Electronic Supplementary Information

For

Ratiometric Electrochemical Detection of Alkaline Phosphatase

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General information:

Proton, carbon and phosphorus nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 300 spectrometer (^{1}H NMR at 300 MHz, ^{13}C NMR at 75.5 MHz and ^{31}P NMR at 121.5 MHz). Chemical shifts for protons are reported downfield from tetramethylsilane and are referenced[1] to residual protium in the solvent (^{1}H NMR: $CHCl_3$ at 7.26 ppm, C_6H_6 at 7.16 ppm, H_2O at 4.79 ppm). Chemical shifts for carbons are reported in parts per million downfield from tetramethylsilane and are referenced[1] to the carbon resonances of the solvent peak (^{13}C NMR: $CDCl_3$ at 77.0 ppm, C_6D_6 at 128.1 ppm). Chemical shifts for phosphorus are reported in parts per million referenced to 85% phosphoric acid. NMR data are represented as follows: chemical shift, integration, multiplicity (s = singlet, bs = broad singlet, d = double, dd = doublet of doublet, t = triplet, q = quartet, sept = septet, m = multiplet), coupling constants (Hz). IR spectra were recorded on a Perkin-Elmer 1600 FT IR spectrophotometer, with absorbencies quoted as ν in cm^{-1}. High resolution mass spectrometry was performed on a µTOF electrospray time-of-flight (ESI-TOF) mass spectrometer (Bruker Daltonik). Melting points were obtained on a Bibby-Sterilin SMP10 melting point machine. Electrochemical analysis was performed on a Metrohm Autolab PGSTAT30 potentiostat using General Purpose Electrochemical System (GPES) software in differential pulse mode (step potential = 3 mV, modulation amplitude 49.95 mV). Analytical thin layer chromatography (TLC) were performed using aluminium-backed plates coated with Alugram® SIL G/UV_{254} purchased from Macherey-Nagel and visualised by UV light (254 nm) and/or $KMnO_4$ or 2,4-DNPH staining. Silica gel column chromatography was carried out using 60 Å, 200-400 mesh particle size silica gel purchased from Sigma-Aldrich. Preparative reverse phase (C18) column chromatography was carried out using VersaPak® 30g C18 cartridges (23 mm × 110 mm) preloaded with 20-45 µm spherical C18 bonded silica purchased from Sigma-Aldrich.

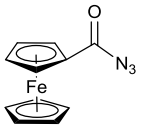
Materials:

All reactions were carried out under an atmosphere of nitrogen, in oven-dried glassware unless otherwise stated. Dichloromethane, tetrahydrofuran (THF) and toluene were dried and degassed by passing through anhydrous alumina columns using an Innovative Technology Inc. PS-400-7 solvent purification system and stored under an atmosphere of argon prior to use. Ferrocenecarboxylic acid was purchased from Alfa Aesar. All other chemicals were purchased from Sigma-Aldrich. *N*-Chlorosuccinimide (NCS) and triethylamine (TEA) were purified by standard published methods.[2] All other chemicals were used as received. Alkaline phosphatase and streptavidin-conjugated alkaline phosphatase were purchased as lyophilised solids from Sigma-Aldrich and stored in a −20 °C freezer. Prior to use, a stock solution of the enzyme was made up using 50 mM pH 9 tris buffer and stored at 4 °C until immediate use. High sensitivity C-reactive protein (CRP) ELISA kit was purchased from Kalon Biological Ltd (Guildford, UK) and stored at 4 °C prior to use.

Electrochemical analysis:

Electrochemical analysis was performed by applying a 20 µL sample to screen-printed electrochemical cell equipped with carbon working and counter electrodes and a silver (pseudo Ag/AgCl) reference electrode. The potential across the cell was powered by a Metrohm Autolab PGSTAT30 potentiostat controlled by a laptop running General Purpose Electrochemical System (GPES) software in differential pulse mode (modulation = 0.04 s, interval = 0.1 s, initial voltage = −300 mV, end voltage = 300 mV, step potential = 3 mV, modulation amplitude 49.95 mV). Post-scan, a baseline correction (moving average: peak width = 0.03) was performed. Peak integrals were obtained using the 'peak search' function and conversions calculated using the equation: $Conversion\ (\%) = \left(\frac{\int \mathbf{3}}{(\int \mathbf{3} + \int \mathbf{1})}\right) \times 100$.

Procedure for the synthesis of ferrocenoyl azide (4)



Ferrocenecarboxylic acid (2.00 g, 8.7 mmol, 1 eq.) was suspended in anhydrous dichloromethane (20 mL) and cooled to 0 °C. Oxalyl chloride (1.5 mL, 17.4 mmol, 2 eq.) was then added dropwise, followed by a drop of DMF. The reaction mixture was allowed to warm to room temperature and stirred for 3 hours, after which the solvent and residual oxalyl chloride were removed *in vacuo*. The red solid obtained was dissolved in anhydrous dichloromethane (20 mL) and cooled to 0 °C. Tetrabutylammonium bromide (0.03 g, 0.1 mmol, 0.01 eq.) was added, followed by a solution of sodium azide (0.85 g, 13.1 mmol,1.5 eq.) in water (4 mL). The reaction mixture was allowed to warm to room temperature and left to stir overnight (~16 hours). Water (50 mL) was then added and the organics separated. The aqueous layer was then extracted with dichloromethane (2 × 20 mL). The combined organics were dried over $MgSO_4$ and filtered, and the solvent removed *in vacuo*. Purification *via* silica gel column chromatography (hexane 1:1 dichloromethane (R_f = 0.45)) gave the title compound as an crystalline orange solid (1.75 g, 79%).

^{1}H NMR (300 MHz, C_6D_6); δ 4.74 (2H, app s), 4.02 (2H, app s), 3.91 (5H, s).

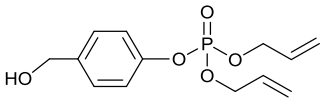
^{13}C NMR (75.5 MHz, C_6D_6); 176.3, 97.7, 72.7, 70.7, 70.4.

IR (solid, cm^{-1}); 2149, 1671.

Mp; 86-87°C (lit.[3] 84-86 °C).

Data in accordance with literature precedent.[3]

Procedure for the synthesis of diallyl (4-(hydroxymethyl)phenyl) phosphate (5)



Phosphorus trichloride (8.7 mL, 100 mmol, 2 eq.) was added to anhydrous THF (70 mL) and cooled to 0 °C. A solution of allyl alcohol (13.6 mL, 200 mmol, 4 eq.) and anhydrous TEA (31 mL, 220 mmol, 4.4 eq.) in anhydrous THF (30 mL) was then added dropwise. The reaction mixture was then allowed to warm to room temperature and stirred for 1 hour before being cooled to 0 °C. Water (50 mL) was then added slowly and the reaction mixture allowed to warm to room temperature before being stirred for 0.5 hours. The organics were removed *in vacuo* and the aqueous residue was extracted with ethyl acetate (3 × 100 mL). The organics were combined, dried over Na_2SO_4 and filtered, and the solvent removed *in vacuo*. The residue was then dissolved in anhydrous toluene (75 ml) and added slowly to a round-bottom flask containing a stirring solution of *N*-chlorosuccinimide (11.7 g, 87.5 mmol, 1.75 eq.) in anhydrous toluene (75 mL) under argon at 0 °C. The reaction mixture was then allowed to warm to room temperature and left to stir overnight (~16 hours) before being filtered *via* gravity filtration. The filtrate was then concentrated *in vacuo*. The residue was then taken up in anhydrous THF (50 mL) and added slowly to a stirring solution of 4-hydroxybenzaldehyde (6.1 g, 50 mmol, 1 eq.) and anhydrous TEA (10.5 mL, 75 mmol, 1.5 eq.) in anhydrous THF (50 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 2 hours before being filtered. The filtrate was then concentrated *in vacuo* before being taken up in ethyl acetate (50 mL), which was then washed with saturated sodium bicarbonate solution (50 mL) and the organics separated. The aqueous layer was then extracted twice with ethyl acetate (2 × 50 mL). The combined organics were then washed with water (3 × 50 mL), dried (Na_2SO_4) and filtered, and the solvent removed *in vacuo*. The residue was then taken up in anhydrous THF (50 mL) and cooled to 0 °C before sodium borohydride (3.8 g, 100 mmol, 2 eq.) was added portion-wise. The reaction mixture was allowed to warm to room temperature and stirred for 2 hours. The reaction mixture was then cooled to 0 °C before being quenched with saturated sodium bicarbonate solution (50 mL). The reaction mixture was then extracted with ethyl actetate (3 × 50 mL). The combined organics were then washed with 1M sodium hydroxide (2 × 50 mL) and water (2 × 50 mL), dried over Na_2SO_4 and filtered, and the solvent removed *in vacuo*. Purification *via* silica gel column chromatography (ethyl acetate 1:1 hexane (R_f = 0.20, $UV_{254\ nm}$ & $KMnO_4$)) gave the title compound as a colourless liquid (4.0 g, 28%).

^{1}H NMR (300 MHz, $CDCl_3$); δ 7.22 (2H, d, *J* = 8.6 Hz), 7.08 (2H, d, *J* = 8.6 Hz), 5.89-5.80 (2H, m), 5.29 (2H, ddd, *J* = 17.1, 2.6, 1.5 Hz), 5.18 (2H, ddd, *J* = 10.4, 2.6, 1.1 Hz), 4.56-4.51 (6H, m), 2.90 (1H, br s).

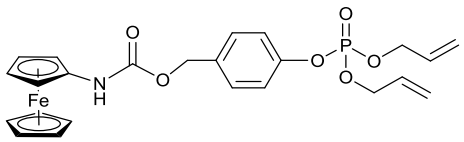
^{13}C NMR (75.5 MHz, $CDCl_3$); δ 149.7 (d, $J_{C\text{-}P}$ = 7 Hz), 138.3 (d, $J_{C\text{-}P}$ = 1 Hz), 132.0 (d, $J_{C\text{-}P}$ = 7 Hz), 128.2, 119.9 (d, $J_{C\text{-}P}$ = 5 Hz), 118.8, 68.9 (d, $J_{C\text{-}P}$ = 6 Hz), 64.2.

^{31}P NMR (121.5 MHz, $CDCl_3$); δ − 5.52.

IR (film, cm^{-1}); ν 3419, 2881, 1651, 1608, 1506, 1459, 1425, 1365, 1267, 1210, 1164, 1097, 1013, 988, 931, 874, 824, 733, 693, 638.

HRMS (ESI); calc'd for $C_{13}H_{17}O_5P$ $[M+Na]^+$: *m/z* 307.0706, found 307.0760.

Procedure for the synthesis of 4-((bis(allyloxy)phosphoryl)oxy)benzyl ferrocenylcarbamate (6)



Ferrocenoyl azide (255 mg, 1 mmol, 1 eq.) was dissolved in anhydrous toluene (3 mL) under argon. Diallyl (4-(hydroxymethyl)phenyl) phosphate (284 mg, 1 mmol, 1 eq.) was then added and the reaction mixture was refluxed for 2 hours. After cooling to room temperature, the solvent was removed *in vacuo*. Purification *via* silica gel column chromatography (hexane 1:1 ethyl acetate (R_f = 0.45, $KMnO_4$)) gave the title compound as a dark orange oil (377 mg, 74%).

^{1}H NMR (300 MHz, C_6D_6); δ 7.26 (2H, d, *J* = 8.3 Hz), 7.12 (2H, d, *J* = 8.3 Hz), 6.81 (1H, br s), 5.71-5.58 (2H, m), 5.14 (2H, dd, *J* = 17.1, 1.3 Hz), 4.99 (2H, s), 4.92 (2H, dd, *J* = 10.4, 1.3 Hz), 4.47-4.33 (4H, m), 4.10 (5H, s), 3.81 (2H, s).

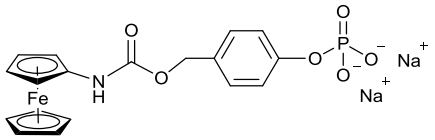
^{13}C NMR (75.5 MHz, C_6D_6); δ 154.1, 150.9 (d, $J_{C\text{-}P}$ = 7 Hz), 134.3, 132.5 (d, $J_{C\text{-}P}$ = 7 Hz), 130.1, 120.5 (d, $J_{C\text{-}P}$ = 5 Hz), 118.4, 97.0, 69.5, 68.9 (d, $J_{C\text{-}P}$ = 6 Hz), 65.9, 64.5, 60.9.

^{31}P NMR (121.5 MHz, C_6D_6); δ − 4.70.

IR (film, cm^{-1}); 3267, 3094, 2953, 1726, 1558, 1508, 1259, 1219, 1017, 951, 818.

HRMS (ESI); calc'd for $C_{24}H_{26}FeNO_6P$ $[M+Na]^+$: *m/z* 534.0745, found 534.0760.

Procedure for the synthesis of sodium 4-(((ferrocenylcarbamoyl)oxy)methyl)phenyl phosphate (1)



4-((bis(allyloxy)phosphoryl)oxy)benzyl ferrocenylcarbamate (377 mg, 0.74 mmol, 1 eq.) was dissolved in anhydrous THF (7.5 mL) and cooled to 0 °C. Polymer-bound tetrakis(triphenylphosphine)palladium (11 mg, 7.4 µmol, 0.01 eq.) was then added, followed by formic acid (0.4 mL, 11.1 mmol, 15 eq.) and anhydrous TEA (1.0 mL, 7.4 mmol, 10 eq.). The reaction mixture was allowed to warm to room temperature and left to stir overnight (~16 hours), after which the reaction mixture was filtered and the filtrate was concentrated *in vacuo*. To the residue was added 1M sodium hydroxide solution (8.8 mL, 8.8 mmol, 12 eq.) at 0 °C. The reaction mixture was allowed to warm to room temperature and stir for an hour before the solvent was removed *in vacuo*. Purification *via* preparative reverse phase (C18) column chromatography (water) gave the title compound as an amorphous orange solid (241 mg, 69%).

^{1}H NMR (300 MHz, D_2O); δ 7.32 (2H, d, *J* = 8.3 Hz), 7.17 (2H, d, *J* = 8.3 Hz), 5.05 (2H, s), 4.47 (2H, s), 4.17 (5H, s), 4.03 (2H, s).

^{13}C NMR (75.5 MHz, D_2O); δ 156.5, 154.2 (d, $J_{C\text{-}P}$ = 6 Hz), 130.3, 129.5, 120.8 (d, $J_{C\text{-}P}$ = 5 Hz), 95.1, 69.7, 67.2, 65.2, 61.8.

^{31}P NMR (121.5 MHz, D_2O); δ 0.95.

IR (solid, cm^{-1}); 1687, 1558, 1510, 1391, 1357, 1233, 1071, 896, 807.

HRMS (ESI); calc'd for $C_{18}H_{18}FeNO_6P$ $[M-H]^-$: *m/z* 430.0143, found 430.0180.

Method for the electrochemical detection of alkaline phosphatase *(optimised conditions)*

A 100 µM stock solution of substrate **1** was prepared using 50 mM pH 9 tris buffer. 500 µL of the stock solution of **1** was added to 500 µL buffered (50 mM pH 9 tris buffer) solution of alkaline phosphatase in a small screw top vial equipped with a small magnetic stirrer. The vial was then placed in a DrySyn® block warmed to 37 °C and stirred. Every 3 minutes for 30 minutes thereafter, a 20 µL sample was subjected to electrochemical analysis.

Method for the electrochemical detection of C-reactive protein (CRP)

To a microtiter well coated with affinity purified sheep anti-CRP antibodies was added 50 µL of a standard concentration of CRP and incubated at room temperature for 60 minutes. After washing, 100 µL of affinity purified sheep anti-CRP antibodies labelled with alkaline phosphatase was added and incubated at room temperature for 60 minutes. After washing, the well was washed further with 50 mM pH 9 tris buffer. 100 µL of a 0.5 mM solution of substrate **1** was added and the well was incubated at 37 °C for 30 minutes. After which, a 20 µL sample was subjected to electrochemical analysis.

Determination of ALP LOD

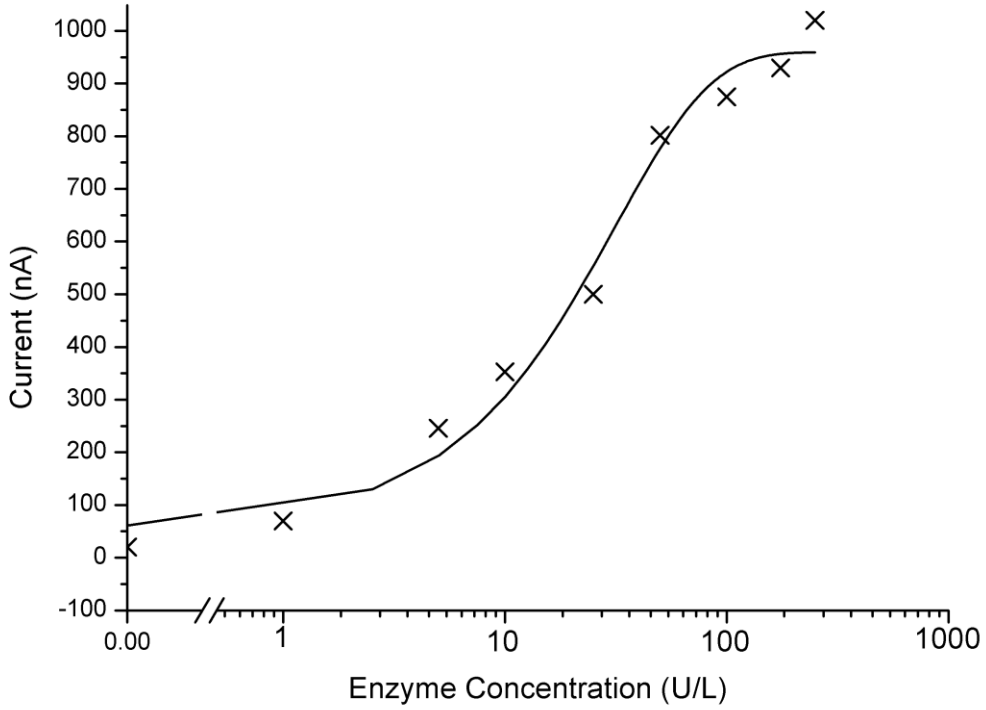


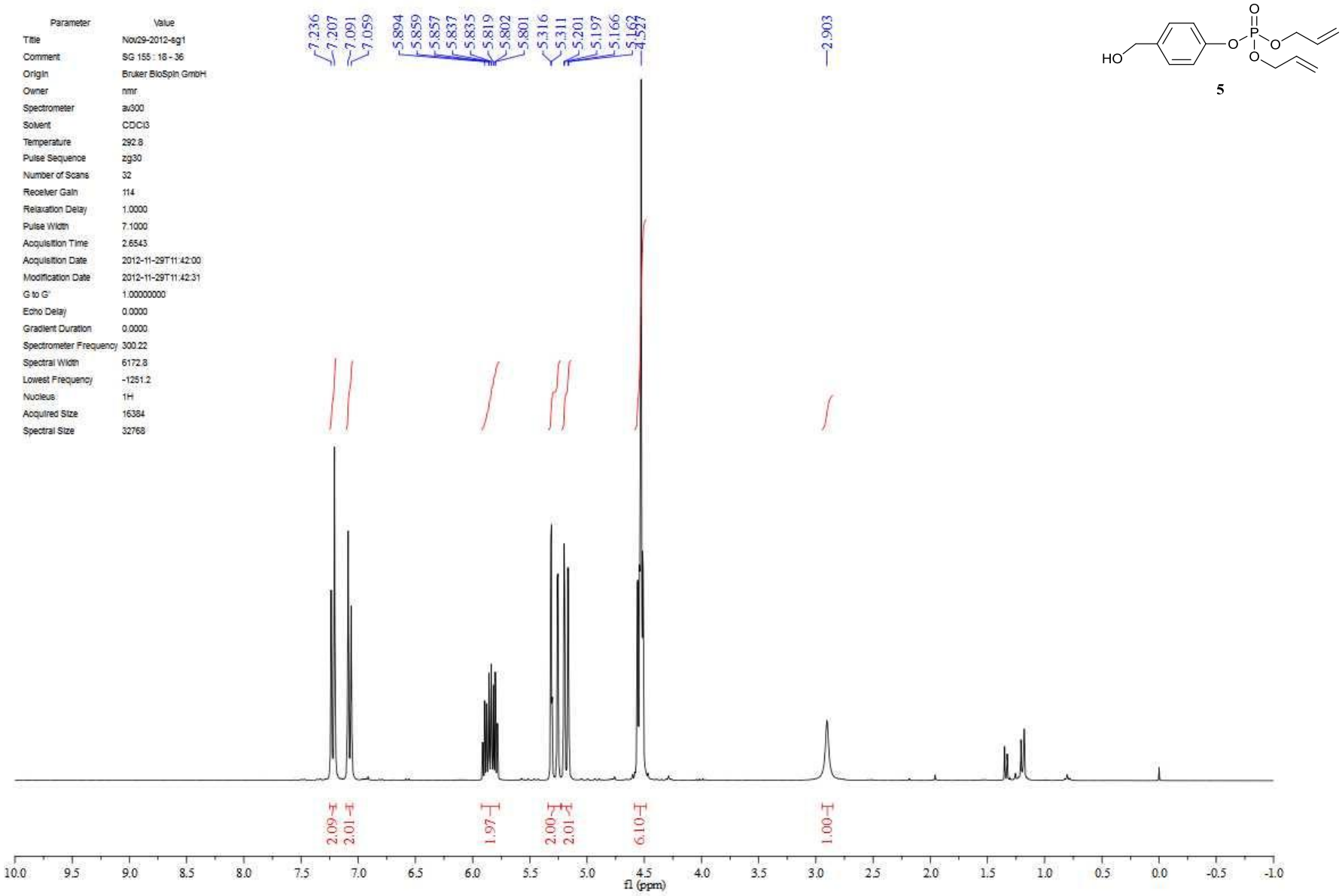
Fig S1 Amperometric response of ferrocenylamine released from substrate 1 (50 µM) after the addition of various concentrations of ALP after incubation at 37 °C for 27 minutes.

Calculation of LOD

Curve fit equation $y = y^0 + A \times e\ (R^0 \times x)$ where; A = –899.22191, R^0 = -0.0318, y^0 = 959.78715. R^2 = 0.98051. When y = LOD (3 × StDev) + Mean (48.6 nA) then x = 0.415 UL^{-1} which equates to 259 fmol assuming MW of ALP is 160000 Da.

References:

1. H.E. Gottlieb, V. Kotlyar and A. Nudelman, *J. Org. Chem.*, 1997, **62**, 7512.
2. *Purification of Laboratory Chemicals* 3rd ed., C. L. L. Chai and W. L. F. Amarego, Pergamon Press, Oxford, 1988.
3. J. Lapić, G. Pavlović, D. Siebler, K. Heinze and V. Rapić, *Organometallics*, 2008, **27**, 726.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5.316
5.311
5.201
5.197
5.166
5.162
4.527
2.903
HO
O
P
O
O
O
5
2.09
2.01
1.97
2.00
2.01
6.10
1.00
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
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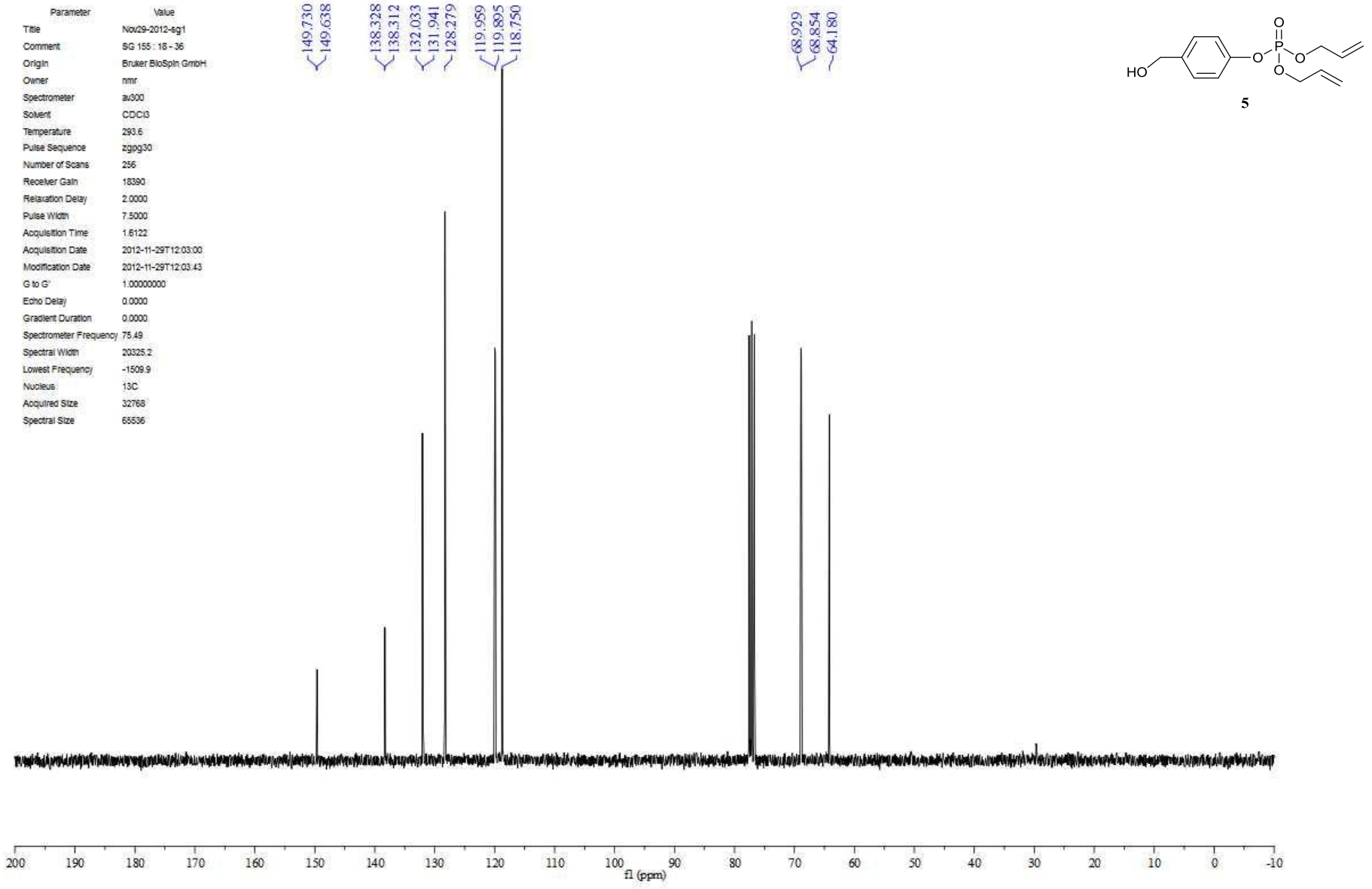
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O
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5
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f1 (ppm)

Parameter	Value
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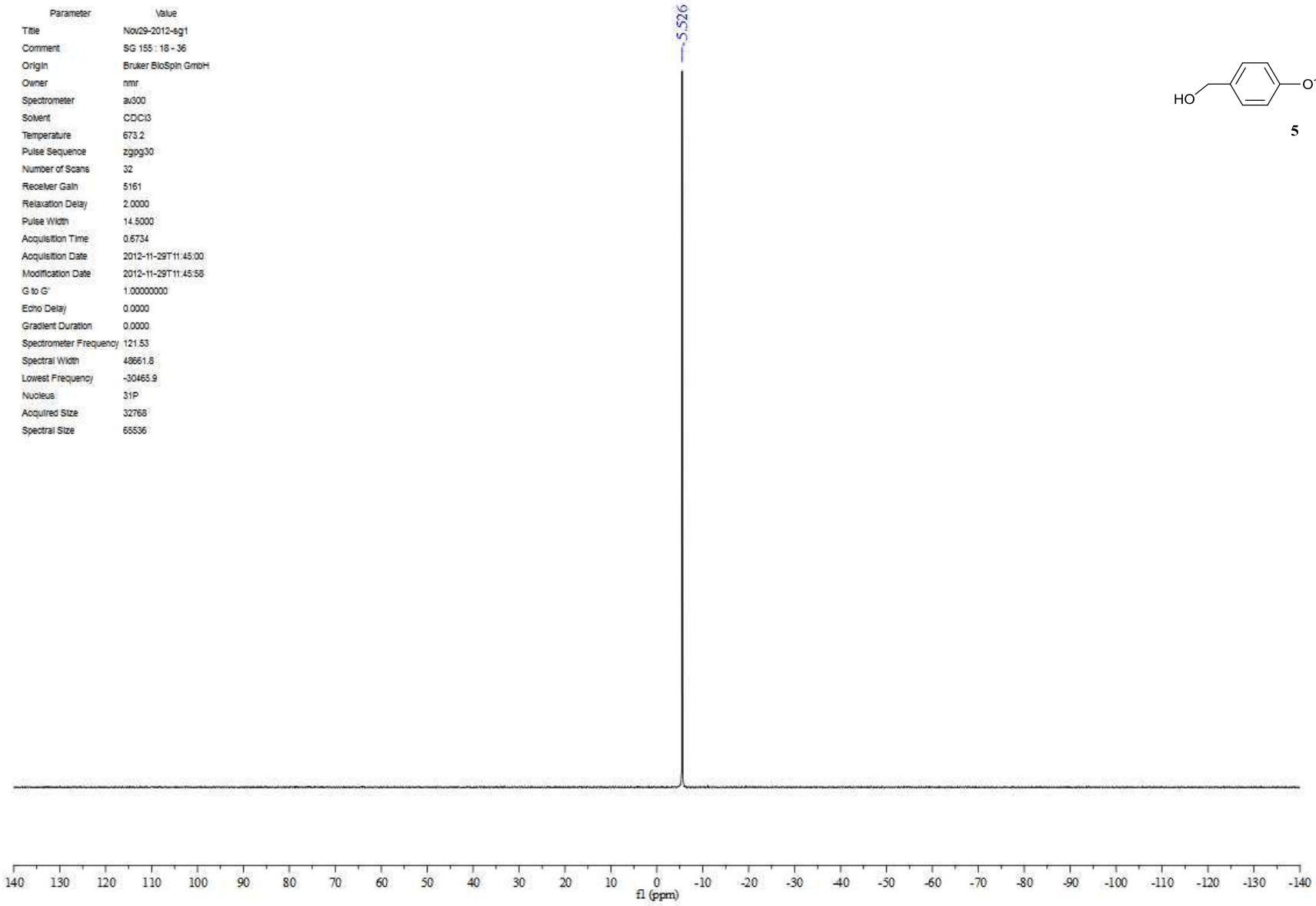

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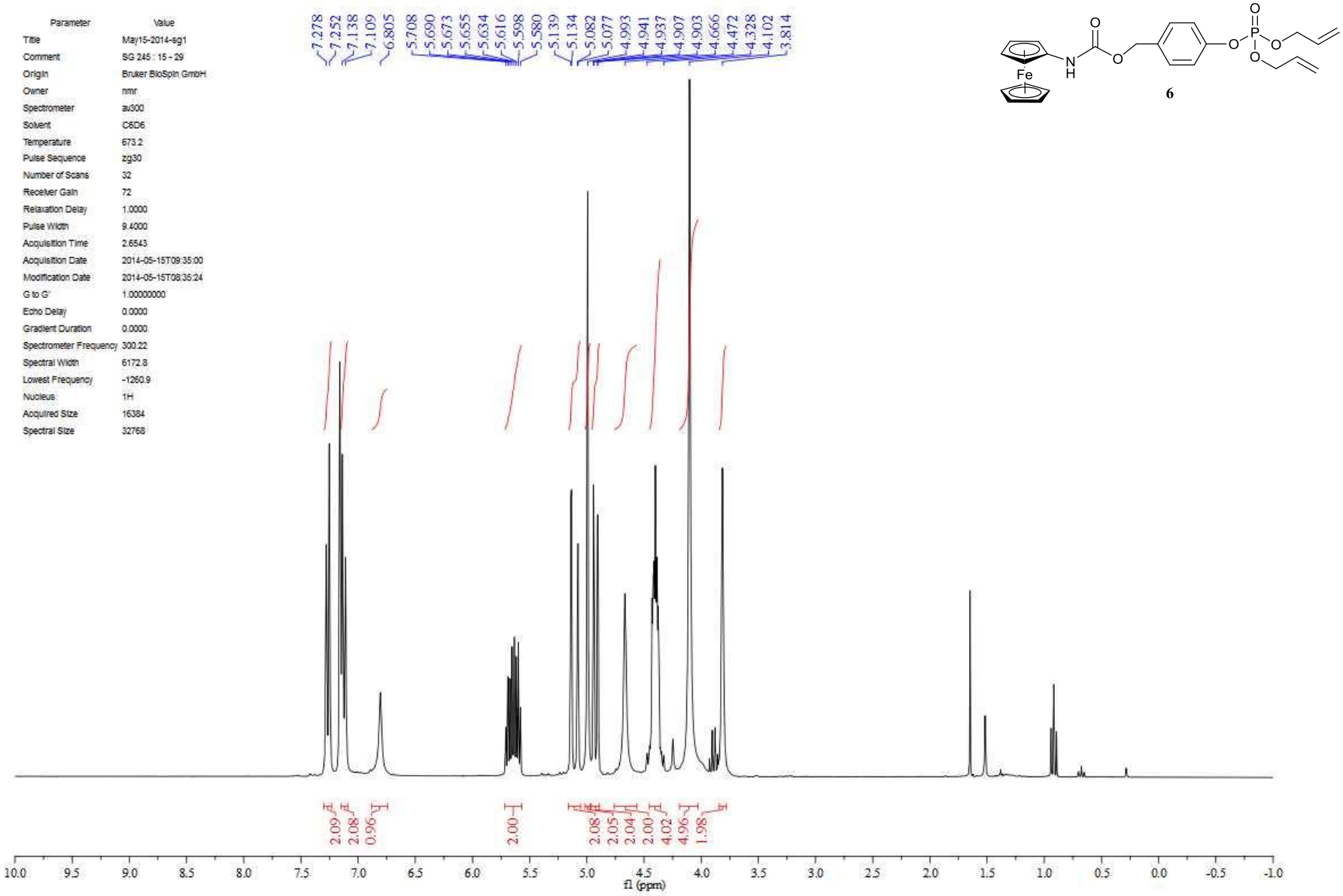
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Acquired Size 16384
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5.598
5.580
5.139
5.134
5.082
5.077
4.993
4.941
4.937
4.907
4.903
4.666
4.472
4.328
4.102
3.814
2.09
2.08
0.96
2.00
2.08
2.05
2.04
2.00
4.02
4.96
1.98
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
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H
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P
O
O
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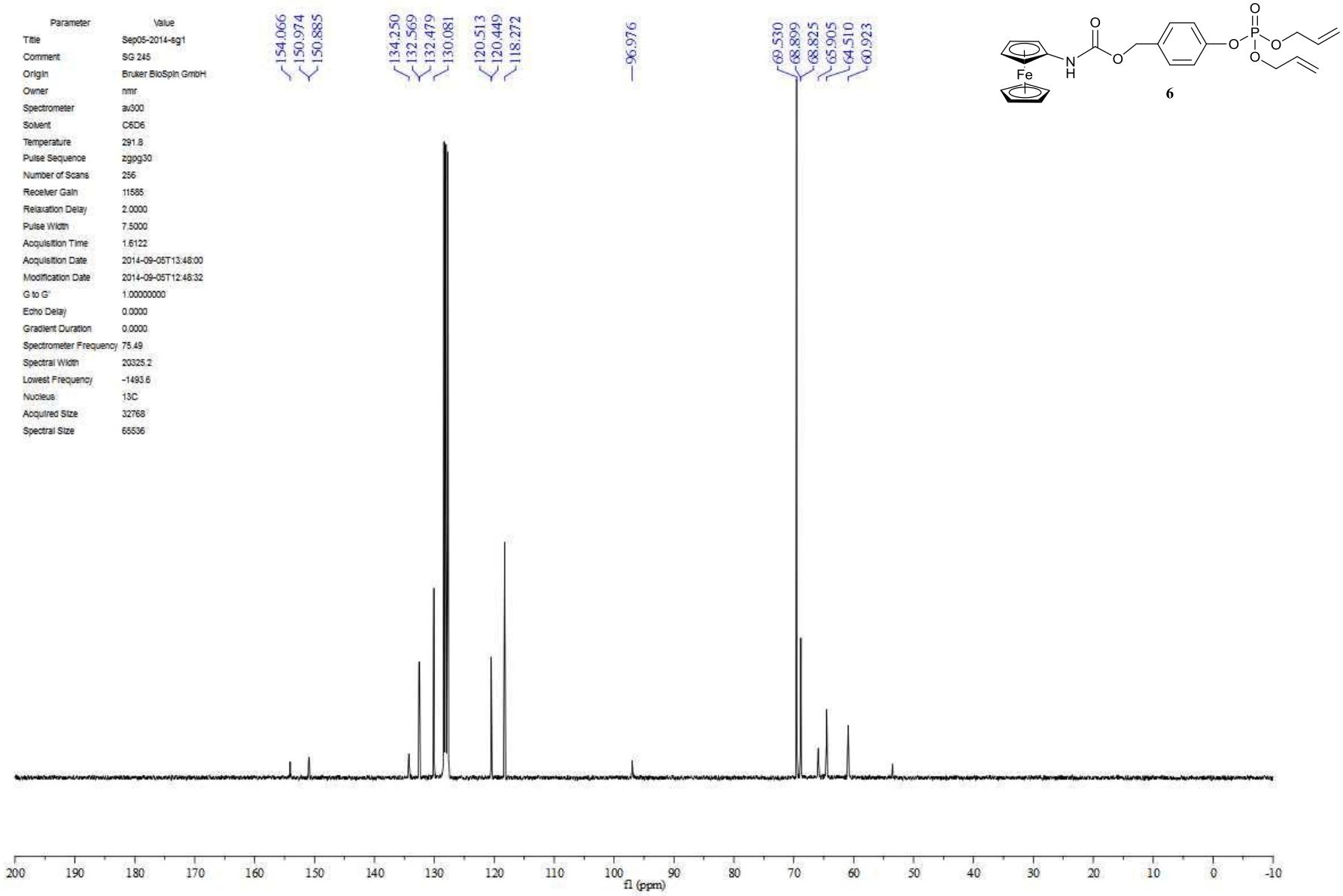
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Acquired Size 32768
Spectral Size 65536
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150.885
134.250
132.569
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f1 (ppm)

Parameter	Value
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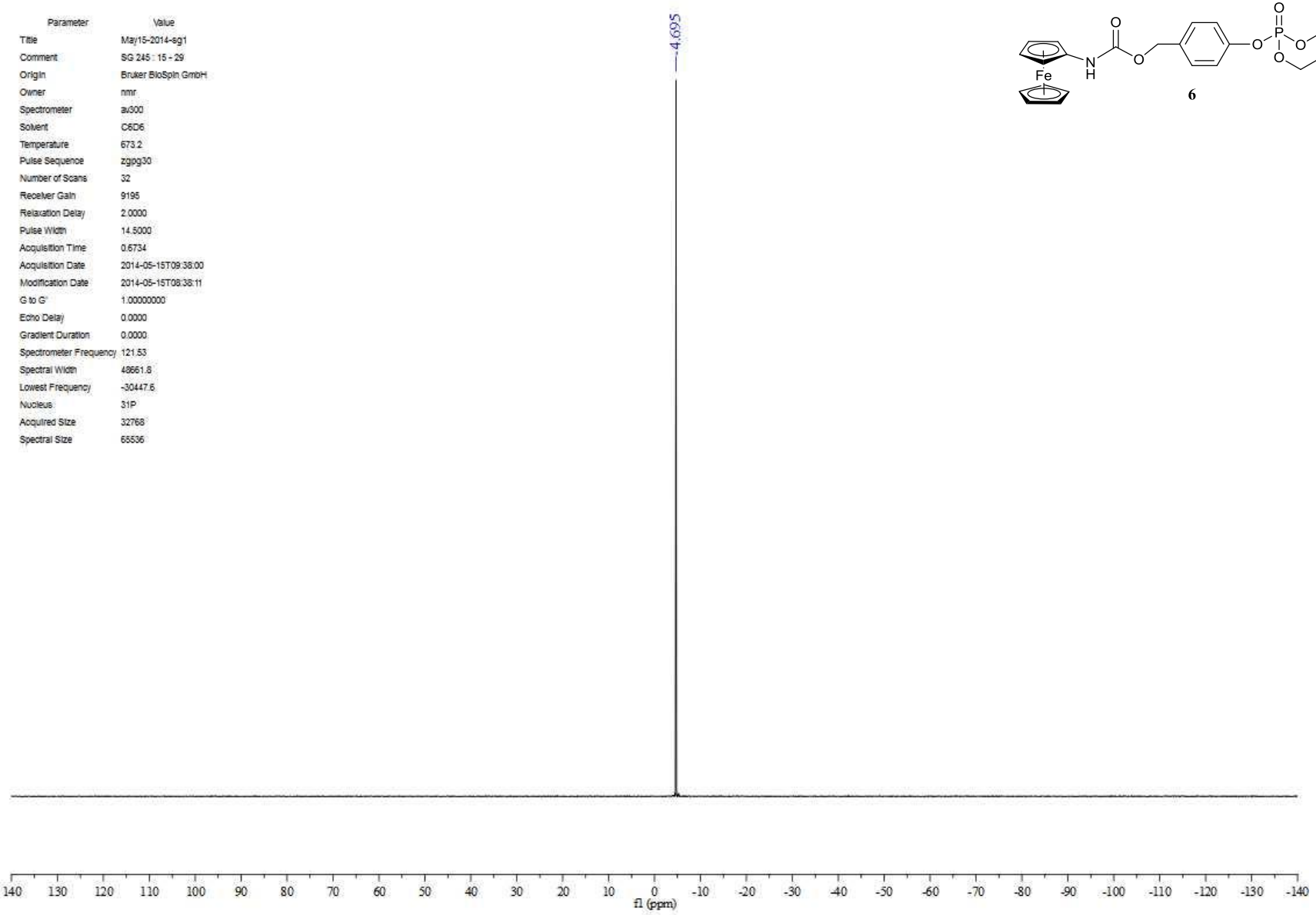

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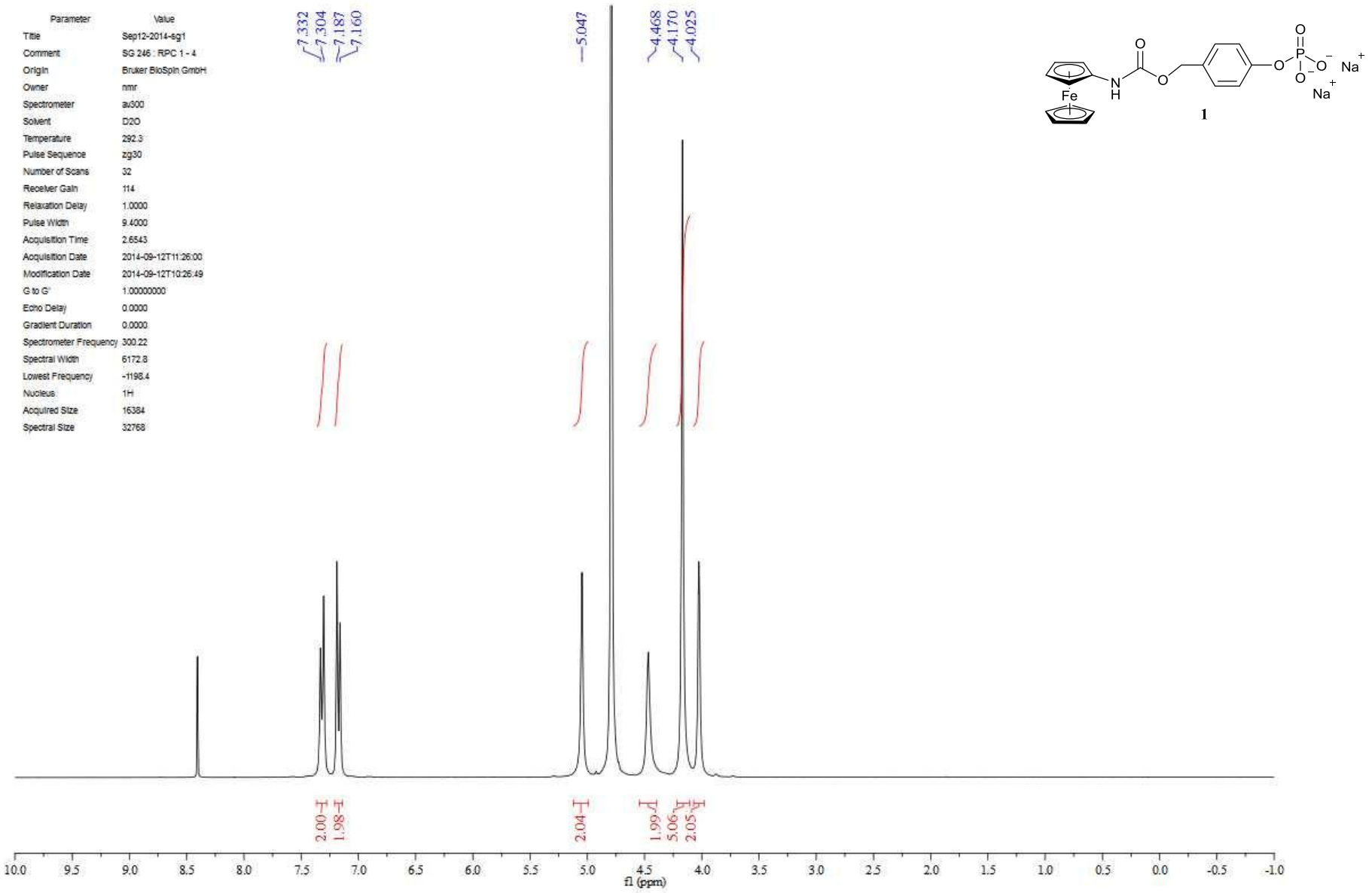
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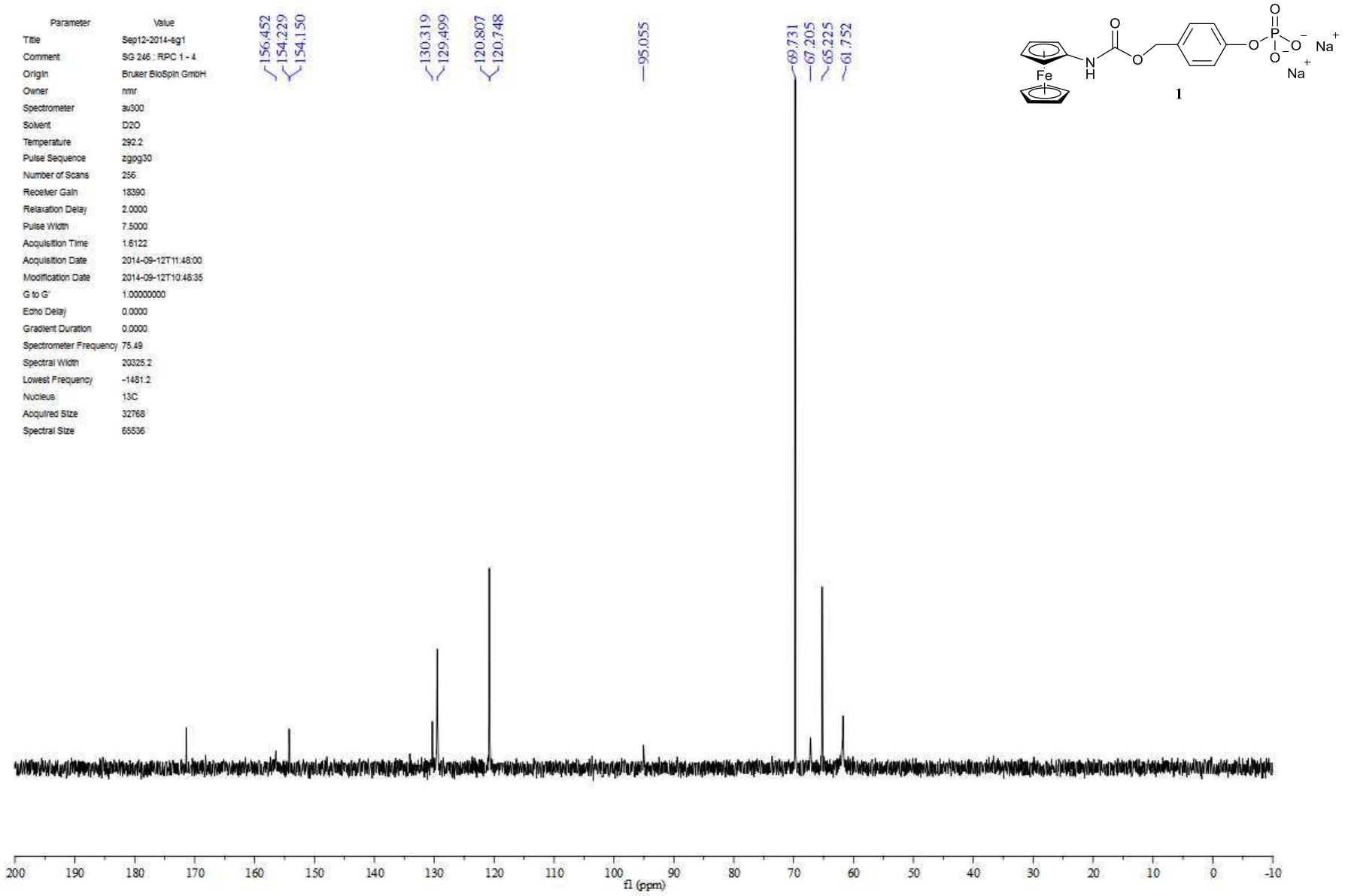
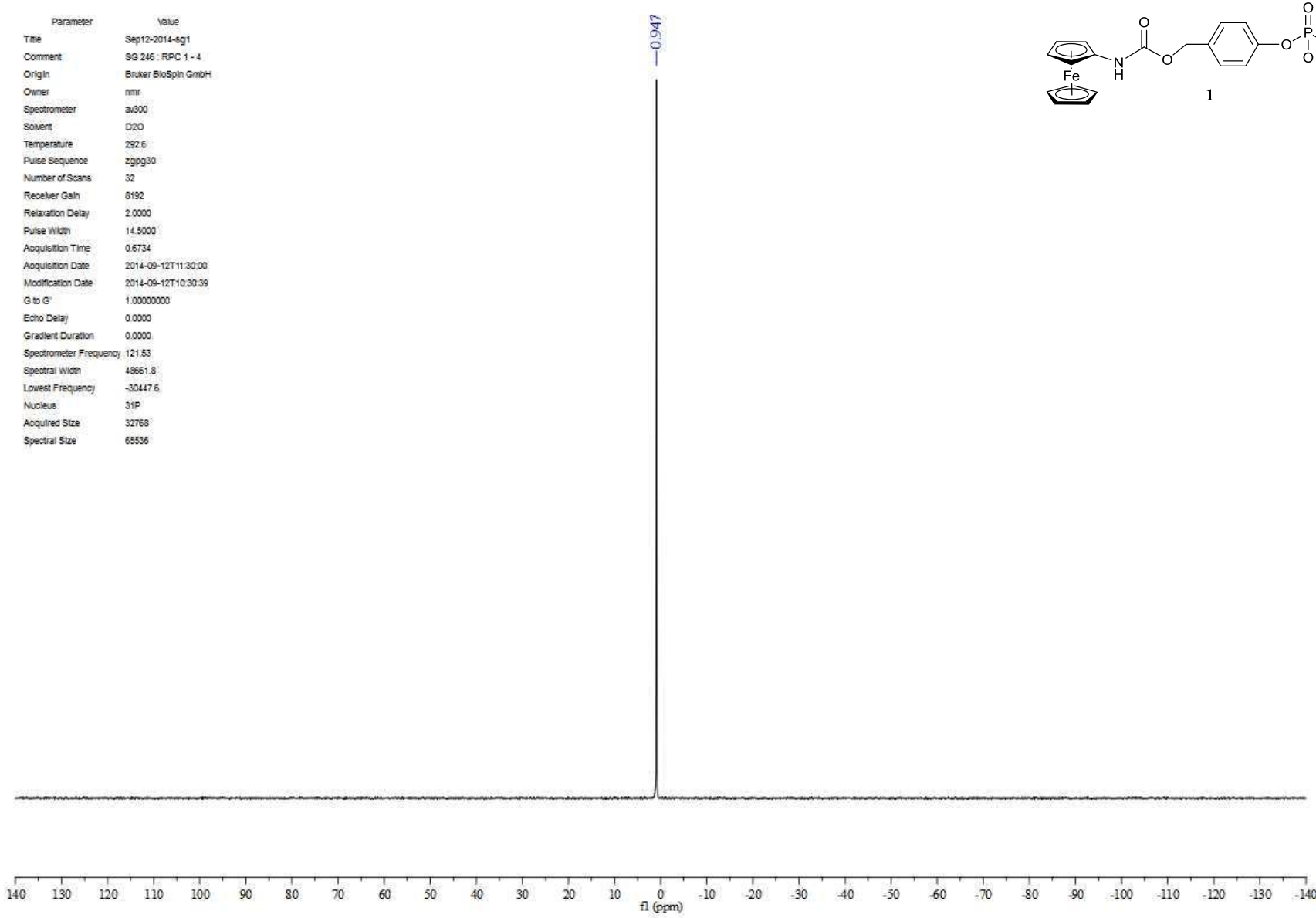
Comment SG 246 : RPC 1 - 4
Origin Bruker BioSpin GmbH
Owner nmr
Spectrometer av300
Solvent D2O
Temperature 292.3
Pulse Sequence zg30
Number of Scans 32
Receiver Gain 114
Relaxation Delay 1.0000
Pulse Width 9.4000
Acquisition Time 2.6543
Acquisition Date 2014-09-12T11:26:00
Modification Date 2014-09-12T10:26:49
G to G' 1.00000000
Echo Delay 0.0000
Gradient Duration 0.0000
Spectrometer Frequency 300.22
Spectral Width 6172.8
Lowest Frequency -1196.4
Nucleus 1H
Acquired Size 16384
Spectral Size 32768
7.332
7.304
7.187
7.160
5.047
4.468
4.170
4.025
2.00
1.98
2.04
1.99
5.06
2.05
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
f1 (ppm)
Fe
N
H
O
O
O
P
O
O
Na
Na
1


Parameter Value
Title Sep12-2014-sg1
Comment SG 246 : RPC 1 - 4
Origin Bruker BioSpin GmbH
Owner nmr
Spectrometer av300
Solvent D2O
Temperature 292.2
Pulse Sequence zgpg30
Number of Scans 256
Receiver Gain 18390
Relaxation Delay 2.0000
Pulse Width 7.5000
Acquisition Time 1.6122
Acquisition Date 2014-09-12T11:48:00
Modification Date 2014-09-12T10:48:35
G to G' 1.00000000
Echo Delay 0.0000
Gradient Duration 0.0000
Spectrometer Frequency 75.49
Spectral Width 20325.2
Lowest Frequency -1481.2
Nucleus 13C
Acquired Size 32768
Spectral Size 65536
156.452
154.229
154.150
130.319
129.499
120.807
120.748
95.055
69.731
67.205
65.225
61.752
Fe
N
H
O
O
O
P
O
O
Na
Na
1
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

Parameter	Value
Title	Sep12-2014-sg1
Comment	SG 246 : RPC 1 - 4
Origin	Bruker BioSpin GmbH
Owner	nmr
Spectrometer	av300
Solvent	D2O
Temperature	292.6
Pulse Sequence	zgpg30
Number of Scans	32
Receiver Gain	8192
Relaxation Delay	2.0000
Pulse Width	14.5000
Acquisition Time	0.6734
Acquisition Date	2014-09-12T11:30:00
Modification Date	2014-09-12T10:30:39
G to G'	1.00000000
Echo Delay	0.0000
Gradient Duration	0.0000
Spectrometer Frequency	121.53
Spectral Width	48661.8
Lowest Frequency	-30447.6
Nucleus	31P
Acquired Size	32768
Spectral Size	65536



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