Supplementary information

On resin synthesis of phosphoethanolamine cellulose

Jhih-Yi Huang[a,b] and Martina Delbianco*[a]

a. Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, Am Mühlenberg 1, 14476 Potsdam, Germany.

b. Department of Chemistry and Biochemistry, Freie Universitäte Berlin, Arnimallee 22, 14195 Berlin, Germany.

Table of Contents

1. Chemical synthesis

All chemicals used were reagent grade and used as supplied unless otherwise noted. The automated syntheses were performed on a home-built synthesizer developed at the Max Planck Institute of Colloids and Interfaces[1], or the commercial synthesizer Glyconeer 3.1 (GlycoUniverse, Germany). Analytical thin-layer chromatography (TLC) was performed on Merck silica gel 60 F254 plates (0.25 mm). Compounds were visualized by UV irradiation or dipping the plate in a staining solution (sugar stain: 10% H_2SO_4 in EtOH; CAM: 48 g/L ammonium molybdate, 60 g/L ceric ammonium molybdate in 6% H_2SO_4 aqueous solution). Flash column chromatography was carried out by using forced flow of the indicated solvent on Fluka Kieselgel 60 M (0.04 – 0.063 mm). Analysis and purification by reverse phase HPLC was performed by using an Agilent 1260 series equipped with an evaporative light scattering detector (ELSD). Products were lyophilized using a Christ Alpha 2-4 LD plus freeze dryer. ^{1}H, ^{13}C, ^{31}P and HSQC NMR spectra were recorded on a Bruker 400-MR (400 MHz), Varian 600-MR (600 MHz), or Bruker Biospin AVANCE700 (700 MHz) spectrometer. Spectra were recorded in $CDCl_3$ or D_2O using the solvent as the internal standard in ^{1}H NMR ($CDCl_3$,: 7.26 ppm ^{1}H, D_2O: 4.79 ppm ^{1}H). The ^{1}H NMR were acquired without heteroatom decoupling. The ^{13}C and ^{31}P were acquired with hydrogen atom decoupling. ^{1}H NMR integrals of the resonances corresponding to residues at the reducing end are reported as non-integer numbers and the sum of the integrals of α and β anomers is set to 1. NMR spectra were processed using MestreNova 14.3 (MestreLab Research). High resolution mass spectra were obtained using a 6210 ESI-TOF mass spectrometer (Agilent) and a MALDI-TOF autoflex™ (Bruker). MALDI and ESI mass spectra were run on IonSpec Ultima instruments.

2. Building blocks for AGA

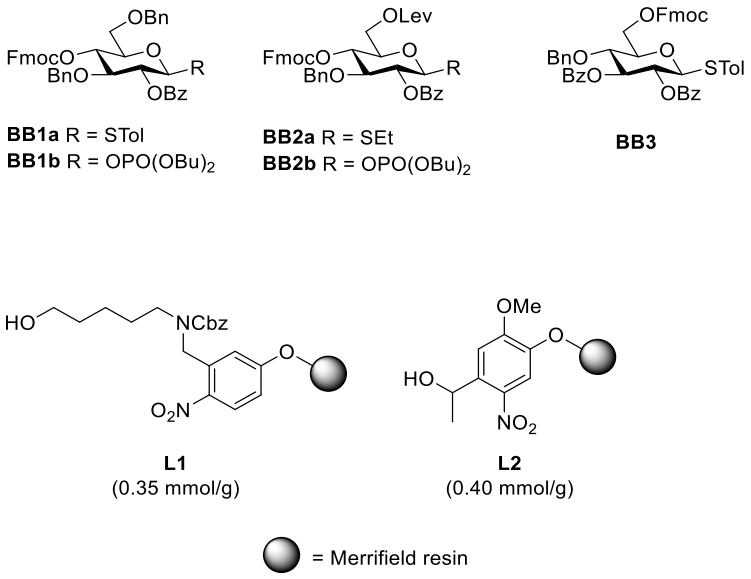


Figure S1 BBs and solid supports used in this work.

Building block **BB1a** and **BB3** were purchased from GlycoUniverse (Germany). Building block **BB1b**, **BB2a** and **BB2b** were synthesized according to previously reported procedures.[2, 3] Merrifield resin equipped with a photocleavable linker (**L1**, loading 0.35 mmol/g and **L2**, loading 0.40 mmol/g) was prepared according to previous literature.[4]

3. Automated glycan assembly

3.1 General materials and methods

The automated syntheses were performed on a home-built synthesizer developed at the Max Planck Institute of Colloids and Interfaces or the commercial synthesizer Glyconeer 3.1 (GlycoUniverse, Germany). All solvents used were HPLC-grade. The solvents used for the building blocks, activator, TMSOTf and capping solutions were taken from an anhydrous solvent system (J.C. Meyer). The building blocks were co-evaporated three times with toluene and dried for 1 h on high vacuum before use. Oven-heated, argon-flushed flasks were used to prepare all moisture-sensitive solutions. Activator, capping, deprotection, acidic wash and building block solutions were freshly prepared and kept under argon during the automation run. All yields of products obtained by AGA were calculated on the basis of resin loading. Resin loading was determined following previously established procedures.[5]

3.2 Preparation of stock solutions

- **Building block solution**: Between 0.05 and 0.10 mmol of building block (depending on the BB, see Module C1 and C2) was dissolved in CH_2Cl_2 (1 mL).
- **NIS/TfOH activator solution**: 1.35 g (6.0 mmol) of recrystallized NIS was dissolved in 40 mL of a 2:1 v/v mixture of anhydrous CH_2Cl_2 and anhydrous dioxane. Then triflic acid (55 µL, 0.6 mmol) was added. The solution was kept at 0 °C (ice bath) for the duration of the automation run.
- **Fmoc deprotection solution**: A solution of 20%$_{v/v}$ piperidine in DMF was prepared.
- **Lev deprotection solution**: Hydrazine acetate (550 mg, 5.97 mmol) was dissolved in pyridine/AcOH/H_2O (40 mL, v/v, 32:8:2) and sonicated for 10 min.
- **TMSOTf solution**: TMSOTf (0.45 mL, 2.49 mmol) was added to CH_2Cl_2 (40 mL).
- **TMSOTf solution 2**: TMSOTf (0.2 mL, 1.1 mmol) was added to CH_2Cl_2 (80 mL). This solution was used for the synthesis of **APA_4** and **$(AP)_3$** with **BB1b** and **BB2b**.
- **Capping solution**: A solution of 10%$_{v/v}$ acetic anhydride and 2%$_{v/v}$ methanesulfunic acid in CH_2Cl_2 was prepared.

3.3 Modules for automated synthesis

Module A: Resin preparation
All automated syntheses were performed on 0.0125 mmol scale. Resin (**L1** or **L2**) is placed in the reaction vessel and swollen in CH_2Cl_2 for 20 min at room temperature prior to the synthesis. During this time, all reagent lines needed for the synthesis are washed and primed. After the swelling, the resin is washed with DMF, THF, and CH_2Cl_2 (three times each with 2 mL for 25 s).

Module B: Acidic wash with TMSOTf solution (20 min)
The resin is swollen in 2 mL CH_2Cl_2 and the temperature of the reaction vessel adjusted to -20 °C. Upon reaching the low temperature, TMSOTf solution (1 mL) is added dropwise to the reaction vessel. After bubbling for 3 min, the acidic solution is drained and the resin is washed with 2 mL CH_2Cl_2 for 25 s.

Action	Cycles	Solution	Amount	T (°C)	Incubation time
Cooling	-	-	-	-20	(15 min)*

Deliver	1	CH_2Cl_2	2 mL	-20	-
Deliver	1	TMSOTf solution	1 mL	-20	3 min
Wash	1	CH_2Cl_2	2 mL	-20	25 sec

*Time required to reach the desired temperature.

Module C1: Thioglycoside glycosylation (35 min)

The building block solution (0.10 mmol of BB in 1 mL of CH_2Cl_2 per glycosylation) is delivered to the reaction vessel. After the set temperature is reached, the reaction is started by dropwise addition of the NIS/TfOH activator solution (1.0 mL, excess). The glycosylation conditions are building block dependent[6] and reported for each synthesis. After completion of the reaction, the solution is drained and the resin is washed with CH_2Cl_2, CH_2Cl_2:dioxane (1:2, 3 mL for 20 s) and CH_2Cl_2 (two times, each with 2 mL for 25 s). The temperature of the reaction vessel is increased to 25 °C for the next module.

Action	Cycles	Solution	Amount	T (°C)	Incubation time
Cooling	-	-	-	T_1	-
Deliver	1	BB solution	1 mL	T_1	-
Deliver	1	NIS/TfOH activator solution	1 mL	T_1	-
Reaction	1	-	-	T_1 to T_2	t_1 t_2
Wash	1	CH_2Cl_2	2 mL	T_2	5 sec
Wash	1	CH_2Cl_2 : Dioxane (1:2)	2 mL	T_2	20 sec
Heating	-	-	-	25	-
Wash	2	CH_2Cl_2	2 mL	> 0	25 sec

Module C2: Glycosyl phosphate glycosylation (45 min)

The building block solution (0.05 mmol of BB in 1 mL of CH_2Cl_2 per glycosylation) is delivered to the reaction vessel. After the set temperature is reached, the reaction is started by dropwise addition of the TMSOTf solution (1.0 mL, stoichiometric or catalytic). After completion of the reaction, the solution is drained and the resin is washed with CH_2Cl_2 (six times, each one is with 2 mL for 25 s). The temperature of the reaction vessel is increased to 25 °C for the next module.

Action	Cycles	Solution	Amount	T (°C)	Incubation time
Cooling	-	-	-	T_1	-
Deliver	1	BB solution	1 mL	T_1	-
Deliver	1	TMSOTf solution	1 mL	T_1	-
Reaction time	1	-	-	T_1 to T_2	t_1 t_2
Wash	1	CH_2Cl_2	2 mL	T_2	5 sec
Heating	-	-	-	25	-
Wash	6	CH_2Cl_2	2 mL	> 0	25 sec

Module D: Capping (30 min)

The resin is washed with DMF (two times with 2 mL for 25 s) and the temperature of the reaction vessel adjusted to 25 °C. A pyridine solution (2 mL, 10%$_{v/v}$ in DMF) is delivered into the reaction vessel. After 1 min, the reaction solution is drained and the resin is washed with CH_2Cl_2 (three times with 3 mL for 25 s). Capping solution (4 mL) is delivered into the reaction vessel. After 20 min, the reaction solution is drained and the resin is washed with CH_2Cl_2 (three times with 3 mL for 25 s).

Action	Cycles	Solution	Amount	T (°C)	Incubation time
Heating	-	-	-	25	(5 min)*
Wash	2	DMF	2 mL	25	25 sec
Deliver	1	10% Pyridine in DMF	2 mL	25	1 min
Wash	3	CH_2Cl_2	2 mL	25	25 sec
Deliver	1	Capping Solution	4 mL	25	20 min
Wash	3	CH_2Cl_2	2 mL	25	25 sec

*Time required to reach the desired temperature.

Module E1: Fmoc deprotection (9 min)

The resin is washed with DMF (three times with 2 mL for 25 s) and the temperature of the reaction vessel is adjusted to 25 °C. Fmoc deprotection solution (2 mL) is delivered to the reaction vessel and kept under Ar bubbling. After 5 min, the reaction solution is drained and the resin is washed with DMF (three times with 3 mL for 25 s) and CH_2Cl_2 (five times each with 2 mL for 25 s). The temperature of the reaction vessel is decreased to -20 °C for the next module.

Action	Cycles	Solution	Amount	T (°C)	Incubation time
Wash	3	DMF	2 mL	25	25 sec
Deliver	1	Fmoc depr. solution	2 mL	25	5 min

Wash	1	DMF	2 mL		
Cooling	-	-	-	-20	-
Wash	3	DMF	2 mL	< 25	25 sec
Wash	5	CH_2Cl_2	2 mL	< 25	25 sec

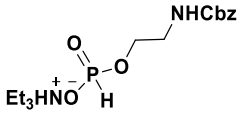
Module E2: Lev deprotection (90 min)

The resin is washed with CH_2Cl_2 (three times with 2 mL for 25 s). CH_2Cl_2 (1.3 mL) is delivered to the reaction vessel and the temperature of the reaction vessel is adjusted to 25 °C. Lev deprotection solution (2 mL) is delivered to the reaction vessel, kept under Ar bubbling for 30 min. This procedure is repeated twice. The reaction solution is drained and the resin is washed with DMF (three times with 3 mL for 25 s) and CH_2Cl_2 (five times each with 2 mL for 25 s).

Action	Cycles	Solution	Amount	T (°C)	Incubation time
Wash	3	DMF	2 mL	25	25 sec
Deliver	2	Lev depr. solution	2 mL	25	30 min
Wash	1	DMF	2 mL	-	-
Cooling	-	-	-	-20	-
Wash	3	DMF	2 mL	< 25	25 sec
Wash	5	CH_2Cl_2	2 mL	< 25	25 sec

3.4 Post-AGA manipulations

Module F1: On-resin phosphorylation using H-phosphonate 4

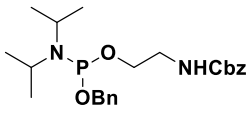


4

4 was prepared according to a previously established procedure.[7]

A solution of **4** (5 equiv. per hydroxyl group) in anhydrous pyridine (4 mL) is evaporated and dried *in vacuo* overnight. The vacuum-dried resin-bound oligosaccharide is places in a 5 mL fritted syringe and washed with anhydrous CH_2Cl_2 (5 x 4 mL) to ensure high swelling of resin. After removal of CH_2Cl_2, a solution of **4** in anhydrous pyridine (1 mL) is added to the resin-bound oligosaccharide, followed by pivaloyl chloride (5 equiv. per hydroxyl group) in anhydrous pyridine (3 mL) and the suspension is gently shaken at room temperature for 5 h. The reaction solution is drained and the resin is washed with CH_2Cl_2 (5 x 4 mL). A solution of iodine (20 equiv. per hydroxyl group) in pyridine/H_2O (4 mL, v/v, 19:1) is added and the mixture is gently shaken at room temperature for another 2 h. The reaction solution is drained and the resin is repeatedly washed with CH_2Cl_2 (5 x 4 mL).

Module F2: On-resin phosphorylation using phosphoramidite 5



5

5 was prepared according to a previously established procedure.[8]

A solution of **5** (5 equiv. per hydroxyl group) in anhydrous pyridine (4 mL) is evaporated and dried *in vacuo* overnight. The vacuum-dried resin-bound oligosaccharide is places in a 5 mL fritted syringe and washed with anhydrous CH_2Cl_2 (5 x 4 mL) to ensure high swelling of resin. After removal of CH_2Cl_2,a solution of **5** in anhydrous CH_2Cl_2 (2 mL) is added to the resin-bound oligosaccharide, followed by tetrazole, 3 to 4 wt.% solution in MeCN (5 equiv. per hydroxyl group) in anhydrous CH_2Cl_2 (2 mL). The suspension is gently shaken at room temperature for 5 h. The reaction solution is drained and the resin is washed using CH_2Cl_2 (5 x 4 mL). A solution of iodine (20 equiv. per hydroxyl group) in pyridine/H_2O (4 mL, v/v, 19:1) is added and the mixture is gently shaken at room temperature for another 2 h. The reaction solution is drained and the resin is repeatedly washed with CH_2Cl_2 (5 x 4 mL).

Module G: On-resin methanolysis

The resin is suspended in THF (4 mL). MeONa in MeOH (0.5 M, 0.4 mL) is added and the suspension is gently shaken at room temperature. After micro-cleavage (see *Module H2*) indicates the complete removal of all ester groups, the reaction solution is drained and the resin is repeatedly washed with MeOH (5 x 4 mL) and CH_2Cl_2 (5 x 4 mL).

Module H1: Cleavage from solid support

The oligosaccharide is cleaved from the solid support using a continuous-flow photoreactor as described previously.[9]

Module H2: Micro-cleavage from solid support

Trace amount of resin (around 20 beads) is dispersed in CH_2Cl_2 (0.1 mL) and irradiated with a UV lamp (6 W, 356 nm) for 10 min. MeCN (10 µL) is then added to the resin and the resulting solution is analyzed by MALDI.

Module I: Hydrogenolysis at ambient pressure

The crude compound obtained from *Module H1* is dissolved in 2 mL of EtOAc:*t*BuOH:H_2O (2:1:1). $Pd(OH)_2/C$ (2.5 times the weight of the starting material) is added to the stirred vial, the reaction is purged for 5 min with a N_2 balloon, and equipped with a H_2 balloon. The reaction progress is monitored to avoid undesired side products formation (*i.e.* degradation of reducing end).[10] Upon completion, prior to filtration, thiourea (10 equiv.) is added to the reaction. The mixture is filtered (PTFE 0.45 µm 25 mm syringe filter, Fisher scientific) and washed with EtOAc, H_2O, and MeCN (4 mL each). The filtrates are concentrated *in vacuo*.

Module J: Purification

The final compounds are analyzed using analytical reversed phase HPLC (Agilent 1200 Series, Methods J3, J5 and J7). The purification of each crude is conducted using manual Sephadex® LH-20 column (Methods J1 and J2) and reverse phase HPLC (Agilent 1200 Series, Methods J4, J6 and J8).

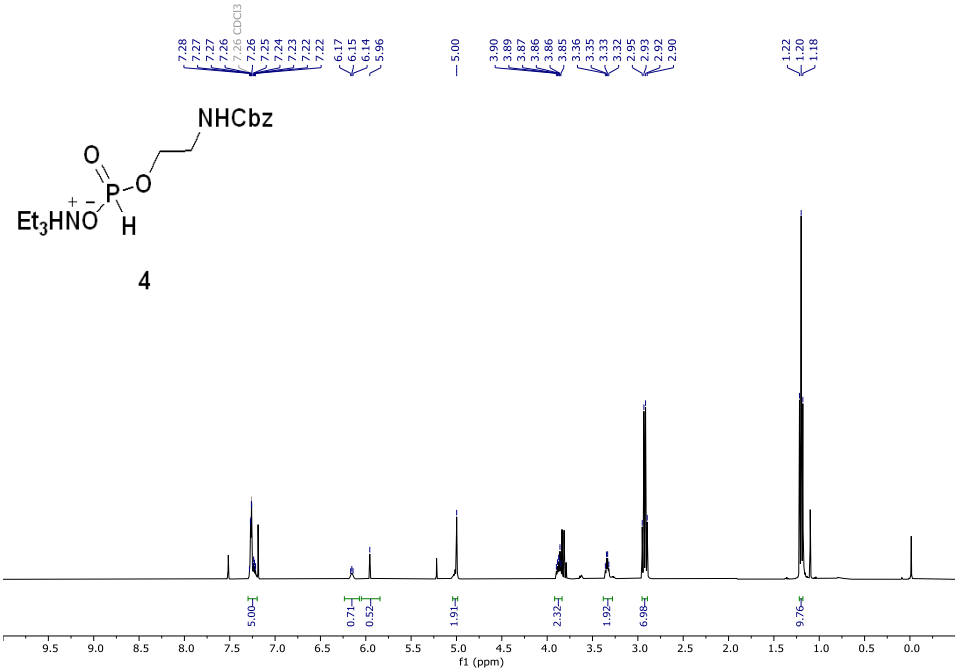
- **Method J1**: Sephadex® LH-20 column with CH_2Cl_2:MeOH (1:1) as eluent, isocratic.
- **Method J2**: Sephadex® LH-20 column with H_2O:MeOH (1:1) as eluent, isocratic.
- **Method J3**: (Hypercarb column, ThermoFisher scientific, 150 x 4.6 mm, 3 µm) flow rate of 0.7 mL/min with H_2O (0.1% formic acid) and MeCN as eluents [isocratic (5 min), linear gradient to 50% MeCN (30 min), linear gradient to 100% MeCN (5 min), isocratic 100% MeCN (10 min)].
- **Method J4 (Prep)**: (Hypercarb column, ThermoFisher scientific, 150 x 10 mm, 5 µm), flow rate of 3 mL/min with H_2O (0.1% formic acid) and MeCN as eluents [isocratic (5 min), linear gradient to 50% MeCN (30 min), linear gradient to 100% MeCN (5 min), isocratic 100% MeCN (10 min)].
- **Method J5**: (Synergi Hydro RP18 column, Phenomenex, 250 x 4.6 mm), flow rate of 1.0 mL/min with H_2O (0.1% formic acid) and MeCN as eluents [isocratic (5 min), linear gradient to 5% MeCN (10 min), linear gradient to 20% MeCN (10 min), isocratic 100% (MeCN 15 min)].
- **Method J6 (Prep)**: (Synergi Hydro RP18 column, Phenomenex, 250 x 10 mm) flow rate of 4.0 mL/min with H_2O (0.1% formic acid) and MeCN as eluents [isocratic (5 min), linear gradient to 5% MeCN (10 min), linear gradient to 100% MeCN (5 min), isocratic 100% MeCN (15 min)].
- **Method J7**: (Hypercarb column, ThermoFisher scientific, 150 x 4.6 mm, 3 µm) flow rate of 0.7 mL/min with H_2O (0.1% formic acid) and MeCN as eluents [isocratic 20% MeCN (5 min), linear gradient to 50% MeCN (20 min), linear gradient to 100% MeCN (10 min)].

- **Method J8 (Prep)**: (Hypercarb column, ThermoFisher scientific, 150 x 10 mm, 5 μm), flow rate of 3 mL/min with H_2O (0.1% formic acid) and MeCN as eluents [isocratic 20% MeCN (5 min), linear gradient to 50% MeCN (20 min), linear gradient to 100% MeCN (10 min)].
- **Method J9**: (Synergi Hydro RP18 column, Phenomenex, 250 x 4.6 mm), flow rate of 1.0 mL/min with H_2O (0.1% formic acid) and MeCN as eluents [isocratic (5 min), linear gradient to 5% MeCN (10 min), linear gradient to 30% MeCN (20 min), isocratic 100% (MeCN 5 min)].

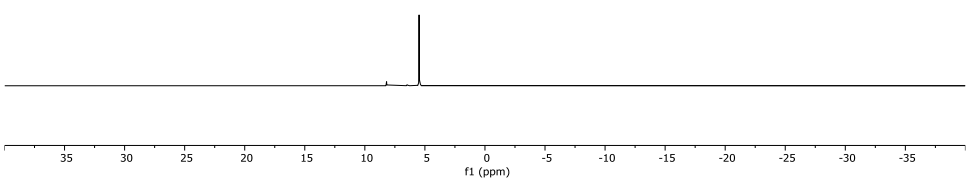
3.5 Characterization of phosphorylating reagent 4 and 5

Characterization of 4: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28 – 7.22 (m, 5H), 6.17 (br, 1H), 5.96 (br, 1H), 5.00 (s, 2H), 3.90 – 3.85 (m, 2H), 3.36 (dd, *J* = 10.8 Hz, 5.6 Hz, 2H), 2.95 (dd, *J* = 15.6 Hz, 7.6 Hz, 7H), 1.22 (t, *J* = 7.2 Hz, 9H). ^{31}P NMR (162 MHz, $CDCl_3$) δ 5.49.

^{1}H NMR of 4 (400 MHz, $CDCl_3$)

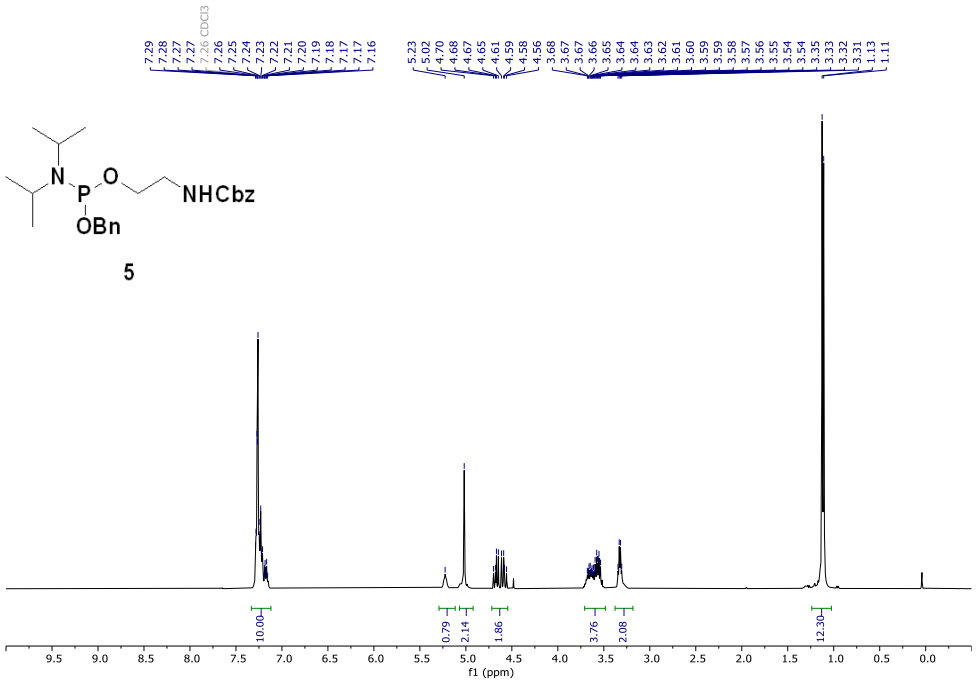


^{31}P NMR of 4 (400 MHz, $CDCl_3$)

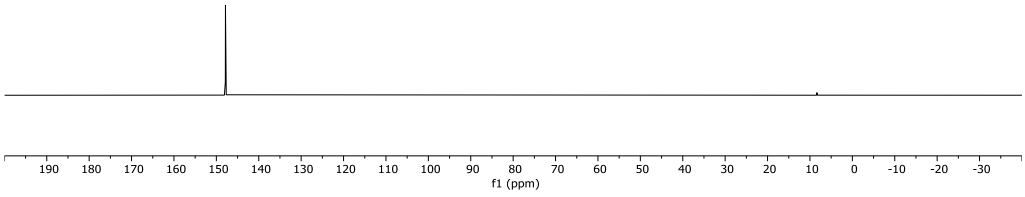


Characterization of 5: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29 – 7.16 (m, 10H), 5.23 (br, 1H), 5.02 (s, 2H), 4.70 – 4.56 (m, 2H), 3.68 – 3.54 (m, 4H), 3.35 (q, J = 5.2 Hz, 2H), 1.13 (d, J = 6.8 Hz, 12H). ^{31}P NMR (162 MHz, $CDCl_3$) δ 147.85.

^{1}H NMR of 5 (400 MHz, $CDCl_3$)



^{31}P NMR of 5 (400 MHz, $CDCl_3$)



4. Oligosaccharide synthesis

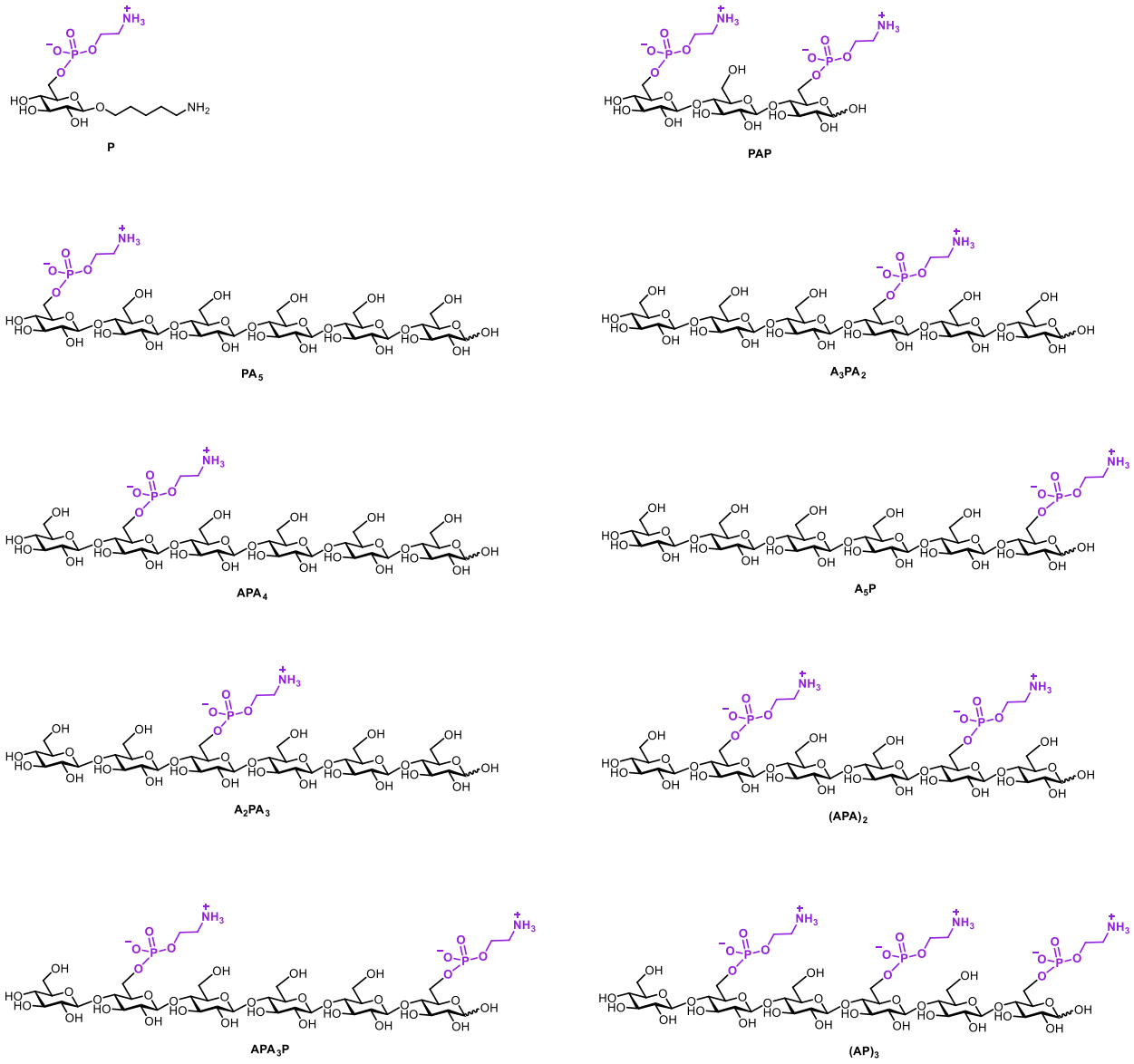
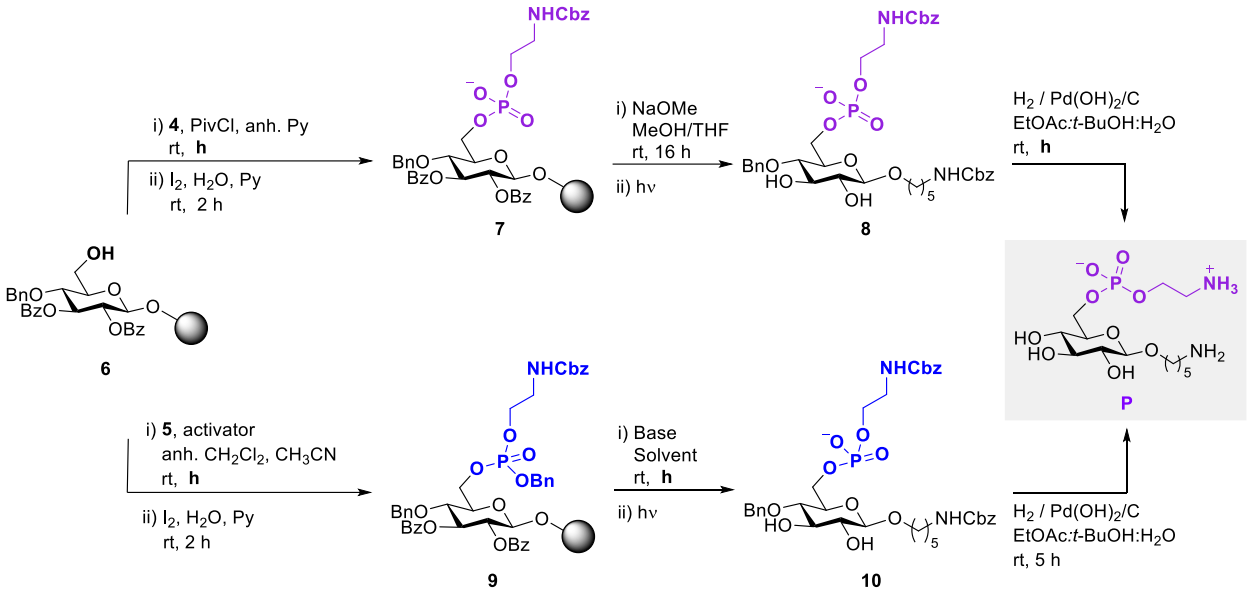


Figure S2 Collection of pEtN-cellulose analogues synthesized by AGA.

4.1 Optimization of solid-phase synthesis of pEtN-modified glucoside P



Scheme S1 Synthetic routes for **P**

Table S1 Screening of condition using **4**.

Phosphorylation

Entry	4 (equiv.)	PivCl (equiv.)	Time (h)	Result[a]
1	50	50	16	Partial phosphorylation
2	25	25	16	Complete phosphorylation
3	25	25	5	Complete phosphorylation
4	5	5	5	Complete phosphorylation
5	5	5	1	Partial phosphorylation

[a] Results analyzed after oxidation step.

Hydrogenation

Entry	Time (h)	Additive (equiv.)[a]	Overall yield[b]
6	3	-	Incomplete conversion
7	5	-	6%
8	5	Thiourea (6)	19%
9	5	Thiourea (10)	36%

[a] Upon completion of hydrogenation, prior to filtration, the crude mixture was treated with thiourea. [b] Overall yield includes AGA, phosphorylation and deprotections.

Table S2 Screening of condition using **5**.

Phosphorylation

Entry	5 (equiv.)	Activator (equiv.)	Time (h)	Result[a]
10	25	*1H*-tetrazole (25)	16	Complete phosphorylation
11	5	*1H*-tetrazole (5)	5	Complete phosphorylation
12	5	BTT (5)	5	Complete phosphorylation

[a] Results analyzed after oxidation step.

Hydrolysis

Entry	Base	Solvent	Time (h)	Result
13	LiOH (0.3 M)	4:1 THF/MeOH	48	No conversion
14	NaOMe (0.5 M)	4:1 THF/MeOH	3	Partial hydrolysis
15	NaOMe (0.5 M)	4:1 THF/MeOH	16	Complete hydrolysis

4.1.1 Mass spectrometry and additional information

Analysis of intermediate 7

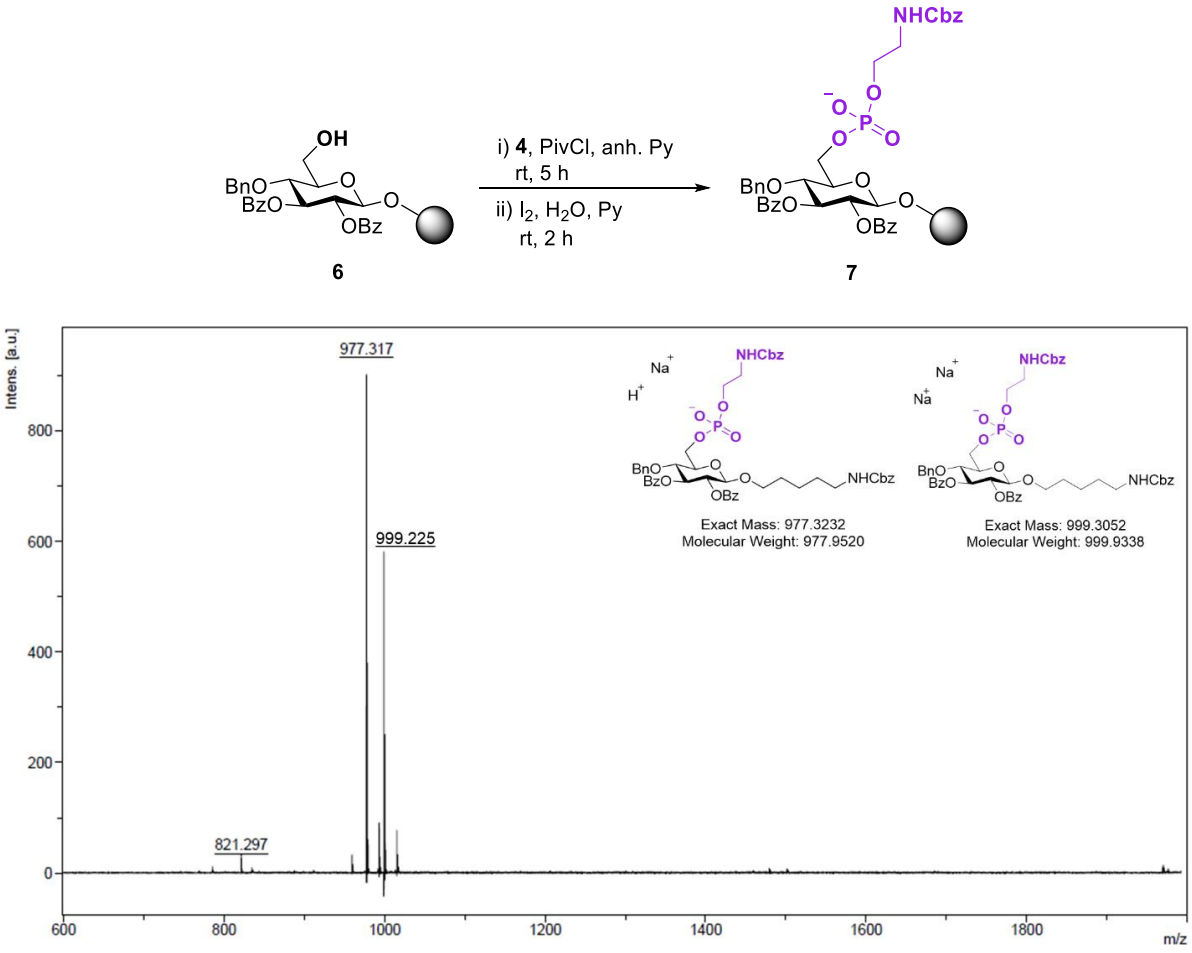
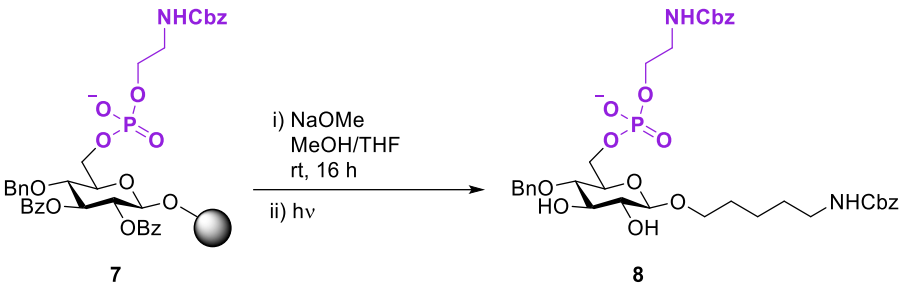


Figure S2 MALDI-ToF of compound **7** after microcleavage (positive mode).

Analysis of intermediate 8



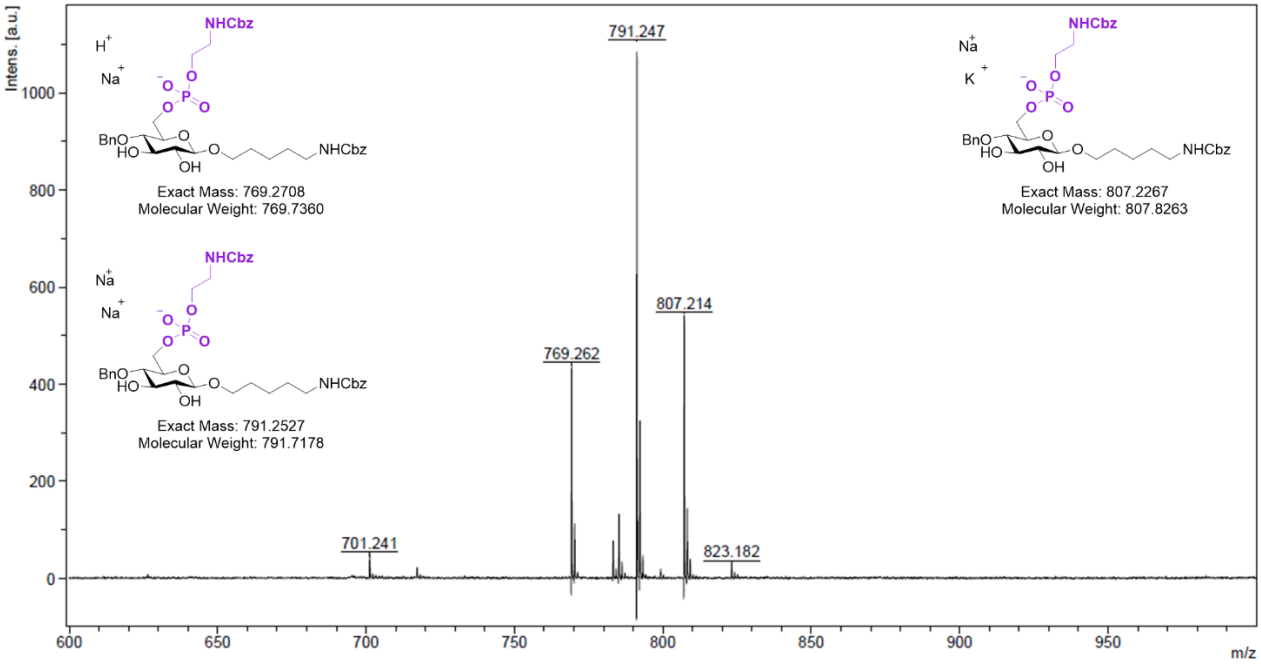


Figure S3 MALDI-ToF of compound **8** after microcleavage (positive mode).

Analysis of the intermediate 9

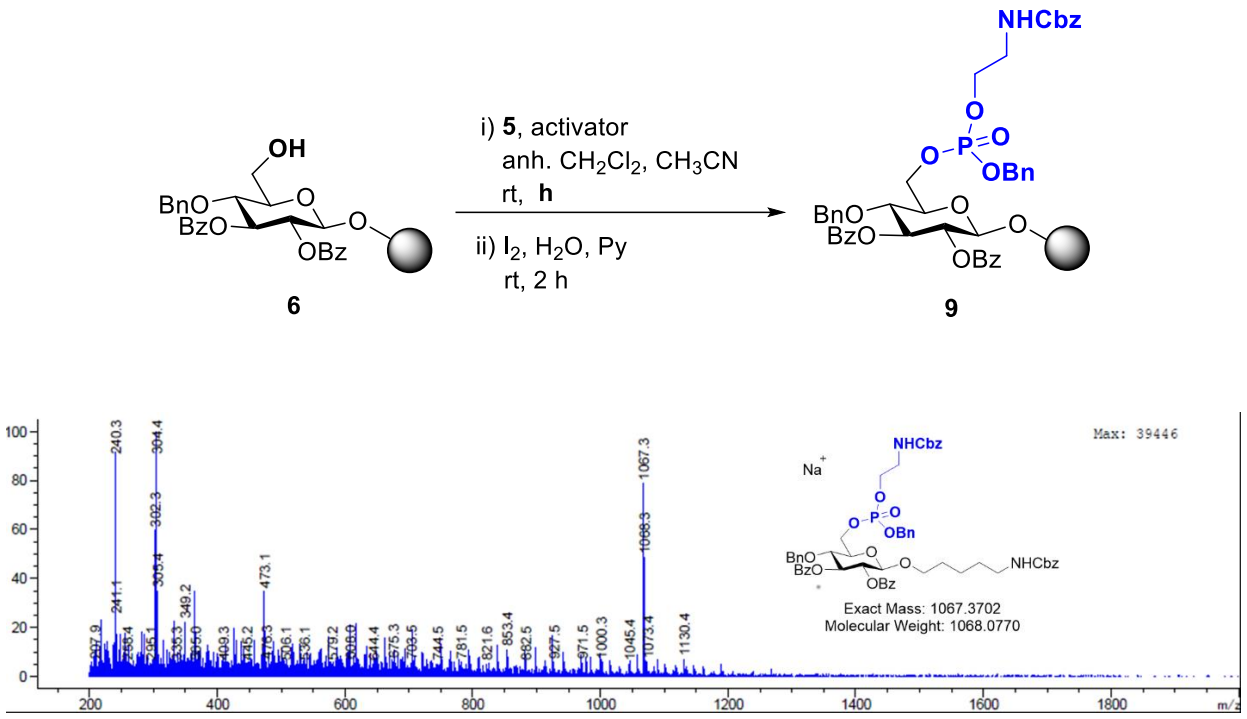
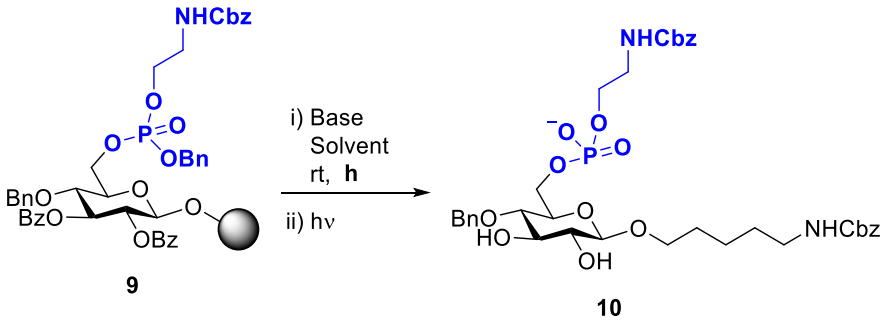


Figure S4 ESI-MS of compound **9** after microcleavage (positive mode).

Analysis of the intermediate 10



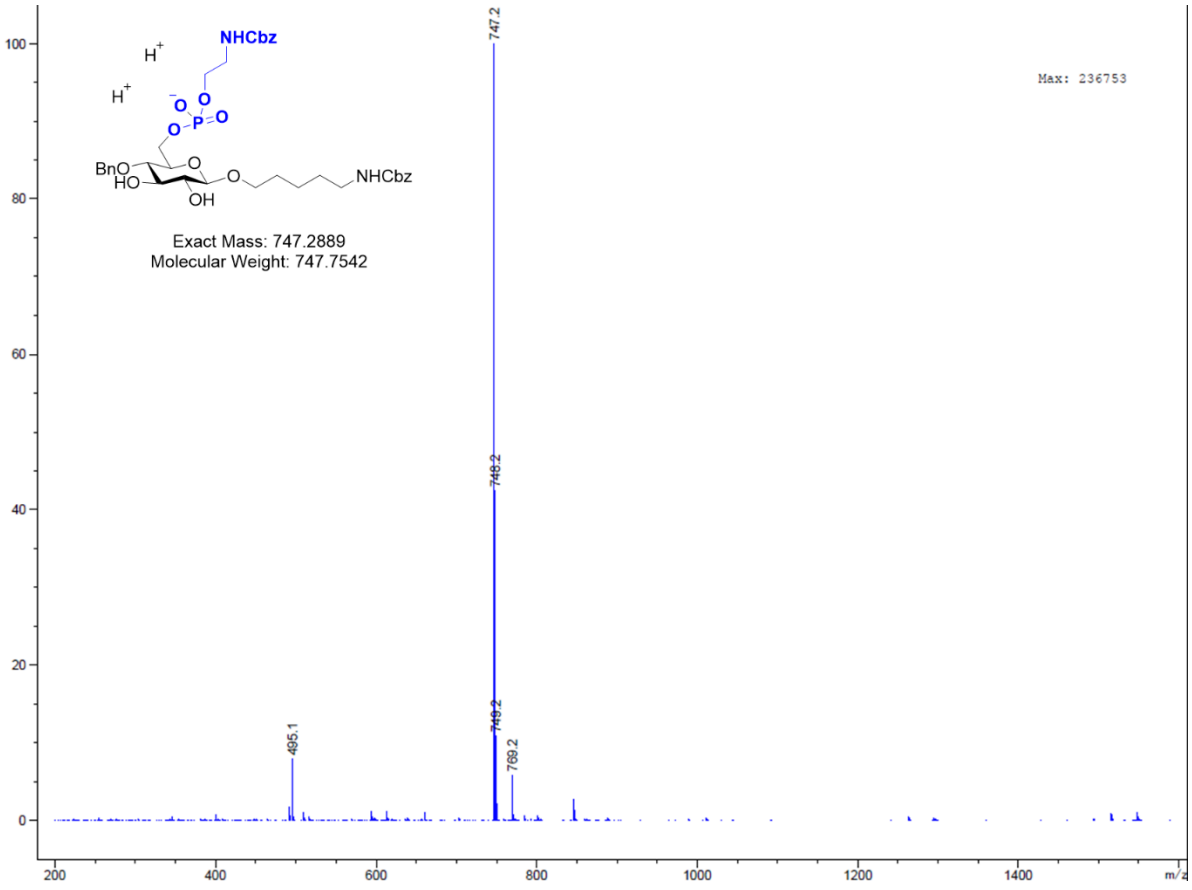
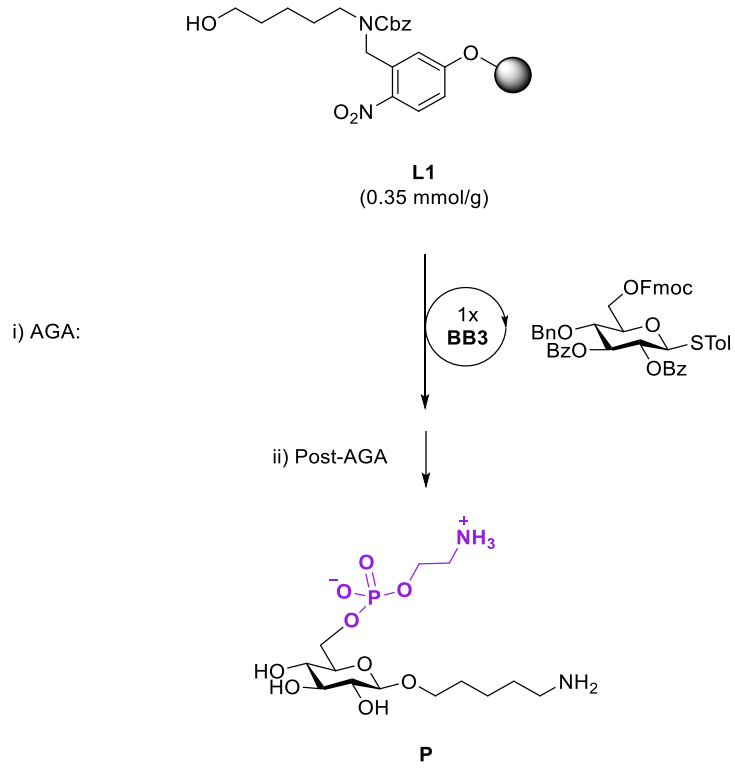


Figure S5 ESI-MS of compound **9** after photocleavage (positive mode).

4.2 Synthesis of P

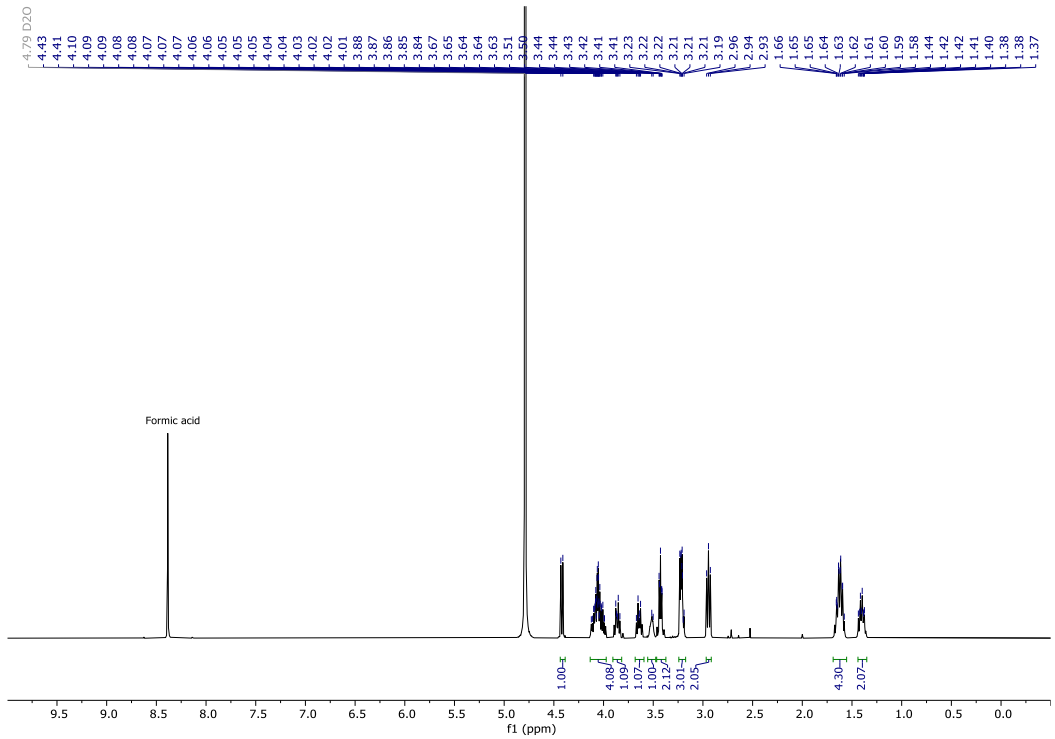


Step		Modules	Notes
AGA		**A**	**L1** swelling
	BB3	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
Post-AGA	**Phosphorylation**	**F**	
	Hydrolysis	**G**	**G:** (16 h)
	Hydrogenolysis	**I**	**I:** (5 h)
	Purification	**J**	**J:** (Method J4: 13.5 min)

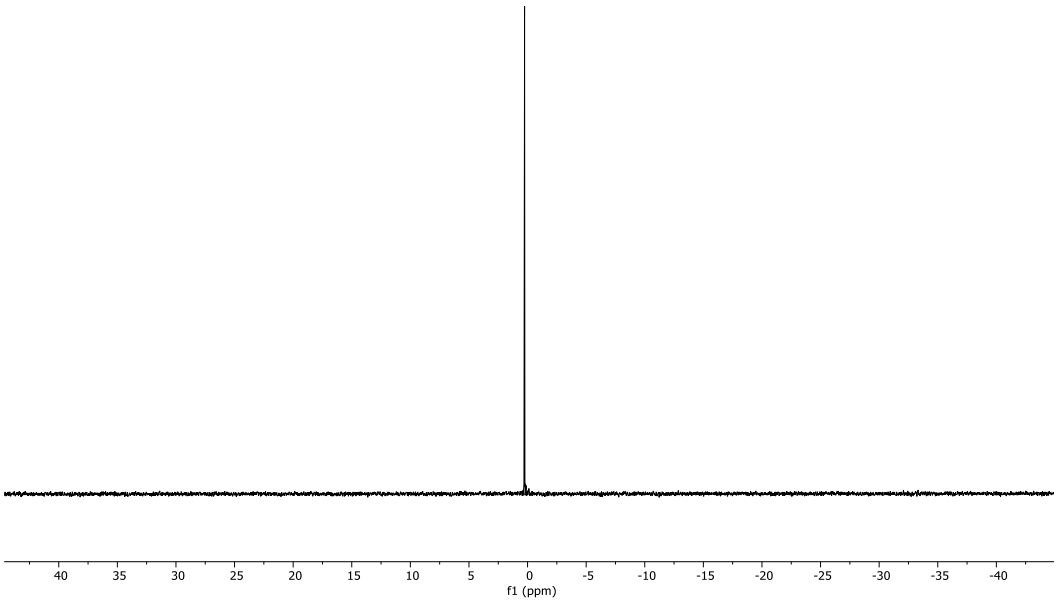
Automated synthesis, global deprotection, and purification afforded **P** as white solid (1.9 mg, 36% overall yield).

^{1}H NMR (400 MHz, D_2O) δ 4.43 (d, *J* = 8.0 Hz, 1H), 4.10 – 3.99 (m, 4H), 3.88 – 3.84 (m, 1H), 3.67 – 3.63 (m, 1H), 3.51 – 3.50 (br, 1H), 3.44 – 3.41 (m, 2H), 3.23 – 3.19 (m, 3H), 2.96 (t, *J* = 7.6 Hz, 2H), 1.66 – 1.58 (m, 4H), 1.44 – 1.37 (m, 2H). ^{31}P NMR (162 MHz, D_2O) δ 0.25. ESI-MS m/z 389.1 $[M+H]^+$ ($C_{13}H_{30}N_2O_9P$ requires 389.1683). Analytical data of **P** are in good agreement with previously reported data.[11]

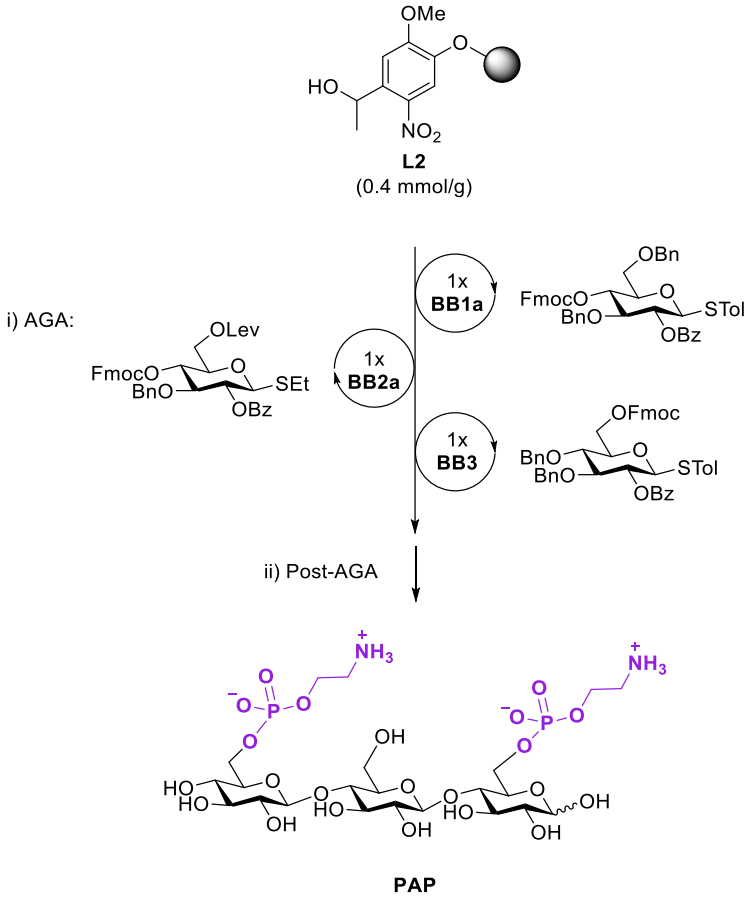
^{1}H NMR of P (400 MHz, D_2O)



^{31}P NMR of P (162 MHz, D_2O)



4.3 Synthesis of PAP

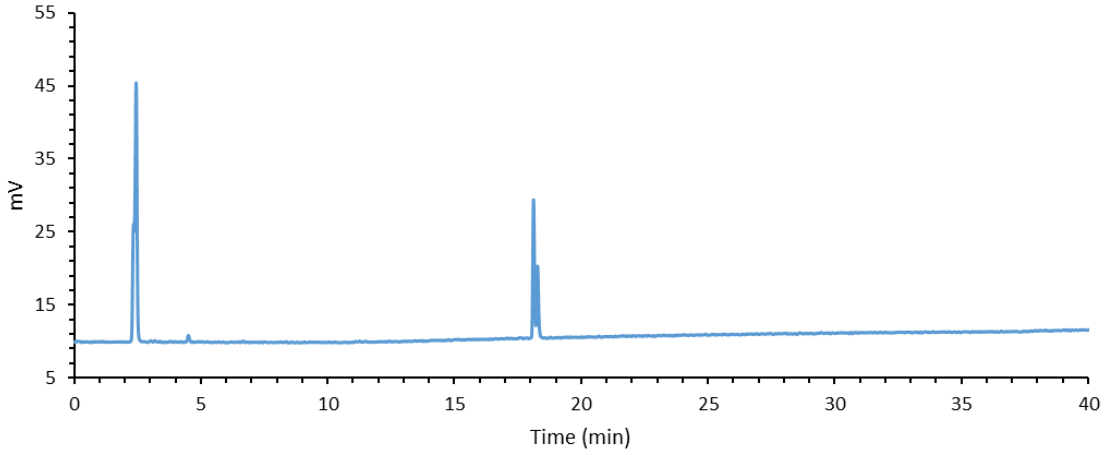


Step		Modules	Notes
AGA		**A**	**L2** swelling
	BB1a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	BB2a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	BB3	**B, C1, D, E1, E2**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
Post-AGA	**Phosphorylation**	**F**	
	Hydrolysis	**G**	**G:** (2 h)
	Purification	**J**	**J:** (Method J1)
	Hydrogenolysis	**I**	**I:** (4 h)
	Purification	**J**	**J:** (Method J4: 19.4 min)

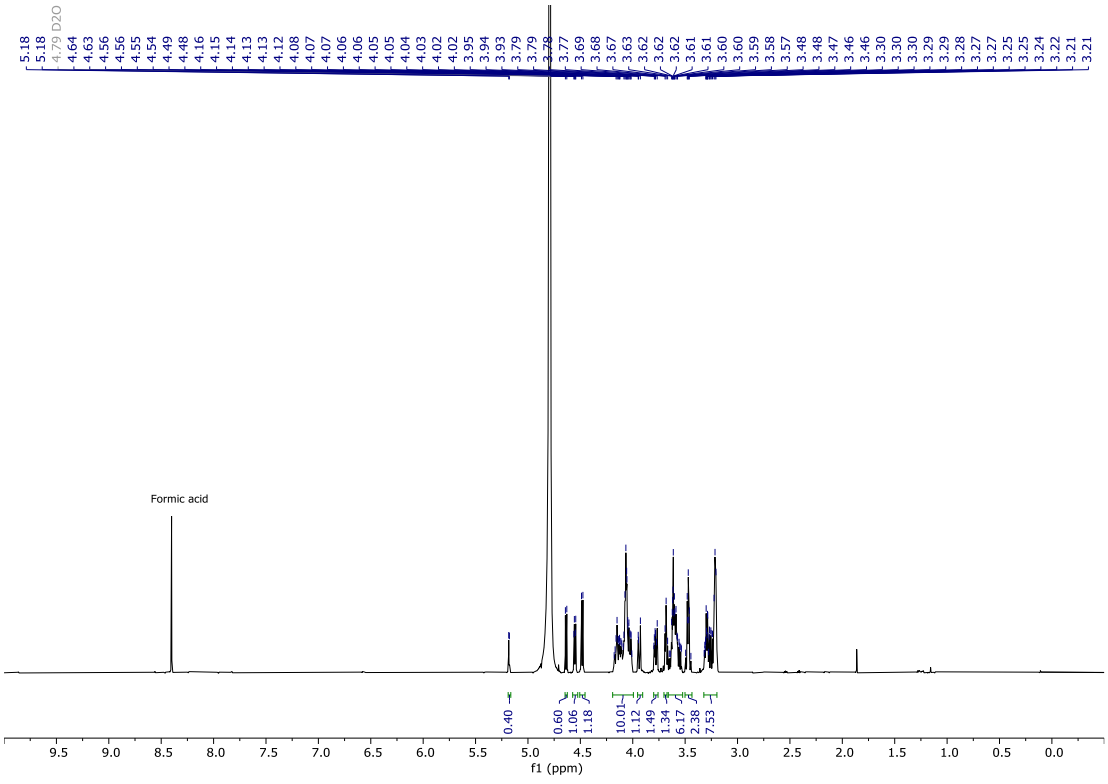
Automated synthesis, global deprotection, and purification afforded **PAP** as white solid (0.7 mg, 7% overall yield).

^{1}H NMR (600 MHz, D_2O) δ 5.18 (d, *J* = 4.2 Hz, 0.4H, H1-α), 4.64. (d, *J* = 7.8 Hz, 0.6H, H1-β), 4.56 (dd, *J*= 7.8 Hz, 2.4 Hz, 1H), 4.49 (d, *J*= 7.8 Hz, 1H), 4.16 – 4.01 (m, 10H), 3.95 – 3.93 (d, *J*= 12.6 Hz, 1H), 3.80 – 3.77 (m, 1H), 3.69 – 3.67 (m, 1H), 3.65 – 3.54 (m, 6H), 3.50 – 3.45 (m, 2H), 3.32 – 3.21 (m, 7H). ^{31}P NMR (243 MHz, D_2O) δ 0.34, 0.04, -0.01. HRMS (QToF) m/z 751.1946 $[M+H]^+$ ($C_{22}H_{45}N_2O_{22}P_2$ requires 751.1934).

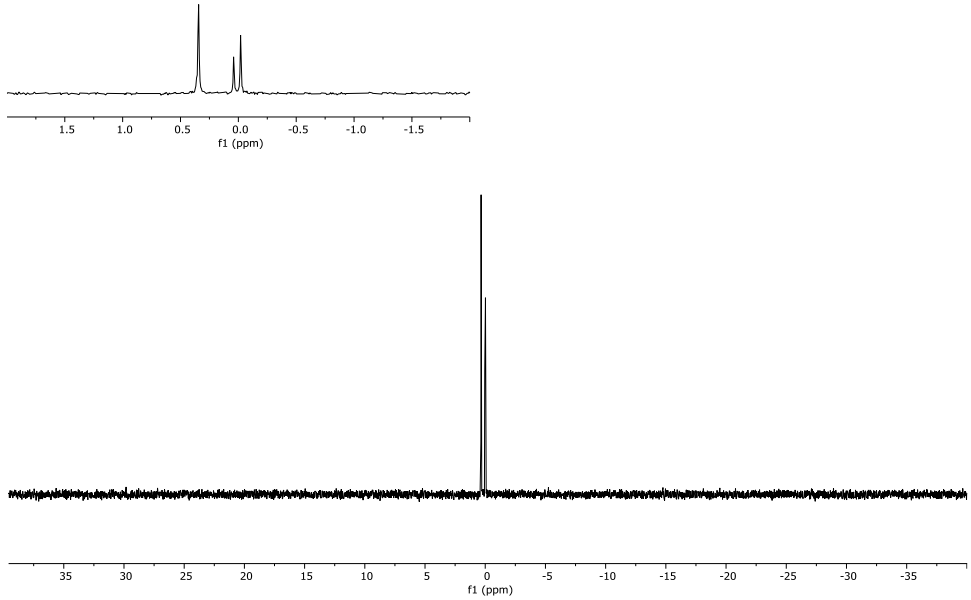
RP-HPLC of PAP (ELSD trace, Method J3, t_R = 18.1, 18.3 min)



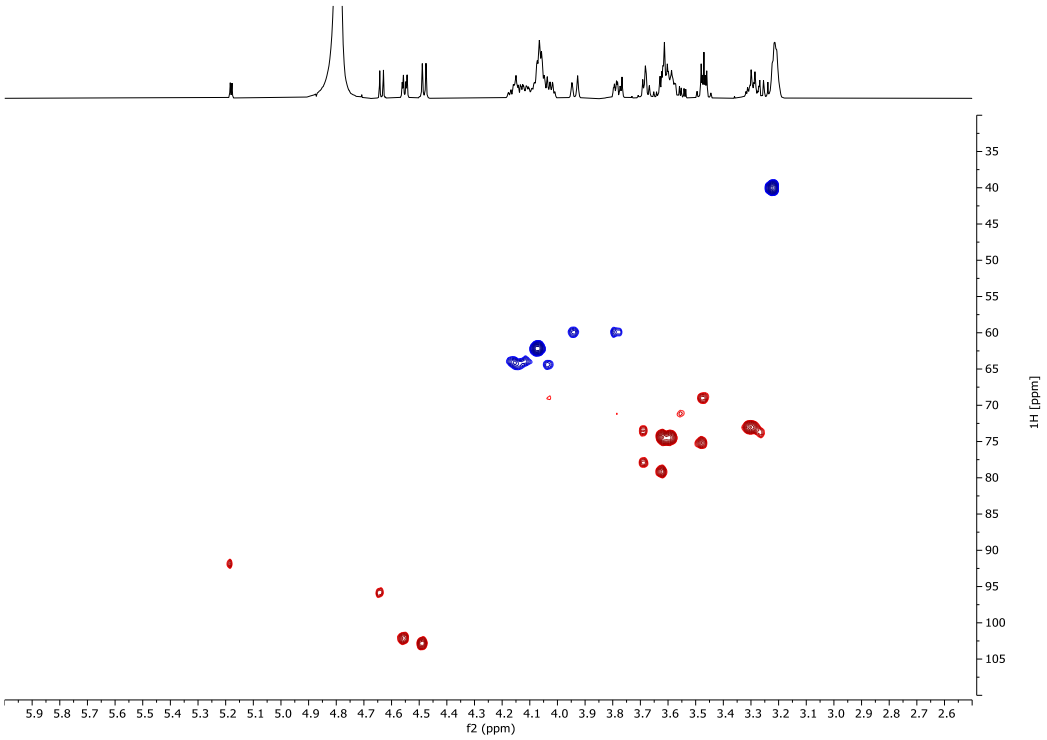
^{1}H NMR of PAP (600 MHz, D_2O)



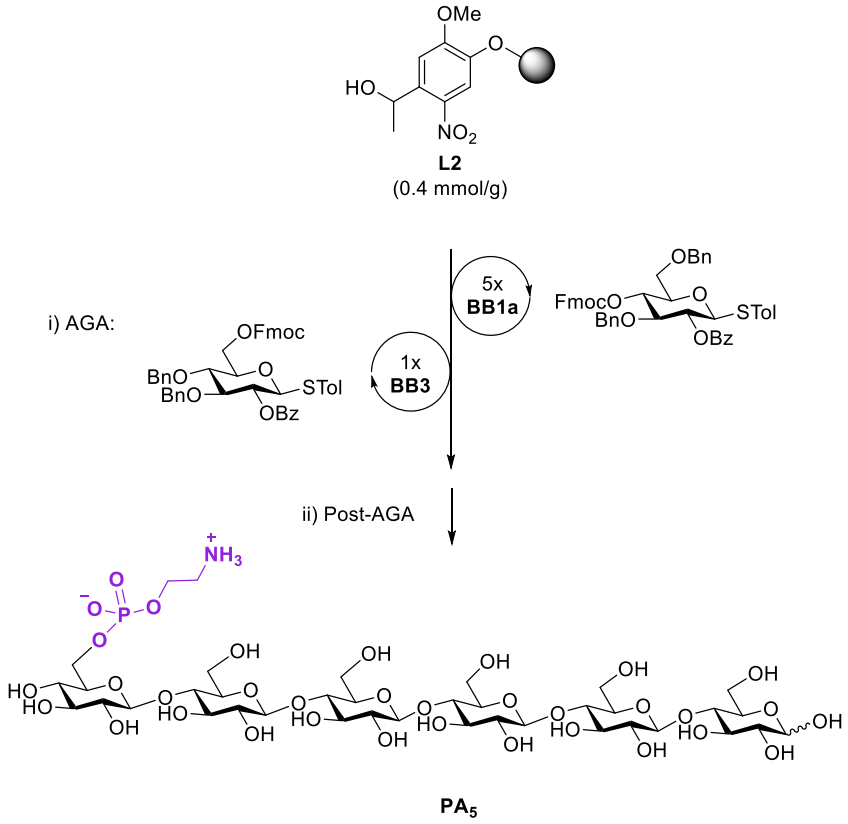
^{31}P NMR of PAP (243 MHz, D_2O)



HSQC NMR of PAP (D_2O)



4.4 Synthesis of PA_5

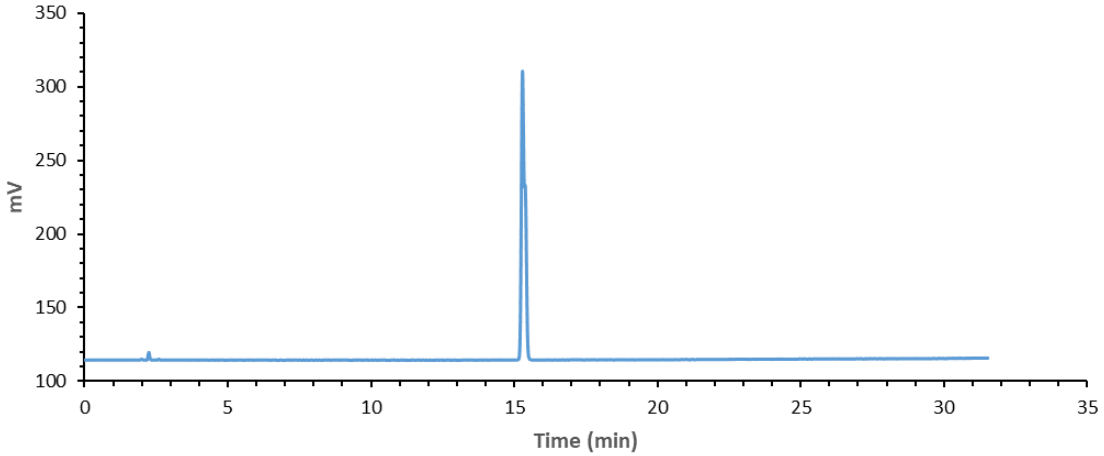


Step		Modules	Notes
AGA		**A**	**L2** swelling
	5 x BB1a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	BB3	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
Post-AGA	**Phosphorylation**	**F**	
	Hydrolysis	**G**	**G:** (2 h)
	Purification	**J**	**J:** (Method J1)
	Hydrogenolysis	**I**	**I:** (4 h)
	Purification	**J**	**J:** (Method J6: 16.2 min)

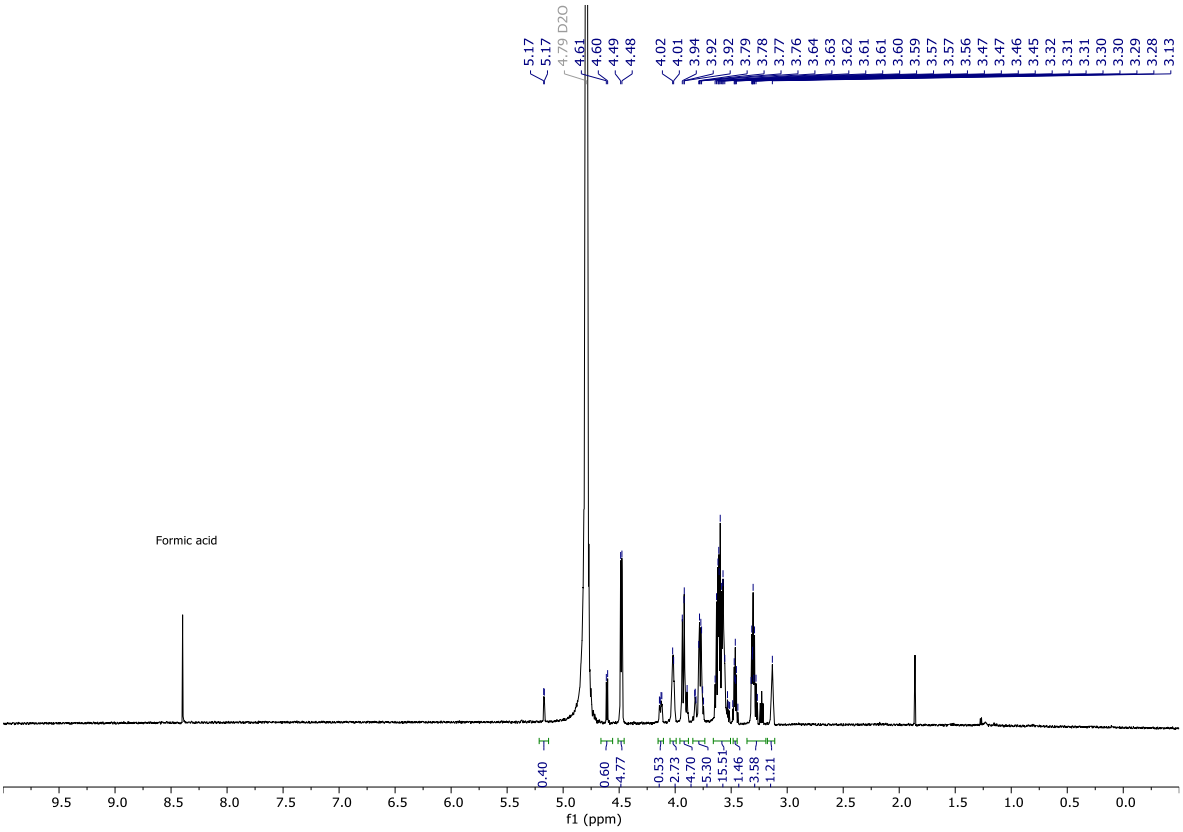
Automated synthesis, global deprotection, and purification afforded **PA_5** as white solid (0.6 mg, 4% overall yield).

^{1}H NMR (700 MHz, D_2O) δ 5.17 (d, J = 4.9 Hz, 0.4H, H1-α), 4.61 (d, J = 9.1 Hz, 0.6H, H1-β), 4.49 (d, J= 9.1 Hz, 5H), 4.02 – 4.01 (m, 1H), 3.94 – 3.89 (m, 3H), 3.83 – 3.75 (m, 5H), 3.64 – 3.51 (m, 16H), 3.49 – 3.44 (m, 1H), 3.32 – 3.27 (m, 4H), 3.13 – 3.20 (m, 1H). ^{31}P NMR (243 MHz, D_2O) δ 0.29. HRMS (QToF) m/z 1114.346 $[M+H]^+$ ($C_{38}H_{69}NO_{34}P$ requires 1114.343).

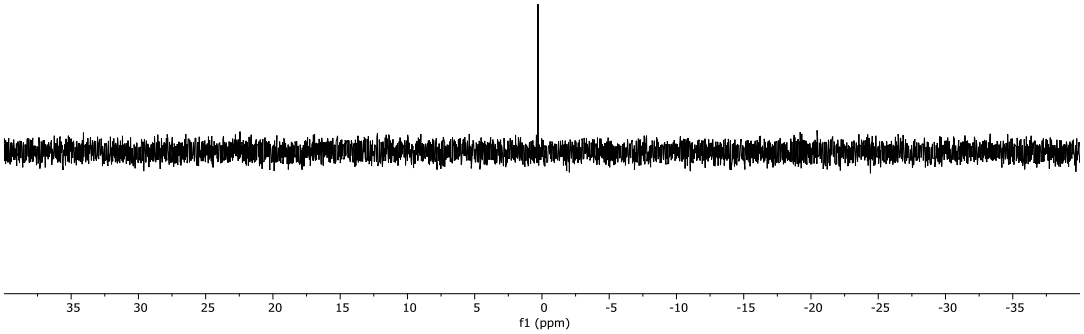
RP-HPLC of PA_5 (ELSD trace, Method J5, t_R = 15.2, 15.3 min)



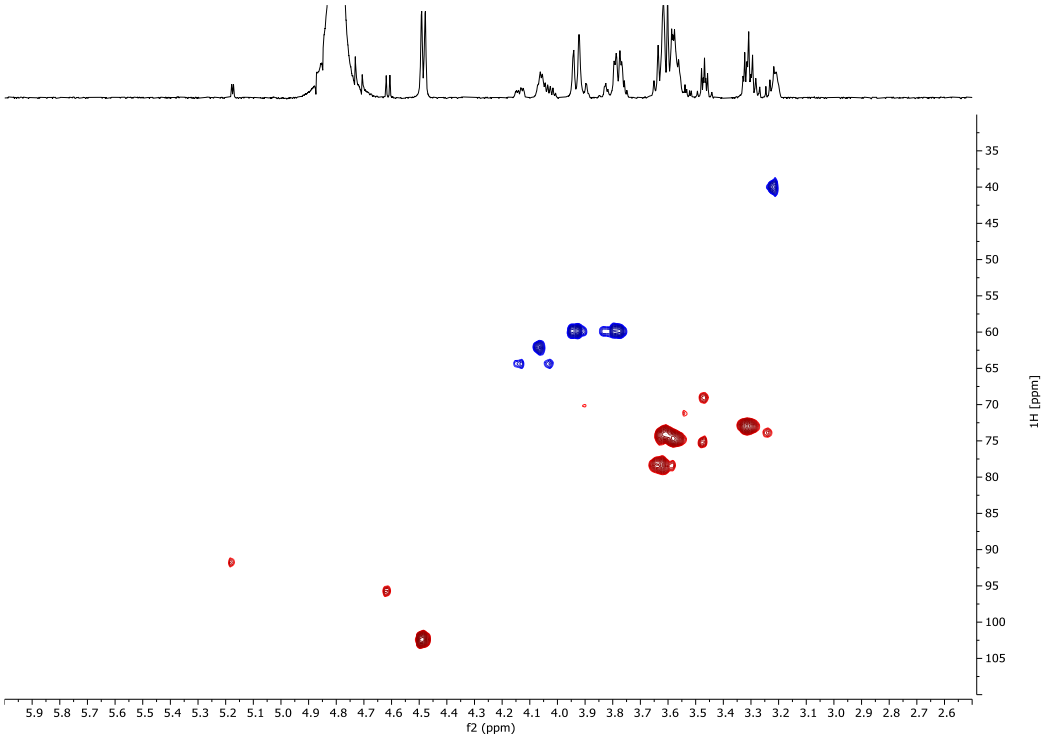
^{1}H NMR of PA_5 (700 MHz, D_2O)



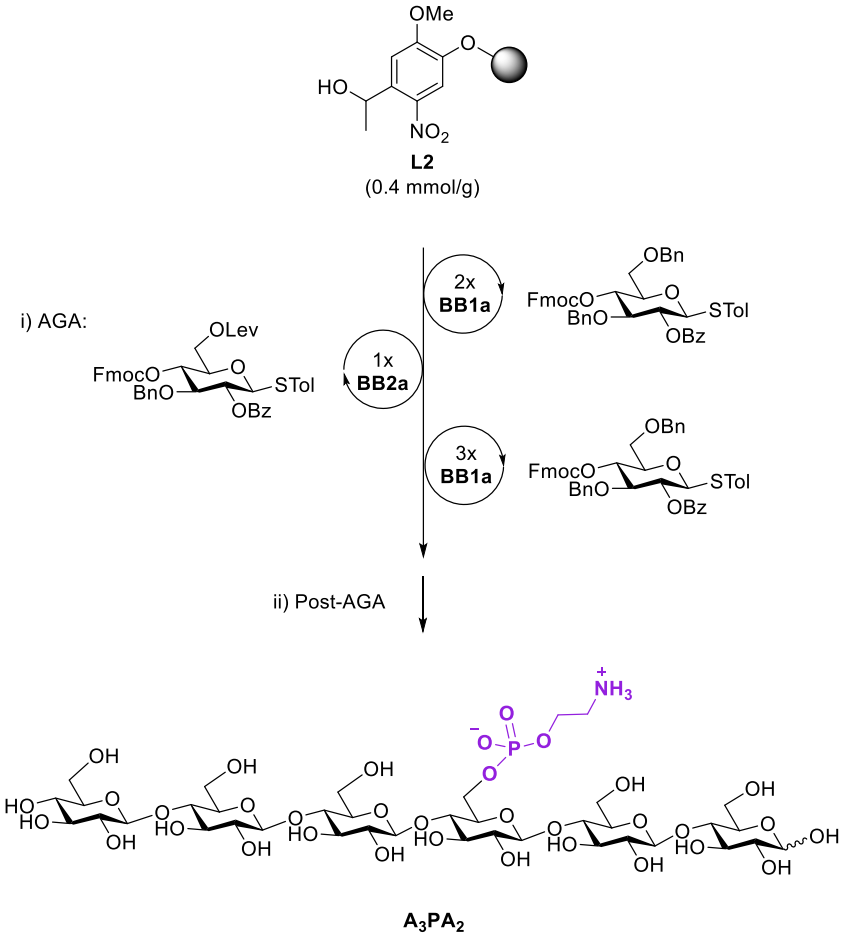
^{31}P NMR of PA_5 (162 MHz, D_2O)



HSQC NMR of PA_5 (D_2O)



4.5 Synthesis of A_3PA_2

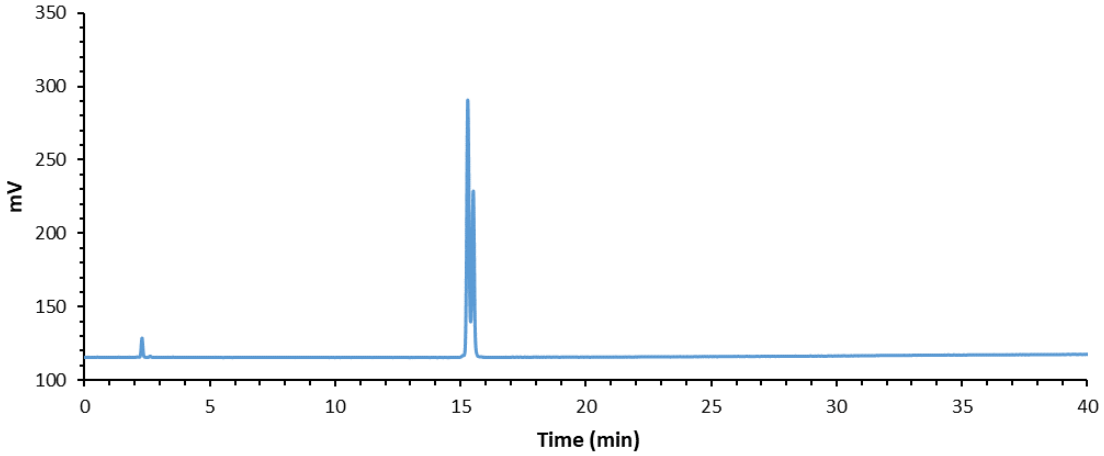


Step		Modules	Notes
AGA		**A**	**L2** swelling
	2 x BB1a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	BB2a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	3 x BB1a	**B, C1, D, E1, D, E2**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
Post-AGA	**Phosphorylation**	**F**	
	Hydrolysis	**G**	**G:** (5 h)
	Purification	**J**	**J:** (Method J1)
	Hydrogenolysis	**I**	**I:** (16 h)
	Purification	**J**	**J:** (Method J6: 15.9, 16.1 min)

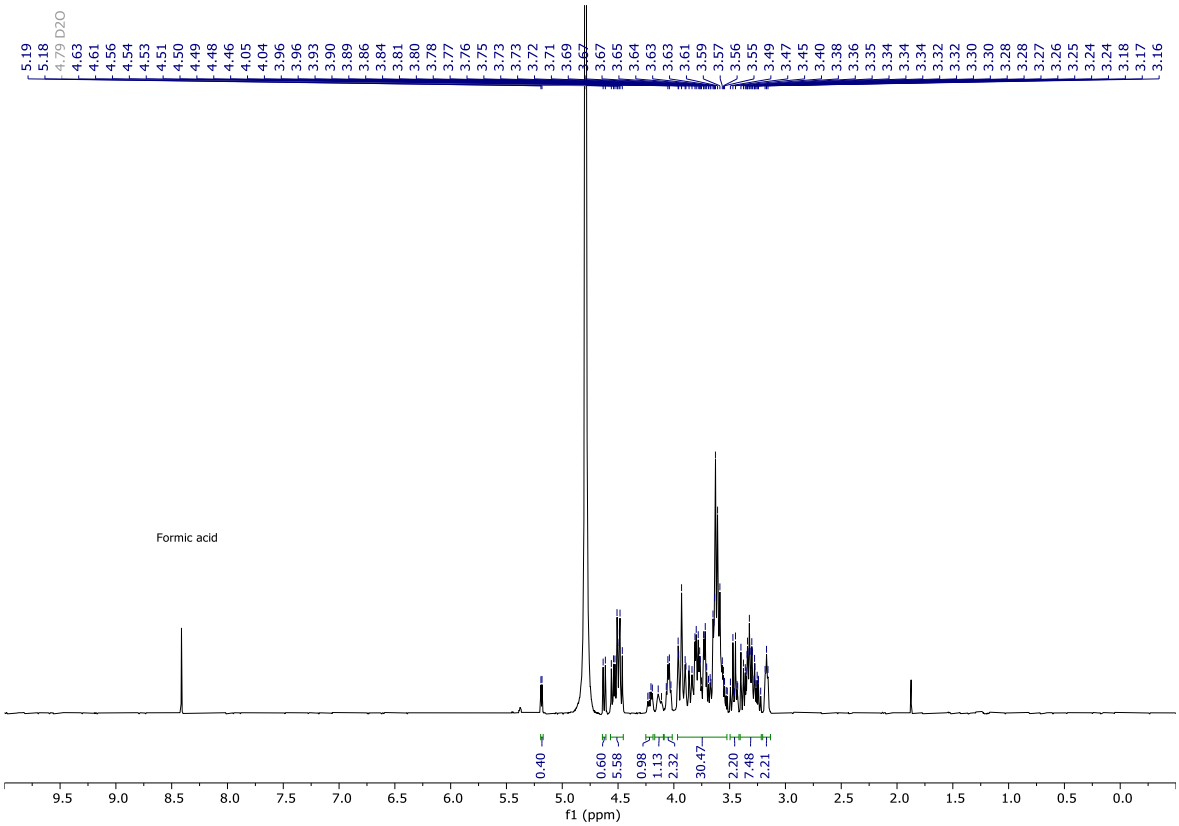
Automated synthesis, global deprotection, and purification afforded **A_3PA_2** as white solid (0.7 mg, 5% overall yield).

^{1}H NMR (400 MHz, D_2O) δ 5.19 (d, *J* = 4.0 Hz, 0.4H, H1-α), 4.63 (d, *J* = 8.4 Hz, 0.6H, H1-β), 4.56 – 4.46 (m, 5H), 4.23 (dd, *J*= 12.4 Hz, 4.4 Hz, 1H) 4.14 – 4.11 (br, 1H), 4.07 (q, *J* = 5.6, 2H), 3.96 – 3.52 (m, 30H), 3.49 – 3.43 (m, 2H), 3.40 – 3.22 (m, 7H), 3.18 (t, *J* = 4.8 Hz, 2H). ^{31}P NMR (162 MHz, D_2O) δ 0.09. HRMS (QToF) m/z 1114.346 $[M+H]^+$ ($C_{38}H_{69}NO_{34}P$ requires 1114.343).

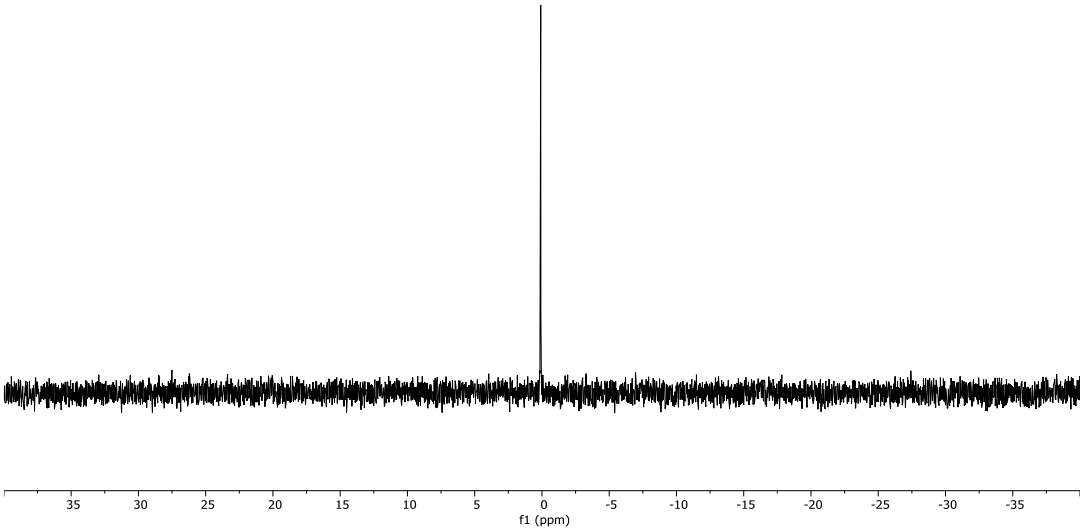
RP-HPLC of A_3PA_2 (ELSD trace, Method J5, t_R = 15.2, 15.5 min)



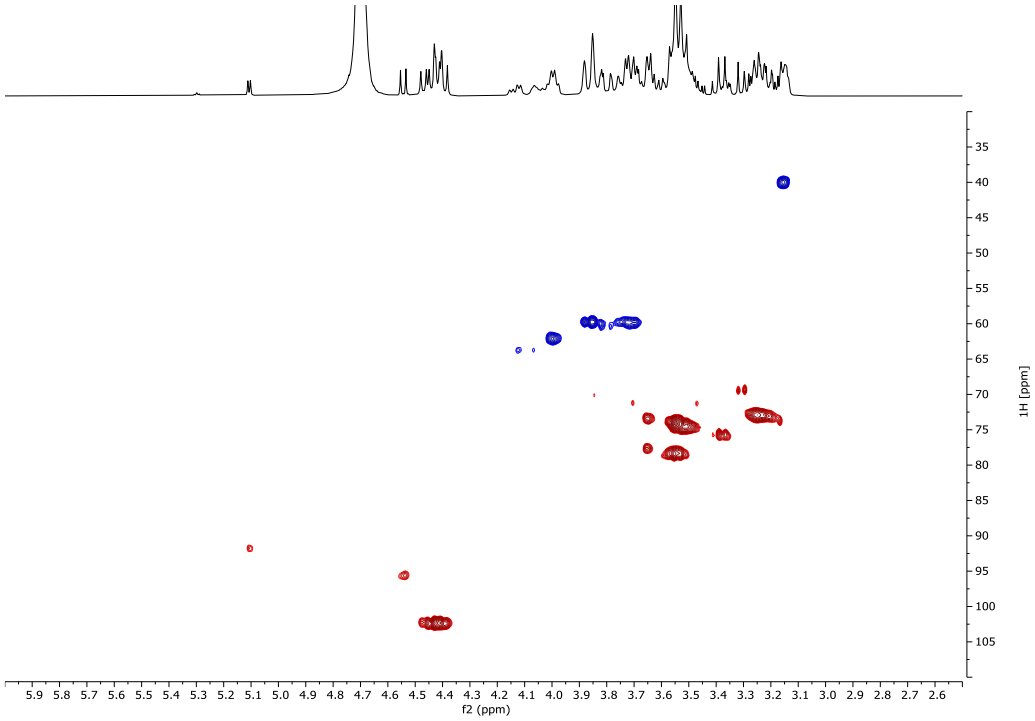
^{1}H NMR of A_3PA_2 (400 MHz, D_2O)



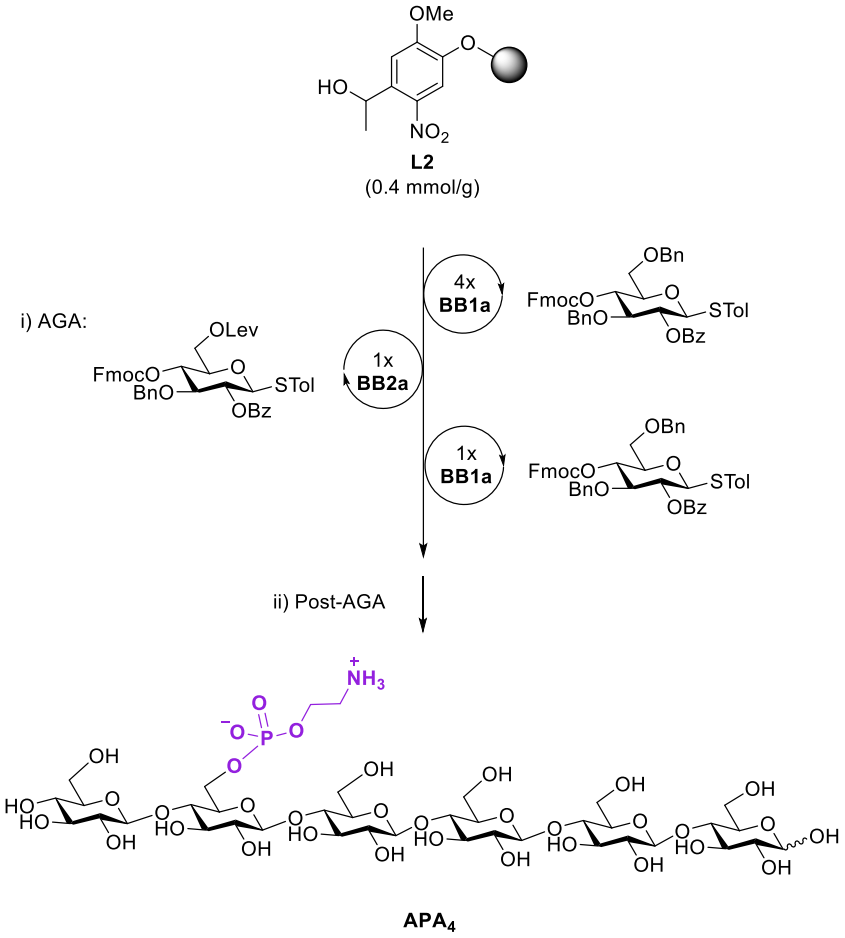
^{31}P NMR of A_3PA_2 (162 MHz, D_2O)



HSQC NMR of A_3PA_2 (D_2O)



4.6 Synthesis of APA_4



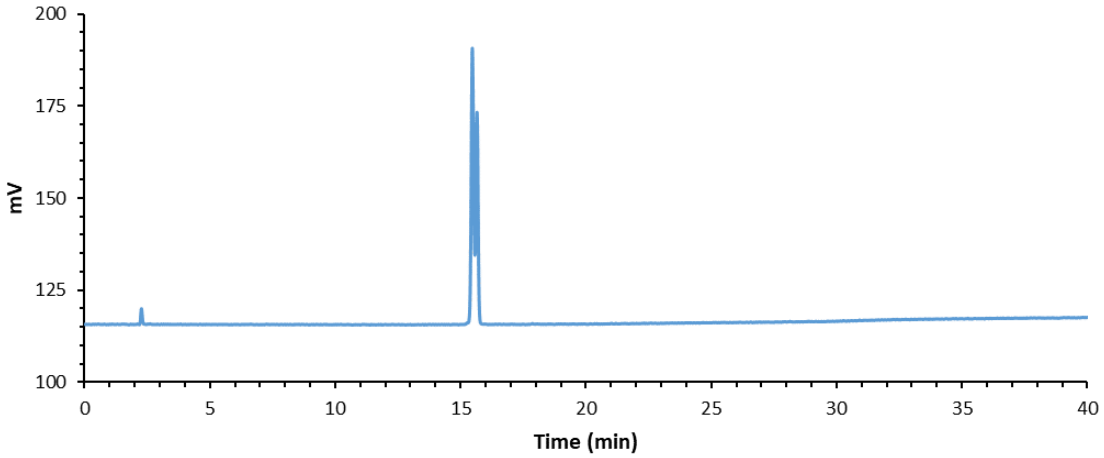
APA_4

Step		Modules	Notes
AGA		**A**	**L2** swelling
	4 x BB1a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	BB2a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	BB1a	**B, C1, D, E1, D, E2**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
Post-AGA	**Phosphorylation**	**F**	
	Hydrolysis	**G**	**G:** (5 h)
	Purification	**J**	**J:** (Method J1)
	Hydrogenolysis	**I**	**I:** (16 h)
	Purification	**J**	**J:** (Method J6: 16.0, 16.1 min)

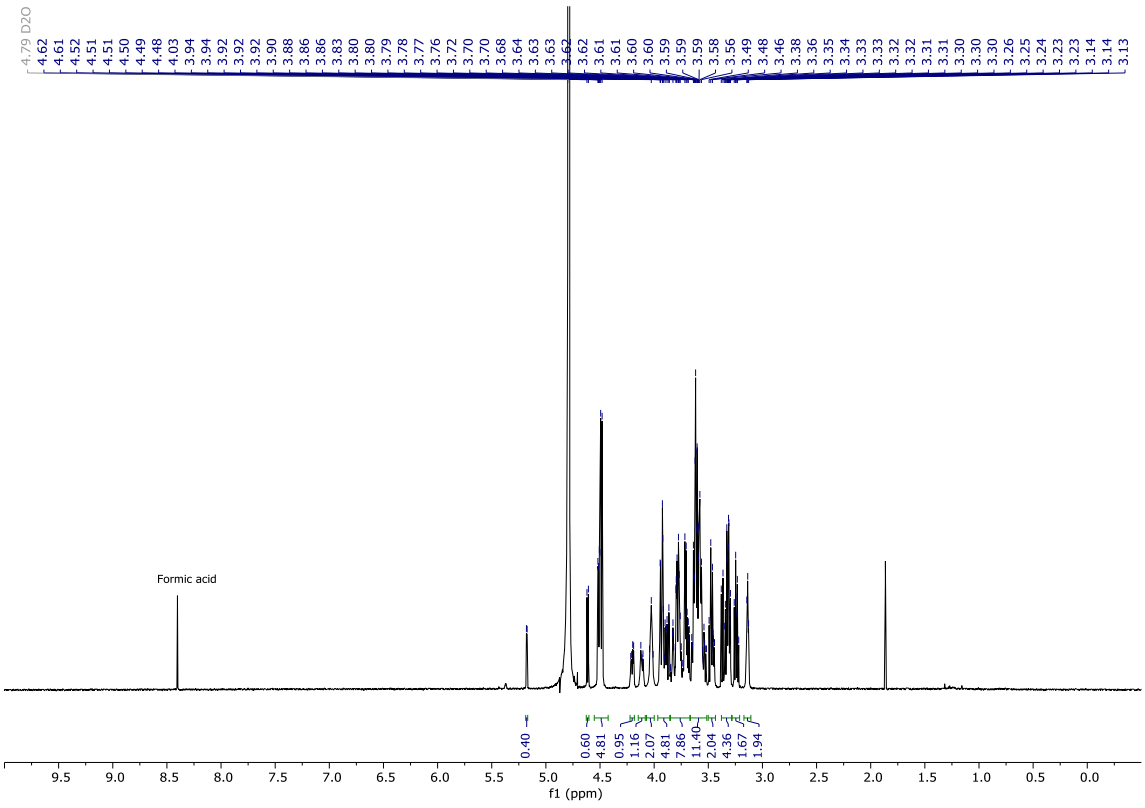
Automated synthesis, global deprotection, and purification afforded **APA_4** as white solid (2.2 mg, 15% overall yield).

^{1}H NMR (600 MHz, D_2O) δ 5.18 (d, *J* = 3.6 Hz, 0.4H, H1-α), 4.62 (d, *J* = 7.8 Hz, 0.6H, H1-β), 4.52 – 4.48 (m, 5H), 4.22 (dd, *J*= 12 Hz, 4.8 Hz, 1H) 4.12 – 4.10 (m, 1H), 4.04 – 4.01 (m, 2H), 3.94 – 3.86 (m, 5H), 3.83 –3.67 (m, 8H), 3.65 – 3.52 (m, 11H), 3.49 – 3.44 (m, 2H), 3.38 – 3.30 (m, 4H), 3.26 – 3.22 (m, 2H), 3.14 (t, *J* = 4.8 Hz, 2H), ^{31}P NMR (243 MHz, D_2O) δ 0.10. HRMS (QToF) m/z 1114.346 $[M+H]^+$ ($C_{38}H_{69}NO_{34}P$ requires 1114.343).

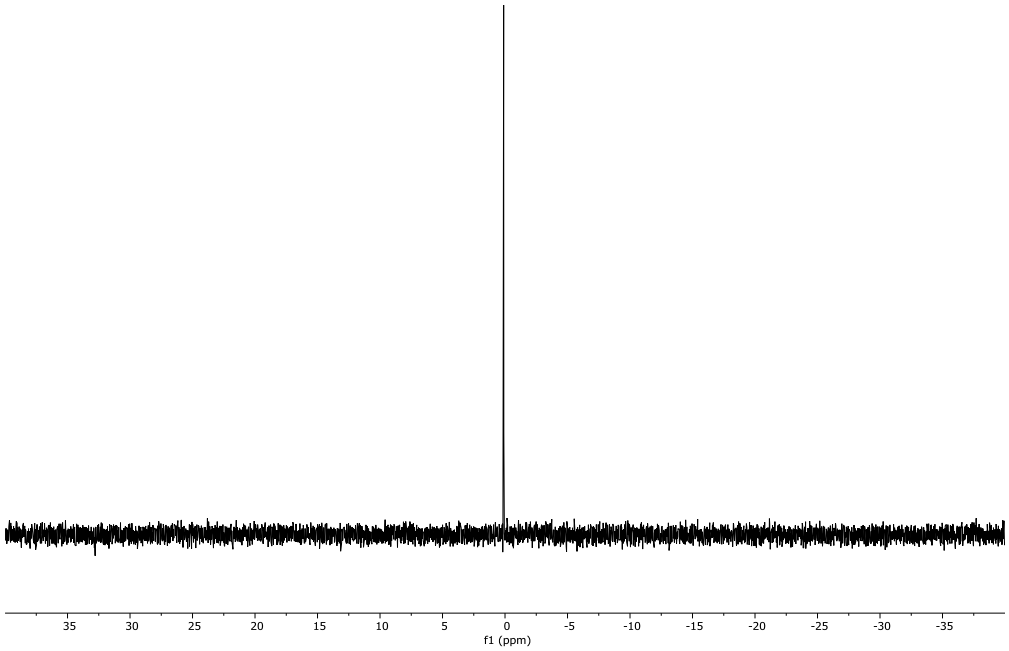
RP-HPLC of APA_4 (ELSD trace, Method J5, t_R = 15.4, 15.6 min)



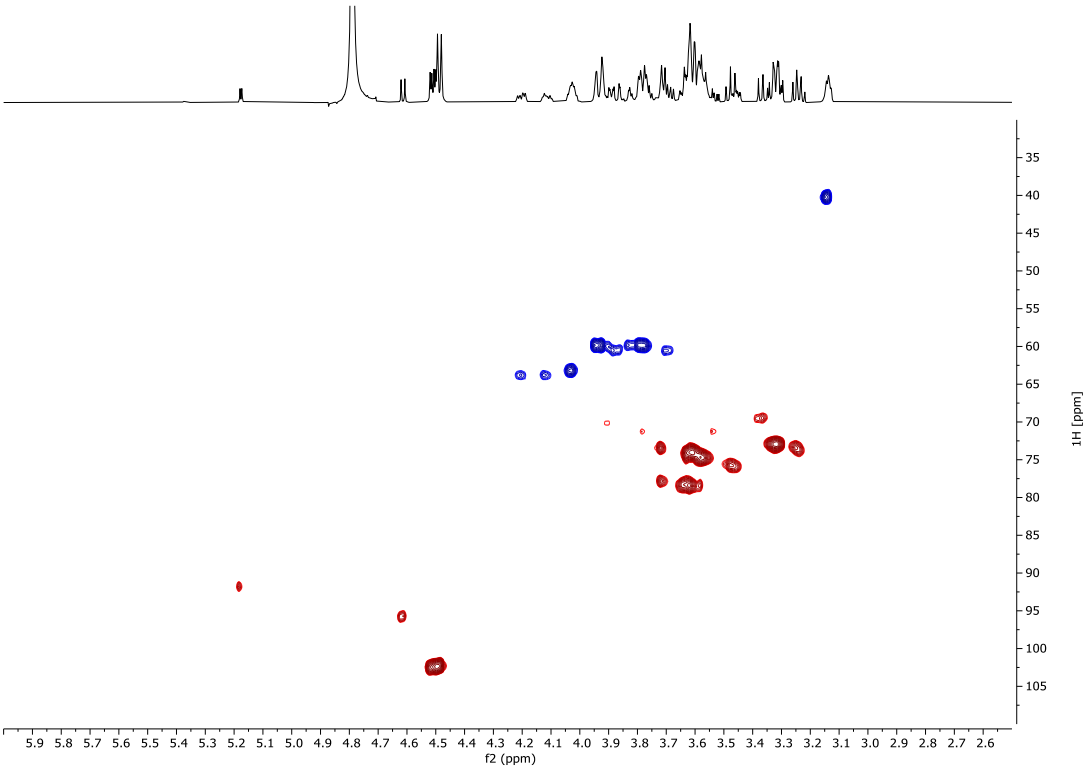
^{1}H NMR of APA_4 (600 MHz, D_2O)



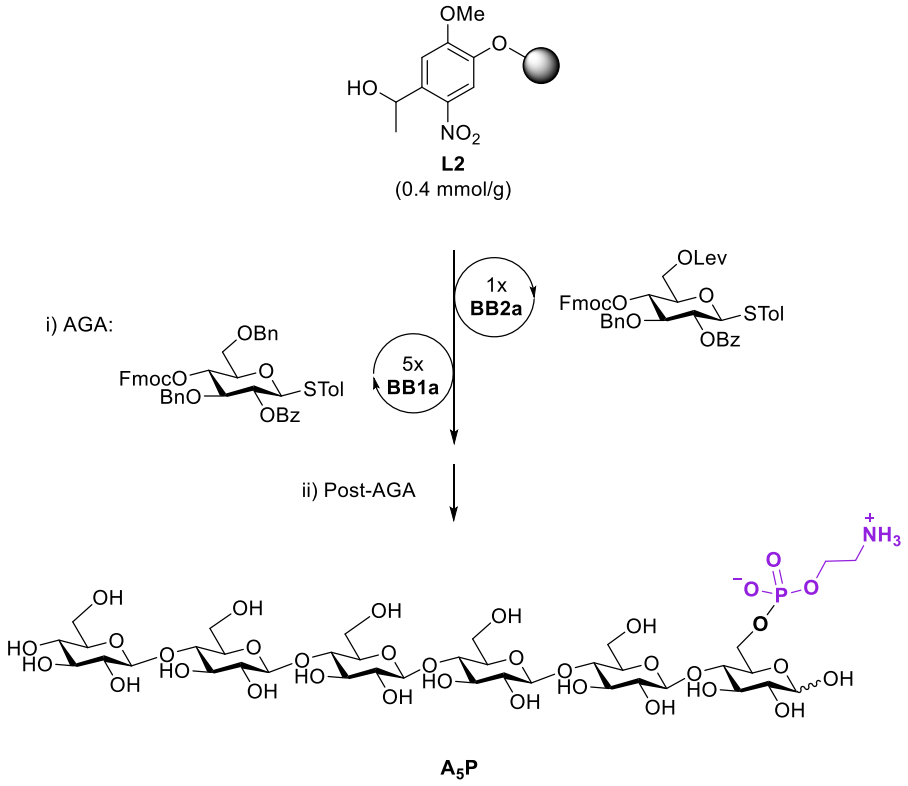
^{31}P NMR of APA_4 (243 MHz, D_2O)



HSQC NMR of APA_4 (D_2O)



4.7 Synthesis of A_5P

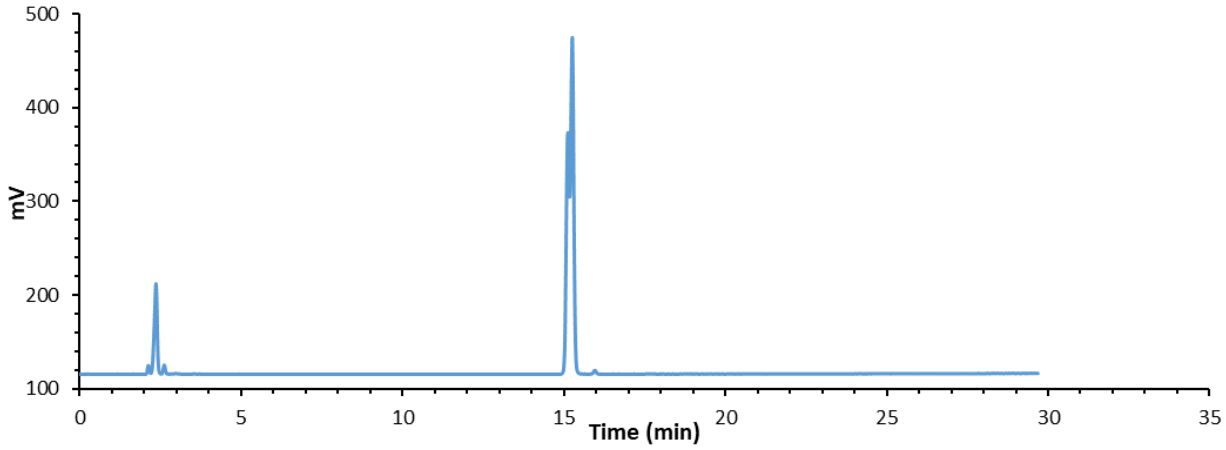


Step		Modules	Notes
AGA		**A**	**L2** swelling
	BB2a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	5 x BB1a	**B, C1, D, E1, D, E2**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
Post-AGA	**Phosphorylation**	**F**	
	Hydrolysis	**G**	**G:** (5 h)
	Purification	**J**	**J:** (Method J1)
	Hydrogenolysis	**I**	**I:** (16 h)
	Purification	**J**	**J:** (Method J6: 15.0 min)

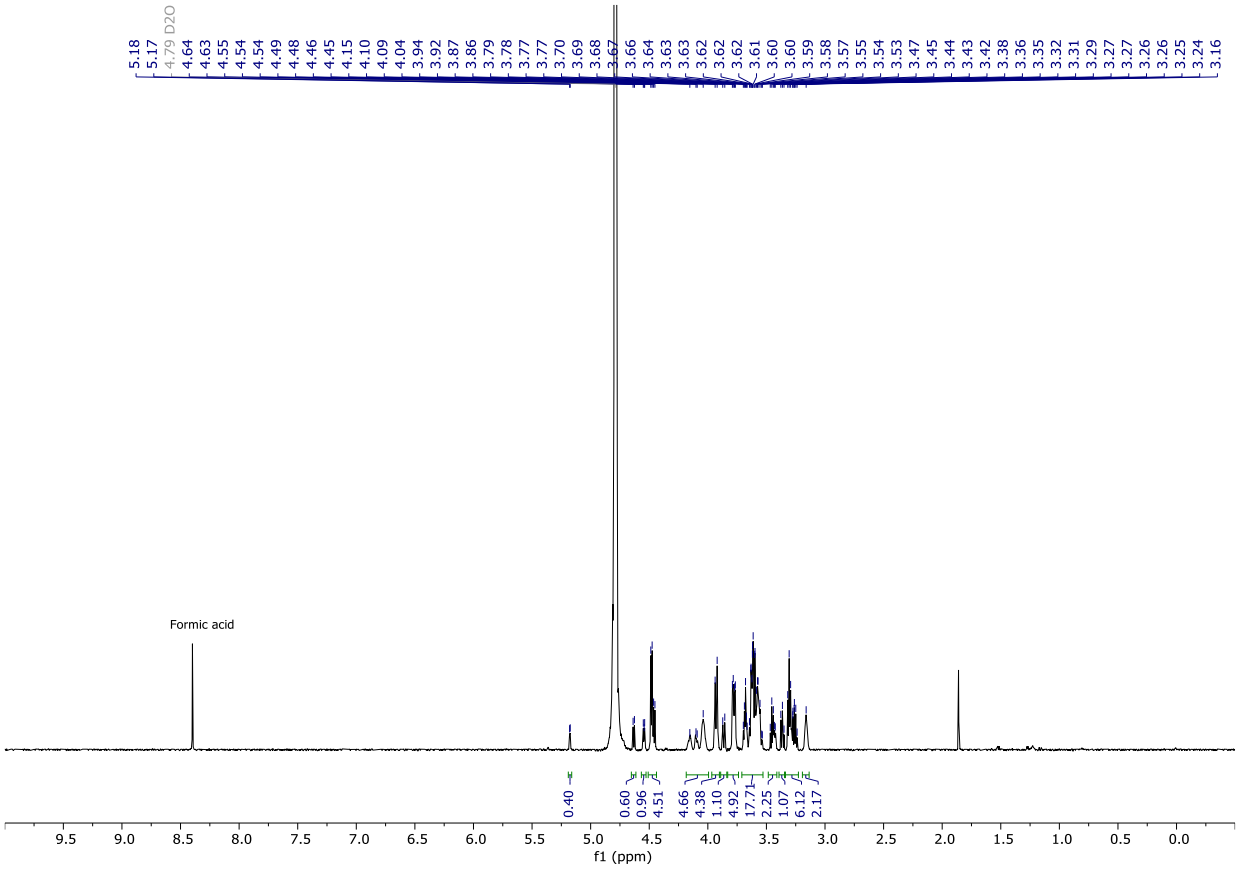
Automated synthesis, global deprotection, and purification afforded **A_5P** as white solid (0.6 mg, 4% overall yield).

^{1}H NMR (700 MHz, D_2O) δ 5.18 (d, *J* = 3.5 Hz, 0.4H, H1-α), 4.64 (d, *J* = 7.7 Hz, 0.6H, H1-β), 4.55 – 4.54 (m, 1H), 4.49 (dd, *J*= 16.8 Hz, 7.7 Hz, 4H) 4.15 – 4.04 (m, 5H), 3.94 (d, *J* = 11.2 Hz, 4H), 3.87 (d, *J* = 12.6 Hz, 1H), 3.79 (dd, *J*= 13.3 Hz, 4.9 Hz, 5H), 3.70 – 3.53 (m, 18H), 3.47 – 3.42 (m, 2H), 3.38 (t, *J*= 9.1 Hz, 1H), 3.32 – 3.24 (m, 6H), 3.16 – 3.15 (br, 2H), ^{31}P NMR (162 MHz, D_2O) δ 0.18, 0.12. HRMS (QToF) m/z 1114.343 $[M+H]^+$ ($C_{38}H_{69}NO_{34}P$ requires 1114.343).

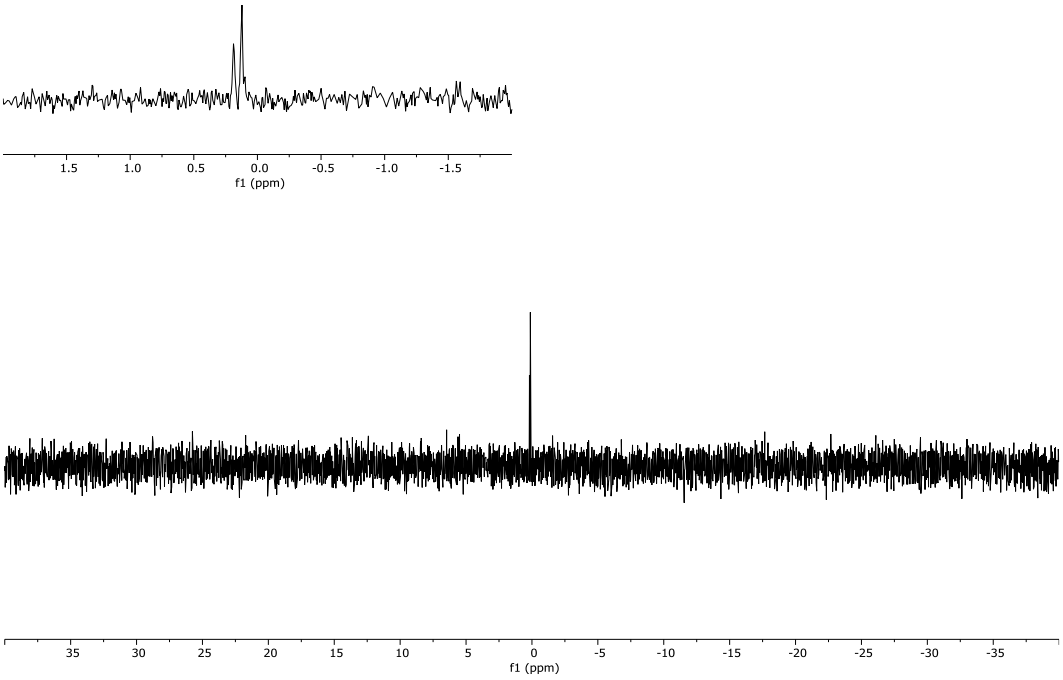
RP-HPLC of A_5P (ELSD trace, Method J5, t_R = 15.1, 15.2 min)



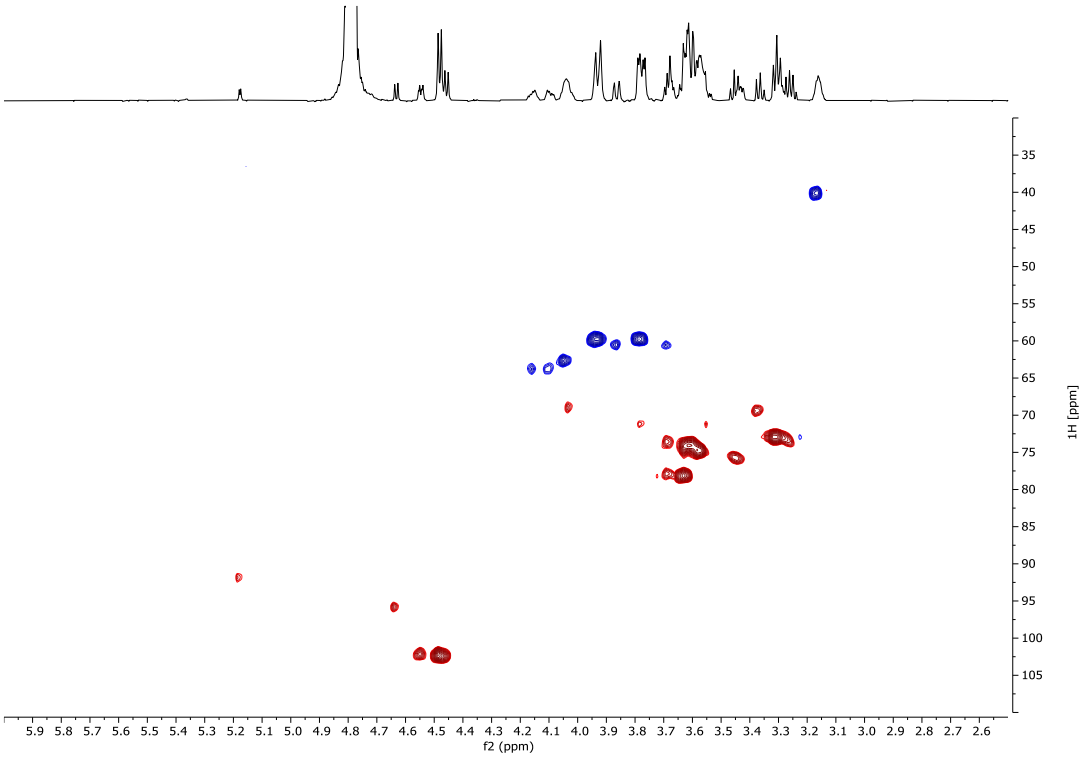
^{1}H NMR of A_5P (700 MHz, D_2O)



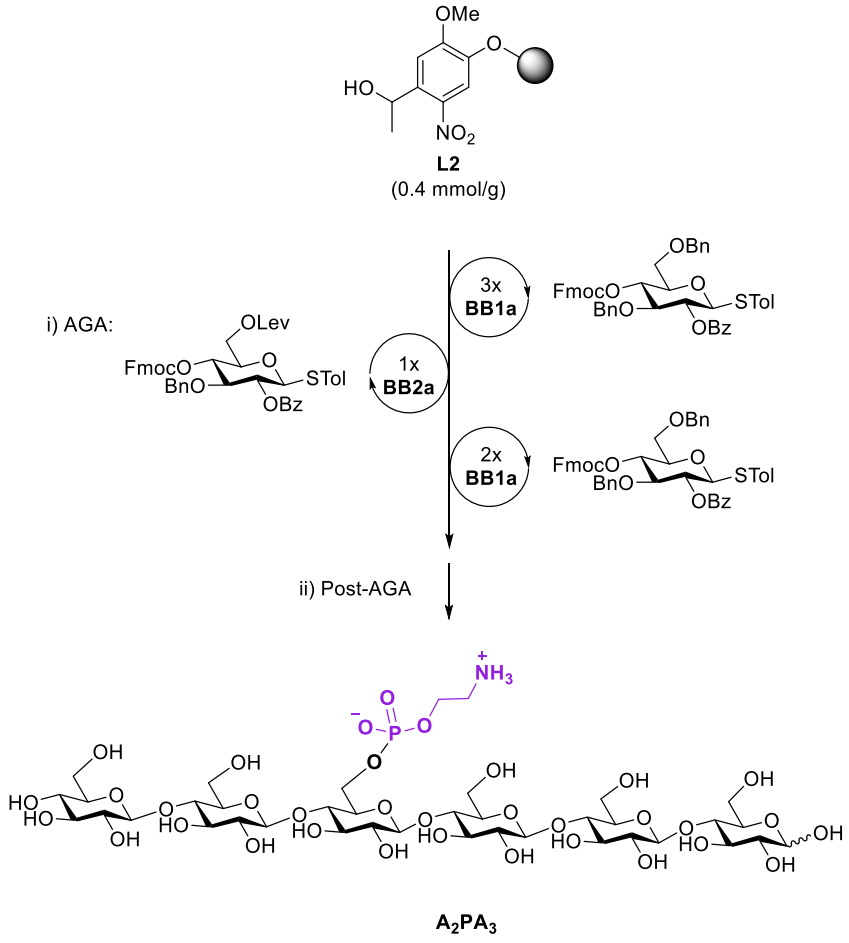
^{31}P NMR of A_5P (162 MHz, D_2O)



HSQC NMR of A_5P (D_2O)



4.8 Synthesis of A_2PA_3

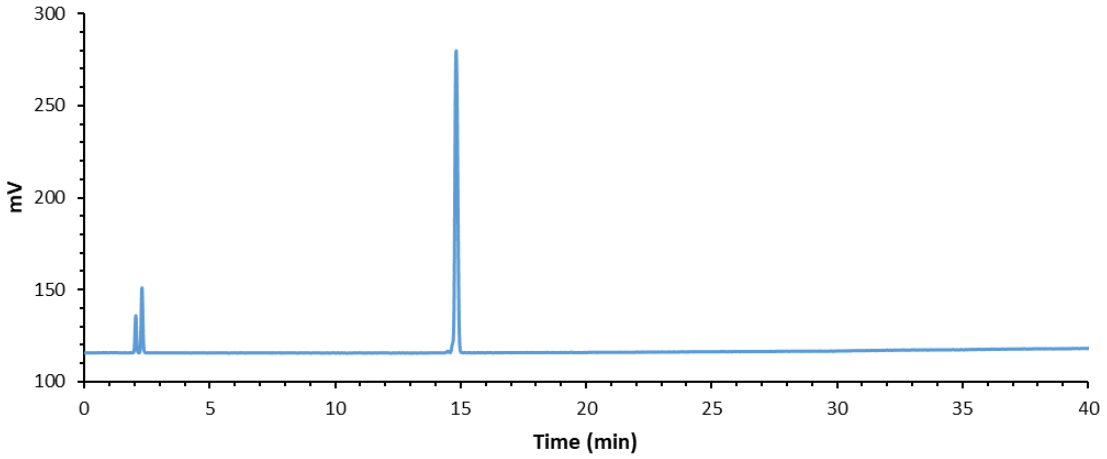


Step		Modules	Notes
AGA		**A**	**L2** swelling
	2 x BB1a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	BB2a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	3 x BB1a	**B, C1, D, E1, D, E2**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
Post-AGA	**Phosphorylation**	**F**	
	Hydrolysis	**G**	**G:** (20 h)
	Purification	**J**	**J:** (Method J1)
	Hydrogenolysis	**I**	**I:** (18 h)
	Purification	**J**	**J:** (Method J6: 16.0 min)

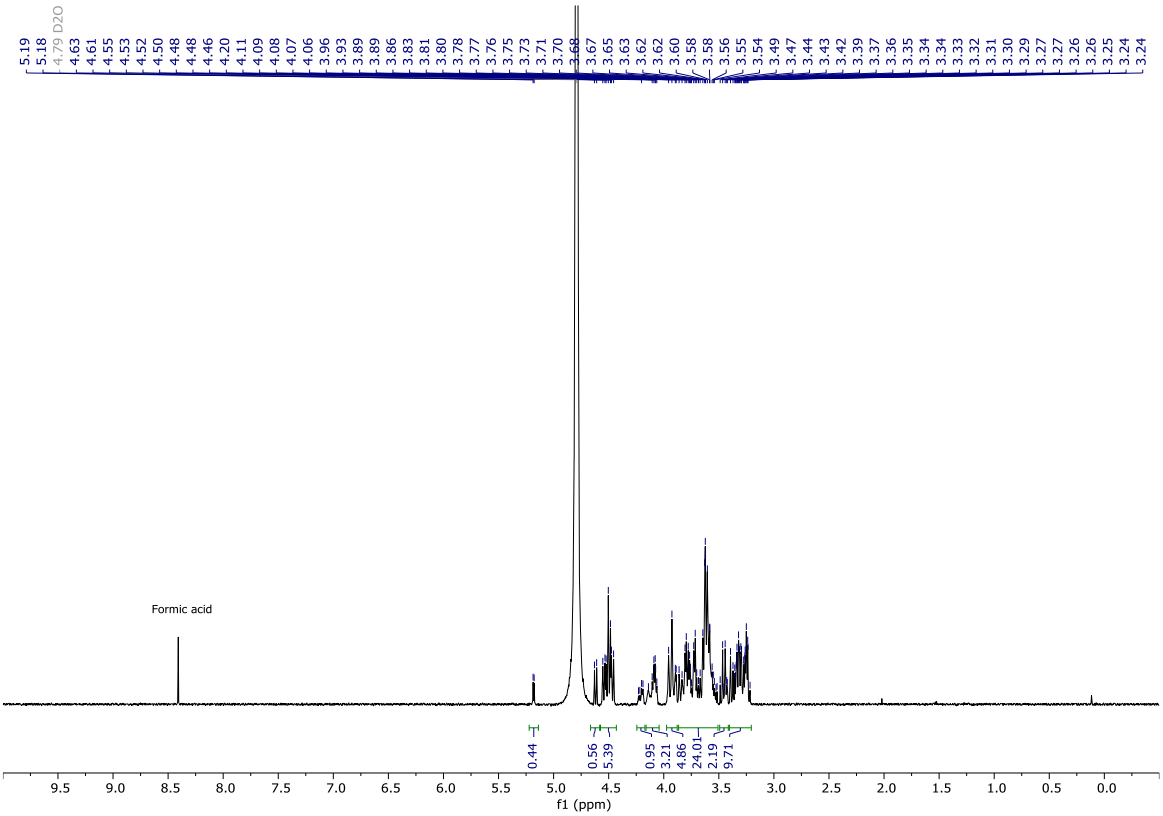
Automated synthesis, global deprotection, and purification afforded **A_2PA_3** as white solid (0.4 mg, 3% overall yield).

^{1}H NMR (600 MHz, D_2O) δ 5.19 (d, *J* = 5.4 Hz, 0.4H, H1-α), 4.63 (d, *J* = 12 Hz, 0.6H, H1-β), 4.55 – 4.46 (m, 5H), 4.23 (dd, *J*= 15.6 Hz, 7.8 Hz, 1H), 4.14 – 4.06 (m, 3H), 3.96 – 3.89 (m, 5H), 3.86 – 3.52 (m, 24H), 3.49 – 3.42 (m, 2H), 3.39 – 3.22 (m, 10H), ^{31}P NMR (243 MHz, D_2O) δ 0.04. HRMS (QToF) m/z 1114.351 $[M+H]^+$ ($C_{38}H_{69}NO_{34}P$ requires 1114.343).

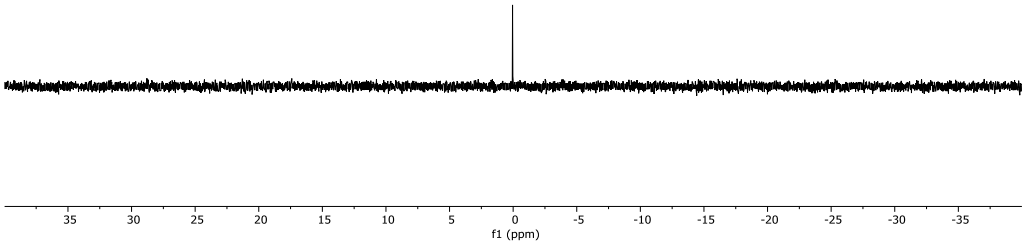
RP-HPLC of A_2PA_3 (ELSD trace, Method J5, t_R = 14.8 min)



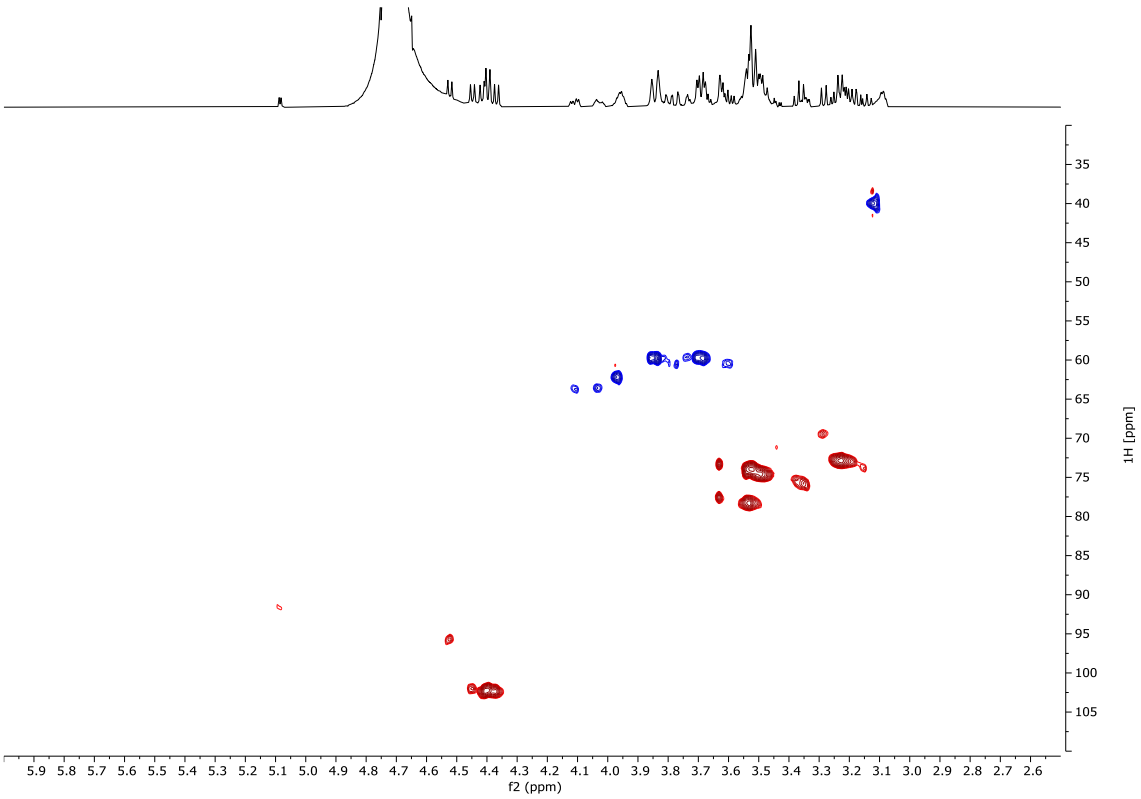
^{1}H NMR of A_2PA_3 (600 MHz, D_2O)



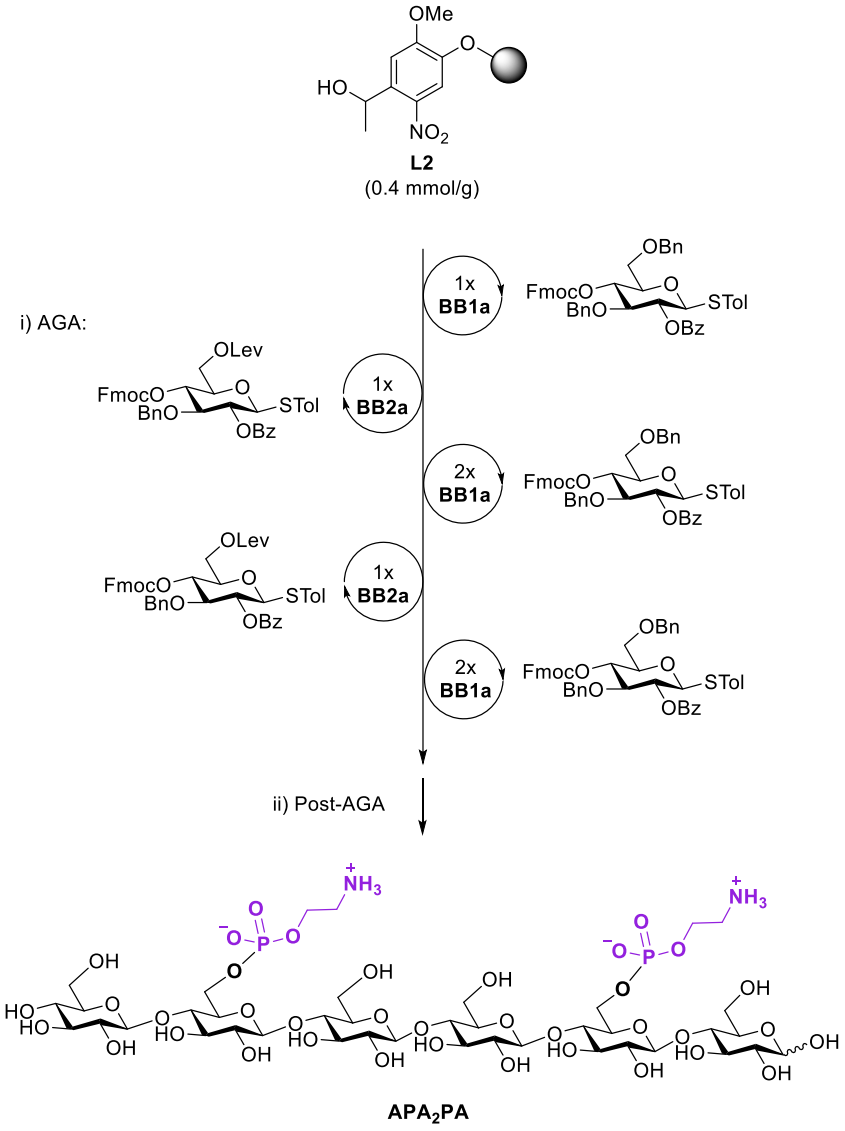
^{31}P NMR of A_2PA_3 (243 MHz, D_2O)



HSQC NMR of A_2PA_3 (D_2O)



4.9 Synthesis of APA_2PA



APA_2PA

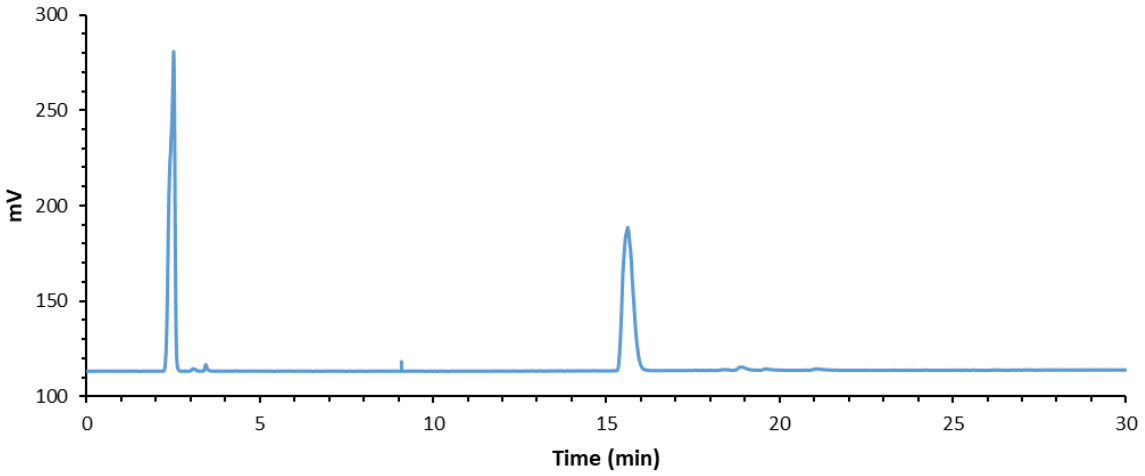
Step		Modules	Notes
AGA		**A**	**L2** swelling
	BB1a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	BB2a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	2 x BB1a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	BB2a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	BB1a	**B, C1, D, E1, D, E2**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
Post-AGA	**Phosphorylation**	**F**	
	Hydrolysis	**G**	**G:** (22 h)
	Purification	**J**	**J:** (Method J1)

	Hydrogenolysis	**I**	**I:** (20 h)
	Purification	**J**	**J:** (Method J8: 16.5 min)

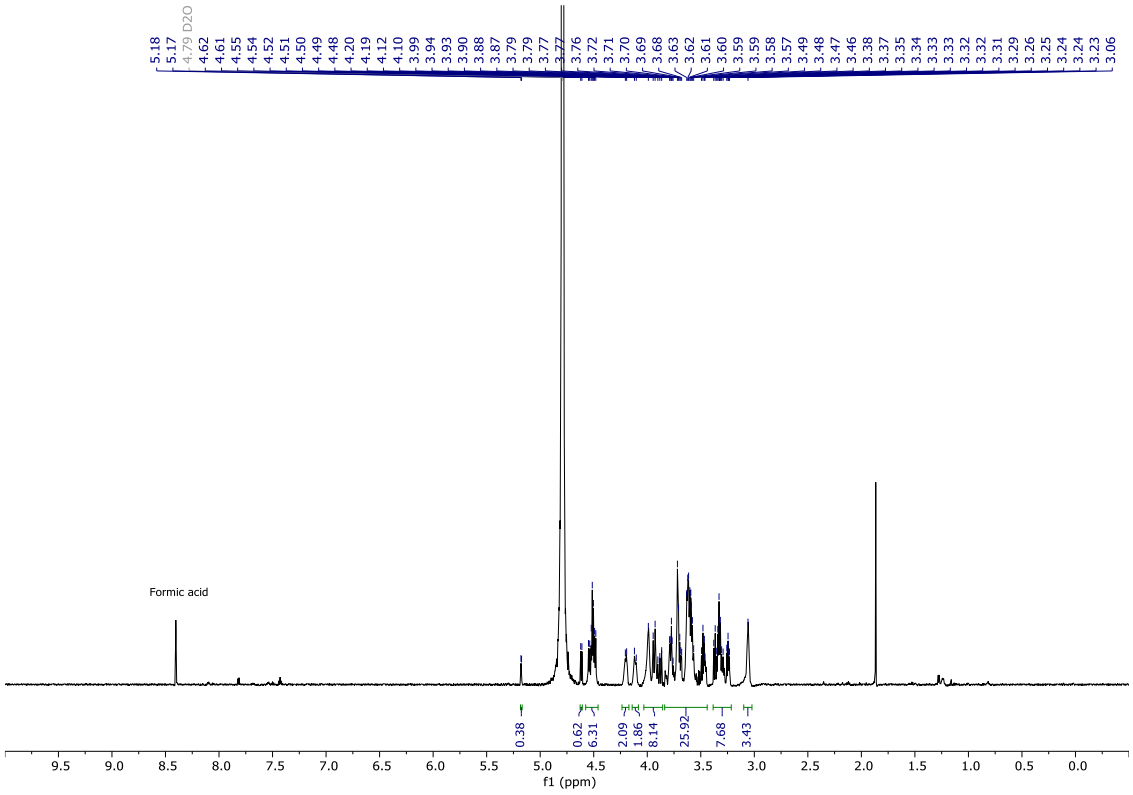
Automated synthesis, global deprotection, and purification afforded **APA$_2$PA** as white solid (0.4 mg, 2% overall yield).

^{1}H NMR (700 MHz, D_2O) δ 5.18 (d, *J* = 3.6 Hz, 0.4H, H1-α), 4.62 (d, *J* = 6.6 Hz, 0.6H, H1-β), 4.55 – 4.48 (m, 6H), 4.20 – 4.19 (br, 2H), 4.12 – 4.10 (br, 2H), 3.99 – 3.87 (m, 8H), 3.79 – 3.46 (m, 26H), 3.38– 3.23 (m, 8H), 3.06 (s, 3H), ^{31}P NMR (162 MHz, D_2O) δ 0.25. HRMS (QToF) m/z 1237.351 $[M+H]^+$ ($C_{40}H_{75}NO_{37}P_2$ requires 1237.351).

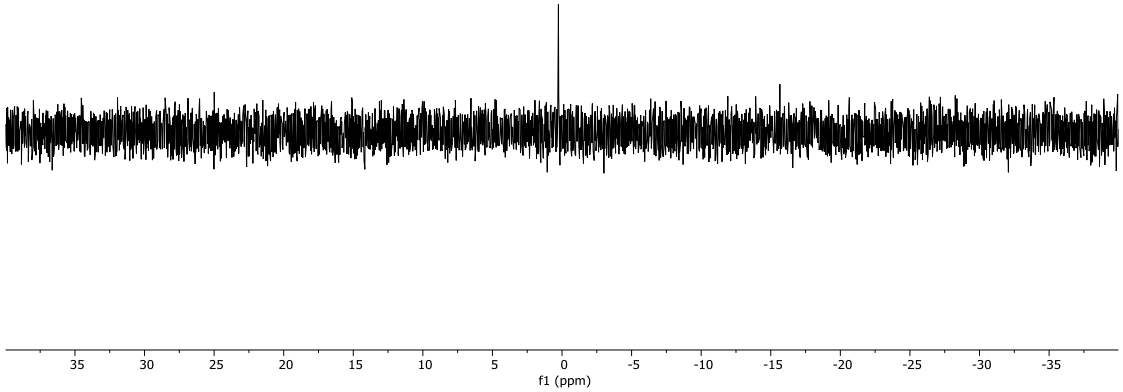
RP-HPLC of APA$_2$PA (ELSD trace, Method J7, t_R = 15.6 min)



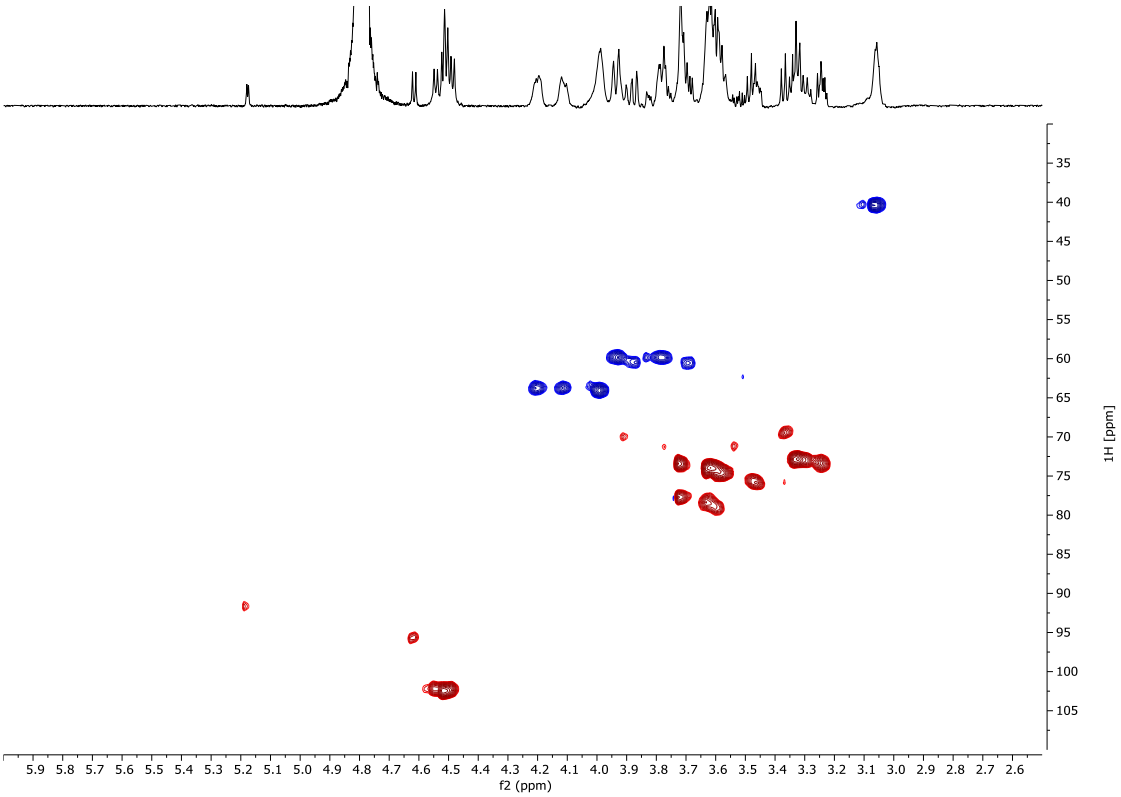
^{1}H NMR of APA$_2$PA (600 MHz, D$_2$O)



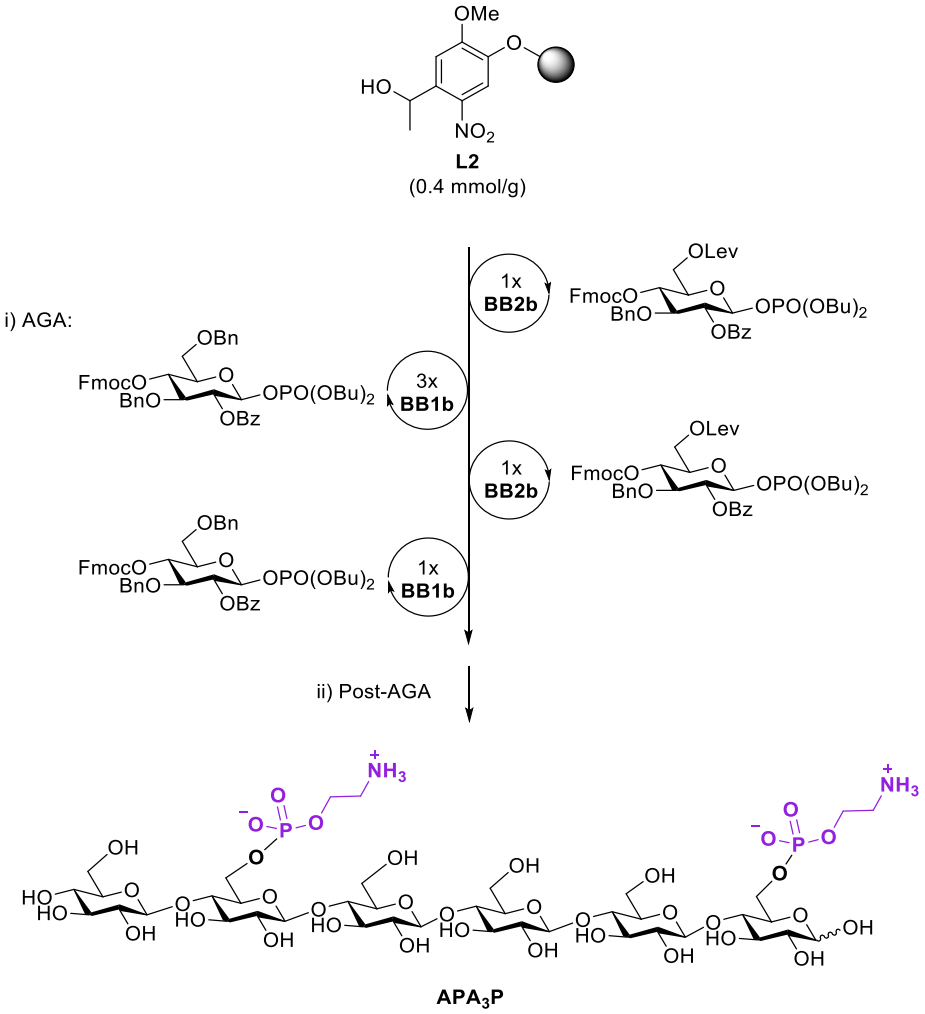
^{31}P NMR of APA$_2$PA (162 MHz, D$_2$O)



HSQC NMR of APA_2PA (D_2O)



4.10 Synthesis of APA_3P

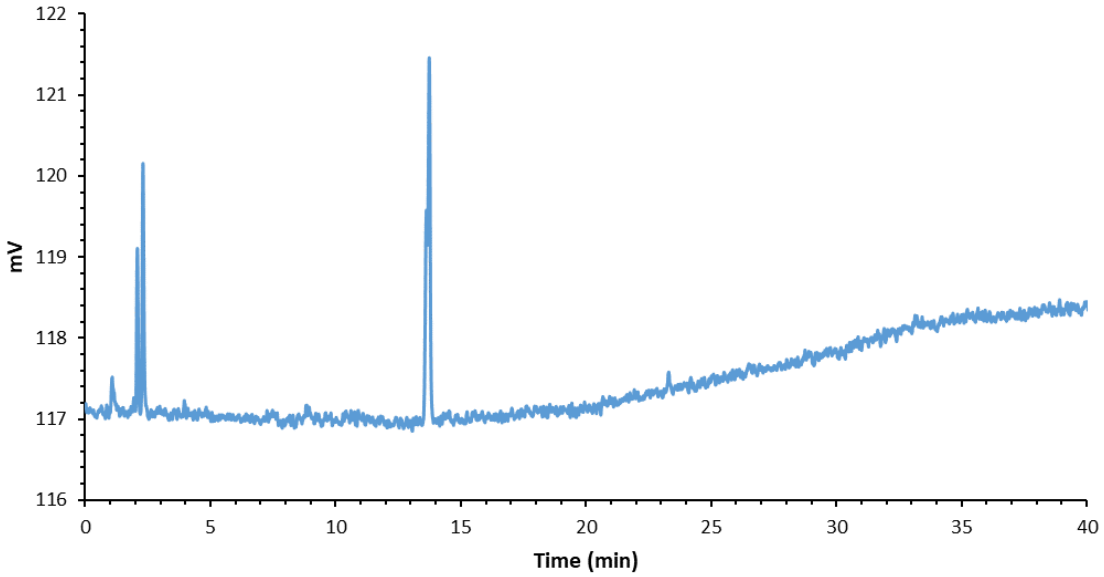


Step		Modules	Notes
AGA		**A**	**L2** swelling
	BB2b	**B, C2, D, E1**	**C2:** (-30 °C for 5 min, -10 °C for 40 min)
	3 x BB1b	**B, C2, D, E1**	**C2:** (-30 °C for 5 min, -10 °C for 40 min)
	BB2b	**B, C2, D, E1**	**C2:** (-30 °C for 5 min, -10 °C for 40 min)
	BB1b	**B, C2, D, E1, D, E2**	**C2:** (-30 °C for 5 min, -10 °C for 40 min)
Post-AGA	**Phosphorylation**	**F**	
	Hydrolysis	**G**	**G:** (4 h)
	Purification	**J**	**J:** (Method J1)
	Hydrogenolysis	**I**	**I:** (20 h)
	Purification	**J**	**J:** (Method J8: 16.0 min)

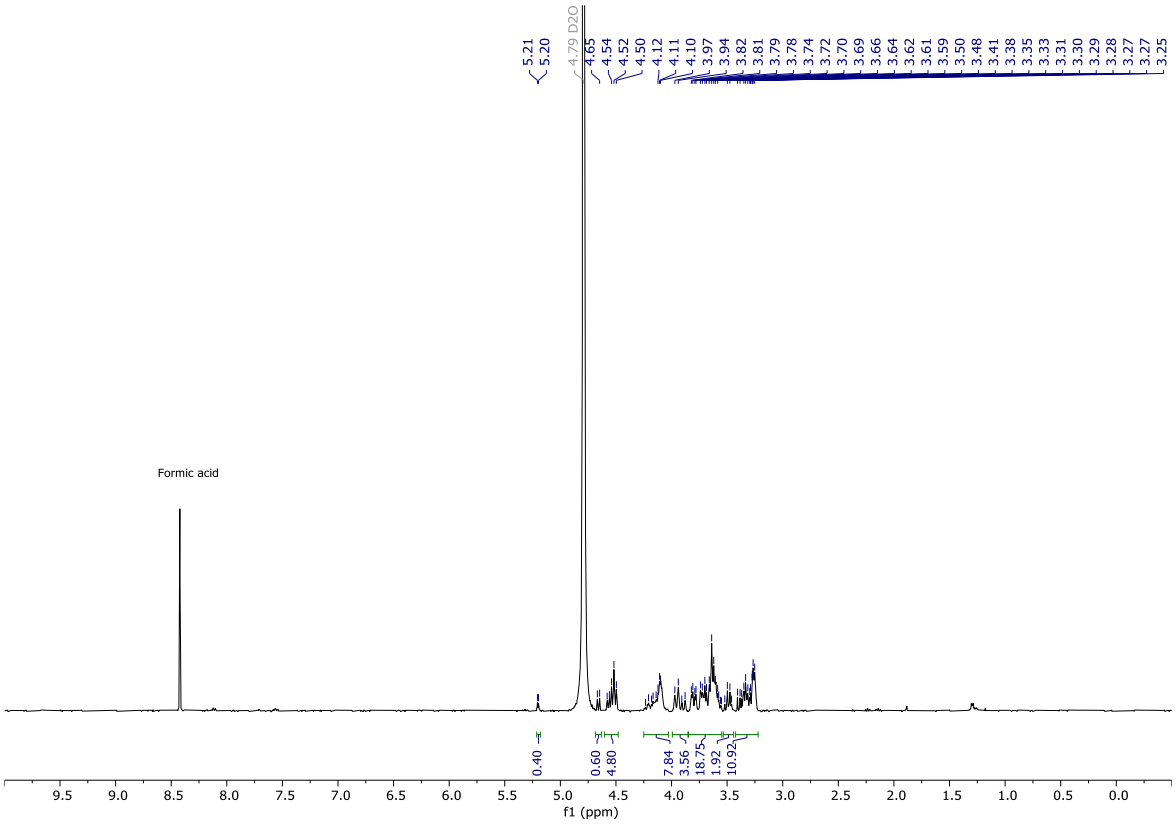
Automated synthesis, global deprotection, and purification afforded **APA$_3$P** as white solid (0.9 mg, 6% overall yield).

^{1}H NMR (400 MHz, D_2O) δ 5.21 (d, *J* = 3.6 Hz, 0.4H, H1-α), 4.67 (d, *J* = 7.6 Hz, 0.6H, H1-β),) 4.58 – 4.50 (m, 5H), 4.23 – 4.10 (m, 8H) 3.97 – 3.88 (m, 3H) 3.82 – 3.55 (m, 19H), 3.52 – 3.46 (m, 2H), 3.41 – 3.25 (m, 11H), ^{31}P NMR (162 MHz, D_2O) δ 0.01, -0.01, -0.04. HRMS (QToF) m/z 1237.354 $[M+H]^+$ ($C_{40}H_{75}NO_{37}P_2$ requires 1237.351).

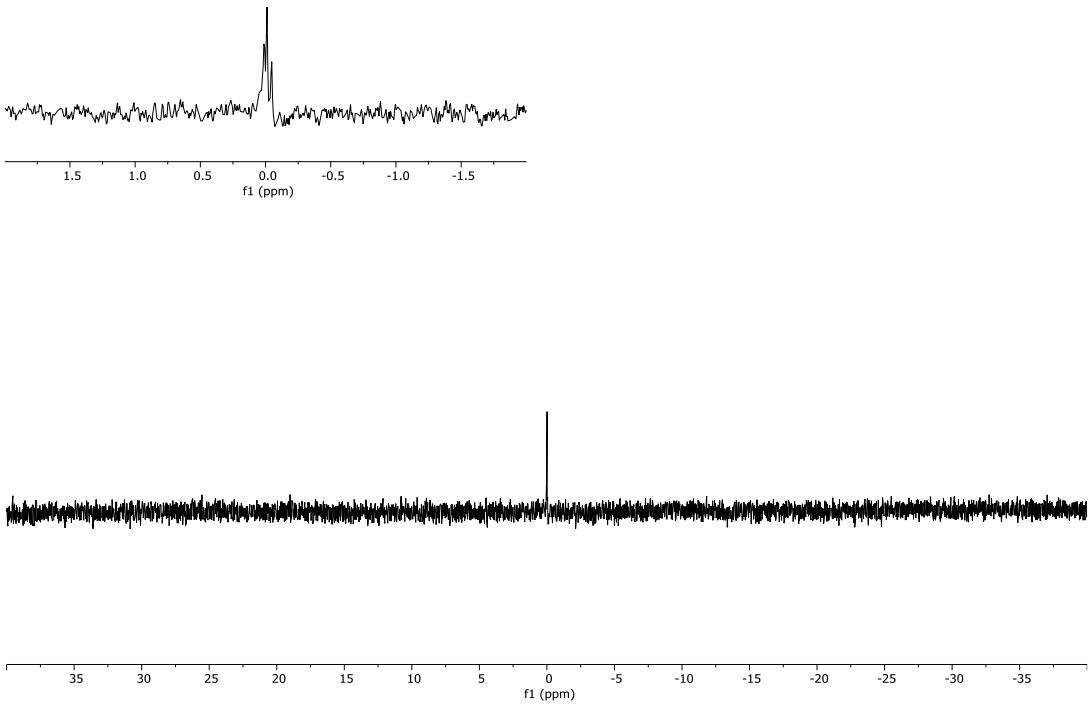
RP-HPLC of APA$_3$P (ELSD trace, Method J5, t_R = 13.6, 13.7 min)



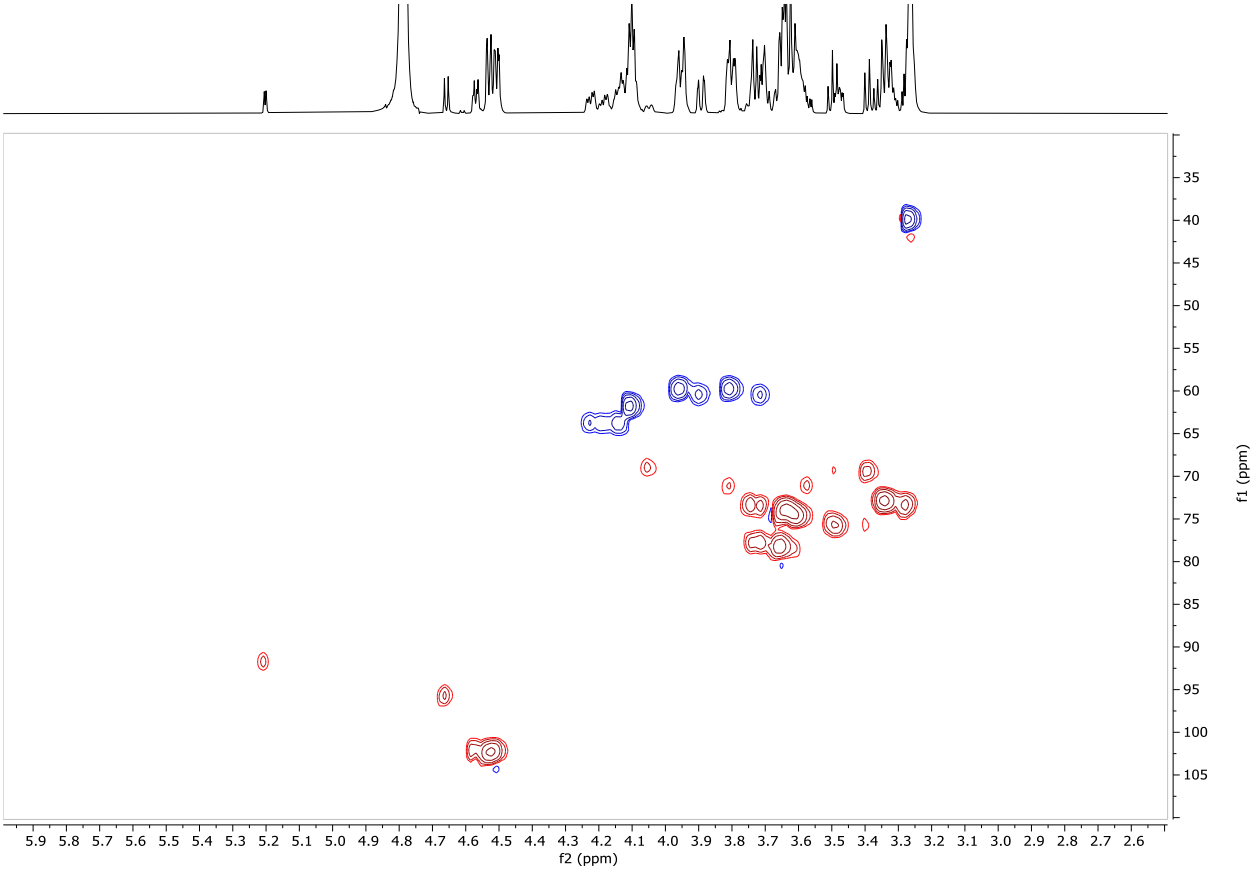
^{1}H NMR of APA$_3$P (400 MHz, D$_2$O)



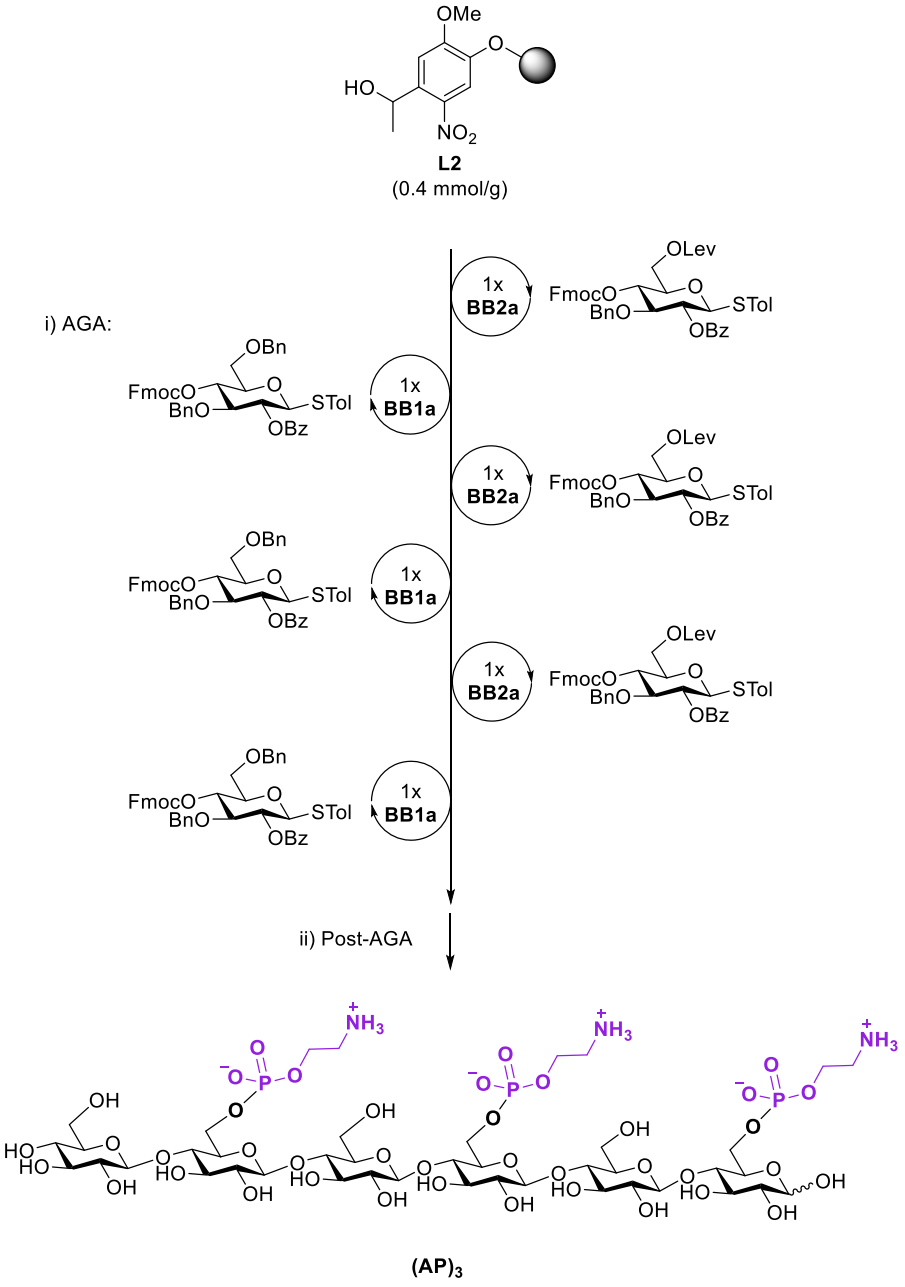
^{31}P NMR of APA$_3$P (162 MHz, D$_2$O)



HSQC NMR of APA_3P (D_2O)



4.11 Synthesis of $(AP)_3$

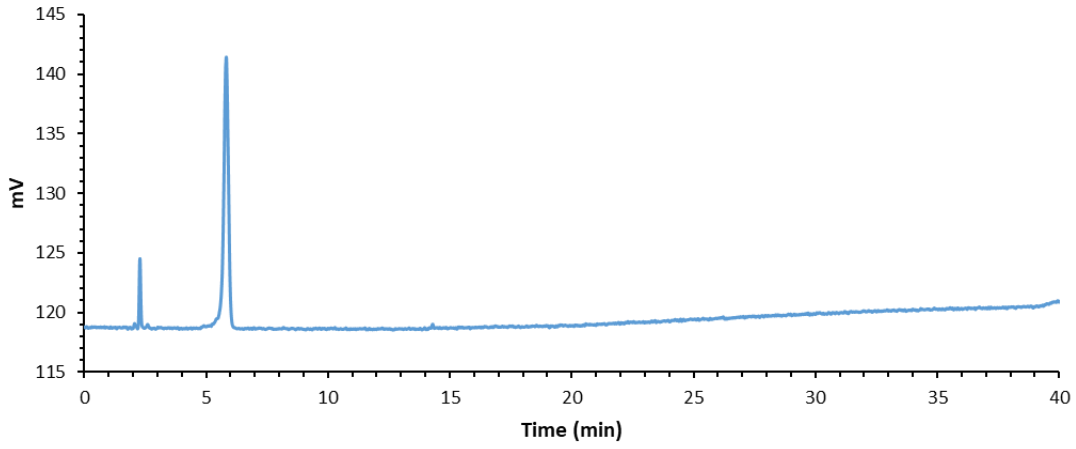


Step		Modules	Notes
AGA		**A**	**L2** swelling
	BB2a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	BB1a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	BB2a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	BB1a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	BB2a	**B, C1, D, E1**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
	BB1a	**B, C1, D, E1, D, E2**	**C1:** (-20 °C for 5 min, 0 °C for 20 min)
Post-AGA	**Phosphorylation**	**F**	
	Hydrolysis	**G**	**G:** (4 h)
	Purification	**J**	**J:** (Method J1)
	Hydrogenolysis	**I**	**I:** (19 h)
	Purification	**J**	**J:** (Method J6: 14.7 min)

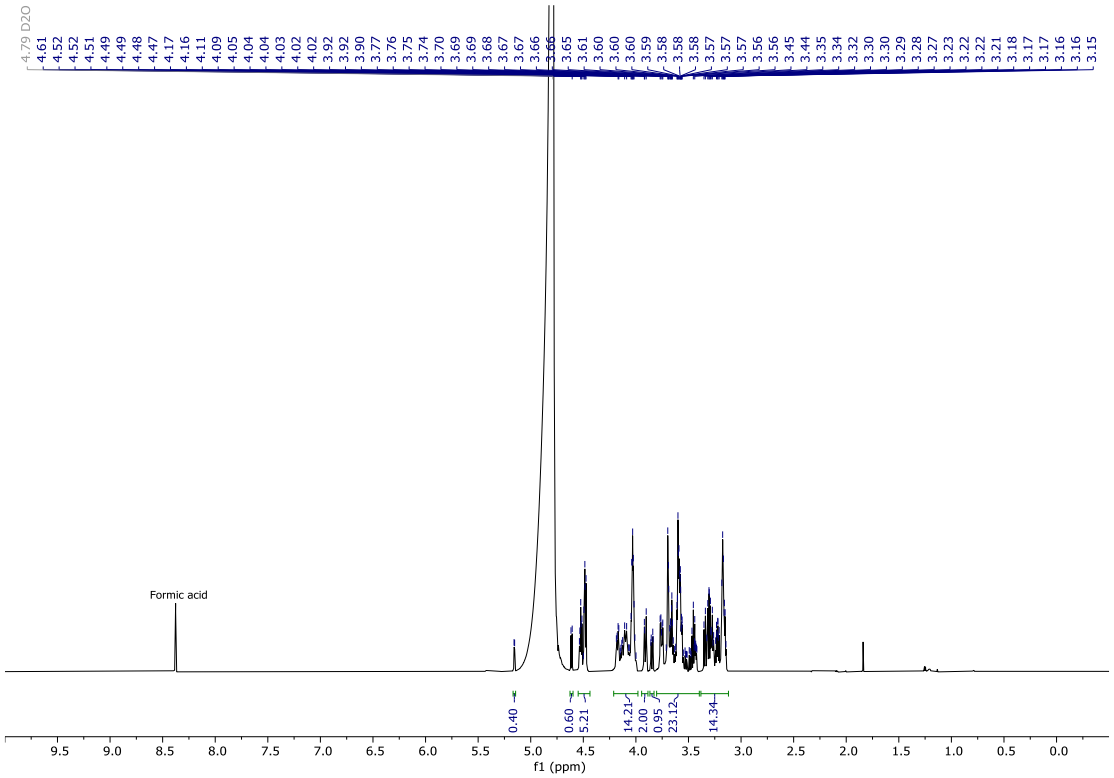
Automated synthesis, global deprotection, and purification afforded **$(AP)_3$** as white solid (0.9 mg, 7% overall yield).

^{1}H NMR (700 MHz, D_2O) δ, 5.16 (d, *J* = 3.5 Hz, 0.4H, H1-α), 4.62 (d, *J* = 7.7 Hz, 0.6H, H1-β),) 4.54 – 4.47 (m, 5H), 4.19 – 4.00 (m, 14H) 3.92 (d, *J* = 14 Hz, 2H), 3.86 (d, *J* = 7 Hz, 1H), 3.77 – 3.42 (m, 23H), 3.35 – 3.14 (m, 14H), ^{31}P NMR (162 MHz, D_2O) δ 0.08, 0.03. HRMS (QToF) m/z 1360.3546 $[M+H]^+$ ($C_{42}H_{81}N_3O_{40}P_3$ requires 1360.3604).

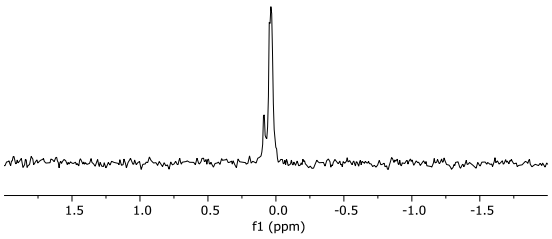
RP-HPLC of $(AP)_3$ (ELSD trace, Method J9, t_R = 5.8 min)

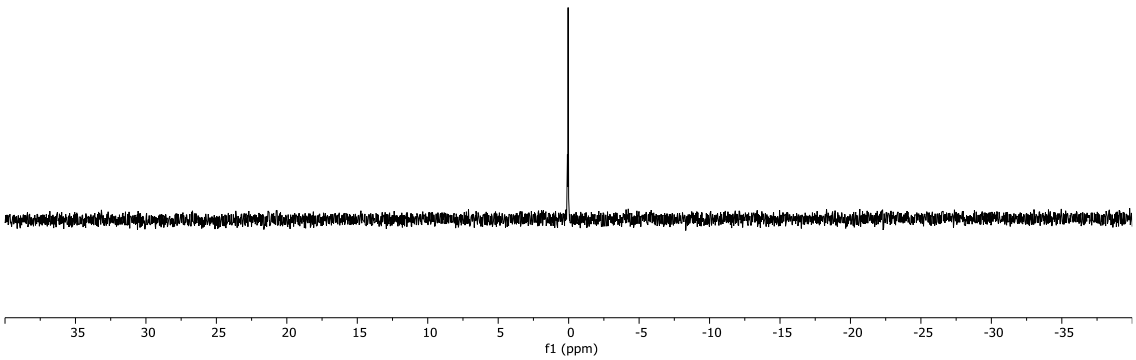


^{1}H NMR of $(AP)_3$ (700 MHz, D_2O)

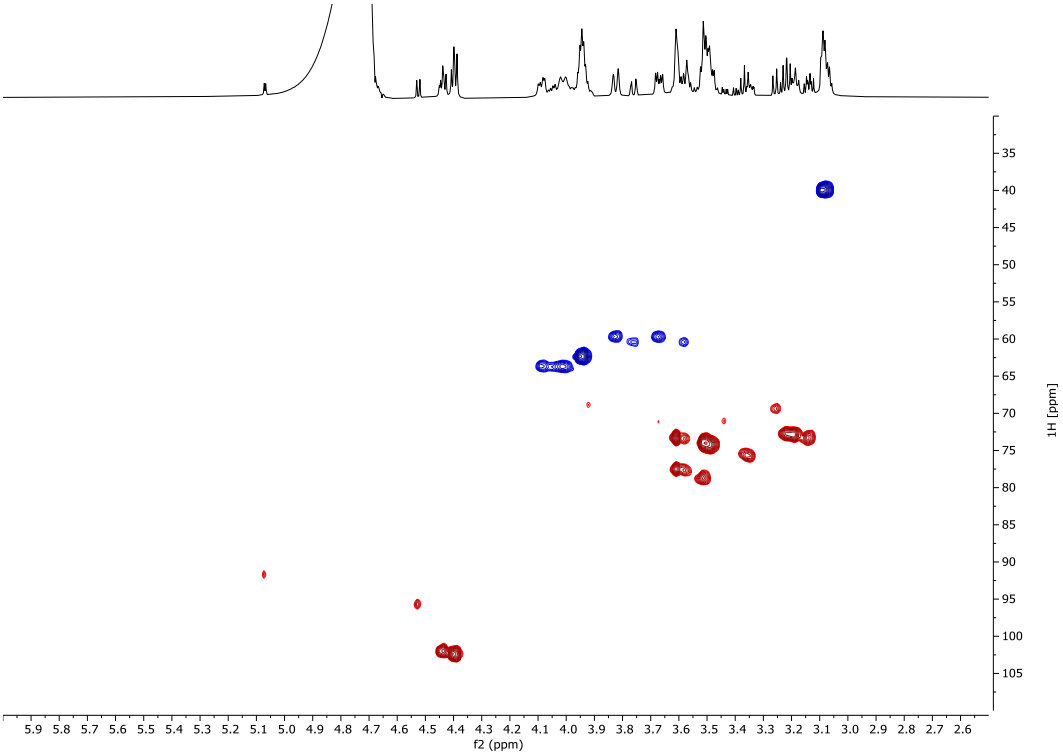


^{31}P NMR of $(AP)_3$ (162 MHz, D_2O)





HSQC NMR of $(AP)_3$ (D_2O)



5. References

(1) Eller, S.; Collot, M.; Yin, J.; Hahm, H. S.; Seeberger, P. H. Automated Solid-Phase Synthesis of Chondroitin Sulfate Glycosaminoglycans. *Angew. Chemie - Int. Ed.* **2013**, *52*, 5858–5861. https://doi.org/10.1002/anie.201210132.

(2) Dallabernardina, P.; Schuhmacher, F.; Seeberger, P. H.; Pfrengle, F. Automated Glycan Assembly of Xyloglucan Oligosaccharides. *Org. Biomol. Chem.* **2016**, *14*, 309–313. https://doi.org/10.1039/C5OB02226F.

(3) Tyrikos-Ergas, T.; Sletten, E. T.; Huang, J.-Y.; Seeberger, P. H.; Delbianco, M. On Resin Synthesis of Sulfated Oligosaccharides. *Chem. Sci.* **2022**, *13*, 2115–2120. https://doi.org/10.1039/D1SC06063E.

(4) Le Mai Hoang, K.; Pardo-Vargas, A.; Zhu, Y.; Yu, Y.; Loria, M.; Delbianco, M.; Seeberger, P. H. Traceless Photolabile Linker Expedites the Chemical Synthesis of Complex Oligosaccharides by Automated Glycan Assembly. *J. Am. Chem. Soc.* **2019**, *141*, 9079–9086. https://doi.org/10.1021/jacs.9b03769.

(5) Gude, M.; Ryf, J.; White, P. D. An Accurate Method for the Quantitation of Fmoc-Derivatized Solid Phase Supports. *Lett. Pept. Sci.* **2002**, *9*, 203–206. https://doi.org/doi.org/10.1023/A:1024148619149.

(6) Tuck, O. T.; Sletten, E. T.; Danglad-Flores, J.; Seeberger, P. H. Towards a Systematic Understanding of the Influence of Temperature on Glycosylation Reactions. *Angew. Chemie Int. Ed.* **2022**, *202115433*. https://doi.org/10.1002/anie.202115433.

(7) Tsai, Y. H.; Götze, S.; Vilotijevic, I.; Grube, M.; Silva, D. V.; Seeberger, P. H. A General and Convergent Synthesis of Diverse Glycosylphosphatidylinositol Glycolipids. *Chem. Sci.* **2013**, *4*, 468–481. https://doi.org/10.1039/c2sc21515b.

(8) Campbell, A. S.; Fraser-Reid, B. Support Studies for Installing the Phosphodiester Residues of the Thy-1 Glycoprotein Membrane Anchor. *Bioorganic Med. Chem.* **1994**, *2*, 1209–1219. https://doi.org/10.1016/S0968-0896(00)82072-6.

(9) Hurevich, M.; Kandasamy, J.; Ponnappa, B. M.; Collot, M.; Kopetzki, D.; McQuade, D. T.; Seeberger, P. H. Continuous Photochemical Cleavage of Linkers for Solid-Phase Synthesis. *Org. Lett.* **2014**, *16*, 1794–1797. https://doi.org/10.1021/ol500530q.

(10) Ohara, K.; Lin, C.-C.; Yang, P.-J.; Hung, W.-T.; Yang, W.-B.; Cheng, T.-J. R.; Fang, J.-M.; Wong, C.-H. Synthesis and Bioactivity of β-(1→4)-Linked Oligomannoses and Partially Acetylated Derivatives. *J. Org. Chem.* **2013**, *78*, 6390–6411. https://doi.org/10.1021/jo4005266.

(11) Tyrikos-Ergas, T.; Gim, S.; Huang, J.; Pinzón Martín, S.; Varón Silva, D.; Seeberger, P. H.; Delbianco, M. Synthetic Phosphoethanolamine-Modified Oligosaccharides Reveal the Importance of Glycan Length and Substitution in Biofilm-Inspired Assemblies. *Nat. Commun.* **2022**, *13*, 3954–3961. https://doi.org/10.1038/s41467-022-31633-5.