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Diazo compounds can be tuned to react selectively with biological phosphates in aqueous buffer

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General Methods

All reagents and solvents used were of analytical grade. Buffers were prepared with nanopure water. All chemicals were purchased from Sigma-Aldrich or TCI and used as received. Peptides were purchased from Bachem, oligonucleotides from Microsynth and used as received without further purification.

^{1}H and ^{13}C NMR were acquired on a Bruker 400 MHz, 500 MHz or AvanceIII+ 600 MHz depending on availability of the machine because photolabile compounds are needed to be measured once obtained. Chemical shifts were referenced to the solvent's residual peak and are reported in ppm.

ESI and ESI-MS-MS spectra were obtained on a Bruker Esquire3000+ spectrometer by direct injection in positive or negative polarity of the ion trap detector. High resolution mass spectra were acquired on a Bruker maXis 4G QTOF ESI mass-spectrometer. MALDI TOF analyses were carried out on a Bruker Microflex mass-spectrometer in linear negative mode using trihydroxyacetophenone as matrix.

Shimadzu preparative HPLC (LC-20AP) equipped with phenomenex column (Gemini® 10 µm C18 110 Å, LC Column 250 x 21.2 mm, AXIA™ Packed) was employed for purification. 100 mM triethylammonium acetate (pH 7.2-7.3) and acetonitrile were used as mobile phases. Flow rate: 20 mL/min, 0% acetonitrile in 2 min, 0-30 % acetonitrile in 22 min. Detection was carried out by monitoring the absorbance of the column effluent at 254 nm. The identity of the product peaks was confirmed by ESI-MS.

The UV measurements were conducted on a Shimadzu UV-1800 UV-Vis spectrophotometer (190-1100 nm, bandwidth 1 nm) using 10 x 10 mm quartz cuvettes from Hellma Analytics (3500 µL).

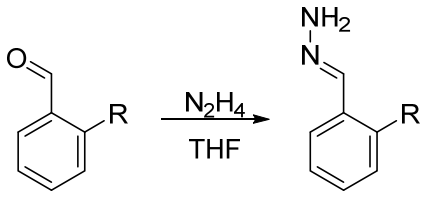
The UV photocleavage reaction was carried out with a CAMAG TLC UV lamp at 366 nm with a sample-lamp distance of ~2 cm.

Alkylation of oligonuceotides was purified and analyzed by HPLC (Agilent 1100 LC system equipped with phenomenex ®Jupiter 3µ C18 300Å, 150x4.6mm column) using 100 mM triethylammonium acetate (pH 7.2-7.3) and acetonitrile as the mobile phase. Flow rate: 1 mL/min. 0-35 % acetonitrile in 12 min, 15 min 80 %. The fractions were analyzed by ESI-MS.

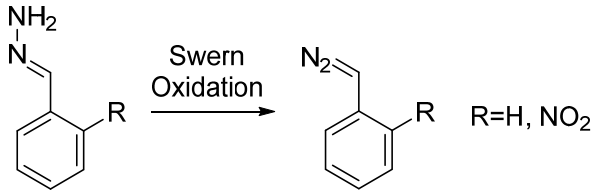
Compeptition alkylations of CMP and benzoic acid were analyzed by UPLC-MS (method A) and peptide alkylation experiments were analyzed by UPLC-MS (method B) using an Agilent 1290 Infinity system, equipped with a Zorbax Eclipse Plus C18 2.1x50 mm column (Agilent), coupled to an Agilent 6130 Quadrupole LC/MS. Elution was performed using 0.1 % formic acid in water/acetonitrile. Method A: flow rate 0.45 mL/min, 5-95 % acetonitrile in 3.5 min; method B: 0% acetonitrile for 0.2 min, 0-54 % acetonitrile in 4 min, 54-95 % acetonitrile in 1 min.

Synthesis of diazo compounds

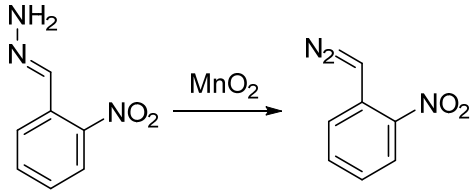
Diazo substrates were synthesized according to published procedures.[1-3] The corresponding aryl aldehyde was first converted into a hydrazone which was then oxidized to the final diazo compound. We tested two oxidation methods: Swern Oxidation and MnO_2 oxidation. They both work well. Oxidations using MnO_2 are fast and simple (~20 min), however during work up the reactive diazo product was found to decompose easily. Swern Oxidation was carried out at -78 °C, and there is less decomposition afterwards, especially for benzyl diazo which is very unstable at room temperature. Once formed, benzyl diazo is used for modification reactions directly after work up and without further purification. 2-nitro-phenyl diazomethane was found to have a higher stability compared to benzyl diazo and thus can be stored for more than 2 weeks at -20 °C.



In a round-bottomed flask containing 12 mL of N_2H_4 (1 M in THF, 12 mmol), a solution of aldehyde (1 M in THF, 10 mmol) was slowly added. The mixture was stirred for 30 min at room temperature. The mixture was evaporated under reduced pressure to affording the desired hydrazone (>95% yield). All hydrazones were used without further purification for the generation of diazo compounds.

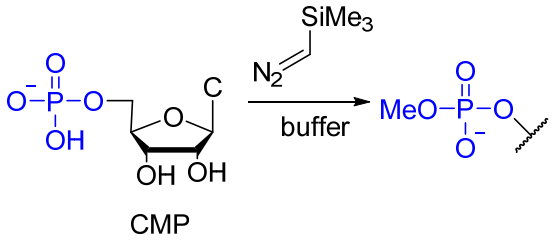


Under a nitrogen atmosphere oxalyl chloride (0.225ml, 1.05 eq.) was added drop wise to a stirred solution of Et_2O (21 mL) containing DMSO (0.195, 1.10 eq.) at -55 °C. After gas evolution ceased (~20 min), the reaction was cooled down to -78 °C. Behind a blast shield, a mixture of Et_3N (0.732ml, 2.10 eq.) and hydrazone (2.50 mmol, 1.00 eq.) in THF (6 mL) was added drop wise over a period of 5-7 min to the activated DMSO solution. An immediate color change and concomitant formation of a white precipitate were observed. The reaction mixture was maintained at -78 °C for ~1 h and allowed to gradually warm up to 0°C followed by the addition of and ice cold half saturated NH_4Cl solution (20ml) was added. The reaction mixture was extracted with cold Et_2O (dry ice was added to reach -30°C, 2 x 50 mL), and the combined organic layers were dried over magnesium sulfate, filtered and concentrated at 15 °C under reduced pressure (>90% yield).



In a 100ml round bottom flask, hydrazone (661 mg, 4.0 mmol, 1.00 eq.) dissolved in 50 mL of $CHCl_3$, was stirred with MnO_2, (2.85 g, 33 mmol, 8.25 eq.) for 5 min in the dark. In the absence of light the mixture was filtered over celite and the filtrate washed with 0.1 M $NaHCO_3$ until pH 7 was reached, dried over $MgSO_4$, filtered and stored at -20°C in a brown glass vial wrapped in black foil.

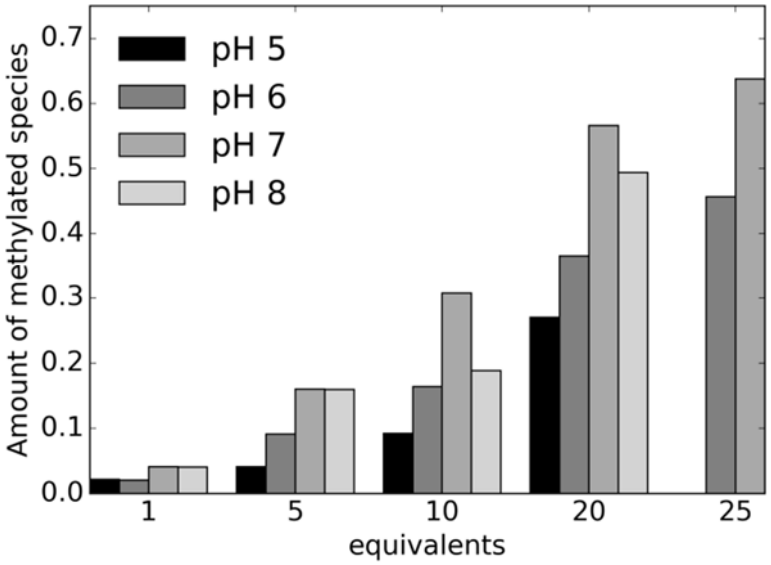
CMP methylation



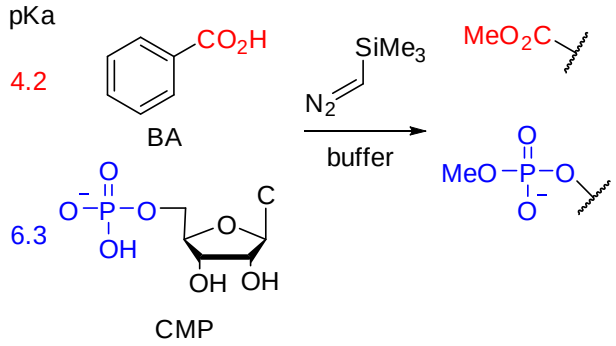
All aqueous buffers were prepared freshly with final concentrations of 200 mM.
Buffer pH 4 and 5: CH_3COOH/CH_3COONa, pH 6: MES/NaOH, pH 7 and 8: MOPS/NaOH.
CMP (20 mg, 54 µmol) was dissolved in the appropriate buffer (2.72 mL) to give a 20 mM stock solution. Then, different equivalents of $TMSCHN_2$ (2.0 M in hexane) were added. Upon disappearance of the yellow layer, UPLC-MS analysis (Method A) was performed. The results are summarized in table S1.

Table S1 CMP methylation conversions at different pH with $TMSCHN_2$

pH	Equiv. Diazo	Conv.
5	1	2%
	5	4%
	10	9%
	20	27%
6	1	21%
	5	9%
	10	16%
	20	36%
	25	37%
	35	46%
7	1	41%
	5	16%
	10	31%
	20	57%
	25	64%
8	1	41%
	5	16%
	10	19%
	20	49%



Methylation selectivity towards CMP and benzoic acid with $TMSCHN_2$



CMP (20 µL, 100 mM in water), benzoic acid (20 µL, 100 mM in CH_3CN), buffer (60 µL, 200 mM) were added into an Eppendorf vial. The vial was closed, vortexed for 5 s and then centrifuged for 10 s. Then $TMSCHN_2$ (2.0 M in hexane, 20 µL) was added and the mixture allowed to react without agitation at room temperature. After the yellow color disappeared, the reaction was analyzed by UPLC-MS (method A). The peak areas of CMP and methylated CMP were measured at 254 nm. The peak areas of benzoic acid and methyl benzoate were measured at 220 nm. The results are summarized in table S2.

Table S2 Competition experiments at varying pH towards diazo compounds

pH	CMP conv.	ø	SD	BA conv.	ø	SD
	[%]					
4	16.1 15.2 14.5	15.2	0.767	33.0 34.9 33.9	34.3	1.49
5	31.0 31.1 30.8	30.9	0.154	23.4 26.2 28.1	25.9	2.37
6	41.8 41.4 41.4	41.6	0.321	7.7 7.6 9.1	8.1	0.86
7	50.9 56.0 46.1	51.0	4.93	10.1 10.9 10.7	10.6	0.356
8	53.8 61.4 49.2	54.9	6.05	6.5 6.4 6.6	6.5	0.056

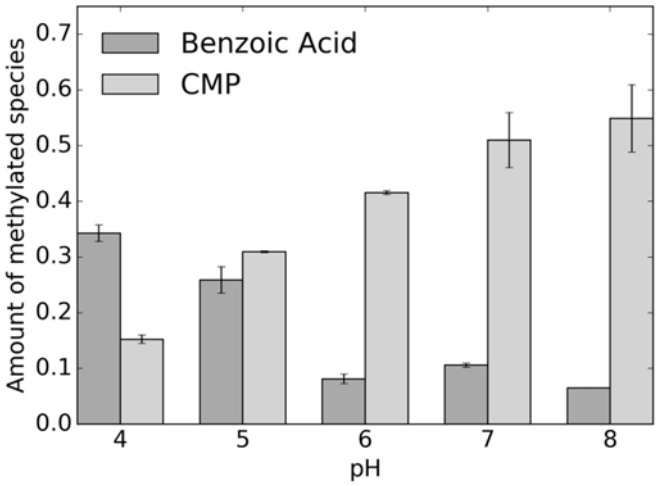


Figure S1 competition experiments at varying pH towards diazo compounds

Decomposition of 2-nitrophenyldiazomethane at different pH

The following stock solutions were freshly prepared:

2-Nitrophenyldiazomethane, 10mM in CH_3CN

pH 5 buffer: Acetate-NaOH, 200 Mm

pH 6 buffer: MES-NaOH, 200 mM

pH 7 buffer: MOPS-NaOH, 200 mM

Background of the UV measurement

2 mL buffer and 0.5 mL CH_3CN were used for background measurement, cuvette (d =1 mm).

Measurements at pH 6 and 7

2 mL buffer and 0.45 mL CH_3CN was put into a UV measuring cuvette, 450 µL CH_3CN was added. Then, 50 µL diazo (which was kept on ice during the procedure) was added. The cuvette was gently shaken and immediately transferred to the cuvette holder. The decay in absorption was measured at 400 nm for 3600 s at an interval of 0.5 s.

Measurement at pH 5

2 mL buffer was put into a UV measuring cuvette and the cuvette was put into the photometer ready for the UV measurement. Diazo was added (500 µL) and at the same time the UV measurement was started. The absorption was recorded at 400 nm for 500 s at an interval of 0.5 s. The amount of diazo was increased in an attempt to give a measurable decay curve, but as the data show the compound is largely consumed on the first measurement.

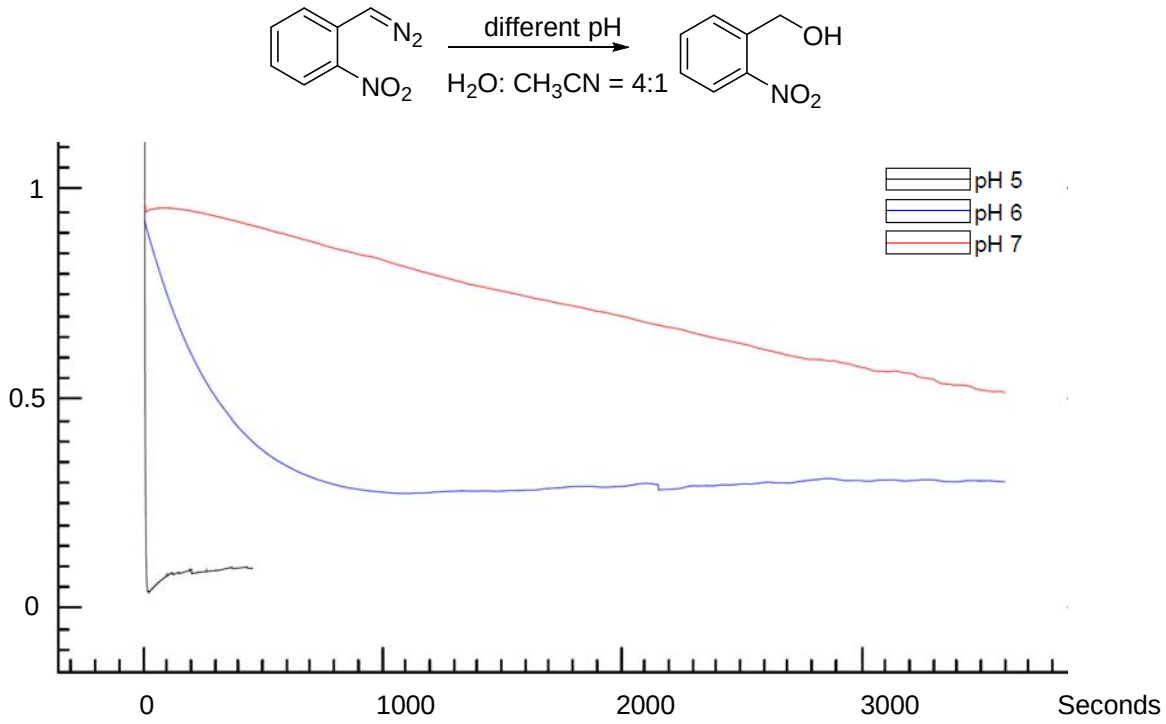
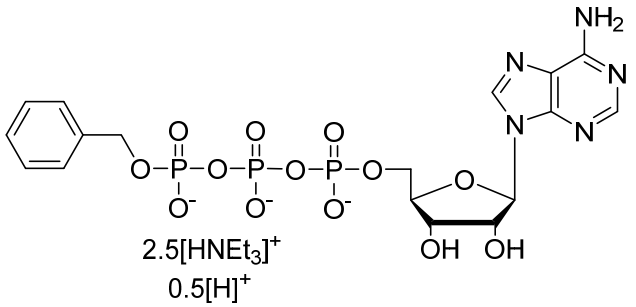


Figure S2 Decomposition of 2-nitrophenyldiazomethane at different pH

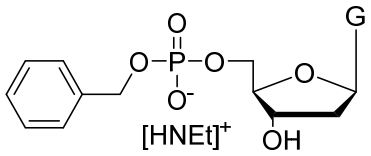
Modification of bioactive phosphate compounds

In a round bottomed flask a 50 mM stock solution of phosphate derivative (~50 mg dissolved in 250 mM MES buffer pH 6) was prepared. To the solution was added freshly prepared phenyl diazomethane (as a solid, 10 eq.) whereupon gas bubbles were formed immediately. The reaction mixture was left for 30 min at room temperature until gas evolution ceased. The side-products were very sticky to the glass wall and the clear solution was purified by preparative HPLC to afford the pure product.

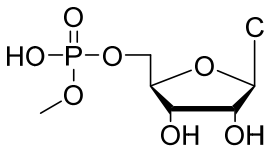
In some cases, the starting material phosphate monoester was only supplied as RPO_4-xH_2O hydrate compounds. The amount of water is unclear. They were co-evaporated with CH_3CN three times to remove the water (100 mg phosphate monoester in 10 ml CH_3CN), Afterwards they were used as anhydrous form to calculate the exact amount of phosphate compound.



40 mg ATP sodium salt was dissolved in 1.45 mL of 250 mM MES buffer pH 6, and freshly prepared benzyl diazo (90 mg) was added to the reaction solution. Gas was formed immediately. The reaction mixture was left for 30 min until the gas formation ceased. The side-products stuck to the glassware and the clear solution was purified by preparative HPLC. The desired product eluted at 15 min. The product fractions were combined and lyophilized to afford the desired product (43 mg, 46%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.84 (s, 2H), 8.48 (s, 1H), 8.14 (s, 1H), 7.37 – 7.26 (m, 6H), 7.24 (m, 1H), 5.91 (d, J_{HH} = 5.1 Hz, 1H), 4.88 (dd, J_{HP} = 6.3, 1.9 Hz, 2H), 4.58 (t, J_{HH} = 5.0 Hz, 1H), 4.34 (dd, J_{HH} = 17.7, 13.4 Hz, 1H), 4.10 – 3.99 (m, 3H), 3.04 – 2.88 (m, 16H), 1.12 (t, J_{HH} = 7.2 Hz, 25H). ^{31}P NMR (202 MHz, DMSO-d_6) δ -12.33, -12.43, -23.47. ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.94, 152.56, 149.51, 139.23, 127.95, 127.03, 118.79, 86.97, 83.35 (d, $^{3}J_{CP}$ = 8.08 Hz), 73.92, 69.97, 66.28 (d, $^{2}J_{CP}$ = 5.35 Hz), 64.42 (d, $^{2}J_{CP}$ = 3.23 Hz), 45.00, 8.61. HR-ESI (Et_3N salt): Exact Mass calcd. for $C_{23}H_{37}N_6O_{13}P_3$ [M·NEt_3+H]$^{+}$: 699.1631, found: 699.1701.

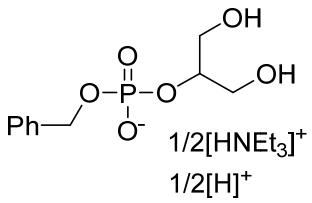


50 mg deoxyguanosine monophosphatephospahte disodium salt was dissolved in 2.5 mL of 250 mM MES buffer pH 6, and freshly prepared benzyl diazo (151 mg) was added to the reaction solution, and gas was formed immediately. The reaction mixture was left for half an hour until the gas formation ceased. The side-products stuck to the glassware and the clear solution was purified by preparative HPLC. The desired product eluted at 15 min. The product fractions were combined and lyophilized to afford the desired product (30 mg, 51%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.69 (s, 1H), 7.92 (s, 1H), 7.36-7.27 (m, 4H), 7.24 – 7.20 (m, 1H), 6.60 (s, 2H), 6.12 (t, J_{HH} = 6.9 Hz, 1H), 5.49 (d, J_{HH} = 2.8 Hz, 1H), 4.71 (d, J_{HP} = 6.6 Hz, 2H), 4.42 (s, 1H), 3.89-3.92 (m, 2H), 3.83 – 3.72 (m, 1H), 2.90 (s, 7H), 2.61 – 2.52 (m, 1H), 2.19 (ddd, J_{HH} = 13.0, 6.1, 3.1 Hz, 1H), 1.11 (t, J_{HH} = 7.0 Hz, 11H). ^{31}P NMR (202 MHz, DMSO-d_6) δ -0.70 (s). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.77, 153.67, 150.88, 139.44 (d, $^{3}J_{CP}$ = 7.57 Hz) , 135.45, 128.01, 126.98, 116.68, 86.07 (d, $^{3}J_{CP}$ = 7.37 Hz) 82.77, 71.22, 65.90 (d, $^{2}J_{CP}$ = 5.35 Hz), 64.80 (d, $^{2}J_{CP}$ = 5.15 Hz), 45.36, 8.78. HR-ESI (Et_3N salt): Exact Mass calcd. for $C_{23}H_{36}N_6O_7P$ [M·Et_3N+H]$^{+}$: 539.2378, found: 539.2378.

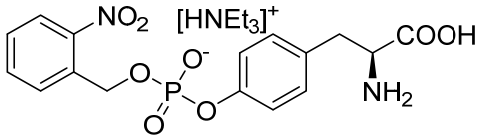


CMP (22 mg, 60 μmol) was dissolved in 1.2 mL of 250 mM MES buffer pH 6. TMS diazomethane was added (100 μL, 200 μmol, 2M in hexane, 3.33 eq.). It was stirred for 20 min at room temperature. According to HPLC, only a minor amount of product has formed. Additional TMS-diazomethane was added (200 μL, 400 μmol, 2M in hexane, 6.67 eq.) and the reaction was stirred until the yellow diazo colour disappeared. The reaction mixture was lyophilized and purified by preparative HPLC. The

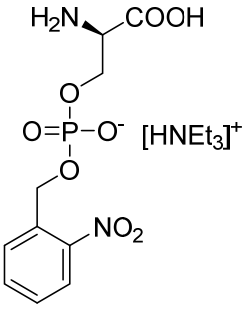
product fractions were combined and lyophilized to afford the desired methylated product (5 mg, 25%) as a white solid. ^{1}H-NMR (500 MHz, D_2O) δ = 7.83 (d, *J* = 7.6Hz, 1H), 6.01 (d, J = 7.6Hz, 1H), 5.90 (d, *J* = 3.9Hz, 1H), 4.25 to 4.19 (m, 2H), 4.19 – 4.13 (m, 1H), 4.09 (ddd, *J* = 4.3, 12.0, 2.3Hz, 1H), 3.99 (ddd, *J* = 5.1, 12.1, 3.1Hz, 1H), 3.51 (d, *J* = 11.0Hz, 3H). ^{13}C-NMR (125 MHz, D_2O) δ 166.2, 157.70, 141.22, 96.43, 89.42, 82.52, 74.11, 69.22, 64.17, 52.87. ^{31}P-NMR (202 MHz, D_2O) δ 1.47. HR-ESI: Exact mass calcd. for $C_{10}H_{15}N_3O_8P$ $[M]^-$: 336.0602, found: 336.0605.



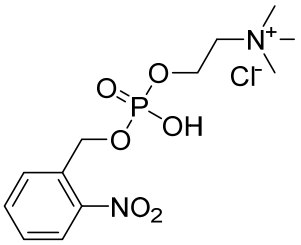
50 mg glycerol phosphate disodium salt was dissolved in 4.6 mL of 250 mM MES buffer pH 6 followed by the addition of freshly prepared benzyl diazo (90 mg). The reaction mixture was left for 30 min until the gas formation ceased. The side-products were stuck to the glassware and the clear solution was purified by preparative HPLC. The desired product eluted at 15 min. The product fractions were combined and lyophilized to afford the desired product (30 mg, 49%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.85 (s, 0.5H), 7.42 – 7.32 (m, 4H), 7.30 (m, 1H), 4.85 (d, J_{HP} = 6.3 Hz, 2H), 4.04 (m, 1H), 3.46 (dd, J_{HH} = 5.4, 2.0 Hz, 4H), 3.05 (dd, J_{HH} = 7.5, 3.2 Hz, 3H), 1.17 (t, J_{HH} = 7.3 Hz, 4H). ^{31}P NMR (202 MHz, DMSO-d_6) δ -0.71. ^{13}C NMR (101 MHz, DMSO-d_6) δ 138.44 (d, $^{3}J_{CP}$ = 8.48 Hz), 128.16, 127.44, 127.26, 77.91 (d, $^{2}J_{CP}$ = 5.76 Hz), 66.60 (d, $^{2}J_{CP}$ = 5.35 Hz), 61.66 (d, $^{3}J_{CP}$ = 4.64 Hz), 45.49, 8.50. HR-ESI: Exact mass calcd. for $C_{10}H_{14}Na_2O_6P$ $[M-H+2Na]^+$: 307.0318, found: 307.0320.



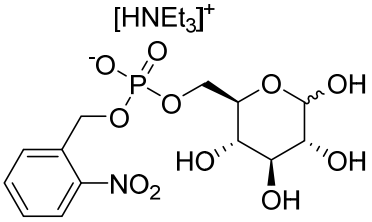
53 mg tyrosine phosphate was dissolved in 4 mL of 250 mM of MES buffer pH 6 followed by the addition of 2-nitro-phenyl diazomethane (350 mg). CH_3CN (1 mL) was added to rinse down the remaining solid particles. After 40 min the gas formation ceased and the aqueous solution was purified by preparative HPLC. The desired product eluted at 17 min. The product fractions were combined and lyophilized to afford the desired product (25 mg, 37%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.07 (dd, J_{HH}= 8.2, 0.9 Hz, 1H), 7.82 (d, J_{HH} = 7.6 Hz, 1H), 7.79 – 7.73 (m, 1H), 7.54 (t, J_{HH} = 7.2 Hz, 1H), 7.10 (d, J_{HH} = 8.5 Hz, 2H), 7.01 (d, J_{HH} = 8.4 Hz, 2H), 5.16 (d, J_{HP} = 7.3 Hz, 2H), 3.39 (dd, J_{HH} = 7.7, 4.8 Hz, 1H), 3.05 (d, J_{HH} = 4.7 Hz, 1H), 2.87 (q, J_{HH} = 7.2 Hz, 7H), 2.83 – 2.78 (m, 1H), 1.10 (t, J_{HH} = 7.2 Hz, 10H). ^{31}P NMR (202 MHz, DMSO-d_6) δ -6.07 (s). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.14, 152.64 (d, $^{2}J_{CP}$ = 6.46 Hz), 146.47, 135.51 (d, $^{3}J_{CP}$ = 7.58 Hz), 133.97, 130.30, 129.77, 128.47, 128.08, 124.35, 119.70-119.65 88 (d, $^{3}J_{CP}$ = 4.75 Hz), 63.25 (d, $^{2}J_{CP}$ = 4.75 Hz), 55.19, 45.31, 35.99, 8.75. HR-ESI: Exact mass calcd. for $C_{16}H_{15}N_2Na_2O_8P$ $[M-H+2Na]^+$: 441.0361, found:441.0432.



The reaction for serine phosphate modification at 50 mM resulted in the formation of a carboxylate ester as a side product. Therefore the reaction was carried out at 10 mM serine phosphate which resulted only in phosphate modification. The freshly prepared 2-nitro phenyl diazomethane (220 mg, 5 eq.) was immediately added to the serine phosphate solution (50 mg in 27 mL 100 mM MES buffer pH 6). After 30 min the gas formation ceased. The side-products stuck to the glassware and the clear solution was purified by preparative HPLC. The desired product eluted at 14 min. The product fractions were combined and lyophilized to afford the desired product (18 mg, 21%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.08 (d, J = 8.1 Hz, 1H), 7.90 – 7.73 (m, 2H), 7.56 (t, J = 7.5 Hz, 1H), 5.09 (d, J_{HP} = 6.8 Hz, 2H), 4.04 (t, J_{HH} = 12.1 Hz, J_{HP} = 12.1 Hz, 1H), 3.89 (dd, J_{HP} = 11.0 Hz, J_{HH} = 7.5 Hz, 1H), 3.41 (d, J_{HH} = 5.4 Hz, 1H), 3.00 (q, J_{HH} = 7.2 Hz, 5H), 1.15 (t, J_{HH} = 7.0 Hz, 8H). ^{31}P NMR (202 MHz, DMSO-d_6) δ 0.61.13C NMR (101 MHz, DMSO-d_6) δ 167.77, 146.68, 135.21, 135.17 (d, $^{3}J_{CP}$ = 8.08 Hz), 134.00, 128.63, 128.28, 124.42, 64.10 (d, $^{2}J_{CP}$ = 5.45 Hz), 63.17 (d, $^{2}J_{CP}$ = 4.04 Hz), 55.73, 45.31, 8.67.

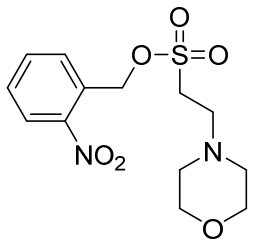


66 mg of choline phosphate chloride salt was dissolved in 4 mL of MES buffer pH 6. Then 327 mg of diazo was added to phosphate aqueous solution. The reaction proceeded smoothly and the aqueous solution was purified by preparative HPLC. The desired product eluted at 15 min. The product fractions were combined and lyophilized to afford the desired product (22 mg, 31%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.07 (d, J_{HH} = 8.0 Hz, 1H), 7.86 (d, J_{HH} = 7.5 Hz, 1H), 7.79 (t, J_{HH} = 7.4 Hz, 1H), 7.55 (t, J_{HH} = 7.5 Hz, 1H), 5.07 (d, J_{HP} = 7.0 Hz, 2H), 4.06 (m, 2H), 3.52 (m, 2H), 3.13 (s, 9H). ^{31}P NMR (202 MHz, DMSO-d_6) δ -0.99. ^{13}C NMR (101 MHz, DMSO-d_6) δ 146.62, 135.89 (d, $^{3}J_{CP}$ = 7.37 Hz), 133.94, 128.58, 128.09, 124.34, 65.46, 62.92 (d, $^{2}J_{CP}$ = 4.44 Hz), 58.36 (d, $^{2}J_{CP}$ = 5.25 Hz), 53.10, 53.07, 53.04, 39.94, 39.73, 39.52, 39.31, 39.10. HR-ESI: Exact mass calcd. for $C_{12}H_{20}N_2O_6P^+$ [M-Cl]$^+$: 319.1053, found: 319.1056.



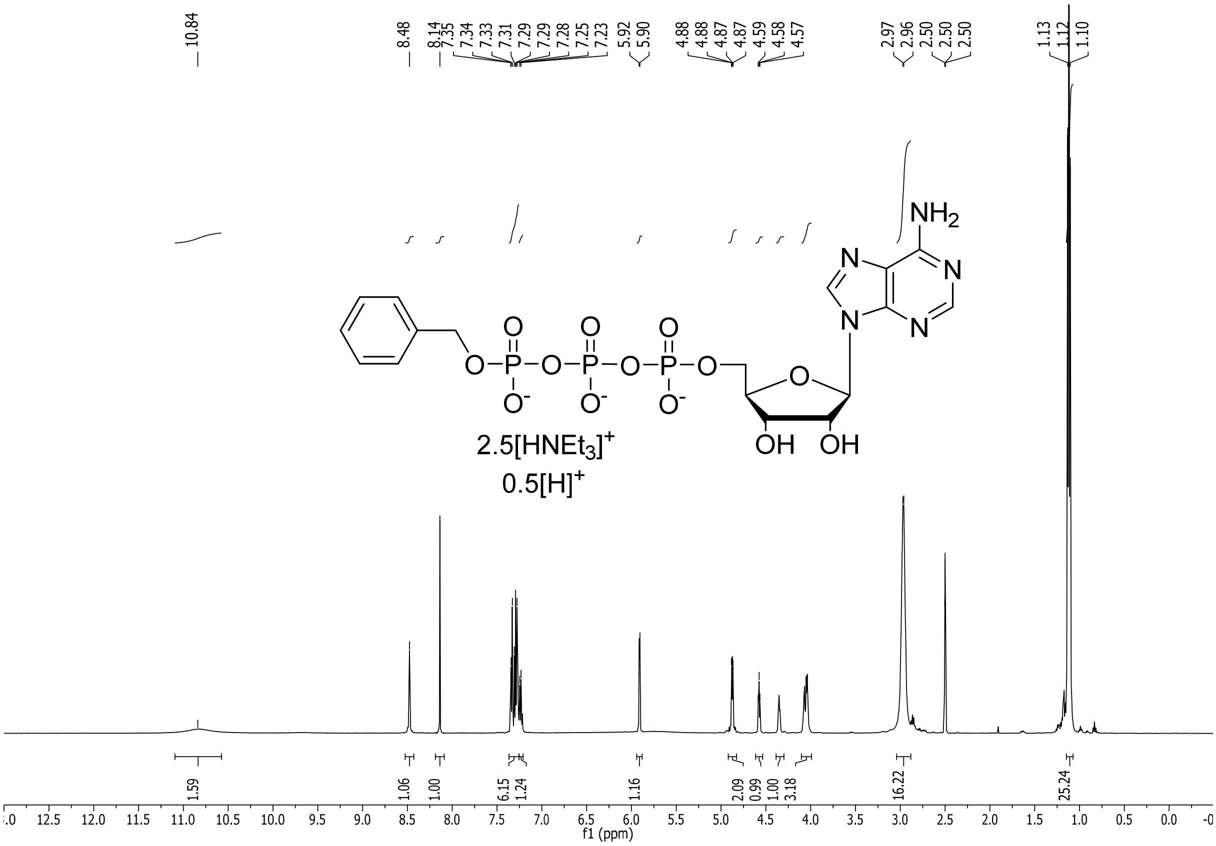
48 mg of glucose monophosphate dipotassium salt was dissolved in 2.85 mL of MES buffer pH 6 followed by the addition of 233 mg of diazo. The reaction proceeded smoothly and the aqueous solution

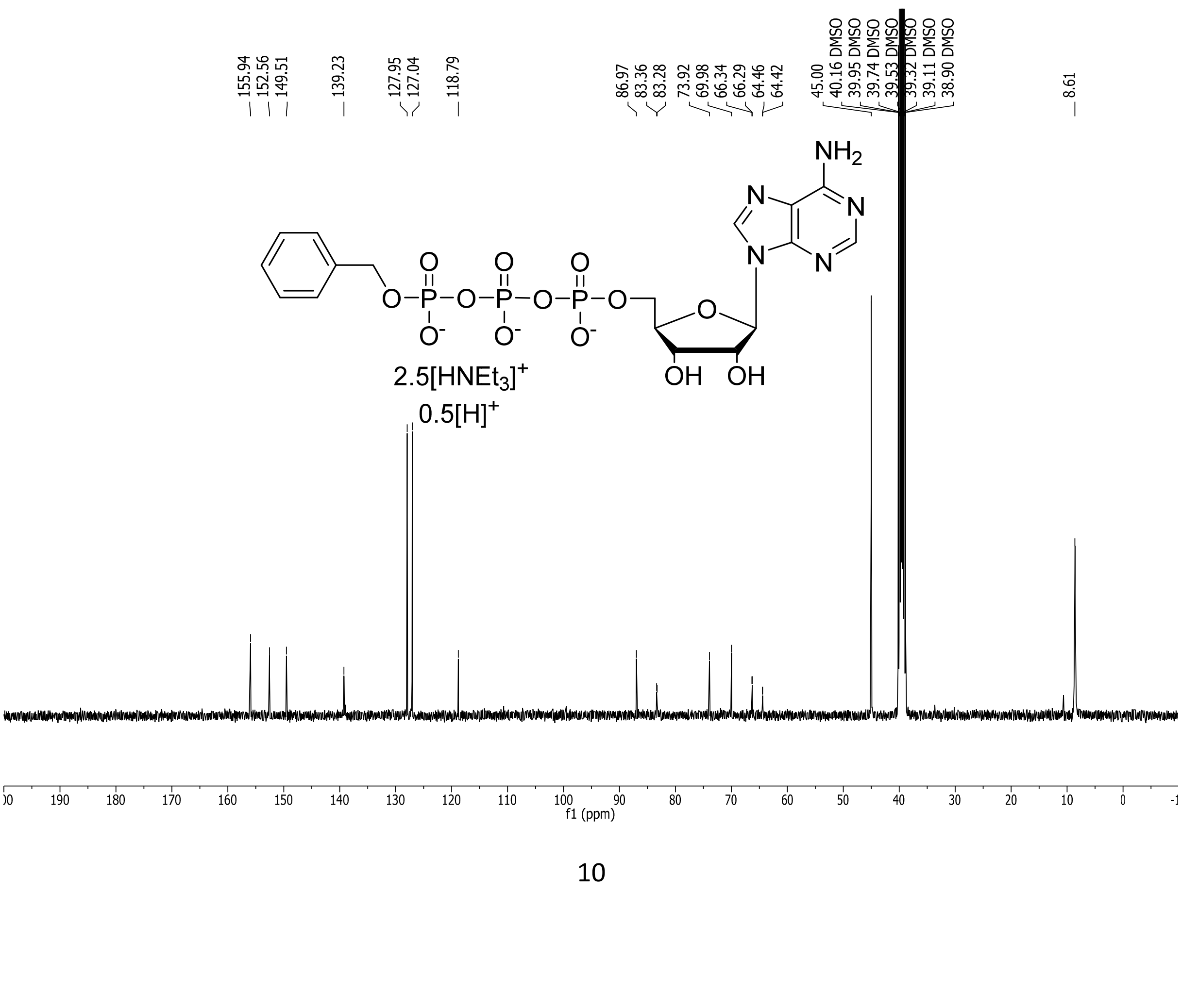
was purified by preparative HPLC. The desired product eluted at 17 min. The product fractions were combined and lyophilized to afford the desired product (42 mg, 74%). The glucose modified product is a mixture of antomer (ratio=1:1.2), but some of the NMR peaks are overlapped. ^{1}H NMR (400 MHz, D_2O) δ 8.19 (d, J_{HH} = 8.1 Hz, 1H), 7.95 – 7.75 (m, 2H), 7.60 (t, J_{HH} = 7.6 Hz, 1H), 5.35 (d, J_{HP} = 7.3 Hz, 2H), 5.14 (d, J_{HH} = 3.7 Hz, 0.49H), 4.61 (d, J_{HH} = 8.0 Hz, 0.59H), 4.20 – 4.00 (m, 2H), 3.88 (d, J_{HH} = 9.6 Hz, 0.5H), 3.69 (t, J_{HH} = 9.5 Hz, 0.5H), 3.57 – 3.39 (m, 3H), 3.22 (q, J_{HH} = 7.3 Hz, 6H), 1.29 (t, J_{HH} = 7.3 Hz, 9H). ^{13}C NMR (101 MHz, D_2O) δ 146.56, 146.51, 134.54, 133.72-133.65 (d, $^{3}J_{CP}$ = 7.37 Hz), 133.62-133.54(d, $^{3}J_{CP}$ = 7.77 Hz), 128.73, 128.70, 128.68, 128.61, 124.96, 124.92, 95.95, 92.08, 75.54, 74.67-74.59 (d, $^{2}J_{CP}$ = 7.77 Hz), 74.03, 72.54, 71.40, 70.35-70.28 (d, $^{2}J_{CP}$ = 7.67 Hz), 69.18, 64.57, 64.53 (d, $^{2}J_{CP}$ = 3.23 Hz), 64.49-64.44 (d, $^{2}J_{CP}$ = 4.84 Hz), 46.66, 8.21. ^{31}P NMR (202 MHz, D_2O) δ 0.61, 0.53. HR-ESI (Et_3N salt): Exact mass calcd. for $C_{19}H_{34}N_2O_{11}P^+$ $[M{\cdot}NEt_3{+}H]^+$: 497.1900, found: 497.1902.

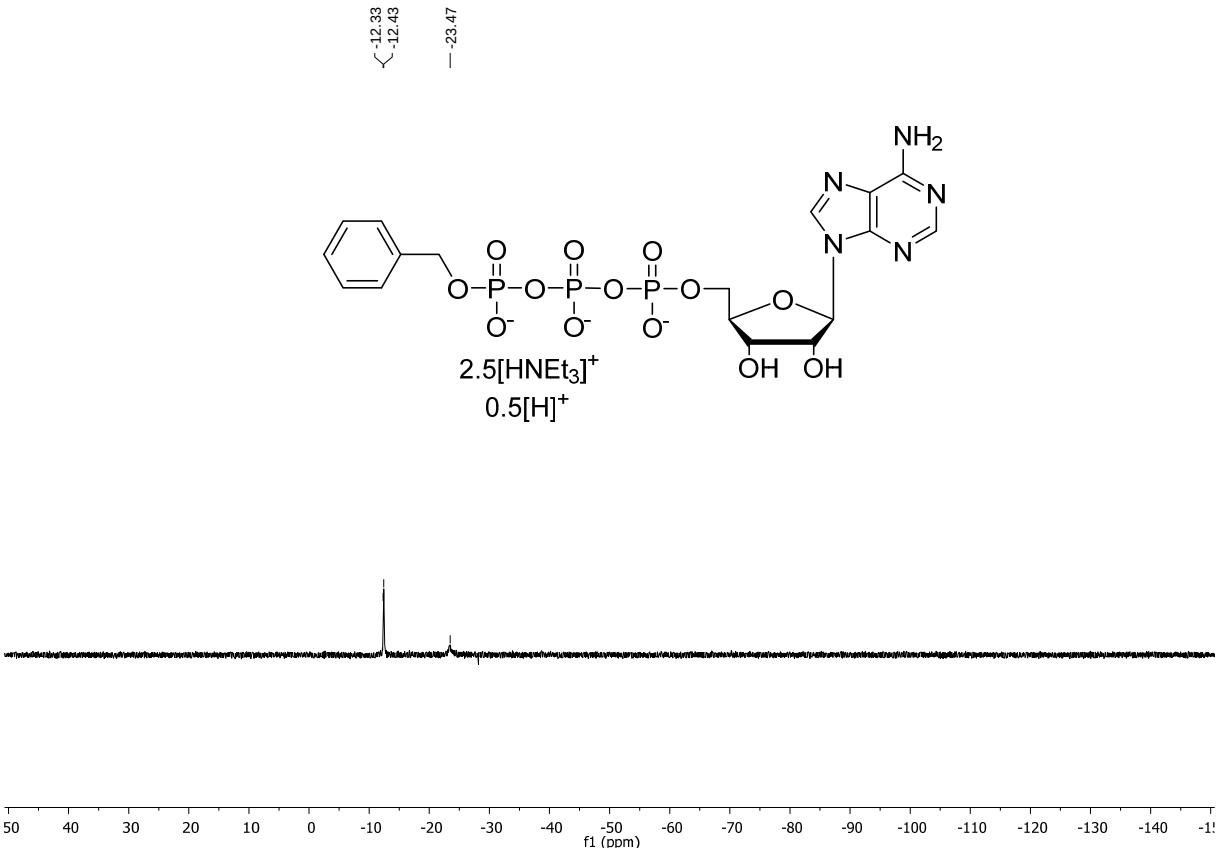


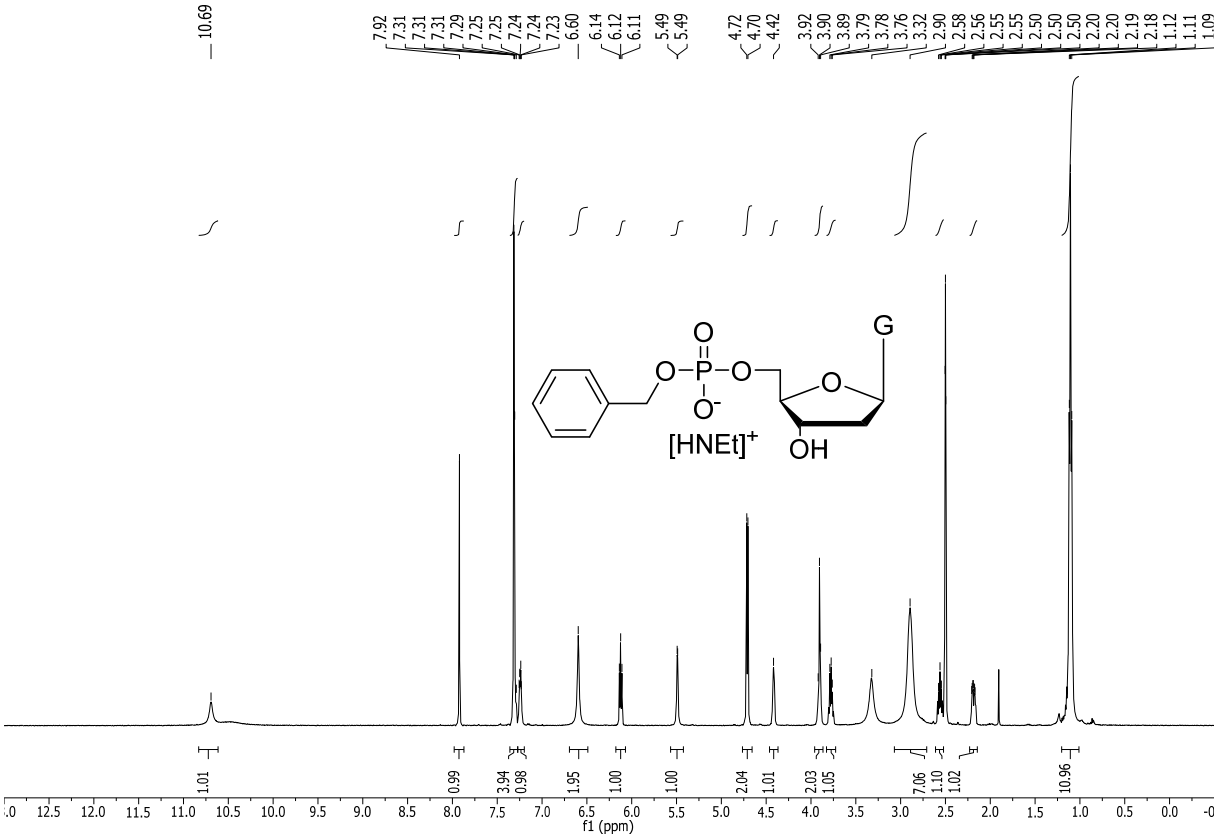
Because MES salt was used as the buffer, it is ten times concentrated than phosphate monoester, so there is also MES esterification reaction observed as the side reaction. But the conversion is very low (Y < 5%). In the next chapter, when diazo was tested with phosphodiester, since there is no phosphate monoester is available, thus the MES reacted with diazo and the sulfate esterification product could be purified. (in the c-di-GMP reaction, MES ester, isolated yield 3%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.21 (dd, J = 7.9, 1.2 Hz, 1H), 7.97 – 7.81 (m, 3H), 5.04 (s, 2H), 3.97 – 3.89 (m, 2H), 3.85 (dd, J = 14.5, 9.2 Hz, 4H), 3.54 (d, J = 12.5 Hz, 2H), 3.30 – 3.26 (m, 2H), 3.16 – 3.08 (m, 2H).^{13}C NMR (151 MHz, DMSO-d_6) δ 150.74, 136.81, 133.95, 132.45, 126.06, 120.49, 60.71, 59.91, 56.31, 53.54, 43.41. HR-ESI (Et_3N salt): Exact mass calcd. for $C_{19}H_{34}N_3O_6S$ $[M{\cdot}Et_3N{+}H]^+$: 432.2163, found: 432.2170.

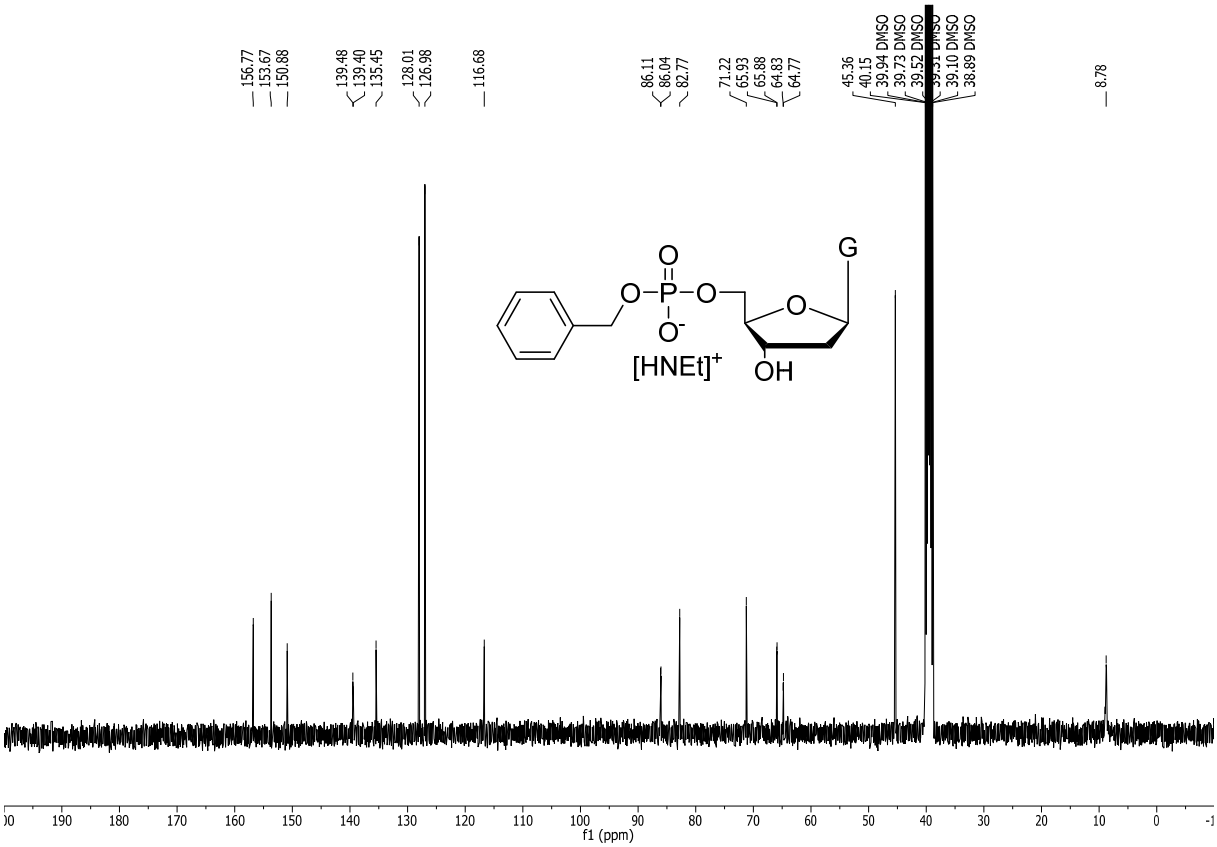
NMR Spectra

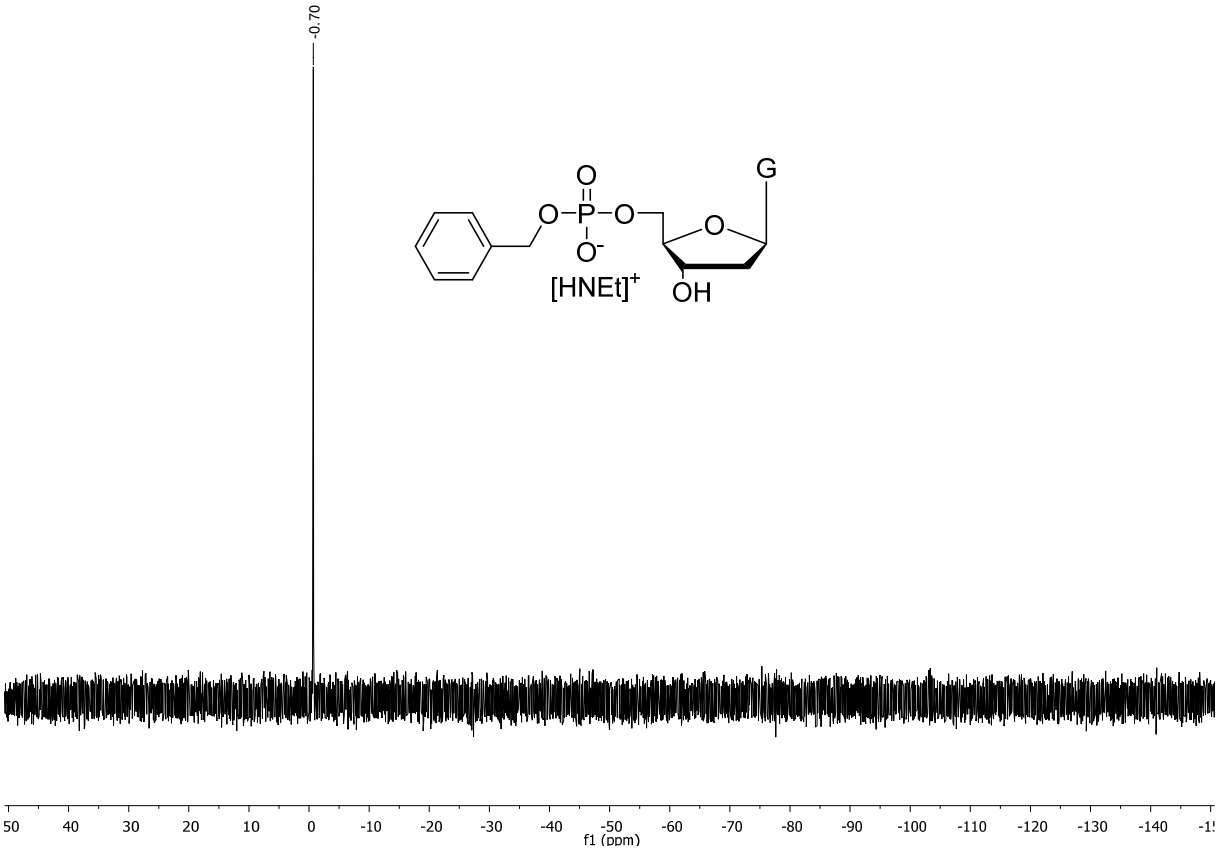

10.84
8.48
8.14
7.35
7.34
7.33
7.31
7.29
7.29
7.28
7.25
7.23
5.92
5.90
4.88
4.88
4.87
4.87
4.59
4.58
4.57
2.97
2.96
2.50
2.50
2.50
1.13
1.12
1.10
NH2
N
N
N
N
O
O
O
O
P
P
P
O-
O-
O-
O
OH
OH
2.5[HNEt3]+
0.5[H]+
1.59
1.06
1.00
6.15
1.24
1.16
2.09
0.99
1.00
3.18
16.22
25.24
f1 (ppm)

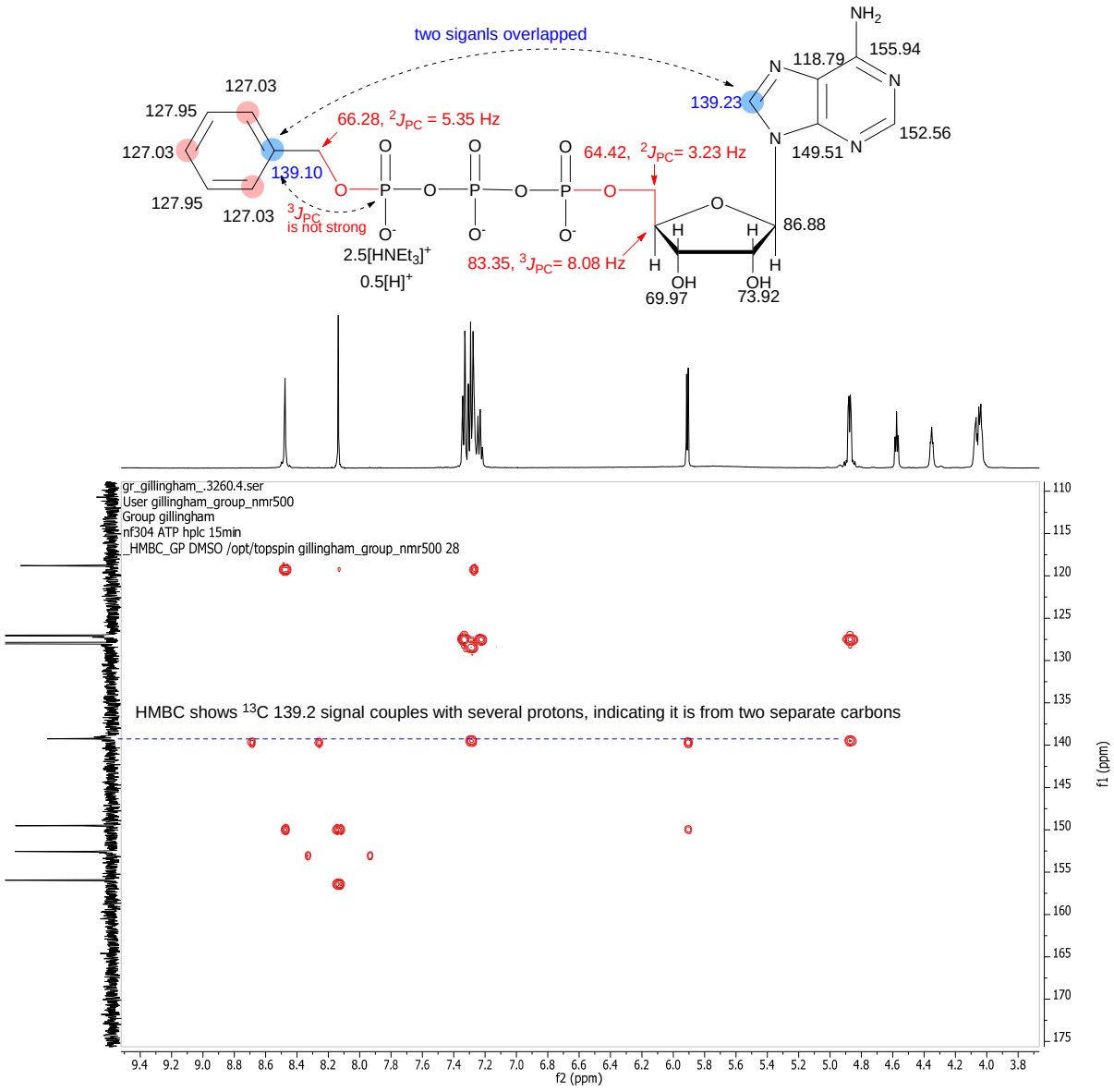

155.94
152.56
149.51
139.23
127.95
127.04
118.79
86.97
83.36
83.28
73.92
69.98
66.34
66.29
64.46
64.42
45.00
40.16 DMSO
39.95 DMSO
39.74 DMSO
39.53 DMSO
39.32 DMSO
39.11 DMSO
38.90 DMSO
8.61
NH2
N
N
N
N
O
O
O
O
P
P
P
O-
O-
O-
O
OH
OH
2.5[HNEt3]+
0.5[H]+
f1 (ppm)

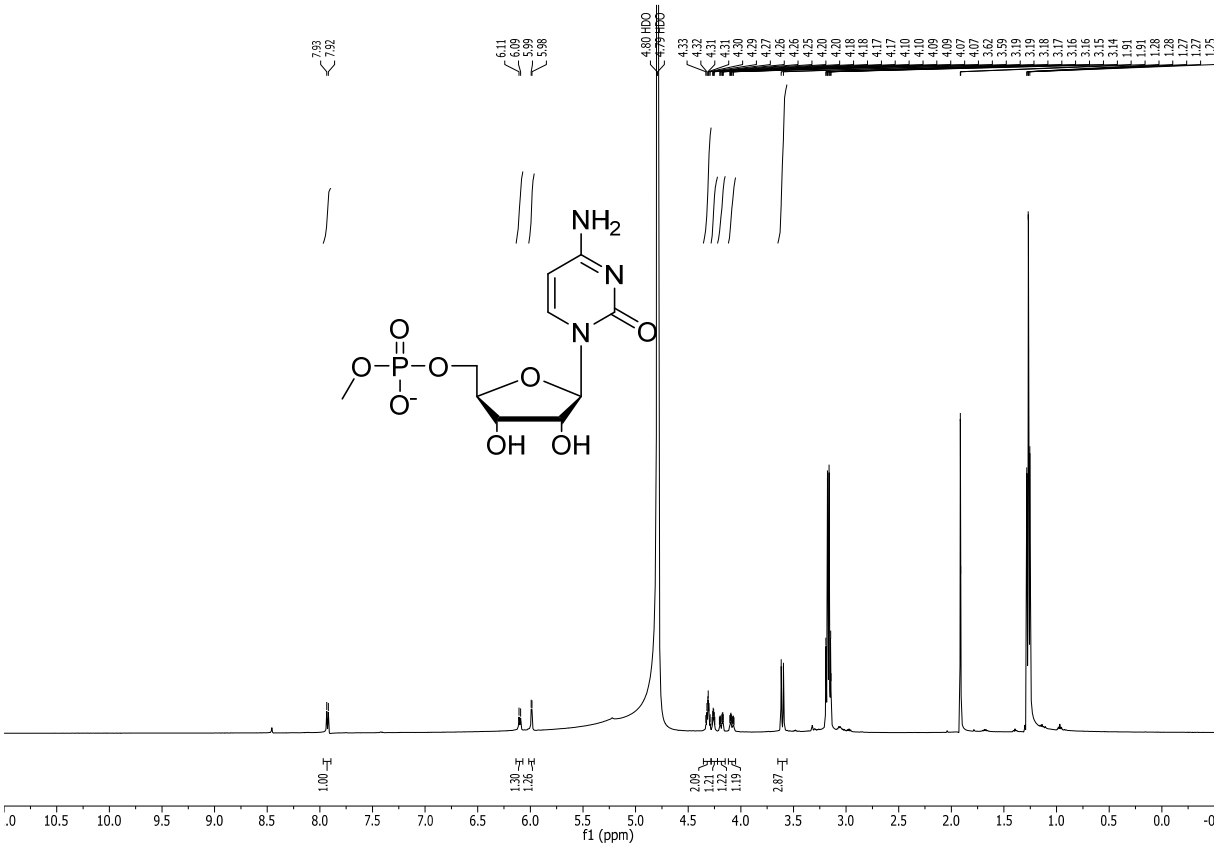

-12.33
-12.43
-23.47
NH2
N
N
N
N
O
O
O
O
O
O
O
O
O
O
O
O
OH
OH
2.5[HNEt3]+
0.5[H]+
f1 (ppm)

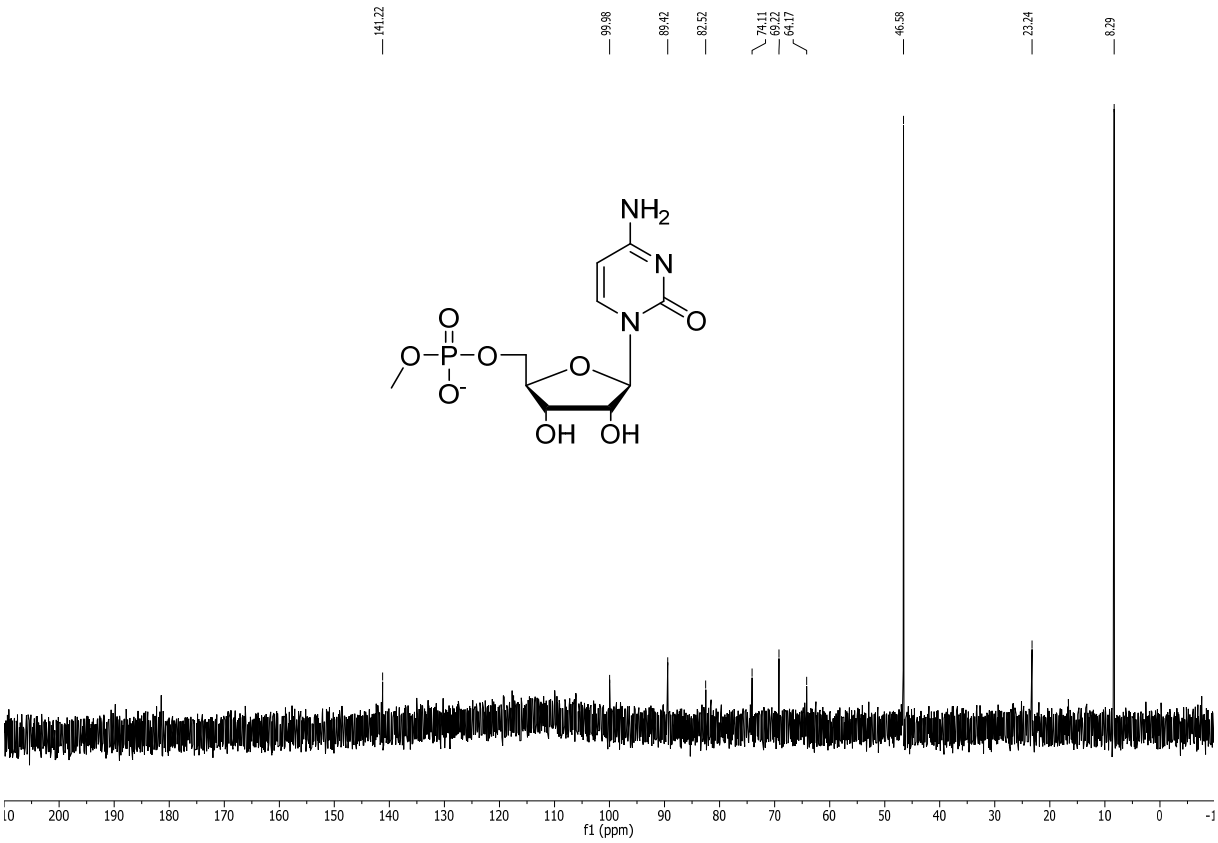

10.69
7.92
7.31
7.31
7.31
7.29
7.25
7.25
7.24
7.24
7.23
6.60
6.14
6.12
6.11
5.49
5.49
4.72
4.70
4.42
3.92
3.90
3.89
3.79
3.78
3.76
3.32
2.90
2.58
2.56
2.55
2.55
2.50
2.50
2.50
2.20
2.20
2.19
2.18
1.12
1.11
1.09
G
O
O
O
O
O
[HNEt]+
OH
1.01
0.99
3.94
0.98
1.95
1.00
1.00
2.04
1.01
2.03
1.05
7.06
1.10
1.02
10.96
f1 (ppm)

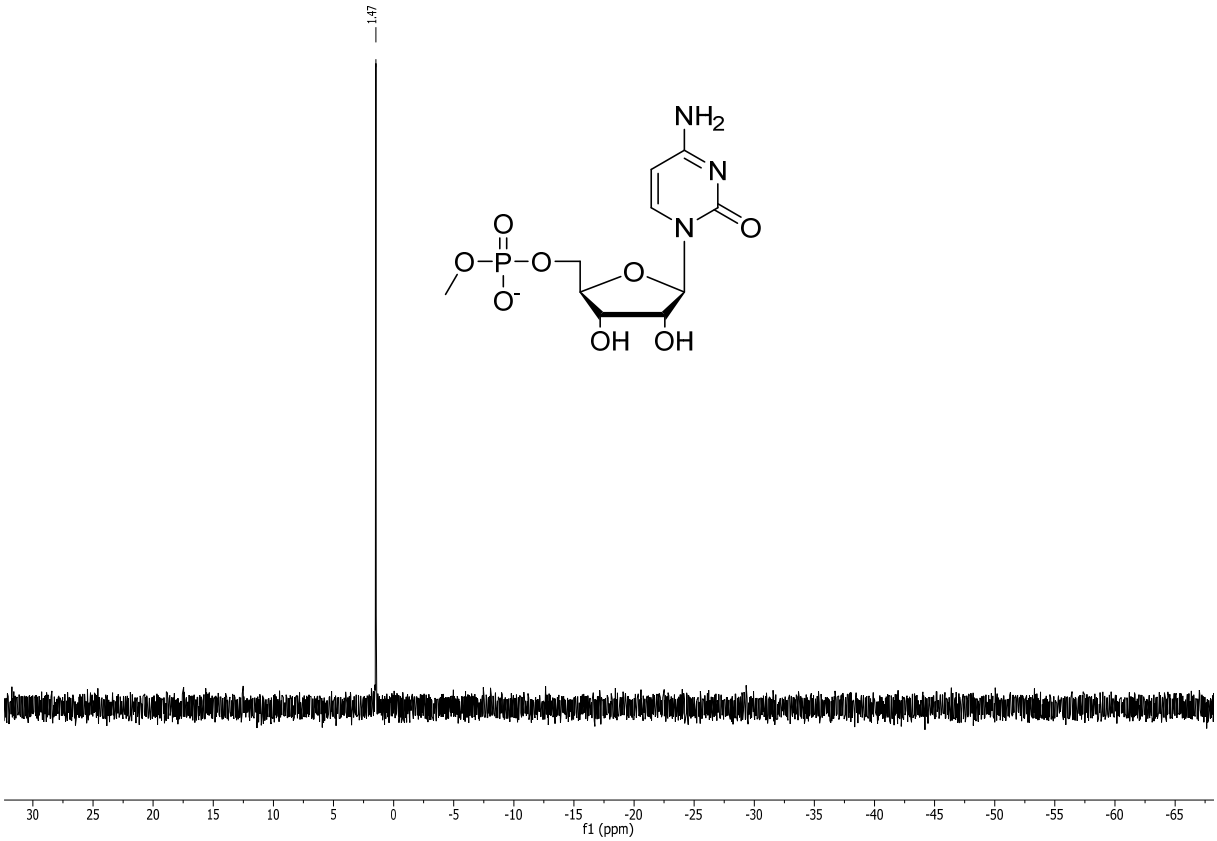

156.77
153.67
150.88
139.48
139.40
135.45
128.01
126.98
116.68
86.11
86.04
82.77
71.22
65.93
65.88
64.83
64.77
45.36
40.15
39.94 DMSO
39.73 DMSO
39.52 DMSO
39.31 DMSO
39.10 DMSO
38.89 DMSO
8.78
O
O-P-O
O-
[HNEt]+
G
O
OH
f1 (ppm)

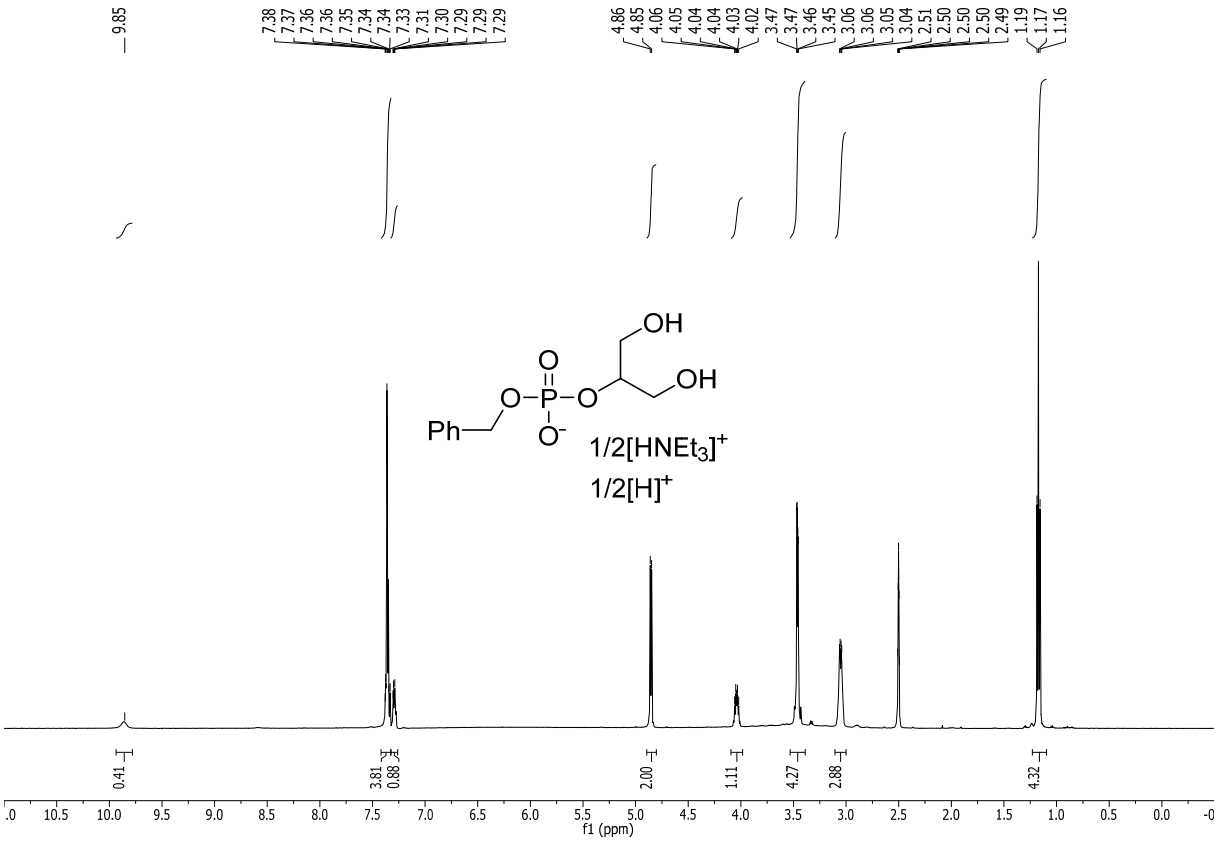

-0.70
O
O-P-O
O-
[HNEt]+
G
O
OH
f1 (ppm)

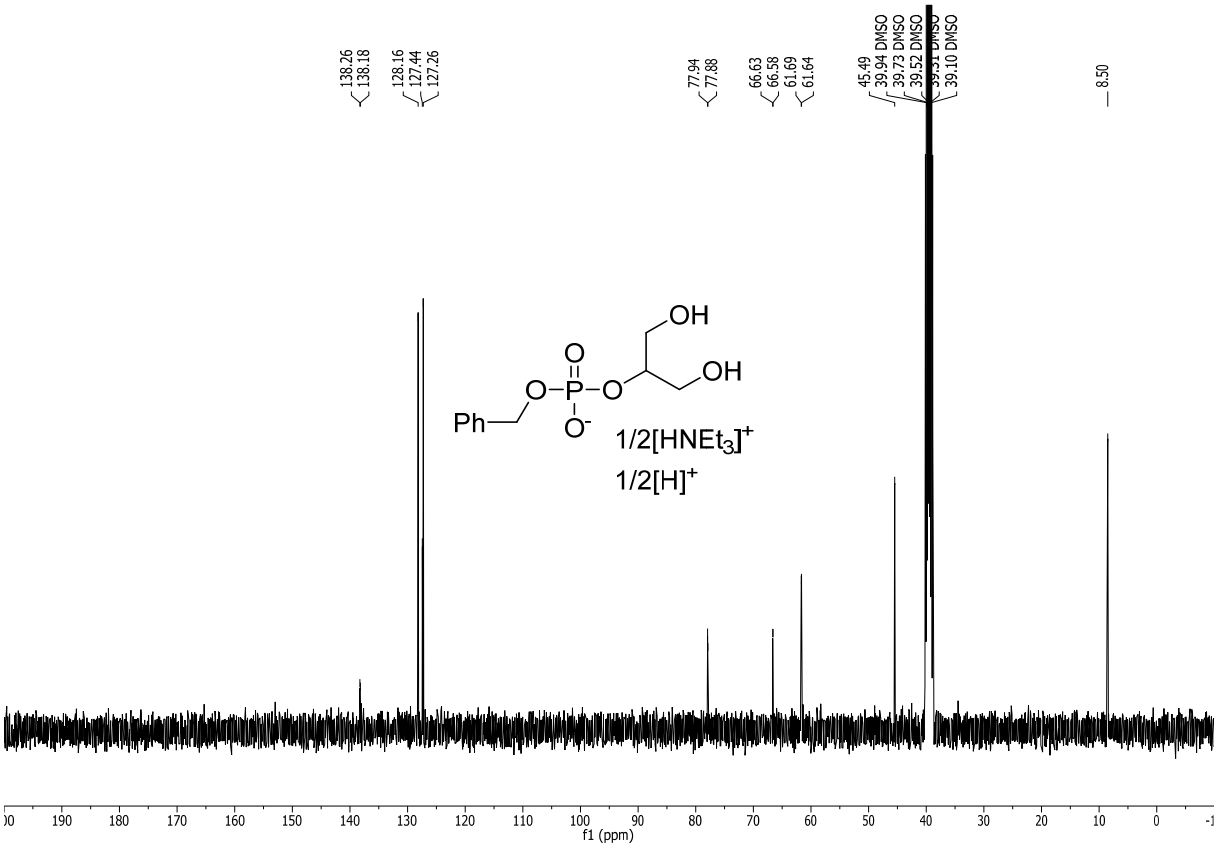

two siganls overlapped
NH2
155.94
118.79
139.23
152.56
149.51
127.03
127.95
127.03
127.95
127.03
139.10
66.28, 2JPC = 5.35 Hz
64.42, 2JPC= 3.23 Hz
3JPC is not strong
2.5[HNEt3]+
0.5[H]+
83.35, 3JPC= 8.08 Hz
86.88
69.97
73.92
gr_gillingham_.3260.4.ser
User gillingham_group_nmr500
Group gillingham
nf304 ATP hplc 15min
_HMBC_GP DMSO /opt/topspin gillingham_group_nmr500 28
HMBC shows 13C 139.2 signal couples with several protons, indicating it is from two separate carbons
f2 (ppm)
f1 (ppm)

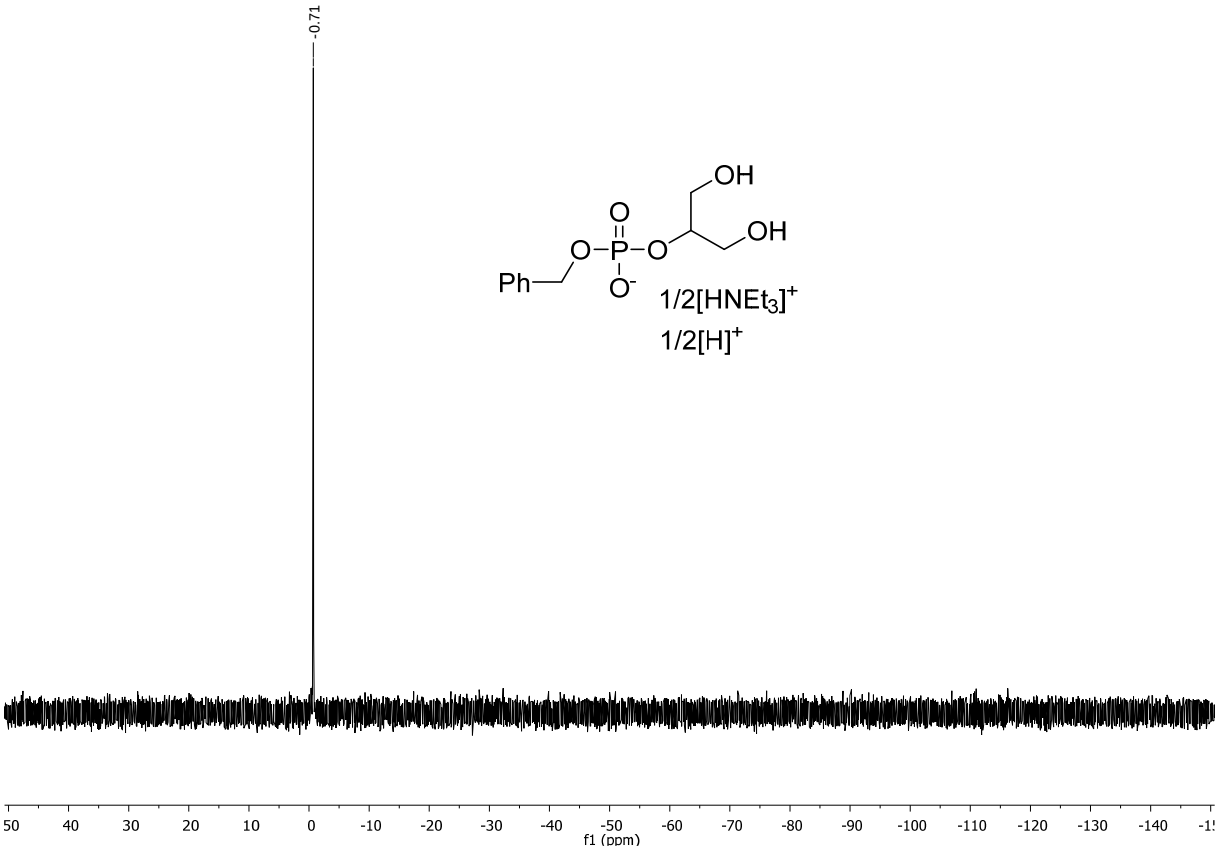

7.93
7.92
6.11
6.09
5.99
5.98
4.80 HDO
4.79 HDO
4.33
4.32
4.31
4.31
4.30
4.29
4.27
4.26
4.26
4.25
4.20
4.20
4.18
4.18
4.17
4.17
4.10
4.10
4.09
4.09
4.07
4.07
3.62
3.59
3.19
3.19
3.18
3.17
3.16
3.16
3.15
3.14
1.91
1.91
1.28
1.28
1.27
1.27
1.25
1.00
1.20
1.26
2.09
1.21
1.22
1.19
2.87
NH2
N
N
O
O
O
P
O
O-
O
OH
OH
f1 (ppm)

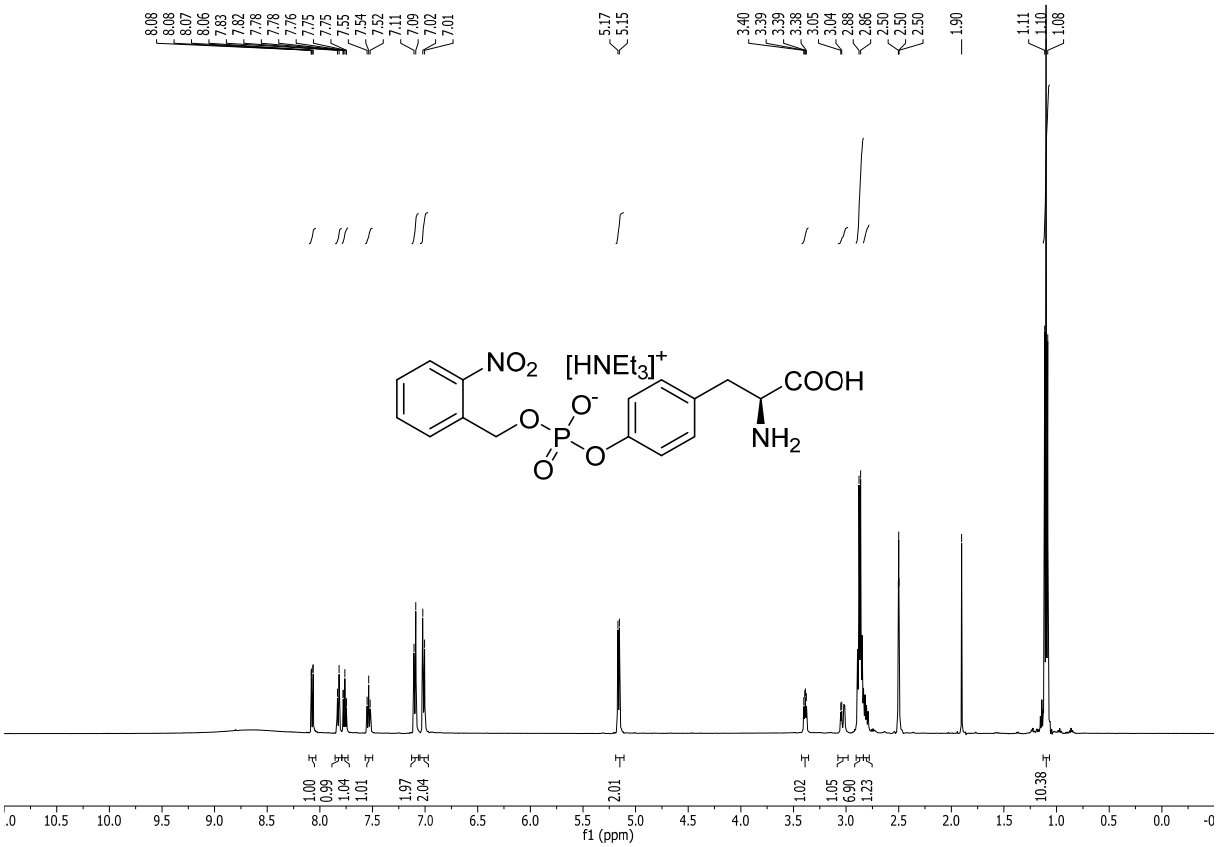

141.22
99.98
89.42
82.52
74.11
69.22
64.17
46.58
23.24
8.29
NH2
N
N
O
O
O
P
O
O-
O
OH
OH
f1 (ppm)

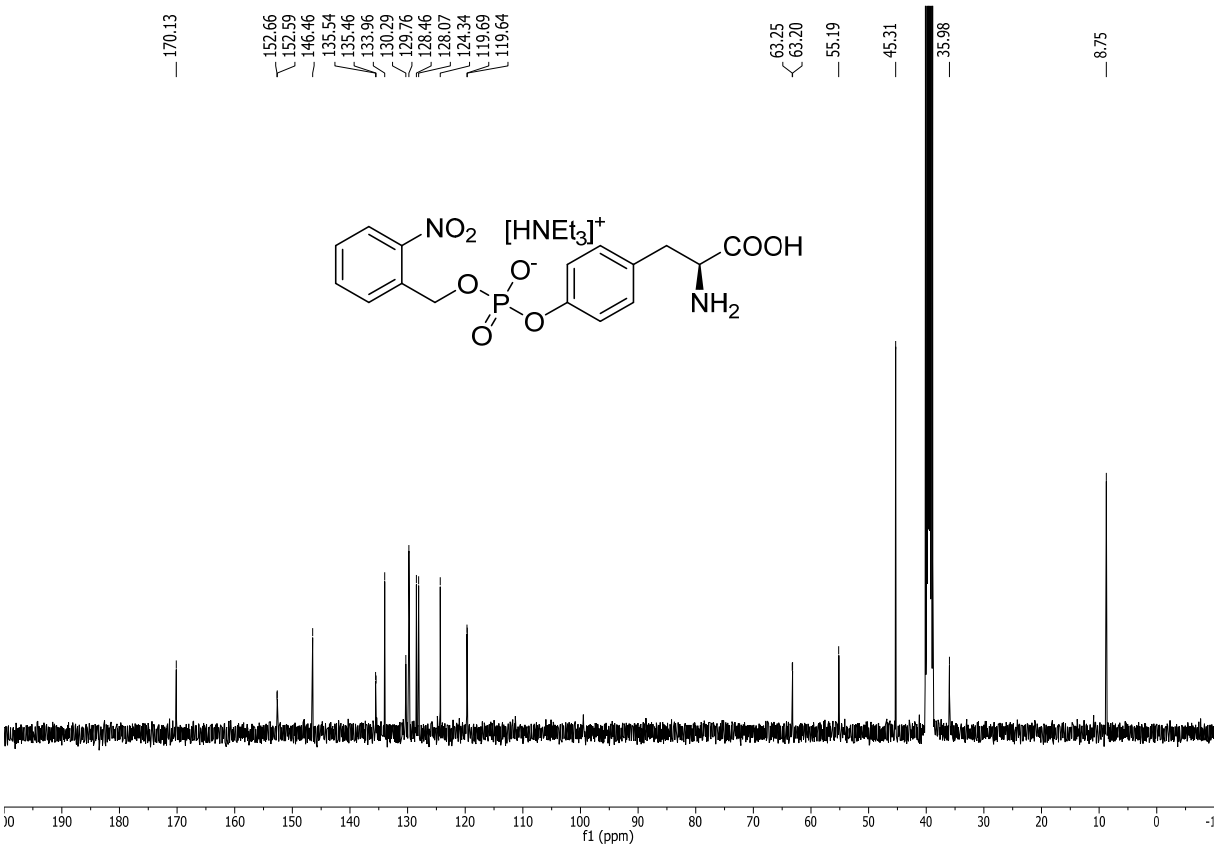

1.47
NH2
N
N
O
O
O-P-O
O-
OH OH
30 25 20 15 10 5 0 -5 -10 -15 -20 -25 -30 -35 -40 -45 -50 -55 -60 -65
f1 (ppm)

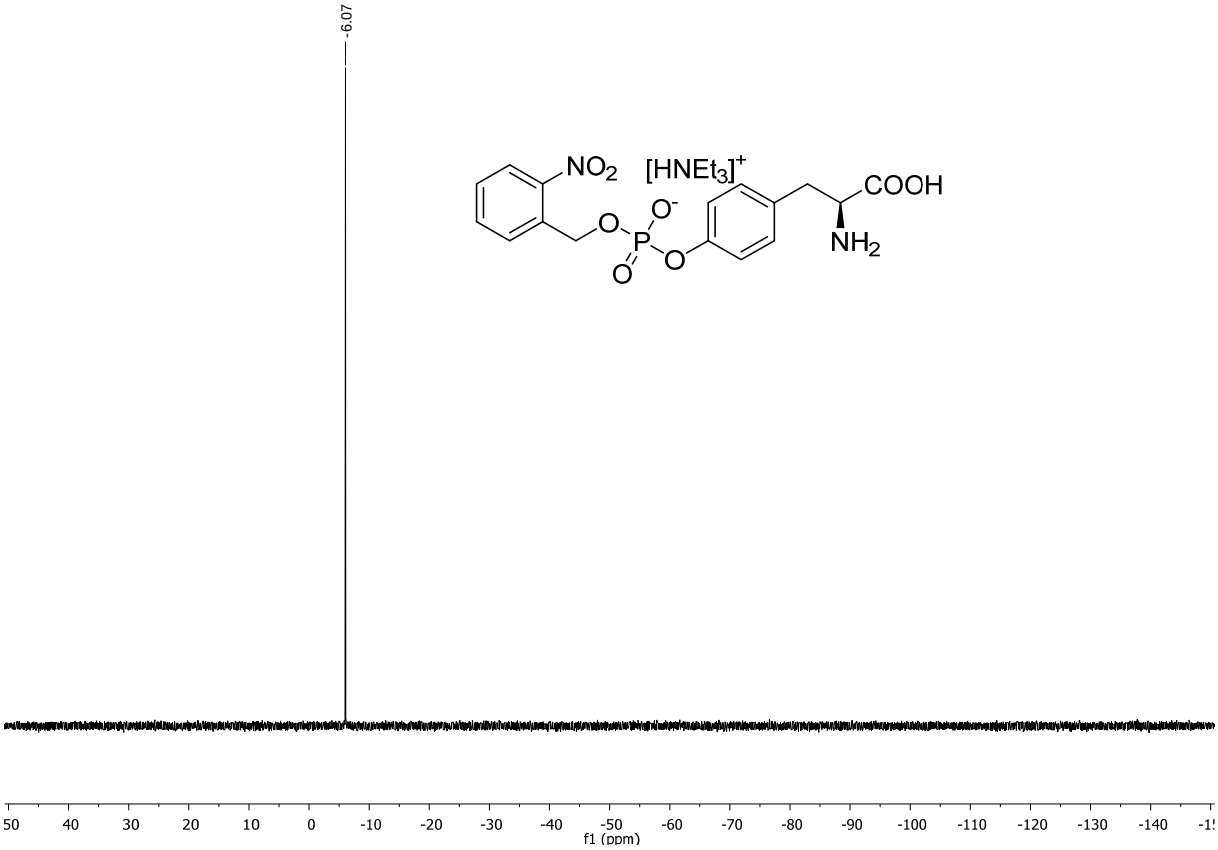

9.85
7.38 7.37 7.36 7.36 7.35 7.34 7.34 7.33 7.31 7.30 7.29 7.29 7.29
4.86 4.85 4.06 4.05 4.04 4.04 4.03 4.02 3.47 3.47 3.46 3.45 3.06 3.06 3.05 3.04 2.51 2.50 2.50 2.49 1.19 1.17 1.16
OH
OH
O-P-O
Ph
O-
1/2[HNEt3]+
1/2[H]+
0.41
3.81
0.88
2.00
1.11
4.27
2.88
4.32
.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 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
f1 (ppm)

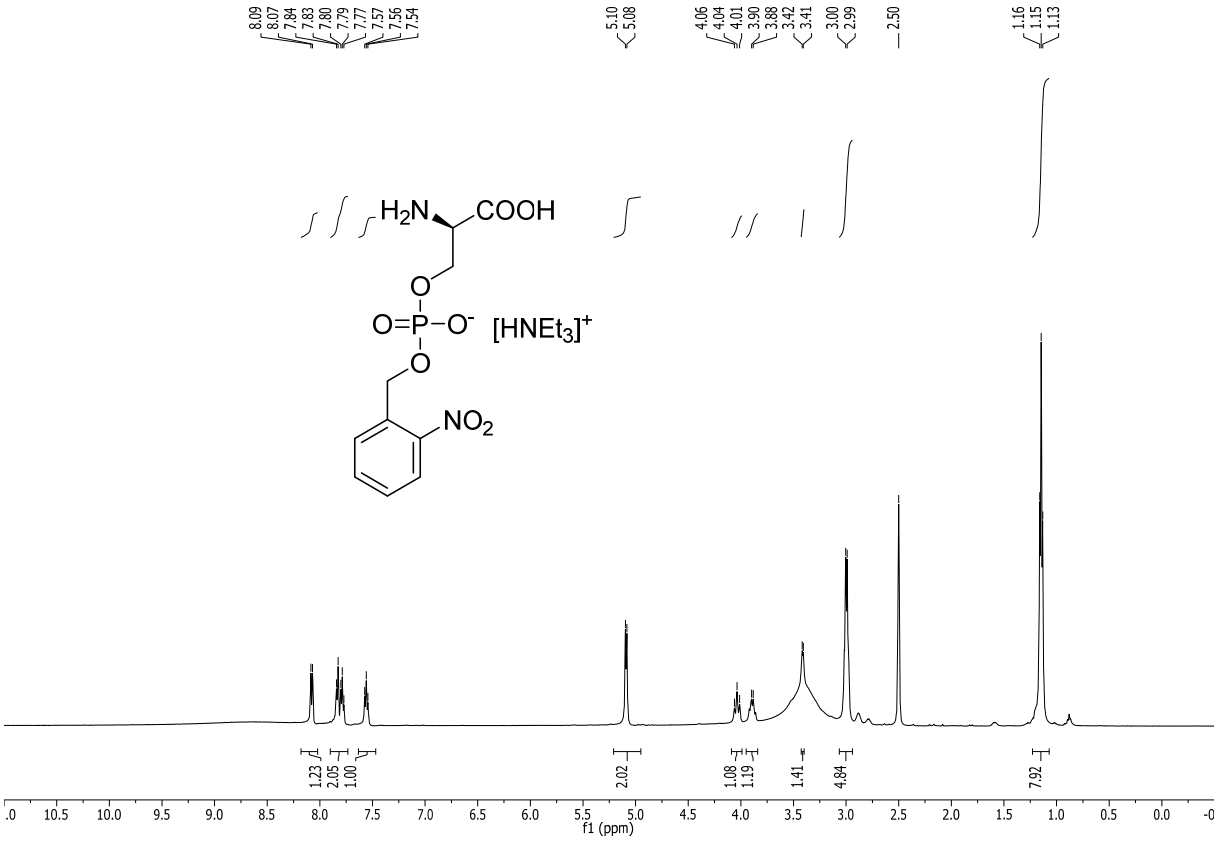

138.26
138.18
128.16
127.44
127.26
77.94
77.88
66.63
66.58
61.69
61.64
45.49
39.94 DMSO
39.73 DMSO
39.52 DMSO
39.31 DMSO
39.10 DMSO
8.50
OH
OH
O
O
P
O
O
Ph
O-
1/2[HNEt3]+
1/2[H]+
f1 (ppm)

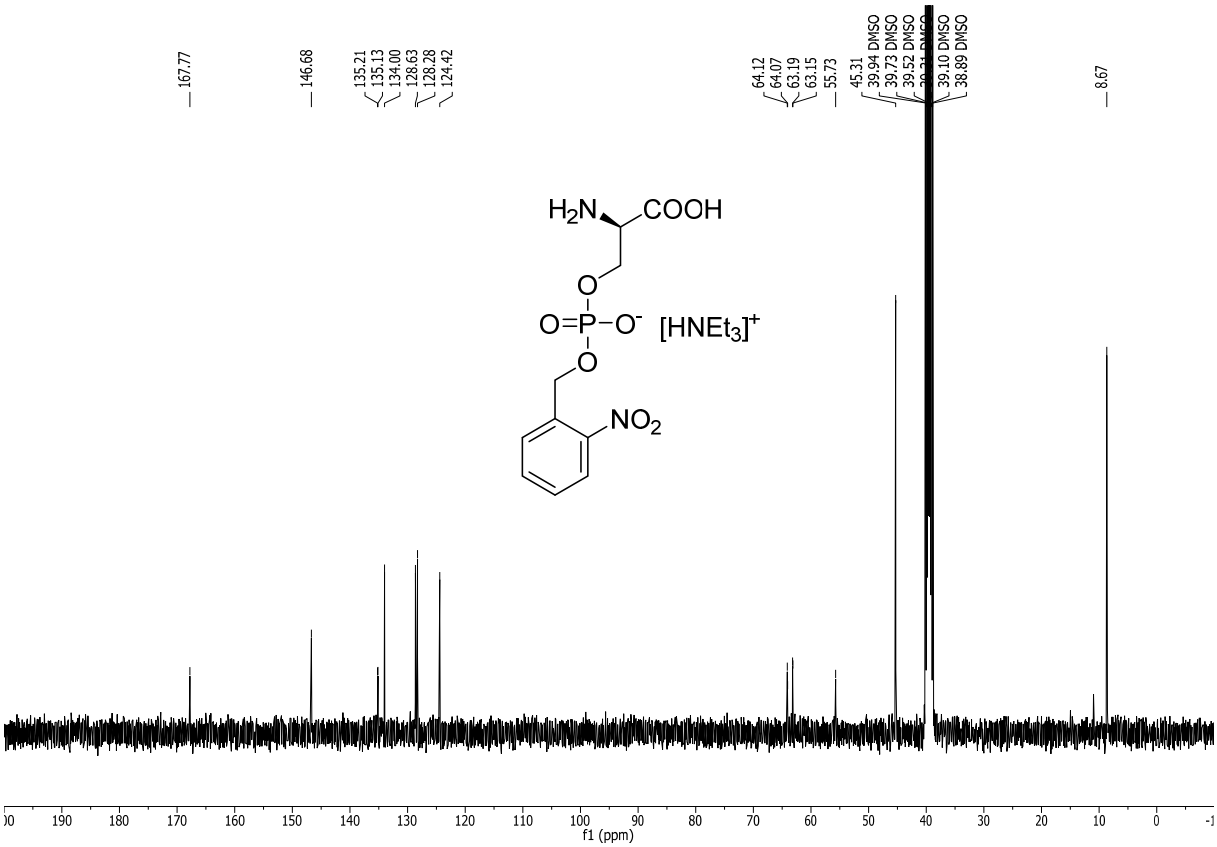

-0.71
OH
OH
O
O
P
O
O
Ph
O-
1/2[HNEt3]+
1/2[H]+
f1 (ppm)

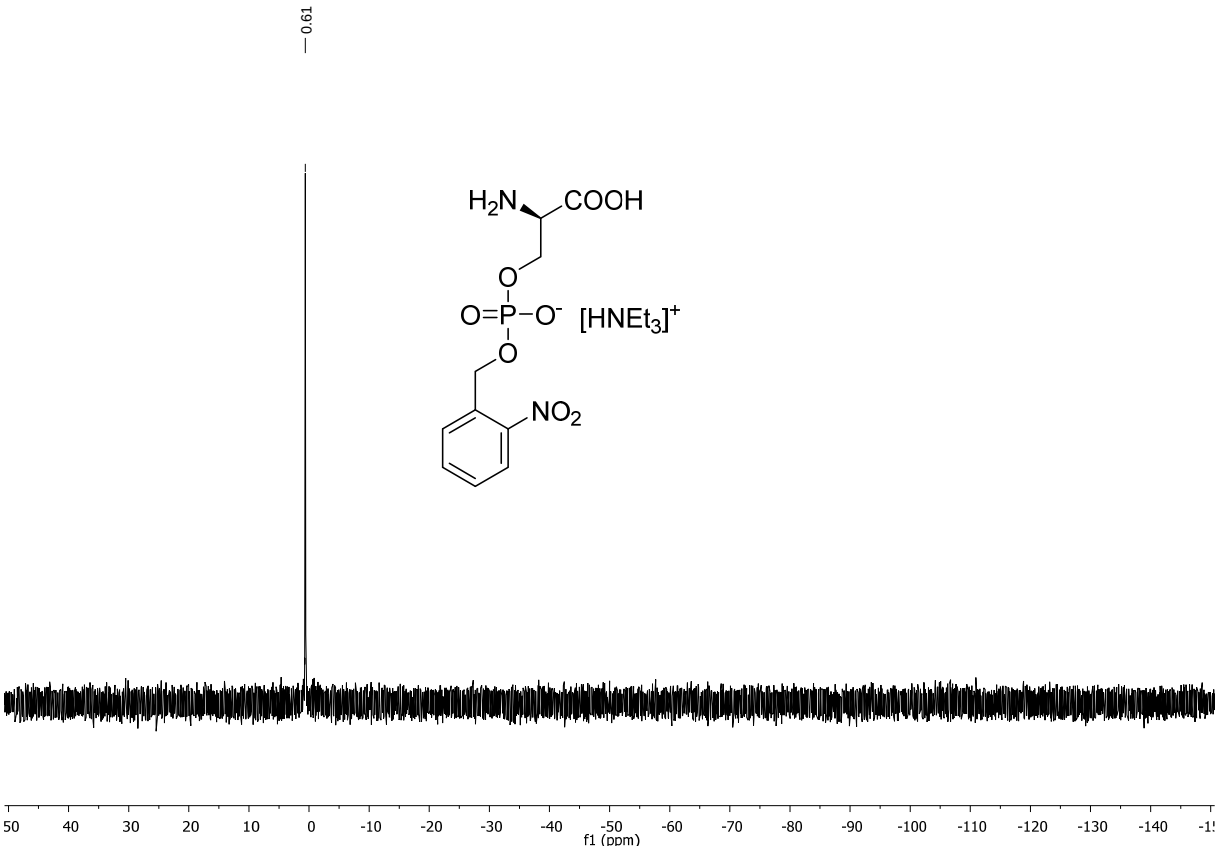

8.08
8.08
8.07
8.06
7.83
7.82
7.78
7.78
7.76
7.75
7.75
7.55
7.54
7.52
7.11
7.09
7.02
7.01
5.17
5.15
3.40
3.39
3.39
3.38
3.05
3.04
2.88
2.86
2.50
2.50
2.50
1.90
1.11
1.10
1.08
NO2
[HNEt3]+
O-
O
P
O
O
O
COOH
NH2
1.00
0.99
1.04
1.01
1.97
2.04
2.01
1.02
1.05
6.90
1.23
10.38
f1 (ppm)

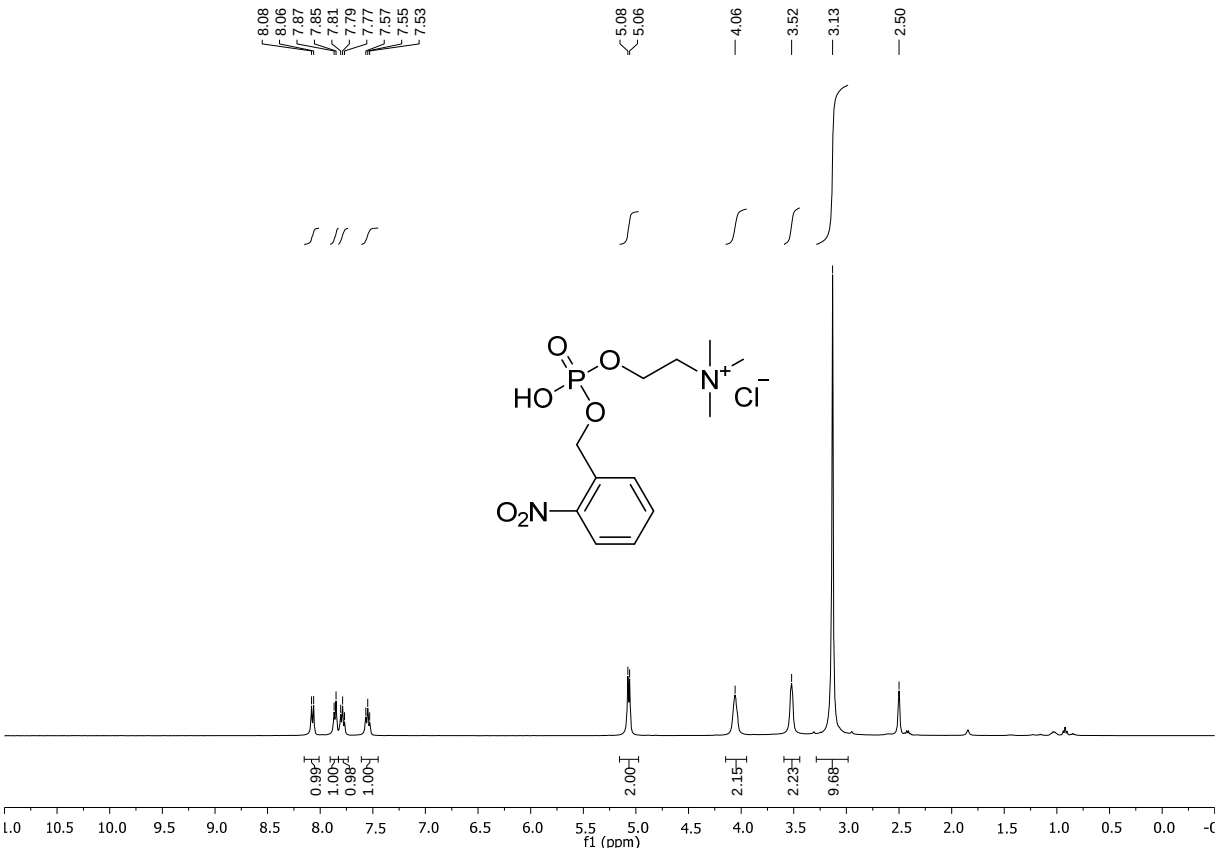

170.13
152.66
152.59
146.46
135.54
135.46
133.96
130.29
129.76
128.46
128.07
124.34
119.69
119.64
63.25
63.20
55.19
45.31
35.98
8.75
NO2
[HNEt3]+
O-
O
P
O
O
O
COOH
NH2
f1 (ppm)


-6.07
NO2
[HNEt3]+
COOH
NH2
O
O-
P
O
O
50 40 30 20 10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90 -100 -110 -120 -130 -140
f1 (ppm)


8.09
8.07
7.84
7.83
7.80
7.79
7.77
7.57
7.56
7.54
5.10
5.08
4.06
4.04
4.01
3.90
3.88
3.42
3.41
3.00
2.99
2.50
1.16
1.15
1.13
H2N
COOH
O
O=P–O-
[HNEt3]+
O
NO2
1.23
2.05
1.00
2.02
1.08
1.19
1.41
4.84
7.92
f1 (ppm)


167.77
146.68
135.21
135.13
134.00
128.63
128.28
124.42
64.12
64.07
63.19
63.15
55.73
45.31
39.94 DMSO
39.73 DMSO
39.52 DMSO
39.10 DMSO
38.89 DMSO
8.67
H2N
COOH
O
O=P−O-
[HNEt3]+
O
NO2
f1 (ppm)


0.61
H2N
COOH
O
O=P−O-
[HNEt3]+
O
NO2
f1 (ppm)


8.08
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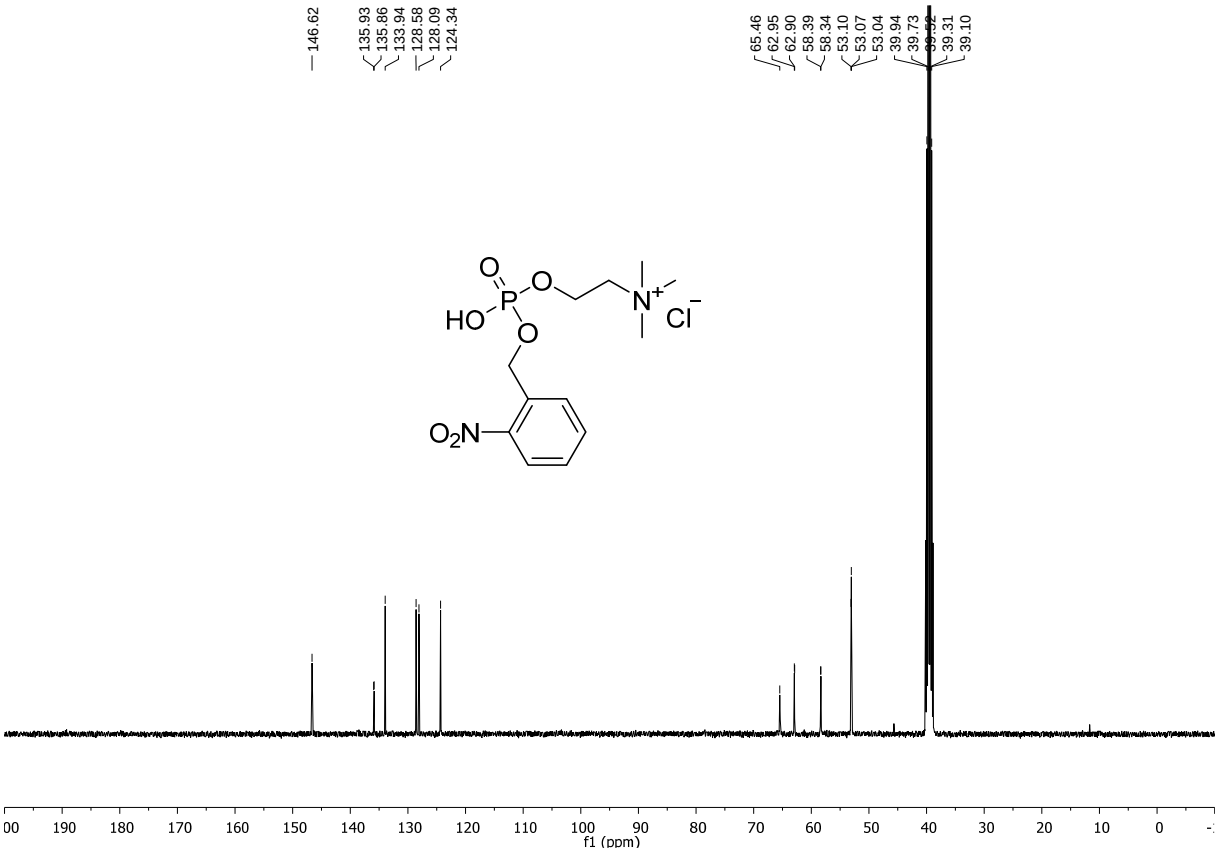
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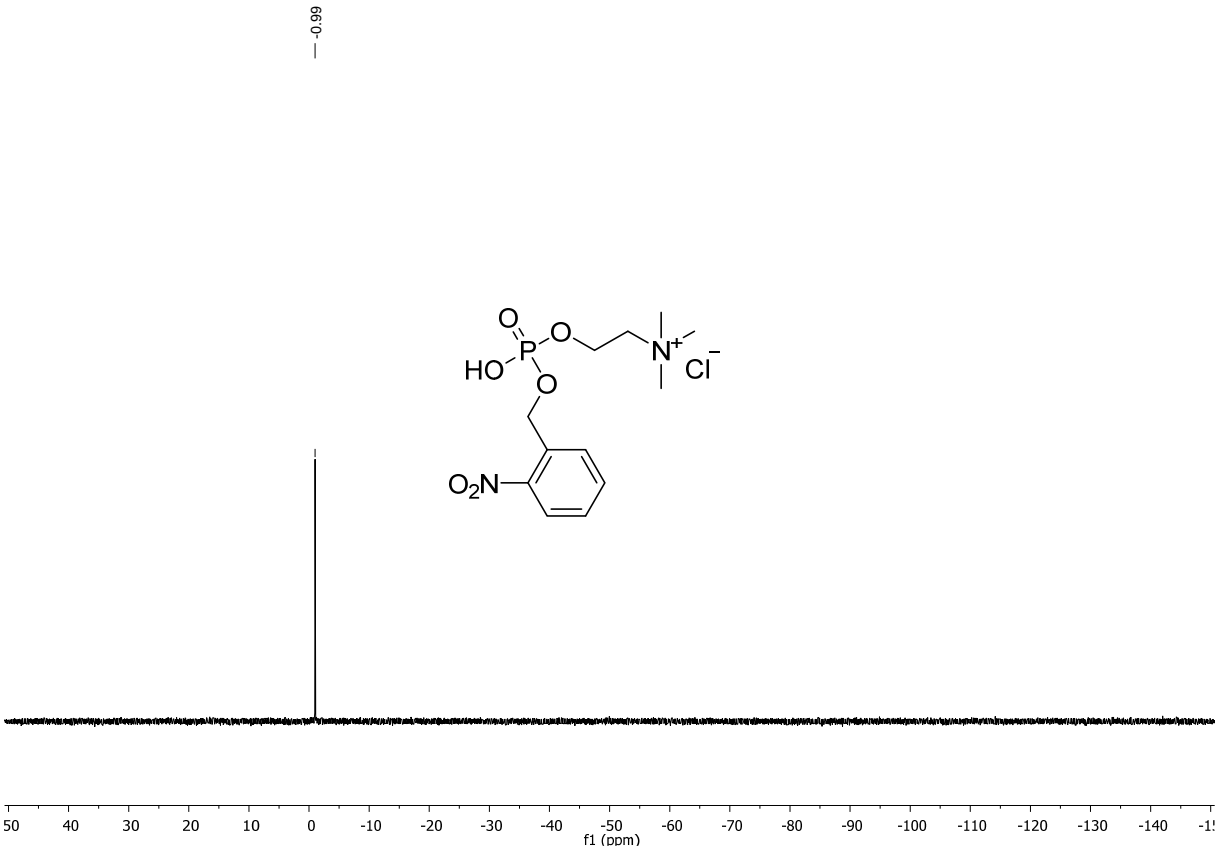
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P
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Cl−
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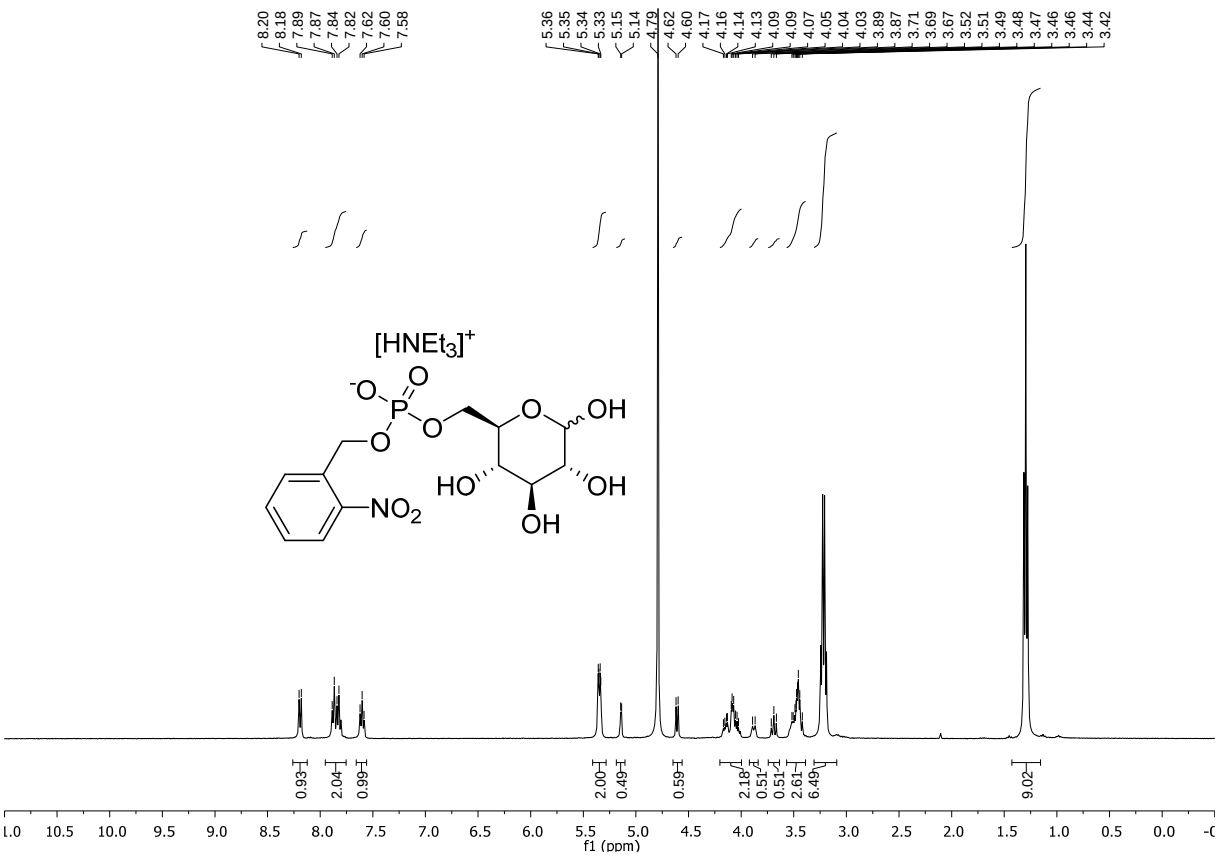
f1 (ppm)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[HNEt3]+
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P
O
O
O
O
OH
HO
OH
OH
NO2
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f1 (ppm)

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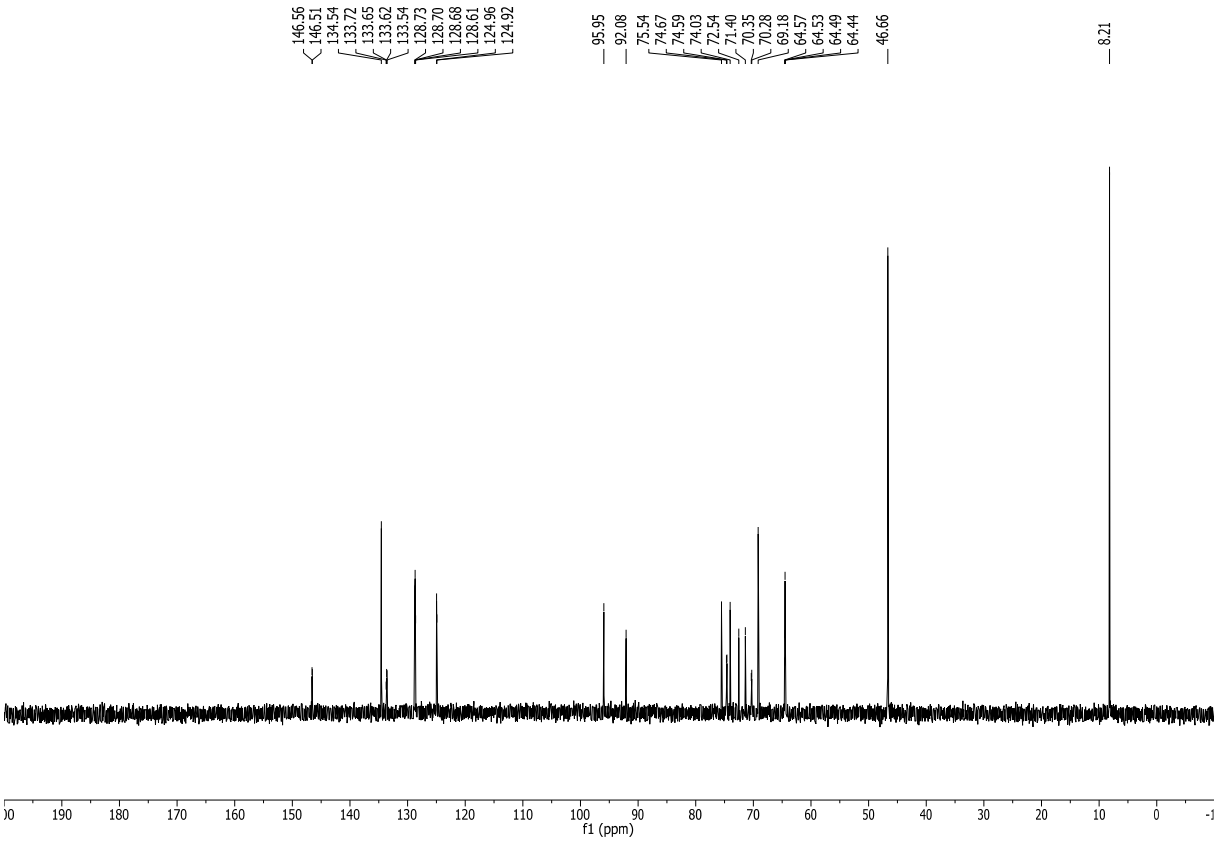
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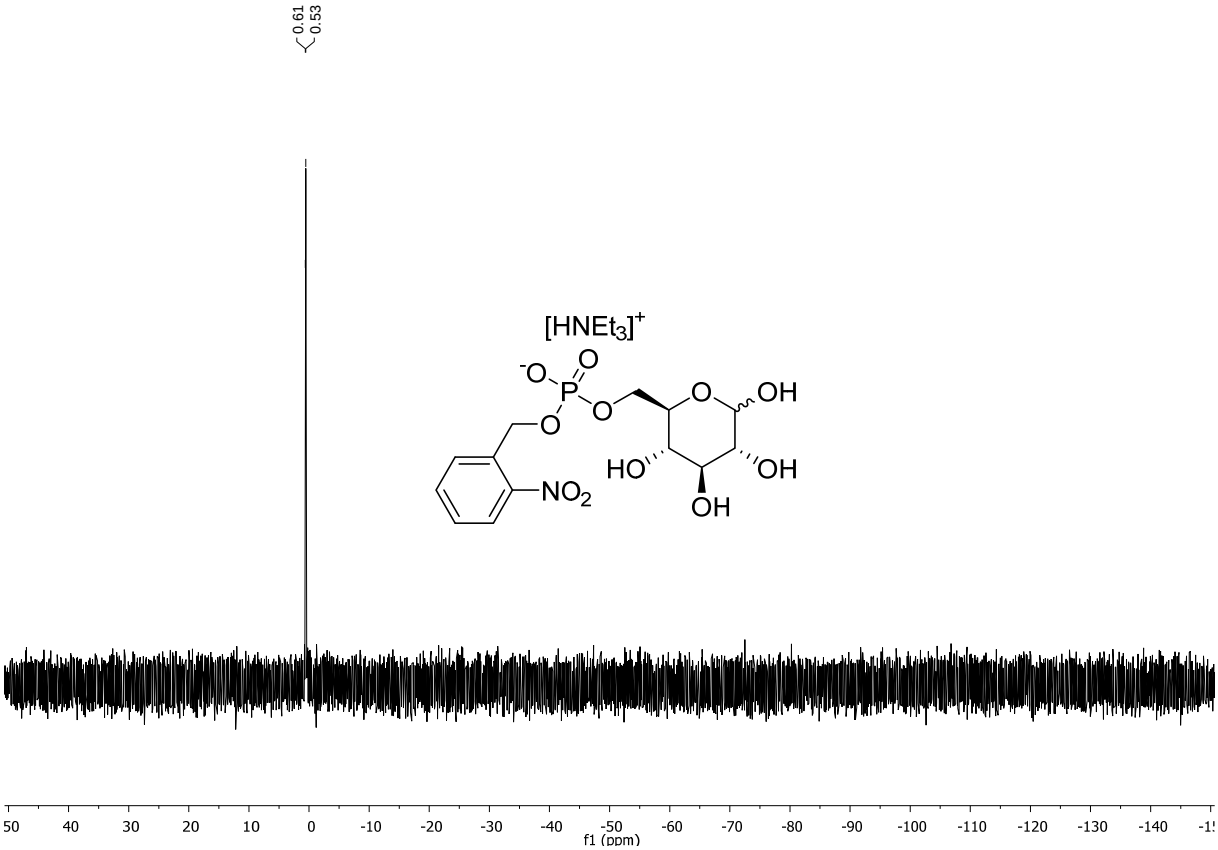
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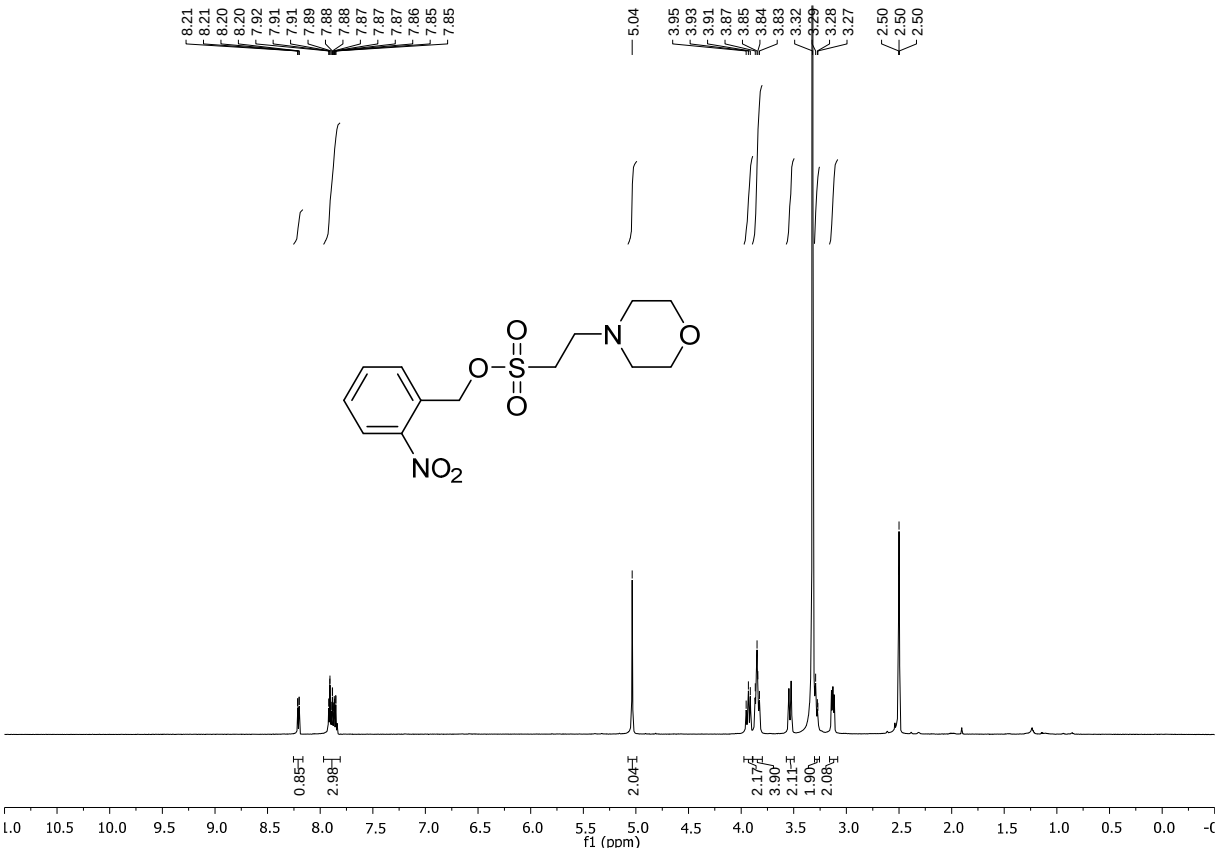
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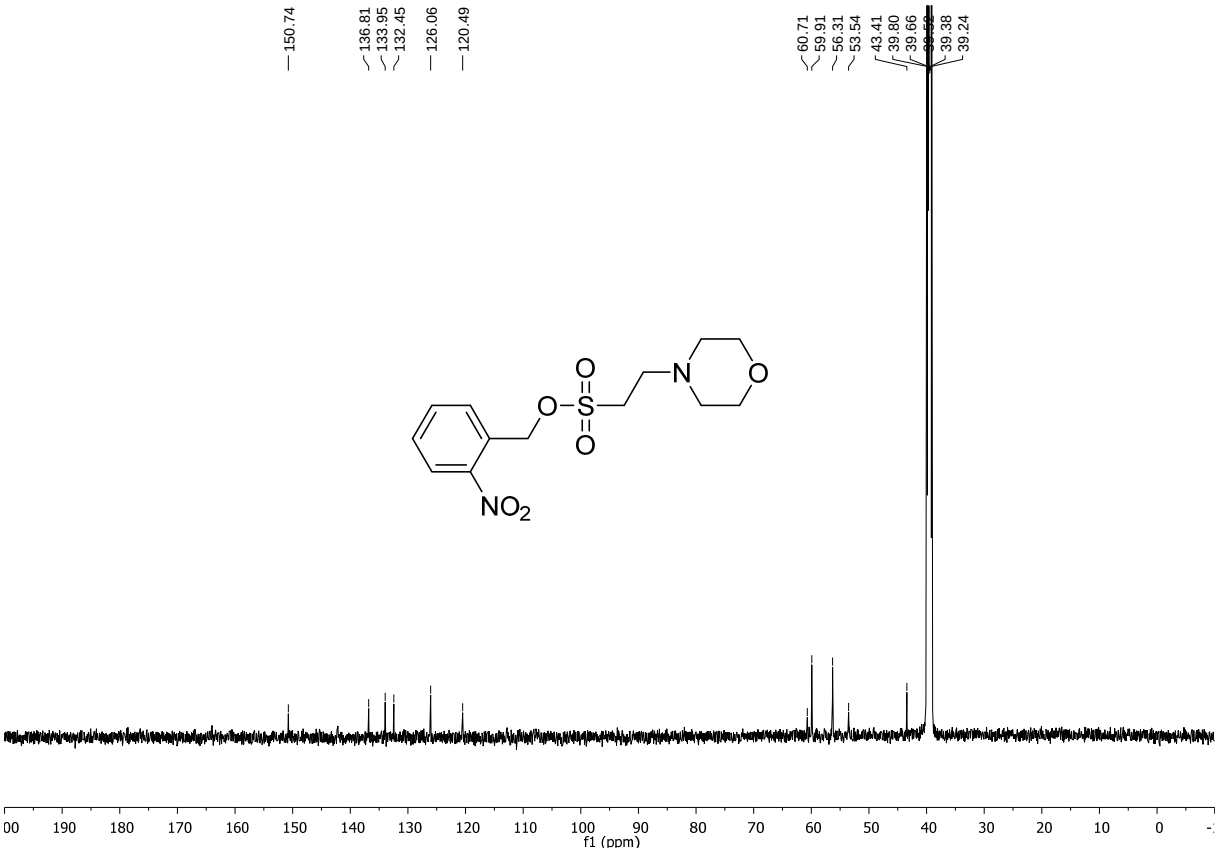
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39.24
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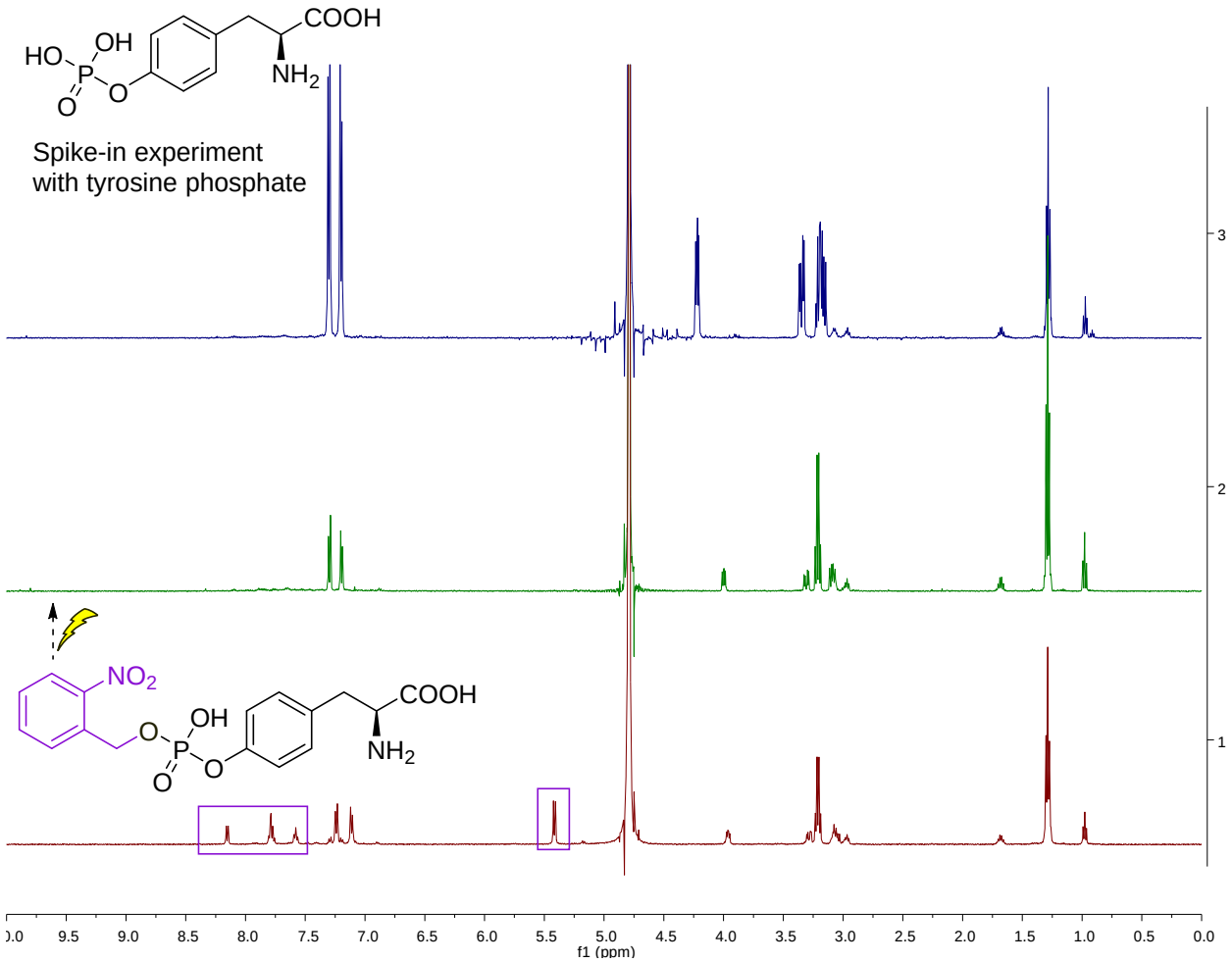
S
O
O
N
O
NO2
f1 (ppm)

Photo cleavage of Tyrosine phosphate 2-nitro benzyl ester

0.8 mg of tyrosine phosphate 2-nitro benzyl ester was dissolved in D_2O in a NMR tube and kept in the dark for measuring NMR spectrum, then it was put under 366 nm UV lamp overnight (NMR spectrum was measured in between). After 15 h, the phosphate 2-nitro-benzyl ester was found to be cleaved according to ^{1}H-NMR spectral data. Afterwards 5 mg of tyrosine phosphate were added to the NMR tube in order to confirm the product peaks. The photo-cleaved side product of the reaction is insoluble in water and therefore not visible in the ^{1}H-NMR spectrum. Please note the presence of triethylamine salt which resulted from the HPLC purification buffer of the phosphate 2-nitro benzyl ester. In these experiments it was used as an internal standard.



Alkylation of phosphate monoester vs phosphodiester

The c-AMP and c-di-GMP were tested under the same condition as before, pH 6, MES buffer. It was found that phosphodiesters remained unconverted employing 10 equivalents of diazo substrate. The hexanucleotides in **table S3** were tested later at pH 7.

Table S3 screening of alkylation of phosphodiester

		Conversion of nulceic acid	
	Reaction concentration	10 eq. diazo	30 eq. diazo
c-AMP	50 mM	0	5%
c-di-GMP	50 mM	0	12%
TACTGC	100 μM	0	0
pTACTGC	100 μM	42%	52%

c-AMP

70 mg c-AMP was dissolved in 4 mL of 250 mM MES buffer pH 6 to give a 50 mM c-AMP stock solution. Then 0.5 mL of this stock solution was incubated with 10 equivalents of 2-nitro-phenyldiazomethane whereupon gas bubbles formed slowly. After 1.5 h the gas formation ceased and UPLC-MS analysis (Method A) indicated no conversion of c-AMP. Another 0.5 mL of c-AMP solution was treated with 30 equivalents of 2-nitro-phenyldiazomethane, however due to the large excess of diazo the reaction became heterogeneous. Although the reagent couldn't interact with all the c-AMP solution, gas formation stopped after 2 h. The aqueous solution was transfer to a new flask and washed with diethyl ether (2 ml) to remove diazo side products. The aqueous solution was submitted to UPLC-MS analysis indicating 5% c-AMP conversion to the corresponding product. When using a huge excess of diazo reagent (50 eq.) for c-AMP modification, conversion didn't exceed 6%.

c-di-GMP

22 mg c-di-GMP was dissolved in 1 mL of 250 mM MES buffer pH 6 to give a 50 mM stock solution. Then 0.5 mL of this stock solution was incubated with 10 equivalents of 2-nitro-phenyldiazomethane, whereupon gas bubbles formed slowly. After 1.5 h the gas formation ceased and UPLC-MS analysis indicated no conversion of c-di-GMP. Another 0.5 mL of c-di-GMP solution was treated with 30 equivalents diazo and gas formation stopped after 2 h. The aqueous solution was transfer to a new flask and washed with diethyl ether to remove the diazo side products. The aqueous solution was submitted to UPLC-MS analysis where 12% of modified product was observed.

Hexanucleotide TACTGC

The purchased oligomers were dissolved in water to give approximately 2 mM stock solutions. The amount of water was calculated according to Microsynth's quality certification. The accurate concentration was determined by NanoDrop measurements. 5'-Phosphorylated oligo (pTACTGC) was determined to be at 1.4 mM and TACTGC to be at 1.7 mM. 10 mg of 2-nitro-phenyl diazomethane was dissolved in CH_3CN to form a 10 mM stock solution.

Reactions

Oligonucleotide alkylations were tested at 100 μM concentration. The oligomer was first mixed with buffer (MOPS, pH 7.15, 5 mM), diluted with water followed by the addition of the diazo. The reaction details and results are summarized in Table S4. The 5'-phospharylated oligomer pTACTGC was found to give a decent conversion using 5 equivalents of diazo and can be increased to 50% using up to 20 equivalents of diazo. Further increase of the stoichiometry didn't have a positive effect on the conversion. The modification of 5'-phospharylated oligomer pTACTGC at 50 μM was also tested with various amounts of diazo compound. Once the diazo stoichiometry was above 10 equivalents, the reaction gave

similar conversions when compared to the modification at 100 μM. With the normal synthetic oligo TACTGC, no conversion was noted even when using 40 equivalents of diazo as determined by HPLC analysis.

In general the major HPLC peaks were collected manually and subjected to ESI-MS and MS/MS analysis. The desired alkylated oligo was further confirmed by HR-ESI: exact mass calcd. for $C_{65}H_{79}N_{21}O_4P_6^{2-}$, $[M]^{2-}$: 989.6687, found: 989.6603. Using MS-MS the exact alkylation site could not be determined due to the limitation of the MS-MS method. The reaction mixture was also submitted to MALDI TOF analysis. The pTACTGC modification showed mainly mono-alkylation product and a small fraction of double modification. This result is in good agreement with the MS-MS data (Figure S4). The minor peak observed (MS 867.07, I/I_{max} 10%) may be [T*.A.C-b(T)]$^-$, [T.A*.C-b(T)]$^-$ or [T.A.C*-b(T)]$^-$, where * is the site of modification, . is the phosphate linkage and -b(T) is the ribose without base. For the TACTGC, no modification was observed by HPLC analysis. Instead MALDI TOF analysis indicated that besides the major oligo peak, a less intense mass peak for the mono-modified oligomer could be identified. This states that a very small portion of oligo is alkylated which could only be observed by MALDI TOF since a new HPLC peak could not be detected under these conditions.

Table S4 Reaction details and alkylation results of 5'-phosphate hexanucleotide

pTACTGC [mM]	TACTGC [mM]	Buffer [mM]	Diazo [mM]	Total [μL]	ACN [%]	equiv.	Conversi [%]
0.1		1	0.1	30	1	1	3.1
	0.1	1	0.1	30	1		0
0.1		1	0.5	30	5	5	26.9
0.05		1	0.25	30	2.5		19.3
	0.1	1	0.5	30	5		0
0.1		1	1.0	30	10	10	42.3
0.05		1	0.5	30	5		38.1
	0.1	1	1.0	30	10		0
0.1		1	1.5	30	15	15	49.5
0.05		1	0.75	30	7.5		49.6
	0.1	1	1.5	30	15		0
0.1		1	2	30	20	20	56.3
0.05		1	1	30	10		54.3
	0.1	1	2	30	20		0
0.1		1	2.5	30	25	25	49.2
0.05		1	1.25	30	12.5		60
	0.1	1	2.5	30	25		0
0.1		1	3.0	30	30	30	52.7
0.05		1	1.5	30	10		52.7
	0.1	1	3.0	30	30		0
0.1		1	4.0	30	40	40	47.6
	0.1	1	4.0	30	40		0

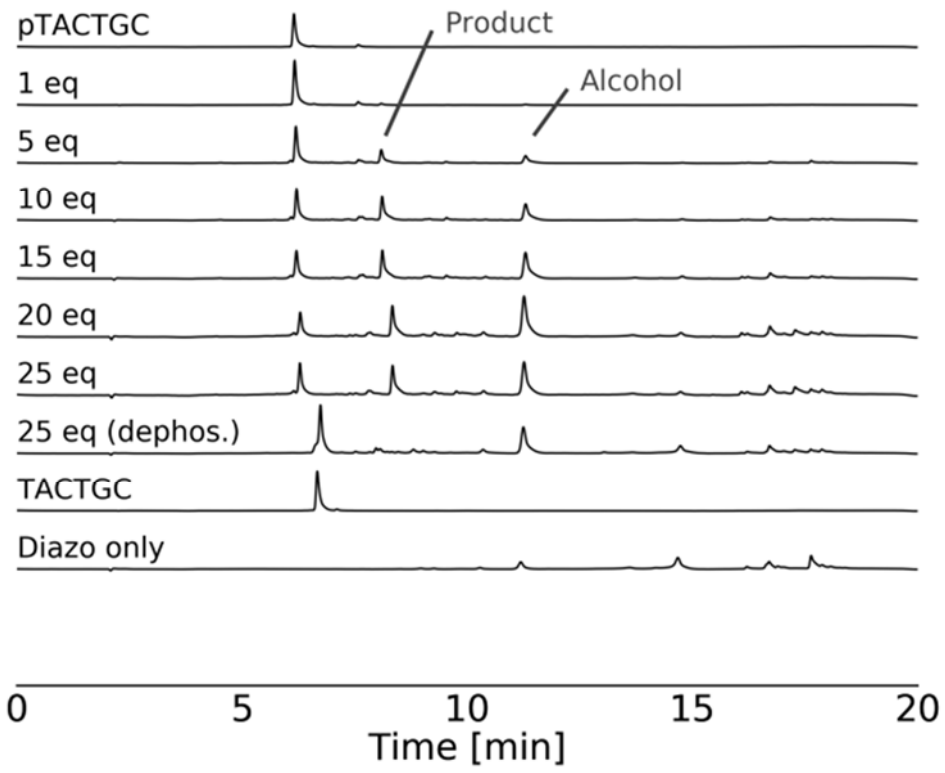


Figure S3 HPLC trace of 0.1 mM 5'-phospahte hexanucleotide modification

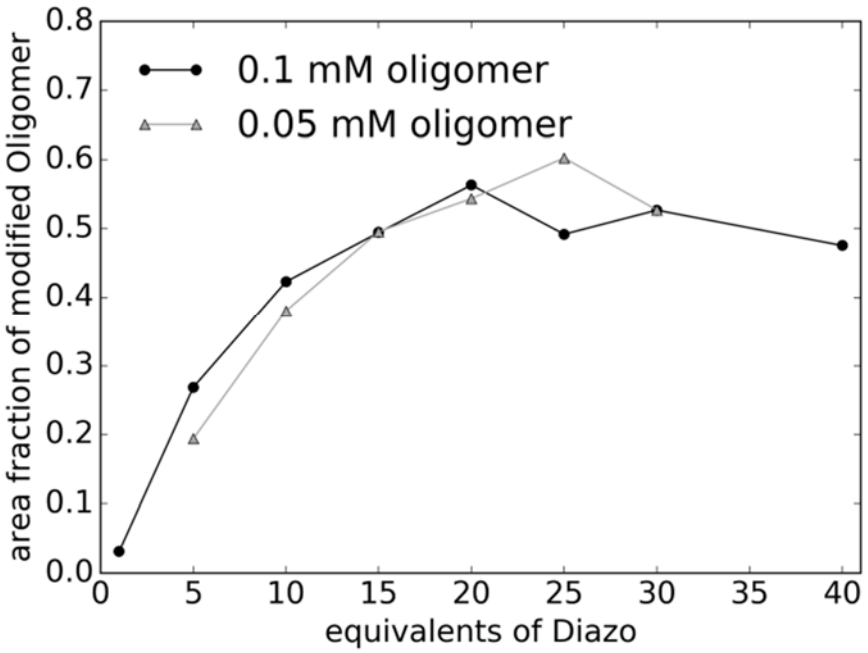


Figure S4 alkylation of 5'-phospahte hexanucleotide with varying equivalents of diazo reagent

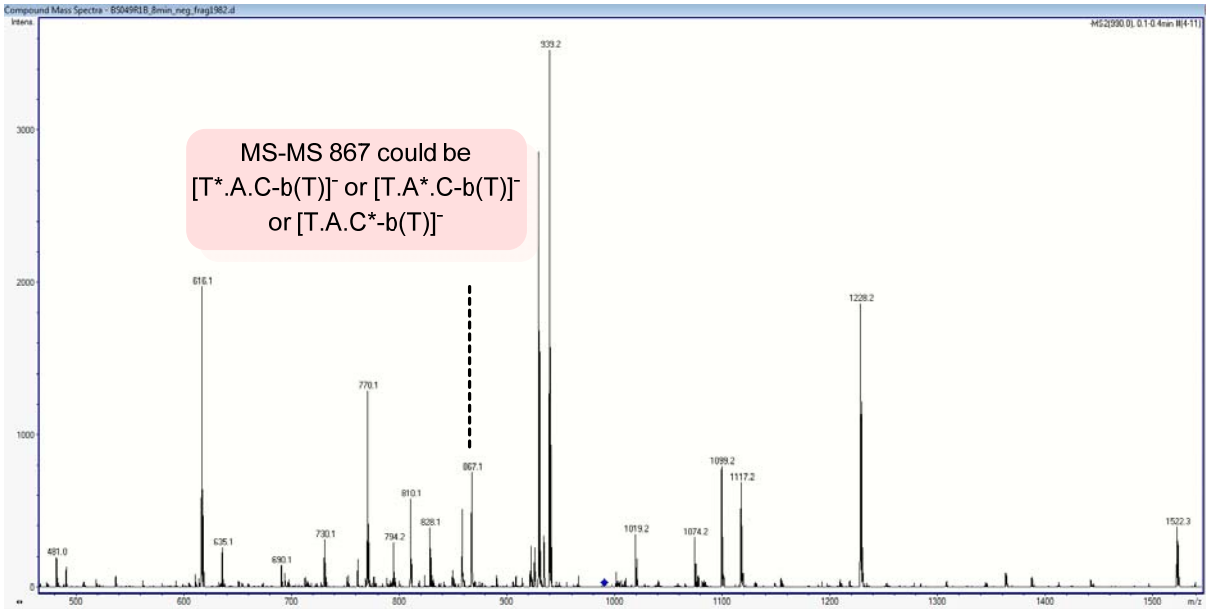


Figure S5 5'-PO_4-TACTGC modification

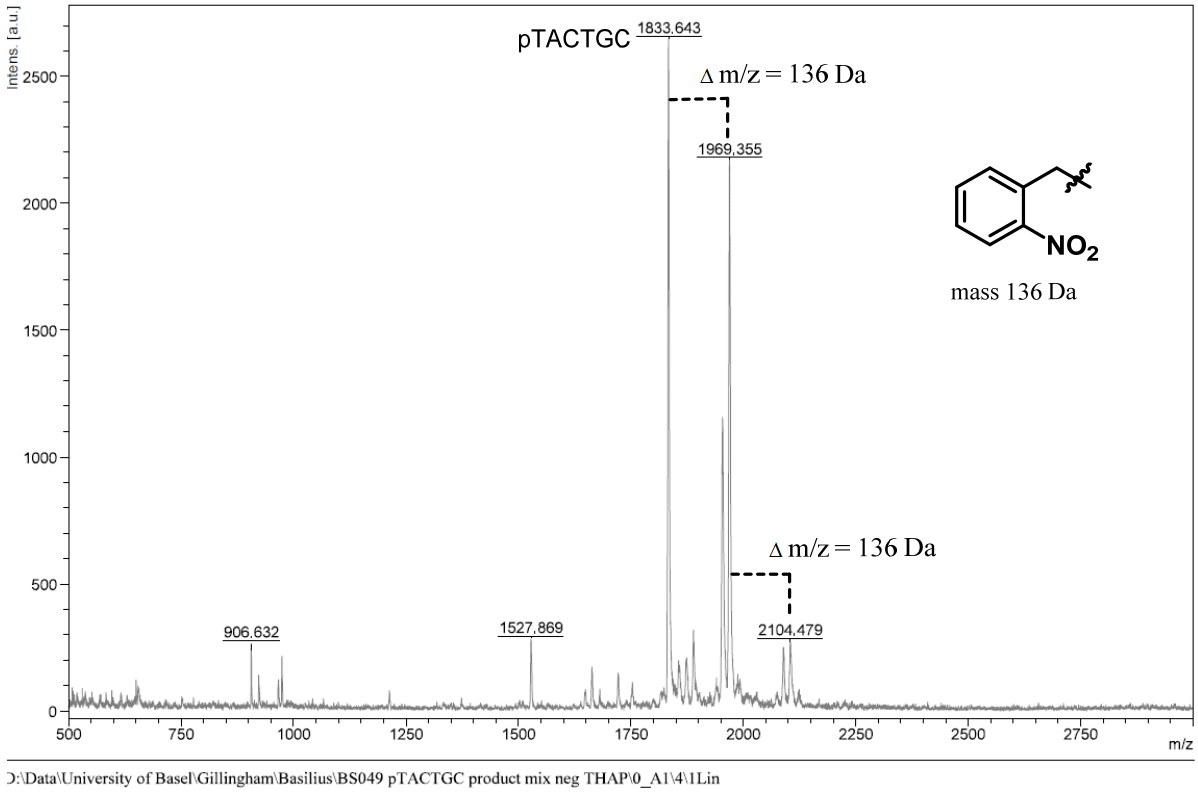


Figure S6 MALDI TOF spectrum of 5'-PO_4-TACTGC modification with 40 equivalents of diazo reagent

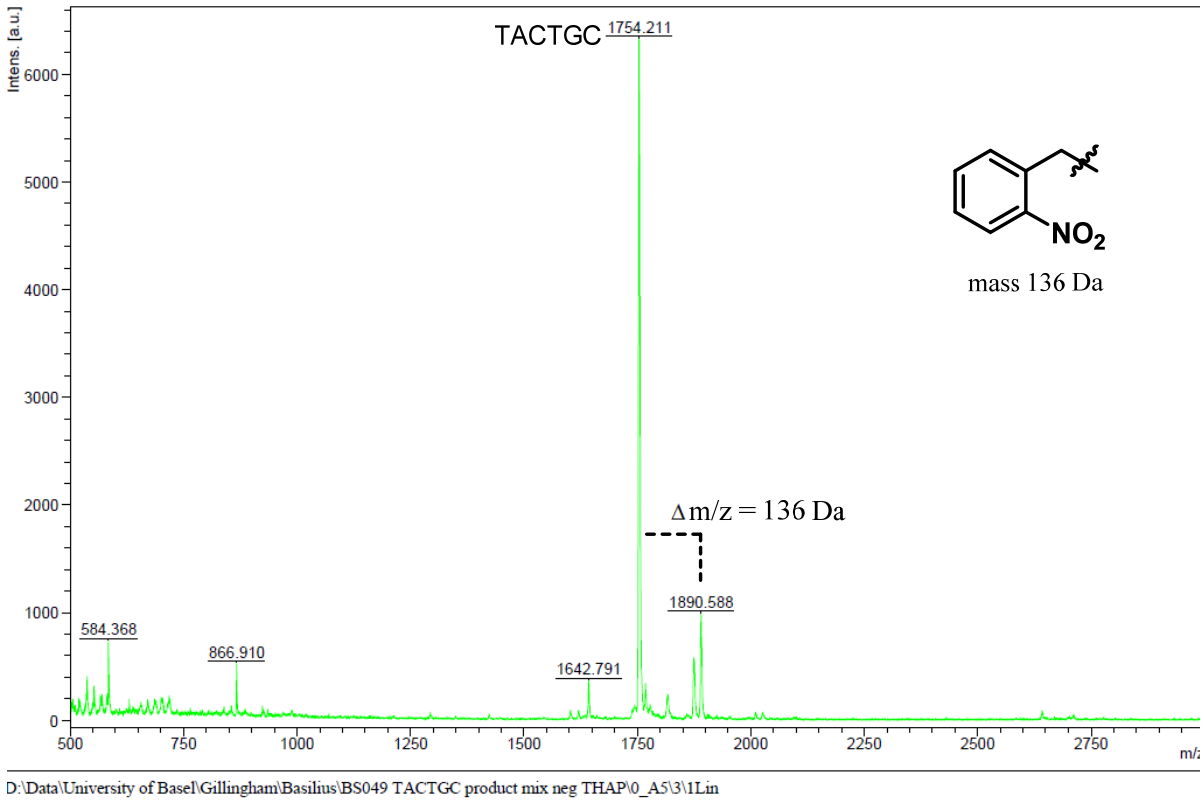
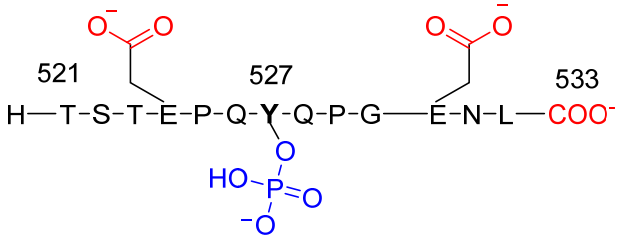


Figure S7 MALDI TOF spectrum of TACTGC modification with 40 equivalents of diazo reagent

Modification of peptide phosphate

All the reactions were carried out by adding the diazo compound to the peptide aqueous solution. The yellow reaction mixture turned colorless upon complete diazo consumption. It took 15-30 min for the following reactions. Since the diazo side products were found to be insoluble in water, diethyl ether (2 x 0.2 mL) was added to wash away the side products. Afterwards the aqueous solution was subjected to a gentle nitrogen flow for a minute to remove residual diethyl ether followed by UPLC-MS analysis (Method B) of the aqueous reaction mixture. The modification position was confirmed by collecting the corresponding HPLC peaks followed by MS-MS analysis. The collected fractions were resubmitted to UPLC-MS to confirm the identity of the purified peak. Sequencing of the modified peptide was carried out by standarded MS/MS method.[4] The calculation was done by mMass software (http://www.mmass.org/).

13mer peptide pp60 Src (521-533)



Phosphorylated peptide pp60 Src (521-533) was purchased from Bachem as TFA salt. It was dissolved in water to give a 5 mM stock solution (pH = 3.3).

The modification of the peptide is carried out at pH 6, however it didn't show selectivity of the phosphate

modification. So the reaction was investigated at different pH.
Stock solution: 5 mM peptide in water, 50 mM diazo in CH_3CN, 5 mM MES buffer pH 6.05, 5 mM MOPS buffer pH 7.05.
1: 190 µL water, 50 µL MES buffer, pH 6.05, 5 µL peptide, 5 µL diazo
2: 190 µL water, 50 µL MOPS buffer, pH 7.05, 5 µL peptide, 5 µL diazo
3: 240 µL water, 5 µL peptide, 5 µL diazo, (pH = 4.2 due to TFA salt of the peptide)

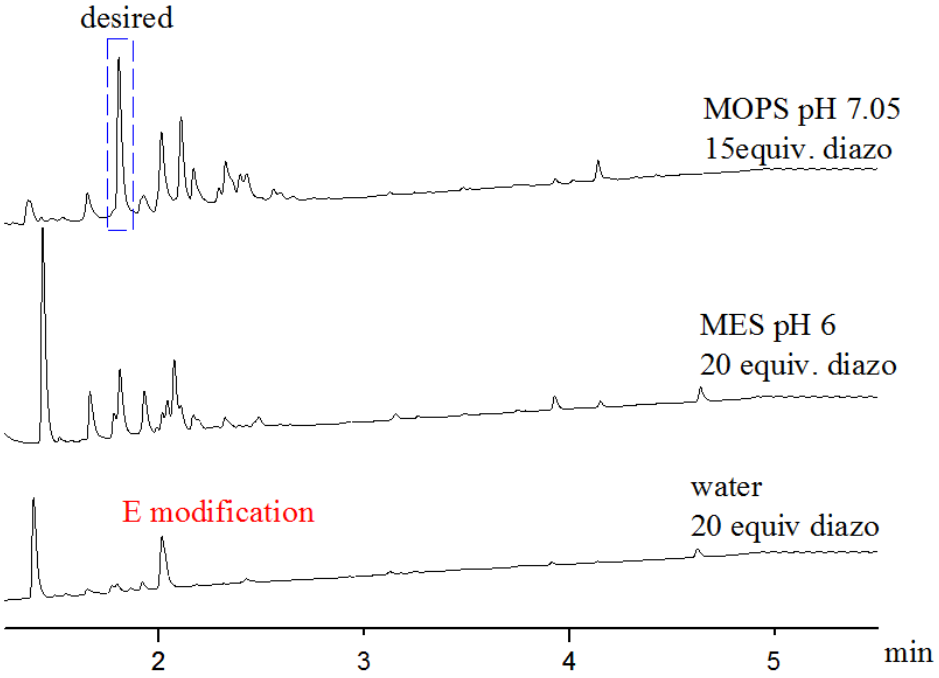


Figure S8 UPLC trace of the Src (521-533) modification at different pH

If the commercially available Src(521-533) TFA salt is dissolved in unbuffered water (final pH 4.2) and treated directly with diazo, alkylation of glutamic acid 531 (E531) was found to be the major product (see Figure S7). Consistent with the pK_a's controlling selectivity, performing the same reaction at pH 6 and 7, the alkylation preference switches to tyrosine 527 (Y527). However the addition of 15 equivalents diazo reagent leads to substantial amounts of double alkylation, thereby reducing the yield of the desired alkylation. The reaction was re-investigated with less equivalents of diazo reagent. (The sites of alkylation have been determined by MS/MS sequencing, see Table S8-S10 for details).

Stock solutions: 5 mM peptide in water, 10 mM diazo in CH_3CN, 5 mM MOPS buffer pH 7.05.
Final reaction mixture: 100 µM peptide, 1 mM MOPS pH 7.05, and diazo were added into the peptide solution one equivalent per time for the first 5 reactions.
1: 192 µL water, 50 µL MOPS buffer, pH 7.05, 5 µL peptide, 2.5 µL 10 mM diazo
2: 190 µL water, 50 µL MOPS buffer, pH 7.05, 5 µL peptide, 5 µL 10 mM diazo
3: 188 µL water, 50 µL MOPS buffer, pH 7.05, 5 µL peptide, 7.5 µL 10 mM diazo
4: 185 µL water, 50 µL MOPS buffer, pH 7.05, 5 µL peptide, 10 µL 10 mM diazo
5: 182 µL water, 50 µL MOPS buffer, pH 7.05, 5 µL peptide, 12.5 µL 10 mM diazo
6: 190 µL water, 50 µL MOPS buffer, pH 7.05, 5 µL peptide, 5 µL 50 mM diazo
7: 188 µL water, 50 µL MOPS buffer, pH 7.05, 5 µL peptide, 7.5 µL 50 mM diazo

Table S5 Src (521-533) alkylation with varying equivalents of diazo reagent

Entry	Diazo Equiv.	Phosphate modification	Conv.
1	1	19%	23%
2	2	35%	43%
3	**3**	**42%**	**52%**
4	4	47%	73%
5	5	44%	68%
6	10	47%	75%
7	15	40%	95%

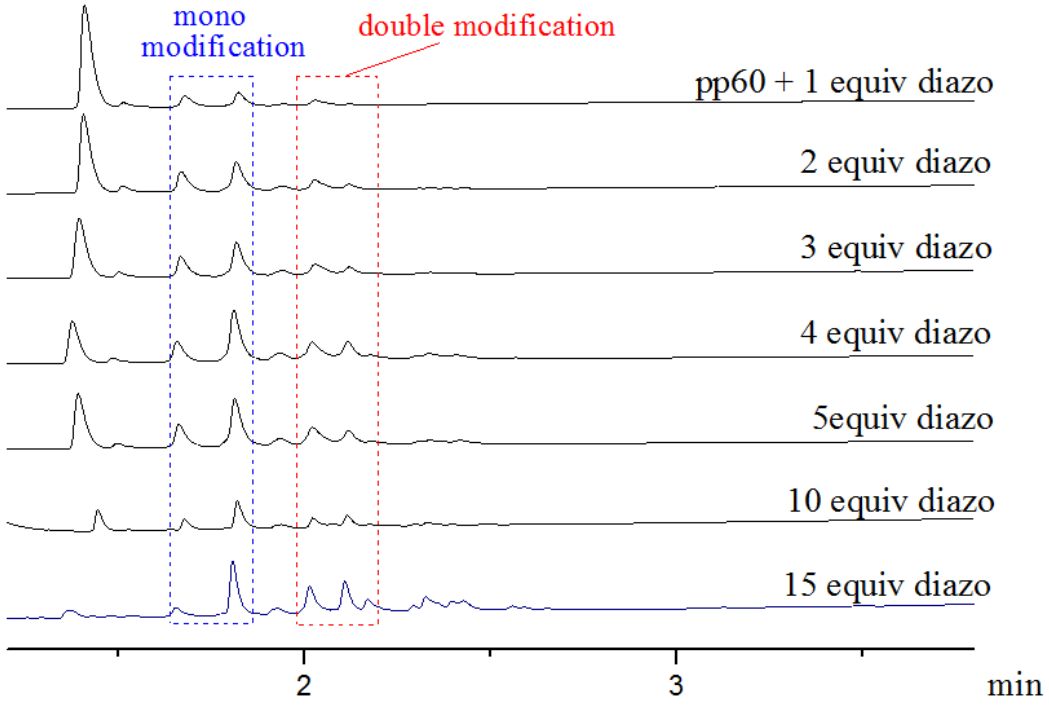
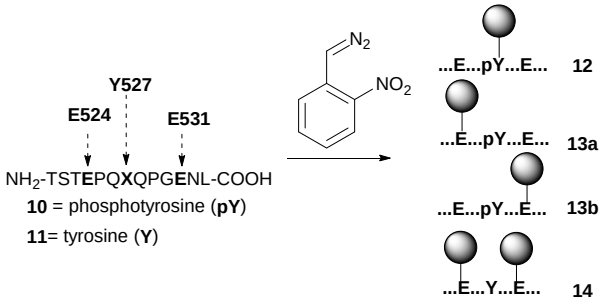


Figure S9 UPLC trace of the Src (521-533) modification with varying equivalents of diazo reagent

Table S6 summary of selective phosphopeptide modification



entry	substrate	pH[a]	equiv. diazo	Major protduct	conv.[b]	Y527: (E524+531)[c]
1	10	4.3	20	13	40	1:9[d]
2	10	7	2	12	43(35)	13:1
3	10	7	3	12	52(42)	15:1
4	10	7	5	12	68(44)	11:1
5	10	7	10	12	75(47)	11:1
6	10	7	15	12	95(40)	11:1
7	11	7	10	14	30	n.d.

[a]Run in 5 mM MOPS buffer at pH 7, pH 4 experiment is run in unbuffered water and the pH is measured after the reaction; [b]first number is conversion of starting peptide; number in parenthesis refers to amount of alkylated phosphate; [c]ratios are determined by HPLC intergration; [d]a small side product obscures phosphate alkylation hence we can only report in a lower limit.

The result of peptide modification with lower amount of diazo reagent is very interesting. With one equivalent diazo, only phosphate modification was achieved. Using three equivalents of diazo, it mainly modifies the phosphate of Y527 with minor double modification (see Figure S7 and Table S6). Although there are three carboxylates available for potential alkylation (C-terminal, E524 and E531), phosphate alkylation is the major product along with small portions of carboxylate modification (Y527: E=15:1, Table S6 entry 2-6). Since the pK_a of the C-terminal carboxylic acid is around 2-3, no alkylation of this position was observed. The increase of diazo equivalents leads to double alkylation as we observed before. The selectivity of phosphate and carboxylic acid alkylation was calculated according to the extracted ion abundance (See Figure S9-S13).

In addition, while 10 equivalents of diazo reagent leads to 75% conversion of the phosphorylated peptide the non phosphorylated peptide is only partially modified under the same conditions (table S7 entry 7).

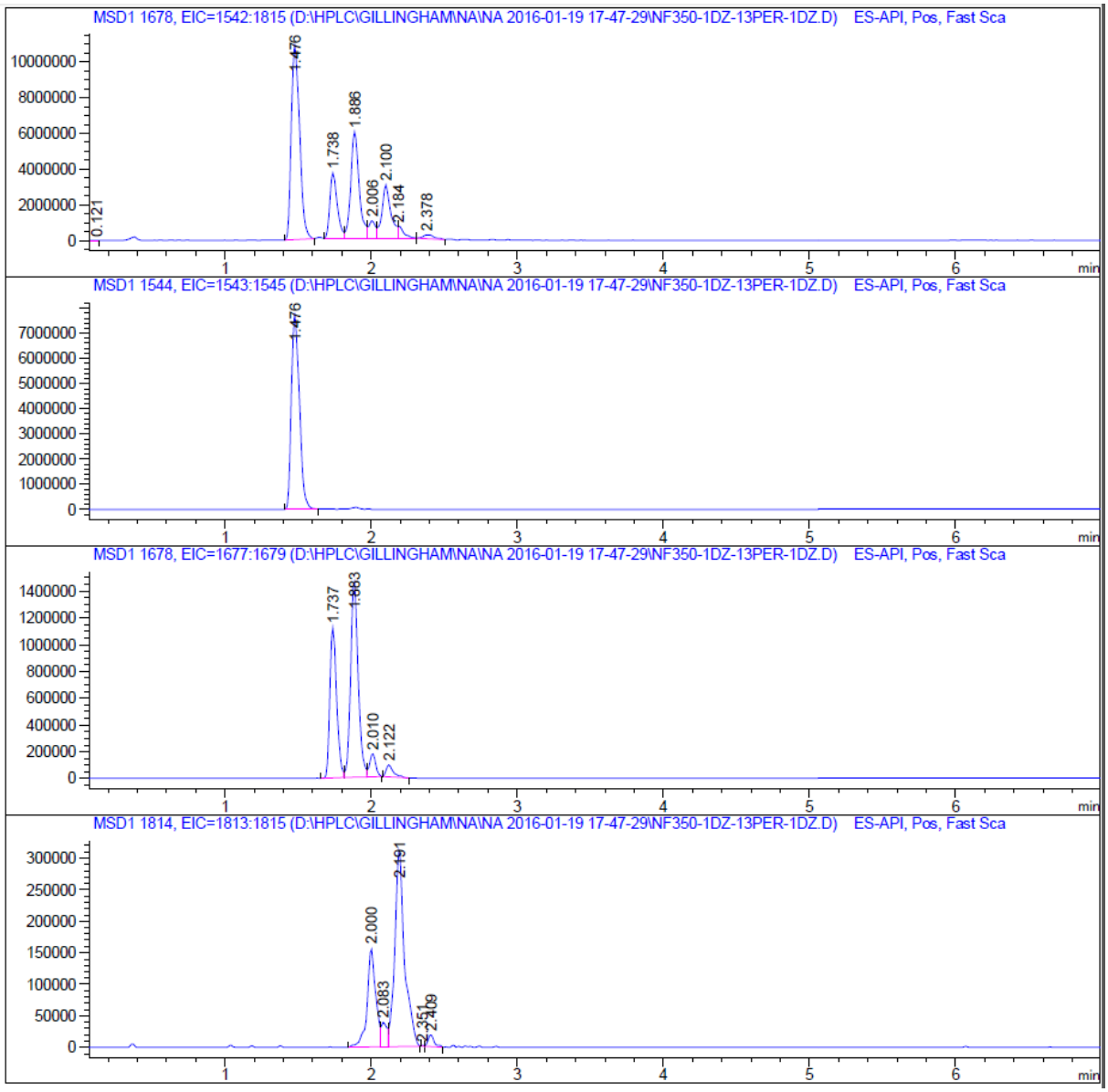


Figure S10 ion extraction of the reaction of Src (521-533) with 2 equivalents of diazo reagent

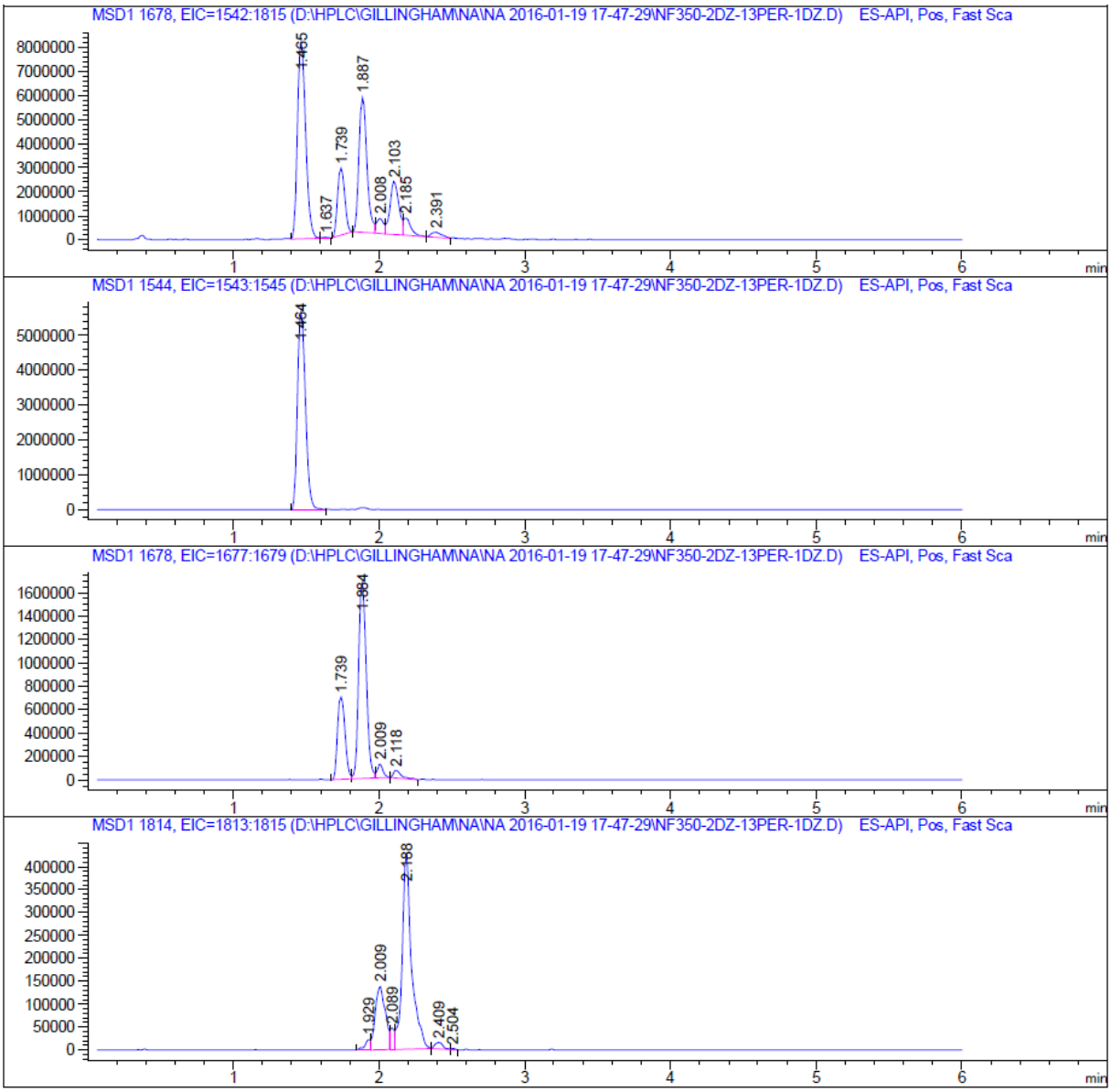


Figure S11 ion extraction of the reaction of Src (521-533) with 3 equivalents of diazo reagent

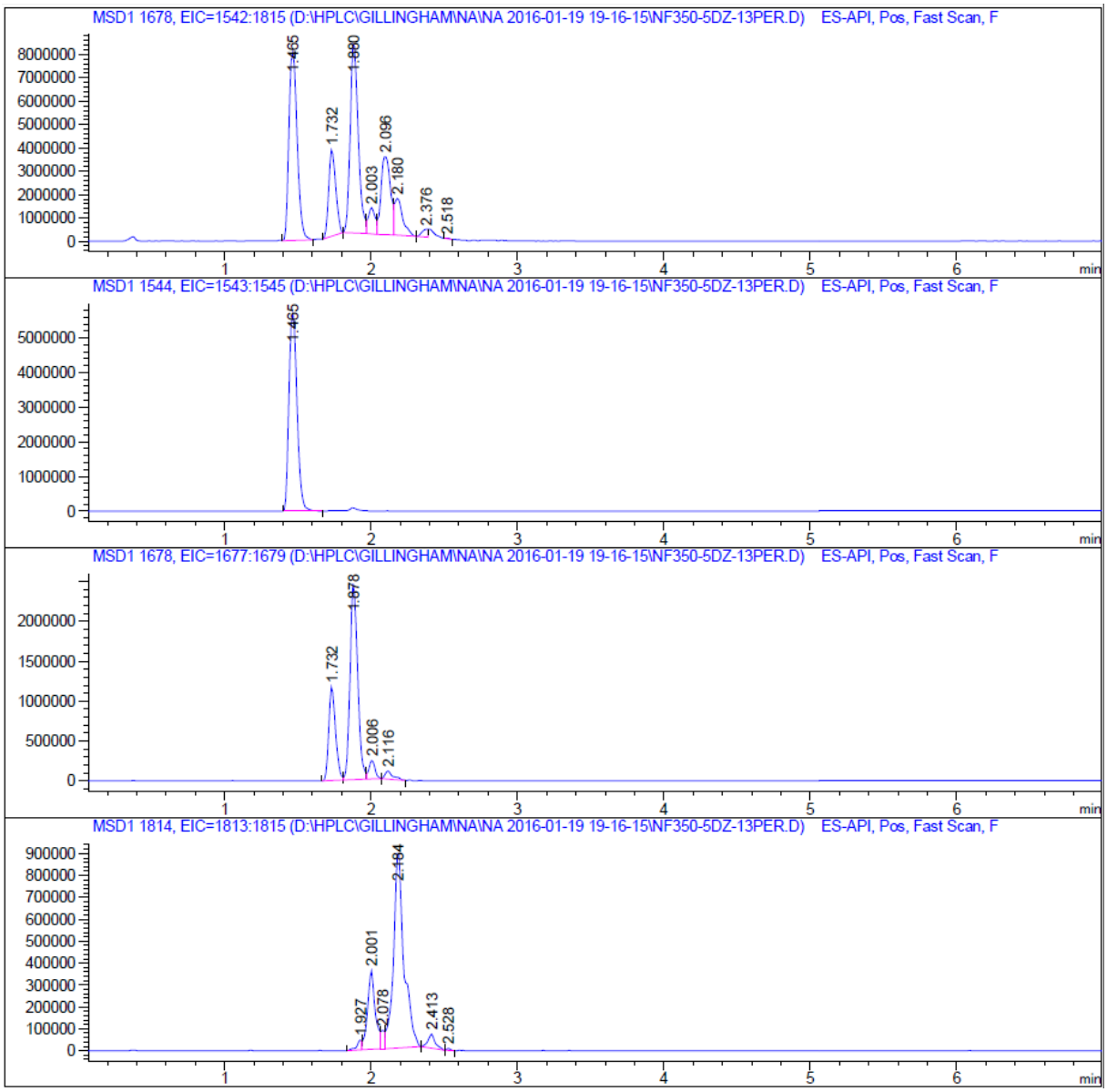


Figure S12 ion extraction of the reaction of Src (521-533) with 5 equivalents of diazo reagent

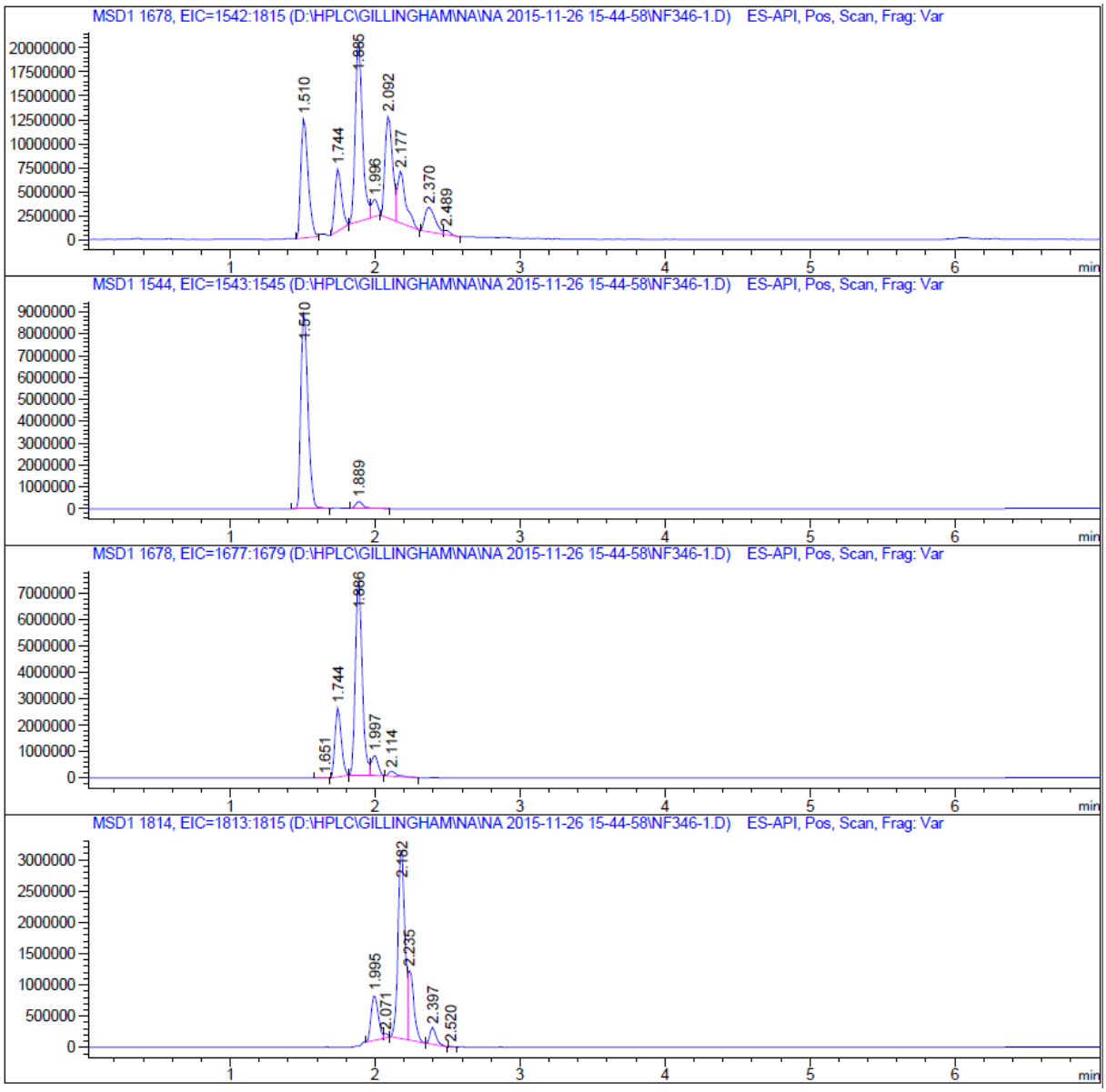


Figure S13 ion extraction of the reaction of Src (521-533) with 10 equivalents of diazo reagent

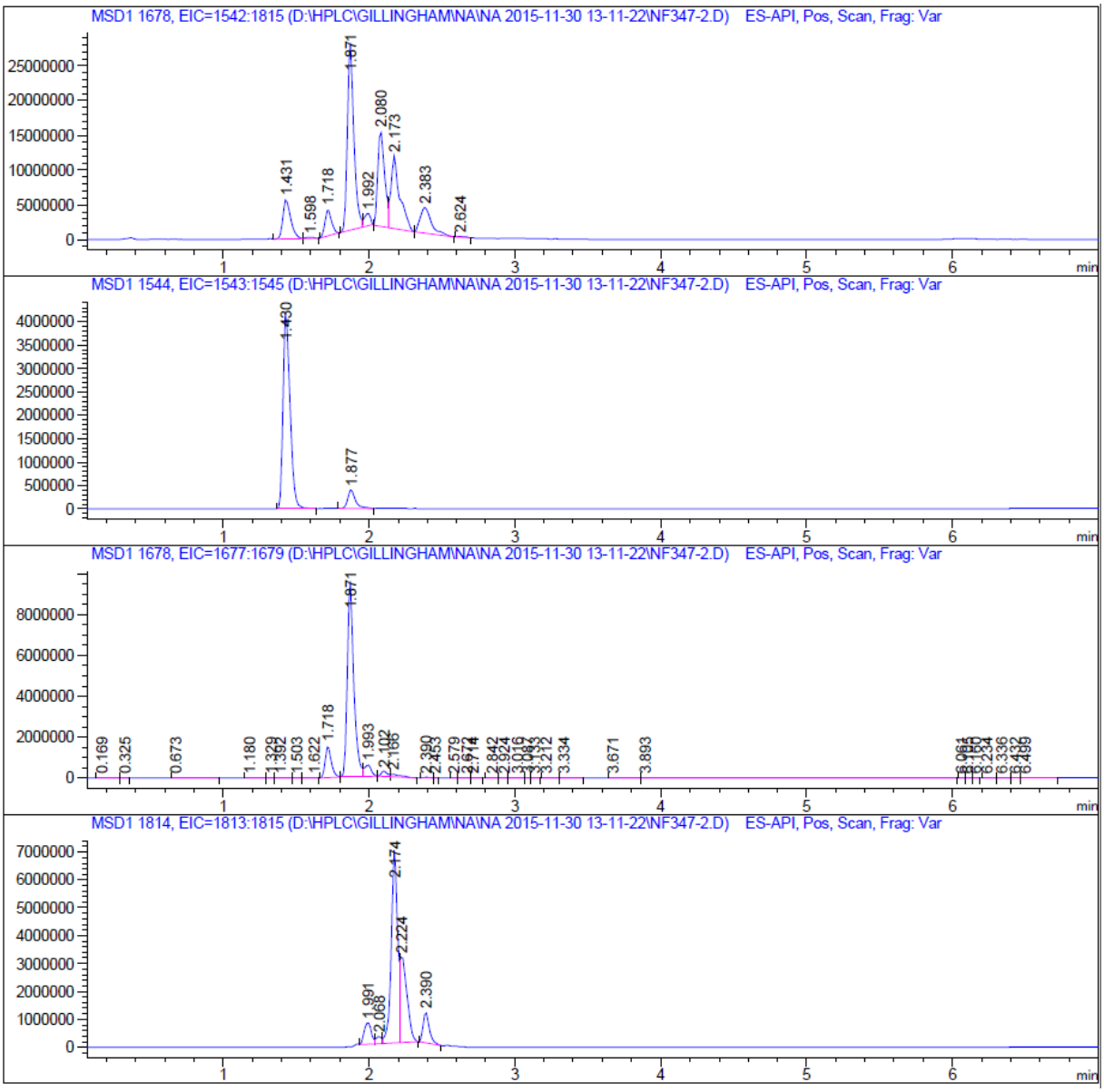
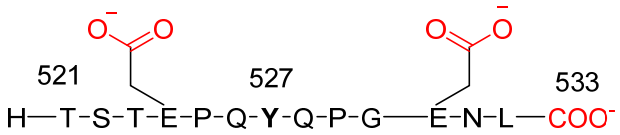


Figure S14 ion extraction of the reaction of Src (521-533) with 15 equivalents of diazo reagent

Peptide pp60 Src (521-533) non phosphorylated



The original peptide without phosphate group was also investigated. The 13mer peptide could be modified with 10 equivalents of diazo reagent. The reaction was carried out as descirbed before, 100 µM peptide, 1 mM MOPS buffer pH 7.05 followed by the addition of the diazo.

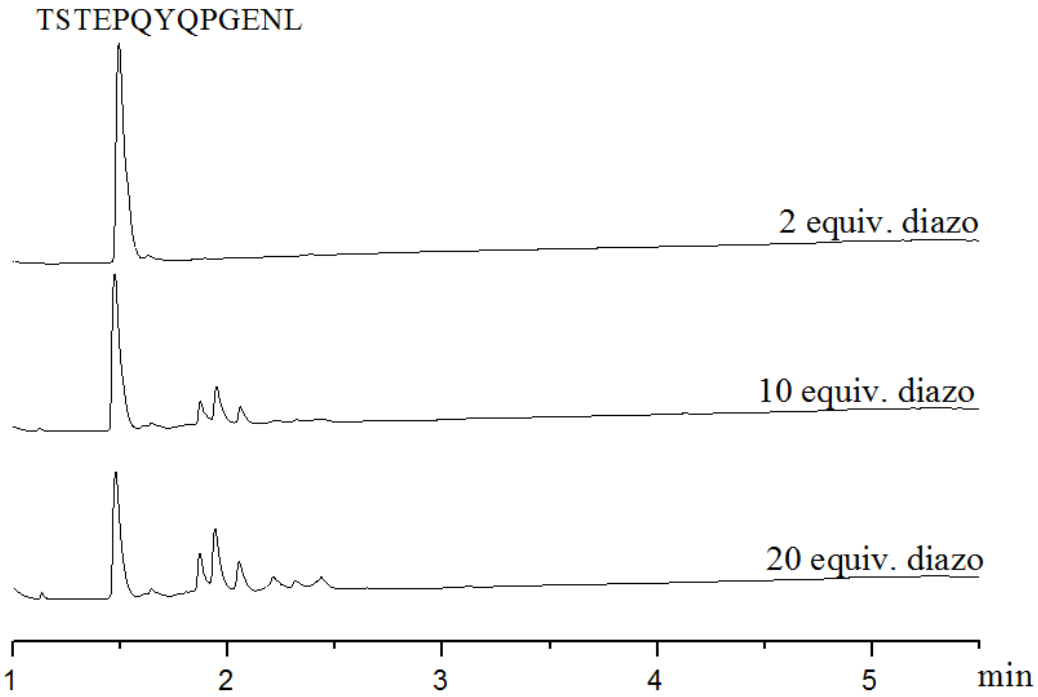


Figure S15 UPLC trace of Src (521-533) of modification

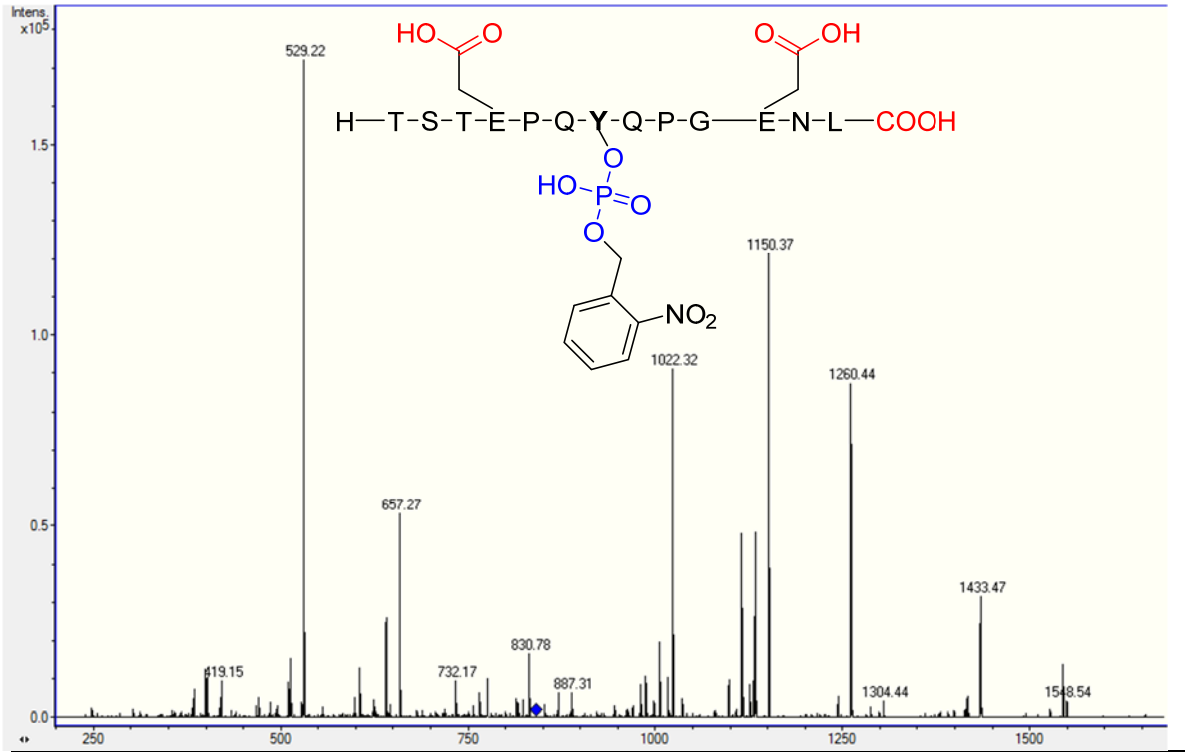
Table S7 Src (521-533 non phosphorylated form) modification

Entry	equiv. diazo	Conv.
1	2	0
2	10	30%
3	20	44%

MS-MS of modified pp60 Src products

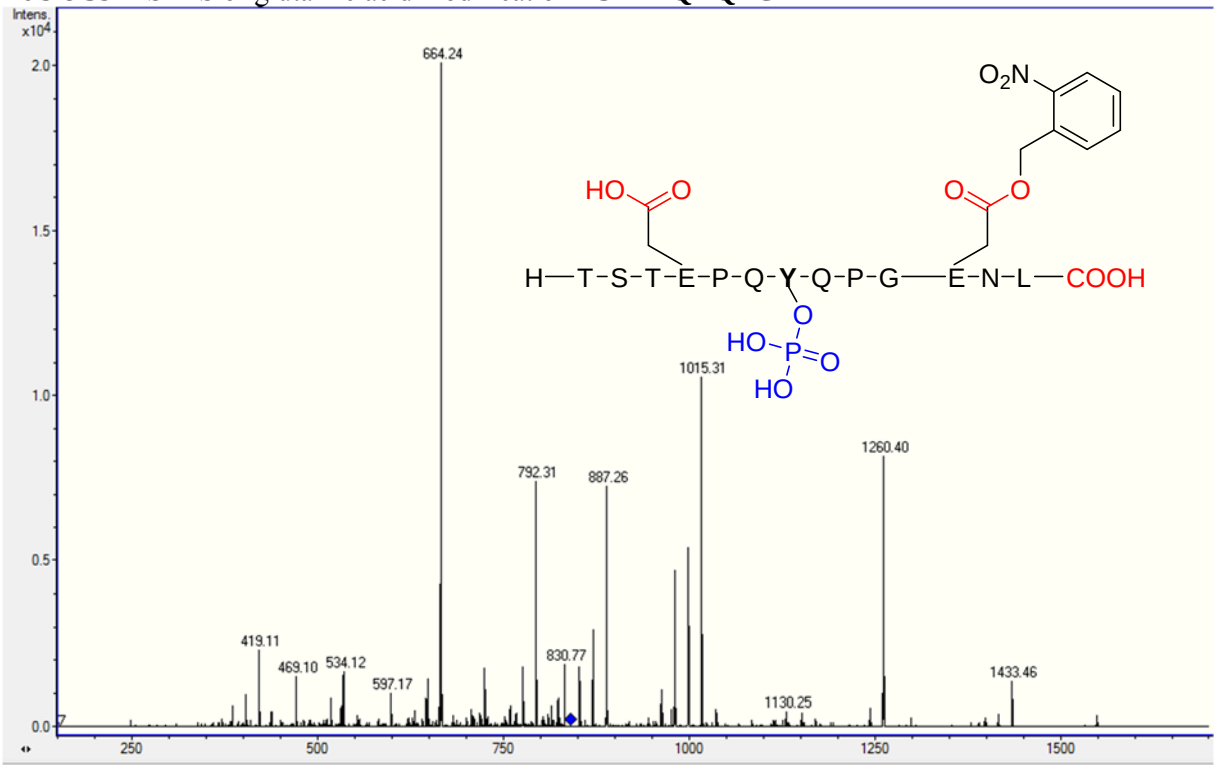
The MS-MS was the fragmentation of the double charged products. In the following table, red is the observed MS which matches the theory MS/MS, the black is the caculated value.

Table S8 MS-MS fragments of phosphate modification **TSTEPQ<u>Y</u>QPGENL**



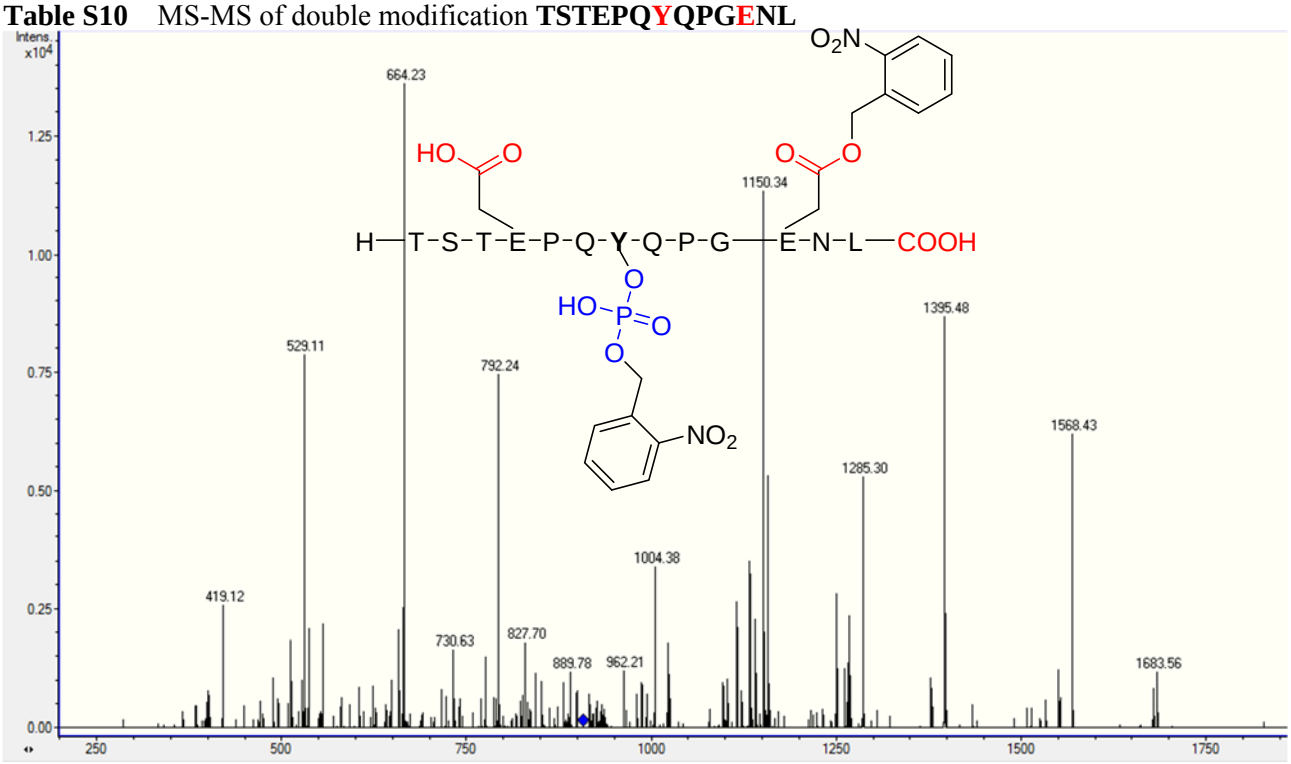
ion	slice	m/z	z	sequence
M	[1-13]	1678.663	1	.TSTEPQYQPGENL. [1x NO_2-Benzyl phosphate]
b2	[1-2]	189.087	1	.TS.t
b3	[1-3]	290.1347	1	.TST.e
b4	[1-4]	419.1773	1	.TSTE.p .TSTEP.
b5	[1-5]	516.23	1	q
b6	[1-6]	644.2886	1	.TSTEPQ.y
b7	[1-7]	1022.35	1	.TSTEPQY.q [1x NO_2-Benzyl phosphate]
b8	[1-8]	1150.409	1	.TSTEPQYQ.p [1x NO_2-Benzyl phosphate]
b9	[1-9]	1247.462	1	.TSTEPQYQP.g [1x NO_2-Benzyl phosphate]
b10	[1-10]	1304.483	1	.TSTEPQYQPG.e [1x NO_2-Benzyl phosphate]
b11	[1-11]	1433.526	1	.TSTEPQYQPGE.n [1x NO_2-Benzyl phosphate]
b12	[1-12]	1547.569	1	.TSTEPQYQPGEN.l [1x NO_2-Benzyl phosphate]
y12	[2-13]	1577.616	1	t.STEPQYQPGENL. [1x NO_2-Benzyl phosphate]
y11	[3-13]	1490.584	1	s.TEPQYQPGENL. [1x NO_2-Benzyl phosphate]
y10	[4-13]	1389.536	1	t.EPQYQPGENL. [1x NO_2-Benzyl phosphate]
y9	[5-13]	1260.493	1	e.PQYQPGENL. [1x NO_2-Benzyl phosphate]
y8	[6-13]	1163.441	1	p.QYQPGENL. [1x NO_2-Benzyl phosphate]
y7	[7-13]	1035.382	1	q.YQPGENL. [1x NO_2-Benzyl phosphate]
y6	[8-13]	657.3202	1	y.QPGENL. q.PGEN
y5	[9-13]	529.2617	1	L
y4	[10-13]	432.2089	1	p.GENL.
y3	[11-13]	375.1874	1	g.ENL.
y2	[12-13]	246.1448	1	e.NL.
y1	[13-13]	132.1019	1	n.L.

Table S9 MS-MS of glutamic acid modification **TSTEPQYQPGENL**



ion	slice	m/z	z	sequence
M	[1-13]	1678.663	1	.TSTEPQYQPGENL. [1xPhosphate; 1x NO_2-benzyl]
b2	[1-2]	189.087	1	.TS.t
b3	[1-3]	290.1347	1	.TST.e
b4	[1-4]	419.1773	1	.TSTE.p
b5	[1-5]	516.23	1	.TSTEP.q
b6	[1-6]	644.2886	1	.TSTEPQ.y
b7	[1-7]	887.3183	1	.TSTEPQY.q [1xPhosphate]
b8	[1-8]	1015.377	1	.TSTEPQYQ.p [1xPhosphate]
b9	[1-9]	1112.43	1	.TSTEPQYQP.g [1xPhosphate]
b10	[1-10]	1169.451	1	.TSTEPQYQPG.e [1xPhosphate]
b11	[1-11]	1433.526	1	.TSTEPQYQPGE.n [1xPhosphate; 1x NO_2-benzyl]
b12	[1-12]	1547.569	1	.TSTEPQYQPGEN.l [1xPhosphate; 1x NO_2-benzyl]
y12	[2-13]	1577.616	1	t.STEPQYQPGENL. [1xPhosphate; 1x NO_2-benzyl]
y11	[3-13]	1490.584	1	s.TEPQYQPGENL. [1xPhosphate; 1x NO_2-benzyl]
y10	[4-13]	1389.536	1	t.EPQYQPGENL. [1xPhosphate; 1x NO_2-benzyl]
y9	[5-13]	1260.493	1	e.PQYQPGENL. [1xPhosphate; 1x NO_2-benzyl]
y8	[6-13]	1163.441	1	p.QYQPGENL. [1xPhosphate; 1x NO_2-benzyl]
y7	[7-13]	1035.382	1	q.YQPGENL. [1xPhosphate; 1xNO_2-benzyl]
y6	[8-13]	792.3523	1	y.QPGENL. [1x NO_2-benzyl]
y5	[9-13]	664.2937	1	q.PGENL. [1x NO_2-benzyl]
y4	[10-13]	567.2409	1	p.GENL. [1x NO_2-benzyl]
y3	[11-13]	510.2195	1	g.ENL. [1x NO_2-benzyl]
y2	[12-13]	246.1448	1	e.NL.
y1	[13-13]	132.1019	1	n.L.

Table S10 MS-MS of double modification **TSTEPQ<u>Y</u>QPG<u>E</u>NL**



ion	slice	m/z	z	sequence
M	[1-13]	1813.695	1	.TSTEPQYQPGENL. [1xBenzyl phosphate; 1x NO_2-benzyl]
b2	[1-2]	189.087	1	.TS.t
b3	[1-3]	290.1347	1	.TST.e
b4	[1-4]	419.1773	1	.TSTE.p
b5	[1-5]	516.23	1	.TSTEP.q
b6	[1-6]	644.2886	1	.TSTEPQ.y
b7	[1-7]	1022.35	1	.TSTEPQY.q [1x NO_2-Benzyl phosphate]
b8	[1-8]	1150.409	1	.TSTEPQYQ.p [1x NO_2-Benzyl phosphate]
b9	[1-9]	1247.462	1	.TSTEPQYQP.g [1x NO_2-Benzyl phosphate]
b10	[1-10]	1304.483	1	.TSTEPQYQPG.e [1x NO_2-Benzyl phosphate]
b11	[1-11]	1568.558	1	.TSTEPQYQPGE.n [1x NO_2-Benzyl phosphate; 1xNO_2-benzyl]
b12	[1-12]	1682.601	1	.TSTEPQYQPGEN.l [1x NO_2-Benzyl phosphate;1xNO_2benzyl]
y12	[2-13]	1712.648	1	t.STEPQYQPGENL. [1x NO_2-Benzyl phosphate;1xNO_2-benzyl]
y11	[3-13]	1625.616	1	s.TEPQYQPGENL. [1x NO_2-Benzyl phosphate; 1x NO_2-benzyl]
y10	[4-13]	1524.568	1	t.EPQYQPGENL. [1x NO_2-Benzyl phosphate; 1x NO_2-benzyl]
y9	[5-13]	1395.525	1	e.PQYQPGENL. [1x NO_2-Benzyl phosphate; 1x NO_2--benzyl]
y8	[6-13]	1298.473	1	p.QYQPGENL. [1x NO_2-Benzyl phosphate; 1x NO_2-benzyl]
y7	[7-13]	1170.414	1	q.YQPGENL. [1x NO_2-Benzyl phosphate; 1x NO_2-benzyl]
y6	[8-13]	792.3523	1	y.QPGENL. [1x NO_2-benzyl]
y5	[9-13]	664.2937	1	q.PGENL. [1x NO_2-benzyl]
y4	[10-13]	567.2409	1	p.GENL. [1x NO_2-benzyl]
y3	[11-13]	510.2195	1	g.ENL. [1x NO_2-benzyl]
y2	[12-13]	246.1448	1	e.NL.
y1	[13-13]	132.1019	1	n.L.

Photo-cleavage of modified pp60 Src (527)

The purified Y527 alkylation product (which is manually collected by HPLC from a 50 nmol reaction setup) was re-dissolved in 100 μL water in an Eppendorf tube. The sample was irradiated with a UV lamp (366 nm) over night. Under this condition the modified product was cleaved affording the original phosphorylated Src (521-533) along with 11% of dephosphorylated peptide. The reaction was monitored by UPLC-MS (method B).

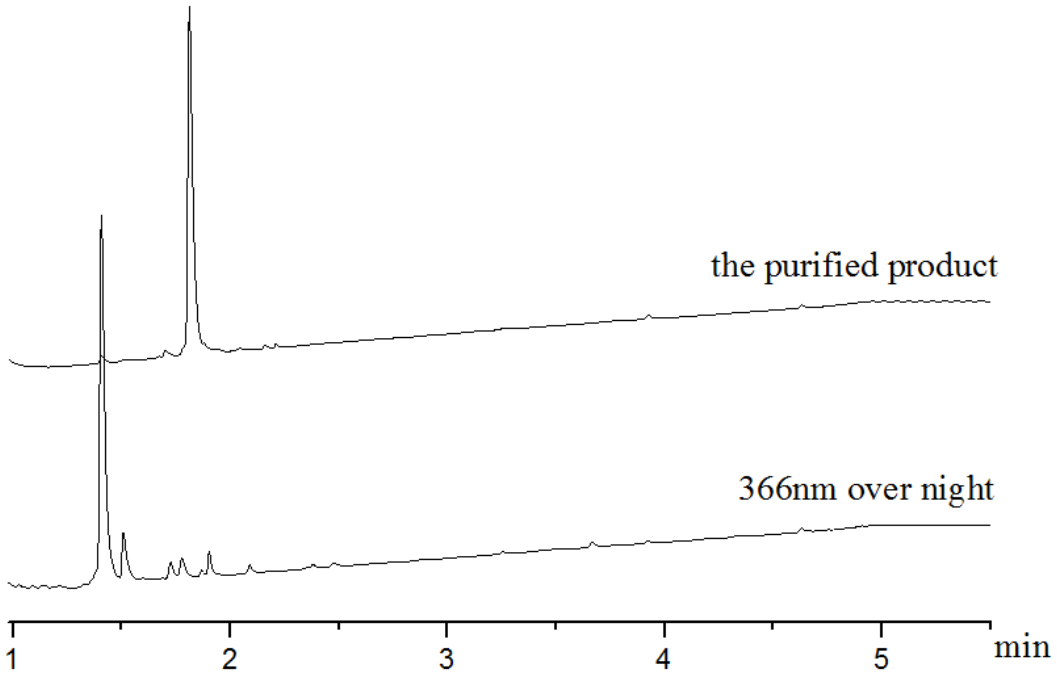
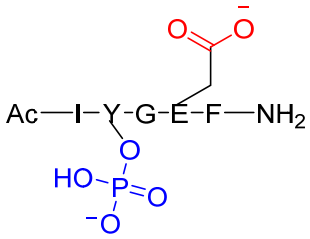
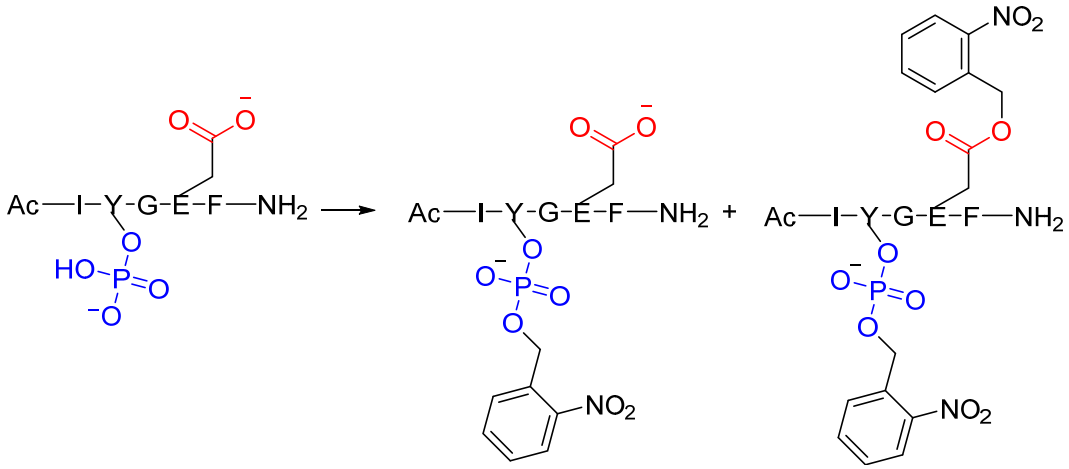


Figure S2 Photocleavage of caged Src (521-533).

5mer peptide



5mer peptide Ac-Ile-Tyr(PO_3H_2)-Gly-Glu-Phe-NH_2 was supplied from Bachem as ammonium salt. It is a phosphorylated synthetic peptide which can be used as an inhibitor of Src. The peptide was dissolved in water to give a 10 mM stock solution. The modification with different equivalents of diazo reagent was carried out as follows:



Stock solution
10 mM peptide, 5 mM MOPS pH 7.1, 10 mM or 100 mM diazo in CH_3CN.
It turned out that, with 3 equivalents diazo, phosphorylated peptide was selectively modified at its phosphate position 43% of conversion (see Figure S16). The desired product was manually collect by HPLC and confirmed using peptide MS-MS sequencing methods (See table S11-12).

1
200 µL reaction volume
4 µL peptide, 80 µL MOPS buffer pH 7.10, 6 µL diazo, 110 µL water
200 µM peptide, 2 mM MOPS, 3 equiv. Diazo
43% of the peptide converted to the desired monophosphate modified product

2
200 µL reaction volume
4 µL peptide, 80 µL buffer pH 7.10, 4 µL diazo, 112 µL water
200 µM peptide, 2mM MOPS, 10 equiv. diazo
56% of peptide converted to desired monophosphate modified product
14% of the peptide converted to double modified product, (both phosphate and glutamic acid)

3
200 µL reaction volume
4 µL peptide, 80 µL buffer pH 7.10, 40 µL diazo, 76 µL water
200 µM peptide, 2 mM MOPS, 100 equiv. Diazo

56% of peptide converted to desired monophosphate modified product
27% of the peptide converted to double modified product, (both phosphate and glutamic acid)

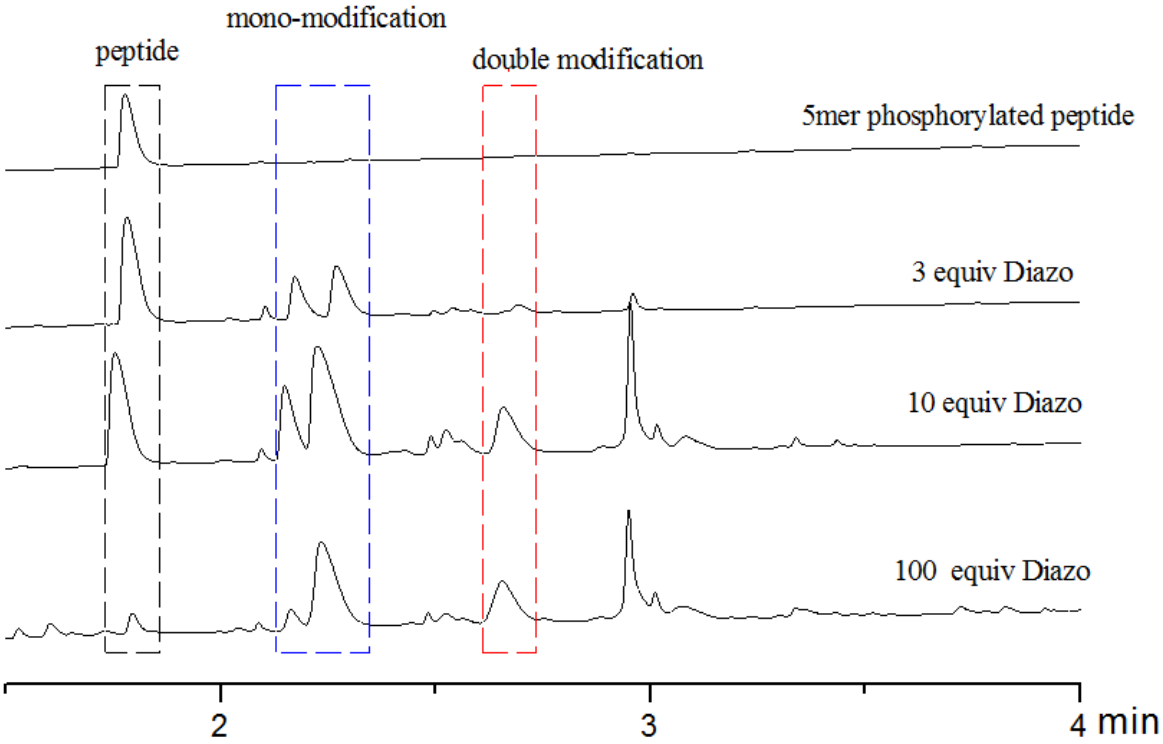
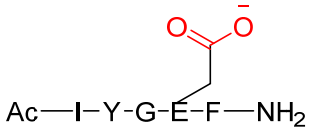


Figure S17 UPLC trace of the modification reaction using the 5mer peptide

5mer peptide dephosphorylated form Ac-IYGEF-NH_2



5mer peptide was obtained from Bachem as ammonium salt and was dissolved in DMSO as stock solution due its low solubility in water. The modification with different equivalents of diazo reagent was carried out in analogy to the setup for the phosphorylated peptide, however no modification was observed even when treated with 20 equivalents of diazo reagent.

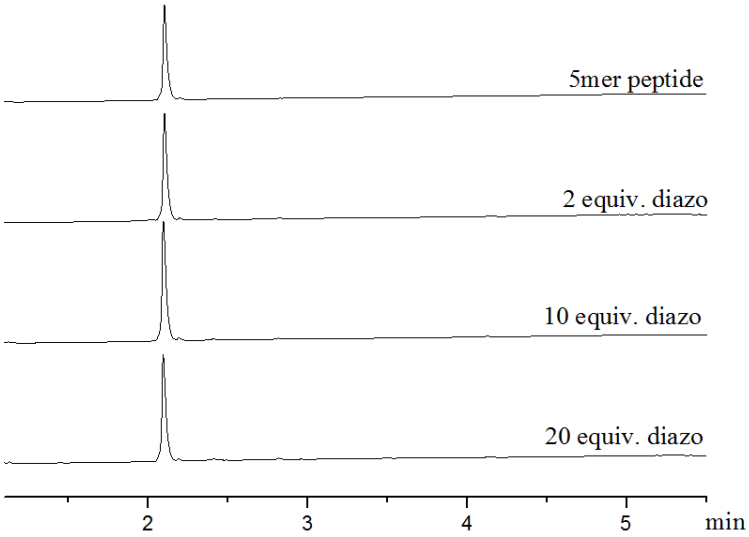
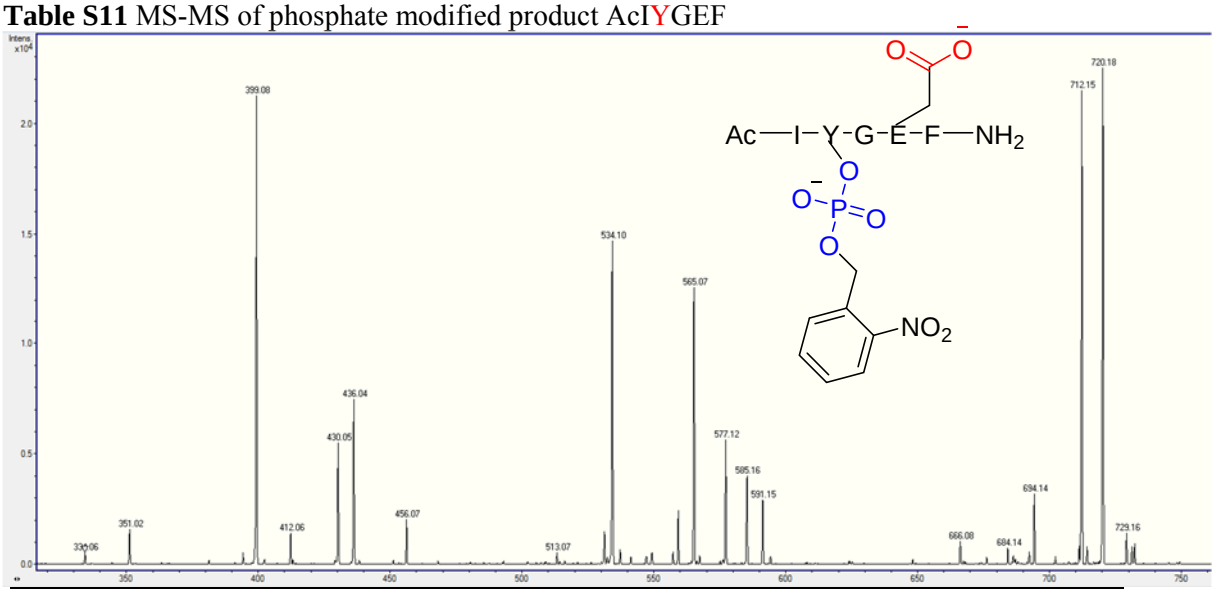


Figure S3 UPLC trace of 5mer peptide modification

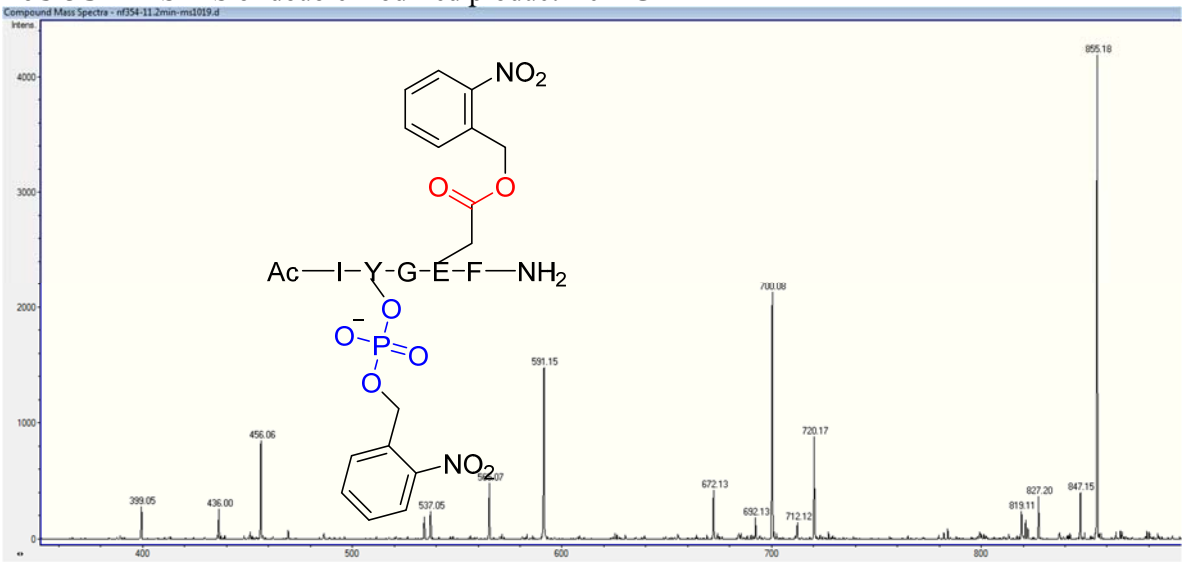
MS/MS of modified products

Table S11 MS-MS of phosphate modified product AcIYGEF



ion	slice	m/z	z	sequence
b2	[1-2]	534.1636	1	.IY.g [1xAcetyl; 1xNO_2-Benzyl phosphate]
b3	[1-3]	591.1851	1	.IYG.e [1xAcetyl; 1xNO_2-Benzyl phosphate]
b4	[1-4]	720.2276	1	.IYGE.f [1xAcetyl; 1xNO_2-Benzyl phosphate]
y4	[2-5]	729.228	1	i.YGEF. [1xAmide; 1xNO_2-Benzyl phosphate]
z4	[2-5]	712.2014	1	i.YGEF. [1xAmide; 1xNO_2-Benzyl phosphate]
y3	[3-5]	351.1663	1	y.GEF. [1xAmide]
z3	[3-5]	334.1397	1	y.GEF. [1xAmide]
y2	[4-5]	294.1448	1	g.EF. [1xAmide]
z2	[4-5]	277.1183	1	g.EF. [1xAmide]
y1	[5-5]	165.1022	1	e.F. [1xAmide]
z1	[5-5]	148.0757	1	e.F. [1xAmide]

Table S12 MS-MS of double modified product AcIYGEF



ion	slice	m/z	z	sequence
b2	[1-2]	534.1636	1	.IY.g [1xAcetyl; 1xNO_2-Benzyl phosphate]
b3	[1-3]	591.1851	1	.IYG.e [1xAcetyl; 1xNO_2-Benzyl phosphate]
b4	[1-4]	855.2597	1	.IYGE.f [1xAcetyl; 1xNO_2-Benzyl phosphate; 1xNO_2-benzyl]
y4	[2-5]	864.26	1	i.YGEF. [1xAmide; 1xNO_2-Benzyl phosphate; 1xNO_2-benzyl]
z4	[2-5]	847.2335	1	i.YGEF. [1xAmide; 1xNO_2-Benzyl phosphate; 1xNO_2-benzyl]
y3	[3-5]	486.1983	1	y.GEF. [1xAmide; 1xNO_2-benzyl]
z3	[3-5]	469.1718	1	y.GEF. [1xAmide; 1xNO_2--benzyl]
y2	[4-5]	429.1769	1	g.EF. [1xAmide; 1xNO_2-benzyl]
z2	[4-5]	412.1503	1	g.EF. [1xAmide; 1xNO_2-benzyl]
y1	[5-5]	165.1022	1	e.F. [1xAmide]
z1	[5-5]	148.0757	1	e.F. [1xAmide]

References

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3. J. W. Walker, G. P. Reid, J. A. McCray and D. R. Trentham, *Journal of the American Chemical Society*, **1988**, *110*, 7170-7177.
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