Supporting Information

New Alkyne and Amine Linkers for Versatile Multiple Conjugation of Oligonucleotides

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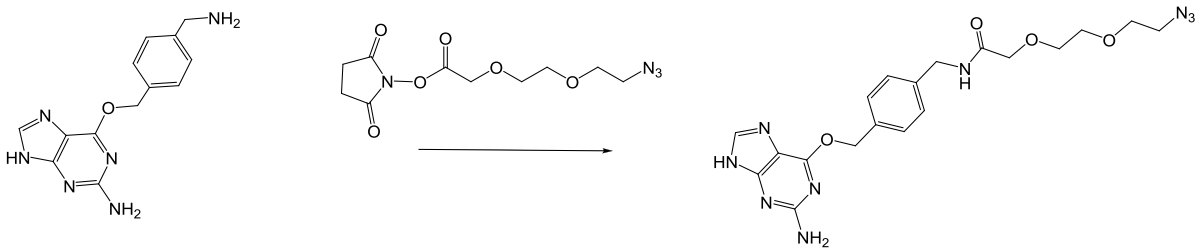
CONTENTS:

(*R*)-5-Aminopentane-1,3-diol (2) $LiAlH_4$ pellets (26.9 g, 708.8 mmol) were suspended in THF (557 ml). A solution of compound **1** (55.7 g, 354.4 mmol) in THF (278.5 mL) was added dropwise to the $LiAlH_4$ at room temperature. The reaction mixture was heated to 70 °C and left to stir for 6 h. Then reaction was chilled to 0 °C and Fieser work-up procedure (dropwise addition of water and NaOH solution followed by water) was done. The solids were removed by filtration, washed with 2-propanol, and the filtrate was concentrated *in vacuo*. The aminodiol **2** recovered (38.5 g, 323.1 mmol, 91%) was used for further steps without additional purification. ^{1}H NMR and ^{13}C NMR were consistent with literature data.

4-[(9*H*-Fluoren-9-yl-methoxycarbonylamino)methyl]benzoic acid (13): To a solution of p-aminomethyl benzoic acid (10 g, 66.15 mmol) and Fmoc-OSu (24.55 g, 72.78 mmol) in THF (200 mL) 10% $NaHCO_3$ aqueous solution (200 mL) was added. The reaction mixture was stirred at room temperature for 4 h. The THF was removed under reduced pressure and water/ethyl acetate work-up was performed. The aqueous solution was acidified with 1 N HCl, the reaction mixture was filtered, and the residue was washed with ethyl acetate and water. The solid residue was dried under vacuum to give **13** (21.76 g, 88%) as a white powder. ^{1}H NMR and ^{13}C NMR found to be consistent with the literature data (Kim, M. H., et al., Simple methods for tracking stem cells with ^{64}Cu-labeled DOTA-hexadecyl-benzoate. *ACS Med. Chem. Lett.*, 2015, *6*, 528–530).



Scheme S1. Synthesis of *N*-(4-(((2-amino-9*H*-purin-6-yl)oxy)methyl)benzyl)-2-(2-(2-azidoethoxy)ethoxy)-acetamide (BG-N_3, SNAP-Tag azide).

***N*-(4-(((2-Amino-9*H*-purin-6-yl)oxy)methyl)benzyl)-2-(2-(2-azidoethoxy)ethoxy)-acetamide (BG-N_3, SNAP-Tag azide).** The 6-((4-(aminomethyl)benzyl)oxy)-9*H*-purin-2-amine (SNAP-Tag) (112.4 mg, 0.417 mmol) was dissolved in 5 ml of acetone/water (1:1 v/v) whereupon 125 mg (0.437 mmol, 1.05 equiv.) 2,5-dioxopyrrolidin-1-yl 2-(2-(2-azidoethoxy)ethoxy)acetate (2-[2-(2-azidoethoxy)ethoxy]acetic acid hydroxysuccinimide ester) was added followed by 87 µl of triethylamine (63 mg, 1.5 equiv.). Reaction was stirred overnight at room temperature. After evaporation of solvent, the crude product was subjected to silica gel flash column chromatography using dichloromethane-methanol mixture (9:1 v/v) as eluent to obtain product BG-N_3 (102 mg, 55% yield). ^{1}H NMR (400.1 MHz, $CDCl_3$ plus CD_3OD): δ = 7.68 (s, 1H), 7.36 (d, J = 8.02 Hz, 2H), 7.20 (d, J = 8.02 Hz, 2H), 5.42 (s, 2H), 4.35 (s, 2H), 3.95 (s, 2H), 3.62-3.52 (m, 4H), 3.48 (t, J = 4.80 Hz, 2H), 3.15 (t, J = 4.80 Hz, 2H) ppm. ES–MS calcd. for $C_{19}H_{24}N_9O_4$ $[M + H]^+$ 442.19, found 442.18.

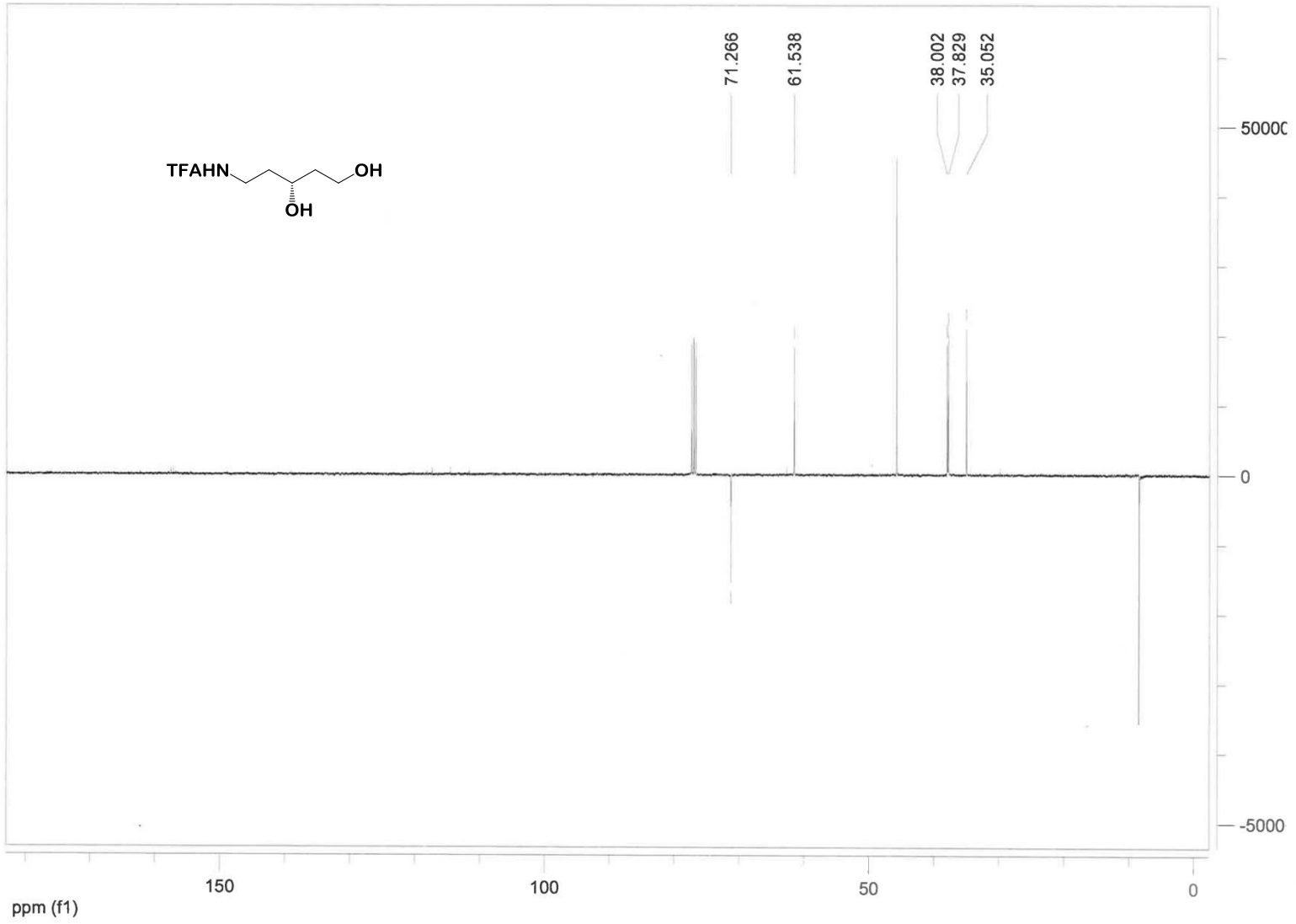


Figure S1. ^{13}C APT NMR spectrum of compound **3** ($CDCl_3$, 100.6 MHz).

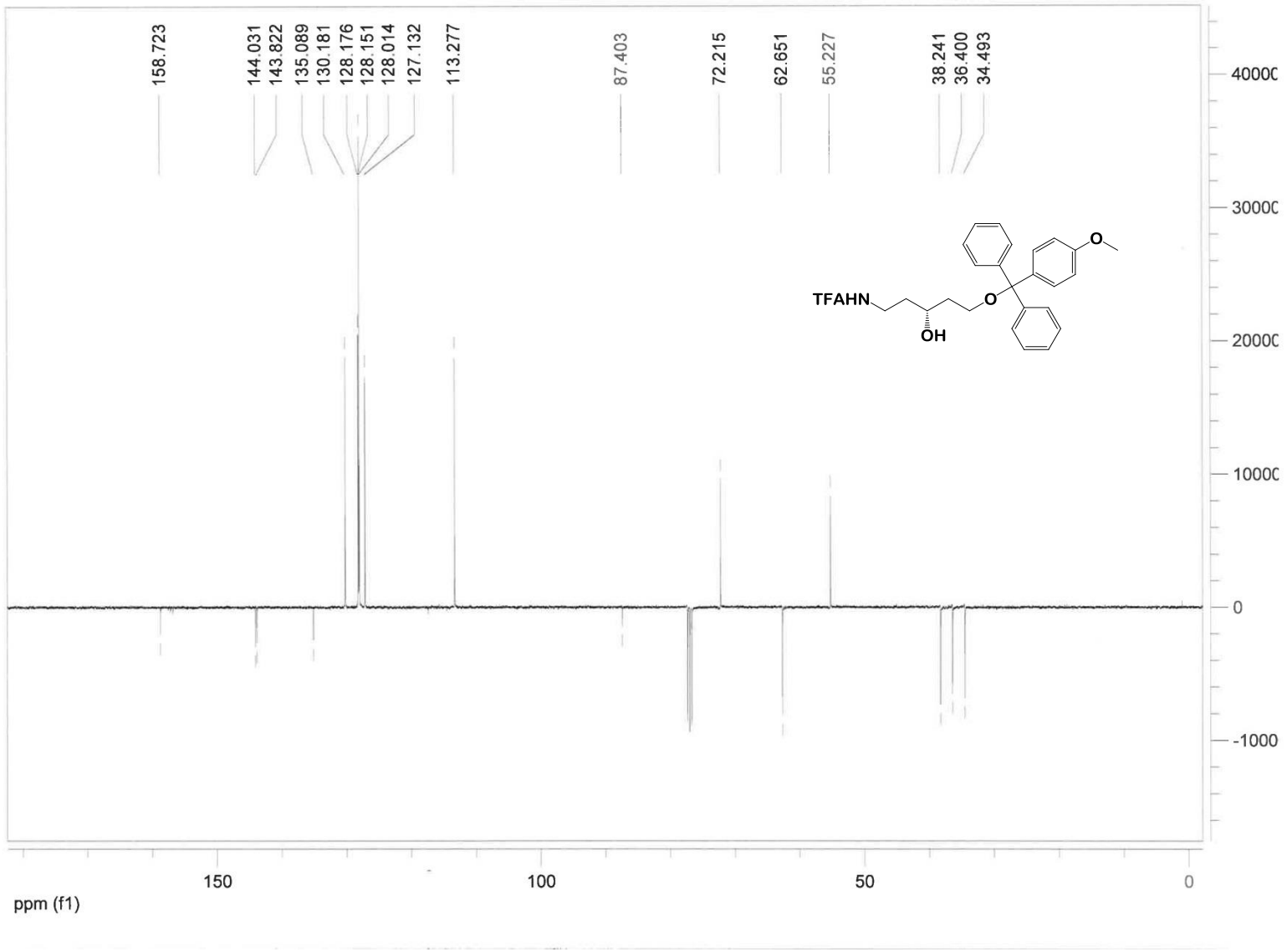


Figure S2. ^{13}C APT NMR spectrum of compound **4** ($CDCl_3$, 100.6 MHz).

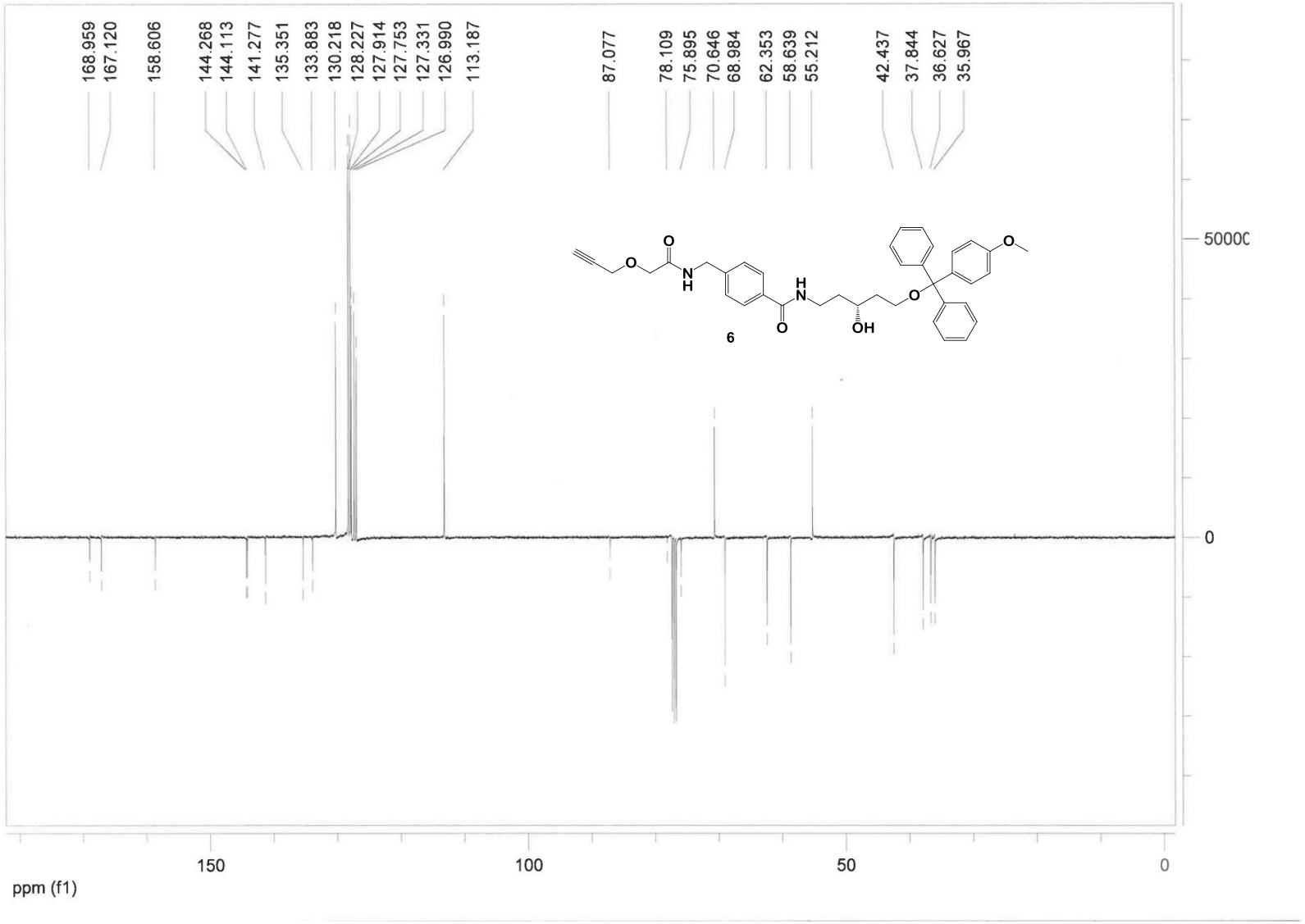


Figure S3. ^{13}C APT NMR spectrum of compound **6** ($CDCl_3$, 100.6 MHz).

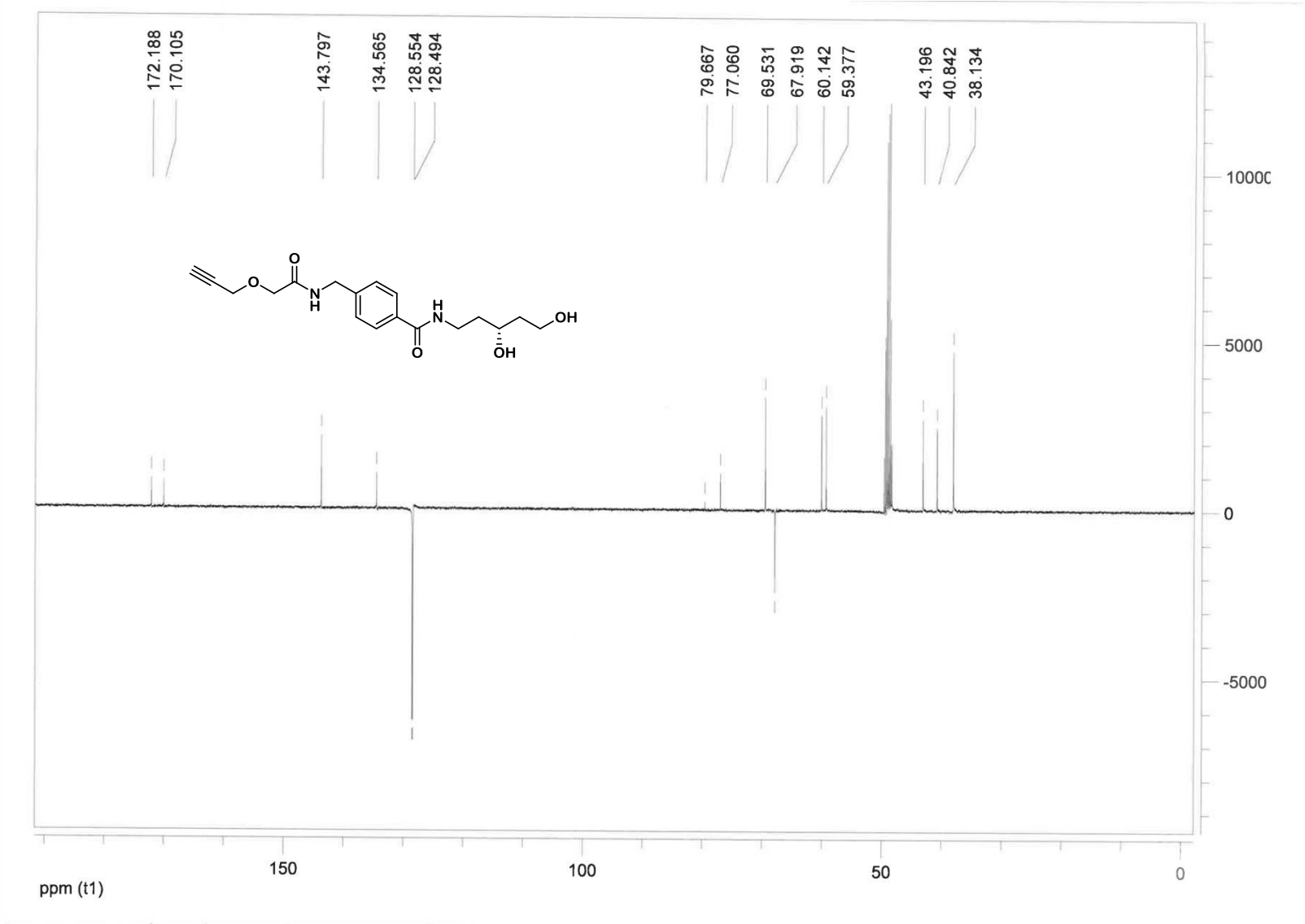


Figure S4. ^{13}C APT NMR spectrum of compound **9** (CD_3OD, 100.6 MHz).

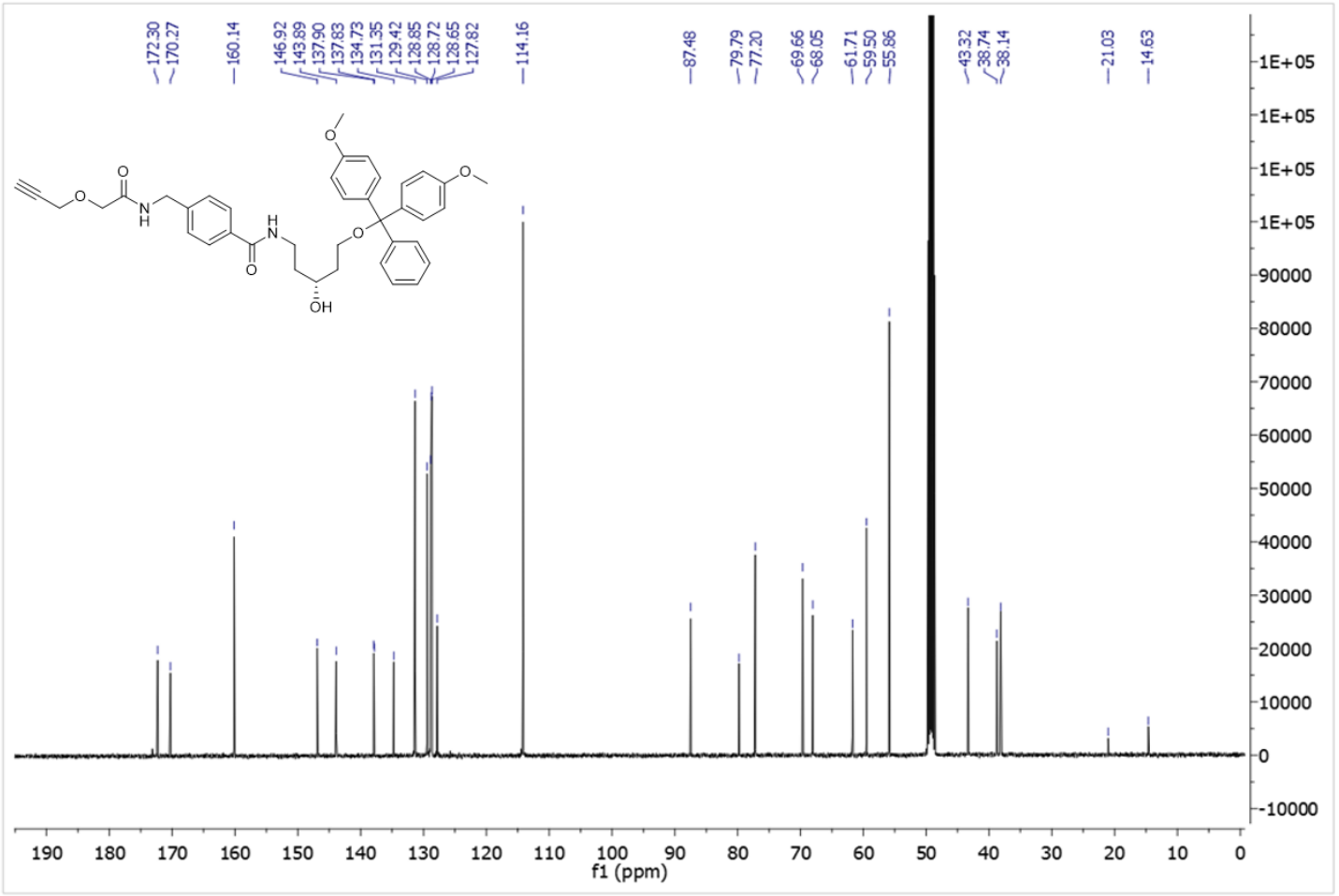


Figure S5. ^{13}C NMR spectrum of compound **10** (CD_3OD, 125.76 MHz).

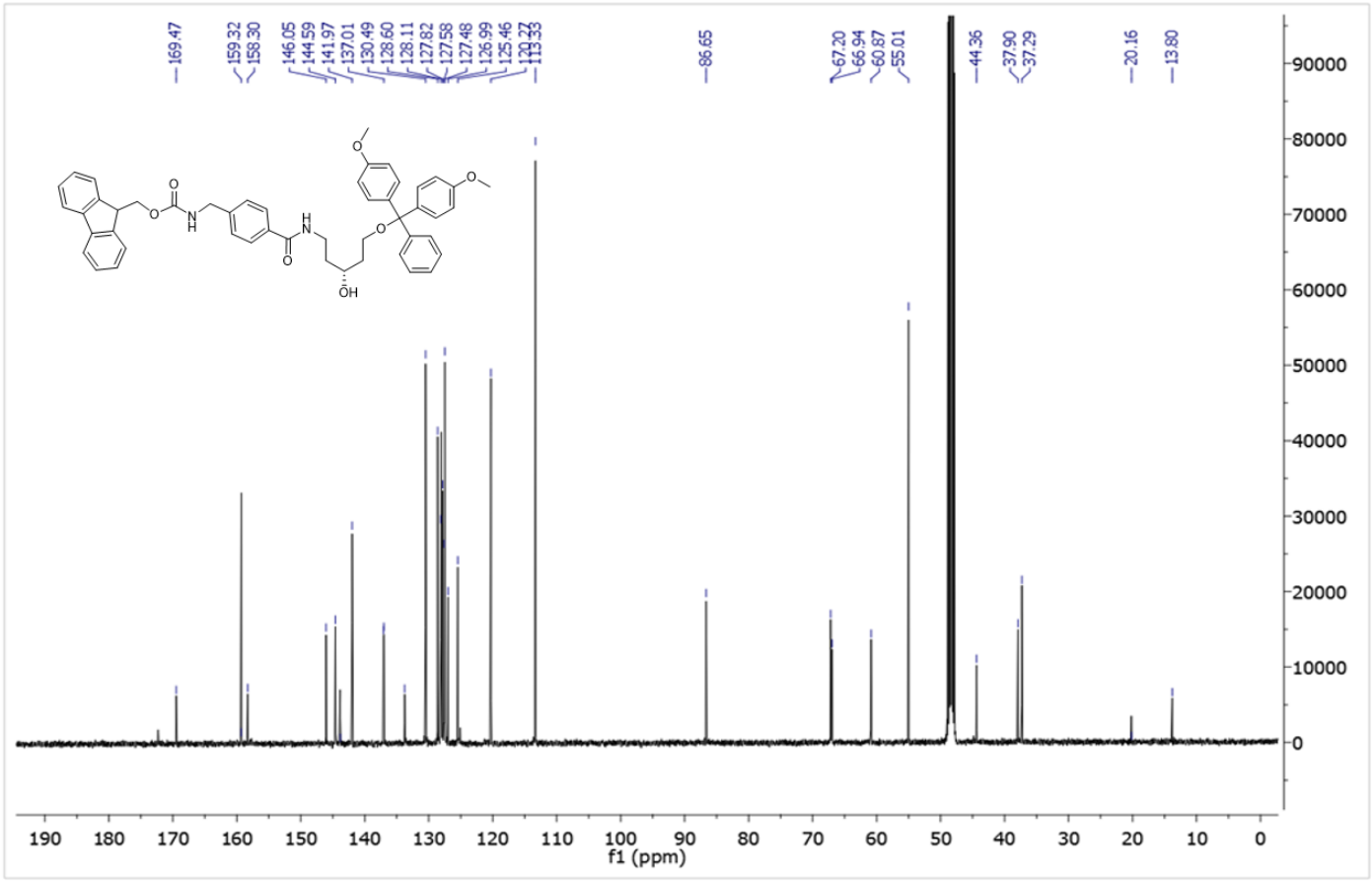


Figure S6. ^{13}C NMR spectrum of compound **15** (CD_3OD, 125.76 MHz).

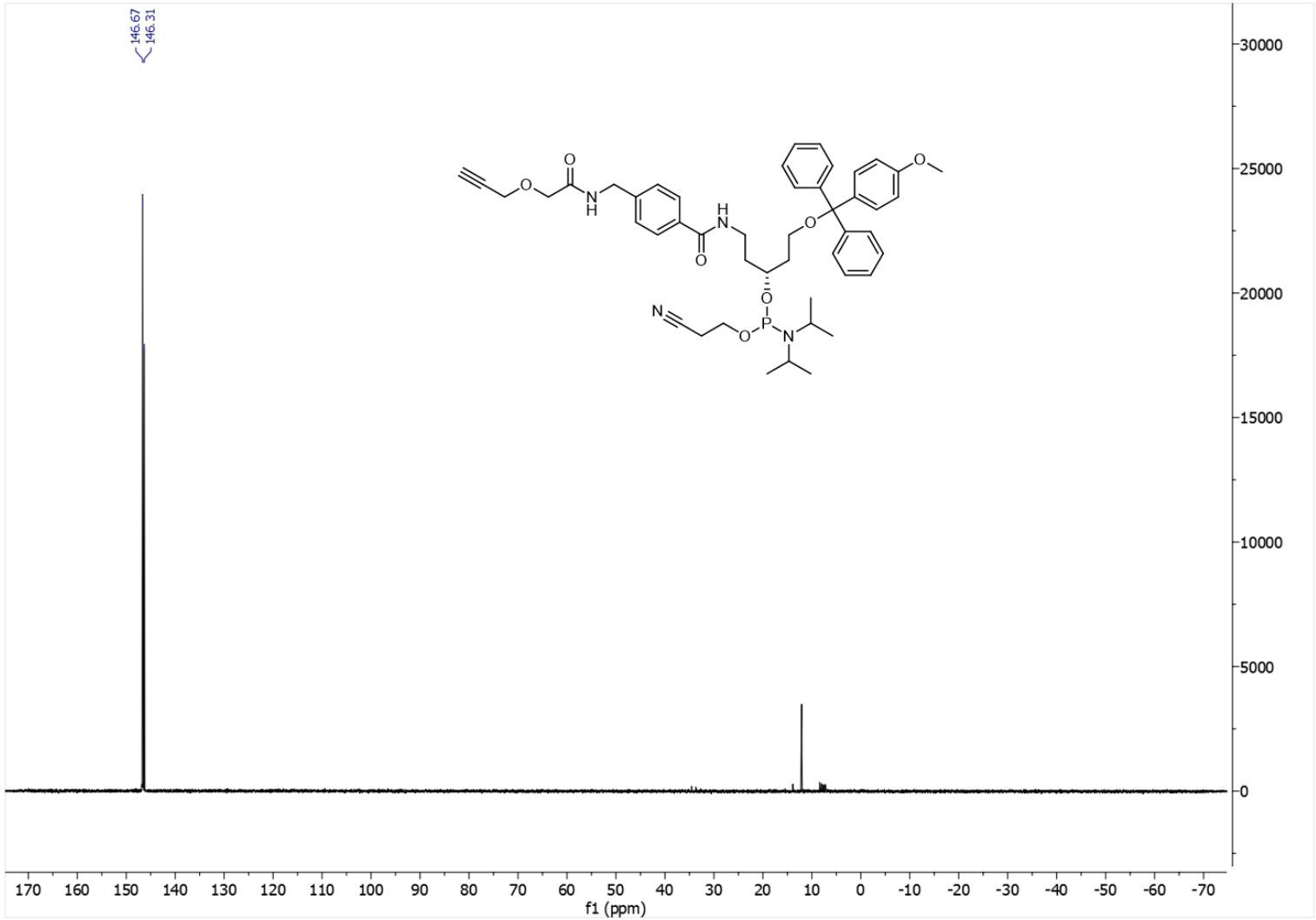


Figure S7. ^{31}P NMR spectrum of compound **7** (DMSO-d6, 202.47 MHz).

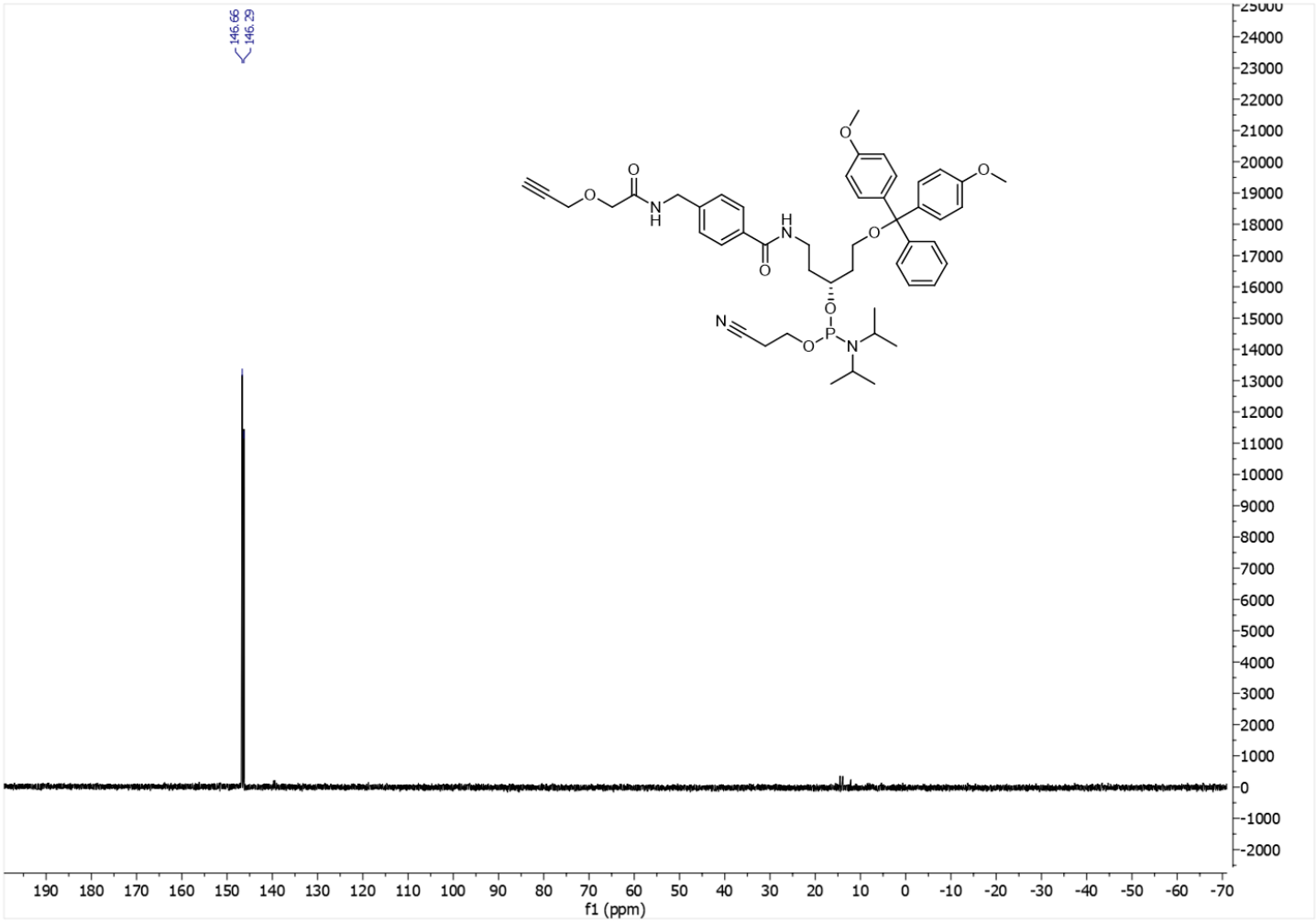


Figure S8. ^{31}P NMR spectrum of compound **11** (DMSO-d6, 202.47 MHz).

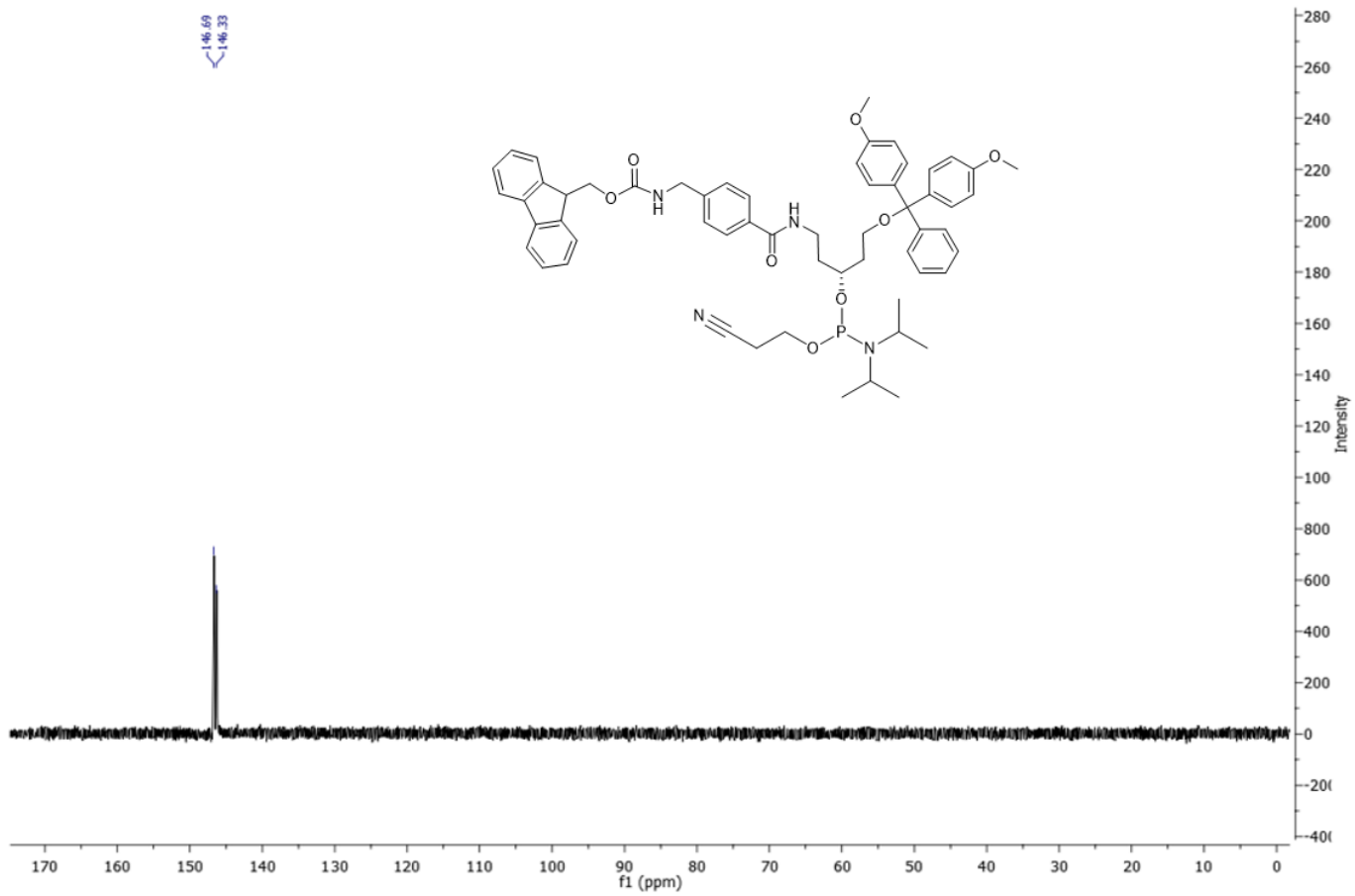


Figure S9. ^{31}P NMR spectrum of compound **16** (DMSO-d6, 202.47 MHz).

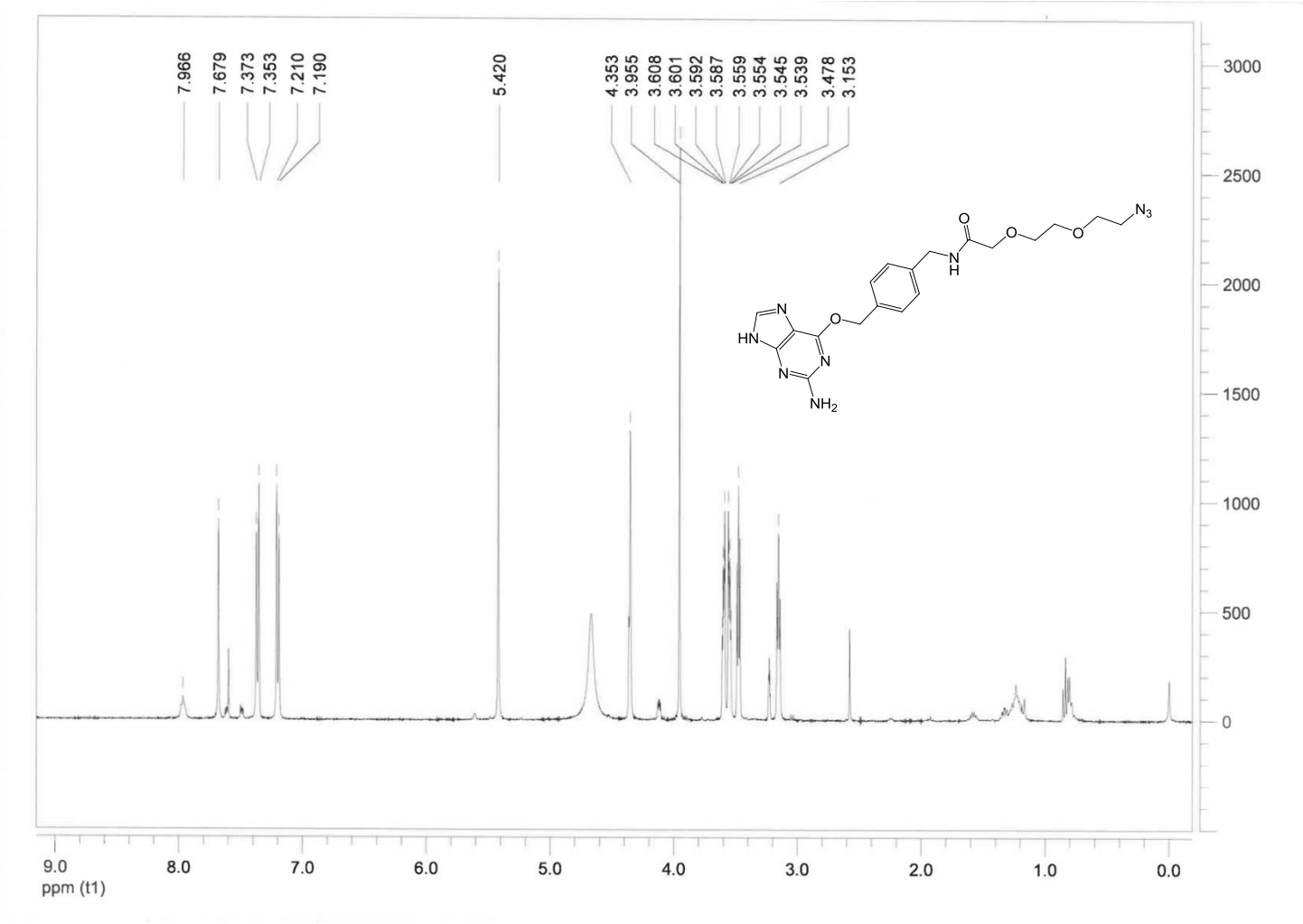


Figure S10. ^{1}H NMR spectrum of BG-N_3 (400.1 MHz, $CDCl_3$ plus CD_3OD).

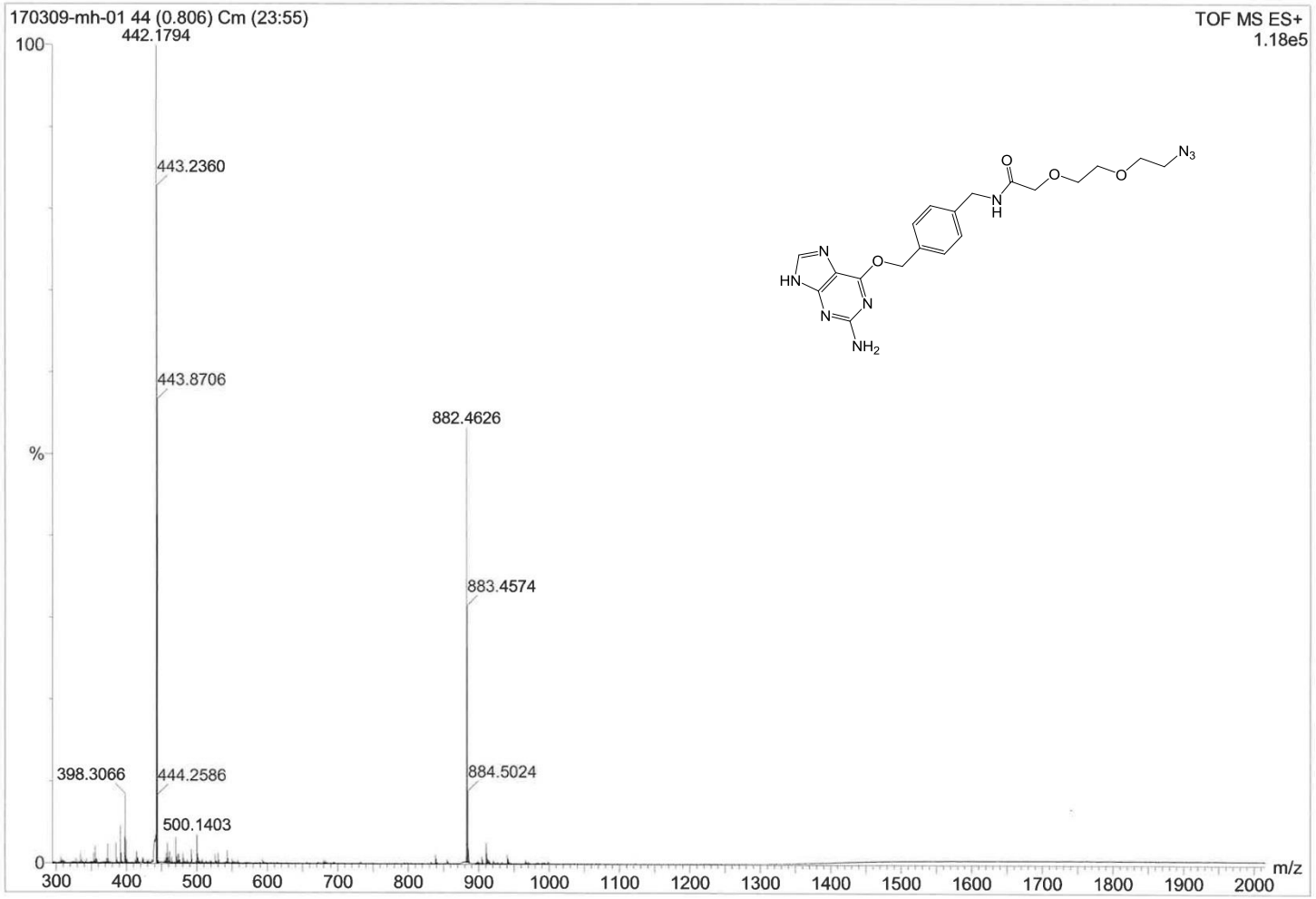


Figure S11. ESI-TOF mass spectrum of compound BG-N_3.

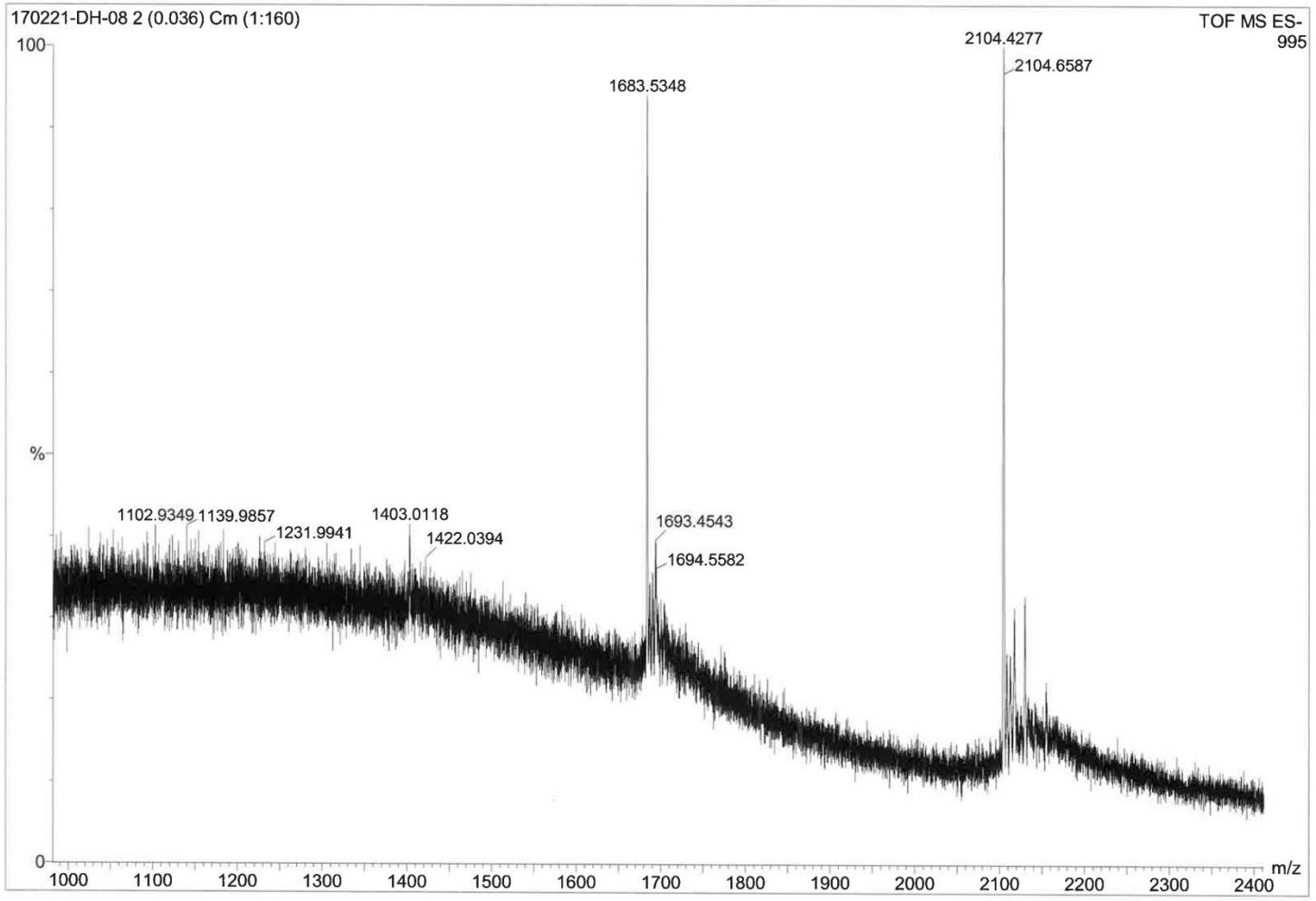


Figure S12. ESI-TOF mass spectrum of ON1.

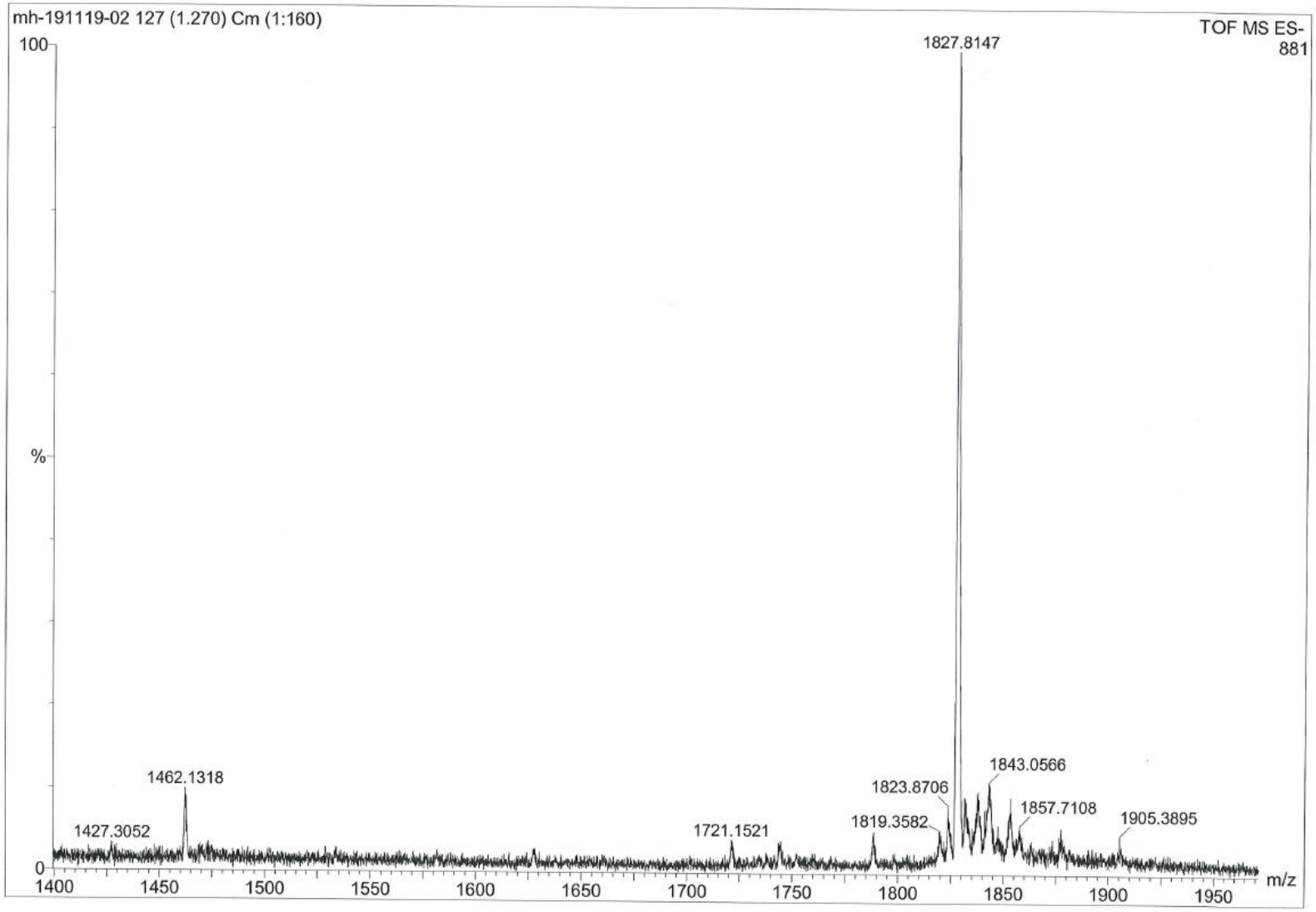


Figure S13. ESI-TOF mass spectrum of ON2.

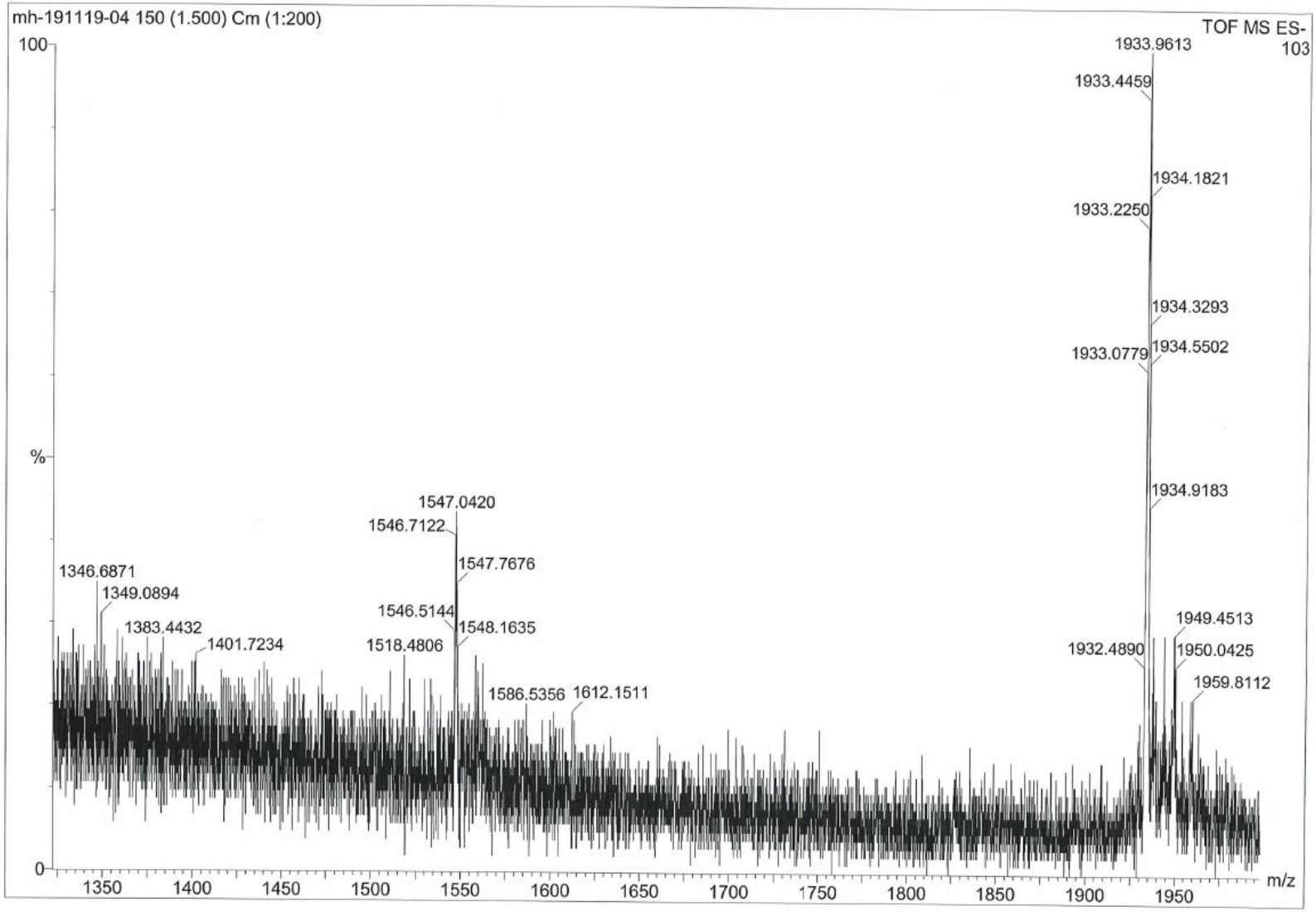


Figure S14. ESI-TOF mass spectrum of ON3.

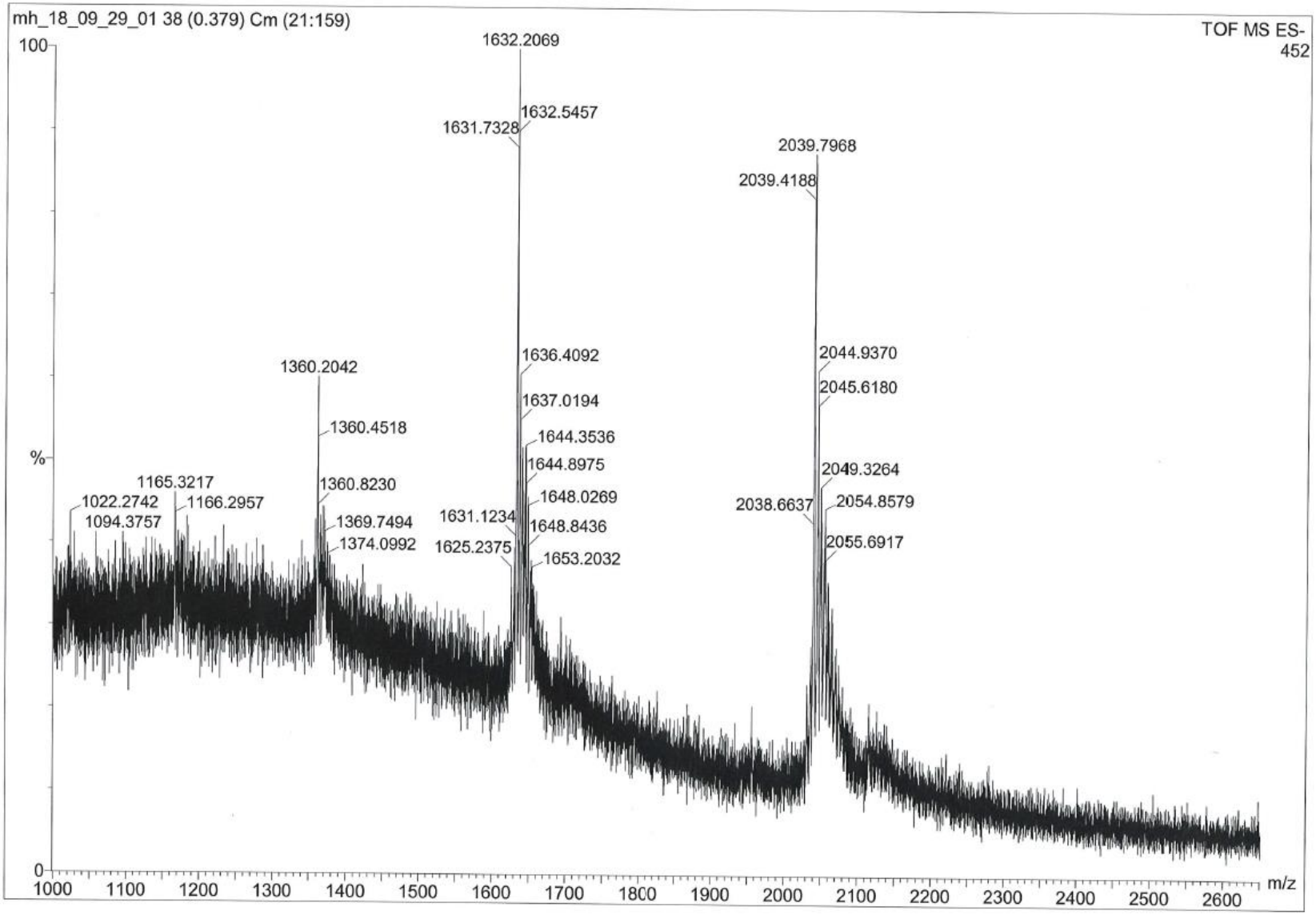


Figure S15. ESI-TOF mass spectrum of ON4.

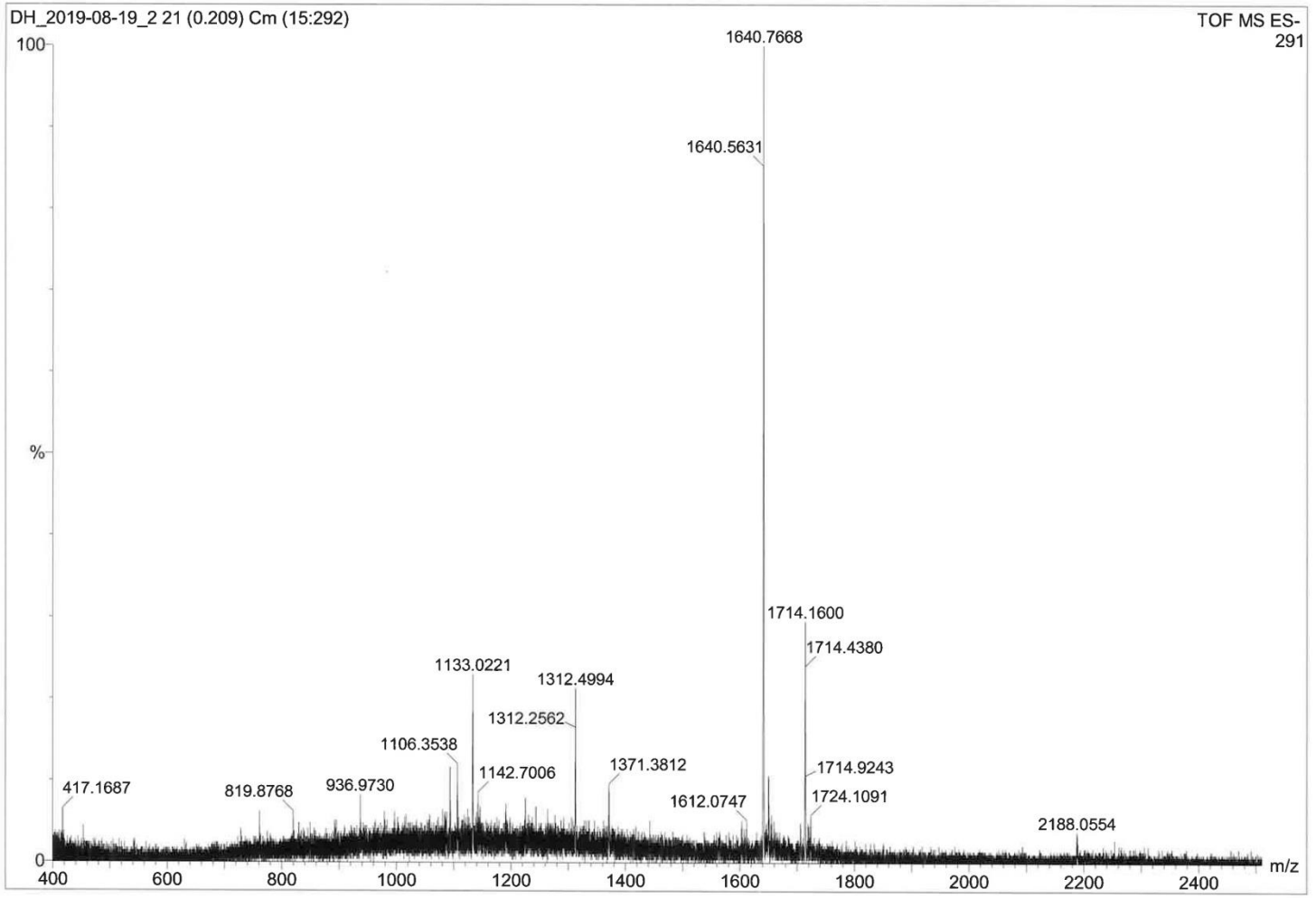


Figure S16. ESI-TOF mass spectrum of ON5.

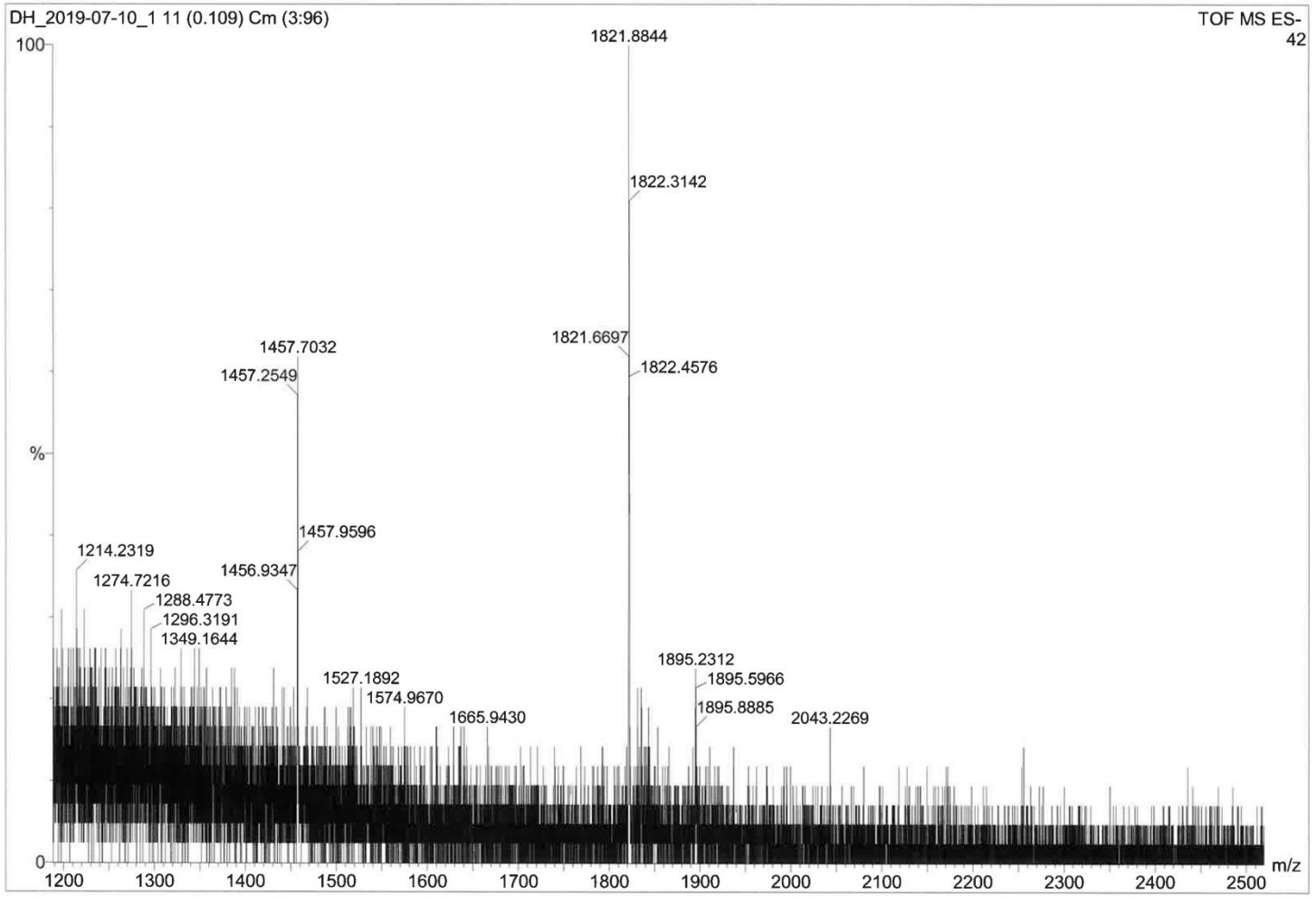


Figure S17. ESI-TOF mass spectrum of ON6.

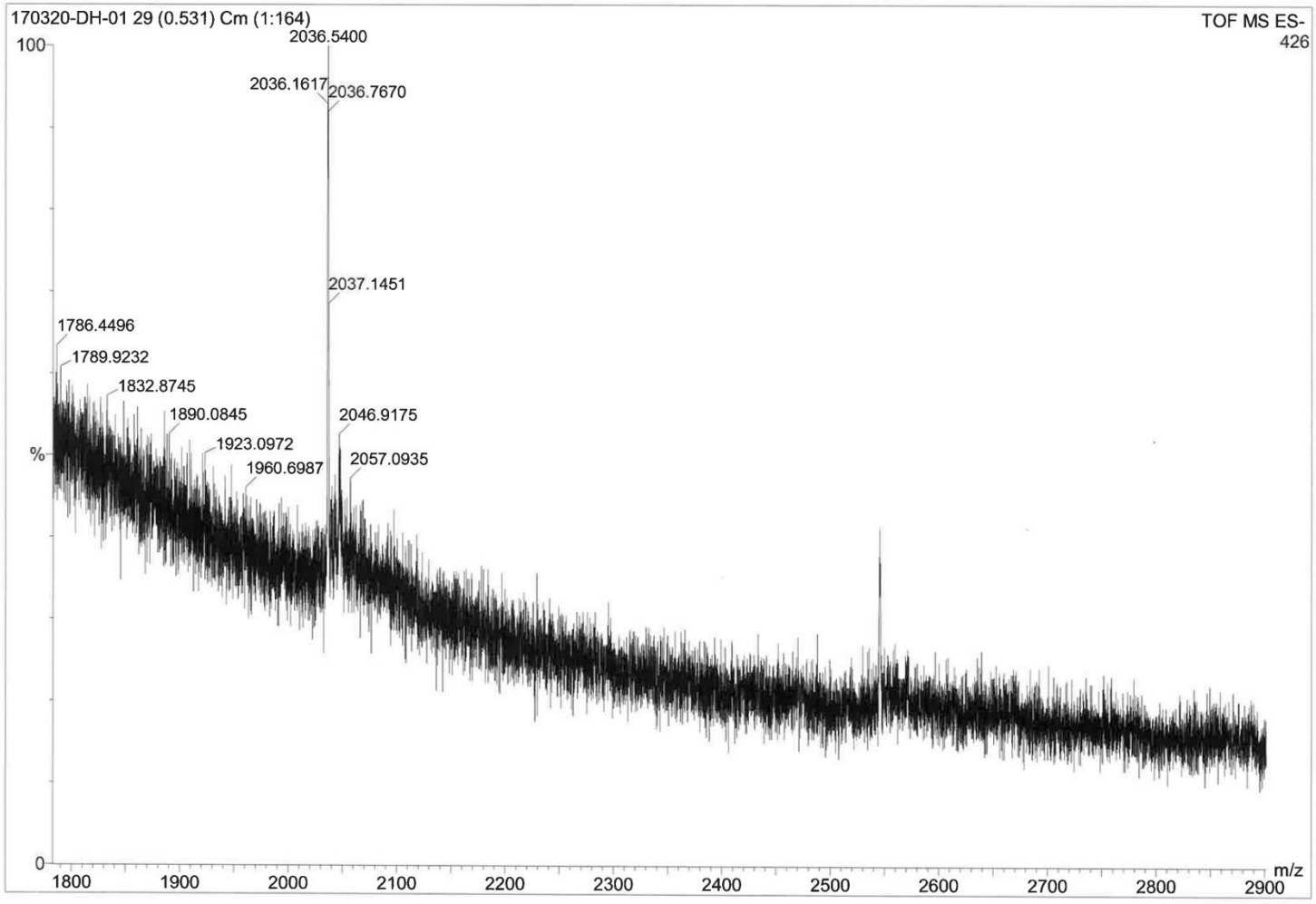


Figure S18. ESI-TOF mass spectrum of ON1-$(BG)_4$.

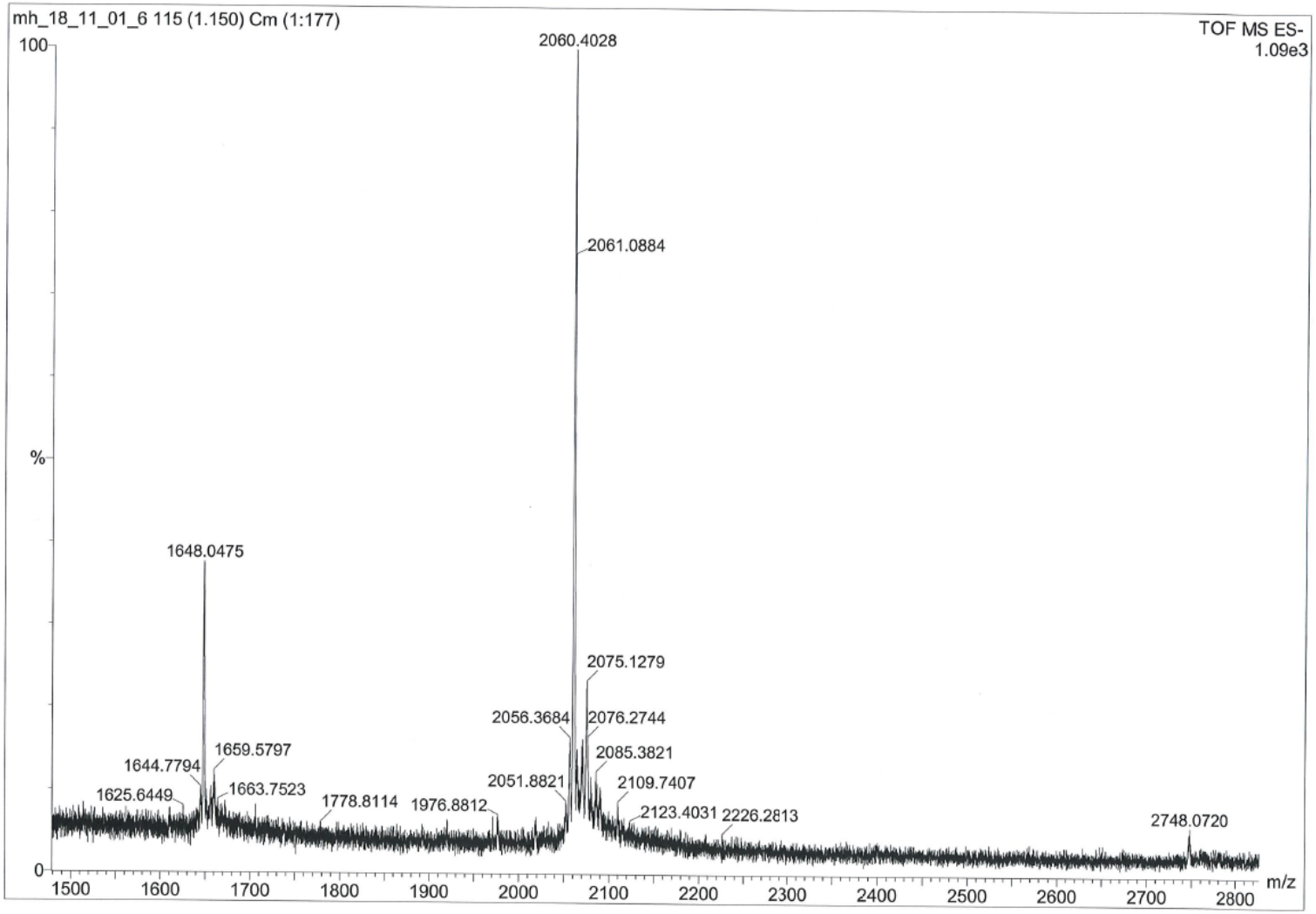


Figure S19. ESI-TOF mass spectrum of ON2-P4.

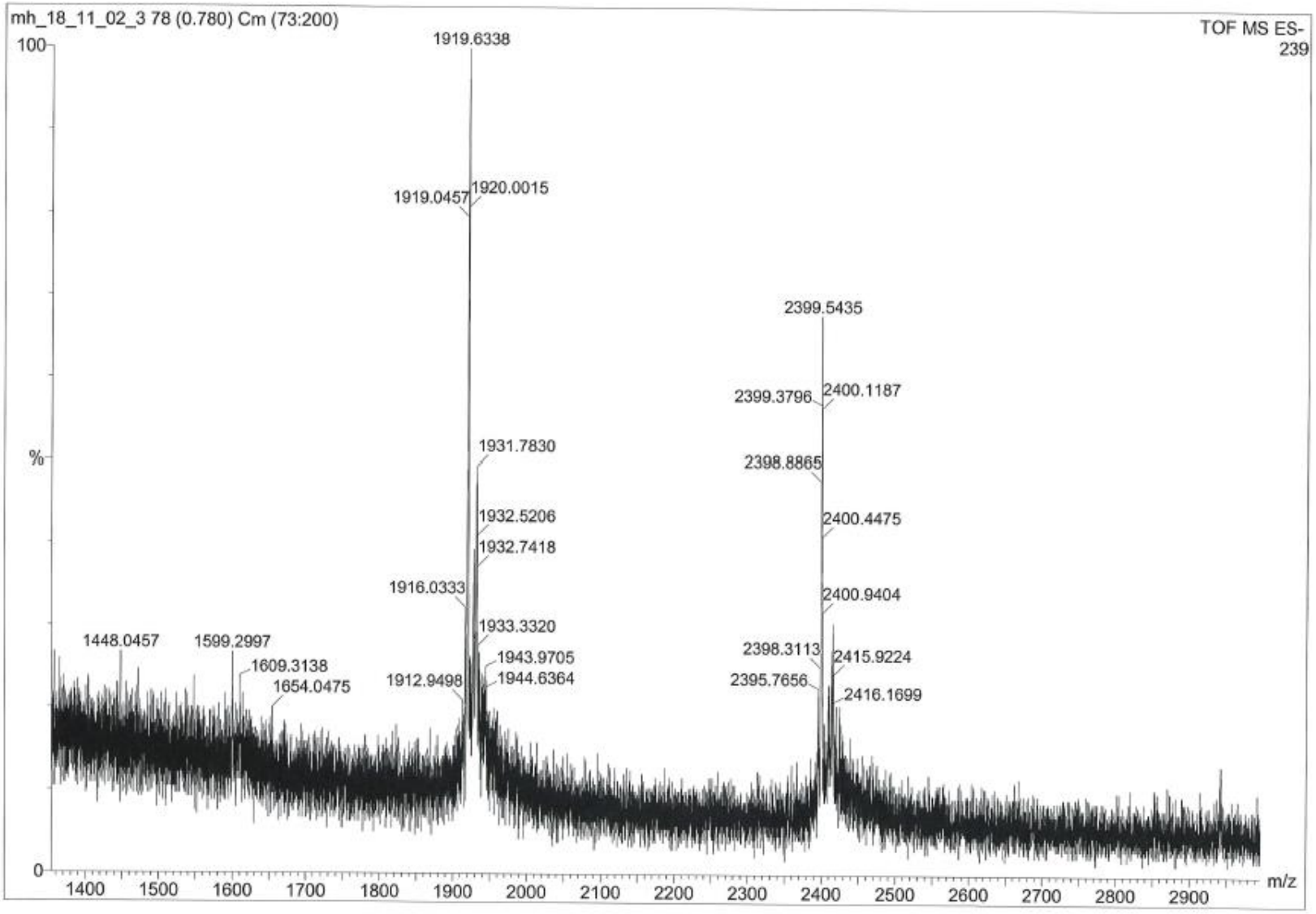


Figure S20. ESI-TOF mass spectrum of ON3-$(P4)_2$.

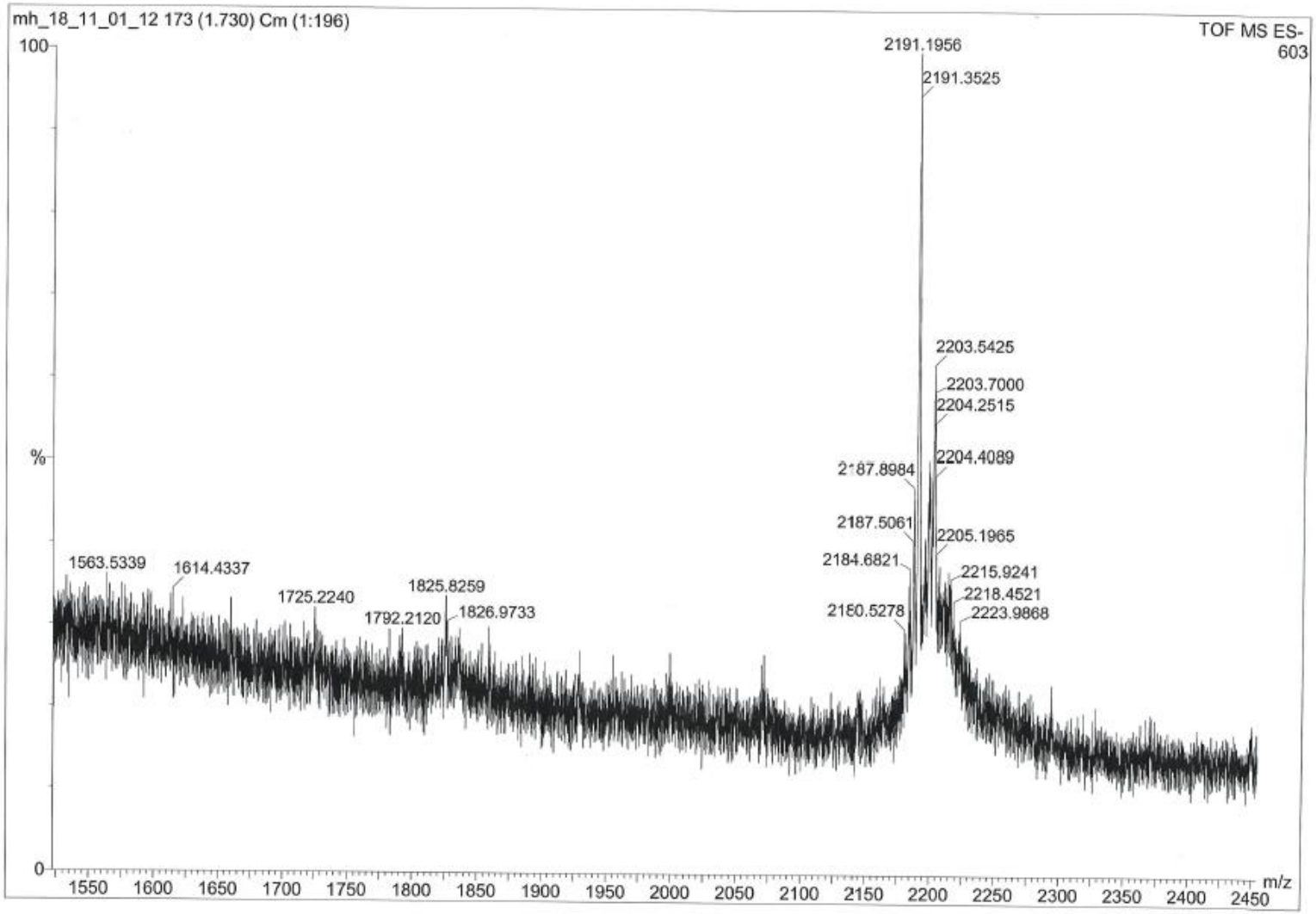


Figure S21. ESI-TOF mass spectrum of ON4-$(P4)_3$.

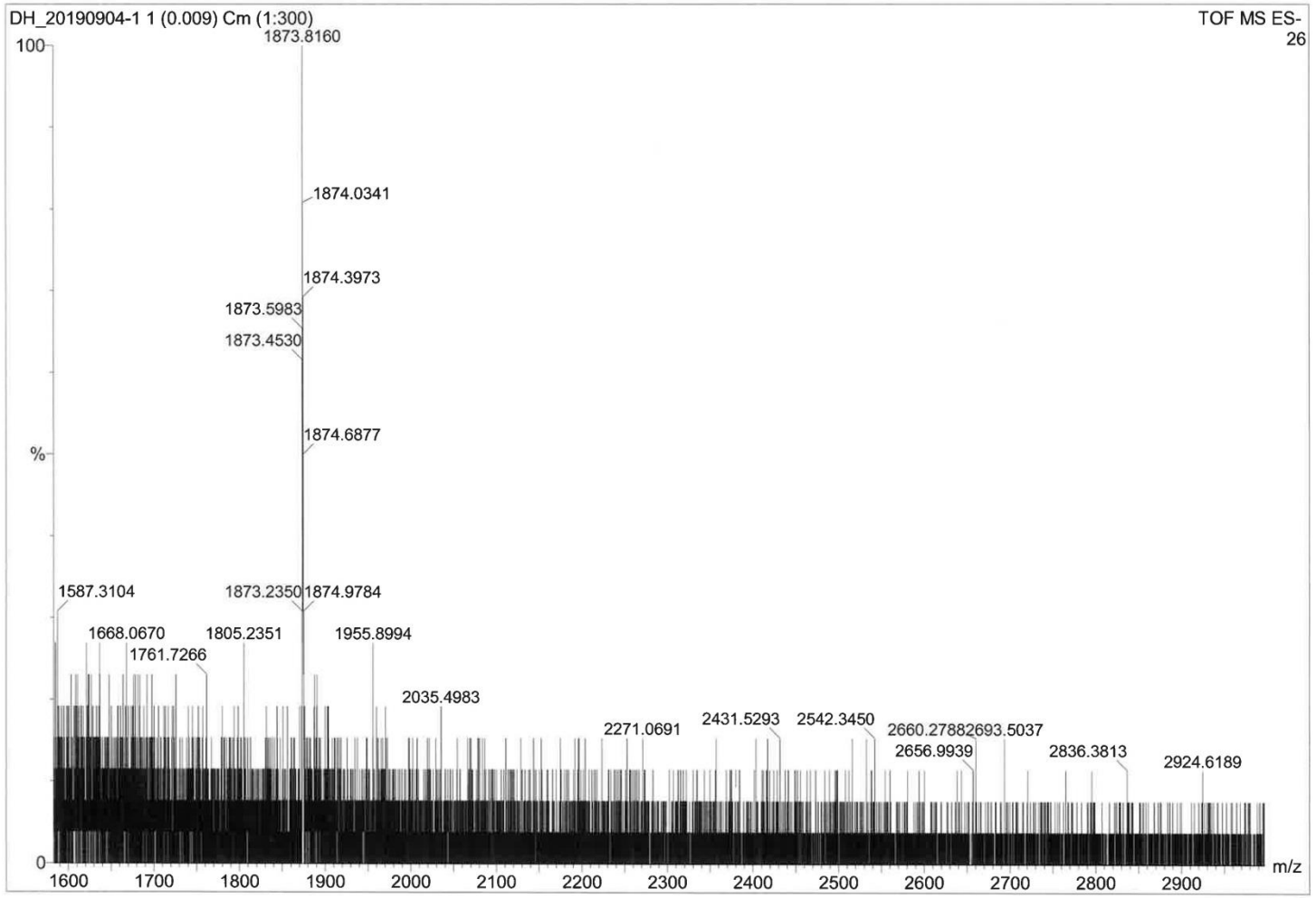


Figure S22. ESI-TOF mass spectrum of ON5-P4.

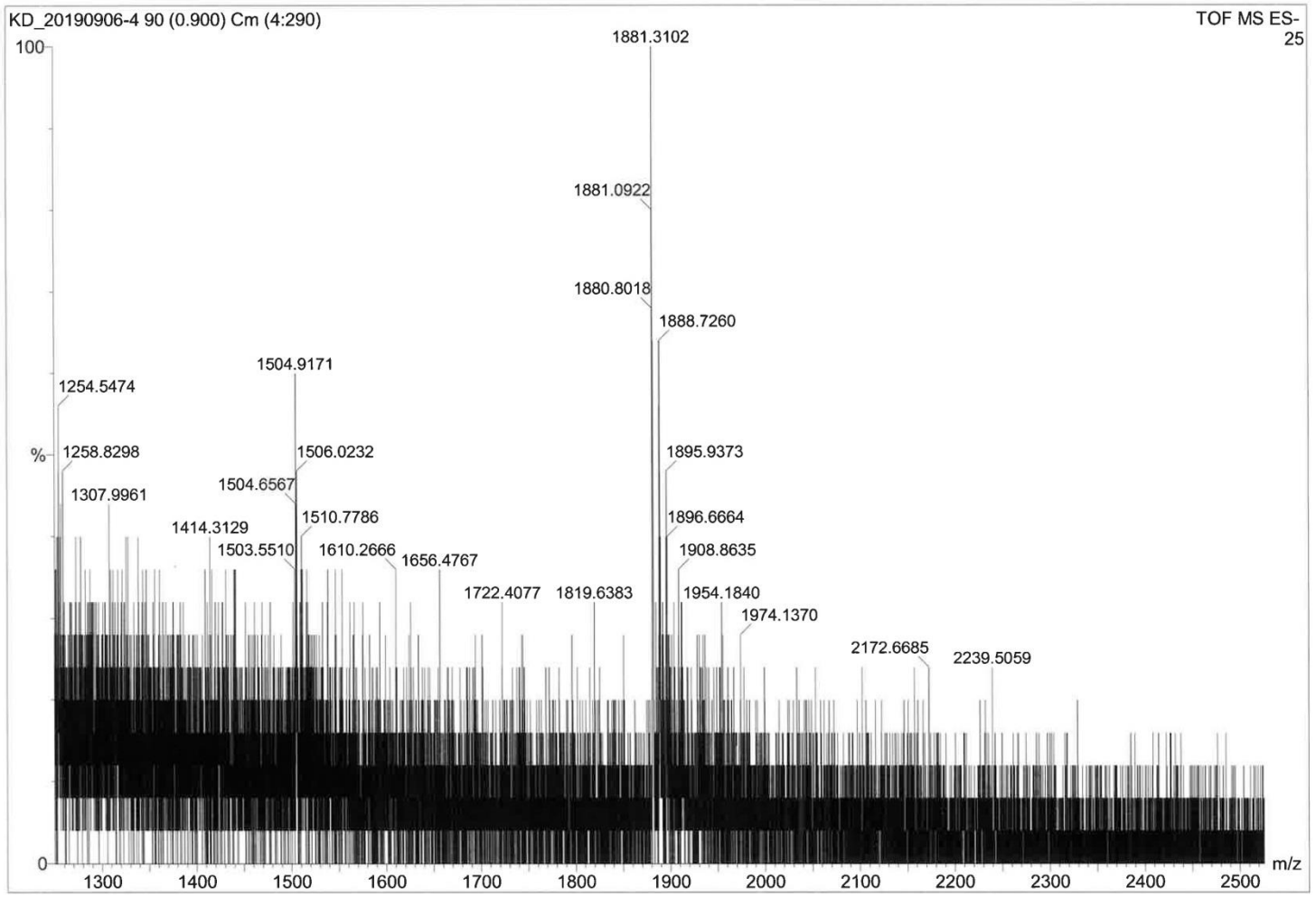


Figure S23. ESI-TOF mass spectrum of ON6-(PA).

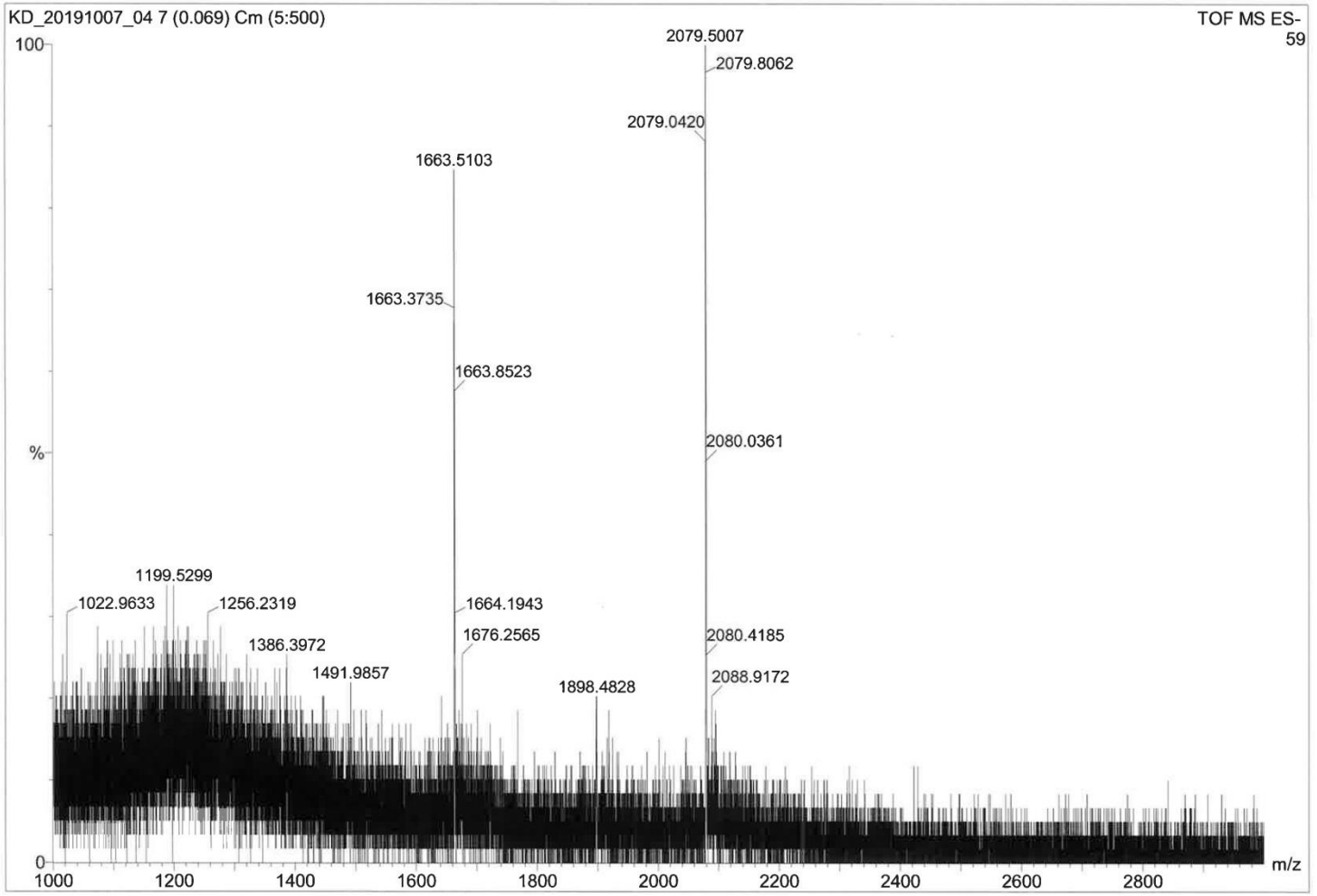


Figure S24. ESI-TOF mass spectrum of ON6-(MIF)-(PA)-(MIF).

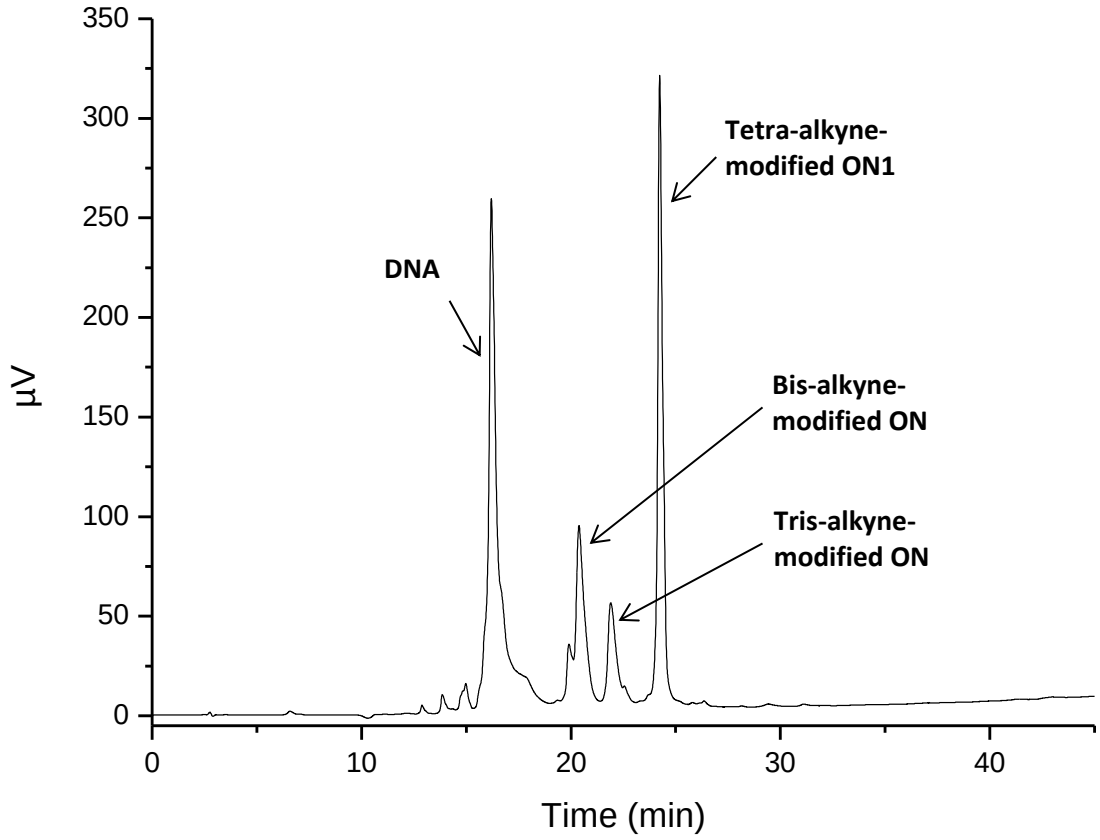


Figure S25. RP-HPLC profile of ON1 using RP HPLC on C18 column (Supelco Discovery BIO Wide Pore C18-5) with a linear gradient from 0 to 100% of buffer B in buffer A over 45 min at 25 °C. Buffer (A): 50 mM triethylammonium acetate (TEAA), pH 6.5; buffer (B): 50 mM TEAA, pH 6.5 in H_2O-CH_3CN (1:1 v/v).

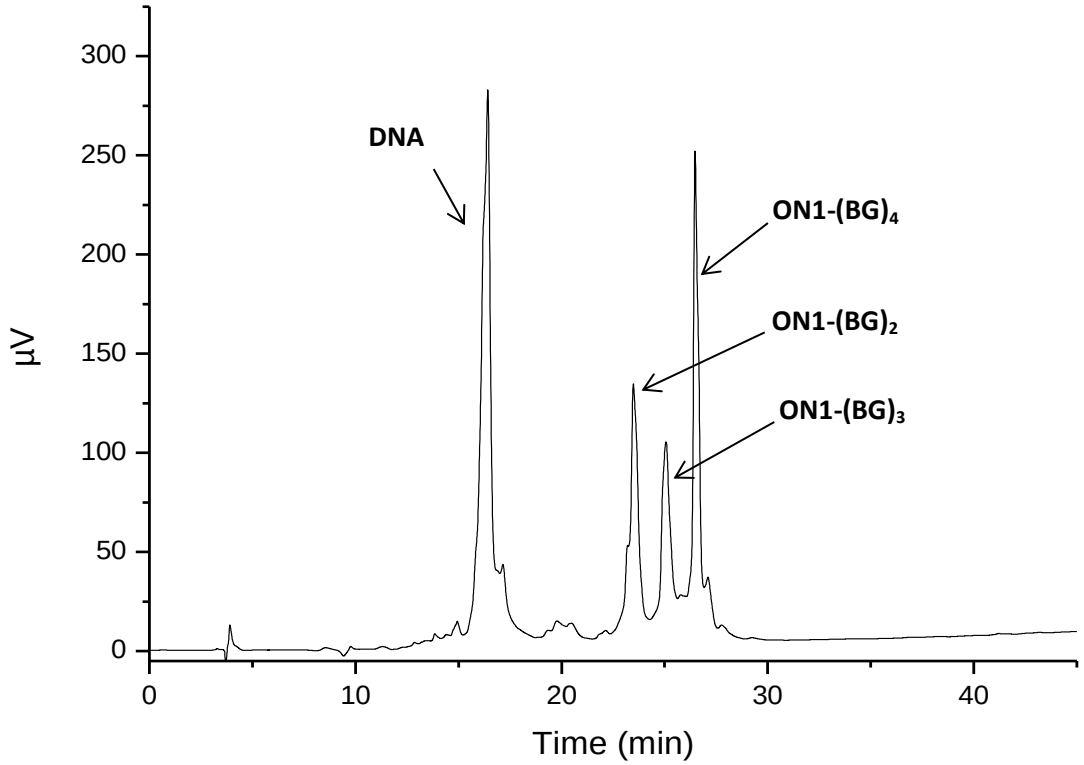


Figure S26. RP-HPLC profile of ON1-$(BG)_4$ using RP HPLC on C18 column (Supelco Discovery BIO Wide Pore C18-5) with a linear gradient from 0 to 100% of buffer B in buffer A over 45 min at 25 °C. Buffer (A): 50 mM triethylammonium acetate (TEAA), pH 6.5; buffer (B): 50 mM TEAA, pH 6.5 in H_2O-CH_3CN (1:1 v/v).

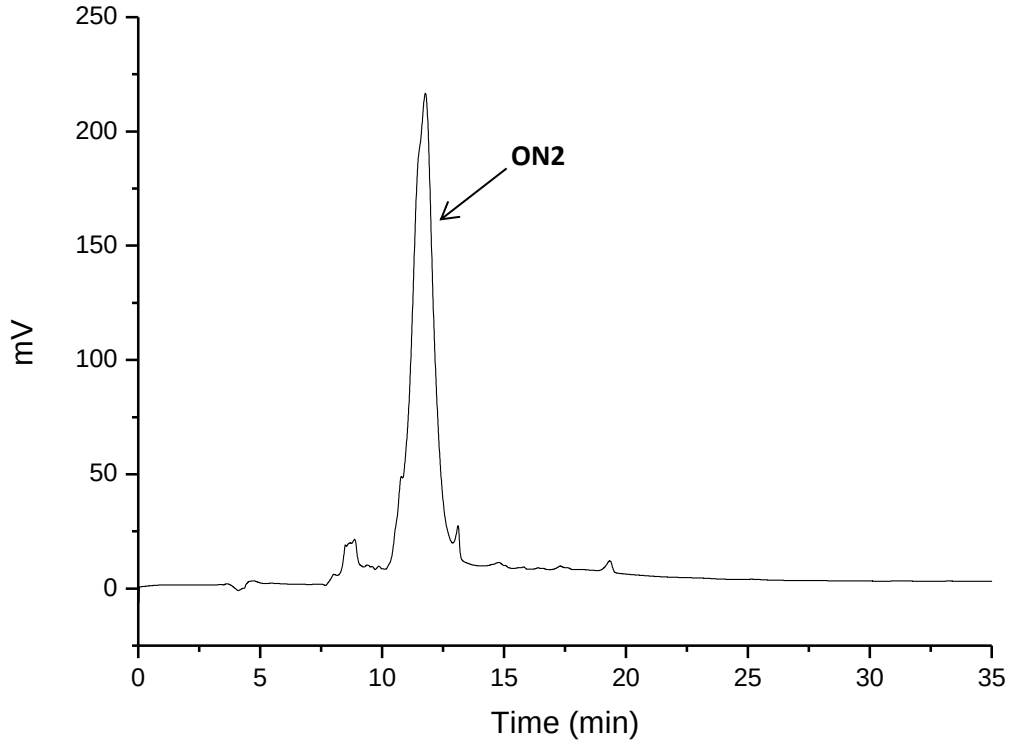


Figure S27. RP-HPLC profile of ON2 using RP HPLC on a C18 column (Supelco Discovery BIO Wide Pore C18-5) with a linear gradient from 0 to 100% of buffer B in buffer A over 30 min at 25 °C. Buffer (A): 50 mM triethylammonium acetate (TEAA), pH 6.5; buffer (B): 50 mM TEAA, pH 6.5 in H_2O-CH_3CN (1:1 v/v).

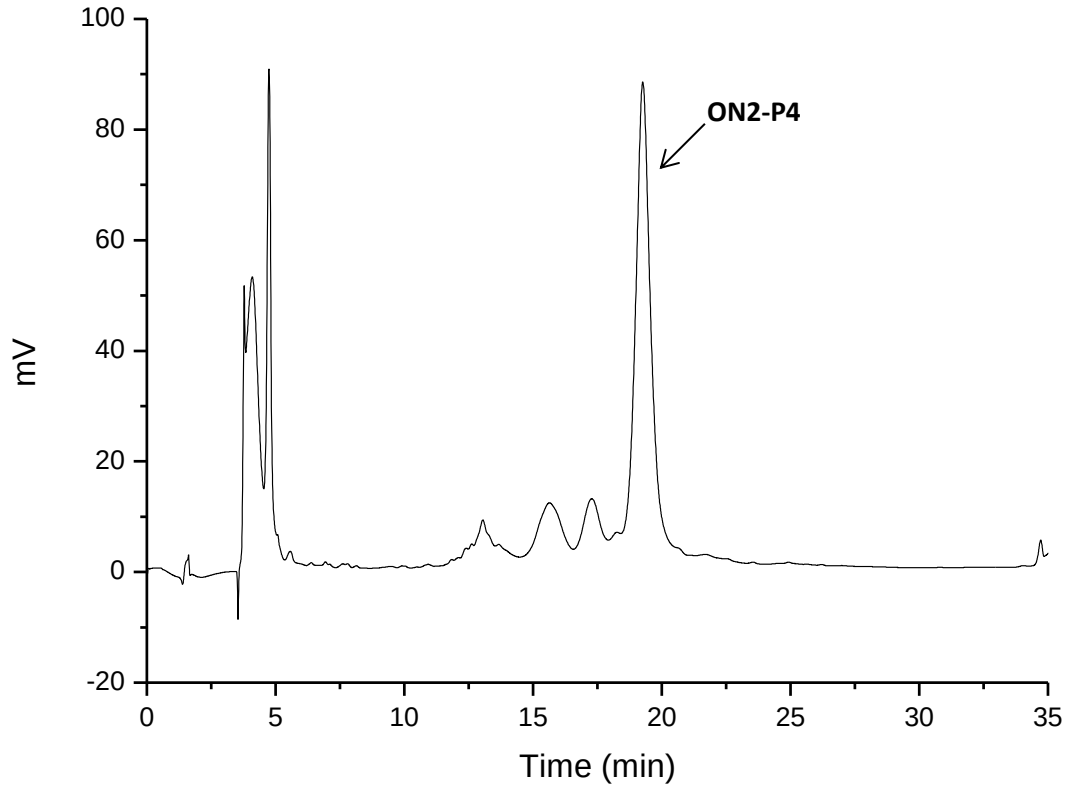


Figure S28. RP-HPLC profile of ON2-P4 using RP HPLC on a C18 column (Supelco Discovery BIO Wide Pore C18-5) with a linear gradient from 10 to 35% of buffer B in buffer A over 30 min at 25 °C. Buffer (A): 50 mM triethylammonium acetate (TEAA), pH 6.5; buffer (B): 50 mM TEAA, pH 6.5 in H_2O-CH_3CN (1:1 v/v).

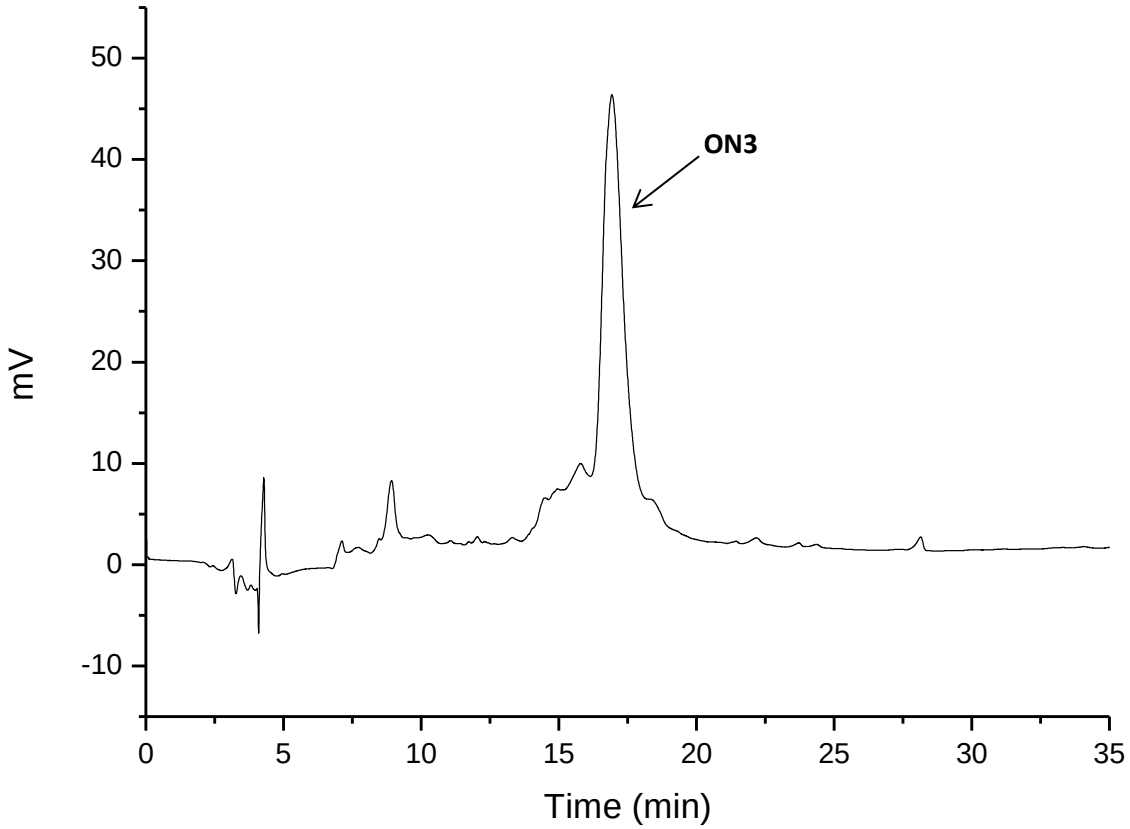


Figure S29. RP-HPLC profile of ON3 using RP HPLC on a C18 column (Supelco Discovery BIO Wide Pore C18-5) with a linear gradient from 0 to 100% of buffer B in buffer A over 30 min at 25 °C. Buffer (A): 50 mM triethylammonium acetate (TEAA), pH 6.5; buffer (B): 50 mM TEAA, pH 6.5 in H_2O-CH_3CN (1:1 v/v).

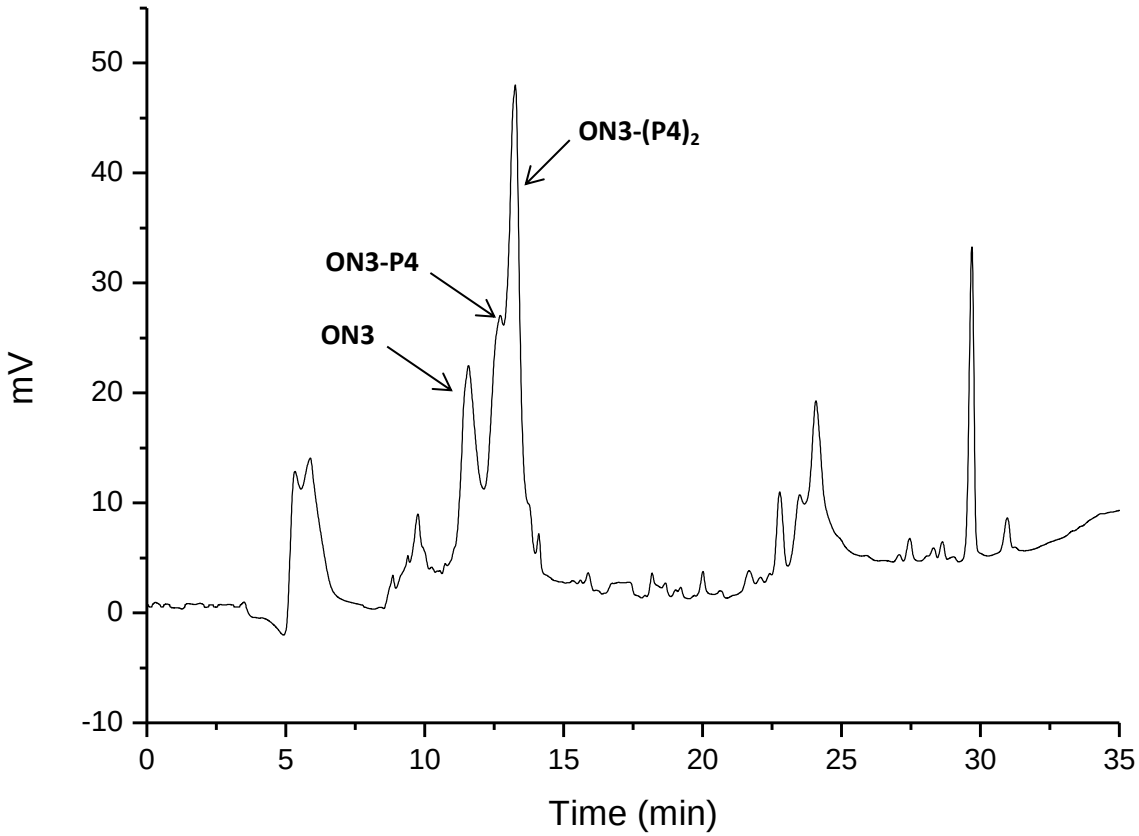


Figure S30. RP-HPLC profile of ON3-$(P4)_2$ using RP HPLC on C18 column (Supelco Discovery BIO Wide Pore C18-5) with a linear gradient from 0 to 100% of buffer B in buffer A over 30 min at 25 °C. Buffer (A): 50 mM triethylammonium acetate (TEAA), pH 6.5; buffer (B): 50 mM TEAA, pH 6.5 in H_2O-CH_3CN (1:1 v/v).

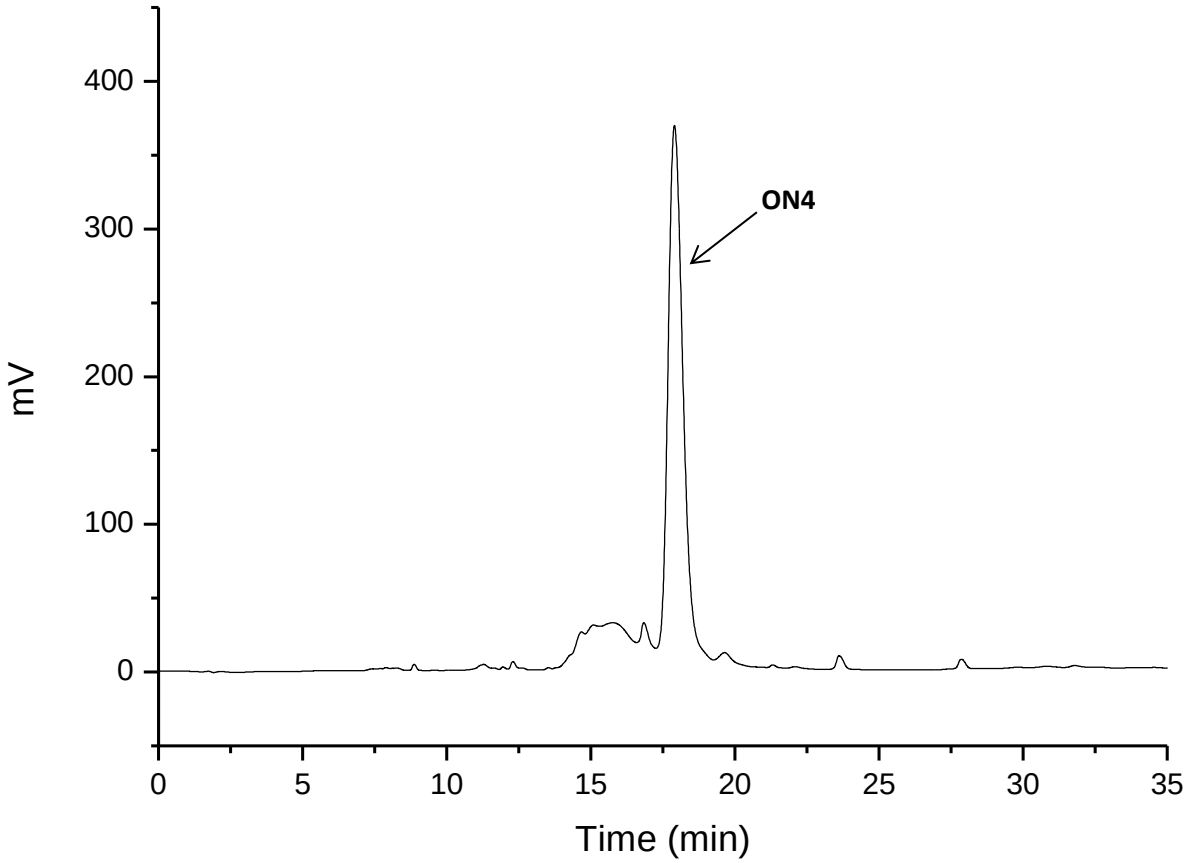


Figure S31. RP-HPLC profile of ON4 using RP HPLC on a C18 column (Supelco Discovery BIO Wide Pore C18-5) with a linear gradient from 0 to 100% of buffer B in buffer A over 30 min at 25 °C. Buffer (A): 50 mM triethylammonium acetate (TEAA), pH 6.5; buffer (B): 50 mM TEAA, pH 6.5 in H_2O-CH_3CN (1:1 v/v).

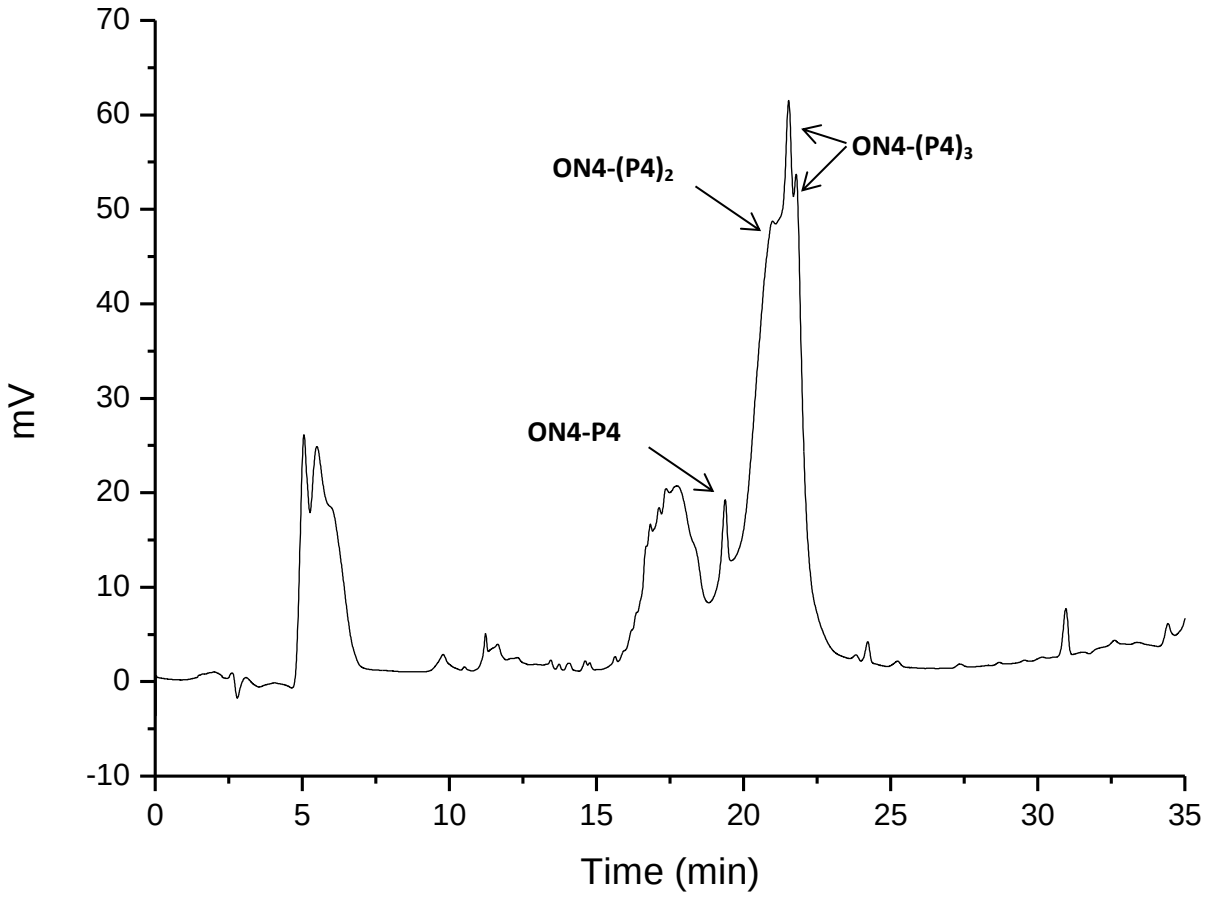


Figure S32. RP-HPLC profile of ON4-$(P4)_3$ using RP HPLC on C18 column (Supelco Discovery BIO Wide Pore C18-5) with a linear gradient from 0 to 50% of buffer B in buffer A over 30 min at 25 °C. Buffer (A): 50 mM triethylammonium acetate (TEAA), pH 6.5; buffer (B): 50 mM TEAA, pH 6.5 in H_2O-CH_3CN (1:1 v/v).

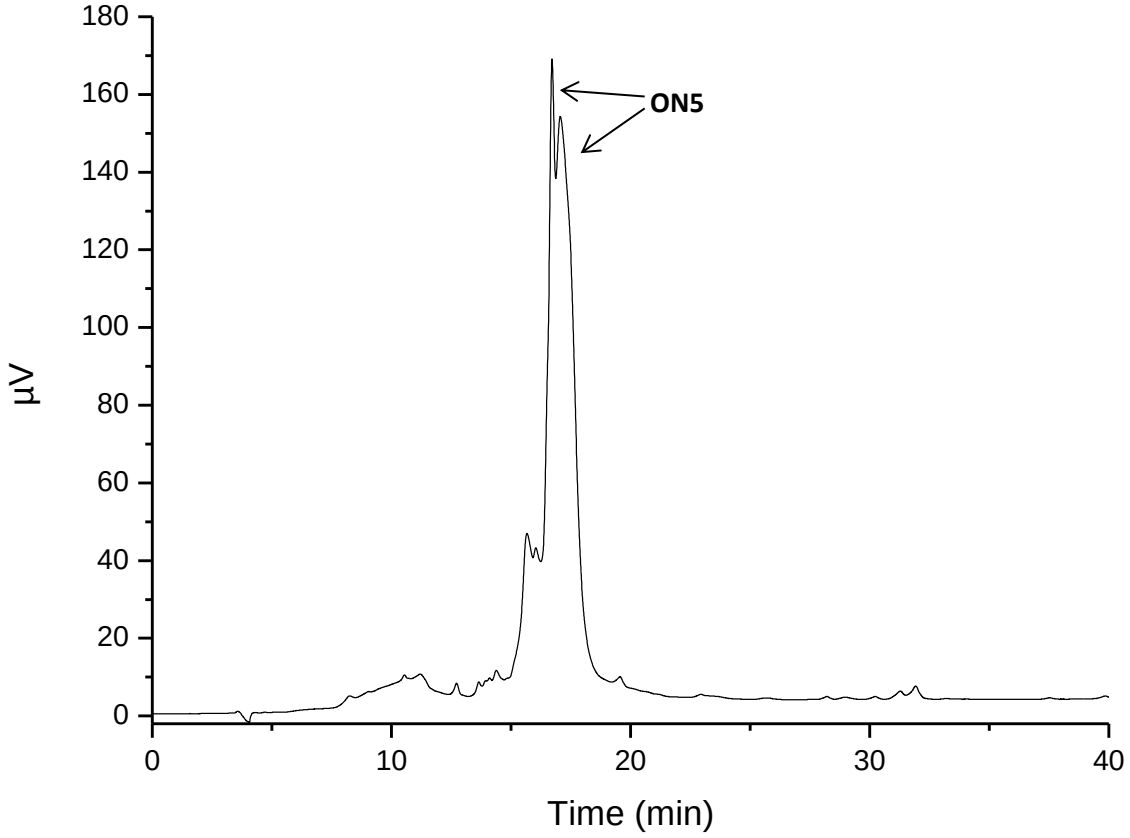


Figure S33. RP-HPLC profile of ON5 using RP HPLC on a C18 column (Supelco Discovery BIO Wide Pore C18-5) with a linear gradient from 0 to 50% of buffer B in buffer A over 45 min at 25 °C. Buffer (A): 50 mM triethylammonium acetate (TEAA), pH 6.5; buffer (B): 50 mM TEAA, pH 6.5 in H_2O-CH_3CN (1:1 v/v).

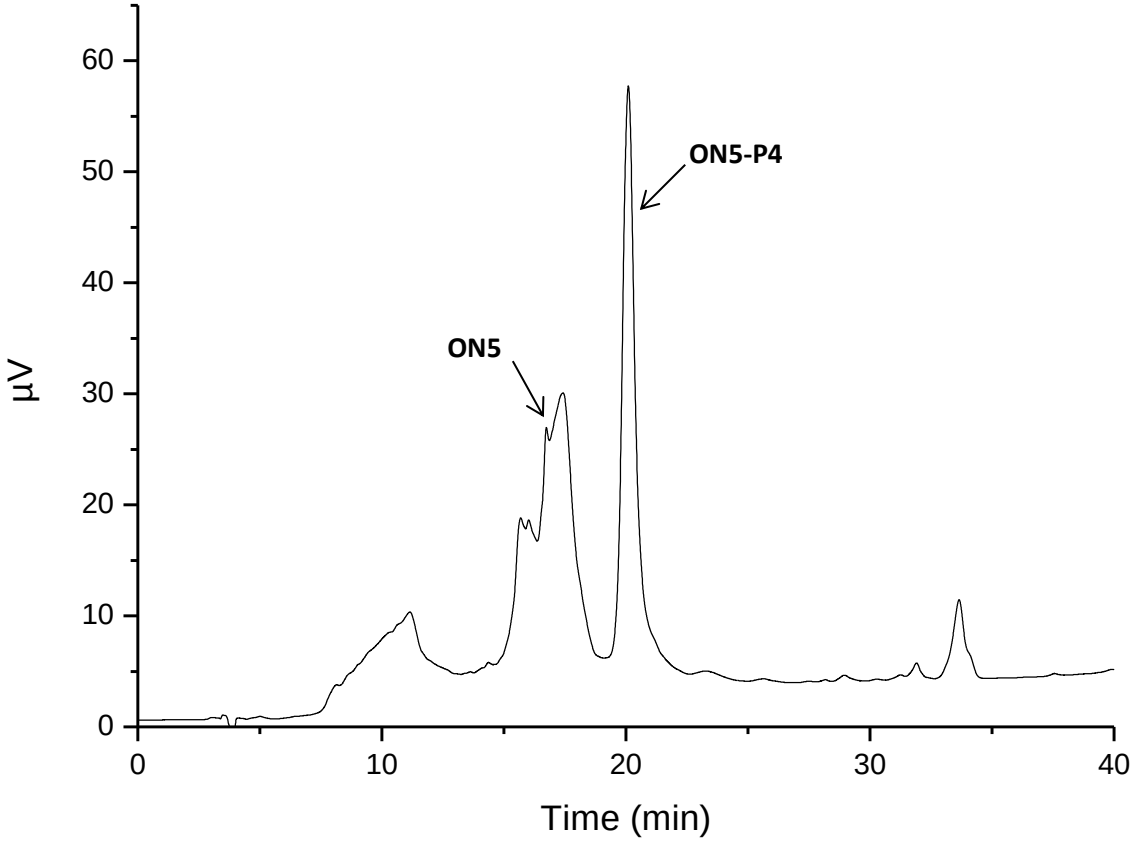


Figure S34. RP-HPLC profile of ON5-P4 using RP HPLC on a C18 column (Supelco Discovery BIO Wide Pore C18-5) with a linear gradient from 0 to 50% of buffer B in buffer A over 45 min at 25 °C. Buffer (A): 50 mM triethylammonium acetate (TEAA), pH 6.5; buffer (B): 50 mM TEAA, pH 6.5 in H_2O-CH_3CN (1:1 v/v).

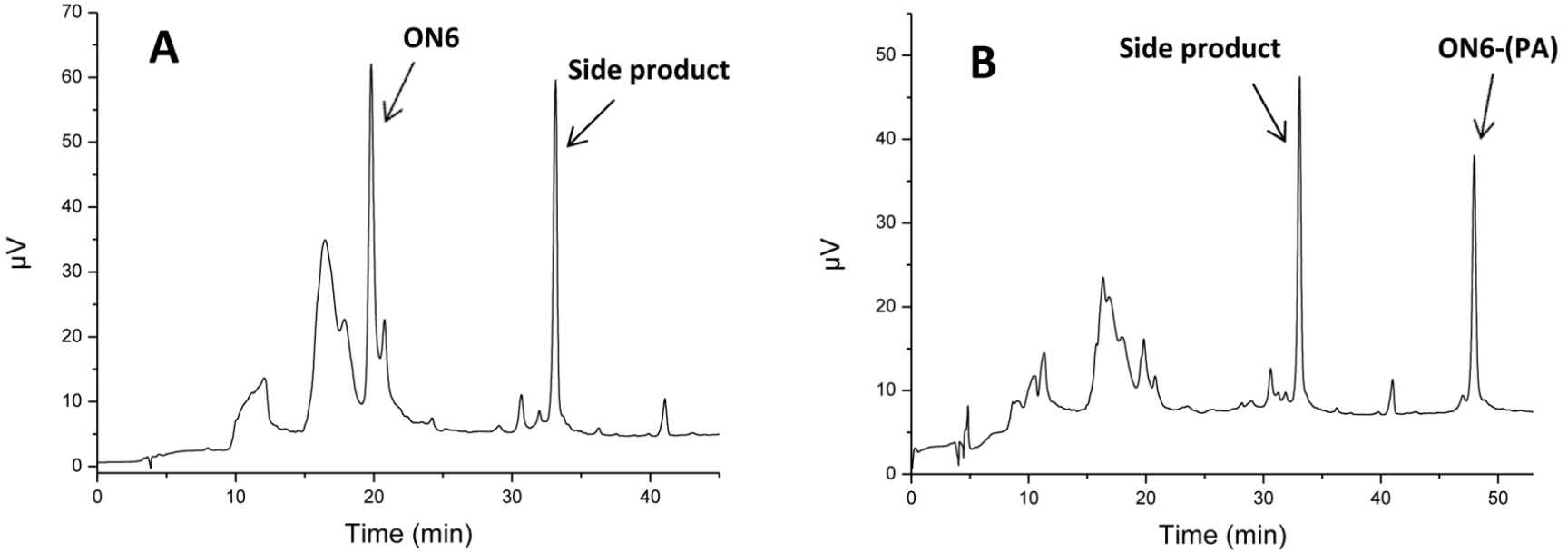


Figure S35. RP-HPLC profiles of (A) ON6 and (B) ON6-(PA) showing formation of side product after deprotection of Fmoc group using 20% piperidine solution in DMF.

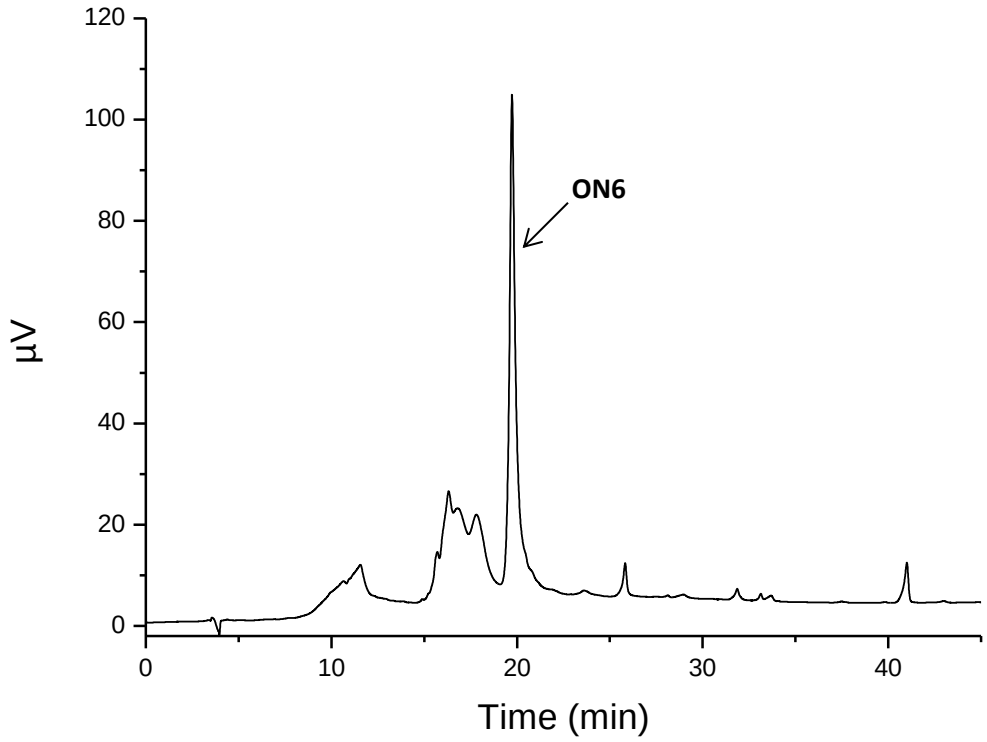


Figure S36. RP-HPLC profile of ON6 when Fmoc is deprotected with a DMF solution of 2% DBU and 5% piperazine, using RP HPLC on a C18 column (Supelco Discovery BIO Wide Pore C18-5) with a linear gradient from 0 to 50% of buffer B in buffer A over 45 min at 25 °C. Buffer (A): 50 mM triethylammonium acetate (TEAA), pH 6.5; buffer (B): 50 mM TEAA, pH 6.5 in H_2O-CH_3CN (1:1 v/v).

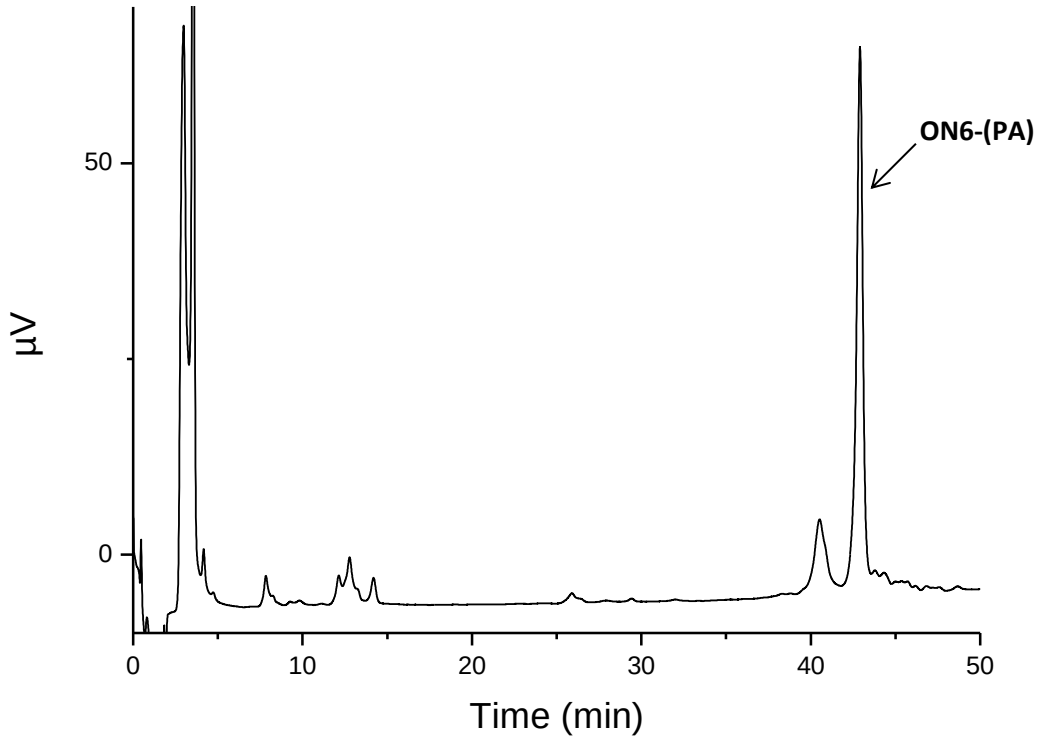


Figure S37. RP-HPLC profile of ON6-(PA) using RP HPLC on a C18 column (Supelco Discovery BIO Wide Pore C18-5) with a linear gradient from 50 to 100% of buffer B in buffer A over 45 min at 25 °C. Buffer (A): 50 mM triethylammonium acetate (TEAA), pH 6.5; buffer (B): 50 mM TEAA, pH 6.5 in H_2O-CH_3CN (1:1 v/v).

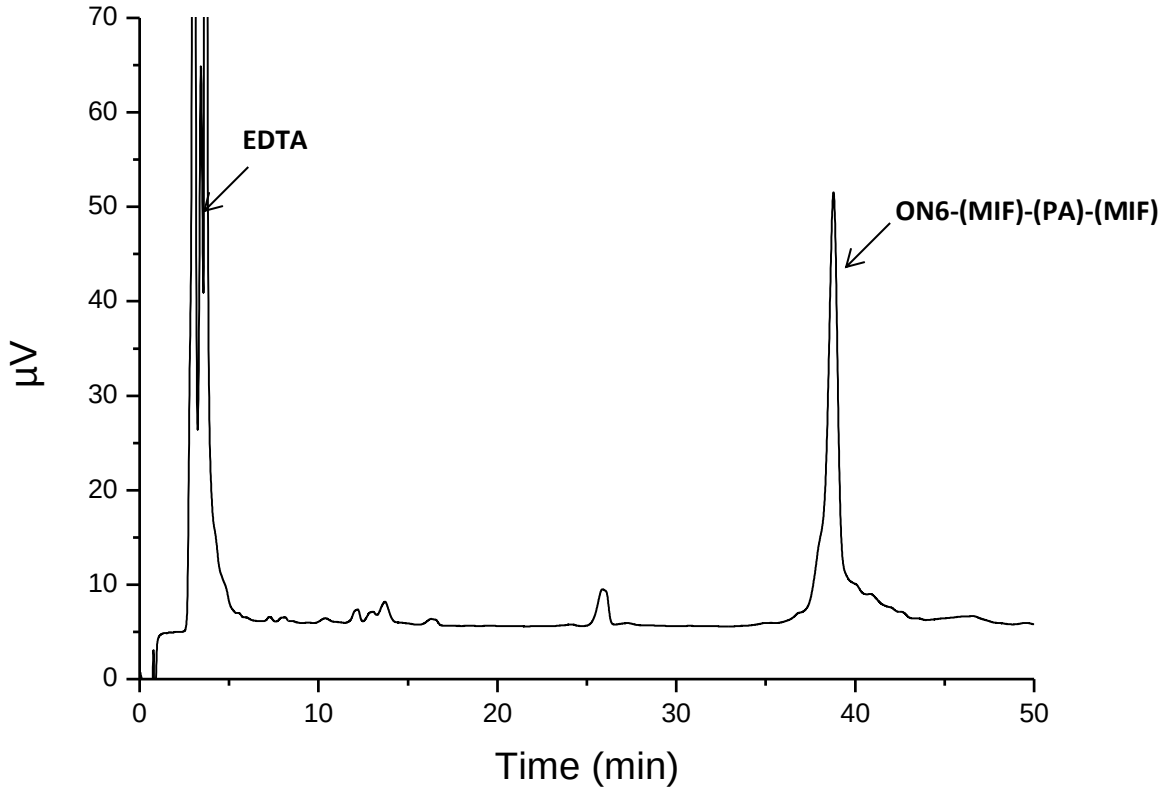


Figure S38. RP-HPLC profile of ON6-(MIF)-(PA)-(MIF) using RP HPLC on C18 column (Supelco Discovery BIO Wide Pore C18-5) with a linear gradient from 50 to 100% of buffer B in buffer A over 45 min at 25 °C. Buffer (A): 50 mM triethylammonium acetate (TEAA), pH 6.5; buffer (B): 50 mM TEAA, pH 6.5 in H_2O-CH_3CN (1:1 v/v).

Table S1. Yields of ON conjugation and total yields of ON conjugates.

ON	Sequence 5′ → 3′[a]	Yield of conjugation[b], (%)	Total yield[c], (%)
ON1-$(BG)_4$	$(BG\text{-}L)_2$-$(BG\text{-}L)_2$-doubler-GCGTTGATGCAATTTCTATGC	76	22
ON2-P4	(P4-L)-*G*G*C*C*A*A*A*C*C*U*C*G*G*C*U*U*A*C*C*U*	79	71
ON3-$(P4)_2$	(P4-L)-(P4-L)-*G*G*C*C*A*A*A*C*C*U*C*G*G*C*U*U*A*C*C*U*	59	44
ON4-$(P4)_3$	(P4-L)-(P4-L)-(P4-L)-*G*G*C*C*A*A*A*C*C*U*C*G*G*C*U*U*A*C*C*U*	63	47
ON5-P4	TCAAGGAAG-(P4-L)-ATGGCATTTCT	40	34
ON6-(PA)	L^{alkyne}-(PA-L)-L^{alkyne}-TCAAGGAAGATGGCATTTCT	65[d]	26
ON6-(MIF)-(PA)-(MIF)	(MIF-L)-(PA-L)-(MIF-L)-TCAAGGAAGATGGCATTTCT	65[e]	17

[a]In all sequences A = 2′-deoxyadenosine, G = 2′-deoxyguanosine, C = 2′-deoxycytidine, T = thymidine, *A* = 2′-OMe-adenosine, *G* = 2′-OMe-guanosine, *C* = 2′-OMe-cytidine, *U* = 2′-OMe-uridine, * = PS linkages. L = linker, BG = benzylguanine, MIF = (Ac)PLG, (N → C), P4 = LGAQSNF, PA = palmitic acid. [b]Yields are calculated for the conversion of linker-containing ONs to corresponding ON conjugates and are based on the HPLC profiles after cleavage of ONs from solid support and deprotection. [c]Yields are based on the HPLC profiles after cleavage of ONs from solid support and deprotection. [d]Yield is calculated for the attachment of palmitoyl moiety. [e]Yield is calculated for the attachment of two MIF moieties.