Staudinger-phosphonite reactions for the chemoselective transformation of azido-containing peptides and proteins

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SUPPORTING INFORMATION

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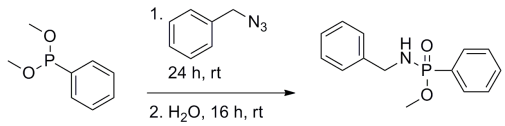
General Information

Materials: All reagents, starting materials, amino acids, and solvents were purchased from commercial suppliers and used without further purification if not further mentioned. 2-Methoxyethanol and 2-(2-Methoxyethoxy)ethanol were dried over molecular sieve for five days. Dry solvents (benzene, DMSO, diethyl ether) were purchased from ACROS ORGANICS. Buffers used for azido-peptide modifications (1 M tris saline pH = 8.2) were prepared from high purity salts purchased from SIGMA-ALDRICH.

Peptide synthesis: Peptides were synthesized on an ABI 433A peptide synthesizer using standard Fmoc based SPPS (0,1 mmol scale), amide coupling conditions HBTU/HOBt utilizing preloaded wang resin (Novabiochem). Fmoc-*p*azido-Phe-OH was coupled manually (3 equiv). Cleavage from the resin was performed with trifluoroacetic acid (95%), and triisopropylsilane/water 1:1 as scavenger for 2 h. Coupling and cleavage procedures are described by Novabiochem. Fmoc-*p*azido-Phe-OH was obtained from BACHEM.

Methodes: HPLC- Fluorescence spectra were recorded on a Waters 600S contoller with a Jasco FP 2020 Plus flourescence detector connected to the waters system. The excitation wavelength was 470 nm, the emission wavelength 530 nm. Separation was performed on an Agilent eclipse XDB C18 5µm column (5µm, 4.6 x 180 mm) at a flow rate of 1 mL/min. The following solvent (A = 1% AcOH in H_2O, B = 1% AcOH in MeCN) and gradient was applied: 0% B 0-5 min; 0-10% B 5-6 min; 10-60% B 6-31 min; 60-100% B 31-34 min; 100% B 34-40 min. Preparative HPLC purification was performed on a JASCO LC-2000 Plus system using a Kromasil RP18 column (25×250 mm) at a flow rate of 16 mL/min. The following solvent (A = 1% AcOH in H_2O, B = 1% AcOH in MeCN) and gradient was applied for peptide purification: 0% B 0-3 min; 0-30% B 3-8 min; 30-100% B 8-35 min; 100% B 35-43 min; 100-20% B 43-45 min. For phosphonite purification the following solvent (A = H_2O, B = MeCN) and gradient was applied: 0-7% B 0-20 min; 7-75% B 7-52 min; 75-100% B 52-60 min. MALDI-MS analysis was performed on an Ultraflex-II TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a 200 Hz solid-state Smart beam™ laser. The mass spectrometer was operated in the positive linear mode. MS spectra were acquired over an m/z range of 4,000-25,000 and data was analyzed using FlexAnalysis®. HPLC-HRMS and conversion studies of azido-peptide **3** were performed on an Agilent 6210 TOF LC/MS system, Agilent Technologies, Santa Clara, CA, USA. Spray voltage was set to 4 kV. Drying gas flow rate was set to 25 psi. Separation of the sample was performed on a Luna 5u C18(2) 100 A column (5 µm, 4.6×150 mm) at a flow rate of 0.6 mL/min. The following solvent (A = 1% AcOH in H_2O, B = 1% AcOH in MeCN) gradient was applied: 0% B 0-5 min; 0-10% B 5-6 min; 10-60% B 6-31 min; 60-100% B 31-34 min; 100% B 34-40 min. An analogous peptide containing deuterated alanine was added before the measurements as an internal standard and conversion was calculated by integration of both. Flash chromatography was performed on silica gel (Acros Silicagel 60 A, 0.035-0.070 mm). TLC was performed on aluminium-backed silica plates (60 F254, 0.2 mm) which were developed using potassium permanganate as visualising agent. ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR spectra were recorded on a Jeol ECX/400 in CD_3CN, $CDCl_3$ or DMSO-D_6. The chemical shifts are reported in ppm relatively to the residual solvent peak.[1] Carbon atoms in borane-protected phosphonites whish are directly bound to the phosphorus could not be detected by standard ^{13}C-NMR measurements because of the coupling to the phosphorus and the borane whish result in very small and broad peaks.

Methyl *N*-benzyl-P-phenylphosphonamidate



Entry	solvent	Yield
1	benzene	72 %
2	CH_2CL_2	80 %
3	DMF	83 %

To a solution of benzyl azide (399.5 mg, 3.000 mmol, 1 equiv) in anhydrous solvent (5 mL) dimethyl phenylphosphonite (510.4 mg, 3.000 mmol, 1 equiv) was added under argon atmosphere. The reaction mixture was stirred for 16 h at room temperature before water (1 mL) was added. After stirring at room temperature

for additional 16 h the solvent was removed under reduced pressure. The crude product was purified by column chromatography to yield methyl *N*-benzyl-P-phenylphosphonamidate as colorless solid. The product was obtained in a yield of 72% (560.7 mg, 2.146 mmol) in benzene, 80% (630.1 mg, 2.412 mmol) in dichloromethane and 83% (652.6 mg, 2.498 mmol) in DMF. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.83-7.77 (m, 2H, 2xCH), 7.53-7.49 (m, 1H, CH), 7.46-7.41 (m, 2H, 2xCH), 7.31-7.21 (m, 5H, 5xCH), 4.06 (ddd, *J* = 8.6, 7.0, 4.2 Hz, 2H, CH_2), 3.70 (d, *J* = 11.1 Hz, 3H, CH_3), 3.20 (s, 1H, NH).^{13}C-NMR (100 MHz, $CDCl_3$): δ = 139.7 (d, *J* = 6.3 Hz, C), 132.0 (d, *J* = 3.0 Hz, CH), 131.5 (d, *J* = 9.8 Hz, CH), 130.4 (d, *J* = 173.6 Hz, CP), 128.6 (2xCH), 128.4 (CH), 127.5 (CH), 127.3 (CH), 51.3 (d, *J* = 5.8 Hz, CH_3), 44.9 (CH_2). ^{31}P-NMR (171.8 MHz, $CDCl_3$): δ = 24.48 (s). MP = 64-65 °C. R_f(ethyl acetate/*n*-hexane : 3/1) = 0.32. HRMS for $C_{14}H_{17}NO_2P^+$, $[M+H]^+$ calced: 262.0991, found: 262.0995.

General procedure 1: Synthesis of tosylated polyethylenglycol-monomethylether

To a solution of polyethylenglycol monomethylether (1 equiv), *p*-toluenesulfonyl chloride (1.3 equiv) and triethylamine (2 equiv) in acetonitrile (0.1 M) at 0 °C was slowly added trimethylaminehydrochloride (1 equiv). 30 min. after addition was completed, the ice bath was removed and the mixture was stirred for 3 h at room temperature. The suspension was filtered and the filtrate was condensed under reduced pressure. The residue was dissolved in toluene and washed twice with hydrochloride acid (10%). The organic layers were dried over magnesium sulfate and concentrated to yield the desired product as colourless oil.

2-(2-(2-methoxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate

2-(2-(2-Methoxyethoxy)ethoxy)ethyl-4-methylbenzenesulfonate was obtained from triethyleneglycol monomethyl ether (6.679 g, 40.68 mmol) according to the general procedure 1 in 100% yield (12.93g, 40.60 mmol) as colourless oil. ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.78 (d, *J* = 8.3 Hz, 2H, 2xCH), 7.43 (d, *J* = 8.6 Hz, 2H, 2xCH), 4.12-4.09 (m, 2H, CH_2), 3.61-3.58 (m, 2H, CH_2), 3.52-3.49 (m, 2H, CH_2), 3.48 (s, 4H, 2xCH_2), 3.46-3.43 (m, 2H, CH_2), 3.28 (s, 3H, CH_3), 2.44 (s, 3H, CH_3). ^{13}C-NMR (100 MHz, CD_3CN): δ = 146.3 (C), 133.8 (C), 131.0 (2xCH), 128.7 (2xCH), 72.5 (CH_2), 71.1 (CH_2), 71.0 (CH_2), 70.9 (CH_2), 70.9 (CH_2), 69.1 (CH_2), 58.8 (CH_3), 21.6 (CH_3). R_f(ethyl acetate/cyclohexane : 1/1) = 0.35. HRMS for $C_{14}H_{22}NaO_6S^+$, $[M+Na]^+$ calced: 341.1035, found: 341.1034.

2,5,8,11-tetraoxatridecan-13-yl 4-methylbenzenesulfonate

2,5,8,11-Tetraoxatridecan-13-yl-4-methylbenzenesulfonate was obtained from tetraethyleneglycol monomethyl ether (4.4089 g, 21.171 mmol) according to the general procedure 1 in 100% yield (7.6236 g, 21.034 mmol) as colourless oil. ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.78 (d, *J* = 8.3 Hz, 2H, 2xCH), 7.43 (d, *J* = 7.9 Hz, 2H, 2xCH), 4.12-4.10 (m, 2H, CH_2), 3.61-3.59 (m, 2H, CH_2), 3.53-3.51 (m, 6H, 3xCH_2), 3.48 (s, 4H, 2xCH_2) 3.46-3.44 (m, 2H, CH_2), 3.28 (s, 3H, CH_3), 2.43 (s, 3H, CH_3). ^{13}C-NMR (100 MHz, CD_3CN): δ = 146.3 (C), 133.8 (C), 131.0 (CH), 128.7 (CH), 72.5 (CH_2), 71.1 (CH_2), 71.1 (CH_2), 71.1 (CH_2), 71.0 (CH_2), 70.9 (CH_2), 70.9 (CH_2), 69.1 (CH_2), 58.8 (CH_3), 21.6 (CH_3). R_f(ethyl acetate/*n*-hexane : 3/1) = 0.49. HRMS for $C_{16}H_{26}NaO_7S^+$, $[M+Na]^+$ calced.: 385.1297, found: 385.1293.

General procedure 2: deprotection of borane-protected phosphonites

A solution of borane protected phosphonite (1 equiv) and 1,4-diazabicyclo-[2.2.2]octane (1.5 equiv) in dry benzene (0.5 M) was warmed for 22 h to 50 °C. The crude product was condensed under reduced pressure. For purification, if desired, the crude product was dissolved in dry *n*-hexane (4 mL/100 mg) and filtered. The solvent was removed under reduced pressure to yield the desired product as colourless oil.

Dimethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite (2b)

Dimethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite (0.0717 g, 0.2157 mmol, 67%) was obtained from dimethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane (**6a**) (0.1121 g, 0.3238 mmol) according to the general procedure 2 for phosphonite-borane deprotection as colourless oil. ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.39-7.35 (m, 1H, CH), 7.13 (ddt, *J* = 7.1, 5.9, 1.1 Hz, 1H, CH), 7.09 (dd, *J* = 6.2, 2.6 Hz, 1H, CH), 6.99 (ddd, *J* = 8.3, 2.6, 1.0 Hz, 1H), 4.13-4.11 (m, 2H, CH_2), 3.78-3.76 (m, 2H,

CH_2), 3.62-3.61 (m, 2H, CH_2), 3.58-3.53 (m, 4H, 2xCH_2), 3.54 (d, *J* = 10.6 Hz, 6H, 2xCH_3), 3.47-3.44 (m, 2H, CH_2), 3.28 (s, 3H, CH_3). ^{31}P-NMR (171.8 MHz, CD_3CN): δ = 160.44 (s).

Dimethyl-4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite (2c)

Dimethyl-4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite (0.1244 g, 0.3743 mmol, 57%) was obtained from dimethyl-4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane (**6b**) (0.2286 g, 0.6604 mmol) according to the general procedure 2 for phosphonite-borane deprotection as colourless oil (+10% starting material[2]). ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.48 (dd, *J* = 8.8, 5.8 Hz, 2H, 2xCH), 6.99 (dd, *J* = 8.8, 1.1 Hz, 2H, 2xCH), 4.14-4.11 (m, 2H, CH_2), 3.79-3.76 (m, 2H, CH_2), 3.62-3.60 (m, 4H, 2xCH_2), 3.57-3.54 (m, 2H, CH_2), 3.51 (d, *J* = 10.9 Hz, 6H, 2xCH_3), 3.45 (m, 2H, CH_2), 3.28 (s, 3H, CH_3). ^{31}P-NMR (171.8 MHz, CD_3CN): δ = 162.29 (s).

Dimethyl (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite (2d)

Dimethyl (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite was obtained (0.0341 g, 0.0906 mmol, 46%) from dimethyl (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite-borane (**6c**) (0.0769 g, 0.1971 mmol) according to the general procedure 2 for phosphonite-borane deprotection as colourless oil. ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.39-7.35 (m, 1H, CH), 7.13 (ddt, *J* = 7.1, 5.8, 1.1 Hz, 1H, CH), 7.09 (ddd, *J* = 6.3, 2.7, 1.2 Hz, 1H, CH), 6.99 (ddd, *J* = 8.2, 2.7, 1.0 Hz, 1H, CH), 4.14-4.11 (m, 2H, CH_2), 3.79-3.77 (m, 2H, CH_2), 3.63-3.61 (m, 2H, CH_2), 3.58-3.57 (m, 2H, CH_2), 3.55-3.53 (m, 6H, 3xCH_2), 3.54 (d, *J* = 10.8 Hz, 6H, 2xCH_3), 3.46-3.44 (m, 2H, CH_2), 3.28 (s, 3H, CH_3). ^{31}P-NMR (171.8 MHz, CD_3CN): δ = 160.57 (s).

Diethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite (2e)

Diethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite (0.0469 g, 0.1301 mmol, 56%) was obtained from diethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane (**6d**) (0.0865 g, 0.2311 mmol) according to the general procedure 2 for phosphonite-borane deprotection as colourless oil. ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.36 (td, *J* = 7.9, 2.0 Hz, 1H), 7.16-7.12 (m, 1H, CH), 7.10 (ddd, *J* = 6.1, 2.7, 1.2 Hz, 1H, CH), 6.97 (dd, *J* = 8.4, 2.9 Hz, 1H, CH), 4.13-4.11 (m, 2H, CH_2), 3.95-3.89 (m, 2H, CH_2), 3.82-3.76 (m, 4H, 2xCH_2), 3.63-3.61 (m, 2H, CH_2), 3.58-3.54 (m, 4H, 2xCH_2), 3.47-3.44 (m, 2H, CH_2), 3.28 (s, 3H, CH3), 1.24 (td, *J* = 7.0, 0.5 Hz, 6H). ^{31}P-NMR (171.8 MHz, CD_3CN): δ = 156.07 (s).

Bis(2-methoxyethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite (2f)

Bis(2-methoxyethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite (0.0907 g, 0.2157 mmol, 54%) was obtained from bis(2-methoxyethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)-phenyl)phosphonite-borane (**6e**) according to the general procedure 2 for phosphonite-borane (0.1739 g, 0.4004 mmol) deprotection as colourless oil. ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.36 (dddd, *J* = 8.1, 7.4, 2.0, 0.5 Hz, 2H, CH), 7.16-7.11 (m, 2H, 2xCH), 6.97 (dddd, *J* = 8.2, 2.7, 1.0, 0.5 Hz, 2H, CH), 4.13-4.11 (m, 2H, CH_2), 4.03-3.95 (m, 2H, CH_2), 3.90-3.83 (m, 2H, CH_2), 3.79-3.76 (m, 2H, CH_2), 3.63-3.61 (m, 2H, CH_2), 3.58-3.51 (m, 8H, 4xCH_2), 3.46-3.44 (m, 2H, CH_2), 3.30 (s, 6H, 2xCH_3), 3.28 (s, 3H, CH_3). ^{31}P-NMR (171.8 MHz, CD_3CN): δ = 160.08 (s).

Bis(2-(2-methoxyethoxy)ethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite (2g)

Bis(2-(2-methoxyethoxy)ethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite (0.0308 g, 0.0606 mmol, 38%) was obtained from bis(2-(2-methoxyethoxy)ethyl) (3-(2-(2-(2-methoxyethoxy)-ethoxy)ethoxy)phenyl)phosphonite-borane (0.0833 g, 0.1595 mmol) according to the general procedure 2 for phosphonite-borane (**6f**) deprotection as colourless oil. ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.36 (td, *J* = 7.8, 1.9 Hz, 1H, CH), 7.17 (ddt, *J* = 7.0, 5.7, 1.0 Hz, 1H, CH), 7.13 (ddd, *J* = 6.3, 2.6, 1.2 Hz, 1H, CH), 6.97 (dd, *J* = 7.9, 2.3 Hz, 1H), 4.13-4.11 (m, 2H, CH_2), 4.00-3.97 (m, 2H, CH_2), 3.90-3.85 (m, 2H, CH_2), 3.79-3.77 (m, 2H, CH_2), 3.62-3.60 (m, 6H, 3xCH_2), 3.57-3.54 (m, 8H, 4xCH_2), 3.47-3.45 (m, 6H, 3xCH_2), 3.28 (s, 3H, CH_3), 2.28 (s, 6H, 2xCH_3). ^{31}P-NMR (171.8 MHz, CD_3CN): δ = 159.96 (s).

Bis(2-(2-methoxyethoxy)ethyl) (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite (2h)

Bis(2-(2-methoxyethoxy)ethyl) (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite (0.0559 g, 0.1012 mmol, 42%) was obtained from bis(2-(2-methoxyethoxy)ethyl) (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite-borane (**6g**) according to the general procedure 2 for phosphonite-borane (0.1351 g, 0.2385 mmol) deprotection as colourless oil. ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.36 (ddd, *J* = 8.4, 7.4, 2.1 Hz, 1H, CH), 7.16 (ddd, *J* = 7.1, 5.9, 1.1 Hz, 1H, CH), 7.13 (ddt, *J* = 7.3, 5.8, 1.1 Hz, 1H, CH), 6.97 (ddd, *J* = 6.1, 2.6, 1.1 Hz, 1H, CH), 4.13-4.11 (m, 2H, CH_2), 4.01-3.96 (m, 2H, CH_2), 3.90-3.85 (m, 2H, CH_2), 3.79-3.77 (m, 2H, CH_2), 3.61-3.52 (m, 18H, 9xCH_2), 3.47-3.44 (m, 6H, 3xCH_2),3.28 (s, 6H, 2xCH_3), 3.28 (s, 3H, CH_3). ^{31}P-NMR (171.8 MHz, CD_3CN): δ = 159.96 (s).

General procedure 3: synthesis of PEGylated bromophenols

A solution of the corresponding bromophenole (1 equiv), the corresponding poly-ethyleneglycole monomethylether toluene-*p*-sulfonate (1 equiv) and potassium carbonate (2.4 equiv) in acetonitrile (0.1 M) was heated to reflux for 16 h. The mixture was brought to room temperature, insoluble material was filtered off, and the filtrate was filtered through a pad of silica. The solvent was removed under reduced pressure to yield the desired product as colourless oil.

1-bromo-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene (5a)

1-Bromo-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene was obtained from 3-bromophenol (3.0071 g, 17.380 mmol) according to the general procedure 3 in 99% yield (5.5043 g, 17.2446 mmol) as colourless oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.08-7.04 (m, 1H, CH), 7.01-6.99 (m, 2H, 2xCH), 6.79-6.76 (m, 1H, CH), 4.04–4.02 (dd, *J* = 5.6, 4.1 Hz, 2H, CH_2), 3.78-3.76 (m, 2H, CH_2), 3.67-3.64 (m, 2H, CH_2), 3.62-3.57 (m, 4H, 2xCH_2), 3.49-3.47 (m, 2H, CH_2), 3.31 (s, 3H, CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 159.4 (C), 130.4 (CH), 123.7 (CH), 122.6 (C), 117.8 (CH), 113.5 (CH), 71.8 (CH_2), 70.7 (CH_2), 70.5 (CH_2), 70.4 (CH_2), 69.4 (CH_2), 67.5 (CH_2), 58.9 (CH_3). HRMS for $C_{13}H_{19}BrNaO_4^+$, $[M+Na]^+$ calced.: 341.0364, found: 341.0373.

1-bromo-4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene (5b)

1-Bromo-4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene was obtained from 4-bromophenol (4.000 g, 23.263 mmol) according to the general procedure 3 in 100% yield (7.4175 g, 23.239 mmol) as colourless oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.25 (d, *J* = 9.2 Hz, 2H, 2xCH), 6.69 (d, *J* = 8.9 Hz, 2H, 2xCH), 4.00–3.94 (m, 2H, CH_2), 3.78–3.69 (m, 2H, CH_2), 3.66–3.59 (m, 2H, CH_2), 3.59–3.52 (m, 4H, 2xCH_2), 3.49–3.40 (m, 2H, CH_2), 3.29–3.18 (s, 1H, CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 157.7 (C), 131.9 (2xCH), 116,2 (2xCH), 112.7 (C), 71.7 (CH_2), 70.6 (CH_2), 70.4 (CH_2), 70.3 (CH_2), 69.4 (CH_2), 67.4 (CH_2), 58.8 (CH_3). HRMS for $C_{13}H_{19}BrNaO_4^+$, $[M+Na]^+$ calced.: 341.0364, found 341.0713.

13-(3-bromophenoxy)-2,5,8,11-tetraoxatridecane (5c)

13-(3-Bromophenoxy)-2,5,8,11-tetraoxatridecane was obtained from 3-bromo-phenol (1.9094 g, 11.036 mmol) according to the general procedure 3 in 93 % yield (3.6958 g, 10.203 mmol) as colourless oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.04-7.00 (m, 1H, CH), 6.98-6.95 (m, 2H, 2xCH2), 6.76-6.73 (m, 1H, CH), 3.99-3.97 (m, 2H, CH_2), 3.74-3.71 (m, 2H, CH_2), 3.61-3.52 (m, 10H, 5x CH_2), 3.44-3.42 (m, 2H, CH_2), 3.26 (s, 3H, CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 159.4 (C), 130.3 (CH), 123.6 (CH), 122.4 (C), 117.7 (CH), 113.4 (CH), 71.7 (CH_2), 70.6 (CH_2), 70.4 (3xCH_2), 70.3 (CH_2), 70.2 (CH_2), 69.3 (CH_2), 67.4 (CH_2), 58.7 (CH_3). HRMS for $C_{15}H_{23}BrNaO_5^+$, $[M+Na]^+$ calced.: 385.0627, found: 385.0624.

General procedure 4: Synthesis of Dimethyl arylphosphonites

To a solution of the functionalized aryl bromide (1 equiv) in dry ether (0.1 M) at –95 °C was added slowly *n*-butyllithium (1.2 equiv). 30 min. after addition trimethyl phosphite (5 equiv) was added. 30 min. after addition the solution was warmed to 0 °C and stirred for additional 4 h. The reaction mixture was condensed under reduced pressure and residue was dissolved in dry ether (0.1 M) again. The solution was cooled to –78 °C

and borane dimethyl sulfide (2.2 equiv) was added slowly. After 30 min. the suspension was warmed to room temperature and stirred for additional 15 h. The reaction was quenched with sat. ammonium chloride and the product was extracted with ethyl acetate (3×). The organic layers were dried over magnesium sulfate, concentrated and purified by column chromatography to yield the desired phosphonite as colourless liquid.

Dimethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane (6a)

Dimethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane was obtained from 1-bromo-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene (**5a**) (1.1386 g, 3.5671 mmol) according to the general procedure 4 in 52% yield (0.6371 g, 1.840 mmol) as colourless oil. ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.48 (td, *J* = 7.9, 3.7 Hz, 1H, CH), 7.35-7.30 (m, 1H, CH), 7.25 (ddd, *J* = 11.4, 2.6, 1.3 Hz, 1H, CH), 7.18 (ddt, *J* = 8.3, 1.8, 0.8 Hz, 1H, CH), 4.16-4.14 (m, 2H, CH_2), 3.80-3.77 (m, 2H, CH_2), 3.72 (dd, *J* = 11.1, 0.7 Hz, 6H, 2xCH_3), 3.64-3.61 (m, 2H, CH_2), 3.58-3.53 (m, 4H, 2xCH_2), 3.45 (dd, *J* = 5.6, 3.4 Hz, 2H, CH_2), 3.28 (s, 3H, CH_3), 0.58 (ddd, *J* = 190.9, 93.8, 18.9 Hz, 3H, BH_3). ^{13}C-NMR (100 MHz, CD_3CN): δ = 159.9 (d, *J* = 13.4 Hz, C), 131.3 (d, *J* = 12.8 Hz, CH), 123.8 (d, *J* = 12.3 Hz, CH), 119.9 (d, *J* = 2.2 Hz, CH), 117.1 (d, *J* = 12.9 Hz, CH), 72.6 (CH_2), 71.4 (CH_2), 71.1 (CH_2), 71.0 (CH_2), 70.1 (CH_2), 68.8 (CH_2), 58.8 (CH_3), 54.5 (d, *J* = 5.8 Hz, 2xCH_3). ^{31}P-NMR (171.8 MHz, CD_3CN): δ = 132.88 (dd, *J* = 167.0, 81.9 Hz). R_f(ethyl acetate/cyclohexane : 3/1) = 0.51.

Dimethyl-4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane (6b)

Dimethyl-4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane was obtained from 1-bromo-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene (**5b**) (1.3348 g, 4.1818 mmol) according to the general procedure 4 in 51% yield (0. 7381 g, 2.132 mmol) as colourless oil. ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.66 (dd, *J* = 10.0, 8.8 Hz, 2H, 2xCH), 7.08 (dd, *J* = 8.9, 2.2 Hz, 2H, 2xCH), 4.18-4.16 (m, 2H, CH_2), 3.80-3.78 (m, 2H, CH_2), 3.68 (d, *J* = 11.2 Hz, 6H, 2xCH_3), 3.63-3.61 (m, 2H, CH_2), 3.58-3.53 (m, 4H, 2xCH_2), 3.46-344 (m, 2H, CH_2), 3.28 (s, 3H, CH_3), 0.58 (ddd *J* = 191.3, 94.4, 14.5 Hz, 3H, BH_3). ^{13}C-NMR (100 MHz, CD_3CN): δ = 163.3 (d, *J* = 2.2 Hz, C), 133.6 (d, *J* = 13.5 Hz, 2xCH), 115.8 (d, *J* = 11.8 Hz, 2xCH), 72.6 (CH_2), 71.3 (CH_2), 71.1 (CH_2), 71.0 (CH_2), 70.0 (CH_2), 68.7 (CH_2), 58.8 (CH_3), 54.2 (d, *J* = 5.7 Hz, 2xCH_3). ^{31}P-NMR (171.8 MHz, CD_3CN): δ = 133.38 (dd, *J* = 172.7, 82.3 Hz). R_f(ethyl acetate/cyclohexane : 2/1) = 0.31.

Dimethyl (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite-borane (6c)

Dimethyl (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite-borane was obtained from 13-(3-bromophenoxy)-2,5,8,11-tetraoxatridecane (**5c**) (0.9831 g, 2.706 mmol) according to the general procedure 4 in 44% yield (0. 4642 g, 1.190 mmol) as colourless oil. ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.48 (td, *J* = 7.9, 3.7 Hz, 1H, CH), 7.32 (ddt, *J* = 9.7, 7.3, 1.1 Hz, 1H, CH), 7.25 (ddd, *J* = 11.5, 2.8, 1.3 Hz, 1H, CH), 7.18 (dd, *J* = 8.3, 2.6 Hz, 1H, CH), 4.16-4.14 (m, 2H, CH_2), 3.80-3.78 (m, 2H, CH_2), 3.71 (d, *J* = 11.2 Hz, 6H, 2xCH_3), 3.63-3.62 (m, 2H, CH_2), 3.58-3.57 (m, 2H, CH_2), 3.54-3.52 (m, 6H, 3xCH_2), 3.46-3.44 (m, 2H, CH_2), 3.28 (s, 3H, CH_3), 0.58 (ddd, *J* = 192.7, 94.4, 16.4 Hz, 3H, BH_3). ^{13}C-NMR (100 MHz, CD_3CN): δ = 159.9 (d, *J* = 13.4 Hz, C), 131.2 (d, *J* = 12.6 Hz, CH), 123.8 (d, *J* = 12.3 Hz, CH), 119.9 (d, *J* = 2.0 Hz, CH), 117.1 (d, *J* = 12.9 Hz, CH), 72.6 (CH_2), 71.3 (CH_2), 71.2 (CH_2), 71.1 (CH_2), 71.1 (CH_2), 71.0 (CH_2), 70.1 (CH_2), 68.8 (CH_2), 58.8 (CH_3), (d, *J* = 5.7 Hz, 2xCH_3). ^{31}P-NMR (171.8 MHz, CD_3CN): δ = 132.89 (dd, *J* = 168.7, 79.0 Hz). R_f(ethyl acetate/cyclohexane : 2/1) = 0.26.

Diethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane (6d)

To a solution of 1-bromo-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene (**5a**) (0.2219 g, 0.6952 mmol, 1 equiv) in dry ether (3 mL) at –95 °C was added slowly *n*-butyllithium (1.6 M in *n*-hexane, 1.2 equiv). 30 min. after addition diethylchloro phosphite (0.11 mL, 0.76 mmol, 1.1 equiv) was added. 30 min. after addition the solution was warmed to 0 °C. After 4 h the solution was cooled to –78 °C and borane dimethyl sulfide (90%, 0.07 mL, 0.84 mmol, 1.2 equiv) was added slowly. After 30 min. the suspension was warmed to room temperature and stirred for additional 15 h. The reaction was quenched with sat. ammonium chloride and the product was extracted with ethyl acetate (3×). The organic layers were dried over magnesium sulfate, concentrated and purified by column chromatography to yield **6d** (0.1651 g, 0.4412 mmol, 63%) as

colourless oil. ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.47 (td, *J* = 7.8, 3.7 Hz, 1H, CH), 7.34 (ddt, *J* = 10.0, 7.5, 1.1 Hz, 1H, CH), 7.25 (ddd, *J* = 11.4, 2.6, 1.2 Hz, 1H, CH), 7.16 (dd, *J* = 8.3, 2.6 Hz, 1H, CH), 4.16-4.14 (m, 2H, CH_2), 4.12-3.97 (m, 4H, 2xCH_2Me), 3.80-3.77 (m, 2H, CH_2), 3.58-3.53 (m, 4H, 2xCH_2), 3.46-3.44 (m, 2H, CH_2), 3.28 (s, 3H, CH_3), 1.30 (t, *J* = 7.1 Hz, 6H, 2xCH_3), 0.96-0.23 (m, 3H, BH_3). ^{13}C-NMR (100 MHz, CD_3CN): δ = 159.9 (d, *J* = 13.4 Hz, C), 131.2 (d, *J* = 12.7 Hz, CH), 123.8 (d, *J* = 12.2 Hz, CH), 119.5 (d, *J* = 2.0 Hz, CH), 117.1 (d, *J* = 12.8 Hz, CH), 72.6 (CH_2), 71.4 (CH_2), 71.1 (CH_2), 71.0 (CH_2), 70.1 (CH_2), 68.7 (CH_2), 64.29 (d, *J* = 5.6 Hz, 2xCH_2), 58.9 (CH_3), 16.7 (d, *J* = 5.7 Hz, 2xCH_3). ^{31}P-NMR (171.8 MHz, CD_3CN): δ = 127.76 (dd, *J* = 168.5, 79.3 Hz). R_f(ethyl acetate/cyclohexane : 2/1) = 0.28.

Bis(2-methoxyethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite-borane (6e)

To a solution of 1-bromo-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene (**5a**) (0.1021 g, 0.3171 mmol, 1 equiv) in dry diethyl ether (5 mL) at –95 °C was added slowly *n*-butyllithium (1.6 M in *n*-hexane, 1.1 equiv). 30 min. after addition bisdiethylamino phosphite (0.081 mL, 0.381 mmol, 1.2 equiv) was added. The solution was stirred for 30 min. at this temperature and then 4 h at room temperature. The precipitate was filtered of and solvent was removed under reduced pressure. Then acetonitrile (5 mL), 2-methoxyethanol (0.065 mL, 0.824 mmol, 2.6 equiv) and tetrazole (0.45 M in acetonitrile, 3.2 µmol, 7.0 µL, 0.01 equiv), were added and heated to reflux for 16 h. The solution was cooled to 0 °C and borane tetrahydrofurane (1 M in tetrahydrofurane, 0.38 mL, 0.38 mmol, 1.2 equiv) was added and slowly warmed up to room temperature. After 12 h the reaction was quenched with sat. ammonium chloride and the product was extracted with ethyl acetate (3×). The organic layers were dried over magnesium sulfate, concentrated and purified by column chromatography to yield **6e** (0.0452 g, 0.1041 mmol, 33%) as colourless oil. ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.47 (dddd, *J* = 8.2, 7.6, 3.9, 0.4 Hz, 1H, CH), 7.34 (ddt, *J* = 10.0, 7.5, 1.1 Hz, 1H, CH), 7.28 (ddd, *J* = 12.1, 2.6, 1.4 Hz, 1H, CH), 7.17 (ddt, *J* = 8.2, 1.9, 0.7 Hz, 1H, CH), 4.19-4.12 (m, 4H, 2xCH_2), 4.09-4.03 (m, 2H, CH_2), 3.80-3.78 (m, 2H, CH_2), 3.63-3.61 (m, 2H, CH_2), 3.59-3.53 (m, 8H, 4xCH_2), 3.47-3.44 (m, 2H, CH_2), 3.32 (s, 6H, 2xCH_3), 3.28 (s, 3H, CH_3), 0.96-0.21 (m, 3H, BH_3). ^{13}C-NMR (100 MHz, CD_3CN): δ = 159.8 (d, *J* = 13.3 Hz, C), 131.2 (d, *J* = 12.5 Hz, CH), 123.9 (d, *J* = 12.2 Hz, CH), 119.8 (d, *J* = 2.0 Hz), 117.1 (d, *J* = 12.9 Hz), 72.6 (CH_2), 72.2 (d, *J* = 6.3 Hz, 2xCH_2), 71.3 (CH_2), 71.1 (CH_2), 71.0 (CH_2), 60.1 (CH_2), 68.7 (CH_2), 67.3 (d, J = 5.8 Hz, 2xCH_2), 58.9 (2xCH_3), 58.8 (CH_3). ^{31}P-NMR (171.8 MHz, CD_3CN): δ = 130.09 (dd, *J* = 164.0, 74.7 Hz). R_f(ethyl acetate/cyclohexane : 5/1) = 0.21.

Bis(2-(2-methoxyethoxy)ethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite-borane (6f)

To a solution of 1-bromo-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene (**5a**) (0.0992 g, 0.3108 mmol, 1 equiv) in dry diethyl ether (5 mL) at –95 °C was added slowly *n*-butyllithium (1.6 M in *n*-hexane, 1.1 equiv). 30 min. after addition bisdiethylamino phosphite (0.080 mL, 0.373 mmol, 1.2 equiv) was added. The solution was stirred for 30 min. at this temperature and then 4 h at room temperature. The precipitate was filtered of and solvent was removed under reduced pressure. Then acetonitrile (5 mL), diethyleneglycol monomethylether (0.095 mL, 0.808 mmol, 2.6 equiv) and tetrazole (0.45 M in acetonitrile, 6.9 µL, 3.1 µmol, 0.01 equiv), was added and heated to reflux for 16 h. The solution was cooled to 0 °C and borane tetrahydrofurane (1 M in tetrahydrofurane, 0.37 mL, 0.37 mmol, 1.2 equiv) were added and slowly warmed up to room temperature. After 12 h the reaction was quenched with sat. ammonium chloride and the product was extracted with ethyl acetate (3×). The organic layers were dried over magnesium sulfate, concentrated and purified by column chromatography to yield **6f** (0.0893 g, 0.171 mmol, 55%) as colourless oil. ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.53 (td, *J* = 7.9, 3.8 Hz, 1H, CH), 7.38-7.35 (m, 1H, CH), 7.28 (ddd, *J* = 11.6, 2.7, 1.3 Hz, 1H, CH), 7.17 (dd, *J* = 8.3, 2.5 Hz, 1H, CH), 4.20-4.13 (m, 4H, 2xCH_2), 4.08-4.04 (m, 2H, CH_2), 3.80-3.78 (m, 2H, CH_2), 3.67-3.65 (m, 4H, 2xCH_2), 3.63-3.61 (m, 2H, CH_2), 3.59-3.53 (m, 8H, 4xCH_2), 3.48-3.44 (m, 6H, 3xCH_2), 3.29 (s, 6H, 2xCH_3), 3.28 (s, 3H, CH_3), 0.99-0.24 (m, 3H, BH_3). ^{13}C-NMR (100 MHz, CD_3CN): δ = 159.9 (d, *J* = 13.4 Hz, 2xCH_2), 131.2 (d, *J* = 12.6 Hz, 2xCH_2), 124.0 (d, *J* = 12.3 Hz, 2xCH_2), 119.7 (d, *J* = 2.3 Hz, 2xCH_2), 117.2 (d, *J* = 13.1 Hz, 2xCH_2), 72.6 (CH_2), 72.5 (2xCH_2), 71.4 (CH_2), 71.1 (CH_2), 71.0 (2xCH_2), 71.0 (CH_2), 70.8 (d, *J* = 6.0 Hz, 2xCH_2), 70.1 (CH_2), 68.7 (CH_2), 67.4 (d, *J* = 5.8 Hz, 2xCH_2), 58.9 (2xCH_3), 58.8 (CH_3). ^{31}P-NMR (171.8 MHz, CD_3CN): δ = 129.93 (dd, *J* = 167.8, 65.1 Hz). R_f(ethyl acetate/cyclohexane : 5/1) = 0.18.

Bis(2-(2-methoxyethoxy)ethyl) (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite-borane (6g)

To a solution of 13-(3-bromophenoxy)-2,5,8,11-tetraoxatridecane (**5c**) (0.1216 g, 0.3357 mmol, 1 equiv) in dry ether (5 mL) at –95 °C was added slowly *n*-butyllithium (1.6 M in *n*-hexane, 1.1 equiv). 30 min. after addition bisdiethylamino phosphite (0.086 mL, 0.403 mmol, 1.2 equiv) was added. The solution was stirred for 30 min. at this temperature and then 4 h at room temperature. The precipitate was filtered of and solvent was removed under reduced pressure. Then acetonitrile (6 mL), diethyleneglycol monomethylether (0.103 mL, 0.873 mmol, 2.6 equiv) and tetrazole (0.45 M in acetonitrile, 7.6 µL, 3.4 µmol, 0.01 equiv), was added and heated to reflux for 16 h. The solution was cooled to 0 °C and borane tetrahydrofurane (1 M in tetrahydrofurane, 0.40 mL, 0.40 mmol, 1.2 equiv) was added and slowly warmed up to room temperature. After 12 h the reaction was quenched with sat. ammonium chloride and the product was extracted with ethyl acetate (3×). The organic layers were dried over magnesium sulfate, concentrated and purified by semi-preparative HPLC to yield **6g** (0.1653 g, 0.2918 mmol, 87%) as colourless oil. ^{1}H-NMR (400 MHz, CD_3CN): δ = 7.47 (td, *J* = 7.9, 3.8 Hz, 1H, CH), 7.36 (ddt, *J* = 9.9, 7.5, 1.1 Hz, 1H, CH), 7.28 (ddd, *J* = 11.6, 2.6, 1.3 Hz, 1H, CH), 7.17 (dd, *J* = 8.2, 2.6 Hz, 1H, CH), 4.21-4.13 (m, 4H, 2xCH_2), 4.11-4.01 (m, 2H, CH_2), 3.82-3.75 (m, 2H, CH_2), 3.66 (dd, *J* = 5.2, 4.0 Hz, 4H, 2xCH_2), 3.62 (dd, *J* = 4.3, 1.9 Hz, 2H, CH_2), 3.60-3.56 (m, 6H, 3xCH_2), 3.55-3.51 (m, 6H, 3xCH_2), 3.50-3.43 (m, 6H, 3xCH_2), 3.29 (s, 6H, 2xCH_3), 3.28 (s, 3H, CH_3), 0.58 (dd, *J* = 182.3, 73.3 Hz, 3H, BH_3). ^{13}C-NMR (100 MHz, CD_3CN): δ = 159.90 (d, *J* = 13.4 Hz,C), 131.45 (d, *J* = 12.7 Hz, CH), 123.98 (d, *J* = 12.2 Hz, CH), 119.78 (d, *J* = 1.9 Hz, CH), 117.22 (d, *J* = 13.0 Hz, CH), 72.6 (CH2), 72.5 (2xCH_2), 71.37 (2xCH_2), 71.20 (CH_2), 71.16 (CH_2), 71.13 (CH_2), 71.07 (2xCH_2), 70.99 (CH_2), 70.81 (d, *J* = 6.2 Hz, 2xCH_2), 70.16 (CH_2), 68.73 (CH_2), 67.46 (d, *J* = 5.8 Hz, 2xCH_2), 58.95 (2xCH_3), 58.88 (CH_3). ^{31}P-NMR (171.8 MHz, CD_3CN): δ = 129.91 (dd, *J* = 161.5, 54.5 Hz).

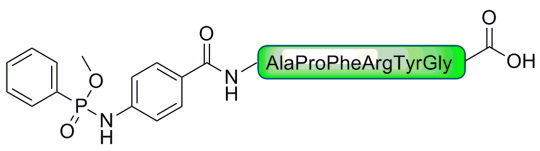
***N*-α-Fluorenylmethyloxycarbonyl-(ε-*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-ylamino)-L-Lysin[3]**

Fmoc-Lys-OH (1289.5 mg, 3.5000 mmol, 1 equiv), 4-chloro-7-nitrobenzofurazan (1047.6 mg, 5.2500 mmol, 1.5 equiv) and sodium hydrogen carbonate (882.1 10.50 mmol, 3 equiv) in water (45 mL / methanol (30 mL) were warmed to 60 °C (oil bath). After 2.5 h the reaction was cooled to room temperature and pH was set to 1 with hydrochloric acid (1N). The reaction mixture was extracted with dichloromethane (3x100 mL) the combined organic layer were washed with brine and dried over magnesium sulphate concentrated and purified by column chromatography to yield Fmoc-Lys(εNBD)-OH (1148.1 mg, 2.1600 mmol, 62%) as orange solid. ^{1}H-NMR (400 MHz, $(CD_3)_2SO$): δ = 12.61 (s, 1H), 9.50 (s, 1H), 8.42 (d, *J* = 8.9 Hz, 1H), 7.84 (d, *J* = 7.6 Hz, 2H), 7.70 (dd, *J* = 7.6, 4.9 Hz, 2H), 7.65 (d, *J* = 8.1 Hz, 1H), 7.38 (t, *J* = 7.5 Hz, 2H), 7.29 (t, *J* = 7.5 Hz, 2H), 6.31 (d, *J* = 9.1 Hz, 1H), 4.30-4.25 (m, 2H), 4.19 (t, *J* = 7.0 Hz, 1H), 4.03-3.92 (m, 1H), 3.48-3.37 (m, 2H), 1.87-1.58 (m, 4H), 1.52-1.40 (m, 2H). ^{13}C-NMR (100 MHz, CD_3CN): δ = 174.0 (C), 156.2 (C), 145.1 (d, *J* = 3.4 Hz, C), 144.4 (C), 144.1 (C), 143.8 (C), 140.7 (d, *J* = 2.5 Hz, C), 137.8 (CH_2), 127.6 (CH_2), 127.1 (CH_2), 125.3 (CH_2), 120.6 (C), 120.1 (CH_2), 99.0 (CH), 65.6 (CH_2), 53.8 (CH), 46.7 (CH), 43.2 (CH_2), 30.5 (CH_2), 27.1 (CH_2), 23.2 (CH_2). R_f(dichloromethane/methanol : 10/1) = 0.22, MP = 116-117 °C. HRMS for $C_{27}H_{24}N_5O_7^-$, [M-H$^+$] calcd.: 530.1681, found: 530.1739.

Hydrolysis Studies (see Figure 1 in manuscript)

A stock solution of phosphonite (**2b**, **2g** or **2f**) (250 mM in DMSO) and disodium hydrogen phosphate (250 mM in 1 M tris/HCl buffer pH = 8.2) wase prepared. The sample was prepared by adding phosphonite stock solution (70 µL) and disodium hydrogen phosphate stock solution (70 µL) to the buffer (1 M tris/HCl pH = 8.2, 540 µL) and instantly measured by ^{31}P-NMR (in 3 minute intervals).

Stability studies of Phosphonamidates



phosphonamidate model peptide

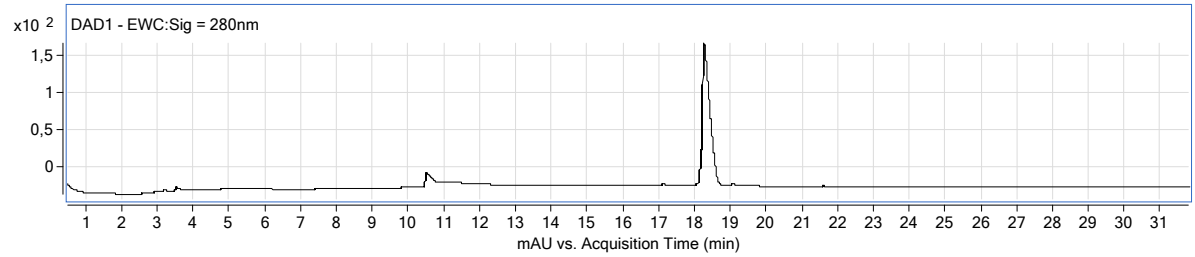
Stability studies were performed with a phosphonamidate containing model peptide, which was purified by preparative HPLC. The purified peptide was:

- reinjected to HPLC/MS: no additional peaks or masses were detected
- incubated in tris/HCl buffer (1 M; pH = 8.2; 100 mM peptide) for 26 h and analysed be HPLC/MS: no additional peaks or masses were detected
- incubated in tris/HCl buffer (1 M; pH = 7.6; 100 mM peptide) for 26 h and analysed be HPLC/MS: no additional peaks or masses were detected
- incubated in TFA (20% in water; 100 mM peptide) for 40 min: one additional peak in HPLC/MS (corresponding amino peptide; approximately 60%)
- incubated in TFA (20% in water; 100 mM peptide) for 2 h: one additional peak in HPLC/MS (corresponding amino peptide; approximately 95%)

Peptides (Pap = *p*-Azidophenylalanine)

PapAlaGluTrpAlaSerLysVal (3a)

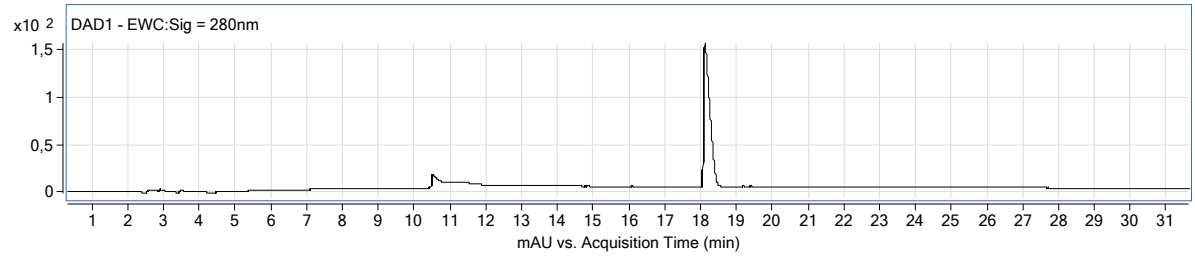
HRMS for $C_{46}H_{66}N_{13}O_{12}^{+}$, [M+H^{+}] calcd.: 992.4948, found: 992.4830.



UV spectrum (280 nm)

PapAla*GluTrpAla*SerLysVal (Ala* = NHCH(CD_3)CO)

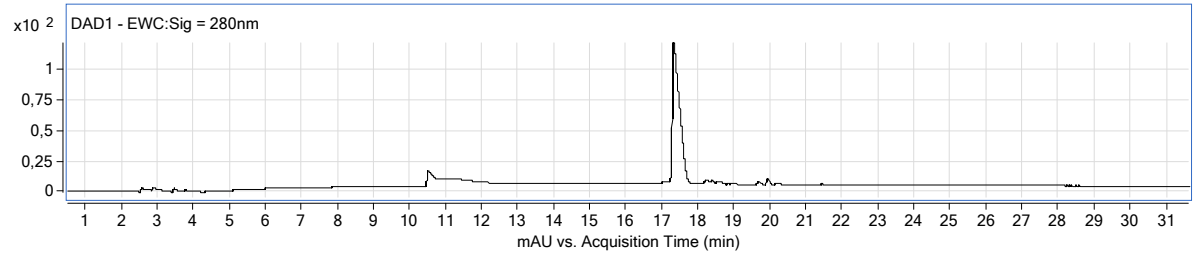
HRMS for $C_{46}H_{60}D_6N_{13}O_{12}^{+}$, [M+H^{+}] calcd.: 998.5325, found: 998.5190.



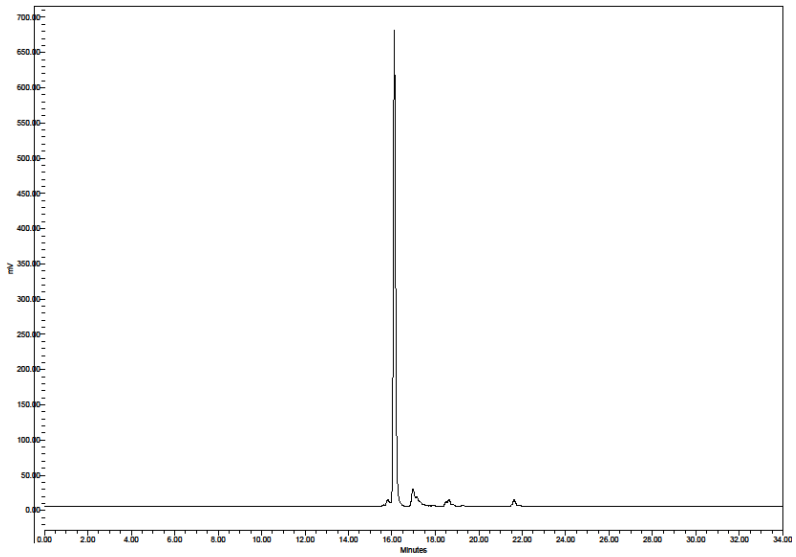
UV spectrum (280 nm)

PapAlaGluAlaSerLysSerLys(NBD)Val (3b)

HRMS for $C_{49}H_{72}N_{17}O_{17}^{+}$, [M+H^{+}] calcd.: 1170.5287, found: 1170.5118.



UV spectrum (280 nm)

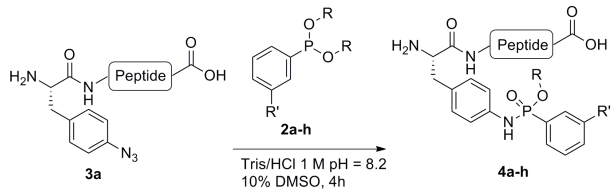


Fluorescence spectrum (530/470 nm) of **3b**

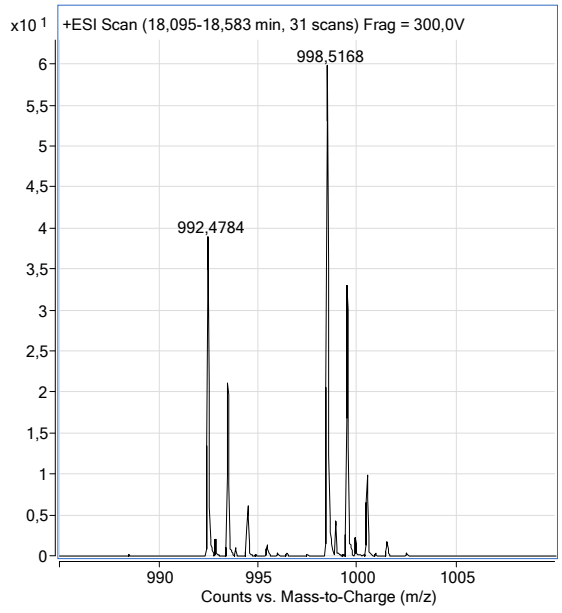
General procedure for Staudinger-phosphonite reaction with peptides for conversion Studies

A stock solution of peptide **3a** (PapAlaGluTrpAlaSerLysVal; 1 mM in tris / HCl buffer 1 M pH = 8.2) and phosphonite (500 mM in dry DMSO) was prepared. Measurements were performed by adding the peptide stock solution (20 µL) and the phosphonite **2a-h** stock solution (20 µL for 500 equiv; 4 µL + 16 µL DMSO for 100 equiv) to the buffer (1 M tris/HCl pH = 8.2, 160 µL) and incubated at room temperature. Then the deuterated analogue of the peptide **3a** (20 µL) was added (see General information).

Conversion Studies (Table 2)



Peptide **3** = PapAlaGluTrpAlaSerLysVal



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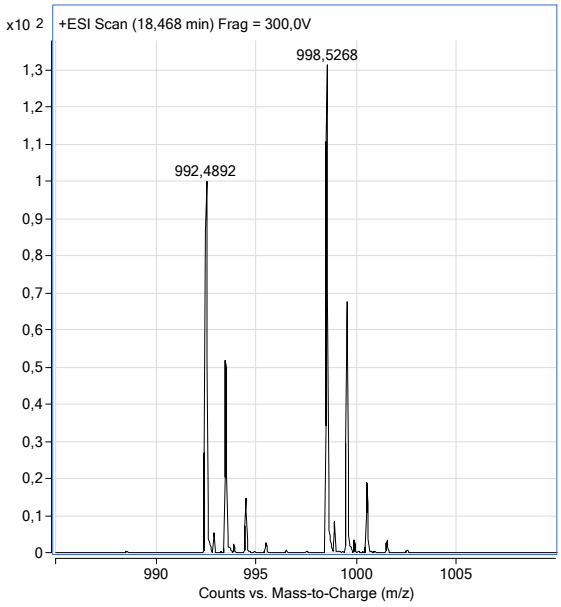

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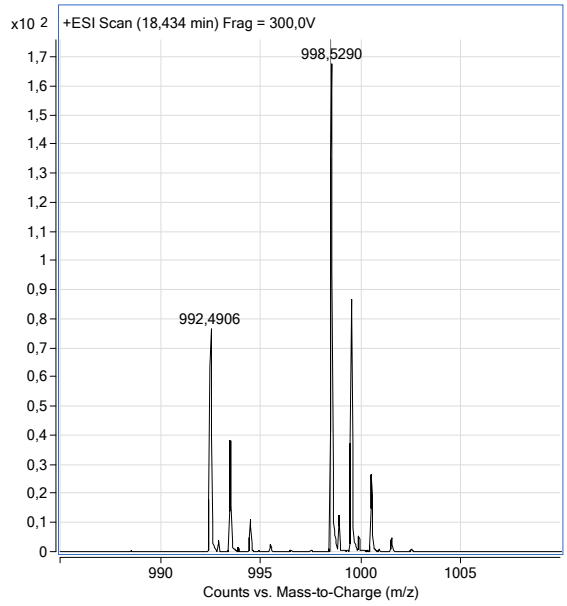

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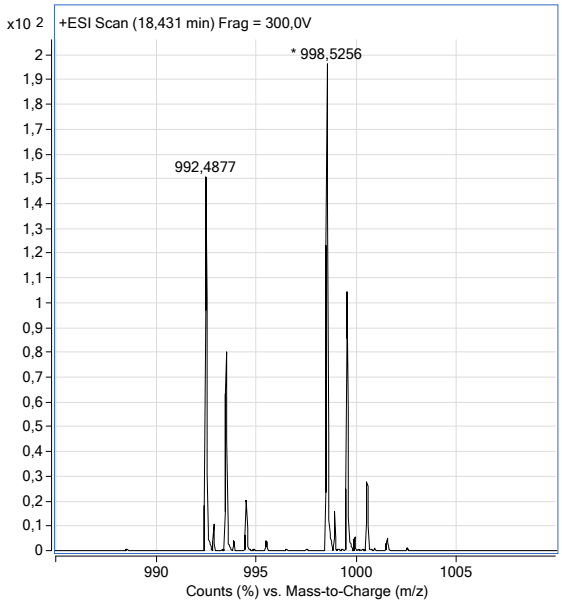

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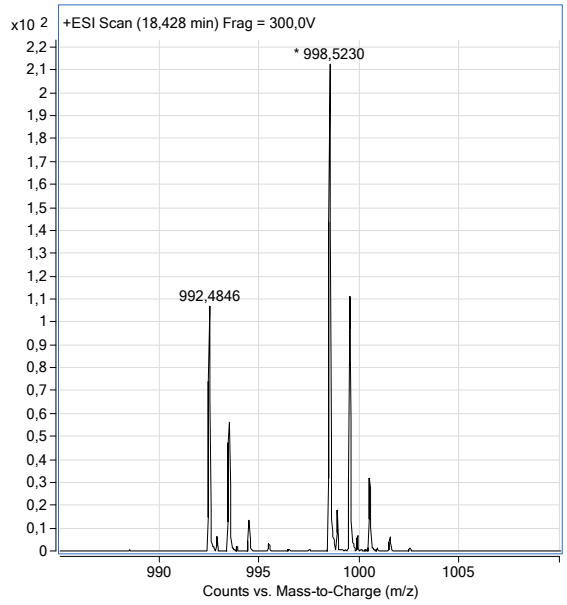

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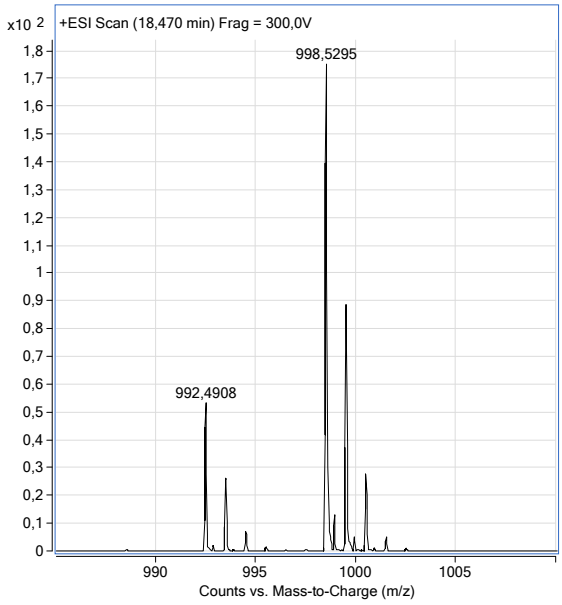

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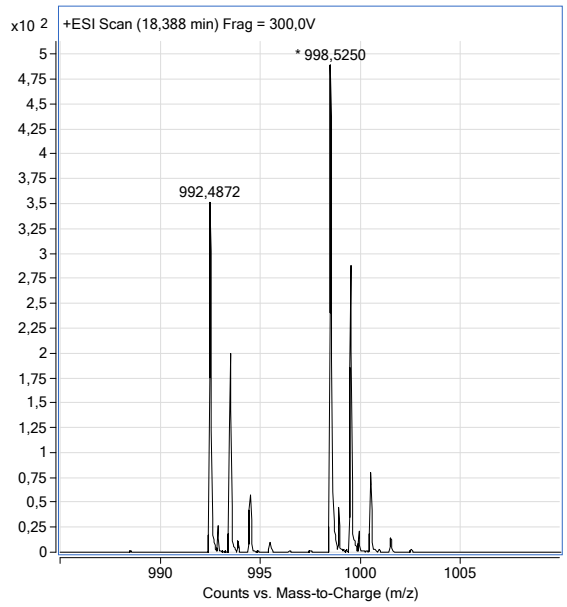

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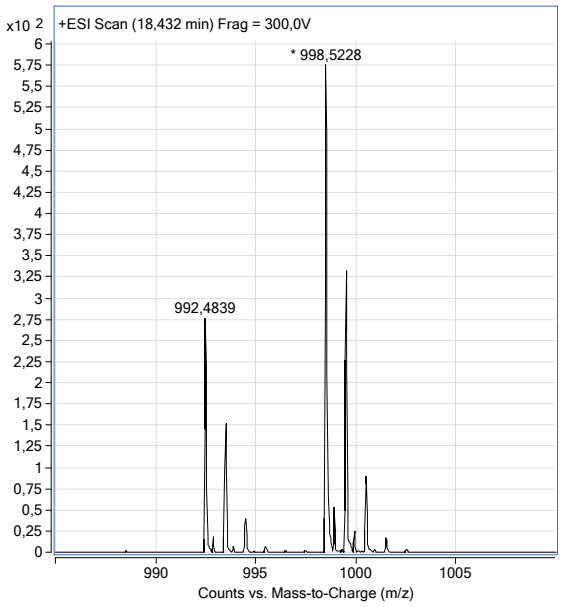

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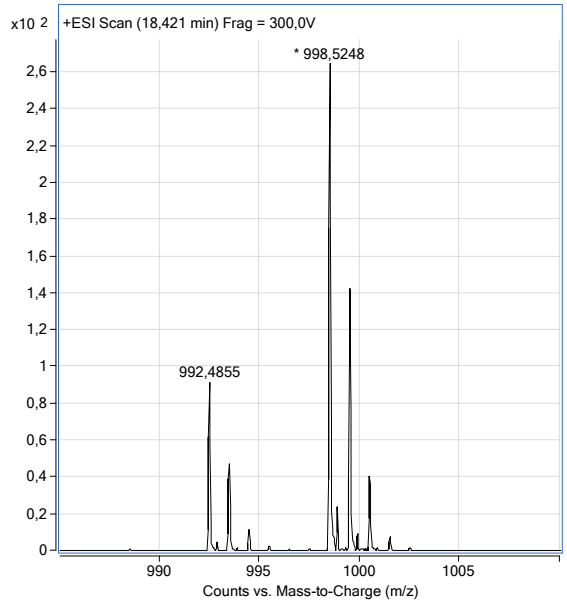

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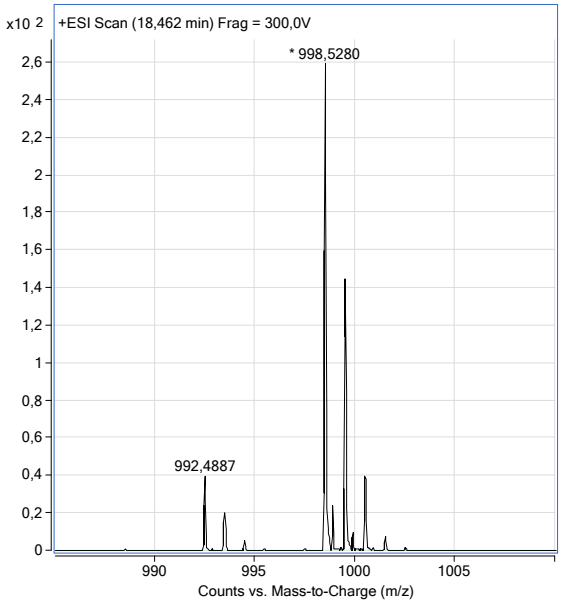

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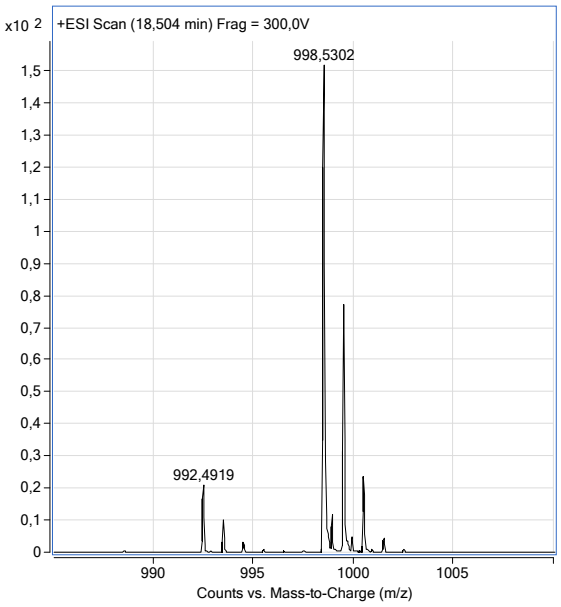

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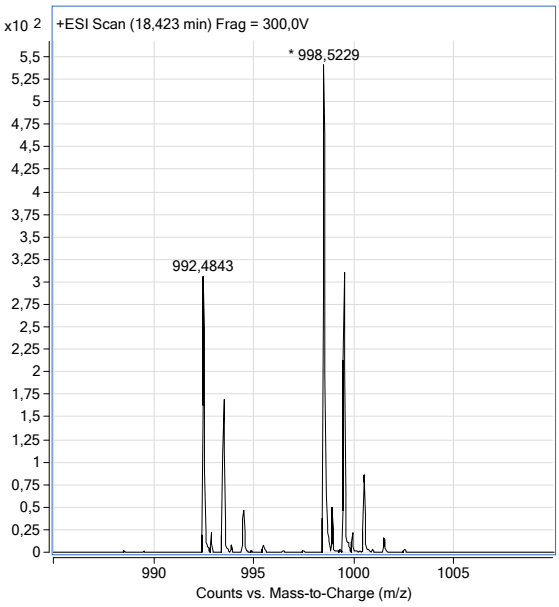

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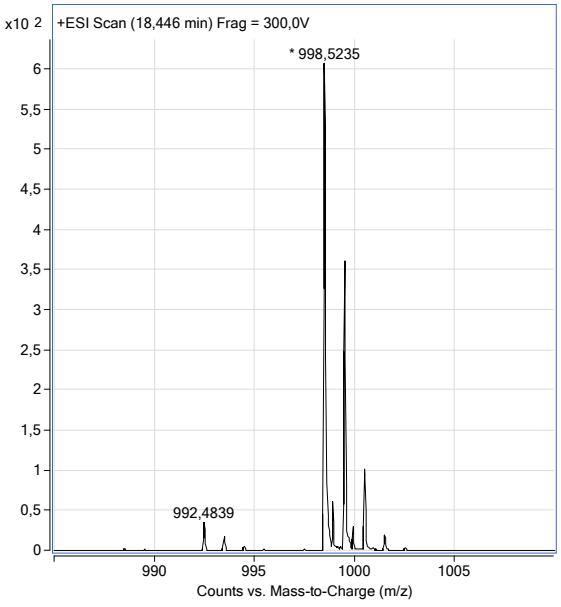

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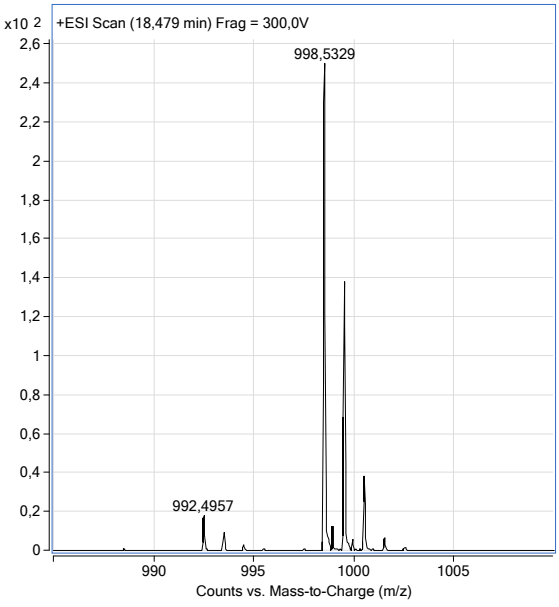

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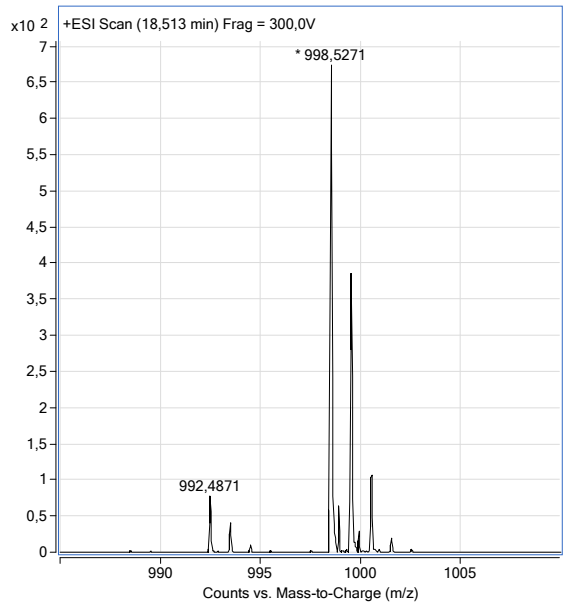
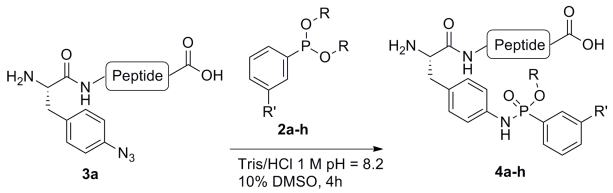


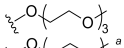
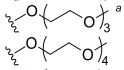
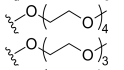
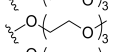
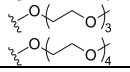
entry 15

Phosphoramidates (Table 2)



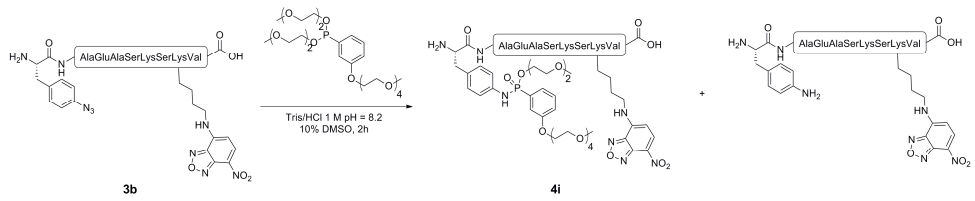
Peptide **3** = PapAlaGluTrpAlaSerLysVal

Table S1: Phosphonamidate productes from in conversion studies (Table 2)

compound	R	R'	HRMS
4a	Me	H	for $C_{53}H_{75}N_{11}O_{14}P^+$, [M+H$^+$] calcd.: 1120.5227, found: 1120.5047
4b	Me		for $C_{60}H_{89}N_{11}O_{18}P^+$, [M+H$^+$] calcd.: 1282.6119, found: 1282.6064
4c	Me	 [a]	for $C_{60}H_{89}N_{11}O_{18}P^+$, [M+H$^+$] calcd.: 1282.6119, found: 1282.6015
4d	Me		for $C_{62}H_{93}N_{11}O_{19}P^+$, [M+H$^+$] calcd.: 1326,6381, found: 1326,6359
4e	Et		for $C_{61}H_{91}N_{11}O_{18}P^+$, [M+H$^+$] calcd.: 1296.6276, found: 1296.6136
4f			for $C_{62}H_{93}N_{11}O_{19}P^+$, [M+H$^+$] calcd.: 1326.6381, found: 1326.6331
4g			for $C_{64}H_{97}N_{11}O_{20}P^+$, [M+H$^+$] calcd.: 1370.6643, found: 1370.6588
4h			for $C_{66}H_{101}N_{11}O_{21}P^+$, [M+H$^+$] calcd.: 1414.6906, found: 1414.6759

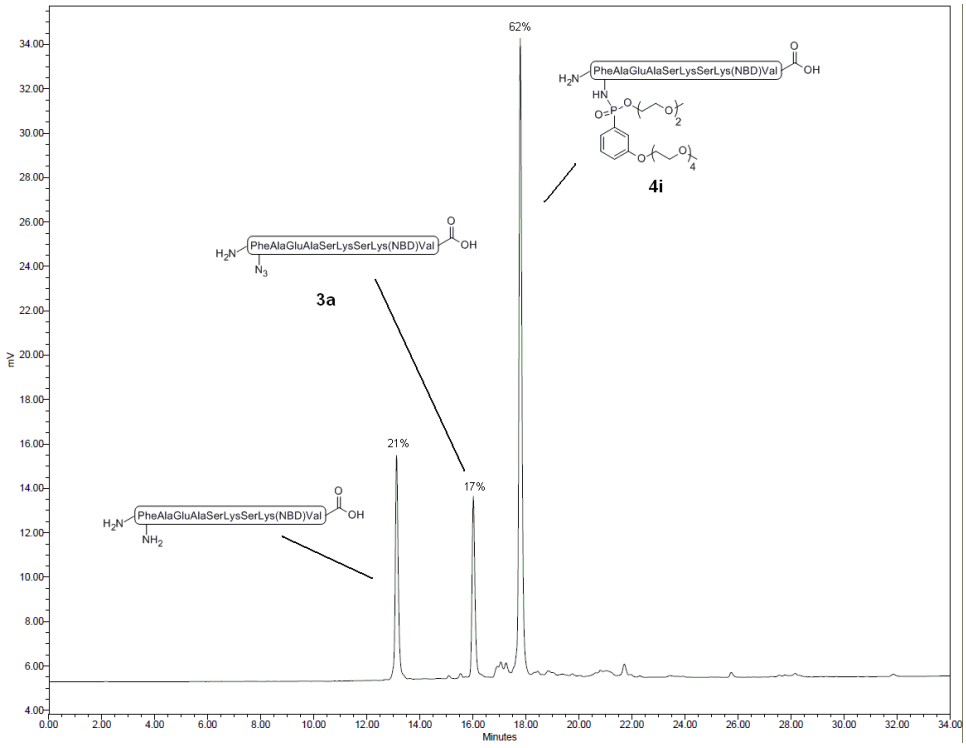
[a] substituent in para position

Staudinger-reaction with fluorescence peptide 3b



A stock solution of peptide **3b** (PapAlaGluAlaSerLysSerLys(NBD)Val; 1 mM in tris / HCl buffer 1 M pH = 8.2) and bis(2-(2-methoxyethoxy)ethyl) (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite (**2h**) (500 mM in dry DMSO) was prepared. The sample were prepared by adding peptide stock solution (20 µL) and phosphonite stock solution (20 µL) to the buffer (1 M tris/HCl pH = 8.2, 160 µL) incubated at room temperature for 1 h and then measured by fluorescence HPLC (530/470 nm).

HRMS for $C_{69}H_{107}N_{15}O_{26}P^+$, [M+H$^+$] calcd.: 1592.2744, found: 1592.7231.



Fluorescence spectrum (530/470 nm)

Protein Synthesis

The 18,9 kD protein CaM from *H. sapiens* contained an amber stop codon on the second position of the reading frame within the initiator sequence, outside the actual protein sequence. For purification, the protein contained a C-terminal His-tag. The reading frame of the gene was cloned into a prokaryotic expression vector.

The protein was synthesized *in-vitro* using the cell-free expression system RTS100 basing on RF-1 deficient *E. coli* cell lysates under dialysis for 24 h (50 µL scale). Introduction of *p*-azido-phenylalanine (Pap) was mediated by the amber suppression methodology; using the Amber stop codon on the DNA-template (UAG) and enriched fractions of orthogonal amber suppressor tRNA [4] and *p*-azido-phenylalanyl-tRNA synthetase [5]. In case of the negative control, CaM containing Ser at position 2 was obtained by *in-vitro*-translation of the same template, using an enriched fraction of Seryl suppressor-tRNA. This tRNA carries an Amber anticodon and is charged by Seryl-tRNA-synthetase.

Protein Purification

The proteins were purified from their crude translation mix via their His_6-tag by Ni-NTA affinity chromatography using Ni-NTA magnetic agarose beads (Qiagen) according to the manufacturer's instructions. After having obtained the purified fractions, the proteins were gel filtrated with Protein Desalting Spin columns (Pierce) and rebuffered in 40 mM Tris/HCl pH = 8.0. These fractions were concentrated by SpeedVac® to roughly one third of the original volume. The concentration of the proteins was determined by UV spectroscopy at 280 nm as c(Pap-CaM **8**) = 90.9 µM (~ 1.7 mg/ml) ; c (Ser-CaM **10**) = 106.1 µM (~ 2 mg/ml)

Specification of the model azido-Calmodulin (8)

f-MXKEKFER**AD QLTEEQIAEF KEAFSLFDKD GDGTITTKEL GTVMRSLGQN PTEAELQDMI NEVDADGNGT IDFPEFLTMM ARKMKDTDSE EEIREAFRVF DKDGNGYISA AELRHVMTNL GEKLTDEEVD EMIREADIDG DGQVNYEEFV QMMTAK**RGSH HHHHH; **X** = Pap

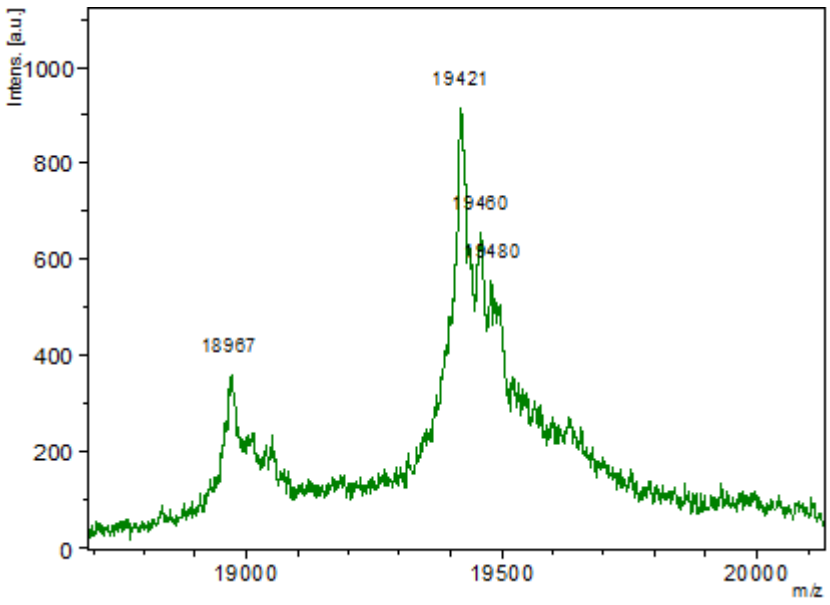
Amino acid count: A = 11; D = 17; E = 23; F = 9; G = 12; H = 7; I = 8; K = 10; L = 9; M = 10; N = 6; P = 2; Q = 6; R = 8; S = 5; T = 12; V = 7; **X** = 1; Y = 2.
Estimated pI = 4.5 (4.2 without HisTag);

Functionalization of Calmadulin (9) and MALDI-TOF analysis

To a solution of protein **8** (90.9 µM in tris / HCl buffer 1 M pH = 8.2, 9 µL) Bis(2-(2-methoxy-ethoxy)ethyl) (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite (**2h**) (500 mM in dry DMSO, 1 µL) was added and incubated at room temperature for 2 h. As control for the chemoselectivity of the Staudinger-phosphonite reaction the Pap2Ser mutant **10** of calmodulin was reacted under the exact same conditions with phosphonite **2h**.

For analysis by SDS-PAGE (separating gel: 15% acrylamide), 1 µl of the crude reaction was added to 15 µl of diluted PAGE running buffer (RotiLoad, Roth) supplemented with 35 µM EGTA. Finally, 8 µL of sample were taken per lane. The bands were visualized by Coomassie staining (see Scheme 3 in the manuscript).

For MALDI-TOF analysis the reaction mix of protein **8** with **2h** was diluted with water to 50 µl and subjected to a buffer exchange towards NH_4HCO_3 (30 mM) using Protein Desalting Spin columns (Pierce) delivering samples for MALDI-TOF mass spectrometry (spectrum see below).



MALDI-TOF

Additionally, the control protein Ser-CaM **10** was digested using trypsin and AspN protease. MS/MS sequencing revealed a modification at the *N*-terminal residue. The mass increase of exactly 28 Da corresponds to formylation at the N-terminal Met during protein translation in E. coli, which led to the following masses:

- functionalized Calmodulin **9** [M+H$^+$]: calcd: 19418, found: 19421
- amino-Calmadulin [M+H$^+$]: calcd: 18970, found: 18967
- azido-Calmodulin **8** [M+H$^+$]: calcd: 18996

Finally, the observed peak ratio of the functionalized Calmodulin **9** to the amino-Calodulin was taken as an estimated value for the conversion of the Staudinger-phosphonite reaction of **8** with **2h** of 70%, assuming that the MALDI-TOF peak for **9** was not significantly influenced by the phosphonamidate moiety as compared to the amino-Calodulin.

References

1 Gottlieb, H. E.; Kotlyar, V.; Nudelman V. A. *J. Org. Chem.* **1997**, *62*, 7512-7515.

2 determined by ^{1}H-NMR integration

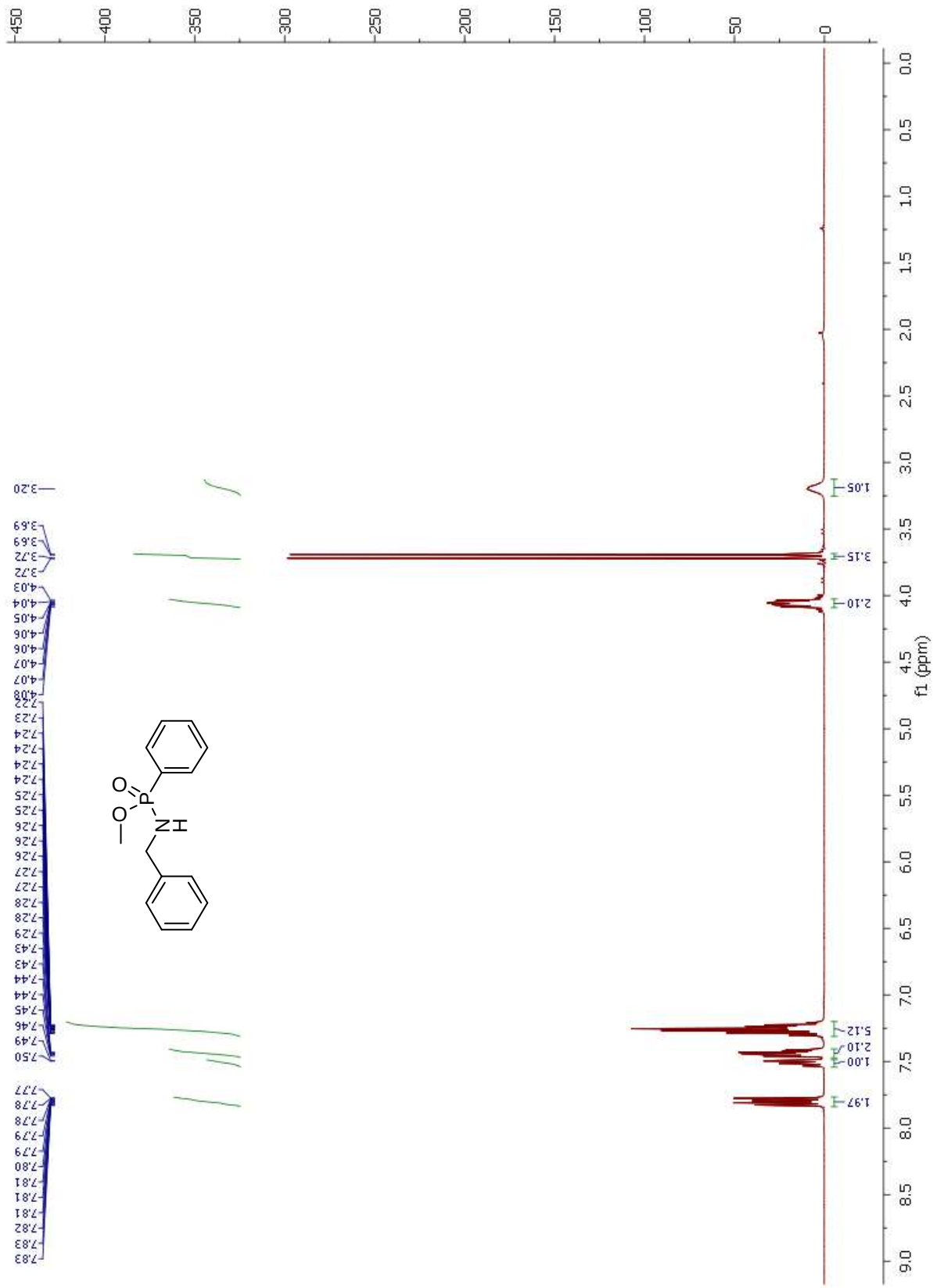
3 Kragol, G.; Lumbierres, M.; Palomo, J. M.; Waldmann, H. *Angew. Chem. Int. Ed.* **2004**, *43*, 5839–5842**.**

4 Gerrits, M.; Strey, J.; Claußnitzer, I.; von Groll, U.; Schäfer, F.; Rimmele, M.; Stiege W. Cell-free Synthesis of Defined Protein Conjugates by Site-directed Cotranslational Labeling. In *Cell-free Expression*; Kudlicki, T., Katzen, F., Bennett, R., Eds.; Landes Bioscience: Austin, 2007, pp 166–180.

5 Chin, J.; Santoro, S.; Martin, A.; King, D.; Wang, L.; Schultz, P. *J. Am. Chem. Soc.* **2002**, *124*, 9026-9027.

NMR spectra

Methyl *N*-benzyl-P-phenylphosphonamidate



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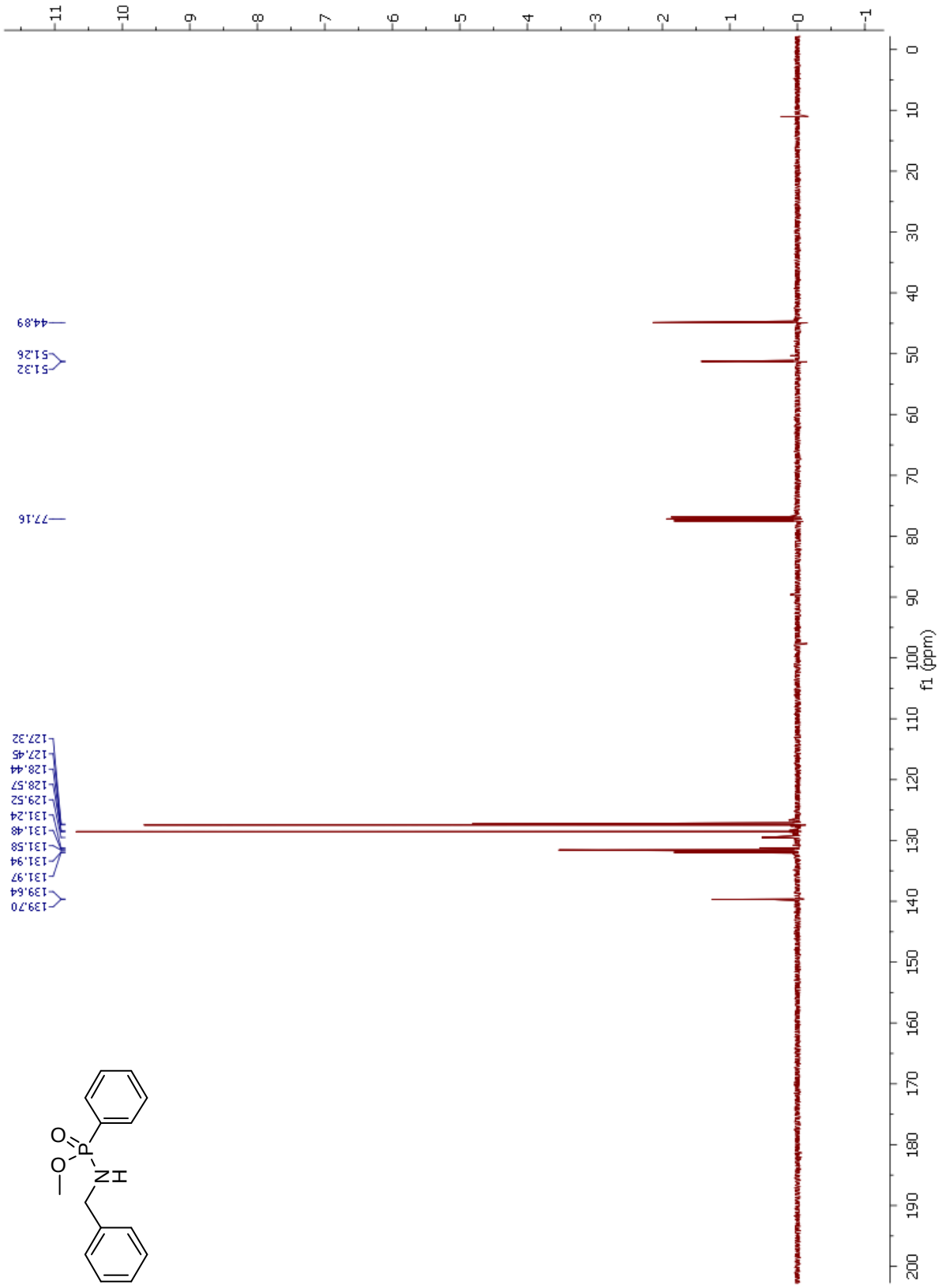

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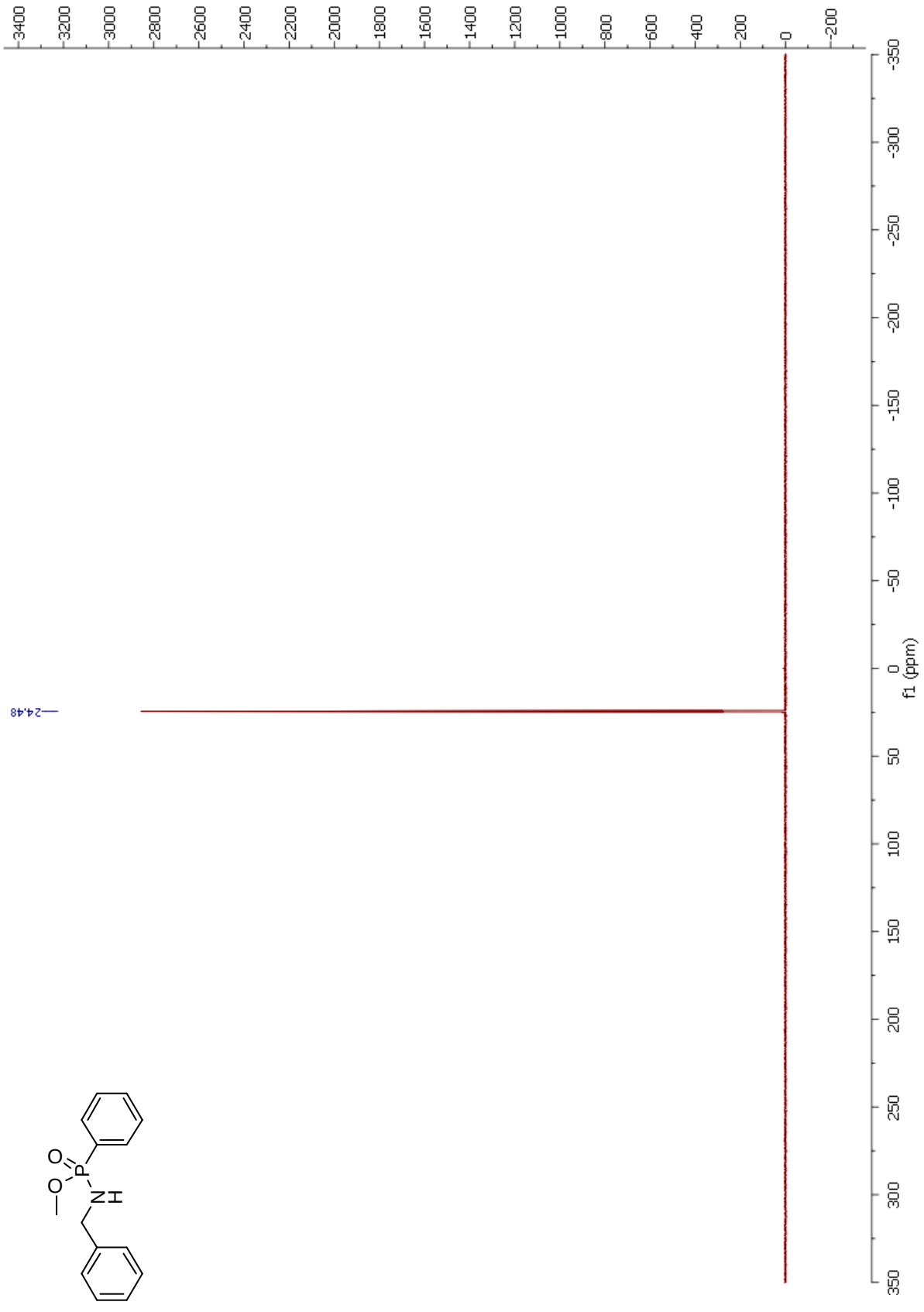
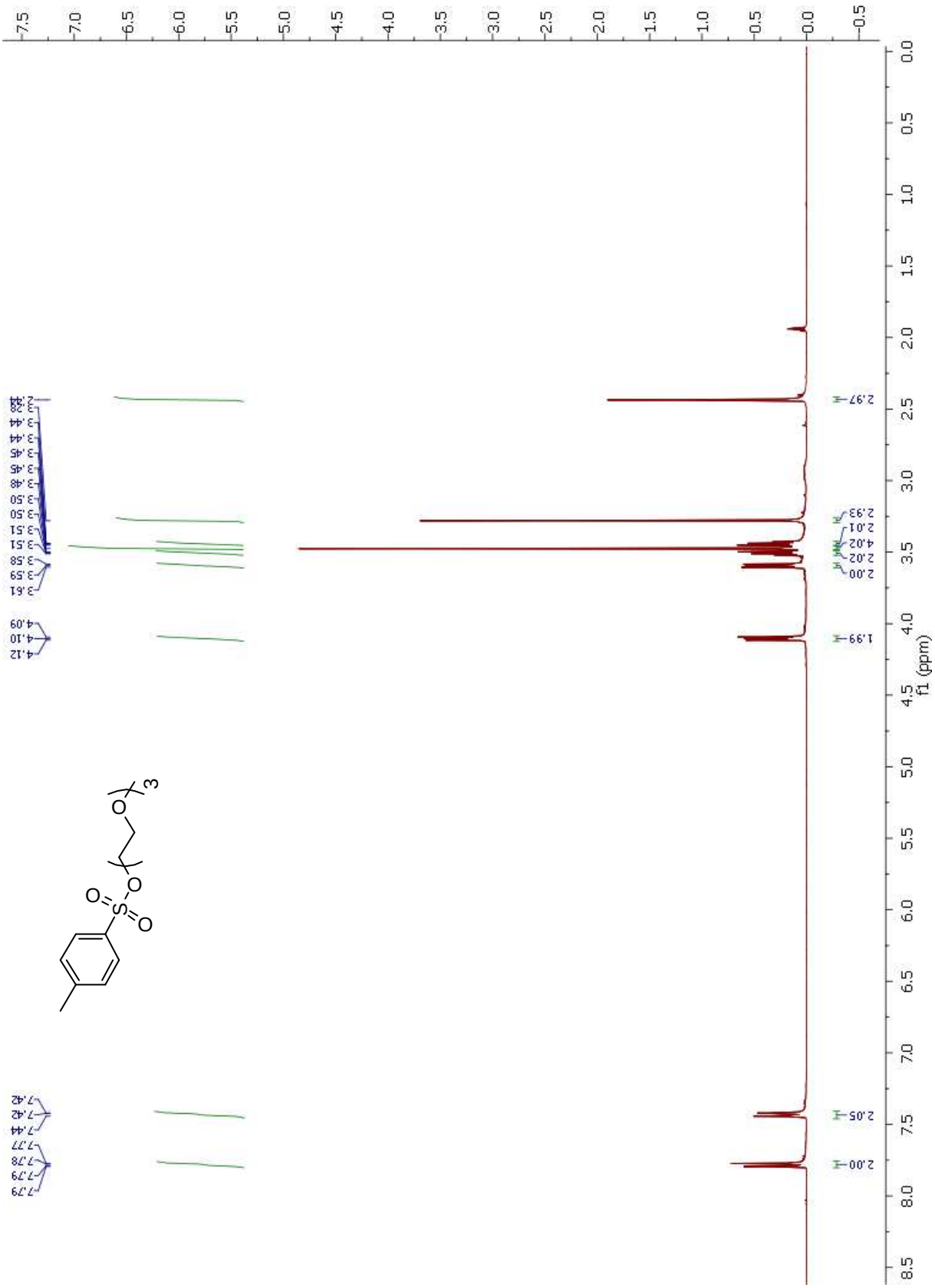


2-(2-(2-methoxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate



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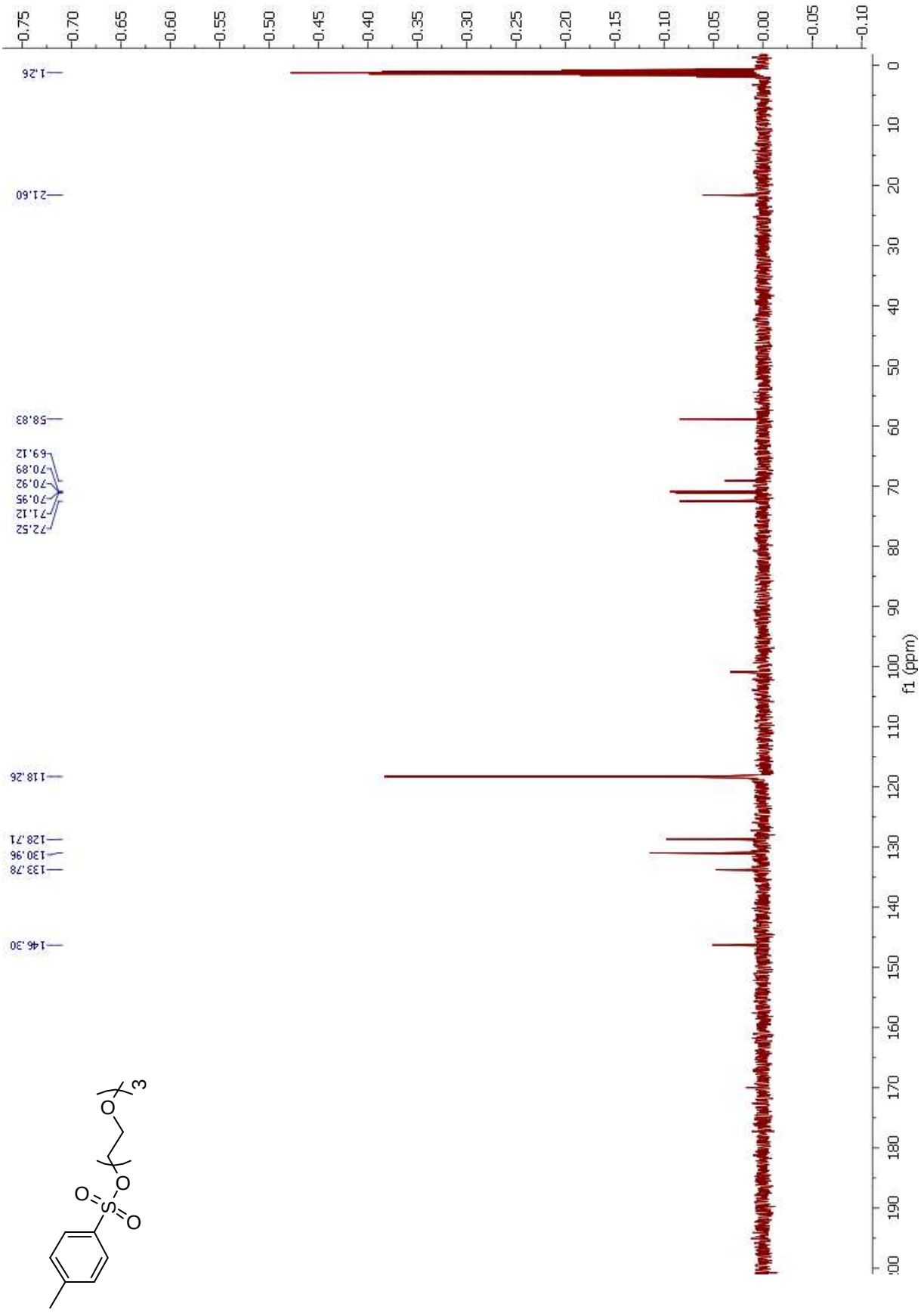
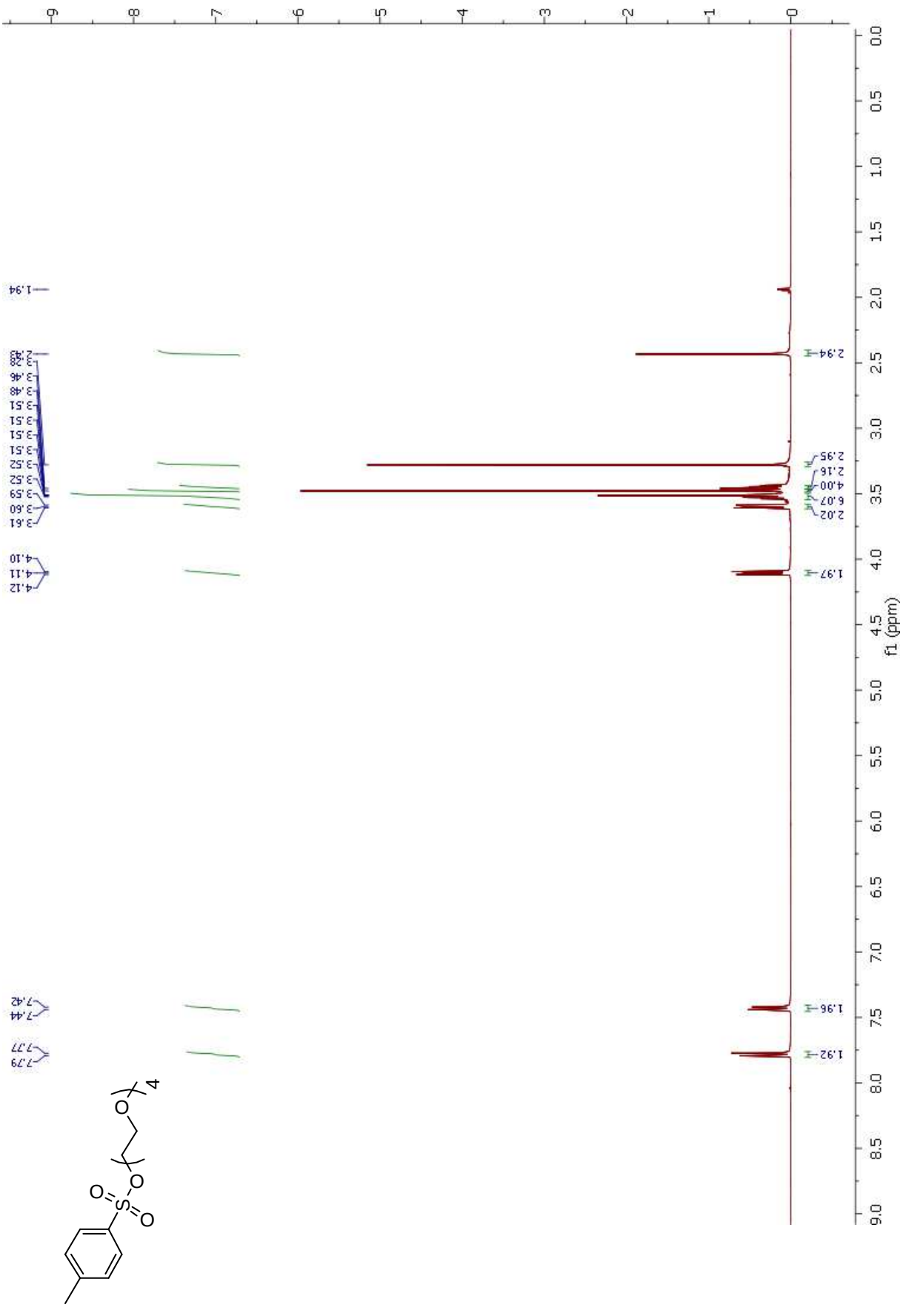
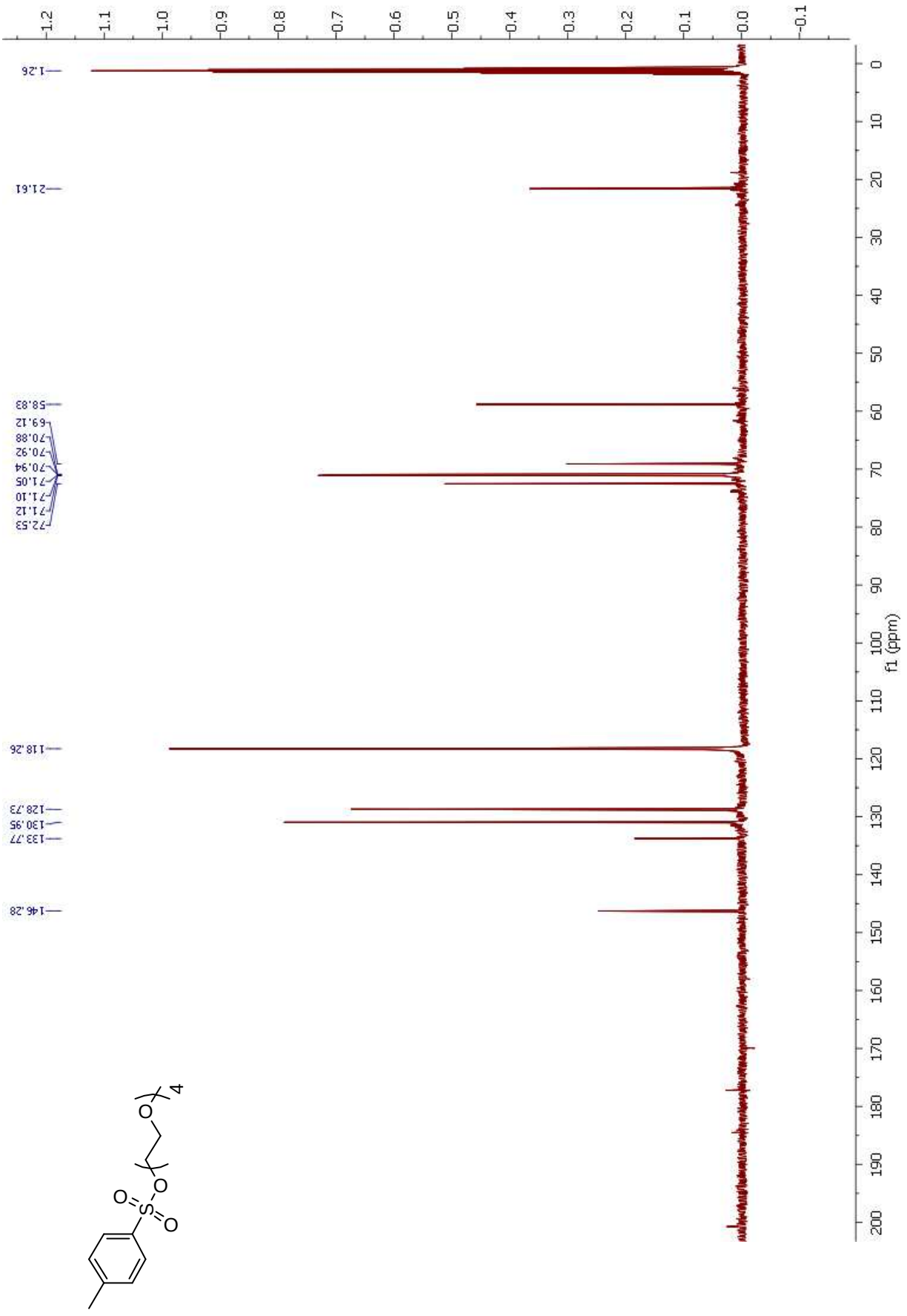


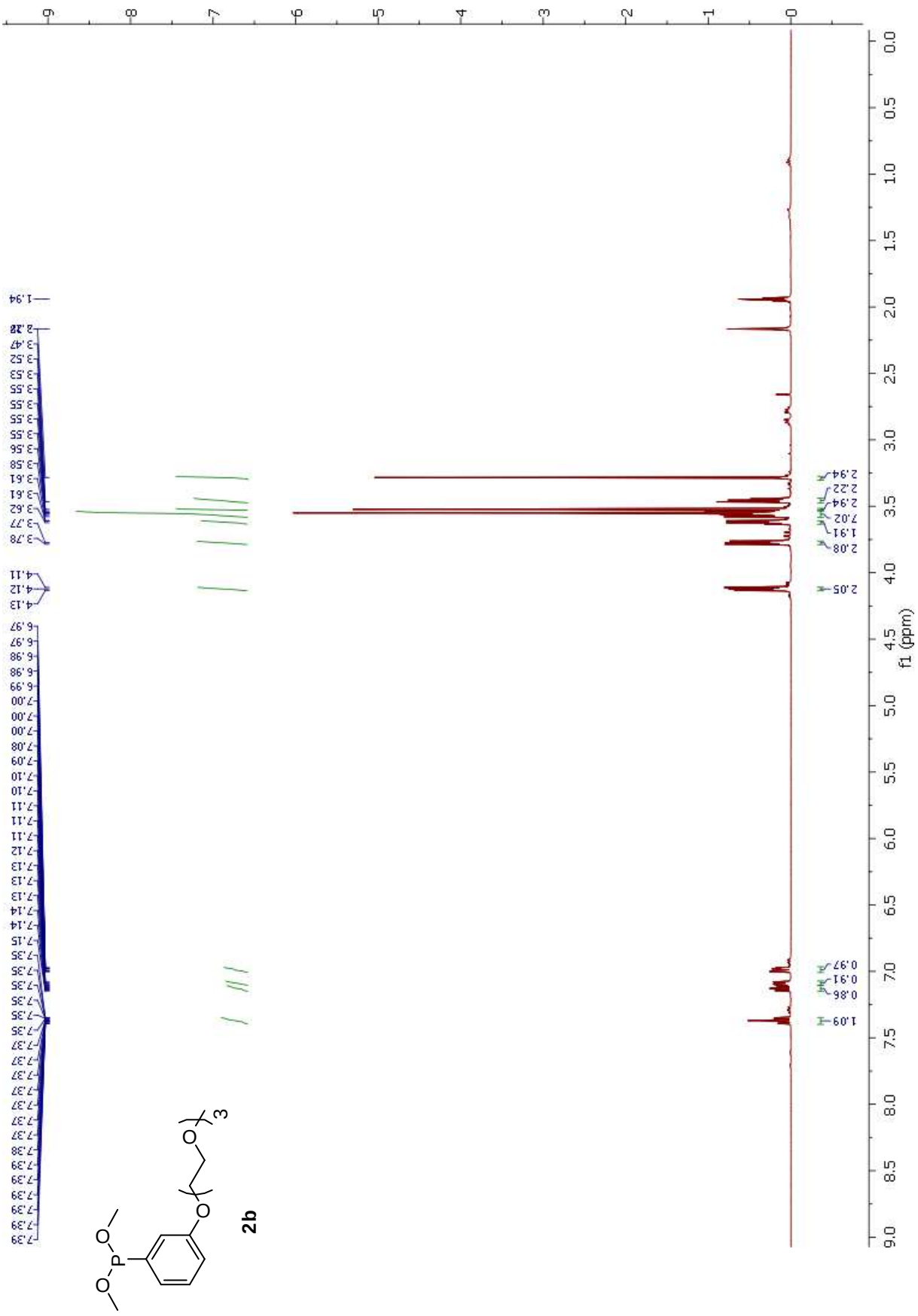
2,5,8,11-tetraoxatridecan-13-yl 4-methylbenzenesulfonate



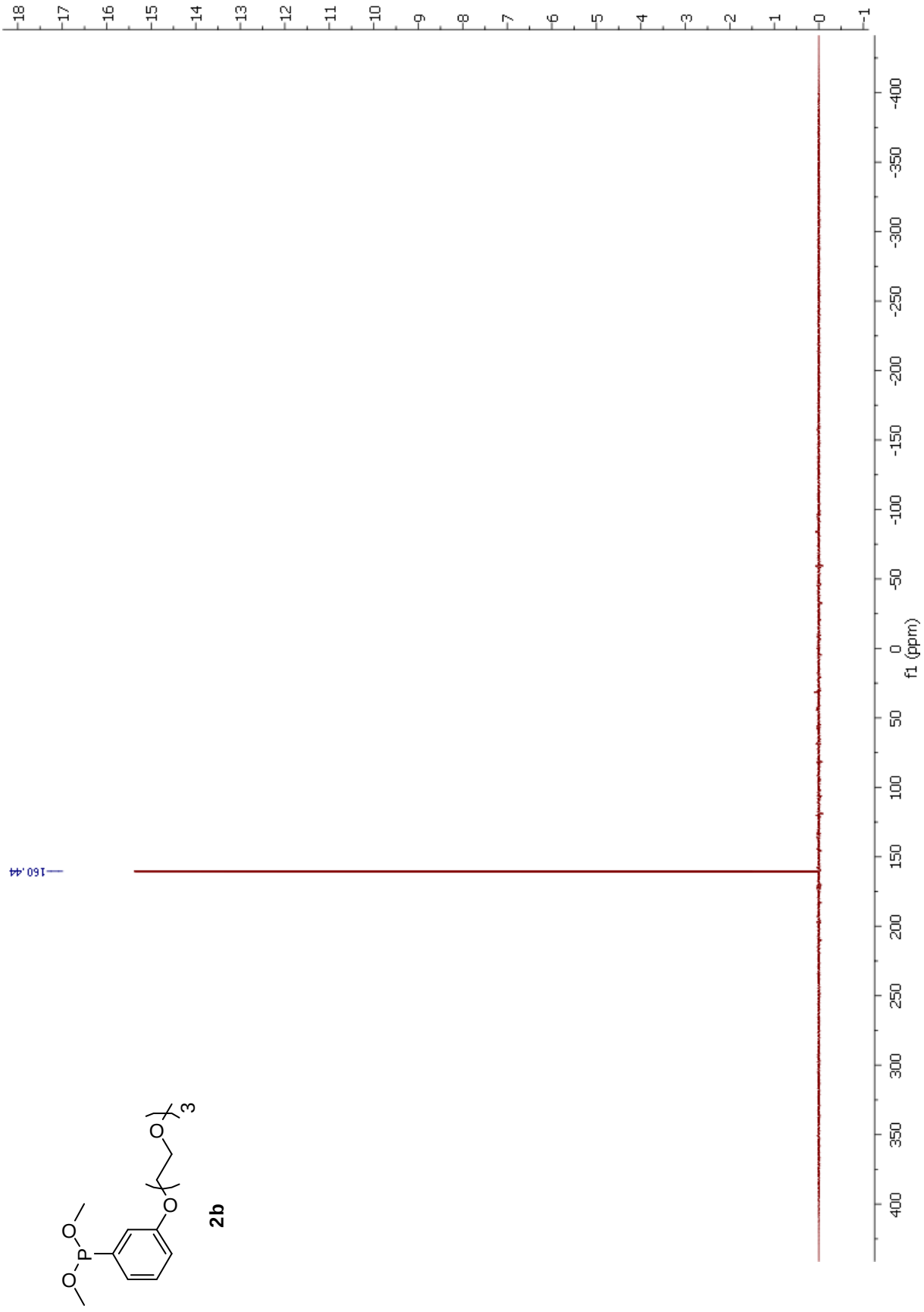
2,5,8,11-tetraoxatridecan-13-yl 4-methylbenzenesulfonate



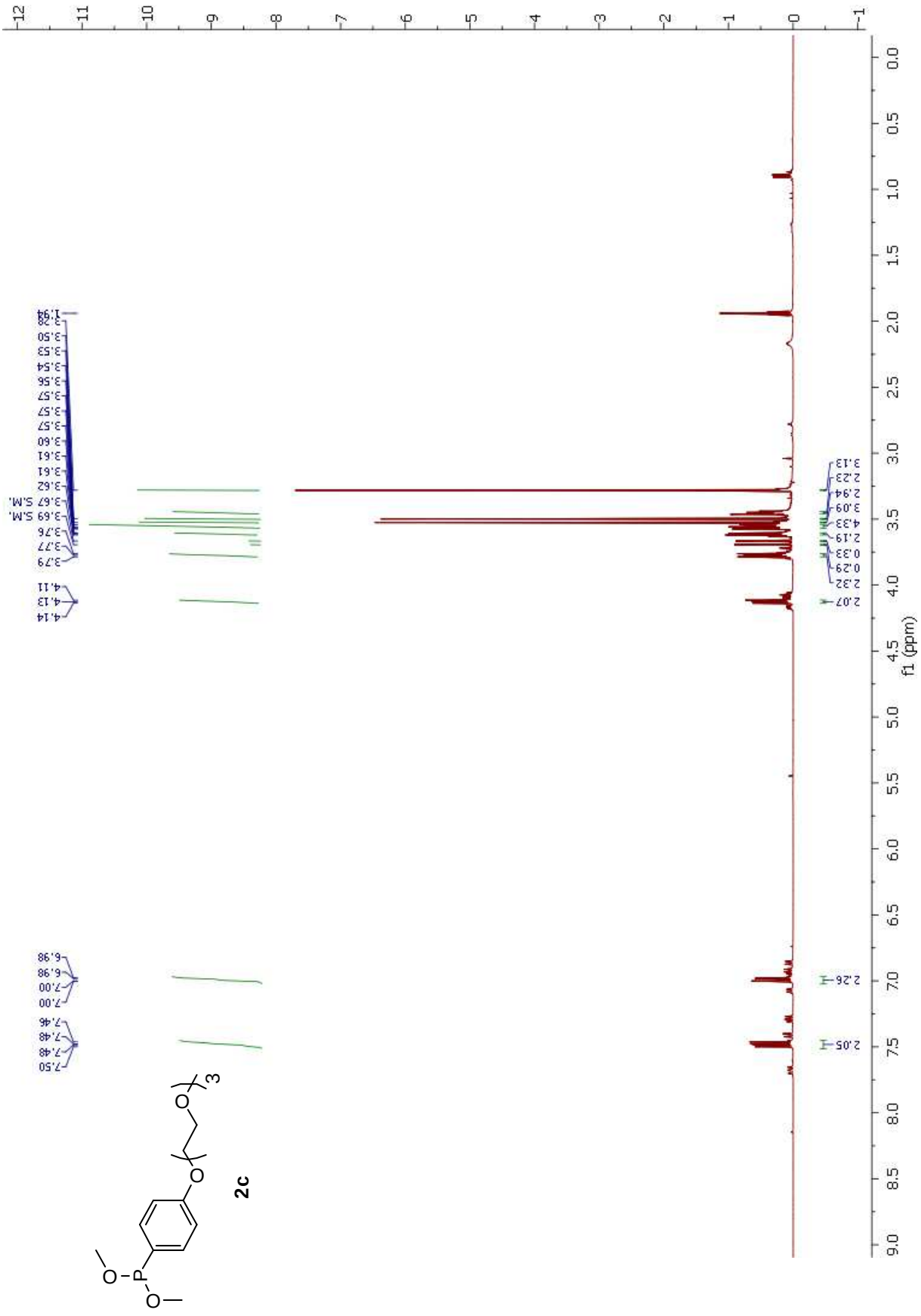
Dimethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite (2b)



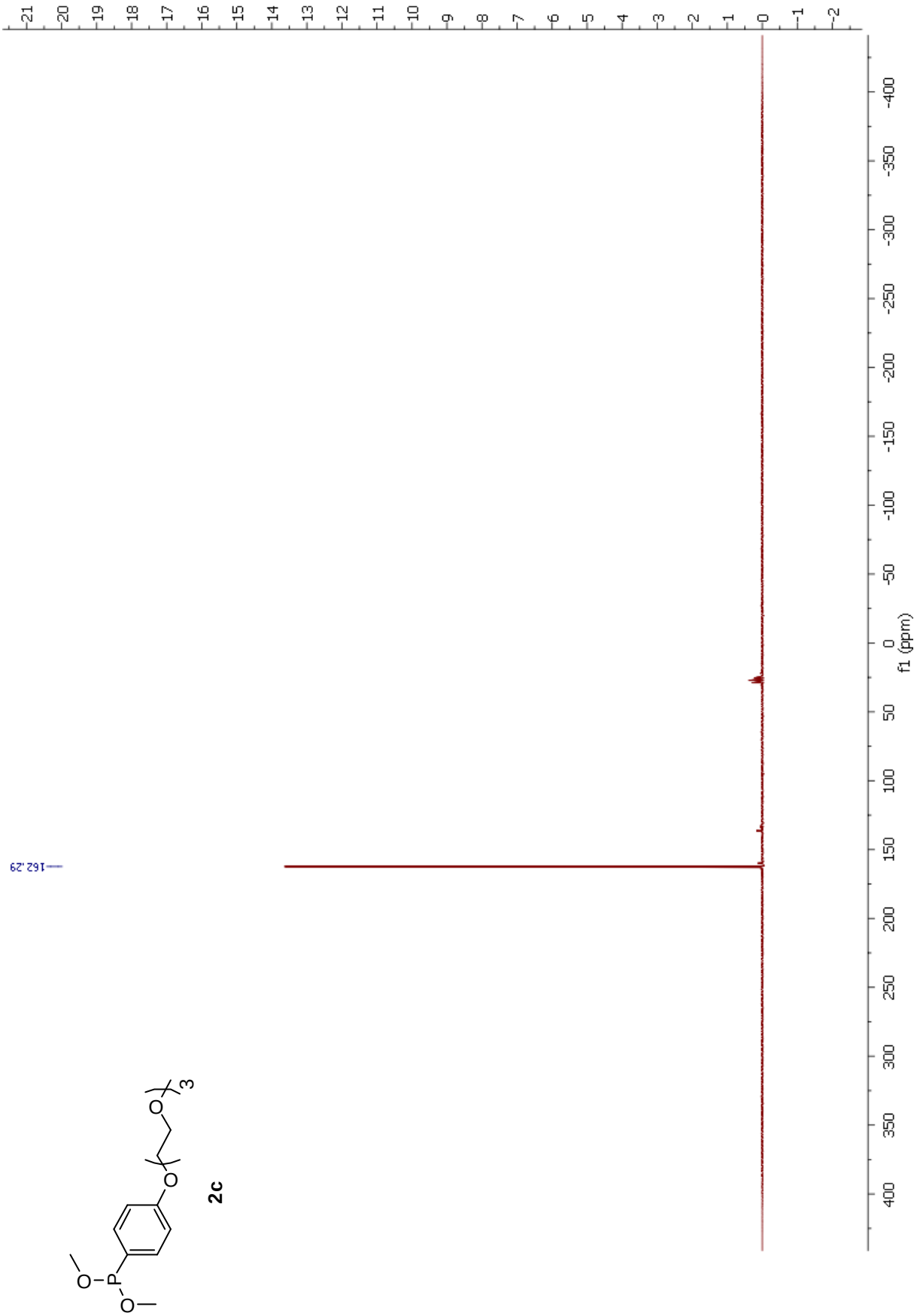
Dimethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite (2b)



Dimethyl-4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite (2c)

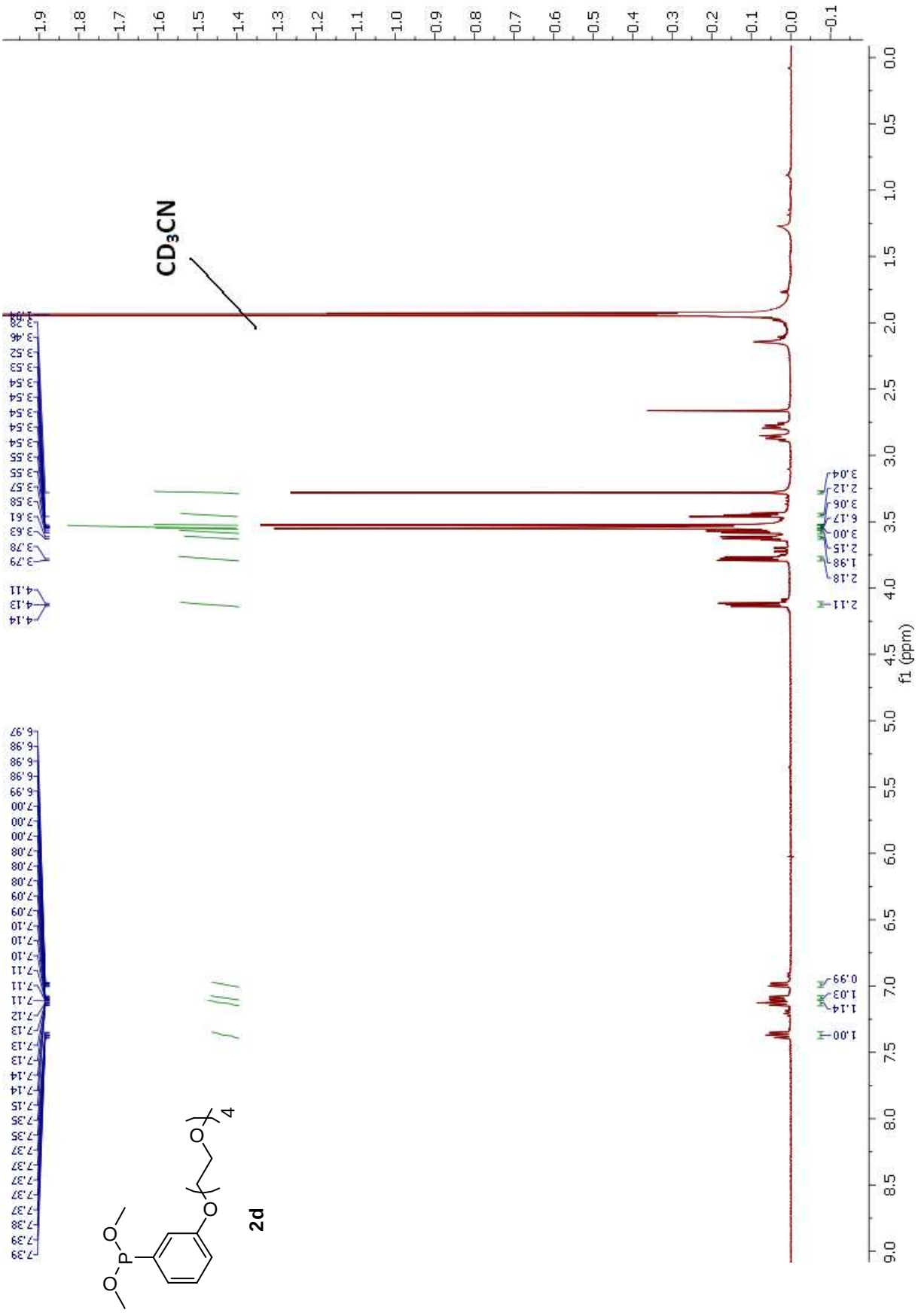


Dimethyl-4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite (2c)

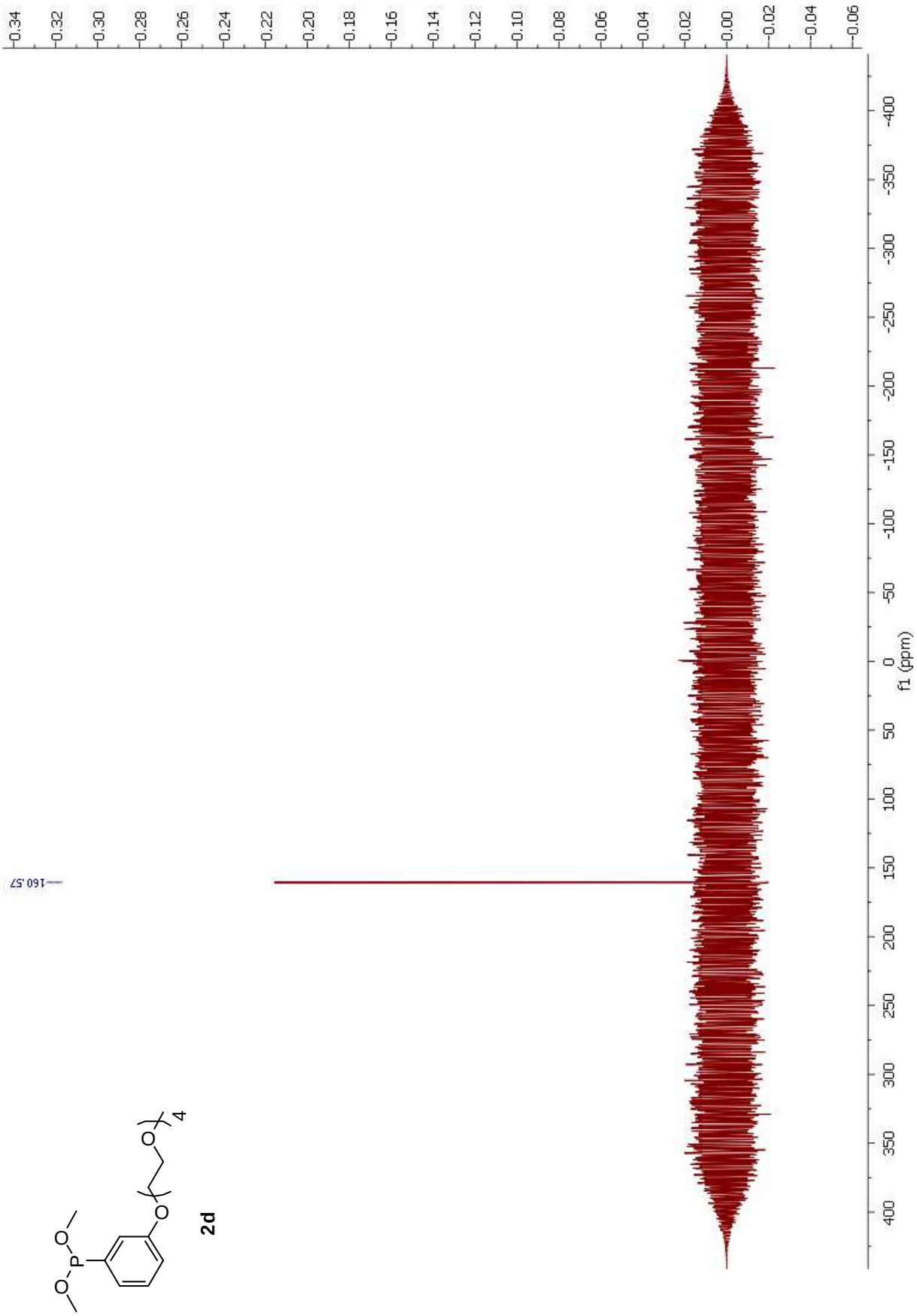


2c

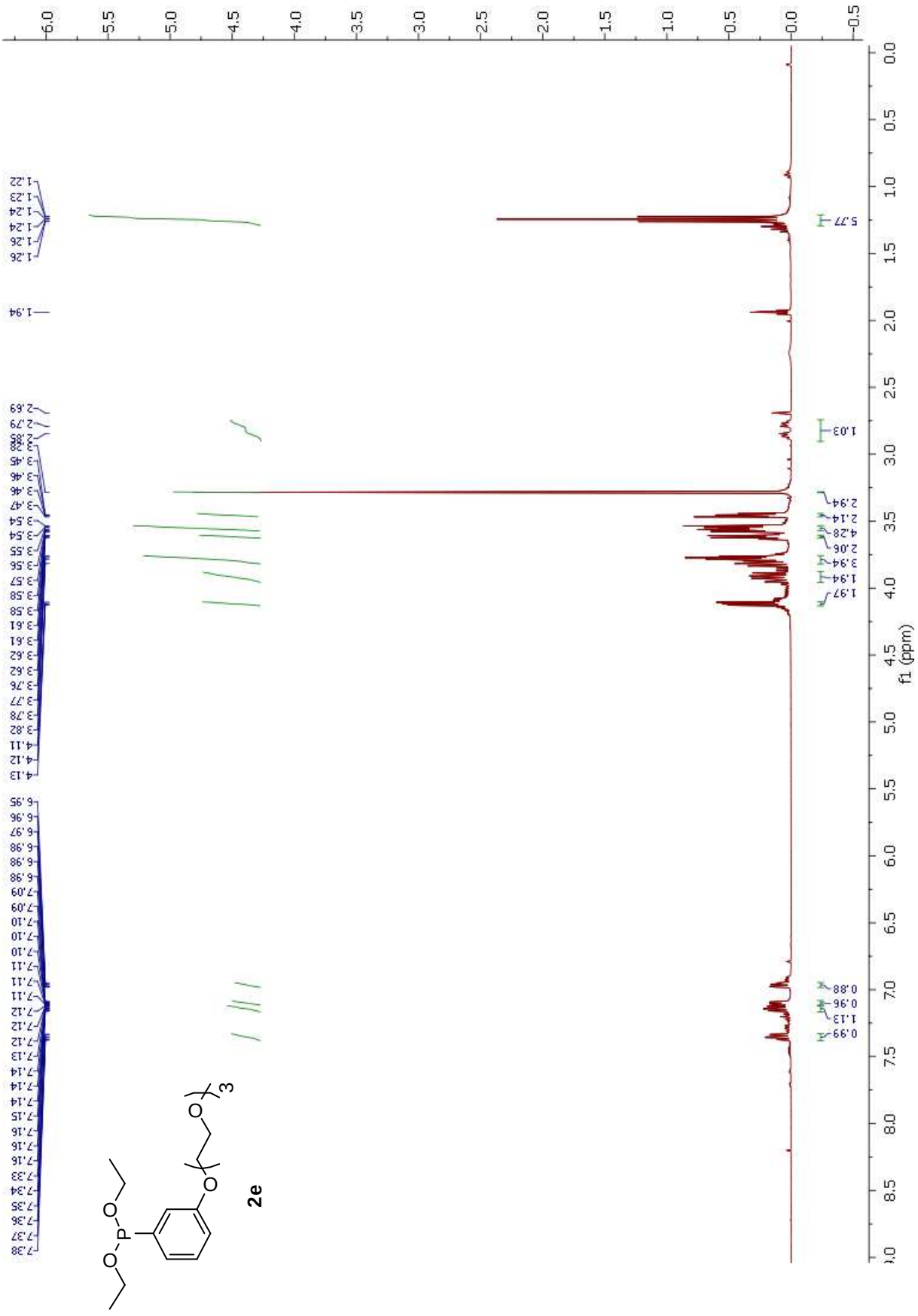
Dimethyl (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite (2d)



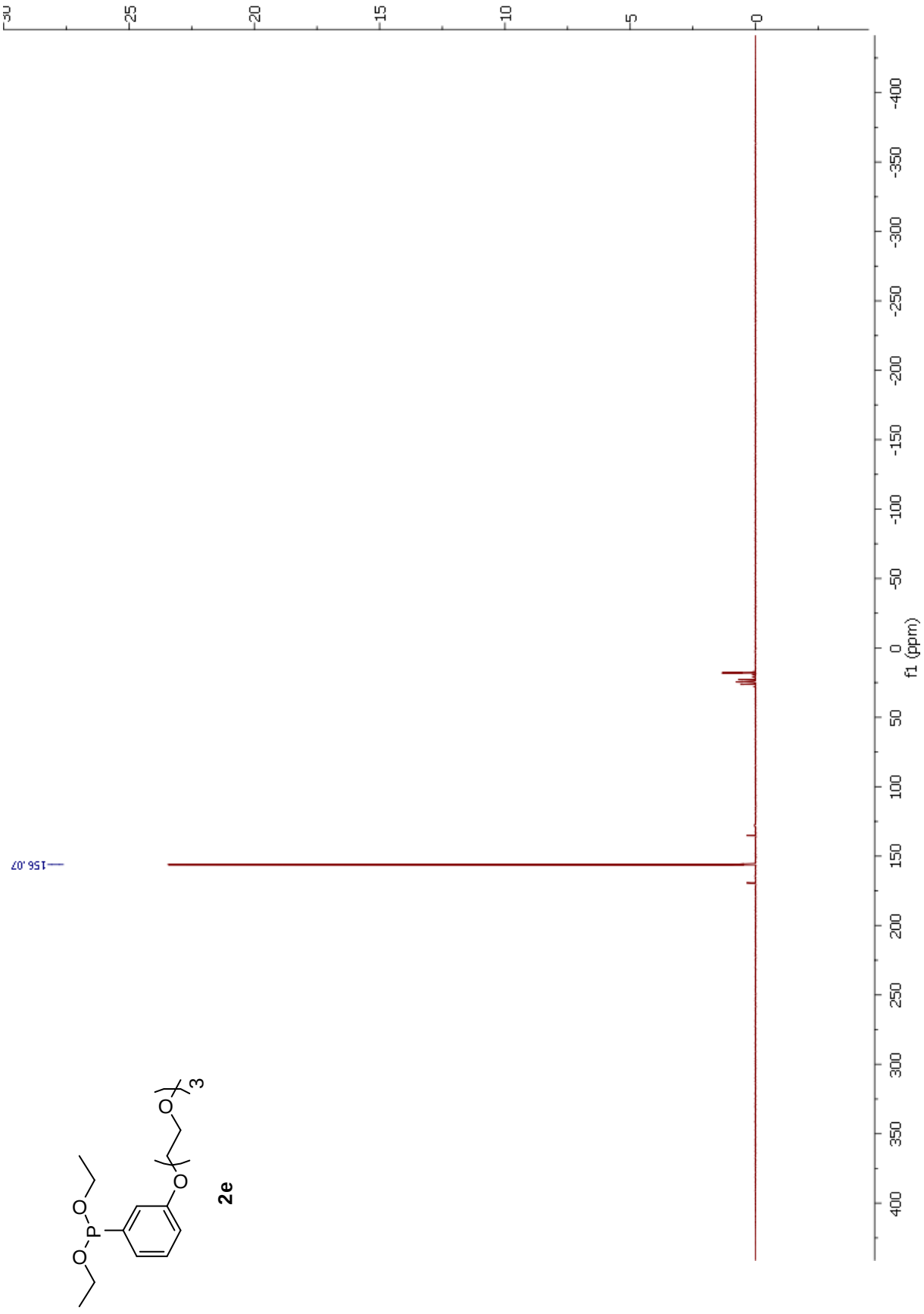
Dimethyl (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite (2d)



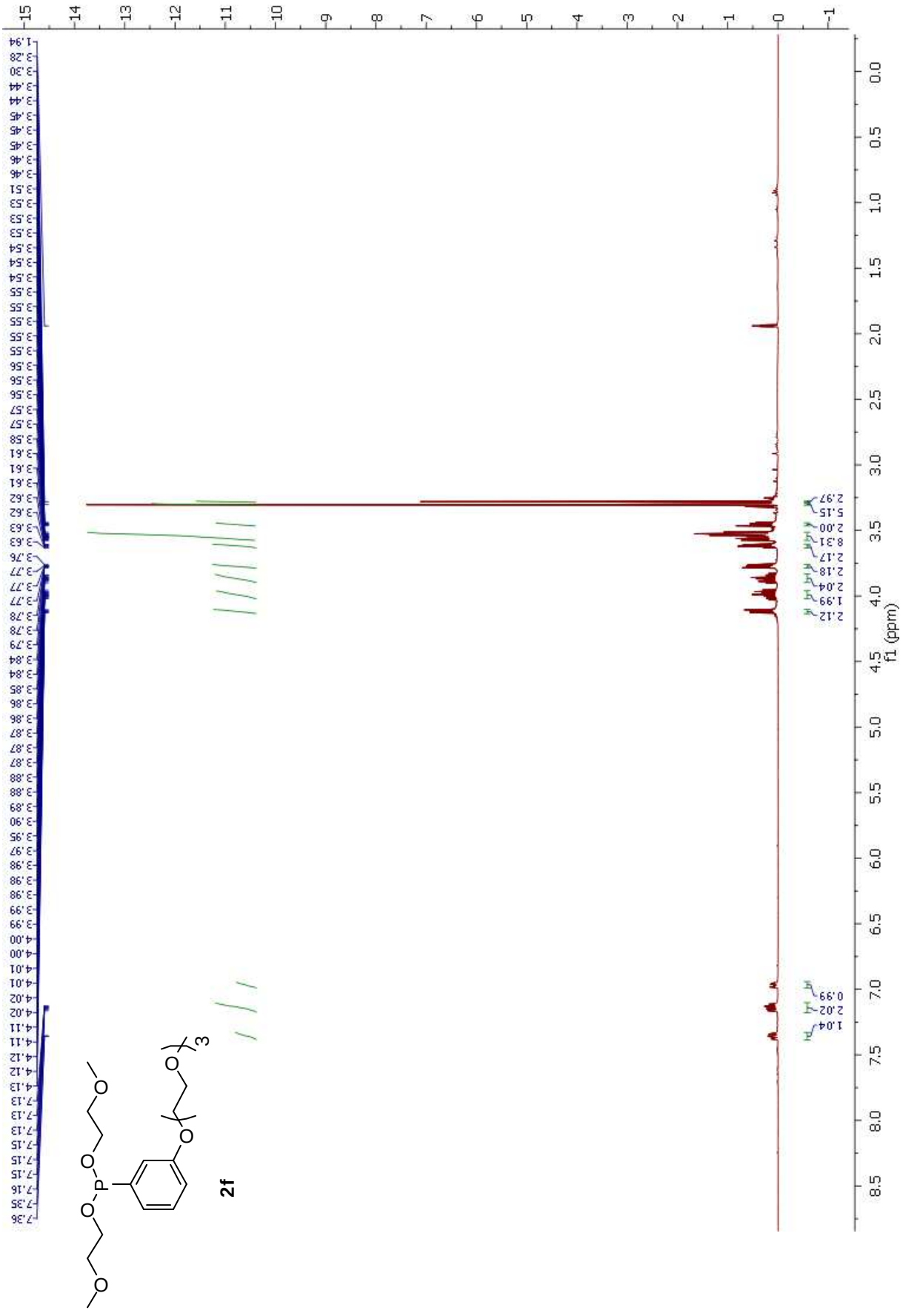
Diethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite (2e)



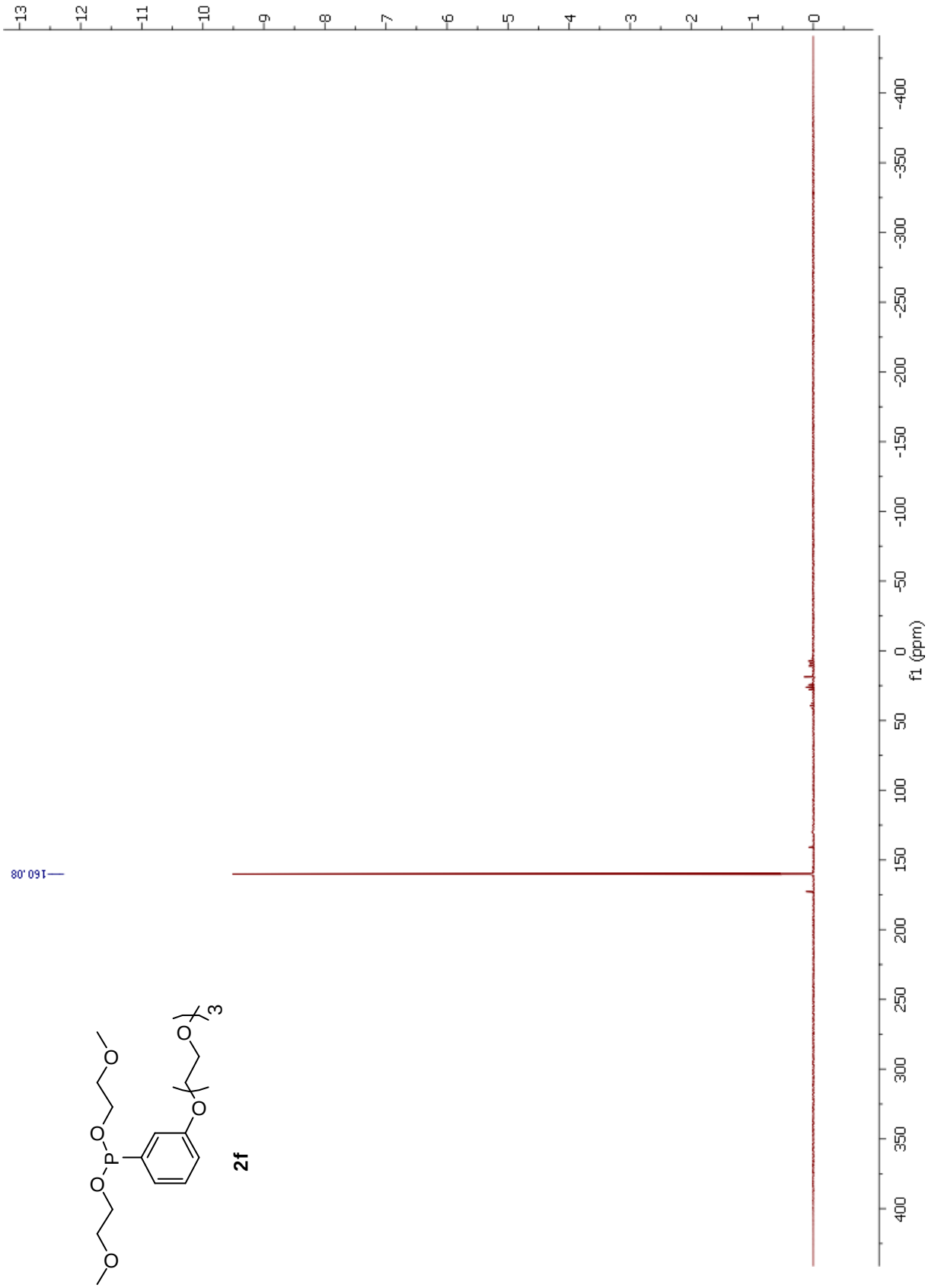
Diethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite (2e)



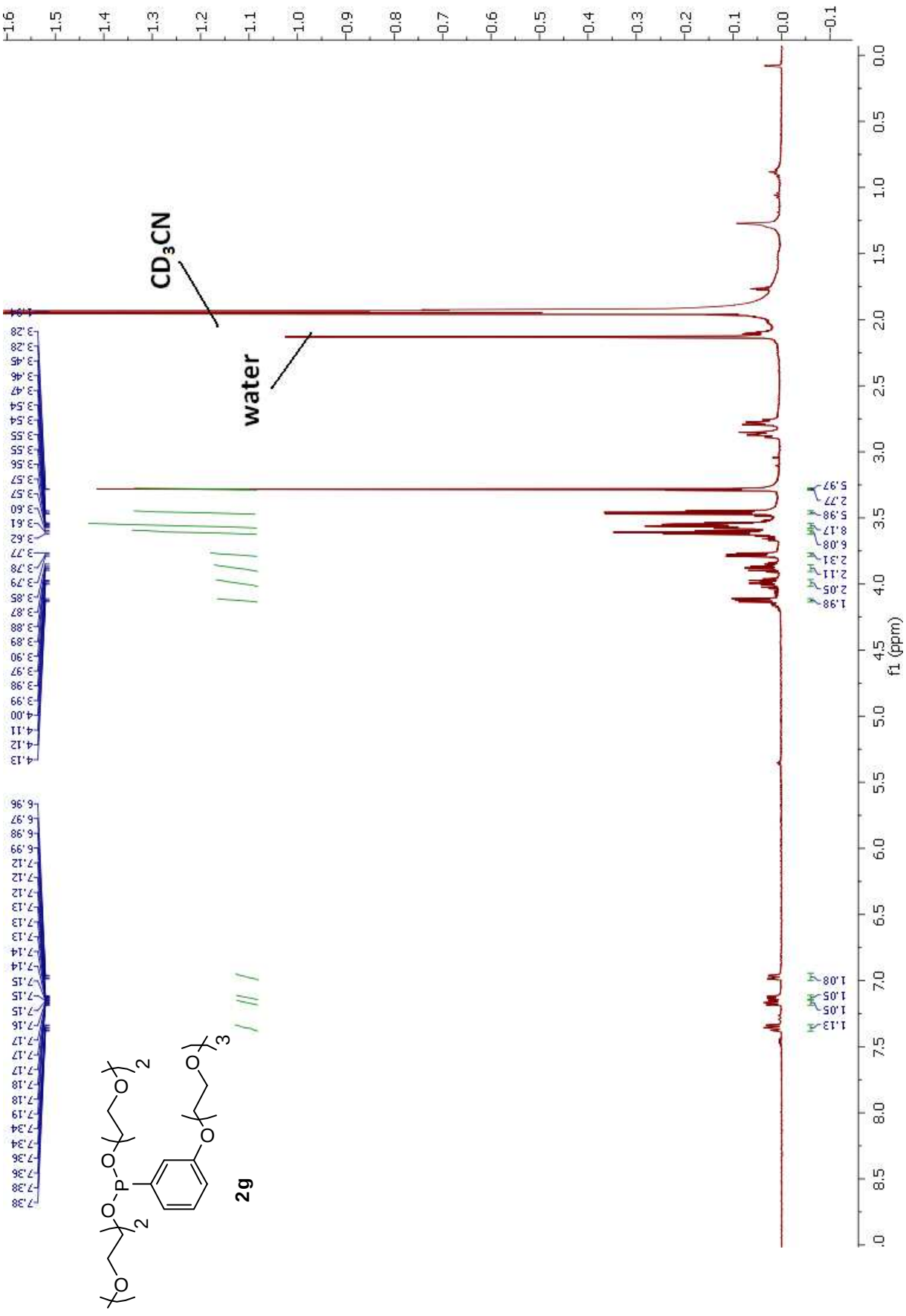
Bis(2-methoxyethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite (2f)



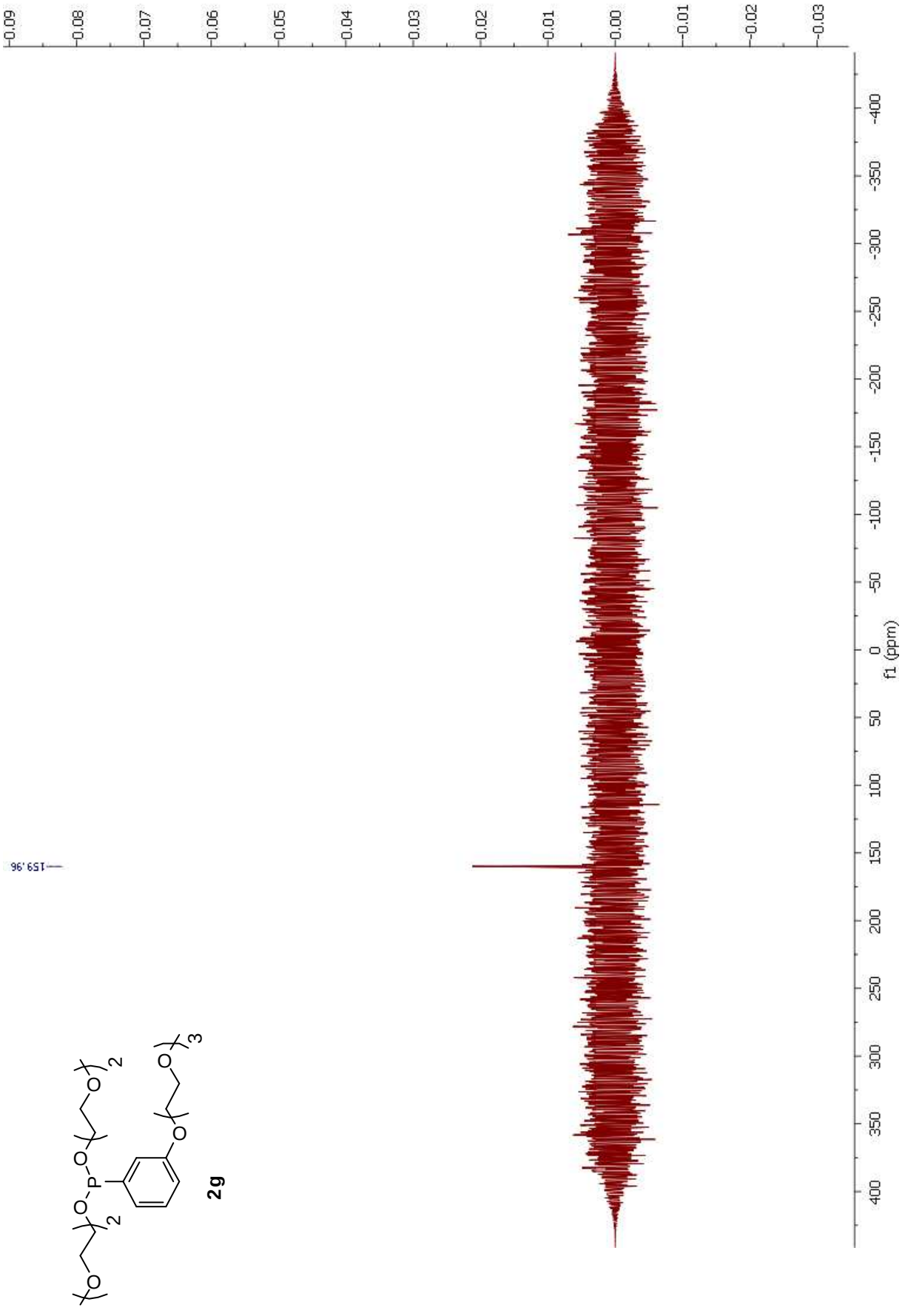
Bis(2-methoxyethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite (2f)



Bis(2-(2-methoxyethoxy)ethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite (2g)

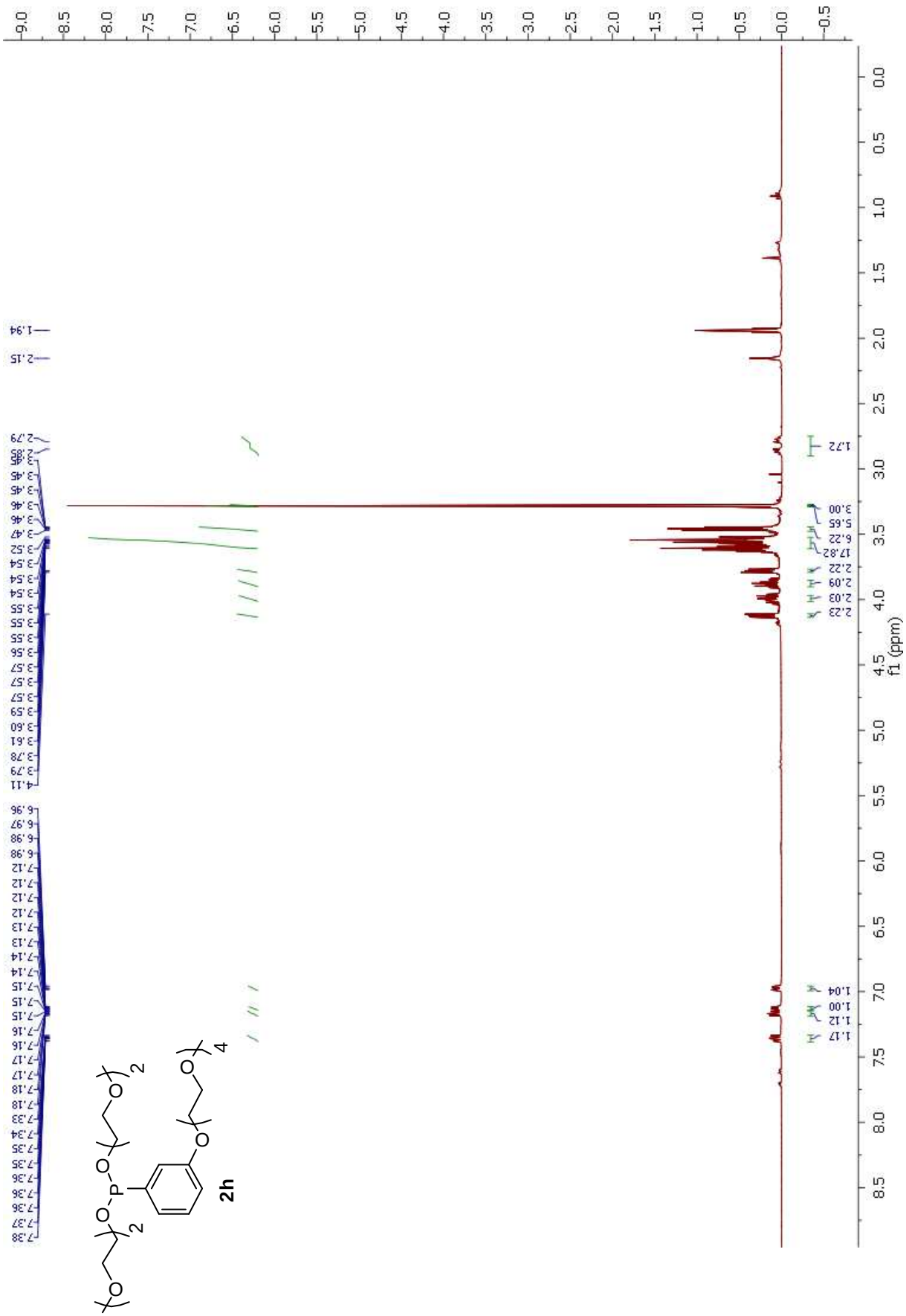


Bis(2-(2-methoxyethoxy)ethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite (2g)

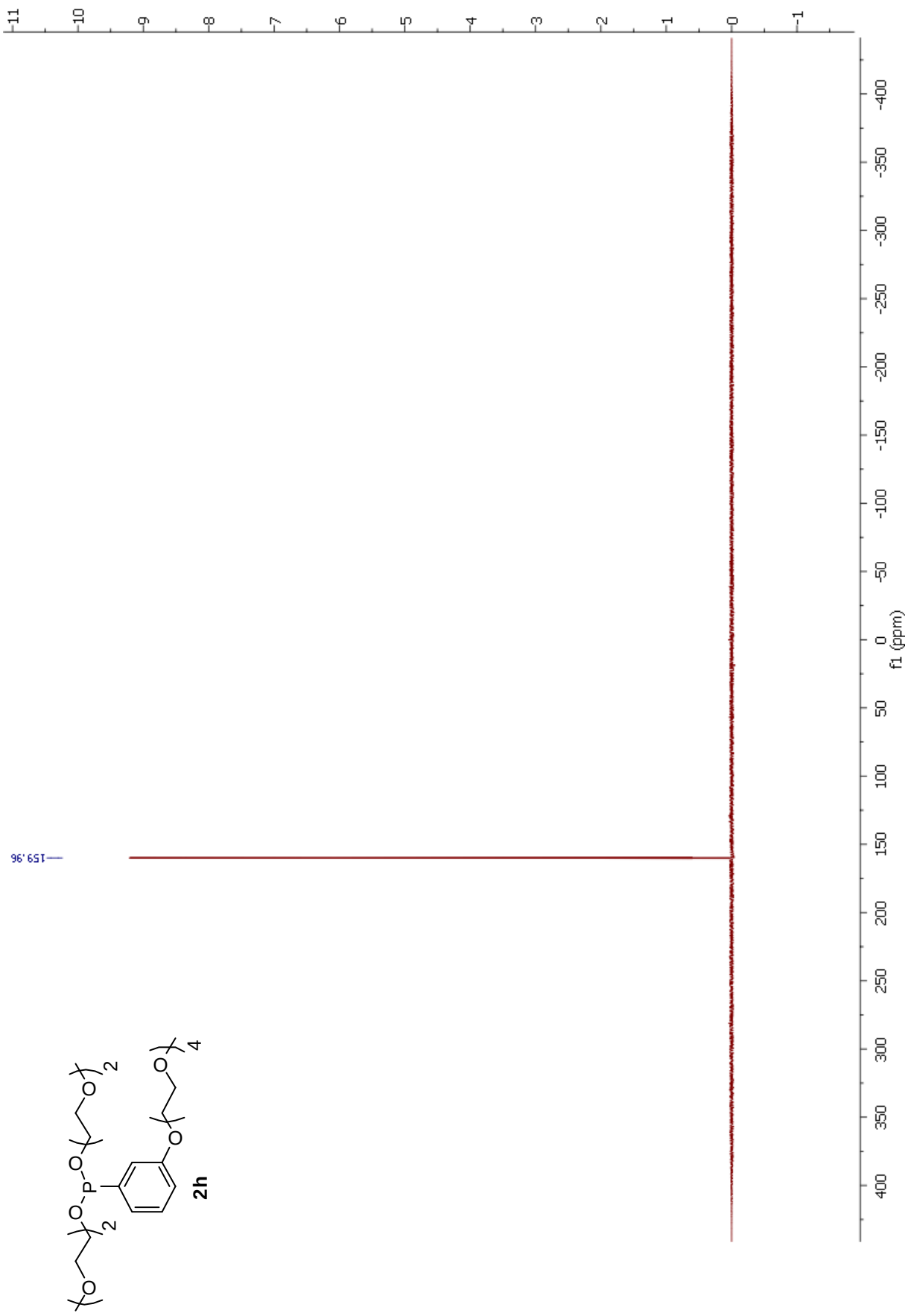


2g

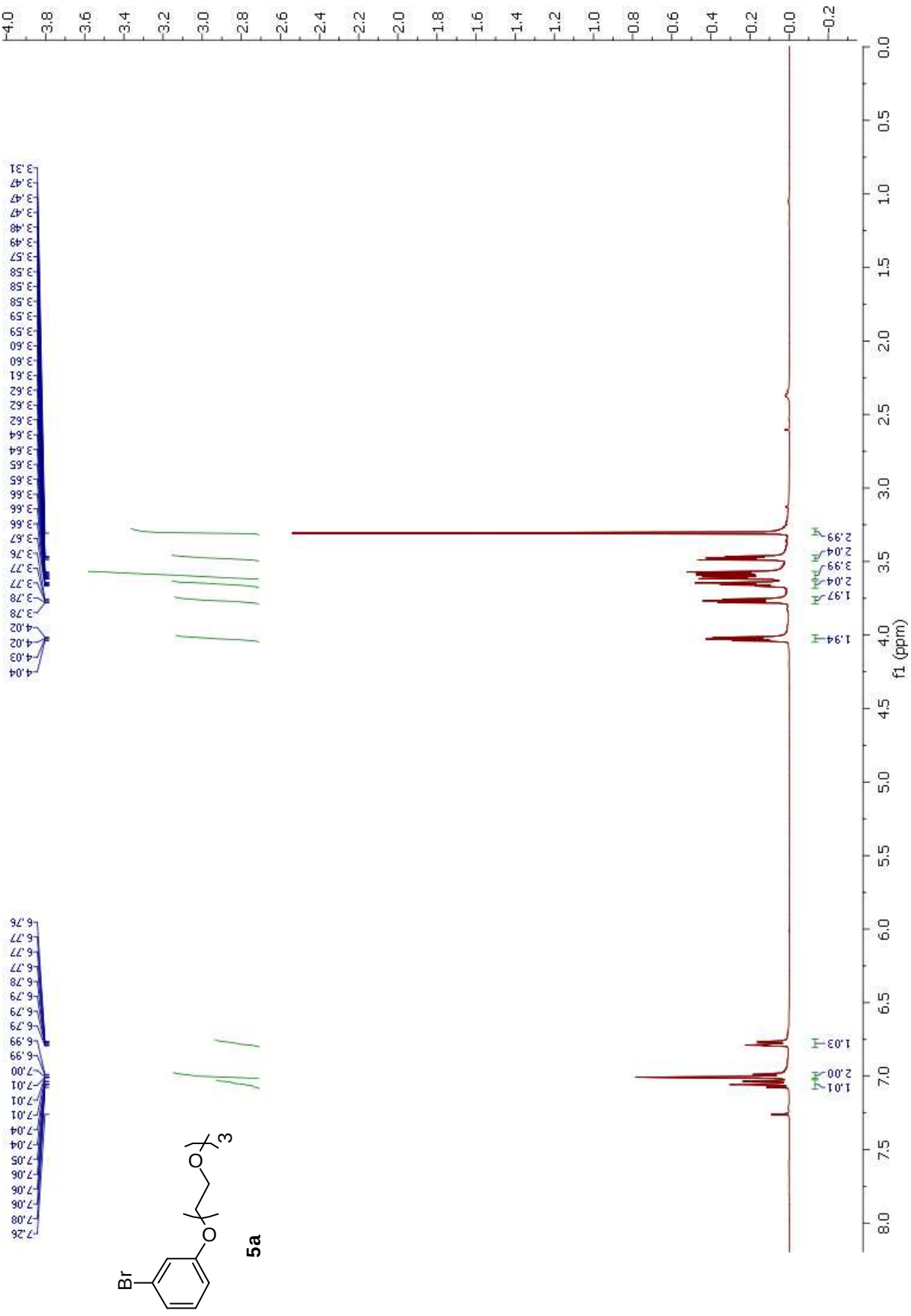
Bis(2-(2-methoxyethoxy)ethyl) (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite (2h)



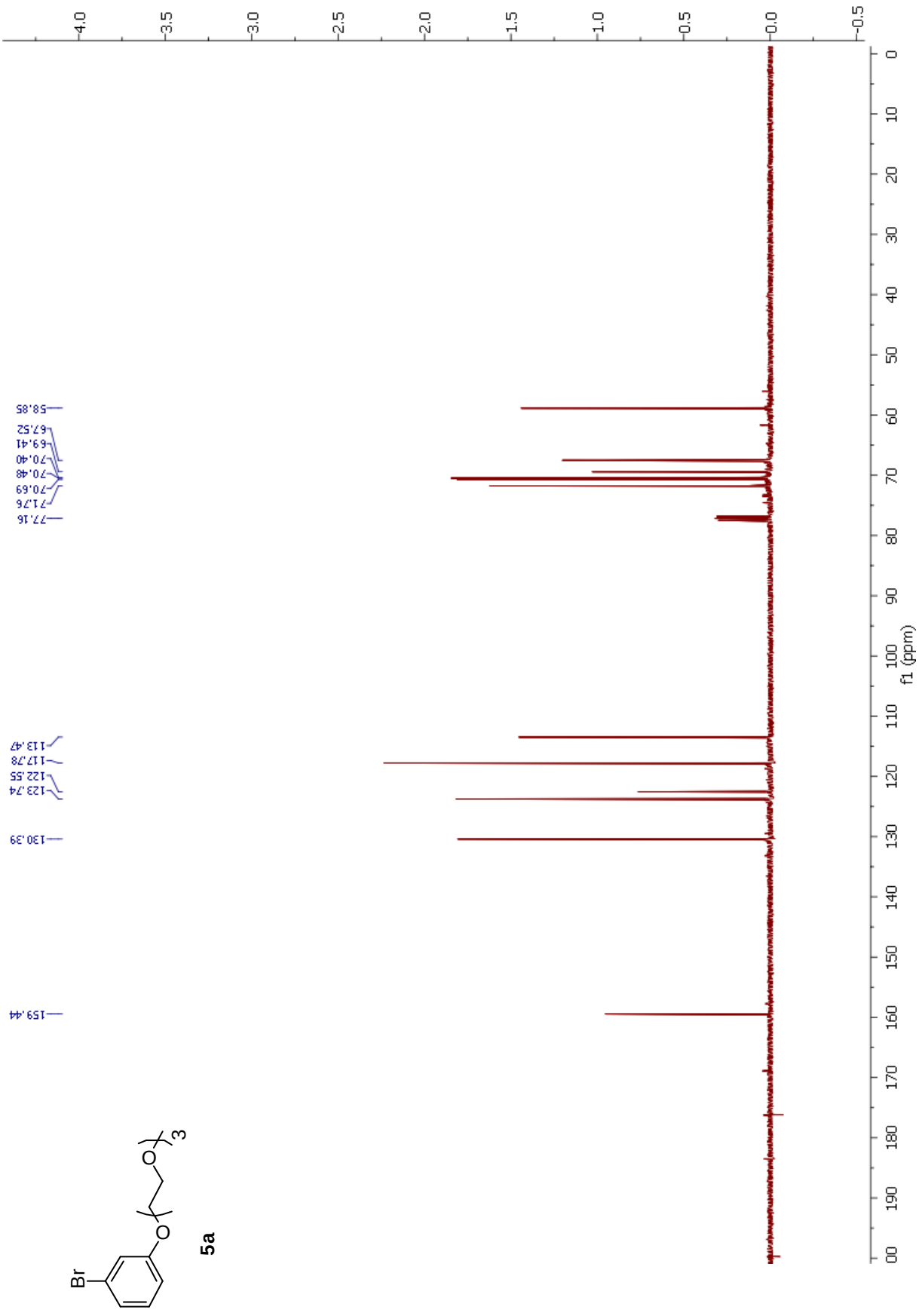
Bis(2-(2-methoxyethoxy)ethyl) (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite (2h)



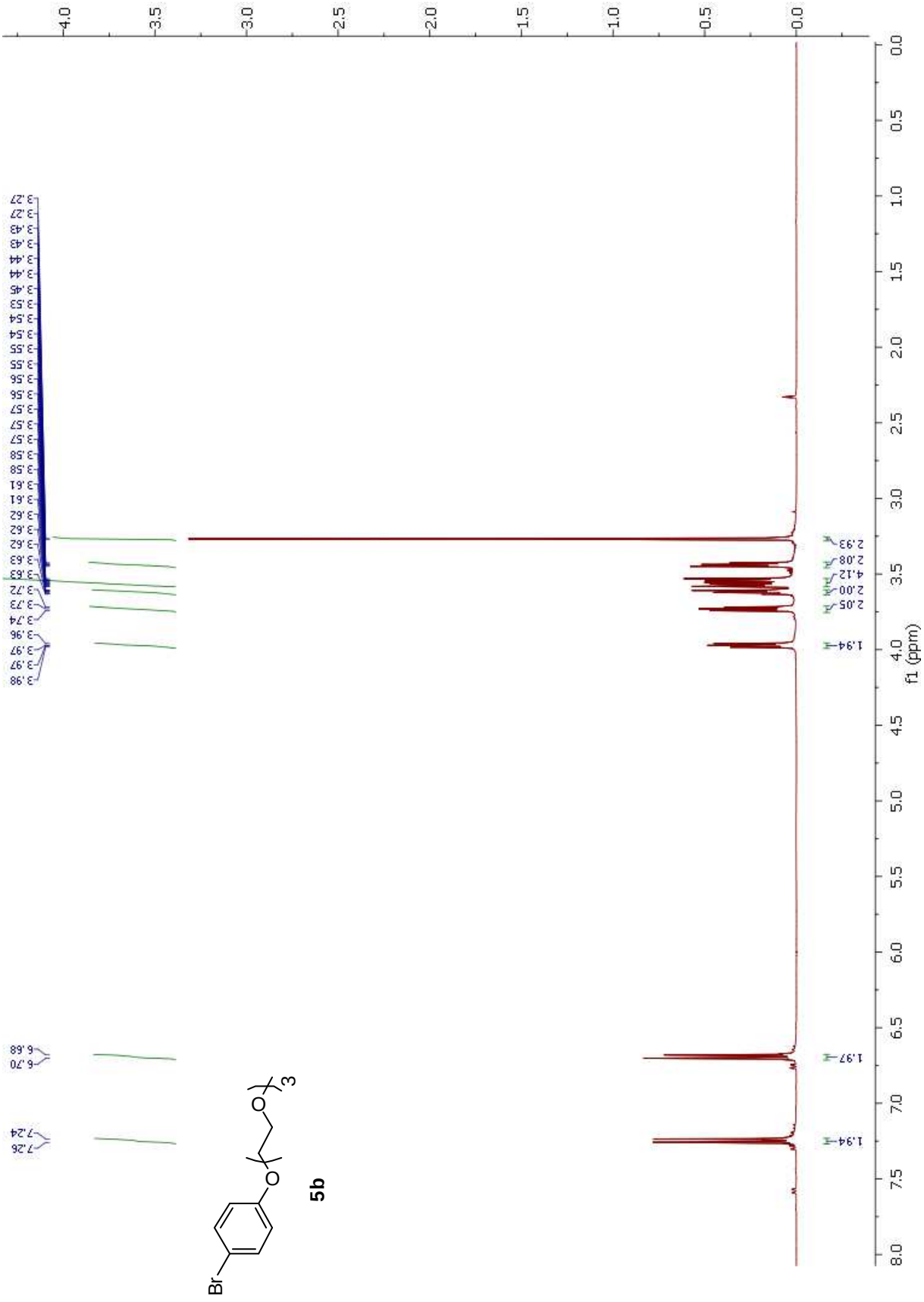
1-bromo-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene (5a)



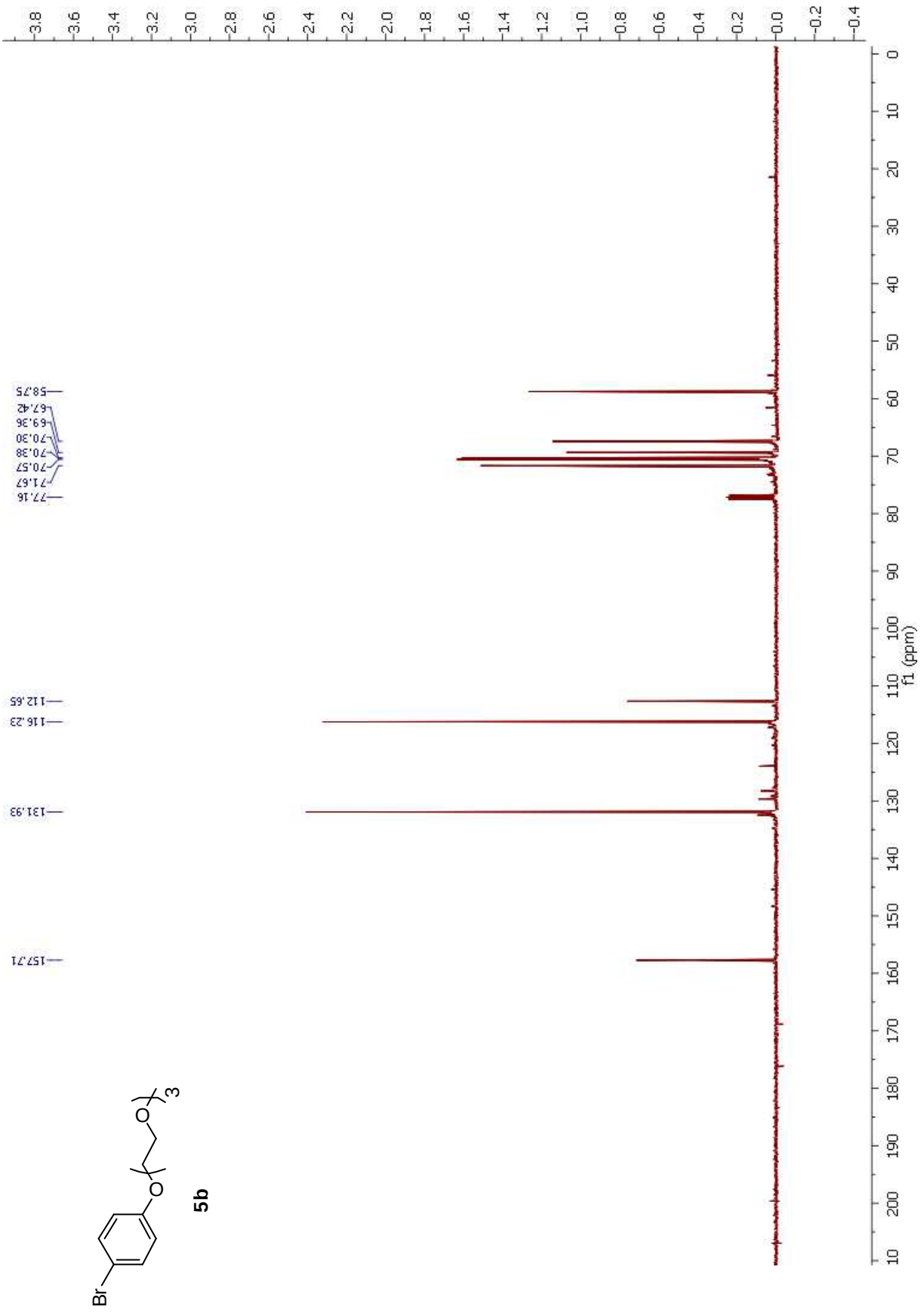
1-bromo-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene (5a)



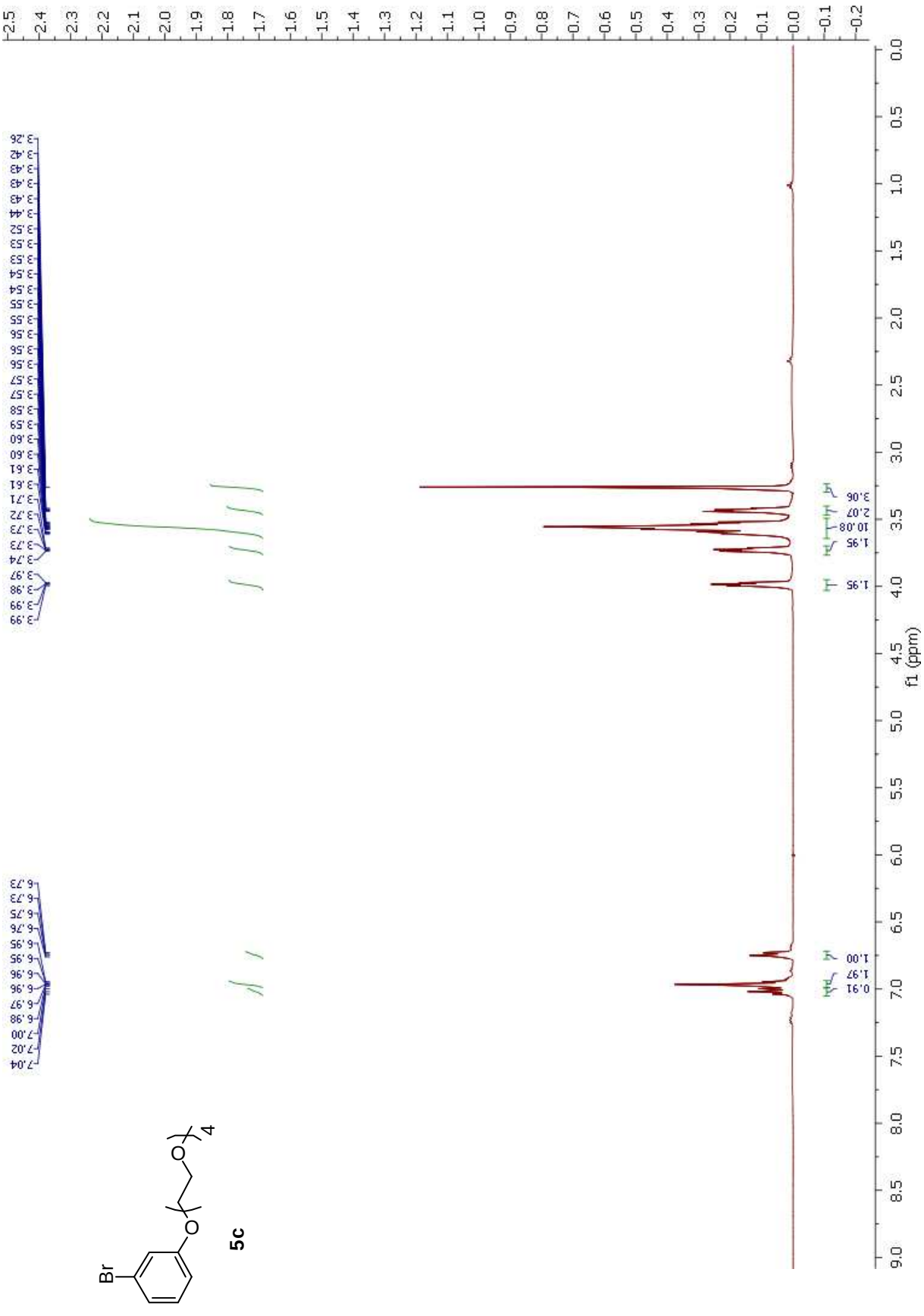
1-bromo-4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene (5b)



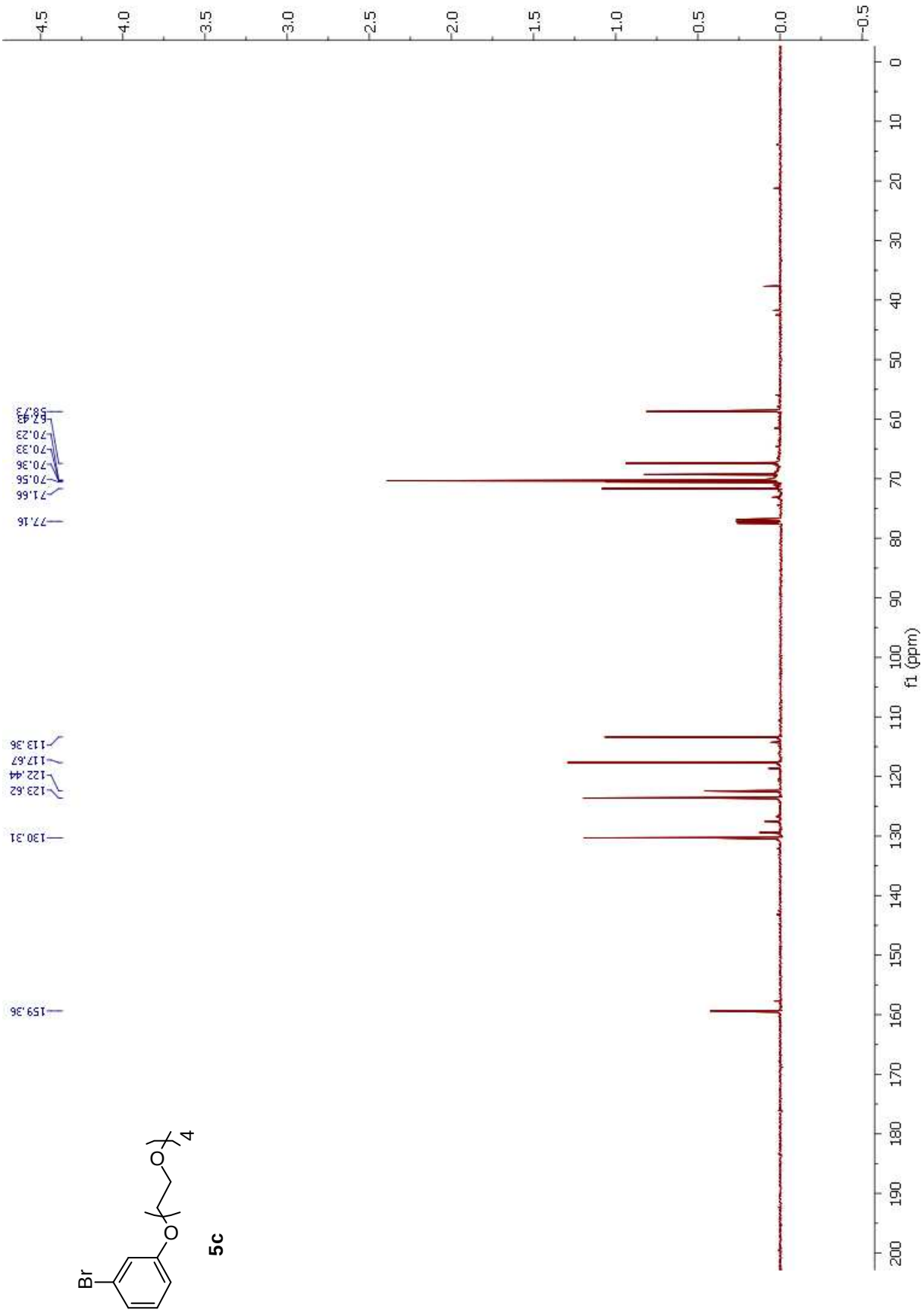
1-bromo-4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene (5b)



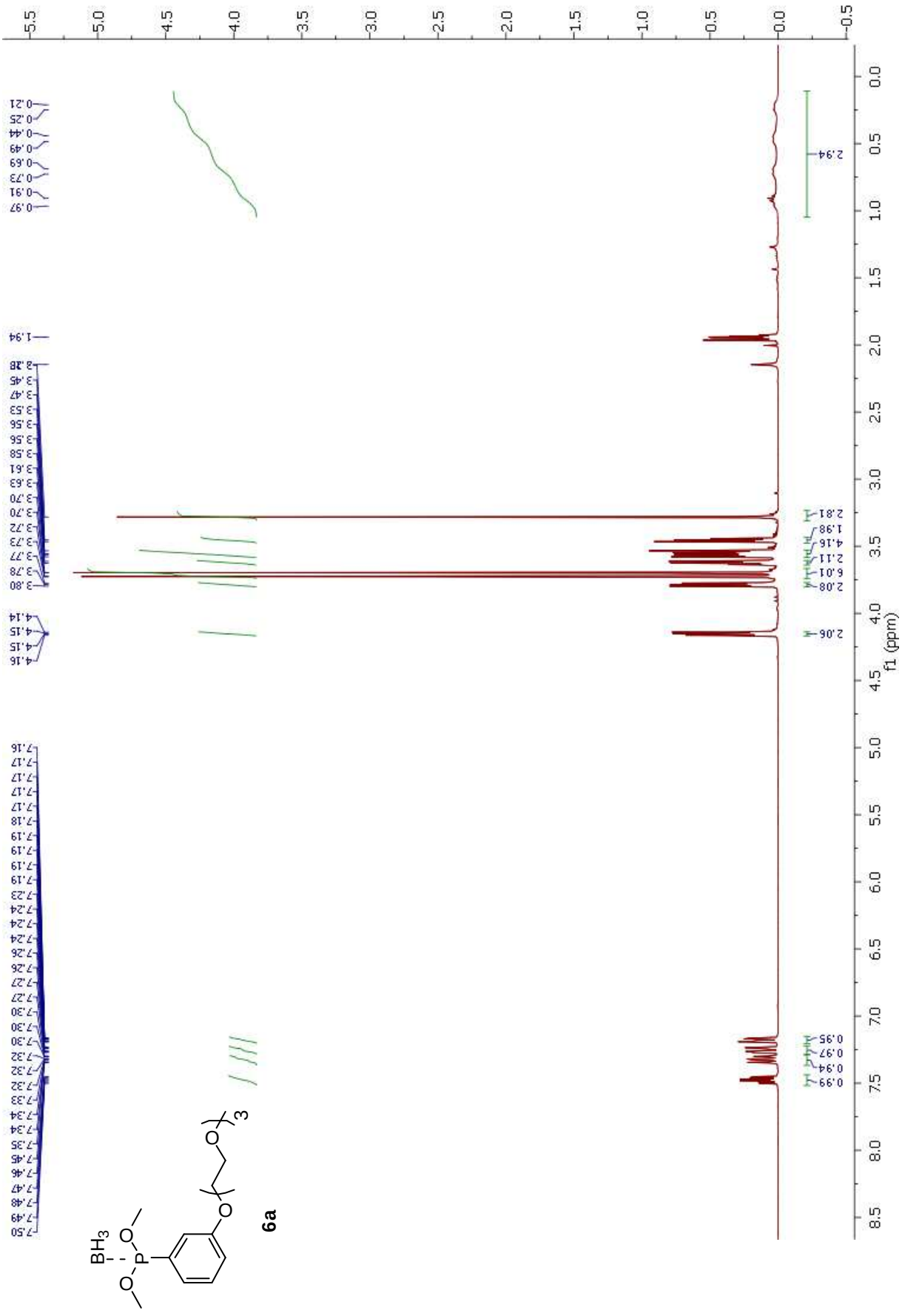
13-(3-bromophenoxy)-2,5,8,11-tetraoxatridecane (5c)



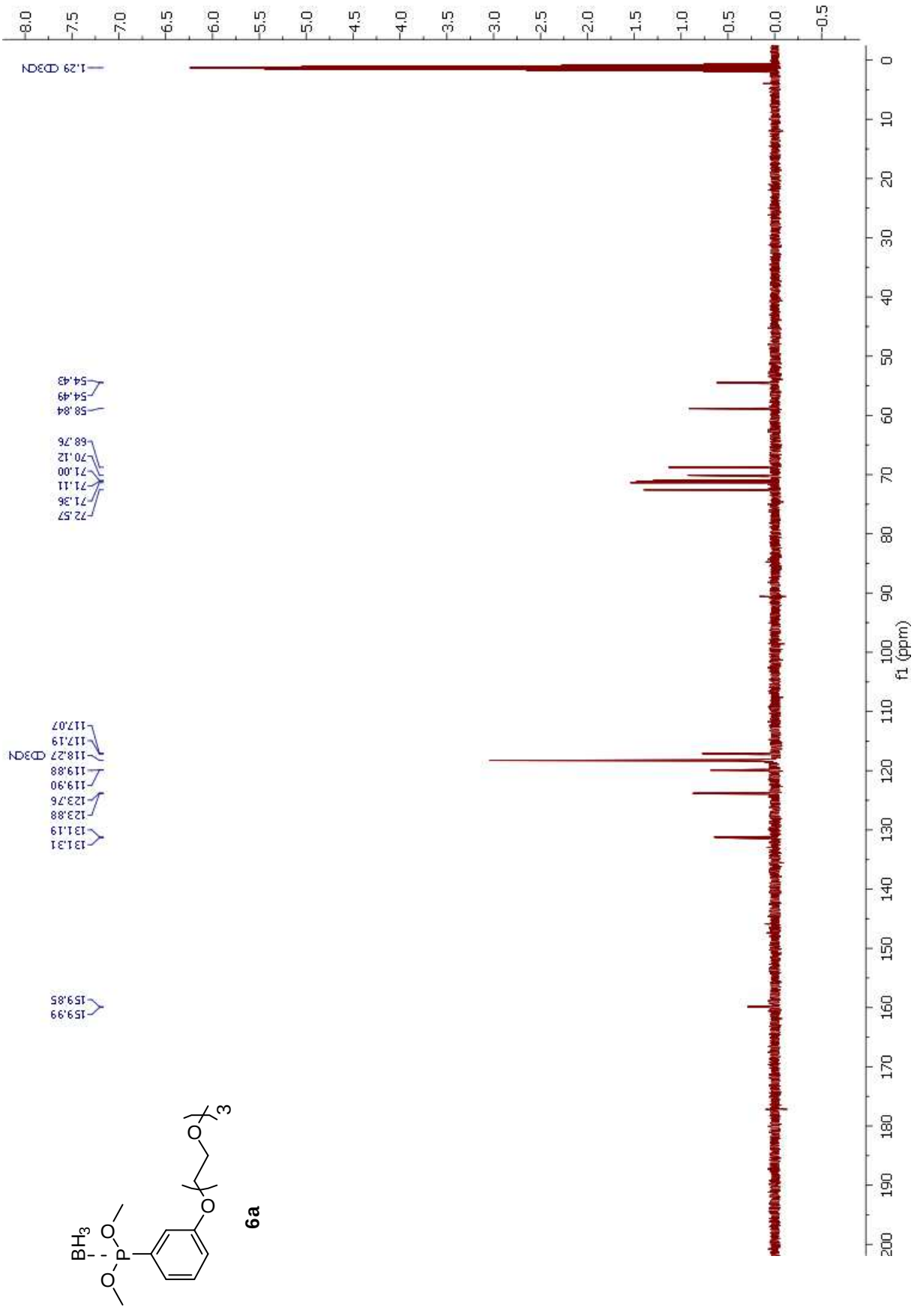
13-(3-bromophenoxy)-2,5,8,11-tetraoxatridecane (5c)



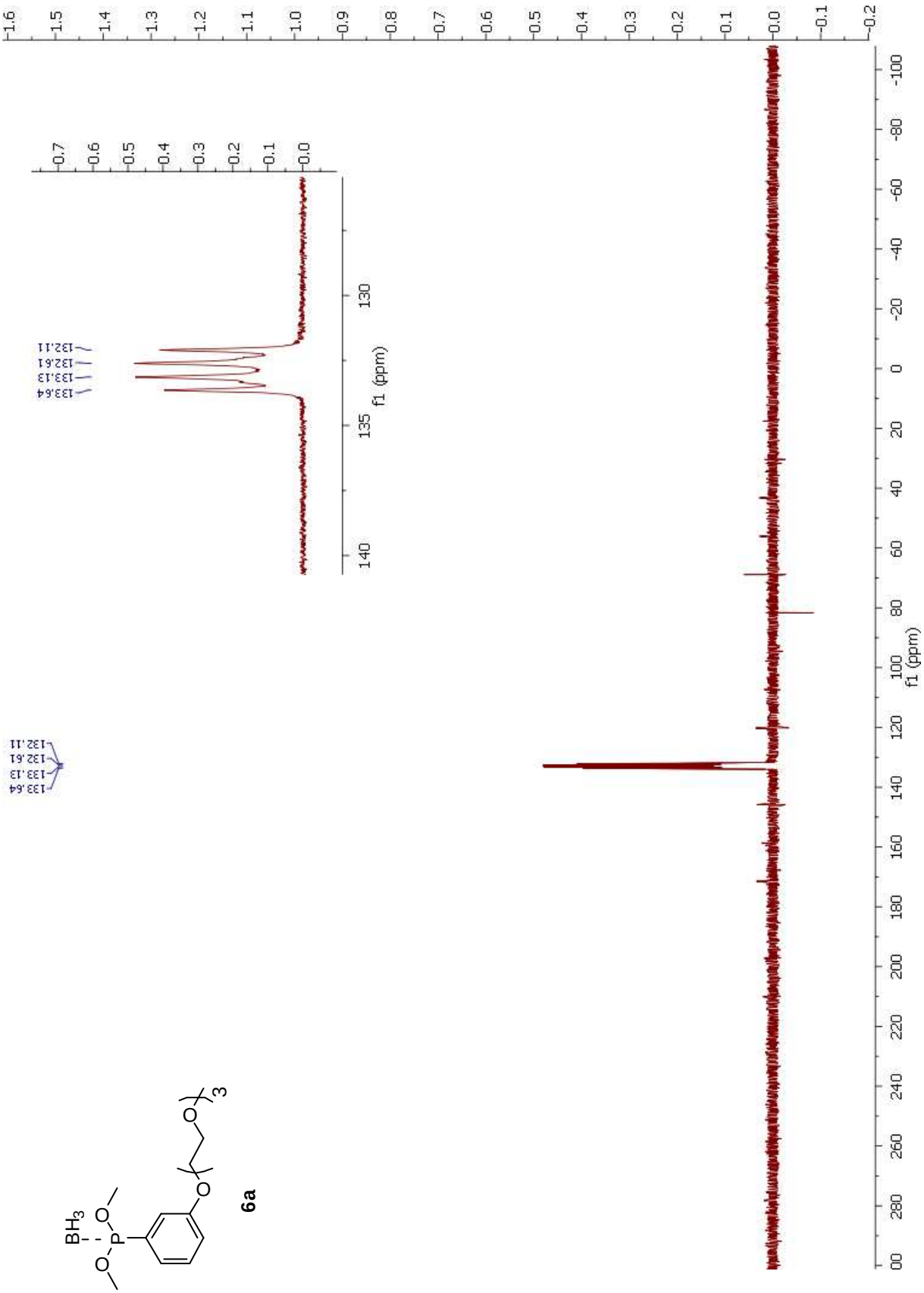
Dimethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane (6a)



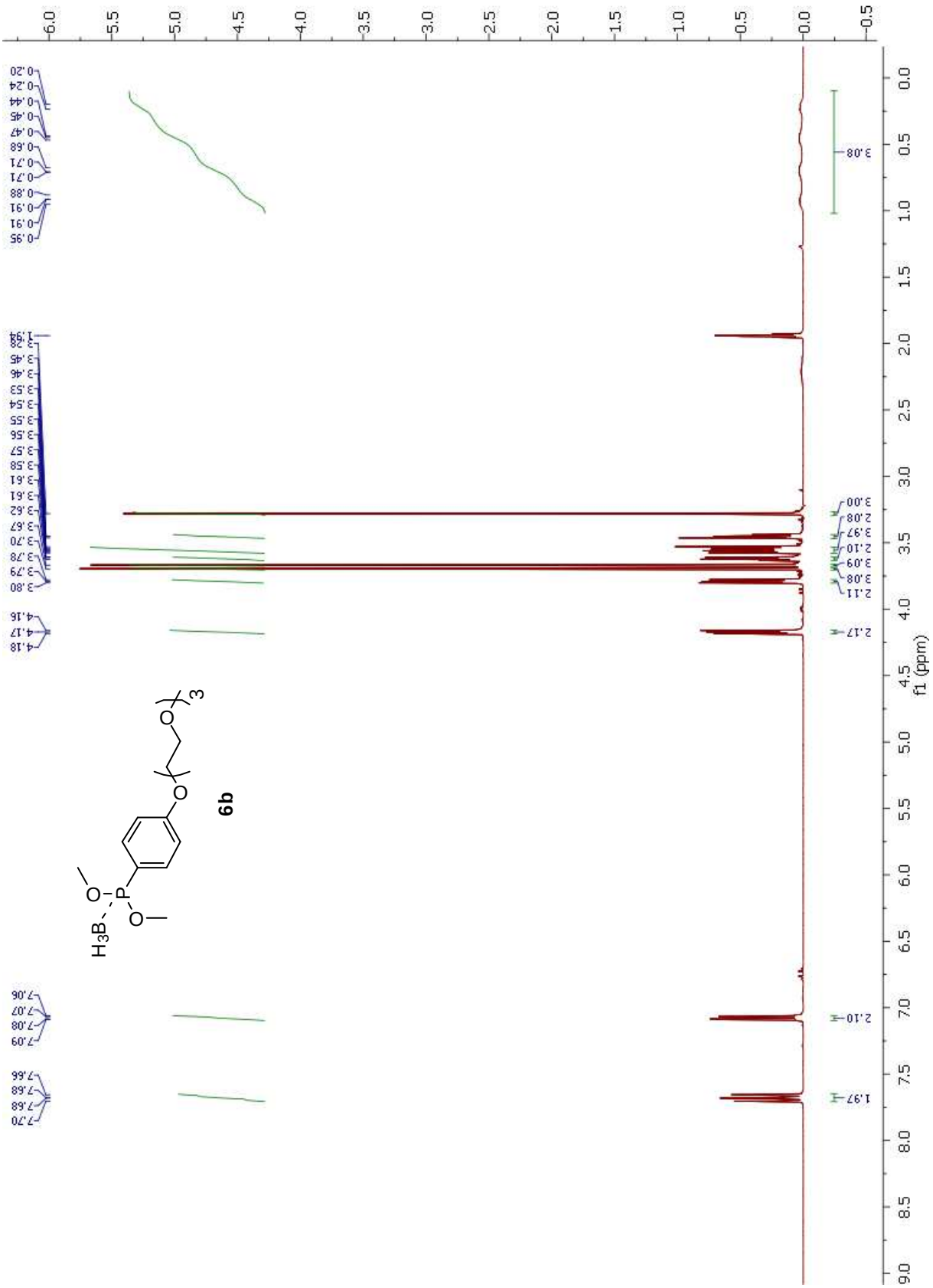
Dimethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane (6a)



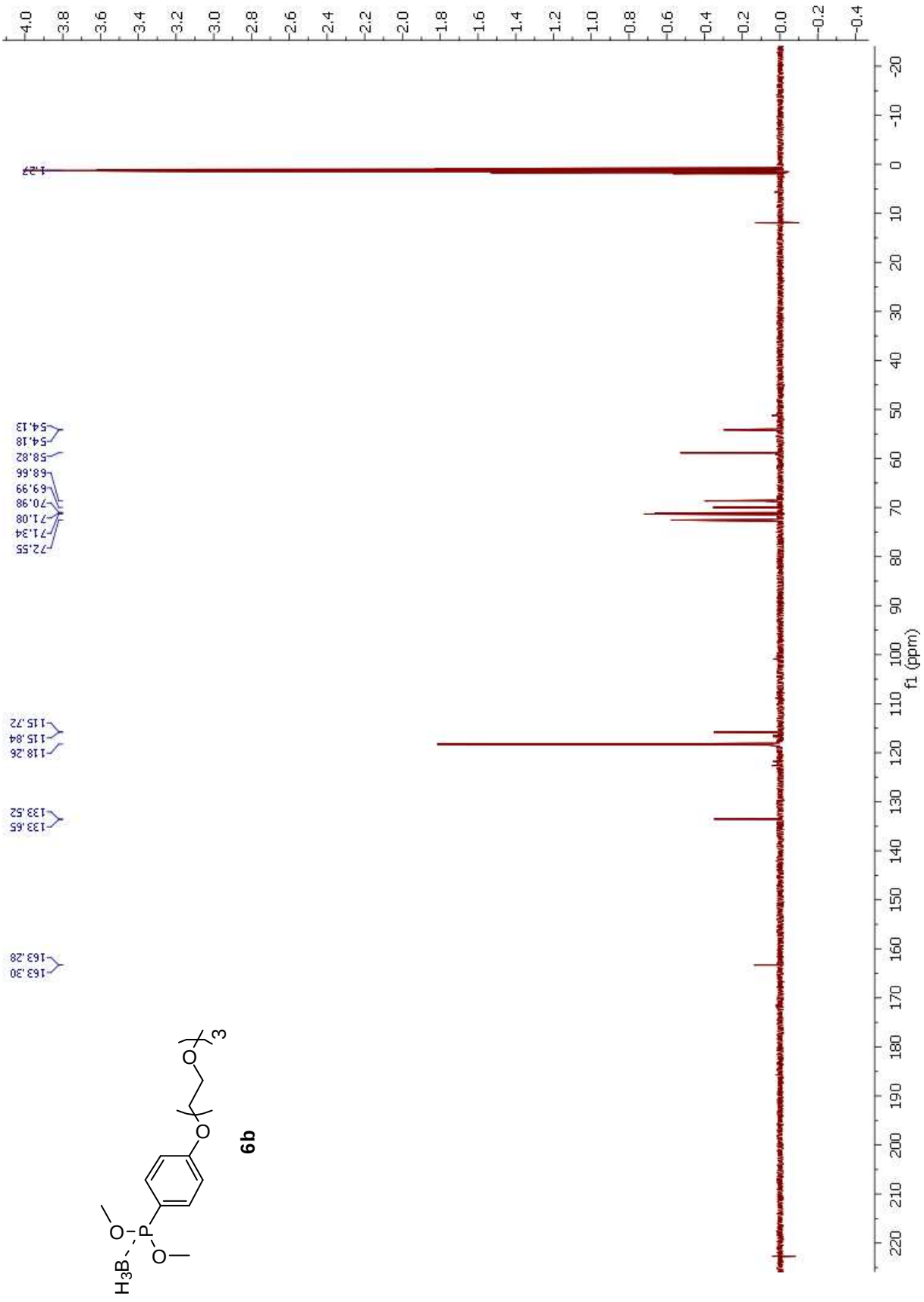
Dimethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane (6a)



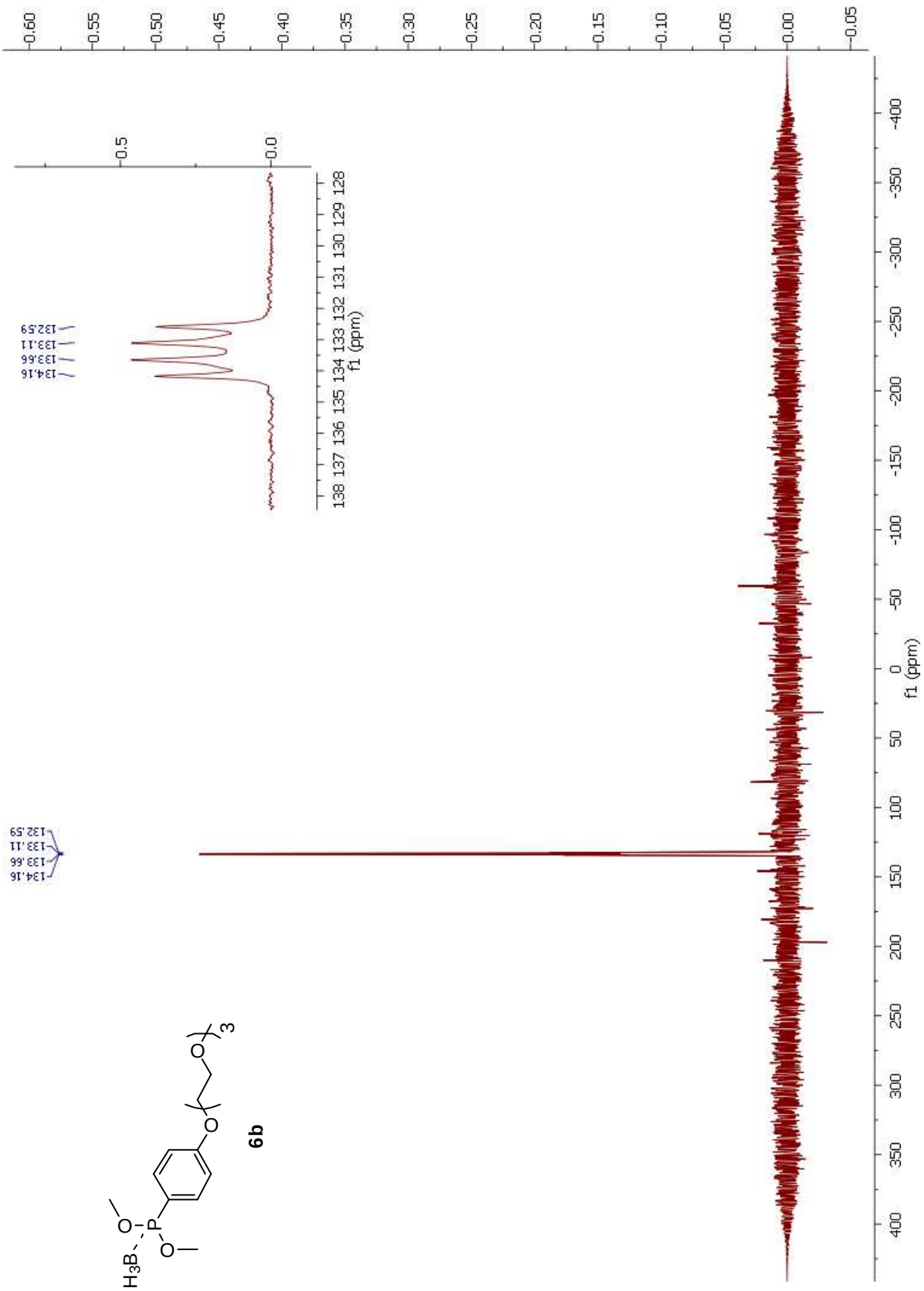
Dimethyl-4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane (6b)



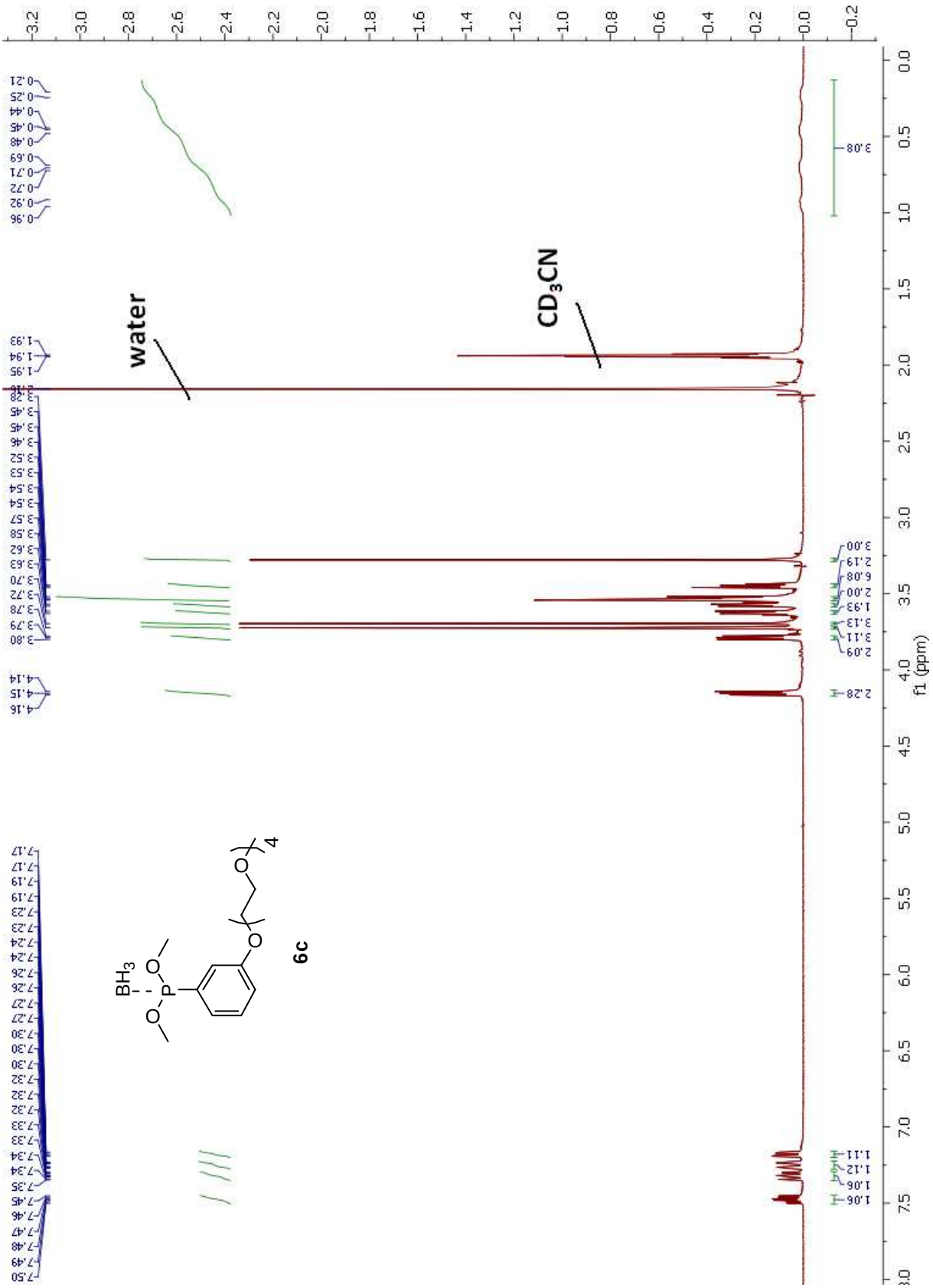
Dimethyl-4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane (6b)



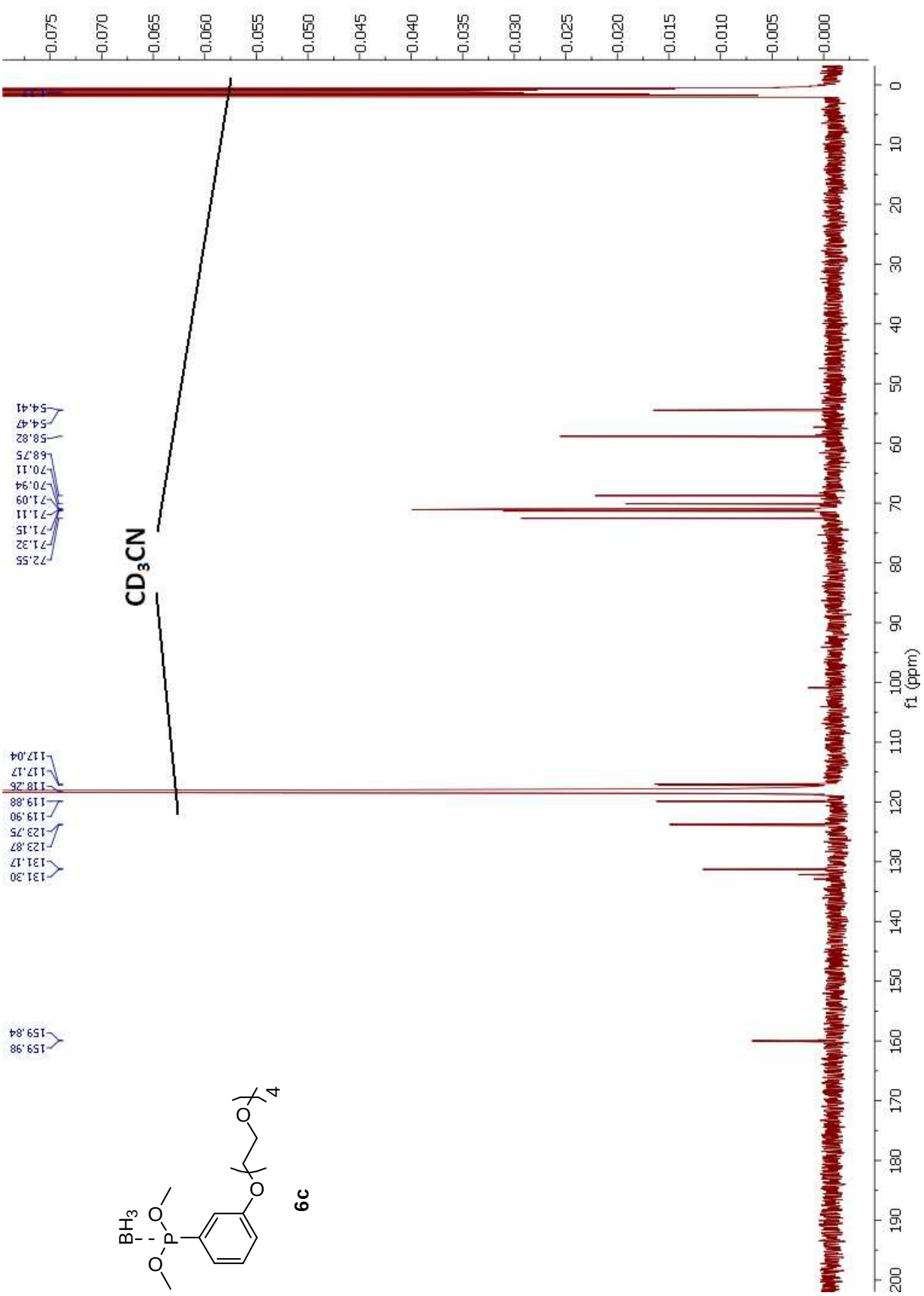
Dimethyl-4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane (6b)



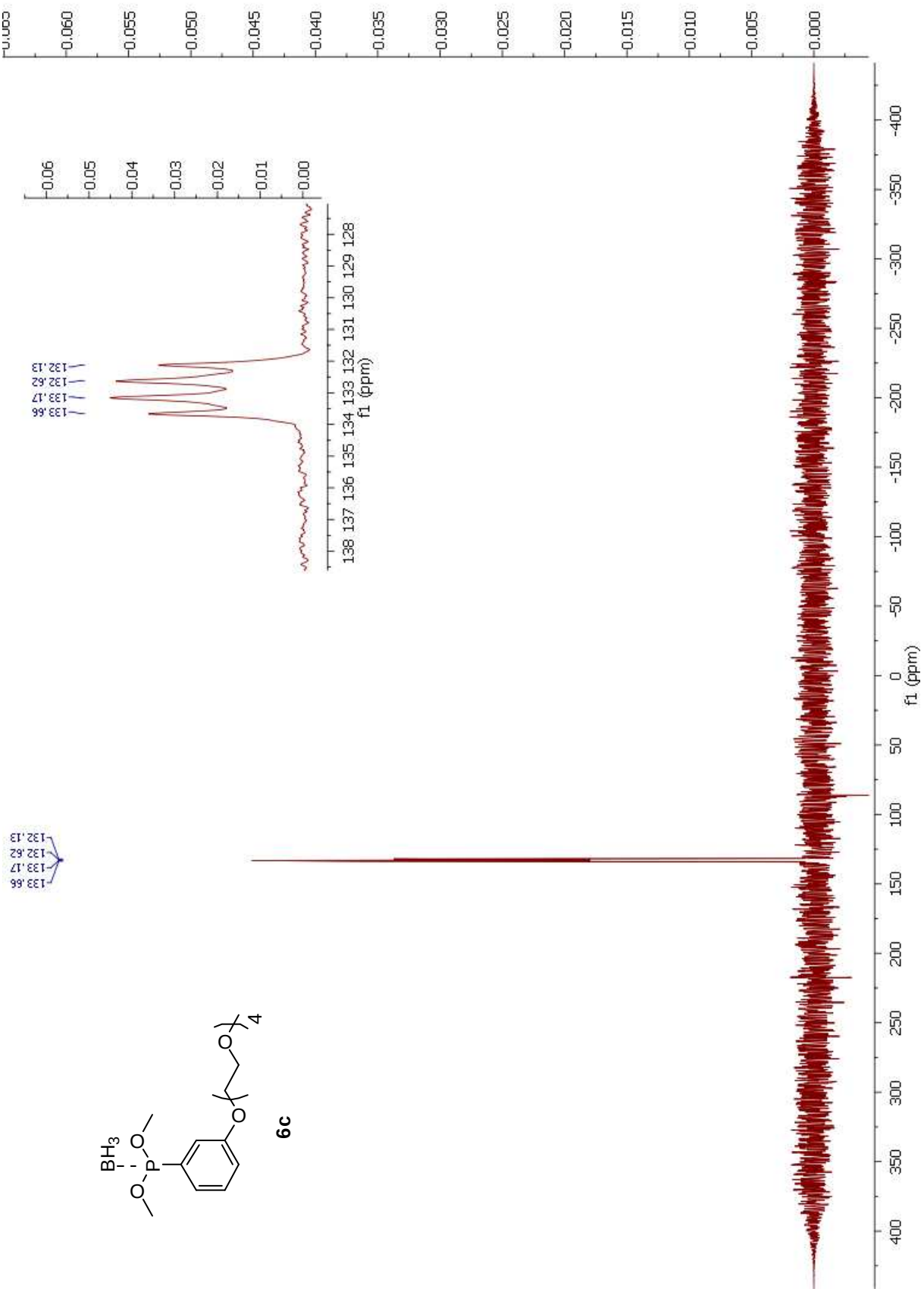
Dimethyl (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite-borane (6c)



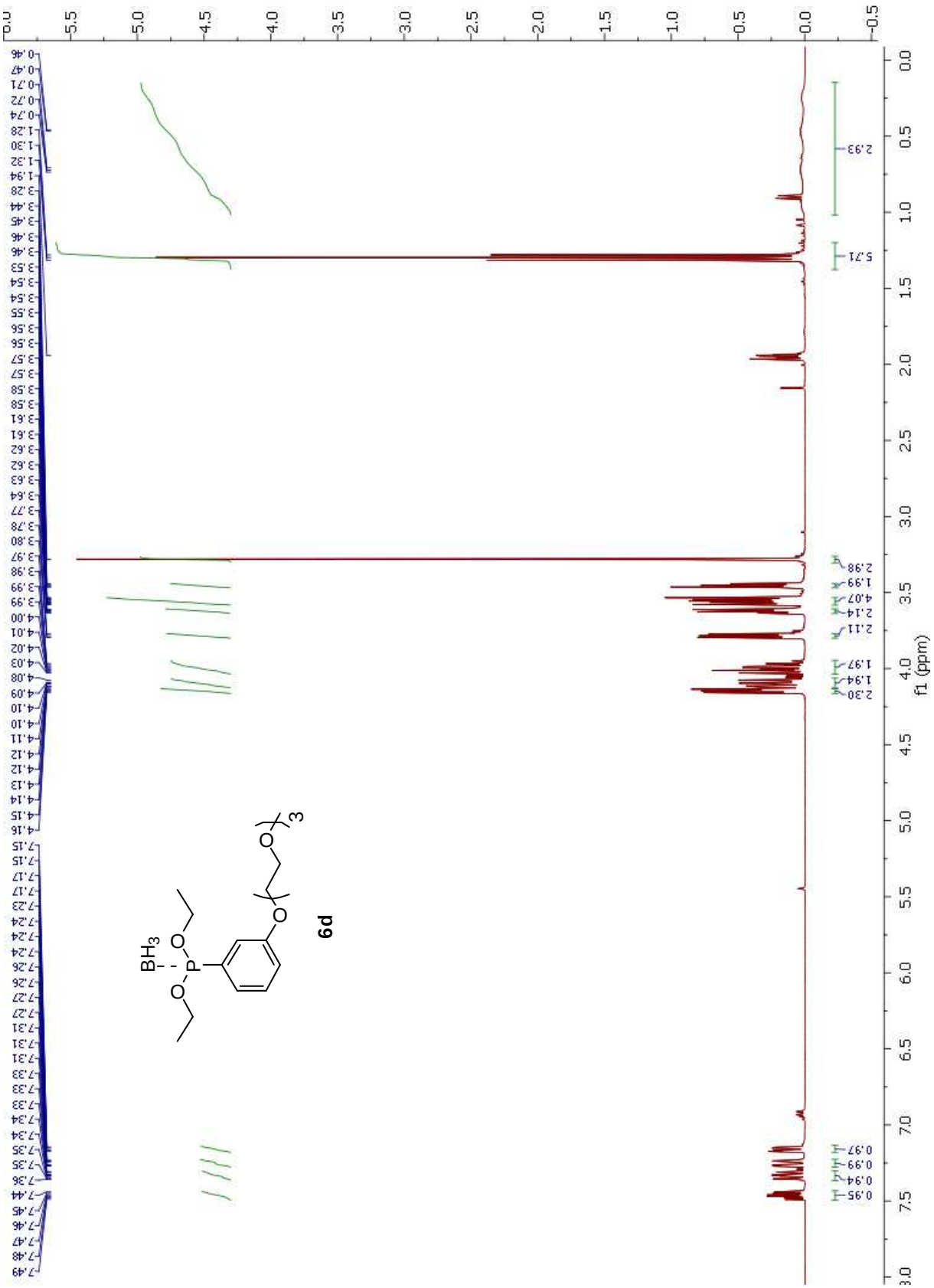
Dimethyl (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite-borane (6c)



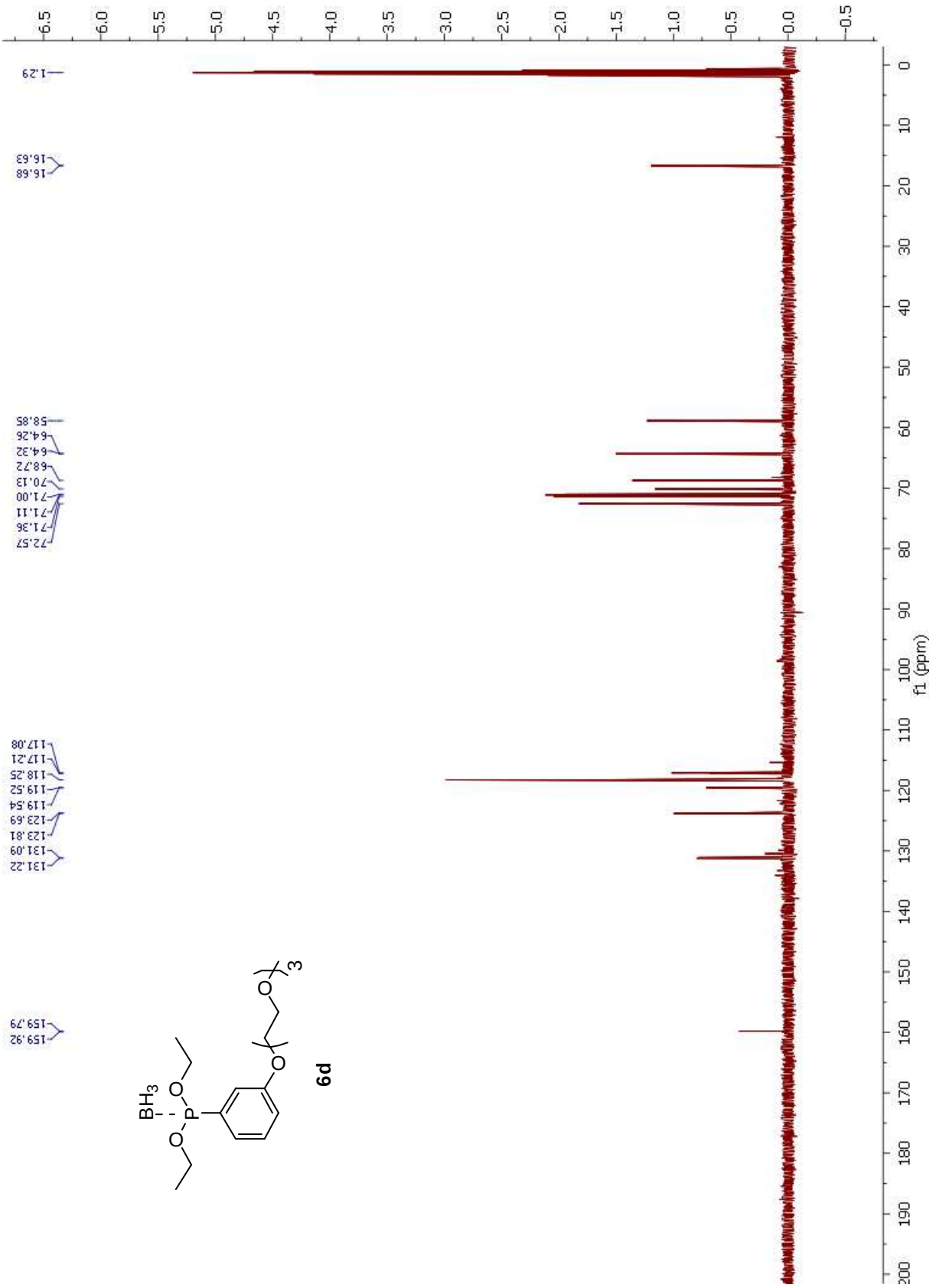
Dimethyl (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite-borane (6c)



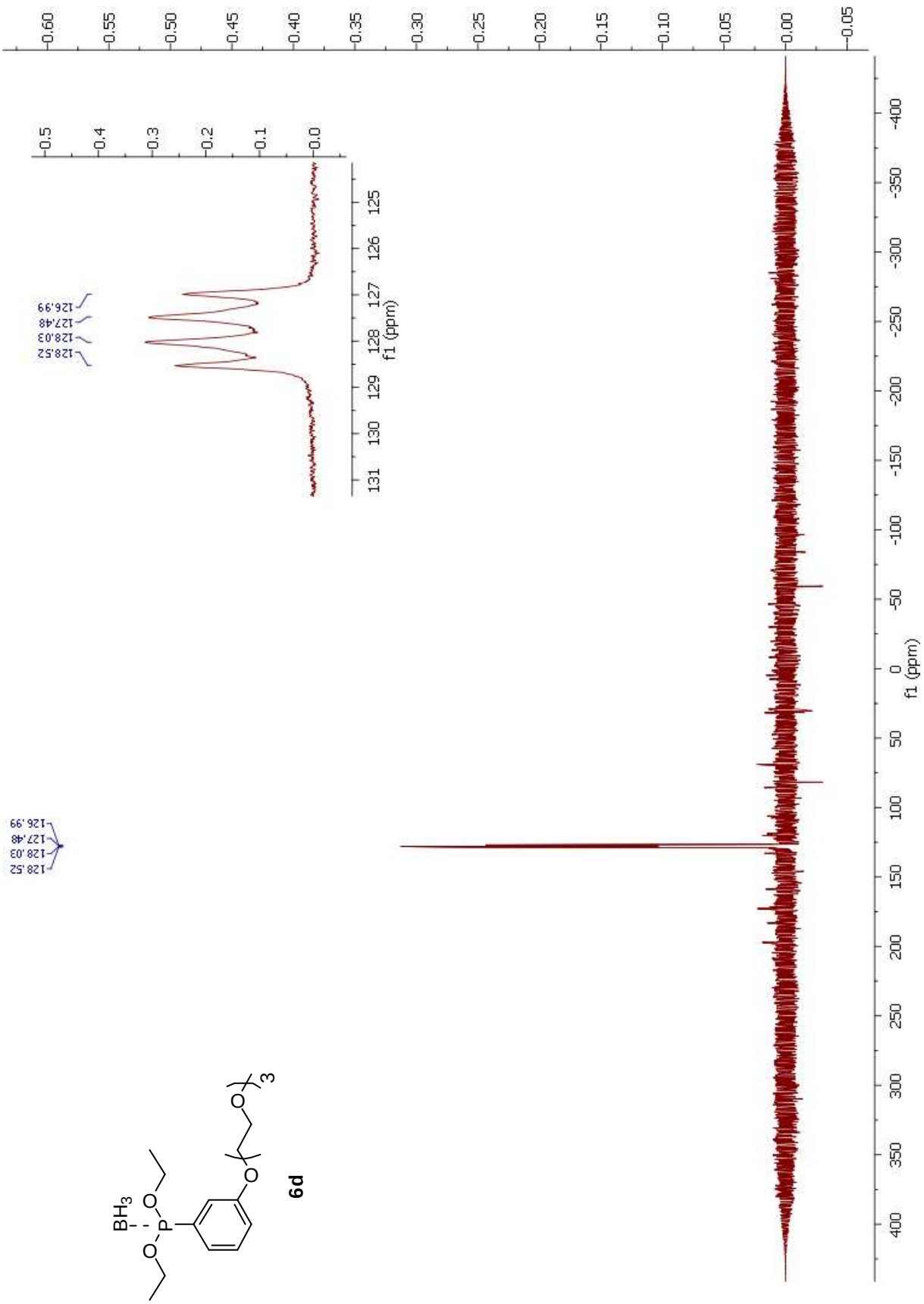
Diethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane (6d)



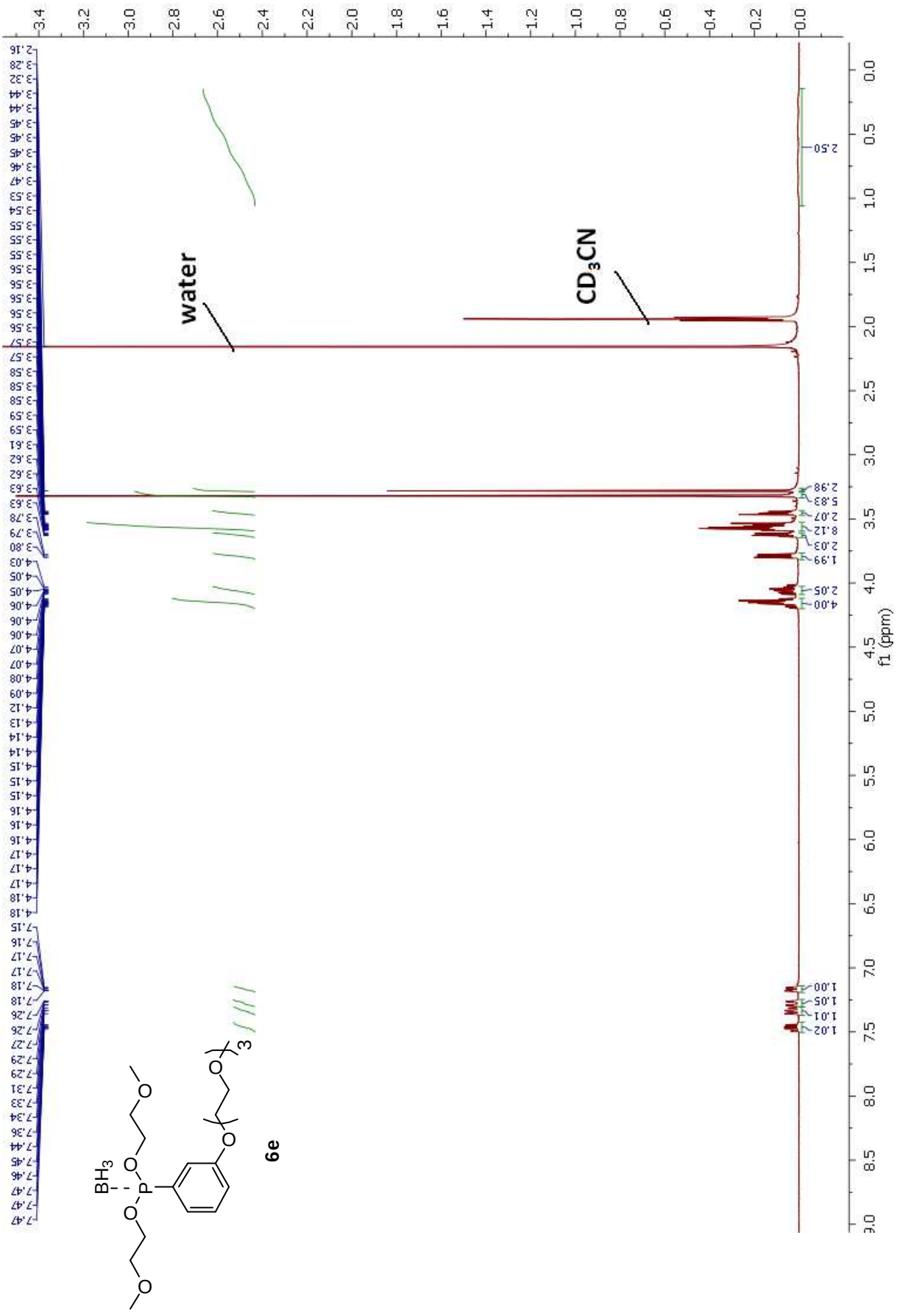
Diethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane (6d)



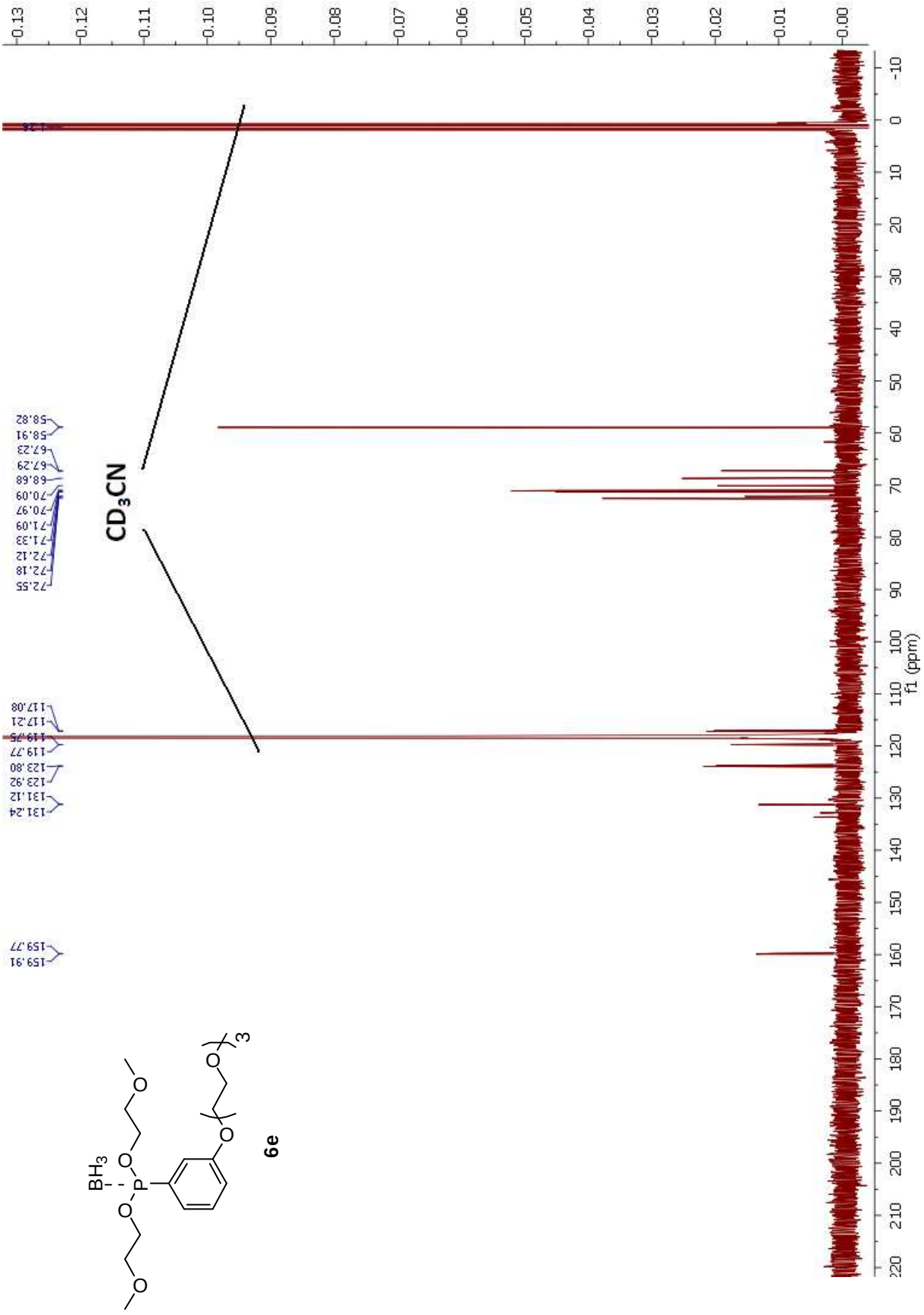
Diethyl-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenylphosphonite-borane (6d)



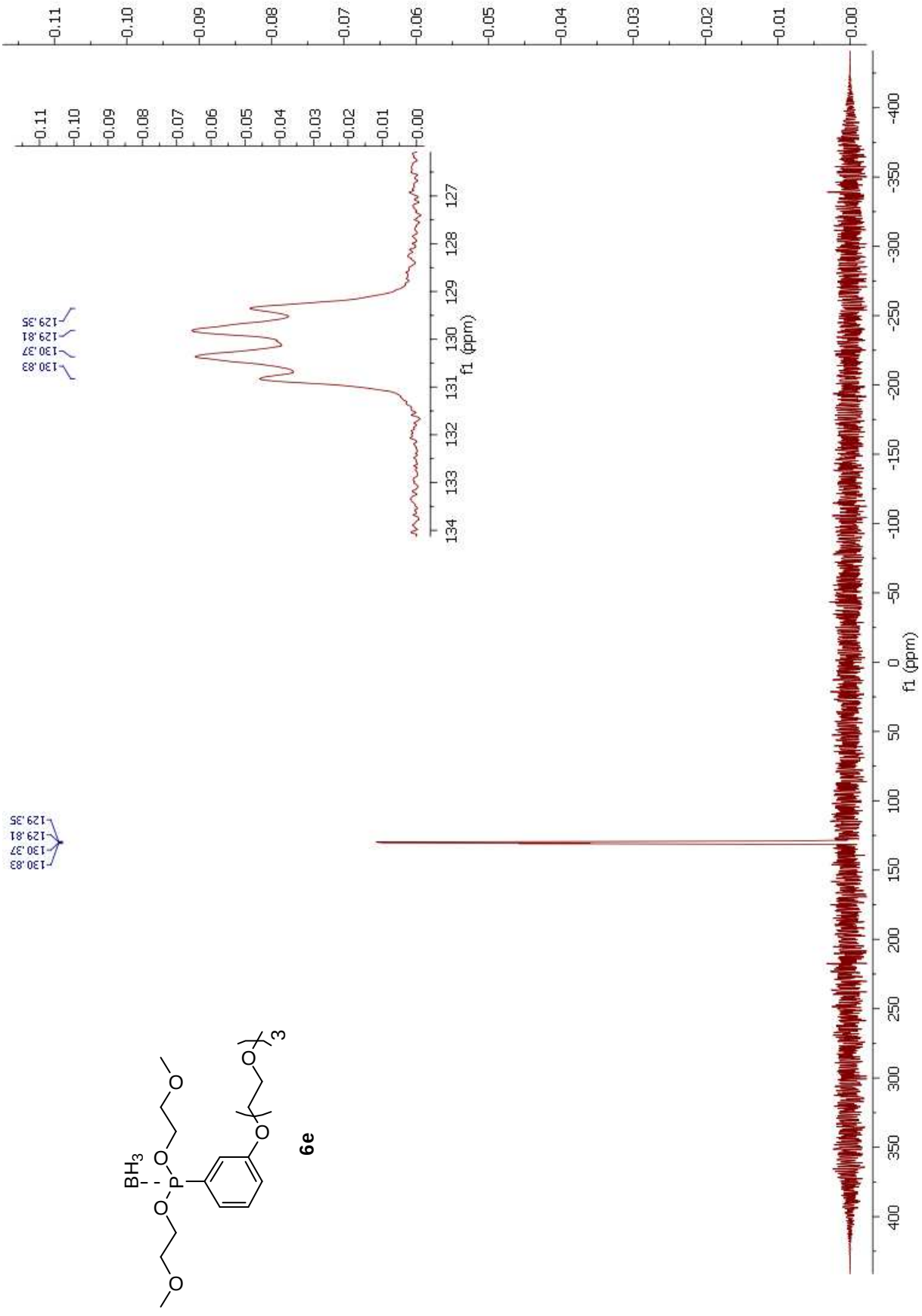
Bis(2-methoxyethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite-borane (6e)



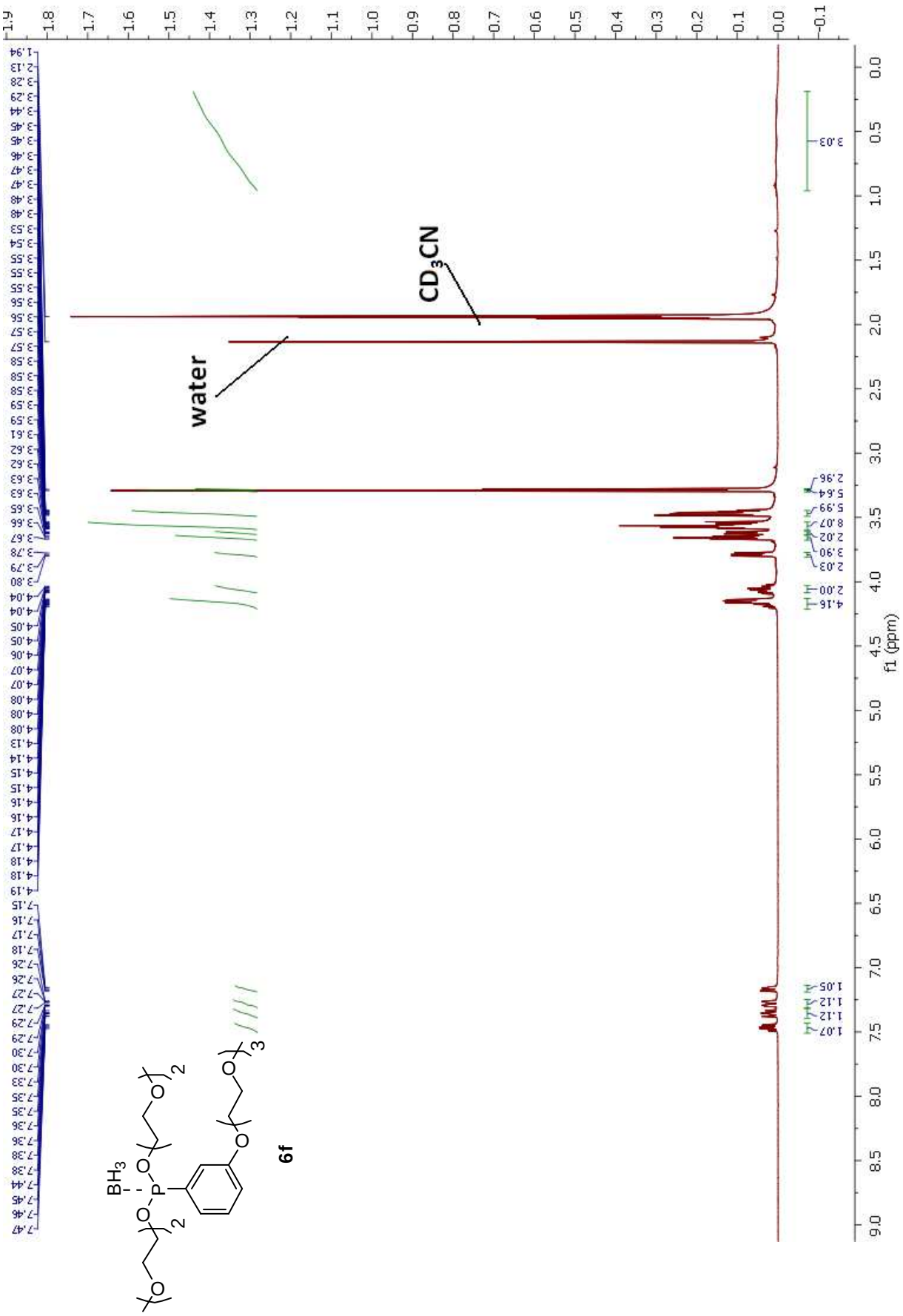
Bis(2-methoxyethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite-borane (6e)



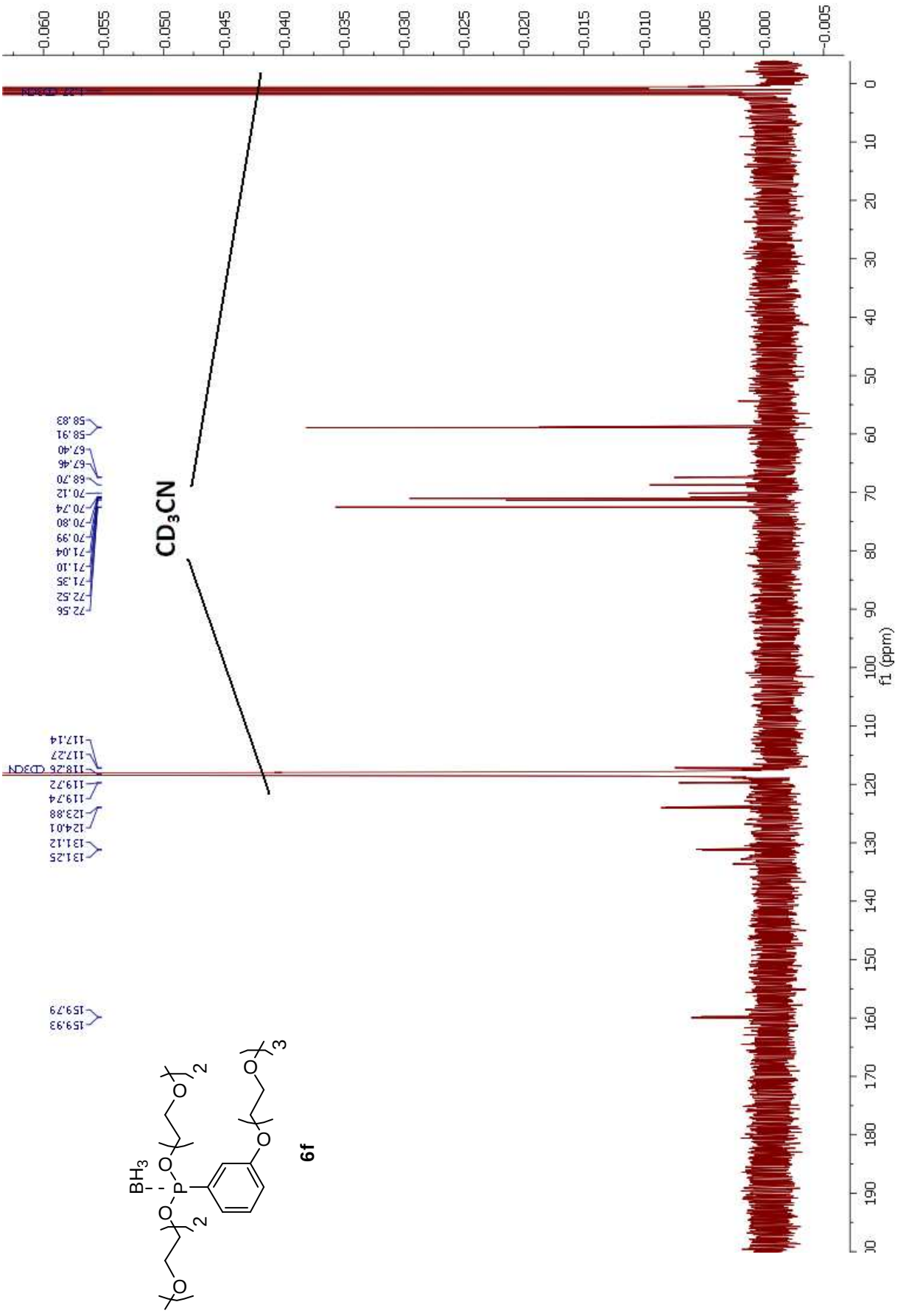
Bis(2-methoxyethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite-borane (6e)



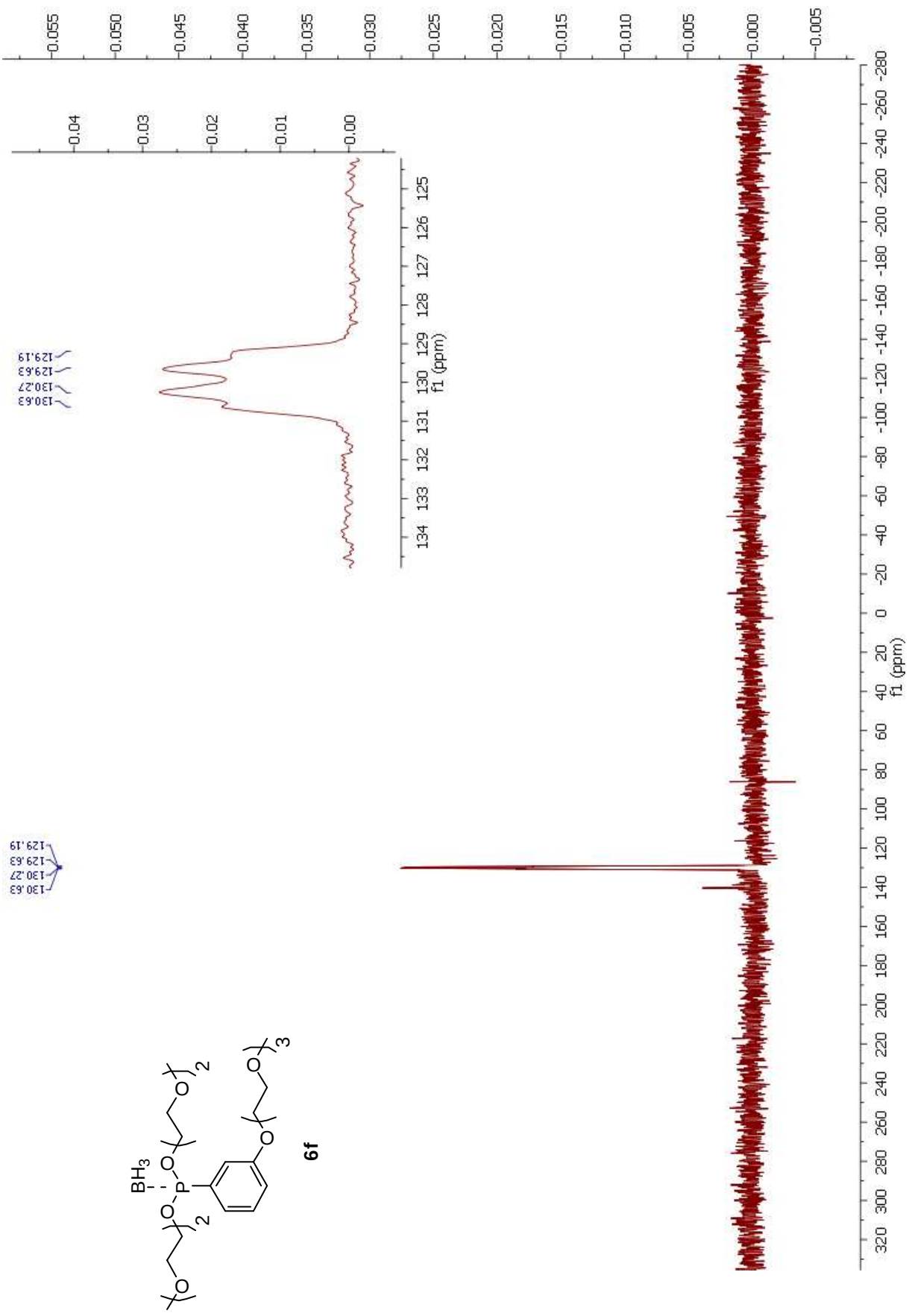
Bis(2-(2-methoxyethoxy)ethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite-borane (6f)



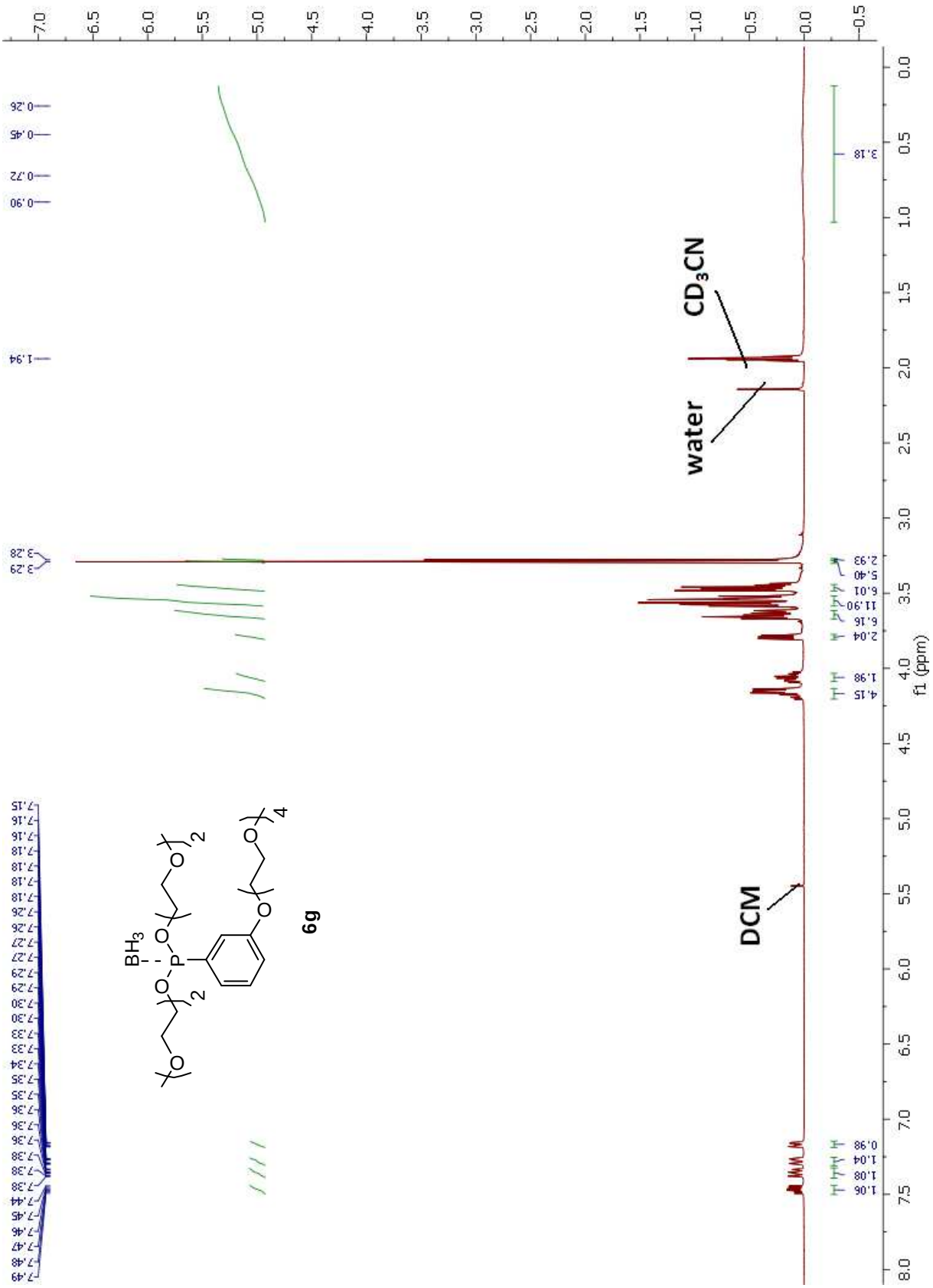
Bis(2-(2-methoxyethoxy)ethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite-borane (6f)



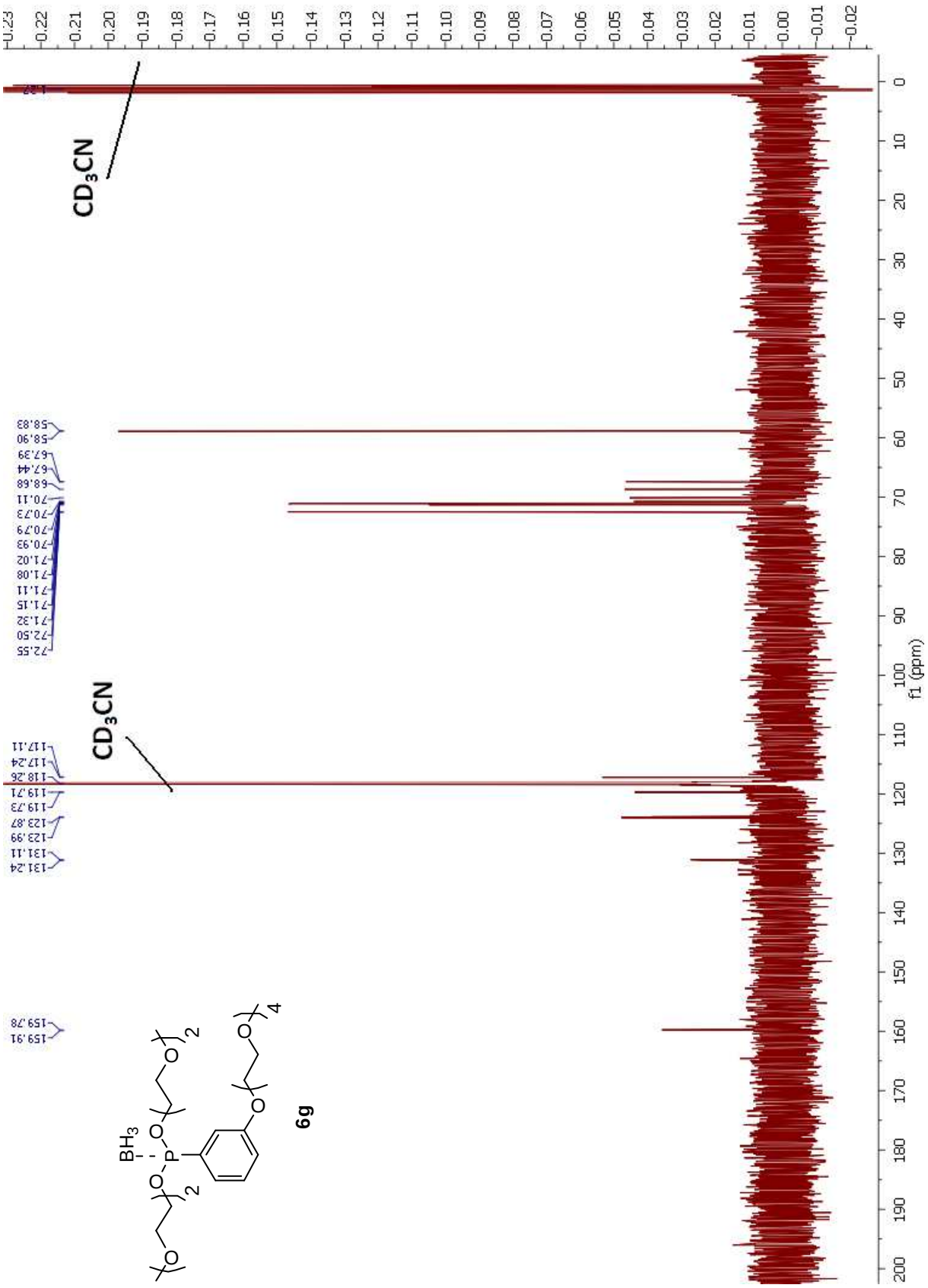
Bis(2-(2-methoxyethoxy)ethyl) (3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)phosphonite-borane (6f)



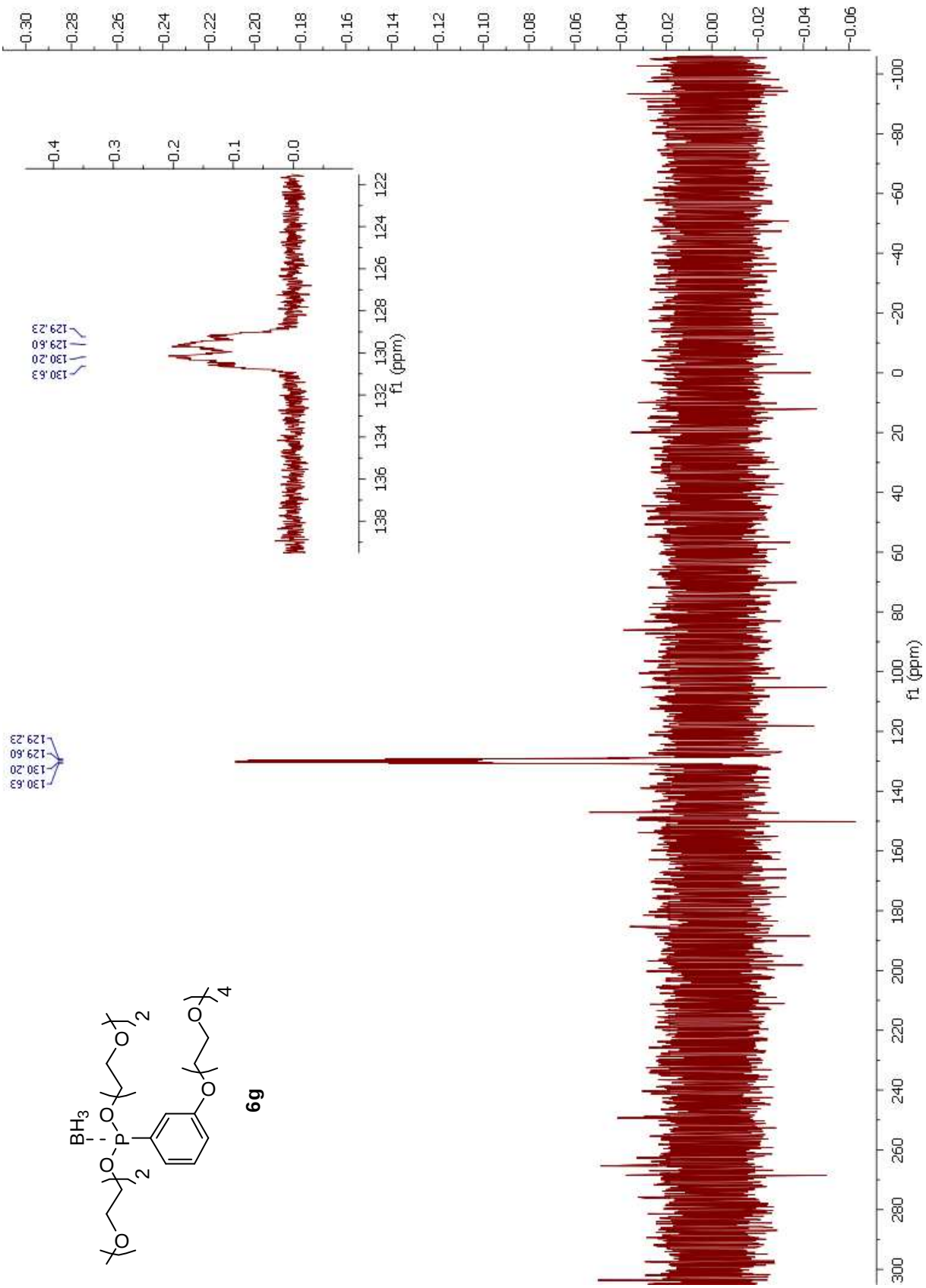
Bis(2-(2-methoxyethoxy)ethyl) (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite-borane (6g)



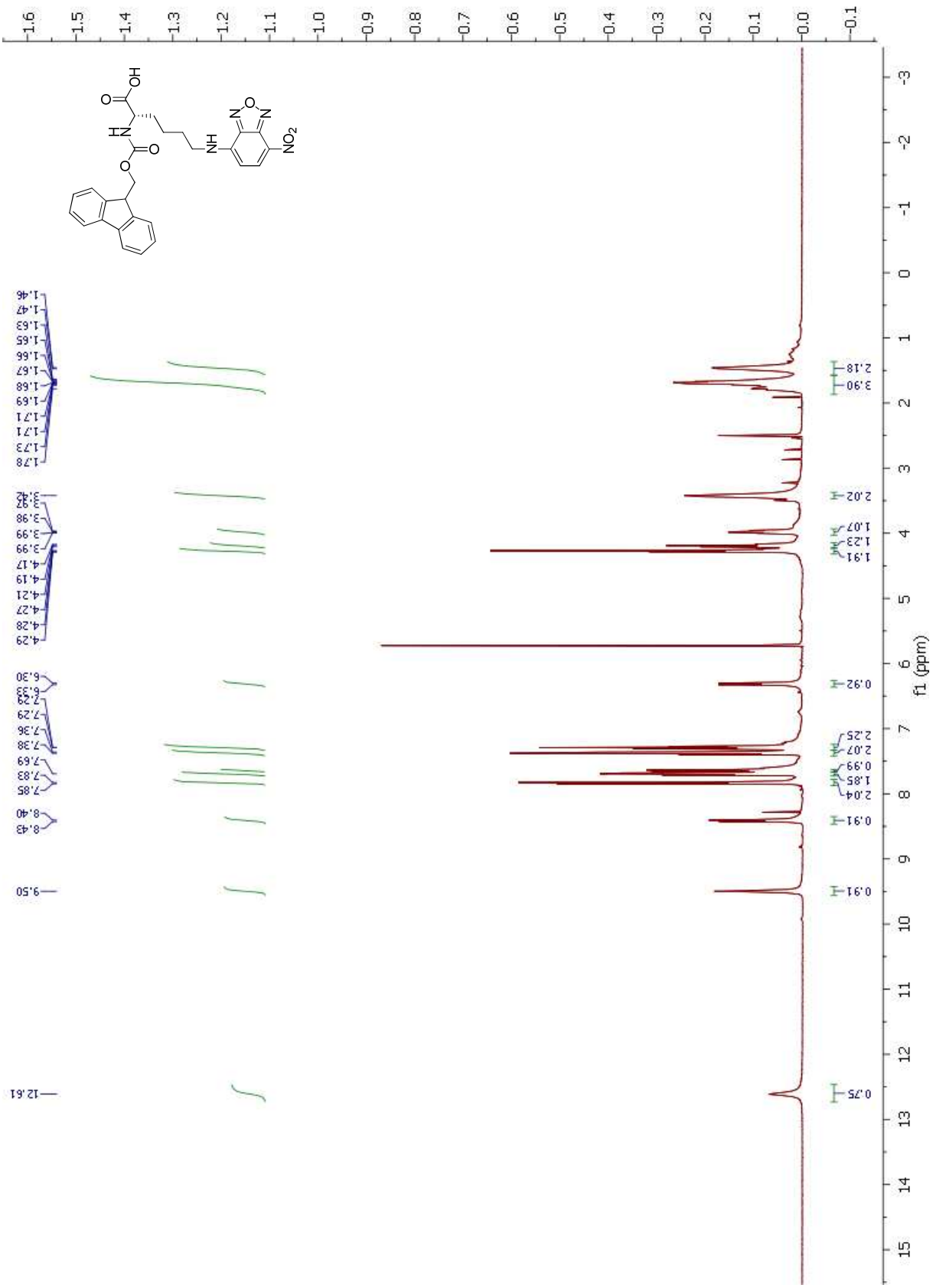
Bis(2-(2-methoxyethoxy)ethyl) (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite-borane (6g)



Bis(2-(2-methoxyethoxy)ethyl) (3-(2,5,8,11-tetraoxatridecan-13-yloxy)phenyl)phosphonite-borane (6g)



***N*-α-Fluorenylmethyloxycarbonyl-(ε-*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-ylamino)-L-Lysin**



***N*-α-Fluorenylmethyloxycarbonyl-(ε-*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-ylamino)-L-Lysin**