6-P), monosodium salt, (G-7879), and cyanogen bromide (CNBr)-activated-Sepharose 4B (C-9142) were purchased from Sigma (St. Louis, MO). IODOGEN™ pre-coated tube iodination reagent was obtained from Pierce (Rockford, IL). Carrier-free $Na^{125}I$, specific activity ~17 Ci/mg, was obtained from Perkin-Elmer (Boston, MA). Pentamannosyl phosphate (PMP) was prepared from the native Y-2448 *O*-phosphomannan of *Hansenula holstii* according to the method of Murray and Neville[1]. This material was coupled to CNBr-activated Sepharose 4B in order to prepare M6P/IGF2R affinity resin as described previously[2]. Pentamannosyl phosphate-bovine serum albumin (PMP-BSA), a pseudoglycoprotein ligand for the M6P/IGF2R was prepared by direct coupling of PMP with BSA in the presence of $NaCNBH_3$ as described previously[2]. PMP-BSA was radioiodinated to a specific activity of 50-74 Ci/g, using IODOGEN™ according to the manufacturer's recommendations.

Preparation of M6P/IGF2R-Sepharose resin: Soluble bovine M6P/IGF2R was purified from fetal bovine serum by the procedure of Valenzano et al.[3] as modified by Byrd et al.[4] In brief, 500 ml of serum was diluted with an equal volume of buffer to yield final concentrations of 25 mM HEPES, pH 7.4, 0.15 M NaCl, 0.1% Triton X-100, 5 mM β-glycerophosphate (column buffer conditions). This diluted serum was pumped at a rate of 5 mL per min over a 10-mL column of PMP-Sepharose. The column was subsequently washed with 5 x 5 mL of column buffer and then eluted with 5 x 5 mL of column buffer supplemented with 10 mM Man-6-P. The fractions containing purified

receptor were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie blue R-250 staining and pooled. The pooled material was dialyzed to remove Man-6-P, lyophilized and stored at –80˚C. Analysis of material purified by these methods using gel filtration chromatography and native gel electrophoresis indicated that the receptor is present as both a monomer (~70%) and a dimer (30%)[4]. M6P/IGF2R-Sepharose resin was used in ligand binding assays and prepared by coupling the purified, redissolved receptor to CNBr-activated Sepharose 4B according to the manufacturer's instructions. For coupling, 1 g of CNBr-activated Sepharose 4B dry resin was rehydrated in 15 mL of 1 mM HCl for 15 min at room temperature then washed with 4 x 10 mL of 1 mM HCl followed by 2 x 10 mL of coupling buffer (0.1 M $NaHCO_3$, pH 8.0, 0.5 M NaCl). The wet resin cake (~3 mL volume) was transferred to a tube containing 1.2-1.4 mg of purified receptor dissolved in 5 mL of coupling buffer. The mixture was incubated on an end-over-end mixer at 4˚C for 16 hours for coupling. Uncoupled sites on the resin were blocked by incubation at 4˚C with 0.5 M glycine, pH 8.0, to produce a final affinity reagent of ~0.5 mg M6P/IGF2R per mL resin beads. This material was stored as a 50% slurry at 4˚C in 25 mM HEPES, pH 7.4, 0.15 M NaCl, 0.02 mM NaN_3, 0.05% Triton X-100 until use.

Competitive Binding Analysis for Man-6-P Analogues: A M6P/IGF2R-Sepharose 4B resin-based radioligand displacement assay was used to evaluate the ability of each of the Man-6-P analogues to bind the receptor. When ^{125}I-PMP-BSA was used as the radioligand, aliquots (10 μL) of receptor resin were incubated with 2 nM of ^{125}I-PMP-BSA in the presence of increasing concentrations of the Man-6-P analogues (0 mM, or

0.0001 to 1 mM) in assay buffer (25 mM HEPES, pH 7.4, 0.15 M NaCl, 0.05% Triton X-100) for 16 h at 10˚C on an end-over-end clinical mixer. As positive and negative controls, respectively, parallel assays were done that contained, increasing concentrations of Man-6-P (0.0001 to 10 mM) or Glc-6-P (1, 3 and 10 mM). The resin pellets were collected by centrifugation for 30 sec at 8,000 x g at 4˚C and the resin pellets were washed with 2 x 1 mL of ice-cold assay buffer. The tips of the tubes bearing the resin pellets were cut and counted in a γ counter. The data were converted into percentage binding values based on comparison with the analogue-free controls (designated as 100% binding). The competitive binding data were graphed as semi-log plots of binding vs. concentration of Man-6-P, Glc-6-P or the analogues. Best-fit curves were generated by nonlinear regression analysis using Prism GraphPad software (San Diego, CA), which also allowed estimation of IC_{50}, the concentration that displaces 50% of radioligand binding. Values for relative binding affinity (RBA) for the Man-6-P analogues were calculated by dividing the IC_{50} value for each analogue into that of Man-6-P for a given experiment.

Displacement Data

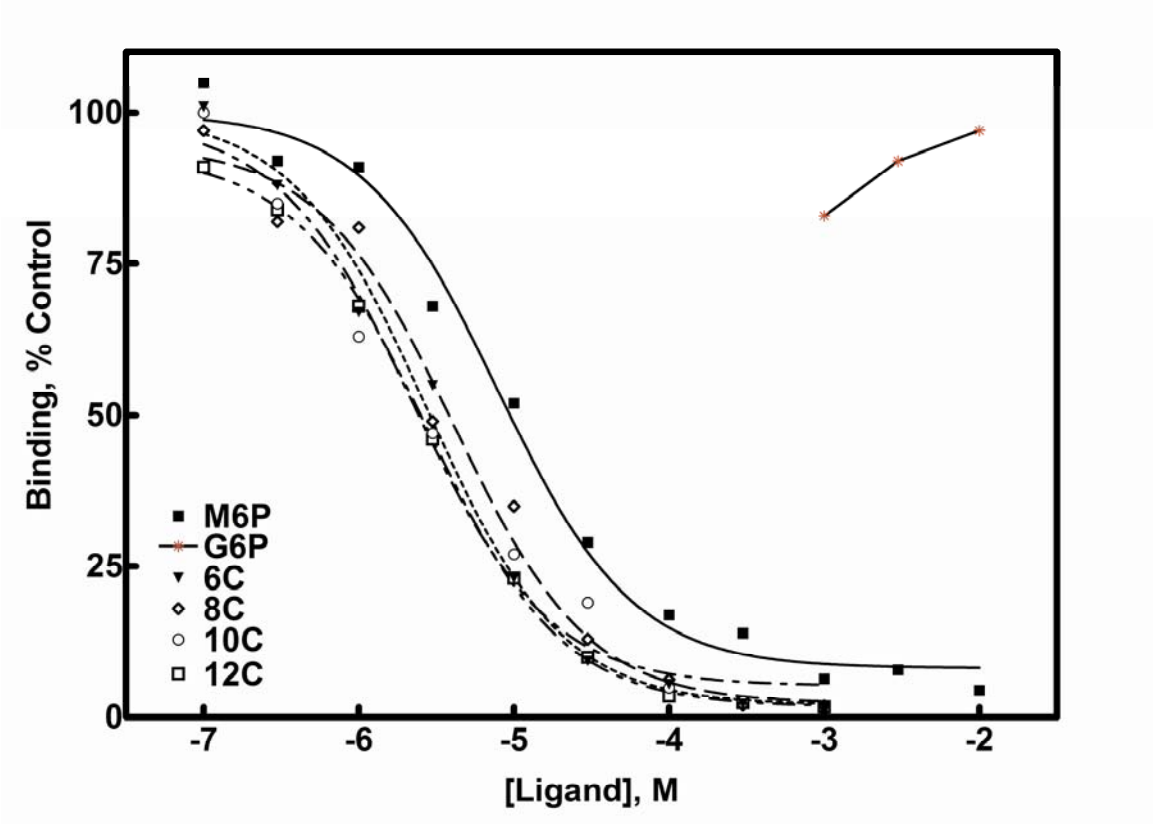


Figure S1. Competitive binding analysis for Man-6-P surrogates in displacement of PMP-BSA from M6P/IGF2R-Sepharose. Each of the compounds was tested at the indicated concentrations as described in Materials and Methods for its ability to displace ^{125}I-PMP-BSA tracer from the M6P/IGF2R-Sepharose resin. Man-6-P and Glc-6-P were analyzed in parallel as positive and negative controls, respectively. Curves were fitted for each data set by nonlinear regression. These data are representative of 4 replicate experiments.

Ligand	Radioligand: PMP-BSA		
	IC_{50}(n) μM	RBA	M_r
M6P	11.5 ± 2.51 (4)	1.0	340
G6P	>10 (4)	N.A.	282
6C	4.76 ± 2.50 (4)	2.63 ± 0.74	666
8C	5.03 ± 1.34 (4)	2.39 ± 0.83	694
10C	4.44 ± 1.40 (4)	2.65 ± 0.52	722
12C	3.70 ± 0.56 (4)	3.02 ± 0.11	750

Table S1. The data have been calculated to determine the IC_{50}, in μM, of each compound that displaced 50% of the radioligand tracer in a binding assay utilizing bovine soluble IGF2R immobilized on Sepharose 4B. Values in parentheses denote the number of replicate experiments done yielding mean ± SEM for each compound. Relative binding affinities (RBAs) were calculated by dividing the IC_{50} value for each compound into that of the IC_{50} of M6P, which was as indicated.

References and notes:

§ *Note: These authors contributed equally to this work.*

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