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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 (¹H NMR at 300 MHz, ¹³C NMR at 75.5 MHz and ³¹P NMR at 121.5 MHz). Chemical shifts for protons are reported downfield from tetramethylsilane and are referenced¹ to residual protium in the solvent (¹H NMR: CHCl₃ at 7.26 ppm, C₆H₆ at 7.16 ppm, H₂O at 4.79 ppm). Chemical shifts for carbons are reported in parts per million downfield from tetramethylsilane and are referenced¹ to the carbon resonances of the solvent peak (¹³C NMR: CDCl₃ at 77.0 ppm, C₆D₆ 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 = doublet, 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 v 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/UV₂₅₄ purchased from Macherey-Nagel and visualised by UV light (254 nm) and/or KMnO₄ 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: $Conversion (\%) = \left(\frac{\int 3}{(\int 3 + \int 1)}\right) \times 100.$

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 MgSO₄ 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, C₆D₆); δ 4.74 (2H, app s), 4.02 (2H, app s), 3.91 (5H, s).

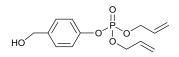
¹³C NMR (75.5 MHz, C₆D₆); 176.3, 97.7, 72.7, 70.7, 70.4.

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 Nchlorosuccinimide (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 \times 50 mL). The combined organics were then washed with water (3 \times 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 $^{\circ}$ 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 Na₂SO₄ 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, CDCl₃); 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).

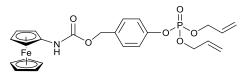
¹³C NMR (75.5 MHz, CDCl₃); δ 149.7 (d, $J_{C-P} = 7$ Hz), 138.3 (d, $J_{C-P} = 1$ Hz), 132.0 (d, $J_{C-P} = 7$ Hz), 128.2, 119.9 (d, $J_{C-P} = 5$ Hz), 118.8, 68.9 (d, $J_{C-P} = 6$ Hz), 64.2.

³¹**P NMR** (121.5 MHz, CDCl₃); δ – 5.52.

IR (film, cm⁻¹); v 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 ($R_f = 0.45$, KMnO₄)) gave the title compound as a dark orange oil (377 mg, 74%).

¹**H NMR** (300 MHz, C₆D₆); 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).

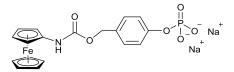
¹³**C NMR** (75.5 MHz, C₆D₆); δ 154.1, 150.9 (d, $J_{C-P} = 7$ Hz), 134.3, 132.5 (d, $J_{C-P} = 7$ Hz), 130.1, 120.5 (d, $J_{C-P} = 5$ Hz), 118.4, 97.0, 69.5, 68.9 (d, $J_{C-P} = 6$ Hz), 65.9, 64.5, 60.9.

³¹**P NMR** (121.5 MHz, C₆D₆); 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, D₂O); 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).

¹³C NMR (75.5 MHz, D₂O); δ 156.5, 154.2 (d, $J_{C-P} = 6$ Hz), 130.3, 129.5, 120.8 (d, $J_{C-P} = 5$ Hz), 95.1, 69.7, 67.2, 65.2, 61.8.

³¹**P NMR** (121.5 MHz, D₂O); 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 μ M stock solution of substrate **1** was prepared using 50 mM pH 9 tris buffer. 500 μ L of the stock solution of **1** was added to 500 μ L 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 μ L 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 μ L of a standard concentration of CRP and incubated at room temperature for 60 minutes. After washing, 100 μ L 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 μ L 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 μ L sample was subjected to electrochemical analysis.

Determination of ALP LOD

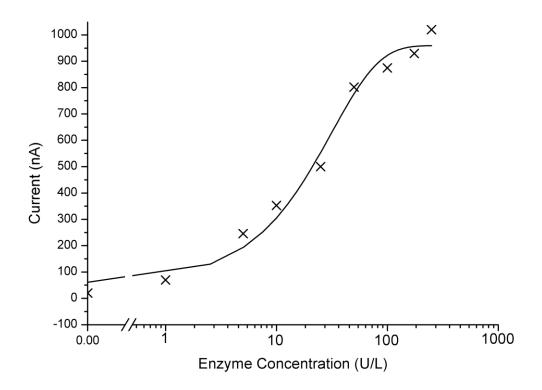


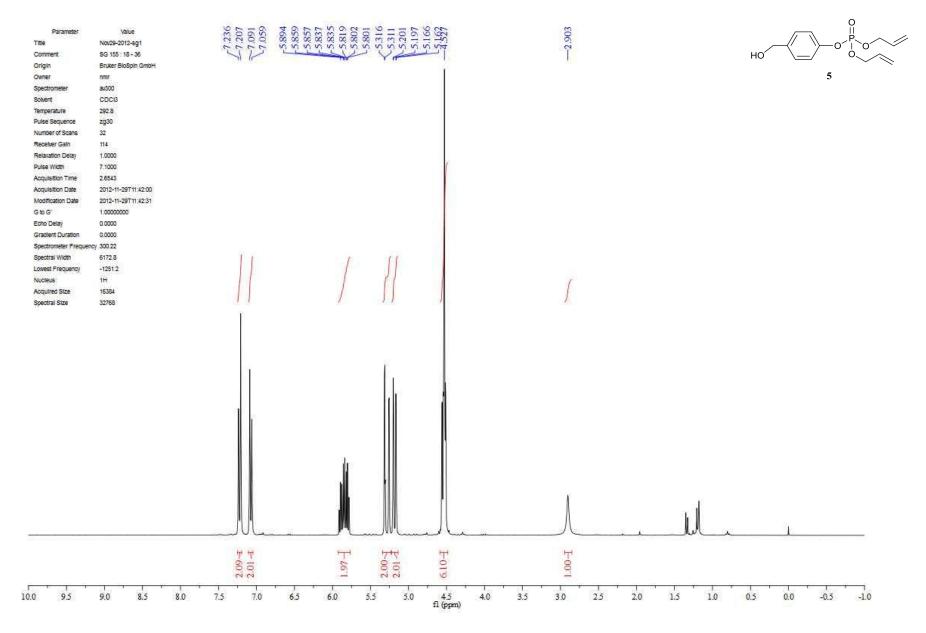
Fig S1 Amperometric response of ferrocenylamine released from substrate 1 (50 μ M) after the addition of various concentrations of ALP after incubation at 37 °C for 27 minutes.

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 $y = LOD (3 \times StDev) + Mean (48.6 nA)$ then $x = 0.415 \text{ UL}^{-1}$ which equates to 259 fmol assuming MW of ALP is 160000 Da.

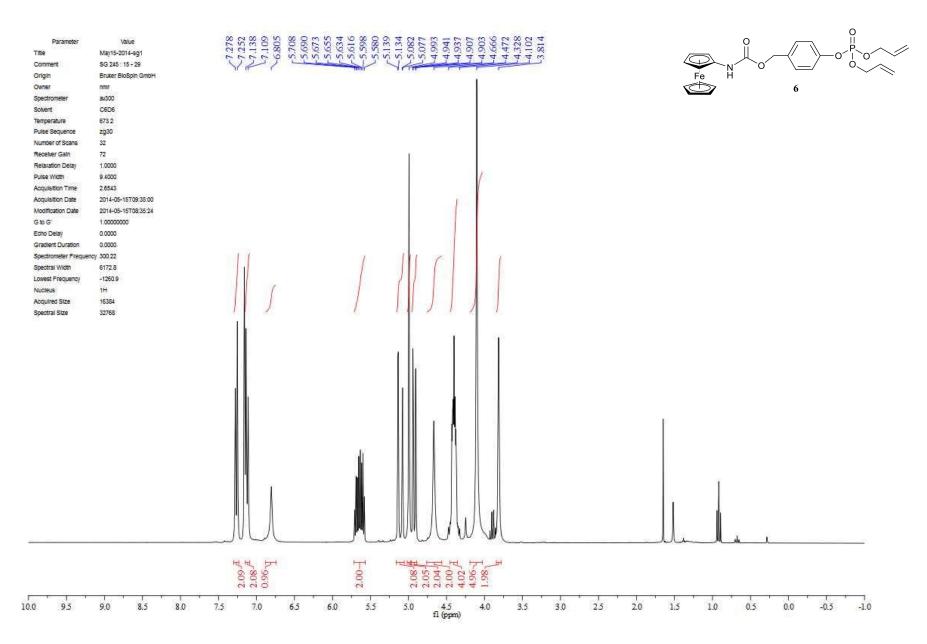
References:

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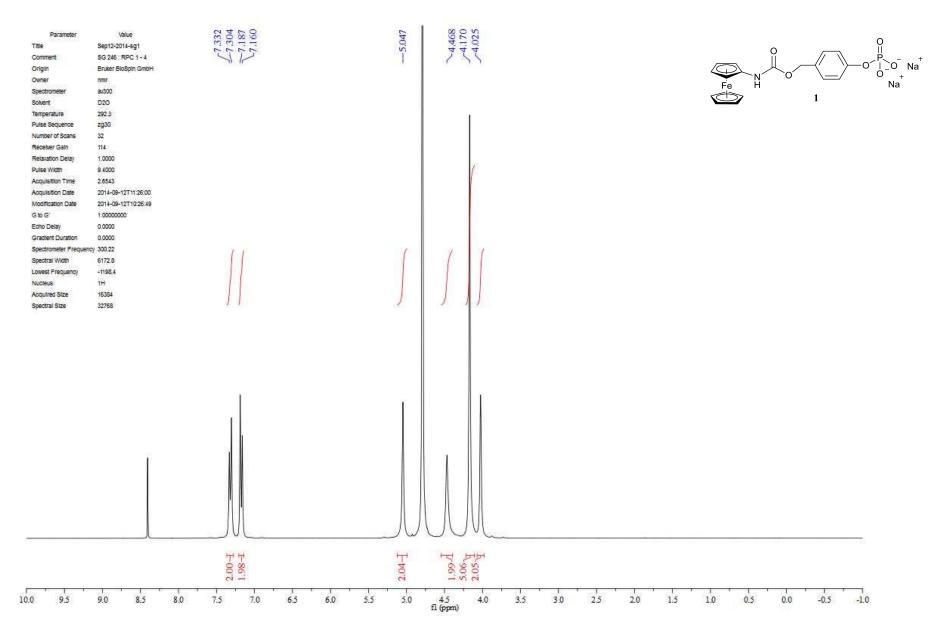
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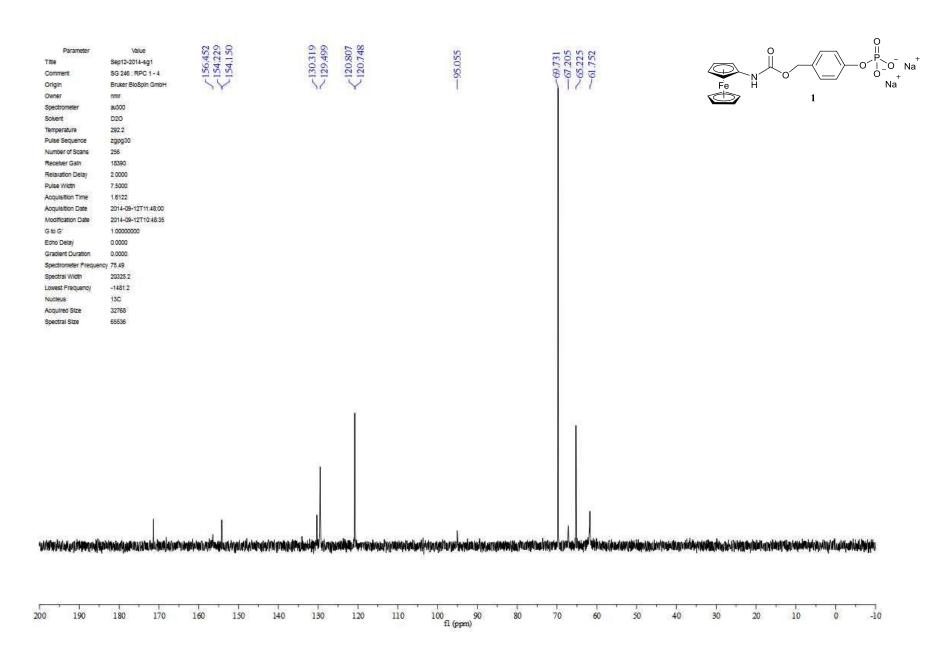


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