Supporting Information for

Dual Colorimetric and Fluorogenic Probes for Visualizing Tyrosine Phosphatase Activity and High Throughput Screening

Suvendu Biswas,[†] Brandon S. McCullough,[†] Elena S. Ma,[†] Dollie LaJoie,[‡] Colin W. Russell,[§] D. Garrett Brown,[§] June L. Round,[§] Katharine S. Ullman,[‡] Matthew A. Mulvey,[§] and Amy M. Barrios*[†]

[†]Department of Medicinal Chemistry, University of Utah College of Pharmacy

[‡]Department of Oncological Sciences, University of Utah School of Medicine

[§]Department of Pathology, University of Utah School of Medicine

Corresponding E-mail: amy.barrios@utah.edu

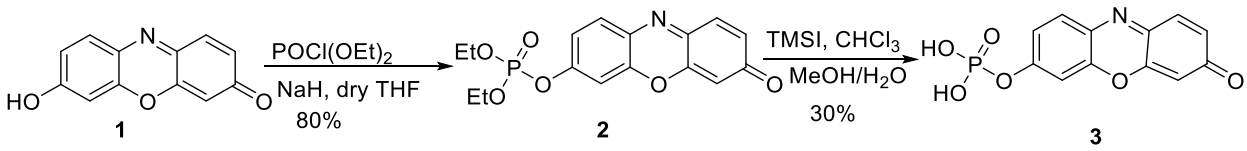
1. General Considerations

The protein tyrosine phosphatases PTP1B, CD45, TCPTP, VHR, YopH, acid phosphatase (from wheat germ) and ALP (Alkaline Phosphatase, from bovine intestinal mucosa) were obtained from commercial sources in purified form and used as received. Chemical reagents and anhydrous solvents were obtained from commercial sources and used without further purification. ^{1}H, ^{13}C, ^{19}F and ^{31}P NMR spectra were recorded on Varian 400 MHz instrument. Spectral data for key compounds can be found in Appendix I. Enzyme assays were performed at room temperature (22°C) in buffers consisting of either Bis-Tris (50 mM, pH 6.5), NaCl (100 mM), EDTA (2 mM), and 0.01% Brij 35 or sodium acetate (100 mM, pH 5.0), NaCl (100 mM), EDTA (1 mM), and 0.01% Brij 35 or tris.HCl (50 mM, pH 7.4). The stock solutions of pRes and F_2pRes were prepared in deionized water. Stock solutions of tris(2-carboxyethyl)phosphine (TCEP), 1,2-naphthoquinone, Res, and F_2Res were prepared in DMSO, and all other stock solutions were prepared in buffer. The final concentration of DMSO in all enzyme assays was less than 0.1% (v/v) unless otherwise noted. Fluorescence and absorbance data were collected by using a Molecular Devices Spectramax M5 plate reader. Initial velocities for enzyme kinetics studies were determined by using the linear region of the initial progress curves and curve fitting was performed using KaleidaGraph software. The ESI-TOF mass spectra were obtained using a Waters LCT Premier™ XE mass spectrometer instrument. Purity of the final probes pRes and F_2pRes were checked on analytical reversed-phase high-performance liquid chromatography (RP-HPLC) performed on a Waters Millennium 2690 HPLC system using a Phenomenex Luna C8 column (250 × 10 mm ID, 5 micron), with a flow rate of 4.0 mL min^{-1} and UV detection at 214 and 255 nm. Elution was achieved with 99.9% H_2O/0.1% TFA ("solvent A") and 100%

CH_3CN ("solvent B") using the following elution protocol: 0–15 min, 0 → 100% A; 15–18 min, 100% B and 18–22 min, 100% B with elution rate 0.1 mL min^{-1}.

2. Synthesis of Phosphorylated Substrates pRes and F_2pRes

The synthesis of pRes was carried out by following a published procedure (see **Scheme S1**).[S1,S2] The fluorinated resorcinol was synthesized using a derivation of a literature procedure (see **Scheme S2)**.[S3] Details of the syntheses are reported below.



Scheme S1. Synthesis of resorufin-7-O-phosphate **3** (pRes).

A. Synthesis of resorufin-7-O-phosphate diethyl ester (2)

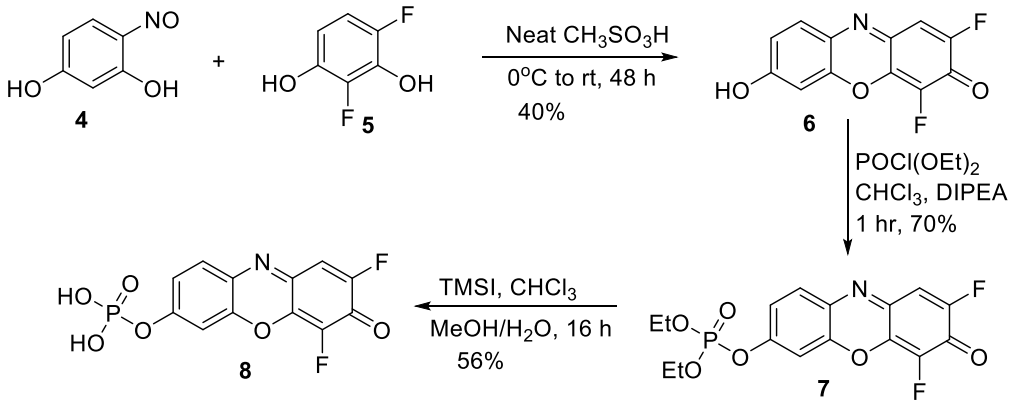
A mixture of resorufin (106 mg, 0.5 mmol) and 60% sodium hydride in mineral oil (240 mg, 6.0 mmol) were dissolved in dry tetrahydrofuran (50 mL) and allowed to stir overnight at room temperature under a N_2 atmosphere. Diethyl chlorophosphate (172 mg, 1.0 mmol) was then added to the reaction mixture and allowed to stir for an additional 10 h. The reaction mixture was concentrated under reduced pressure and purified on a silica column (50:50 ethyl acetate/hexanes) to get protected resorufin phosphate **2** (140 mg, 80%) **(Scheme S1)**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.77 (d, J = 9.6 Hz, 1H), 7.42 (d, J = 10.0 Hz, 1H), 7.25−7.22 (m, 2H), 6.86 (d, J = 10.0 Hz, 1H), 6.33 (s, 1H), 4.30−4.24 (m, 4H), 1.42−1.36 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 186.2, 153.6, 149.2, 147.9, 144.6, 135.0, 134.8, 131.5, 130.6, 117.7, 107.8, 107.2, 65.1, 16.0; ^{31}P NMR (162 MHz, $CDCl_3$): δ −6.98 (s). HRMS (ESI-TOF, $[M+Na]^+$: Calcd. for $C_{16}H_{16}NO_6NaP^+$ 372.0613. Found: 372.0608.

B. Synthesis of resorufin-7-O-phosphate (pRes, 3)

To a solution of **2** (40.14 mg, 0.114 mmol) in 20 mL of dry chloroform was added trimethylsilyliodide (28.0 mg, 0.147 mmol) at 0 ^{o}C. The mixture was allowed to stir at room temperature for 3 h under N_2 atmosphere. The solvent was removed under reduced pressure and 10 mL of aqueous methanol (10% water) was added, followed by stirring for another 2 h at room temperature. After evaporation of the solvent under reduced pressure, the solid obtained was purified by silica column chromatography (50:50 ethyl acetate/methanol), affording the final product, resorufin-7-O-phosphate **3** (10.0 mg, 30%) (**Scheme S1)**. ^{1}H NMR (400 MHz, D_2O): δ 7.68 (d, *J* = 8.8 Hz, 1H), 7.45 (d, *J* = 9.6 Hz, 1H), 7.20 (s, 1H), 7.18 (s, 1H), 6.81 (d, *J* = 9.6 Hz, 1H), 6.32 (s, 1H), ^{31}P NMR (162 MHz, DMSO-d_6): δ −5.30 (s). HRMS (ESI-TOF, $[M-H]^-$: Calcd. for $C_{12}H_7NO_6P^-$ 292.0011. Found: 292.0015.

C. Synthesis of difluororesorufin (F_2Res, 6)

To a solution of 4-nitrosobenzene-1,3-diol **4** (139 mg, 1.0 mmol) in 20 mL of methane sulfonic acid was added 2,4-difluorobenzene-1,3-diol **5** (146.0 mg, 1.0 mmol) at 0 °C. The mixture was allowed to stir at 0 °C for 3 h under N_2 atmosphere followed by stirring for another 2 days at room temperature. The reaction mixture was then poured into 50 mL of ice cold water and extracted with ethyl acetate. After evaporation of the organic solvent under reduced pressure, the solid obtained was purified by silica column chromatography (50:50 ethyl acetate/hexanes) affording the final product (**6**, 100 mg, 40%) (**Scheme S2**). ^{1}H NMR (400 MHz, CD_3OD): δ 7.63 (d, *J* = 9.2 Hz, 1H), 7.28 (d, *J* = 10.4 Hz, 1H), 6.87 (d, *J* = 8.8 Hz, 1H), 6.65 (s, 1H), ^{19}F NMR (376 MHz, CD_3OD): δ −164.36 (s), −164.39 (s) (d). HRMS (ESI-TOF, $[M+Na]^+$: Calcd. for $C_{12}H_5NO_3NaF_2^+$ 272.0135. Found: 272.0138.



Scheme S2. Synthesis of fluorinated resorufin **6** and fluorinated resorufin-7-O-phosphate **8** (F_2pRes).

D. Synthesis of difluoro resorufin-7-O-phosphate diethyl ester (pgF_2pRes, 7)

A mixture of fluorinated resorufin **6** (50 mg, 0.2 mmol) and dry diispropylethylamine (52 mg, 0.4 mmol) was dissolved in dry chloroform (50 mL) and allowed to stir for 15 min at room temperature under a N_2 atmosphere. Diethyl chlorophosphate (52 mg, 0.3 mmol) was then added and the reaction mixture was allowed to stir for an additional 1 h. The reaction mixture was concentrated under reduced pressure and purified on a silica column (20:80 ethyl acetate/hexanes), yielding the protected fluorinated resorufin phosphate (**7,** 54 mg, 70%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85 (d, J = 8.8 Hz, 1H), 7.41 (s, 1H), 7.36−7.32 (m, 1H), 7.16 (d, J = 8.8 Hz, 1H), 4.33−4.24 (m, 4H), 1.40 (t, J = 7.2 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.4, 158.8, 156.1, 154.1, 144.7, 141.4 138.8 133.6, 118.6, 112.7, 108.0, 65.1, 16.0; ^{19}F NMR (376 MHz, $CDCl_3$): δ −115.18 (d), −155.90 (d). ^{31}P NMR (162 MHz, $CDCl_3$): δ −7.1 (s). HRMS (ESI-TOF, $[M+Na]^+$: Calcd. for $C_{16}H_{14}NO_6F_2NaP^+$ 408.0425. Found: 408.0424.

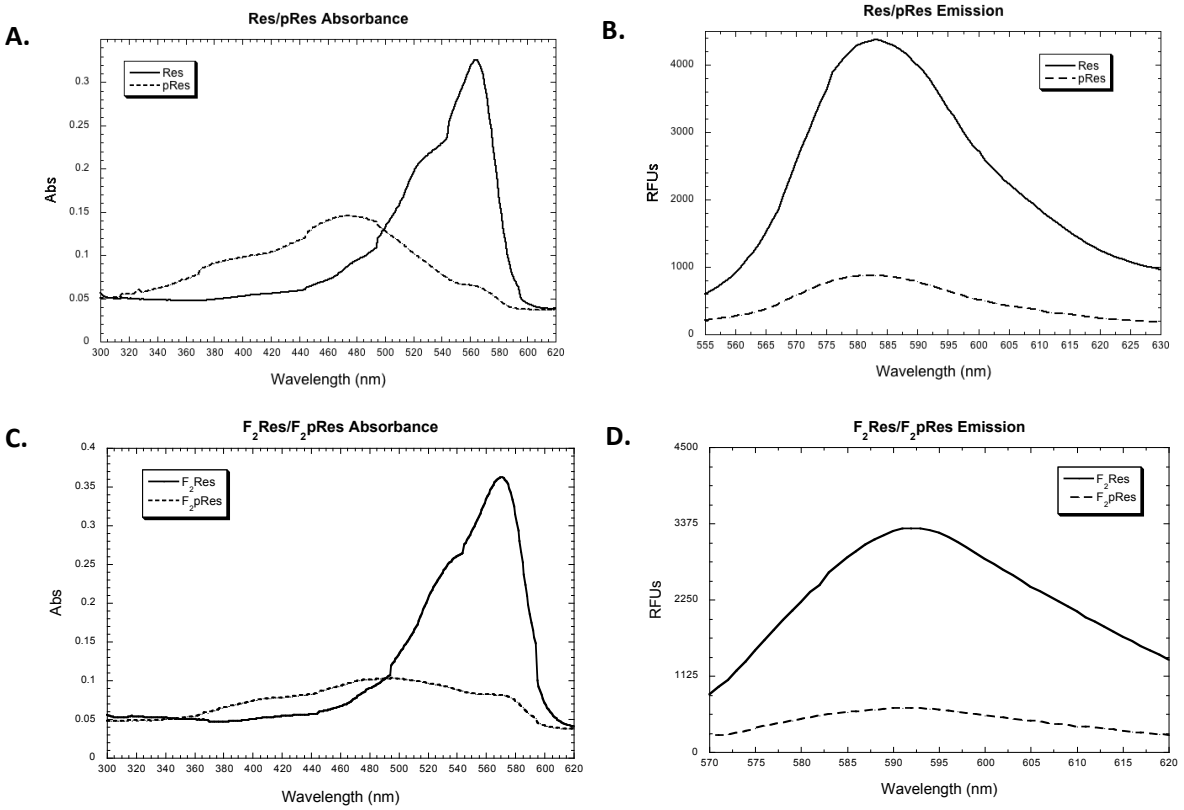
E. Synthesis of difluoro resorufin-7-O-phosphate (F_2pRes, 8)

To a solution of protected resorufin phosphate **7** (30 mg, 0.078 mmol) in 20 mL of dry chloroform was added trimethylsilyliodide (18.0 mg, 0.094 mmol) at 0 °C. The mixture was allowed to stir at room temperature for 3 h under N_2 atmosphere. The solvent was removed under

reduced pressure and 10 mL of aqueous methanol (10% water) was added, followed by stirring for another 2 h at room temperature. After removal of the solvent under reduced pressure, the solid obtained was purified by silica column chromatography (50:50 ethyl acetate/methanol), affording the fluorinated resorufin-7-O-phosphate **8**, (14.0 mg, 56%) (**Scheme S2**). ^{1}H NMR (400 MHz, D_2O): δ 7.76 (d, J = 8.8 Hz, 1H), 7.36 (s, 1H), 7.33−7.26 (m, 2H), ^{19}F NMR (376 MHz, D_2O): δ −119.81 (s), −161.34 (d). ^{31}P NMR (162 MHz, D_2O): δ −0.55 (s). HRMS (ESI-TOF, $[M-H]^-$: Calcd. for $C_{12}H_5NO_6F_2P^-$ 327.9823. Found: 327.9833.

3. Absorbance / Emission Profile

Absorbance and emission readings were performed in a quartz cuvette using 1 mL of 10 μM Res/pRes or F_2Res/F_2pRes compound. The excitation wavelengths were λ_{ex} = 550 nm for Res/pRes and λ_{ex} = 565 nm for F_2Res/ F_2pRes. Data are shown below in **Figure S1.**



E.

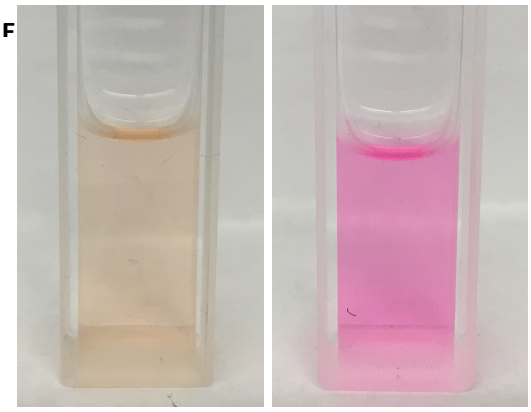
F 

Figure S1. Absorbance and emission spectra of Res/pRes and F_2Res/F_2pRes. **A)** Absorption spectra for Res (solid line) and pRes (dotted line). **B)** Emission spectra for Res (solid line) and pRes (dotted line). **C)** Absorption spectra for F_2Res (solid line) and F_2pRes (dotted line). **D**) Emission spectra for F_2Res (solid line) and F_2pRes (dotted line). **E)** Visible color change of 10 µM pRes (left panel) and pRes after addition of PTP1B (right panel) in pH 6.5 buffer.

4. Enzyme Activity Assays for pRes

Enzyme activity assays were performed in black 96-well plates containing a total volume of 100 µL in each well. Standard conditions employed to determine the kinetic constants with PTP1B, CD45, TCPTP, VHR, and YopH are described below. Final enzyme concentrations were as follows: 4.5 nM PTP1B, 3.8 nM CD45, 16.4 nM VHR, 3.8 nM TCPTP, and 2.2 nM YopH. Substrate pRes was tested at concentrations between 5 µM and 200 µM. Prior to each assay, enzyme was activated by incubating in Bis-Tris buffer with 1 mM TCEP on ice for 30 min. Each substrate concentration was measured in triplicate and averaged to determine initial velocities. Using standard curves the initial velocities were transformed into the rate of product formation and used to generate Michaelis- Menten curves **(Figures S2).** The increase in fluorescence resulting from the turnover of the substrate was measured every 30 s over 30 min using λ_{ex} = 550 nm and λ_{em} = 585 nm. The kinetic parameters were determined from Michaelis-Menten plots and are shown in the Figures S2.

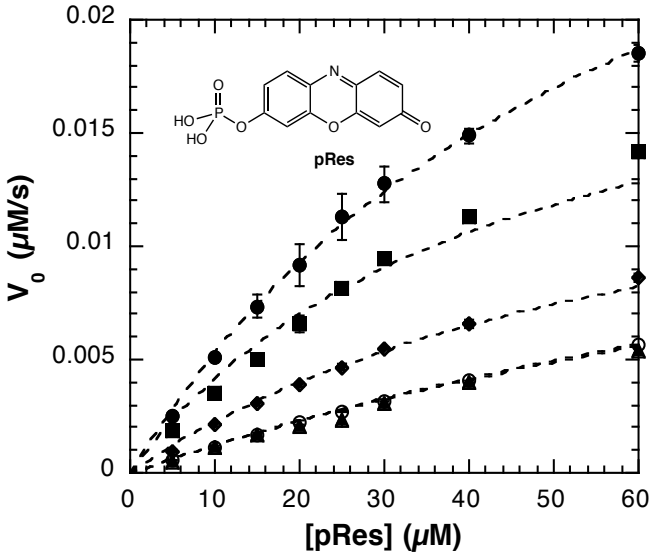


Figure S2. Michaelis-Menten curves for the hydrolysis of pRes by YopH (●), TCPTP (■), PTP1B (◆) VHR (▲), and CD45 (○). The enzymes were used at a final concentration of between 2.2 – 16.4 nM and the reaction was monitored over 30 min with excitation and emission wavelengths of 550 nm and 585 nm. Note that VHR and CD45 have very similar activities against pRes

5. Autohydrolysis Test

No autohydrolysis of either pRes or F_2pRes was observed under the enzyme assay conditions, as shown in **Figure S3**. These experiments were carried out in black 96-well plates containing a total volume of 100 µL in each well. Experiments involving pRes were performed in pH 6.5 buffer while F_2pRes experiments were performed in pH 5.0 buffer. Immediately prior to each experiment a stock solution of either compound (pRes or F_2pRes) dissolved in deionized water was diluted using the appropriate buffer.

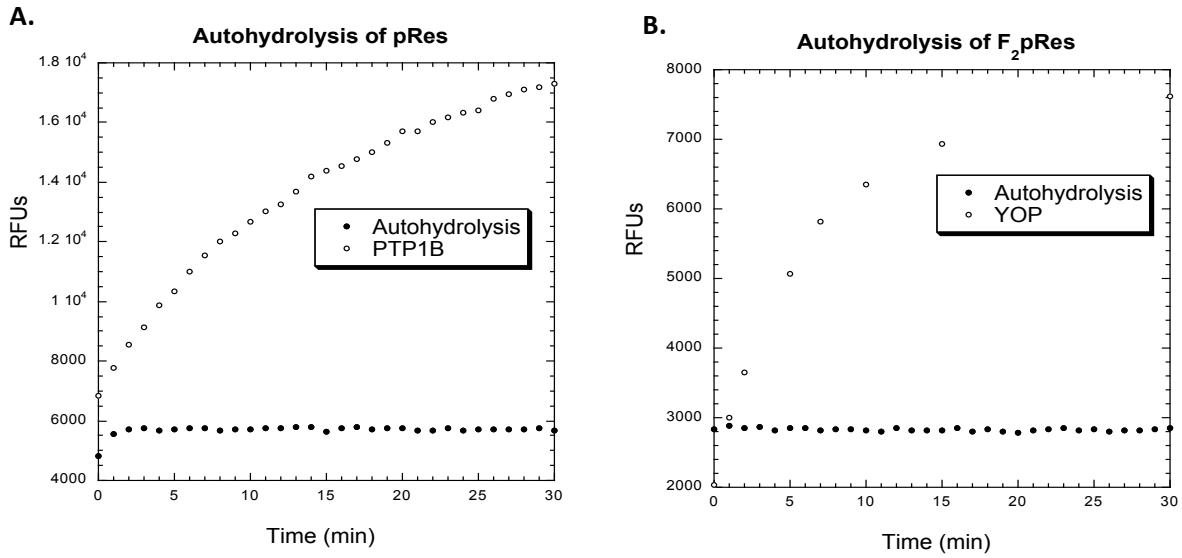


Figure S3. Auto-hydrolysis of **A)** pRes (80 μM) at pH 6.5 and **B)** F_2pRes (80 μM) at pH 5.0. Each time point for the autohydrolysis data is an average of three and two measured fluorescence values for pRes and F_2pRes, respectively. Enzymatic hydrolysis data are from separate experiments with 20 μM pRes/F_2pRes at the respective pH values.

6. Monitoring Enzyme Inhibition

Assays were performed in black 96-well plates containing a total volume of 100 μL in each well, with all reactions performed in triplicate. Inhibitor concentration ranges were 20-1000 μM for sodium orthovanadate and 0.5-100 μM for 1,2-naphthoquinone. DMSO concentrations were kept at 0.1% for sodium orthovanadate and 5.1% for 1,2-naphthoquinone. Enzyme was prepared as described above. Buffer (pH 6.5), enzyme, and inhibitor were added to each well and, after a 30 min pre-incubation, 10 μM pRes was added to initiate the reaction. The observed fluorescence from the hydrolysis of pRes was measured every 30 s over 30 min (λ_{ex} = 550 nm and λ_{em} = 585 nm). IC_{50} values were calculated from the curves shown in **Figure S4** using KaleidaGraph.

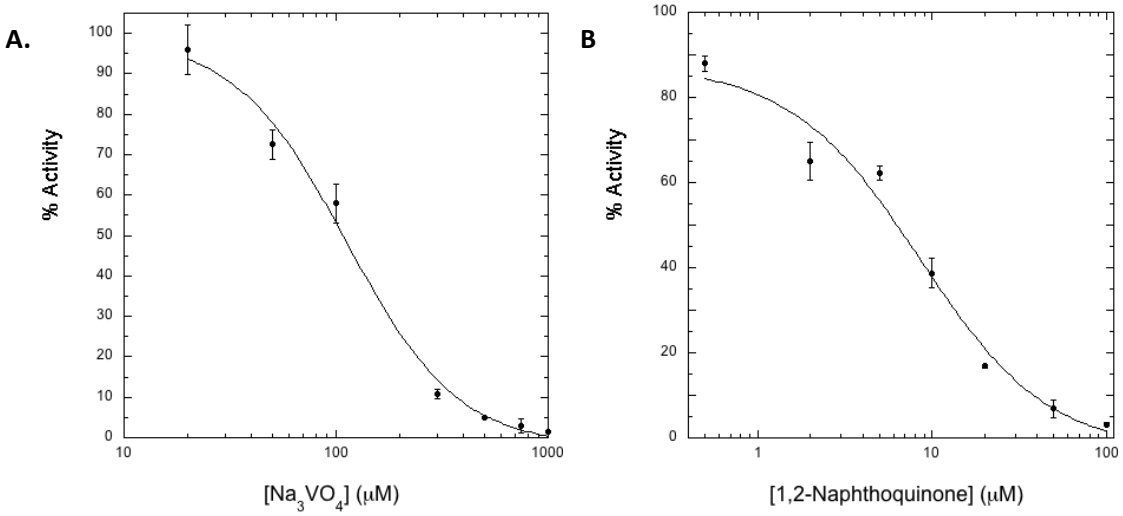


Figure S4. Inhibition of PTP1B by **A)** sodium orthovanadate (IC_{50} = 111 ± 18 μM) and **B)** 1,2-naphthoquinone (IC_{50} = 8.4 ± 2.6 μM).

7. Validation for Use in High-Throughput Screening

Assays were performed in black 96-well plates containing a total volume of 100 μL in each well. Each run consisted of 32 wells each of three different conditions: high, mid, and low. All wells contained 4.5 nM PTP1B and 10 μM pRes, while mid and low wells additionally contained 110 μM sodium orthovanadate and 1 mM sodium orthovanadate, respectively. Buffer (pH 6.5), enzyme, and inhibitor were added to each well as required and allowed to incubate at room temperature for 30 min, after which pRes was added to initiate the reaction. The observed fluorescence resulting from the hydrolysis of pRes was measured every 60 s over a 30 min period. Assays were performed 3 times per day over three days. Results were analyzed using published methods.[S4] Representative data are shown in **Figure S5**, below. Further information on the SW and Z' values used in our analysis can be found in references S5 and S6, respectively.

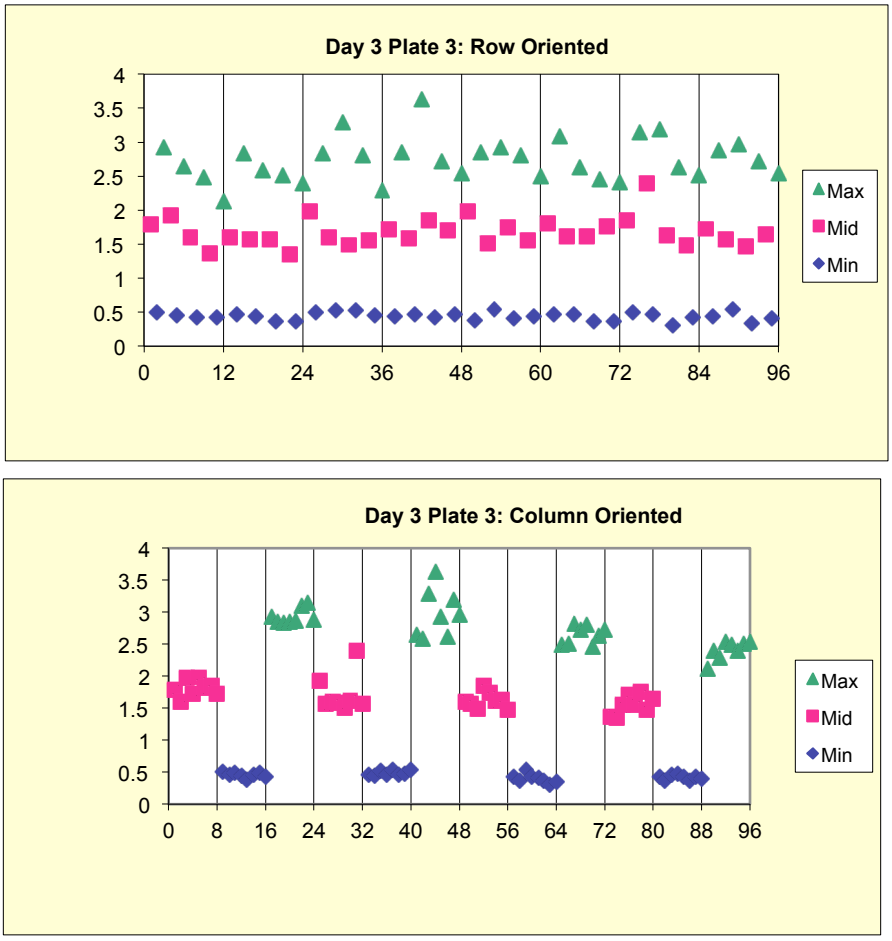


Figure S5. Representative data from the HTS validation of pRes. For this plate, SW = 9.13 and Z' = 0.72. The overall average SW = 10.42 and the average Z' = 0.72.

8. Colorimetric Detection of Bacterial Phosphatase Activity

A significant difference in color between pRes and Res is apparent to the naked eye, as shown in **Figure S1**. Taking advantage of this colorimetric change, we investigated the ability of pRes to serve as a sensor for the presence of secreted bacterial phosphatases during bacterial growth. pRes was mixed to a final concentration of 100 μM with molten LB agar (Fisher Scientific, BP97452) that had been cooled to 55° C. The mixture was poured into petri dishes and allowed

to solidify for 24 h. *Enterococcus faecalis*, *Acinetobacter baumannii*, *Staphylococcus aureus*, and *Staphylococcus saprophyticus* were streaked from frozen stock onto the LB-pRes plates and incubated at 37° C for 24 h. The plates were then imaged with the Eagle Eye II (Stratagene).

9. Live Imaging of PTP Activity in HeLa Cells

HeLa cells were maintained in Dulbecco's modified eagle medium supplemented with 10% FBS and incubated at 37°C with 5% carbon dioxide for the duration of treatments and imaging. Cells were plated in fibronectin-coated Mat-Tek dishes for 80-90% confluence at the time of imaging. Once attached, cells were incubated with either pervanadate or medium alone, as a vehicle control, for up to 3.5 hours. Pervanadate was prepared by mixing equal volumes of 20 mM hydrogen peroxide and 20 mM sodium orthovanadate and incubating at room temperature for ten minutes before diluting 1:100 in medium. Cells were washed once with PBS before adding either 50 μM pRes in PBS, or PBS alone for 10 minutes. Cells were live imaged by spinning disk confocal microscopy using an excitation wavelength centered at 640 nm and emission wavelength range of 700-775 nm. All images had an exposure time of 2 s. Images were adjusted uniformly to subtract background of fluorescent channels and increase the contrast of brightfield channels. While one representative example of live cell PTP activity imaging data is shown in Figure 5 in the main text, additional examples are shown in Figure S6.

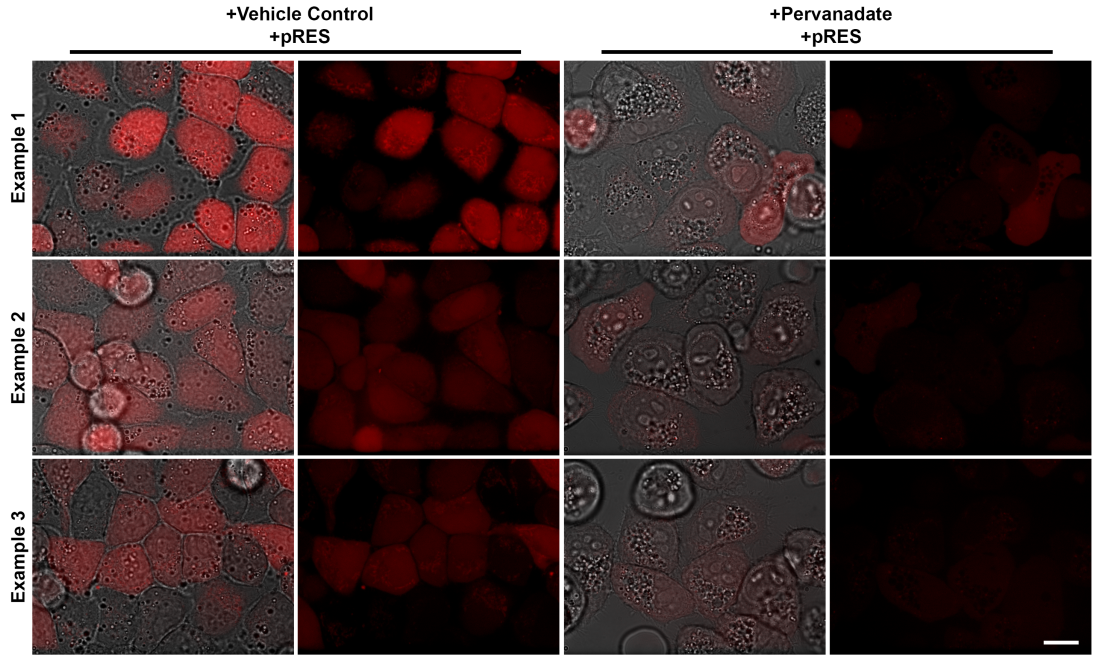
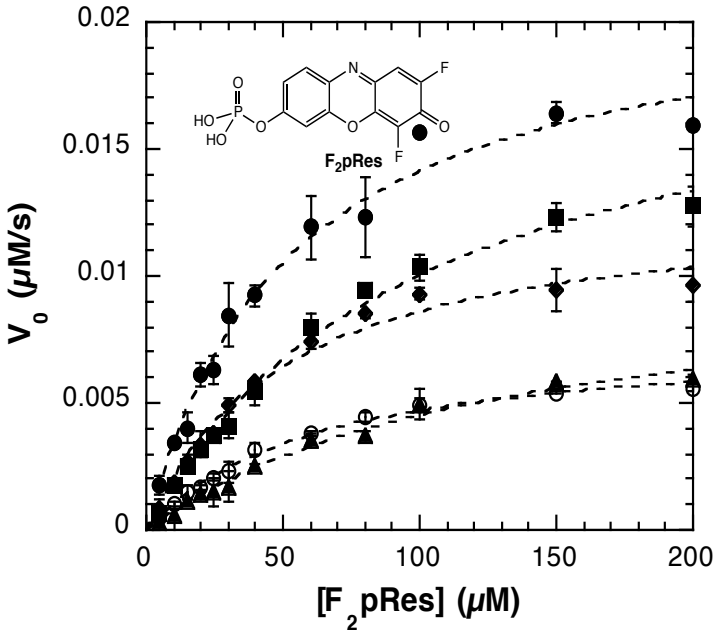


Figure S6. Incubation of cells with pRes results in robust fluorescence that is markedly reduced by pre-treatment with the protein tyrosine phosaphatase inhibitor pervanadate. HeLa cells treated with vehicle control show no autofluorescence. Scale bar represents 20 μm.

10. Enzyme Activity Assays for F_2pRes

Enzyme activity assays were performed in black 96-well plates containing a total volume of 100 μL in each well. Standard conditions employed to determine the kinetic constants with PTP1B, CD45, TCPTP, VHR, and YopH are described below. Final enzyme concentrations were as follows: 4.5 nM PTP1B, 3.8 nM CD45, 16.4 nM VHR, 3.8 nM TCPTP, and 2.2 nM YopH. F_2pRes was tested at concentrations between 5 μM and 200 μM. Prior to each assay, enzyme was activated by incubating in Bis-Tris buffer with 1 mM TCEP on ice for 30 min. The substrate concentration was measured in triplicate and averaged to determine initial velocities.

Using standard curves for each substrate, the initial velocities were transformed into the rate of product formation and used to generate Michaelis- Menten curves **(**Figure **S7).** The increase in fluorescence resulting from the turnover of the substrates were measured every 30 s over 30 min using λ_{ex} = 565 nm and λ_{em} = 595 nm. The kinetic parameters were determined from Michaelis-



Menten plots and are shown in the Figures (m1 = k_{cat} and m2 = K_M).

Figure S7. Michaelis-Menten curves for the hydrolysis of F_2pRes by YopH (●), TCPTP (■), PTP1B (◆) VHR (▲), and CD45 (❍). The enzymes were used at a final concentration of between 2.2 – 16.4 nM and the reaction was monitored over 30 min with excitation and emission wavelengths 565 nm and 595 nm. Note that VHR and CD45 have very similar activities against F_2pRes.

11. pK_a Titrations

Titrations were performed in black 96-well plates containing a total volume of 200 µL in each well. The titration method was adapted from a published protocol.[S4] Buffer solutions with pH ranging from pH 3.0 to 11.0 were prepared as follows: 50 mM citric acid pH 3.0-3.9; 50 mM sodium acetate pH 4.0; 50 mM sodium phosphate monobasic pH 4.5-7.5; 50 mM Tris-HCl pH

8.0; 25 mM Tris free base / 25 mM sodium bicarbonate pH 8.5-9.0; 50 mM sodium bicarbonate pH 9.5; and 50 mM diethanolamine pH 9.6-11.0. Each well contained 25 μM substrate with a DMSO concentration of 1%. Fluorescence observed was measured from pH 4.0-11.0 for Res (λ_{ex} = 550 and λ_{em} = 585) and 3.0-11.0 for F_2Res (λ_{ex} = 565 and λ_{em} = 595). Results were graphed and curve fit using KaleidaGraph, yielding pK_a values of 5.8 and 4.6 for resorufin and difluororesorufin, respectively (**Figure S8**). The data for resorufin (pK_a of 5.8) are in good agreement with the published value of ~6.[S7]

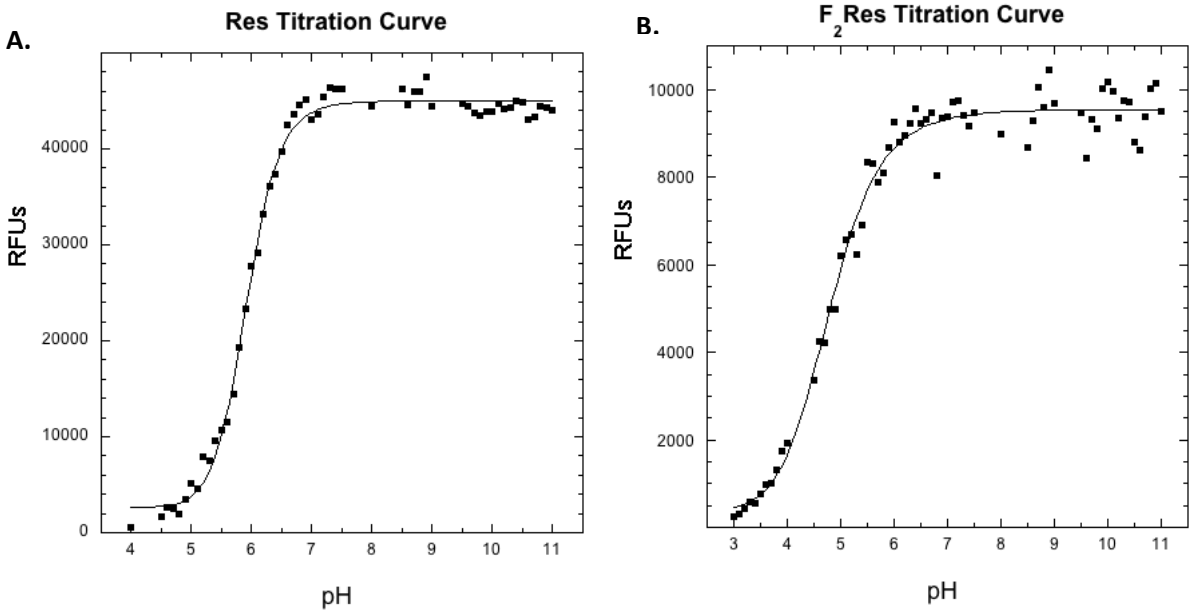


Figure S8. Titration curves of **A.** Res (pK_a = 5.8) and **B.** F_2Res (pK_a = 4.6).

12. Utility of F_2pRes Over pRes at acidic pH

Enzyme activity assays were performed in black 96-well plates containing a total volume of 100 µL in each well. YopH was chosen as it is known to be active at both neutral and acidic pH. The hydrolysis of pRes and F_2pRes was tested with YopH at 20 µM in bis-tris buffer at pH 6.5 and sodium acetate buffer at pH 5.0. Prior to each assay, enzyme was activated by incubating in buffer with 1 mM TCEP on ice for 30 min.

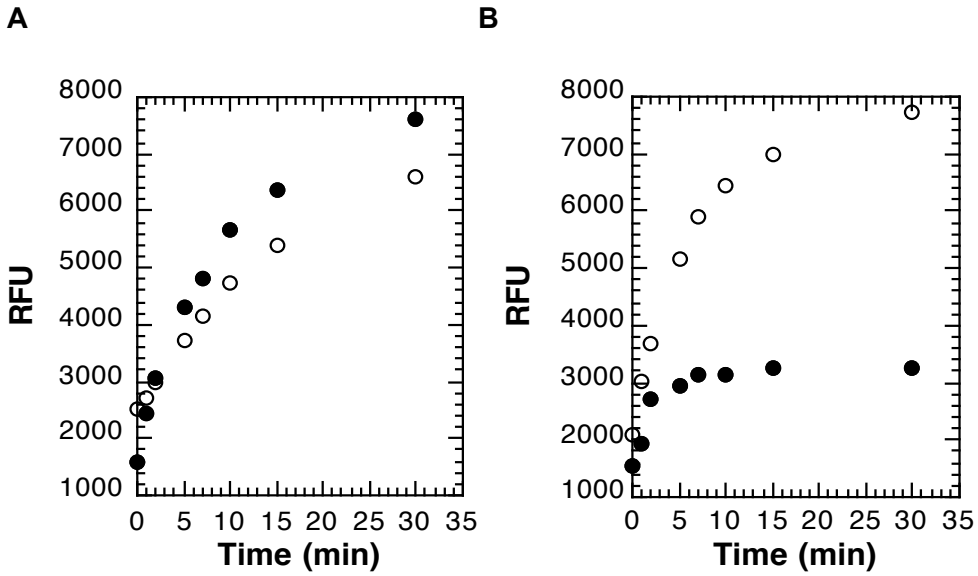


Figure S9. Reaction progress curves for the hydrolysis of pRes (●) and F_2pRes (○) by YopH at (A) pH 6.5 and (B) pH 5.0. The substrate concentration was 20 µM and the YopH concentration was 2.2 nM.

13. Utility of F_2pRes Over pRes in Acid Phosphatase Assays

Assays were performed in a quartz cuvette with a total volume of 1 mL. The concentration of LMW-PTP was 5 nM and acid phosphatse (from wheat germ) was 110 nM. The substrate concentration was kept at 50 µM for both F_2pRes and pRes. Sodium acetate buffer at pH 5.0 was used for LMW-PTP and 90 mM citrate buffer at pH 4.8 was used for acid phosphatases. Data are shown in **Figure S10**, below.

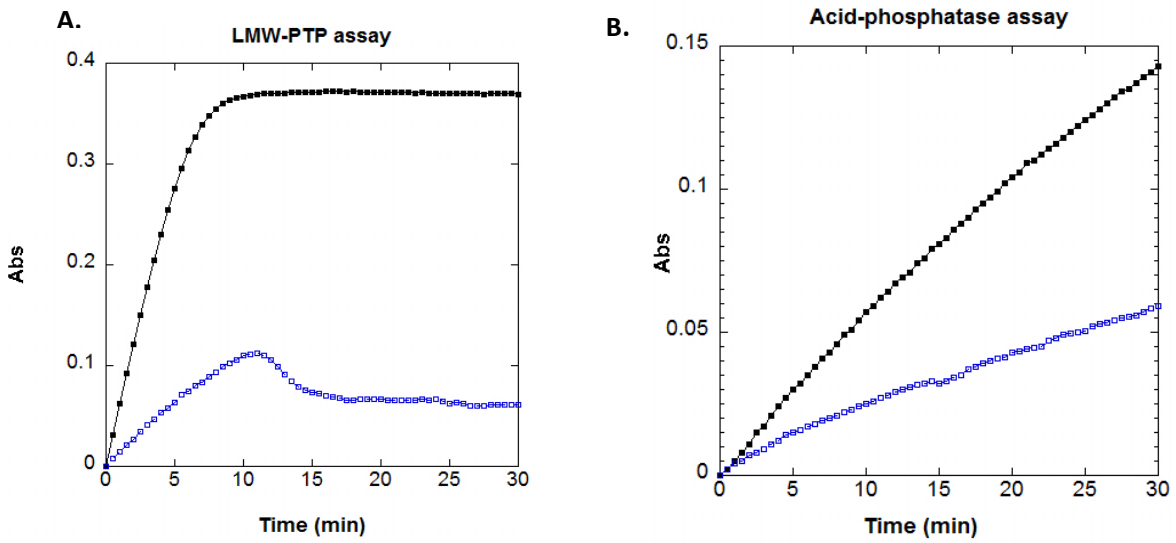


Figure S10. Reaction progress curves for the hydrolysis of pRes and F_2pRes **A)** LMW-PTP assay and **B)** Acid phosphatase assay. F_2pRes (black) is a superior substrate to pRes (blue) at pH 4.8. λ_{abs} = 550 nm for pRes and λ_{abs} = 565 nm for F_2pRes .

14. Enzyme Activity Assays with Acid and Alkaline Phosphatase

Enzyme activity assays were performed in black 96-well plates containing a total volume of 100 µL in each well. Standard conditions were employed to determine the kinetic constants with acid phosphatase (from wheat germ) and ALP (alkaline phosphatase, from bovine intestinal mucosa) are described below. Final enzyme concentrations were as follows: 0.14 nM for ALP and 110 nM for acid phosphatase. pRes was tested at concentrations between 10 µM to 150 µM for acid phosphatase and between 1 µM to 20 µM for ALP. Experiments were performed in in tris.HCl

buffer (50 mM, pH 7.4) for ALP and in bis-tris buffer (50 mM, pH 6.5) for acid phosphatase. Each substrate concentration was measured in triplicate and averaged to determine initial velocities. Using standard curves the initial velocities were transformed into the rate of product formation and used to generate Michaelis- Menten curves **(Figures S11).** The increase in fluorescence resulting from the turnover of the substrate was measured every 30 s over 30 min using λ_{ex} = 550 nm and λ_{em} = 585 nm. The kinetic parameters were determined from Michaelis-Menten plots and are shown in the **Table S1**.

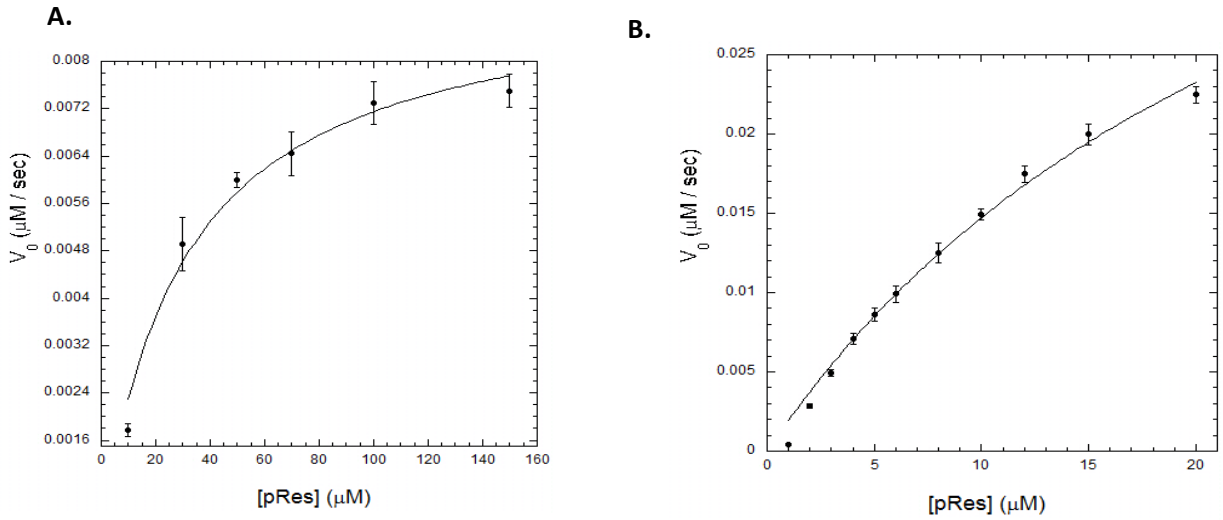


Figure S11. Michaelis- Menten curves for the hydrolysis of pRes **A)** acid phosphatase and **B)** ALP.

	Acid Phosphatase	ALP
K_M	31 ± 6	28 ± 5
k_{cat}	0.085 ± 0.005	400 ± 50
k_{cat}/K_M	$2.8\times10^3 \pm 0.6\times10^3$	$1.5\times10^7 \pm 0.3\times10^7$

Table S1. Kinetic constants (K_M in μM, k_{cat} in s^{-1} and k_{cat}/K_M in $M^{-1}s^{-1}$) for ALP and acid phosphatase assays using pRes as the substrate.

15. References Cited

S1. Muhlegger, K.; von der Eltz, H. Phosphates of resorufin derivatives and compositions thereof for the determination of the activity of phosphatases. U. S. Patent 4,719,097, 1988.

S2. Zhang, H.; Xu, C.; Liu, J.; Li, X.; Guo, L.; Li, X "An Enzyme-Activatable Probe with a Self-Immolative Linker for Rapid and Sensitive Alkaline Phosphatase Detection and Cell Imaging Through a Cascade Reaction" *Chem. Commun.* **2015** *51*, 7031-7034.

S3. Batchelor, R.; Ge, Y.; Gee, K.; Johnson, I.;Leung, W.-Y.; Liu, J.; Patch, B.; Smalley, P.; Steinberg, T. "Fluorinated Resorufin Compounds and their Application" U.S. Patent 7875261 B2. 2011.

S4. Iversen, P. W.; Beck, B.; Chen, Y.-F.; Dere, W.; Devanarayan, V.; Eastwood, B. J.; Farmen, M. W.; Iturria, S. J.; Montrose, C.; Moore, R. A.; Weidner, J. R.; Sittampalam, G. S. *HTS Assay Validation* [online]; Eli Lilly & Company and the National Center for Advancing Translational Sciences, 2012. https://www.ncbi.nlm.nih.gov/books/NBK83783/ (accessed Oct 17, 2016).

S5. Sittampalam, G. S.; Iversen, P. W.; Boadt, J. A.; Kahl, S. D.; Bright, S.; Zock, J. M.; Janzen, W. P.; Lister, M. D. "Design of Signal Windows in High Throughput Screening Assays for Drug Discovery" *J. Biomol. Screen.* **1997** *2*, 159-169.

S6. Zhang, J.-H.; Chung, T. D. Y.; Oldenburg, K. R. "A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays" *J. Biomol. Screen.* **1999** *4*, 67-73.

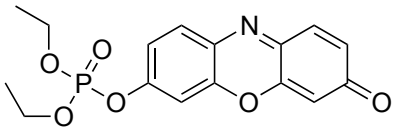
S7. Towne, V.; Will, M.; Oswald, B.; Zhao, Q. "Complexities in Horseradish Peroxidase-Catalyzed Oxidation of Dihydroxyphenoxazine Derivatives: Appropriate Ranges for pH Values and Hydrogen Peroxide Concentrations in Quantitative Analysis" *Anal. Biochem.* **2004** *334*, 290-296.

APPENDIX 1.

Spectral data for compounds 2, 3, 6, 7 and 8

Spectral Data for Compound 2:

1. HRMS
2. ^{1}H NMR
3. ^{13}C NMR
4. ^{31}P NMR



Chemical Formula: $C_{16}H_{16}NO_6P$
Exact Mass: 349.0715
Molecular Weight: 349.2788

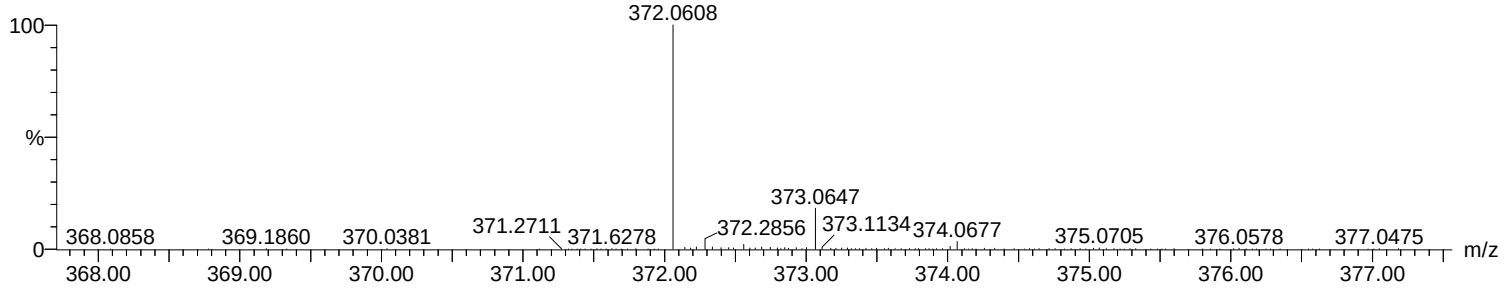
Compound 2

Single Mass Analysis

Tolerance = 5.0 mDa / DBE: min = -100.0, max = 400.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
8171 formula(e) evaluated with 47 results within limits (up to 20 best isotopic matches for each mass)
Elements Used:
C: 2-200 H: 0-120 N: 0-10 O: 0-20 Na: 0-1 P: 1-4

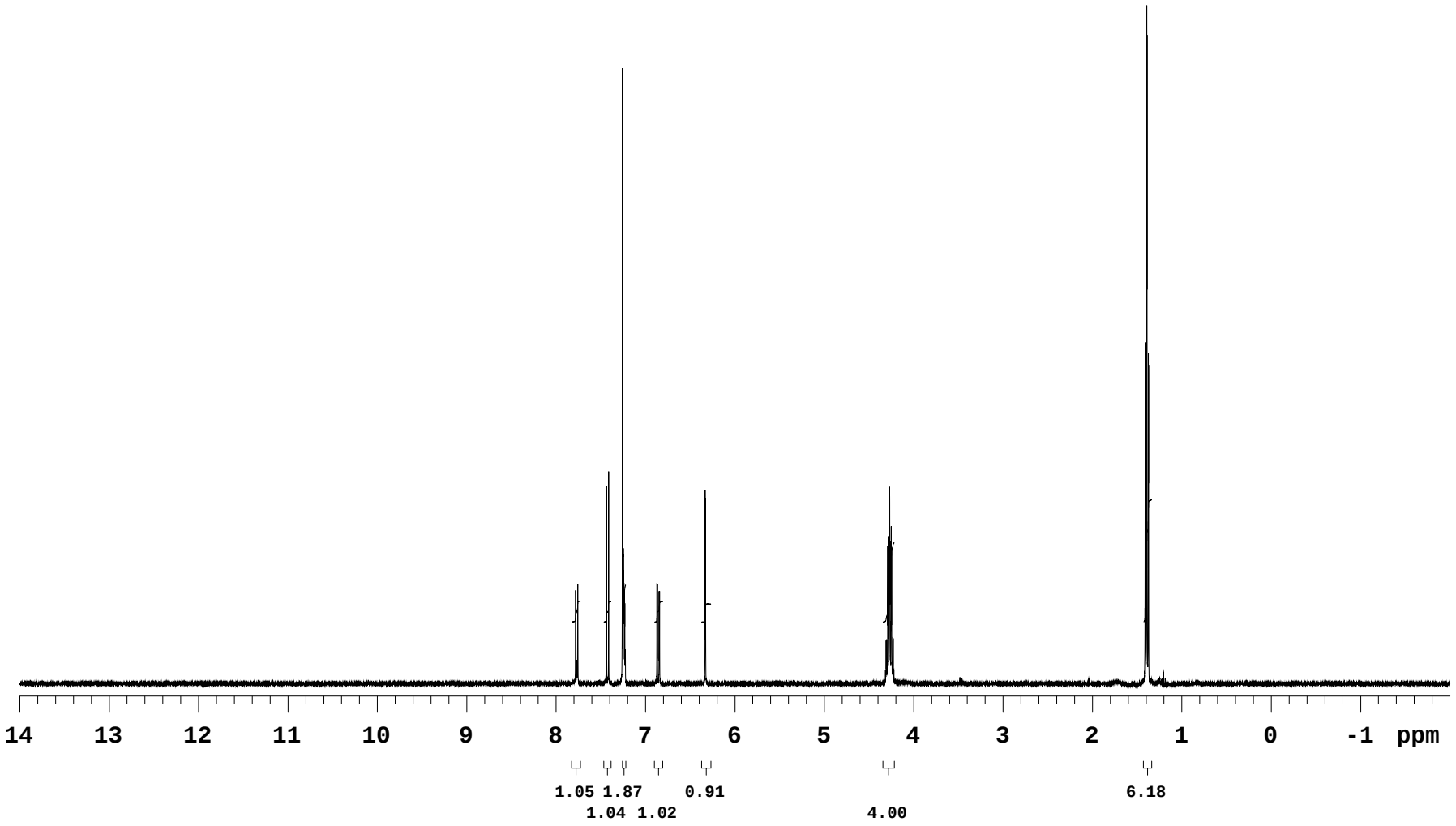
RS-2_HRMS KE319
LCT XE
15Ba1022_LCT_3 22 (0.460) AM (Cen,4, 85.00, Ar,12000.0,490.89,0.70,LS 3); Sm (SG, 2x3.00); Sb (5,40.00); Cm (22:54) 1: TOF MS ES+
1.45e+004



Minimum:				-100.0			
Maximum:		5.0	10.0	400.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
372.0608	372.0613	-0.5	-1.3	9.5	402.5	0.1	C16 H16 N O6 Na P
	372.0597	1.1	3.0	8.5	406.0	3.6	C13 H15 N3 O8 P
	372.0637	-2.9	-7.8	12.5	406.3	3.9	C18 H15 N O6 P
	372.0601	0.7	1.9	7.5	406.4	4.0	C15 H22 N O2 P4
	372.0610	-0.2	-0.5	13.5	406.6	4.2	C14 H11 N7 O4 P
	372.0643	-3.5	-9.4	9.5	407.4	4.9	C15 H17 N3 O3 Na P2
	372.0626	-1.8	-4.8	14.5	407.6	5.2	C17 H12 N5 O2 Na P
	372.0585	2.3	6.2	12.5	407.7	5.3	C17 H17 N3 O P3
	372.0627	-1.9	-5.1	8.5	408.5	6.1	C12 H16 N5 O5 P2
	372.0613	-0.5	-1.3	3.5	408.7	6.3	C11 H20 N O9 P2
	372.0586	2.2	5.9	10.5	409.3	6.9	C12 H12 N7 O4 Na P
	372.0577	3.1	8.3	4.5	409.5	7.0	C13 H23 N O2 Na P4
	372.0560	4.8	12.9	9.5	409.7	7.3	C15 H18 N3 O Na P3
	372.0640	-3.2	-8.6	13.5	409.9	7.5	C13 H12 N9 O P2
	372.0650	-4.2	-11.3	17.5	410.1	7.7	C19 H11 N5 O2 P
	372.0573	3.5	9.4	5.5	410.2	7.8	C11 H16 N3 O8 Na P
	372.0568	4.0	10.8	17.5	410.6	8.2	C19 H12 N5 P2
	372.0603	0.5	1.3	5.5	410.6	8.2	C10 H17 N5 O5 Na P2
	372.0643	-3.5	-9.4	3.5	411.0	8.6	C10 H21 N3 O6 P3
	372.0589	1.9	5.1	0.5	411.1	8.7	C9 H21 N O9 Na P2

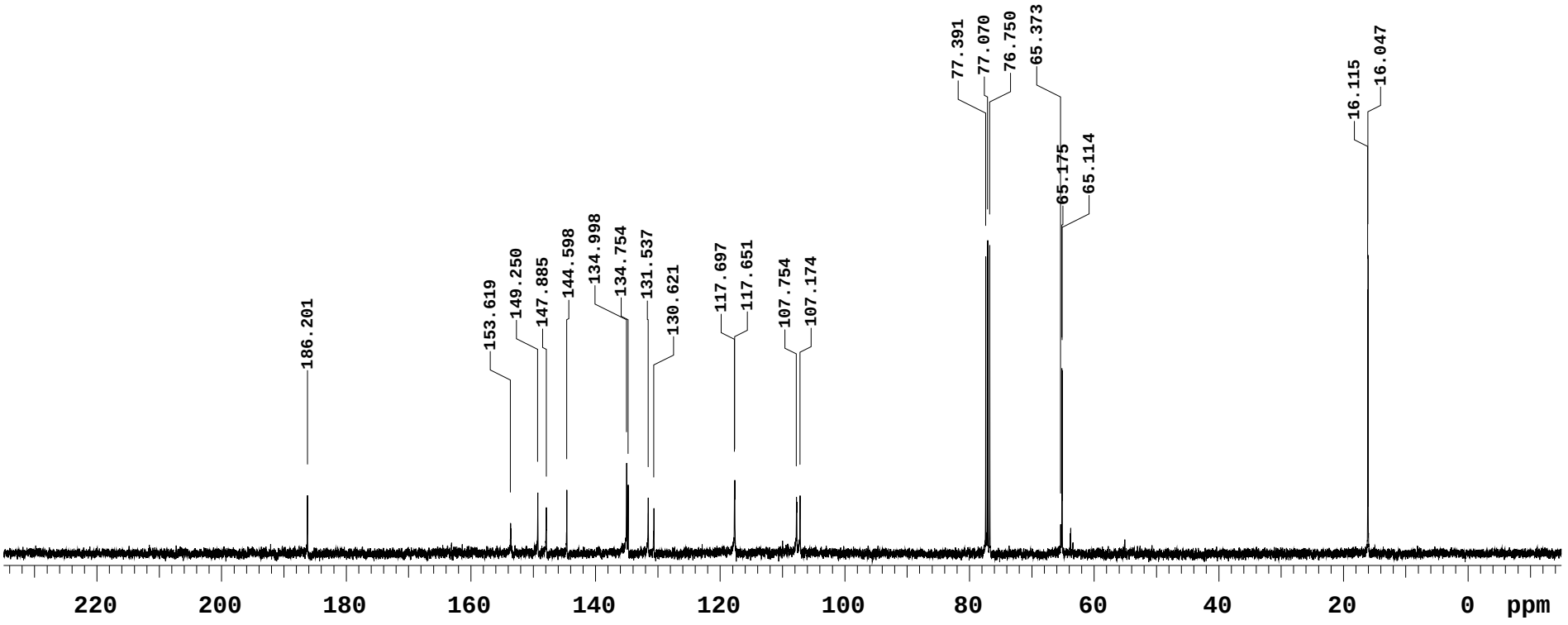
13-reactioninTHF-ether-insoluble

Sample Name	**13-reactioninTHF-ether-insoluble**	Pulse sequence	**PROTON**	Temperature	**22**	Study owner	**suvendu**
Date collected	**2015-03-25**	Solvent	**cdcl3**	Spectrometer	**druidarch-mercury400**	Operator	**suvendu**



13C-PgpRes

Sample Name	**13C-PgpRes**	Pulse sequence	**CARBON**	Temperature	**25**	Study owner	**suvendu**
Date collected	**2016-07-19**	Solvent	**cdcl3**	Spectrometer	**druidarch-mercury400**	Operator	**suvendu**

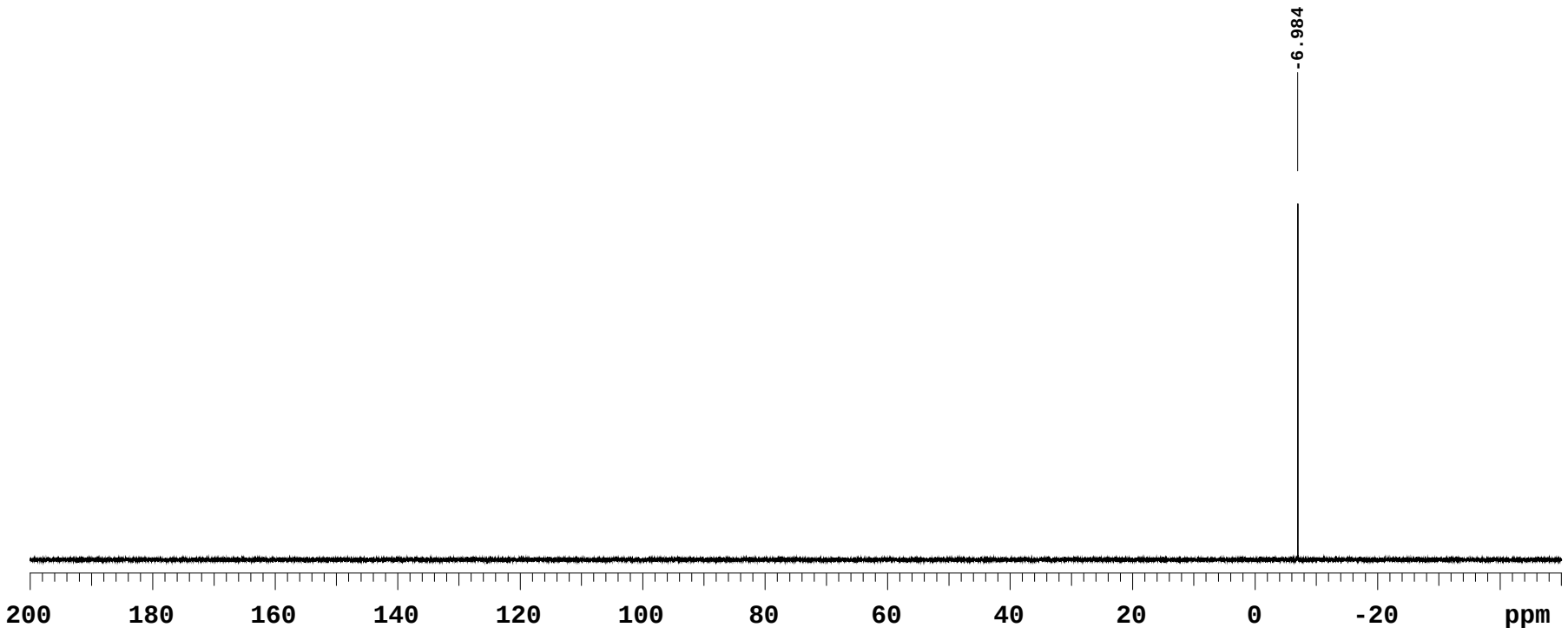


Data file exp

Plot date 2016-07-19

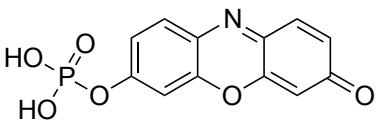
13-reactionin_THF-etherinsuble

Sample Name	**13-reactionin_THF-etherinsuble**	Pulse sequence	**PHOSPHORUS**	Temperature	**22**	Study owner	**suvendu**
Date collected	**2015-03-25**	Solvent	**cdcl3**	Spectrometer	**druidarch-mercury400**	Operator	**suvendu**



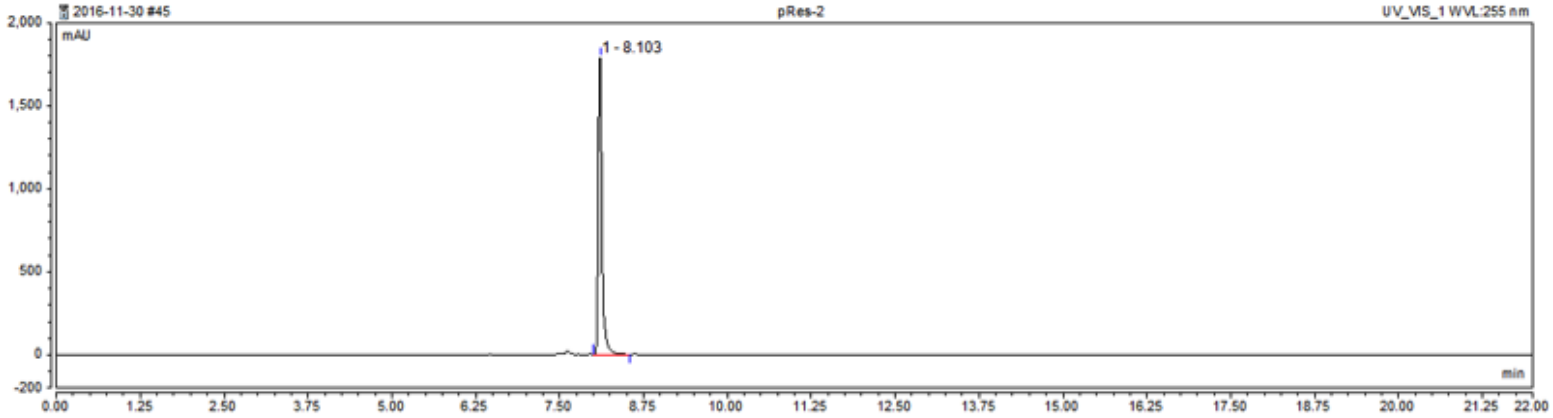
Spectral Data for Compound 3:

1. HRMS
2. ^{1}H NMR
3. ^{31}P NMR



Compound 3

Chemical Formula: $C_{12}H_8NO_6P$
Exact Mass: 293.01
Molecular Weight: 293.17



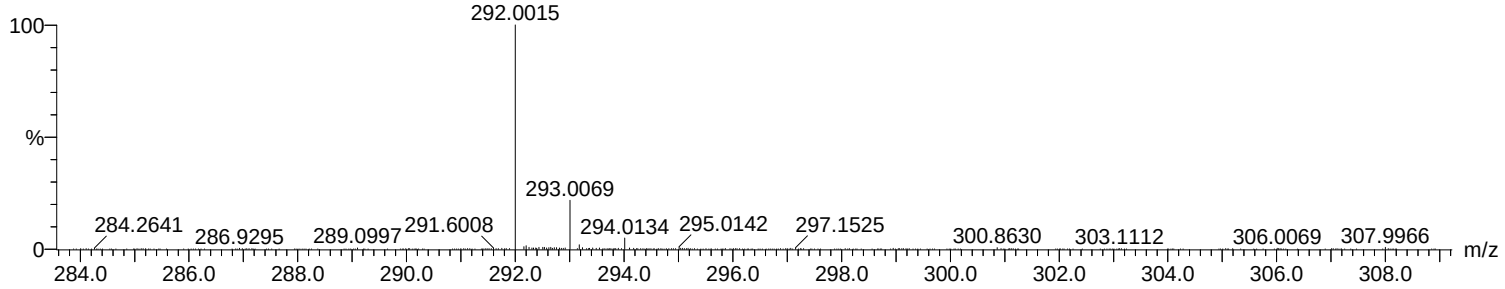
HPLC chromatogram of compound 3.

Single Mass Analysis

Tolerance = 5.0 mDa / DBE: min = -100.0, max = 400.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
1571 formula(e) evaluated with 10 results within limits (up to 20 best isotopic matches for each mass)
Elements Used:
C: 2-200 H: 0-120 N: 0-10 O: 0-20 Na: 0-1 P: 1-1

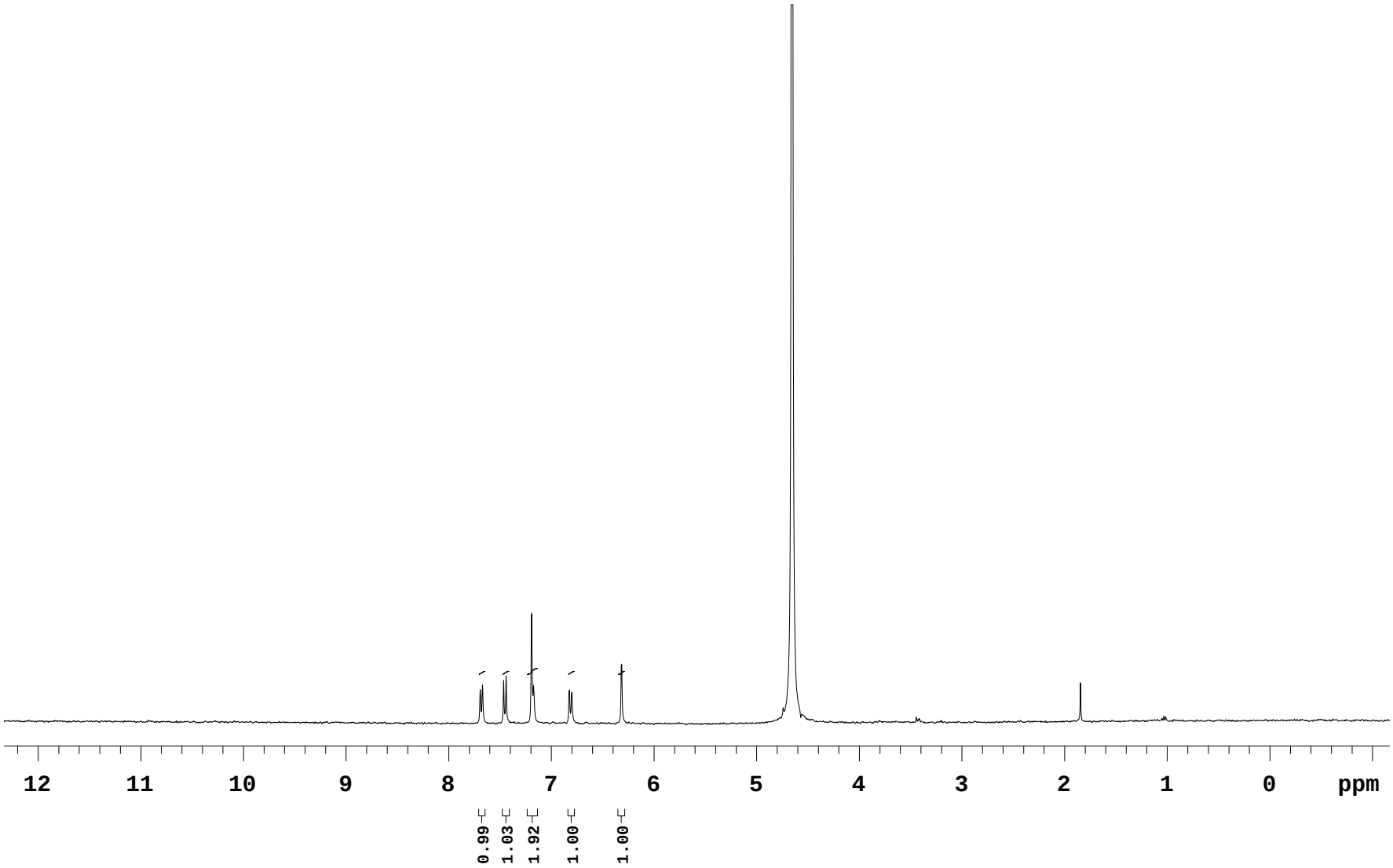
RS-3_HRMS KE319
LCT XE
15Ba1022_LCT_4 13 (0.301) AM (Cen,4, 85.00, Ar,12000.0,390.90,0.70,LS 3); Sm (SG, 2x3.00); Sb (5,40.00); Cm (1:30) 1: TOF MS ES-
2.89e+004



Minimum:				-100.0			
Maximum:		5.0	10.0	400.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
292.0015	292.0011	0.4	1.4	10.5	471.0	2.8	C12 H7 N O6 P
	292.0024	-0.9	-3.1	15.5	470.7	2.6	C13 H3 N5 O2 P
	292.0000	1.5	5.1	12.5	471.8	3.6	C11 H4 N5 O2 Na P
	292.0041	-2.6	-8.9	16.5	469.5	1.4	C16 H4 N3 Na P
	291.9987	2.8	9.6	7.5	472.0	3.9	C10 H8 N O6 Na P
	292.0046	-3.1	-10.6	-1.5	474.2	6.1	C3 H12 N O11 Na P
	291.9984	3.1	10.6	11.5	472.9	4.8	C8 H3 N7 O4 P
	292.0059	-4.4	-15.1	3.5	474.1	6.0	C4 H8 N5 O7 Na P
	291.9971	4.4	15.1	6.5	473.1	5.0	C7 H7 N3 O8 P
	292.0065	-5.0	-17.1	19.5	468.7	0.6	C18 H3 N3 P

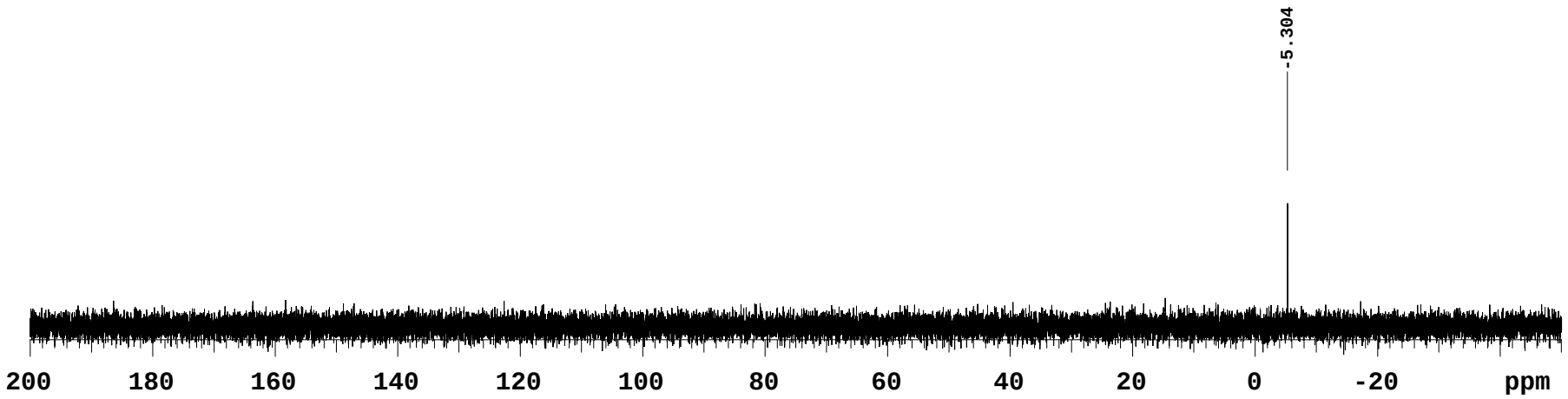
pRes-pure

Sample Name **pRes-pure**	Pulse sequence **PROTON**	Temperature **22**	Study owner **suvendu**
Date collected **2015-10-09**	Solvent **d2o**	Spectrometer **druidarch-mercury400**	Operator **suvendu**



pRes-3rdfrac-2

Sample Name **pRes-3rdfrac-2**	Pulse sequence **PHOSPHORUS**	Temperature **22**	Study owner **suvendu**
Date collected **2015-04-08**	Solvent **dmso**	Spectrometer **druidarch-mercury400**	Operator **suvendu**

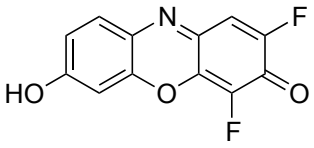


Data file /home/suvendu/vnmrsys/data/pRes-3rdfrac-2_20150408_01/PHOSPHORUS_01.fid

Plot date 2016-02-02

Spectral Data for Compound 6:

1. HRMS
2. ^{1}H NMR
3. ^{19}F NMR



Compound 6

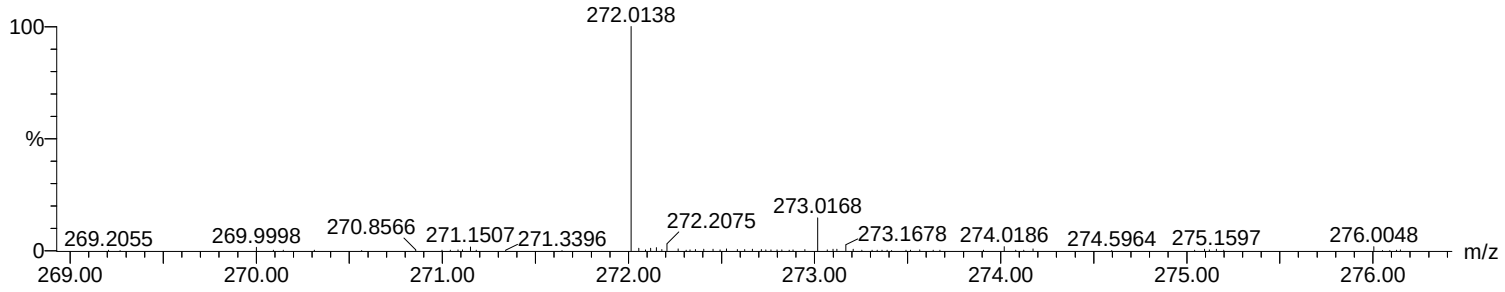
Chemical Formula: $C_{12}H_5F_2NO_3$
Exact Mass: 249.02
Molecular Weight: 249.17

Single Mass Analysis

Tolerance = 5.0 mDa / DBE: min = -100.0, max = 400.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
5372 formula(e) evaluated with 39 results within limits (up to 20 best isotopic matches for each mass)
Elements Used:
C: 2-200 H: 0-120 N: 0-10 O: 0-20 Na: 0-1 F: 0-3

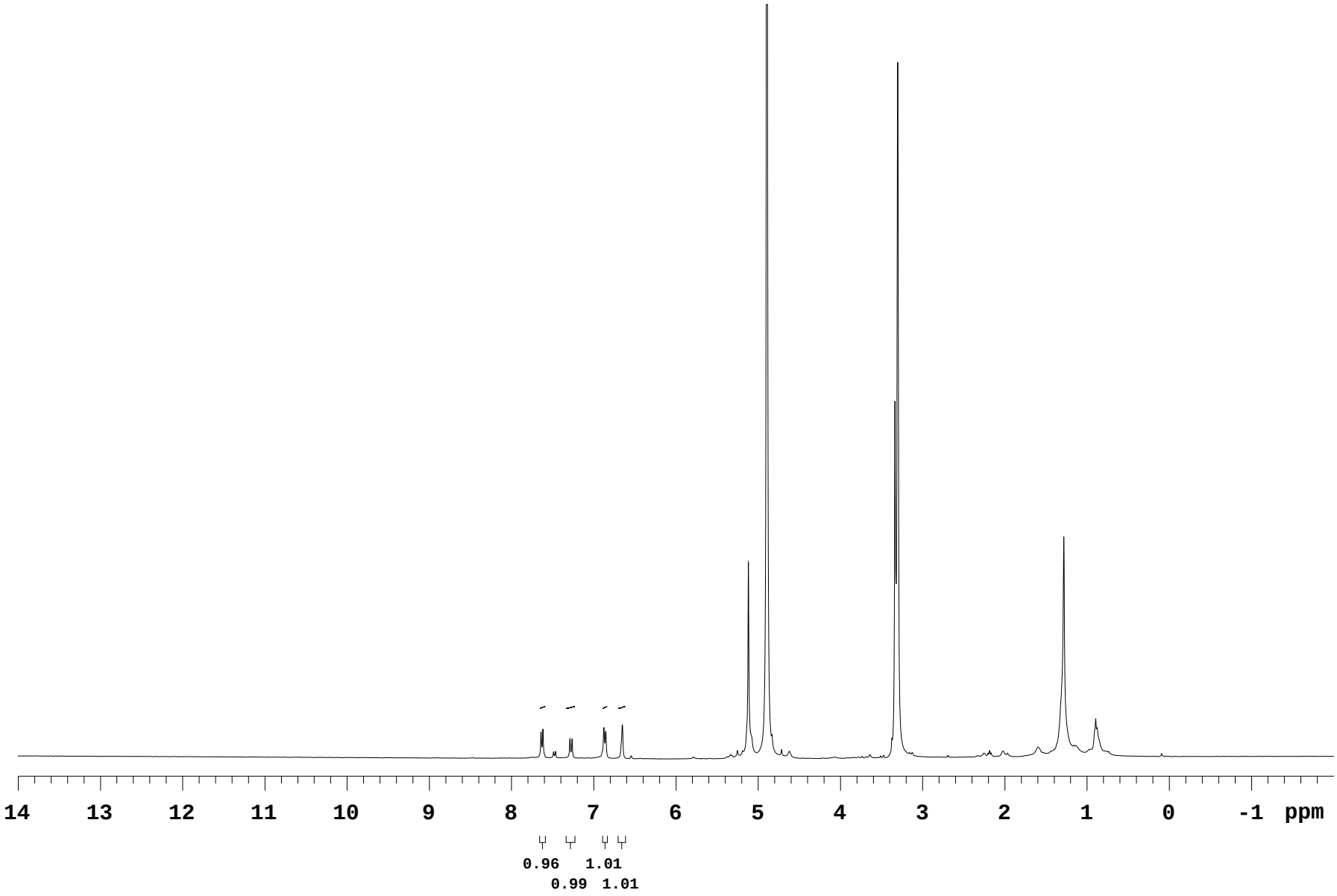
DRS-1_HRMS KE319
LCT XE
15Ba1022_LCT_1 22 (0.460) AM (Cen,4, 85.00, Ar,12000.0,490.89,0.70,LS 3); Sm (SG, 2x3.00); Sb (5,40.00); Cm (3:33) 1: TOF MS ES+
8.60e+003



Minimum:				-100.0			
Maximum:		5.0	10.0	400.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
272.0138	272.0135	0.3	1.1	9.5	306.5	0.5	C12 H5 N O3 Na F2
	272.0108	3.0	11.0	12.5	307.3	1.3	C12 H3 N3 O4 F
	272.0159	-2.1	-7.7	12.5	309.4	3.4	C14 H4 N O3 F2
	272.0184	-4.6	-16.9	13.5	309.6	3.6	C12 H3 N5 O2 Na
	272.0132	0.6	2.2	13.5	310.4	4.4	C10 N7 O F2
	272.0171	-3.3	-12.1	8.5	310.8	4.7	C11 H5 N O4 F3
	272.0124	1.4	5.1	13.5	310.9	4.9	C15 H4 N O2 Na F
	272.0171	-3.3	-12.1	8.5	311.2	5.2	C11 H7 N O6 Na
	272.0168	-3.0	-11.0	12.5	311.4	5.4	C9 H2 N7 O4
	272.0119	1.9	7.0	8.5	312.0	6.0	C9 H4 N3 O5 F2
	272.0184	-4.6	-16.9	13.5	312.2	6.2	C12 H N5 F3
	272.0160	-2.2	-8.1	10.5	312.3	6.3	C10 H2 N5 Na F3
	272.0147	-0.9	-3.3	5.5	312.4	6.3	C9 H6 N O4 Na F3
	272.0097	4.1	15.1	14.5	312.4	6.4	C11 N7 Na F
	272.0155	-1.7	-6.2	7.5	313.2	7.2	C8 H6 N3 O8
	272.0148	-1.0	-3.7	16.5	313.4	7.4	C17 H3 N O2 F
	272.0096	4.2	15.4	16.5	314.2	8.1	C15 H2 N3 O3
	272.0144	-0.6	-2.2	9.5	314.4	8.4	C7 H3 N7 O4 Na
	272.0131	0.7	2.6	4.5	314.6	8.6	C6 H7 N3 O8 Na
	272.0130	0.8	2.9	4.5	314.6	8.6	C6 H5 N3 O6 F3

3-F2Res

Sample Name **3-F2Res**	Pulse sequence **PROTON**	Temperature **22**	Study owner **suvendu**
Date collected **2015-11-24**	Solvent **cd3od**	Spectrometer **druidarch-mercury400**	Operator **suvendu**

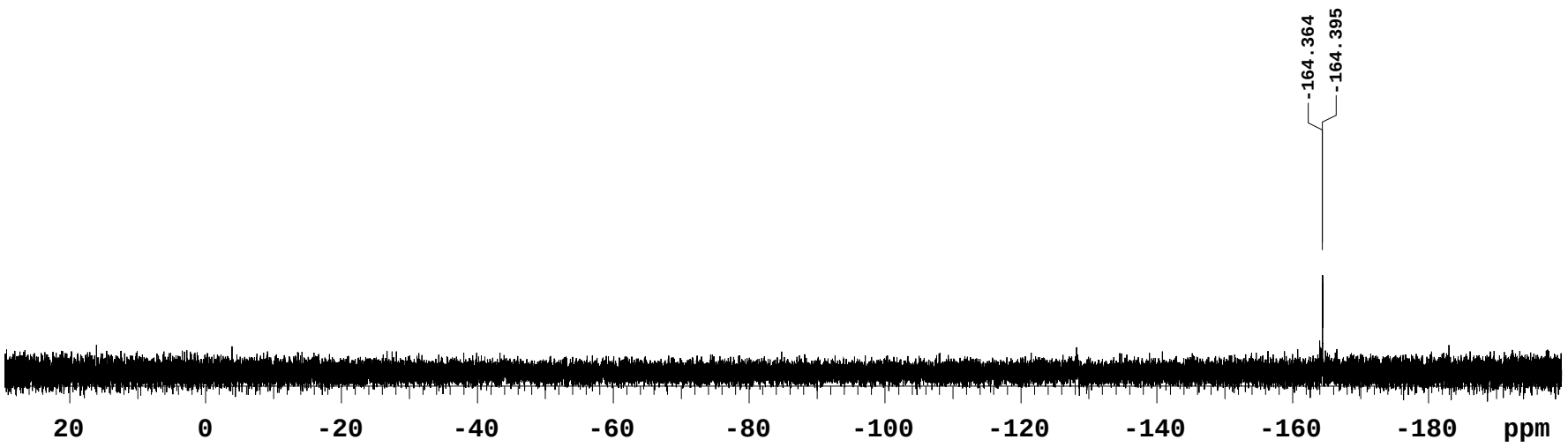


Data file /home/suvendu/vnmrsys/data/3-F2Res_20151124_01/PROTON_01.fid

Plot date 2016-02-02

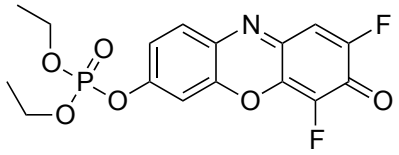
3-19F-F2Res

Sample Name **3-19F-F2Res**	Pulse sequence **FLUORINE**	Temperature **22**	Study owner **suvendu**
Date collected **2015-11-24**	Solvent **cd3od**	Spectrometer **druidarch-mercury400**	Operator **suvendu**



Spectral Data for Compound 7:

1. HRMS
2. ^{1}H NMR
3. ^{13}C NMR
4. ^{19}F NMR
5. ^{31}P NMR



Compound 7

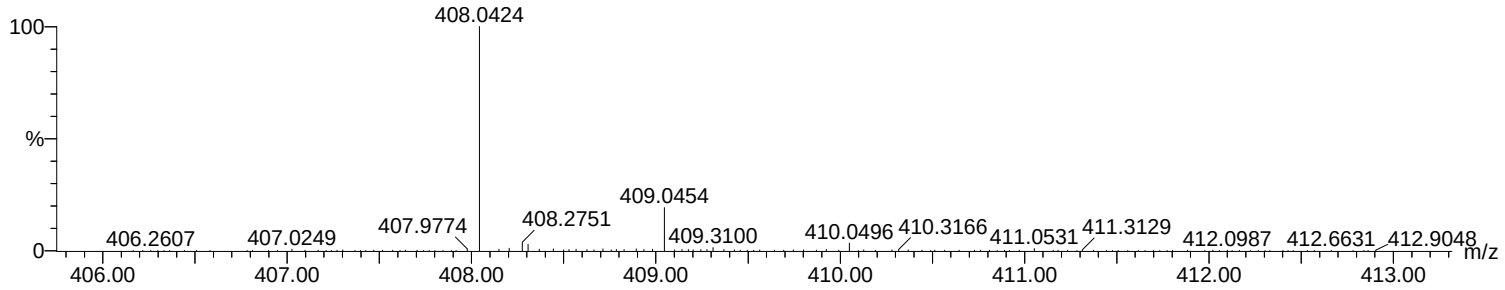
Chemical Formula: $C_{16}H_{14}F_2NO_6P$
Exact Mass: 385.05
Molecular Weight: 385.26

Single Mass Analysis

Tolerance = 5.0 mDa / DBE: min = -100.0, max = 400.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
15073 formula(e) evaluated with 89 results within limits (up to 20 best isotopic matches for each mass)
Elements Used:
C: 2-200 H: 0-120 N: 0-10 O: 0-20 F: 2-3 Na: 0-1 P: 1-4

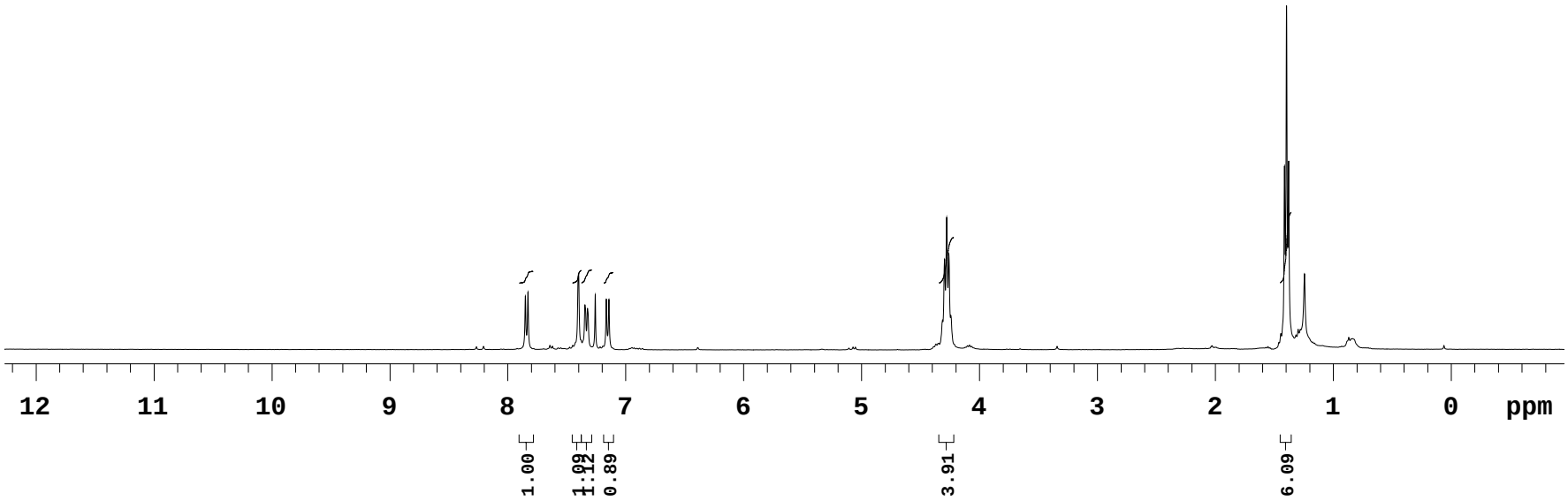
DRS-2_HRMS KE319
LCT XE
15Ba1022_LCT_2 26 (0.549) AM (Cen,4, 85.00, Ar,12000.0,490.89,0.70,LS 3); Sm (SG, 2x3.00); Sb (5,40.00); Cm (26:92) 1: TOF MS ES+
2.61e+004



Minimum:				-100.0			
Maximum:		5.0	10.0	400.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
408.0424	408.0425	-0.1	-0.2	9.5	432.4	0.5	C16 H14 N O6 F2 Na P
	408.0449	-2.5	-6.1	12.5	433.7	1.8	C18 H13 N O6 F2 P
	408.0424	0.0	0.0	9.5	435.0	3.1	C17 H17 N F3 Na P3
	408.0460	-3.6	-8.8	8.5	435.3	3.4	C15 H14 N O7 F3 P
	408.0438	-1.4	-3.4	14.5	435.5	3.6	C17 H10 N5 O2 F2 Na P
	408.0448	-2.4	-5.9	12.5	435.9	4.0	C19 H16 N F3 P3
	408.0422	0.2	0.5	13.5	435.9	4.0	C14 H9 N7 O4 F2 P
	408.0413	1.1	2.7	7.5	436.2	4.3	C15 H20 N O2 F2 P4
	408.0396	2.8	6.9	12.5	436.2	4.3	C17 H15 N3 O F2 P3
	408.0454	-3.0	-7.4	9.5	436.5	4.6	C15 H15 N3 O3 F2 Na P2
	408.0473	-4.9	-12.0	13.5	436.5	4.6	C16 H10 N5 O3 F3 P
	408.0408	1.6	3.9	8.5	437.1	5.2	C13 H13 N3 O8 F2 P
	408.0378	4.6	11.3	8.5	437.1	5.2	C15 H15 N O5 F3 P2
	408.0449	-2.5	-6.1	10.5	437.1	5.2	C14 H11 N5 O3 F3 Na P
	408.0391	3.3	8.1	13.5	437.2	5.3	C16 H11 N5 O F3 P2
	408.0408	1.6	3.9	8.5	437.3	5.4	C14 H16 N3 O2 F3 P3
	408.0401	2.3	5.6	17.5	437.6	5.7	C22 H10 N O2 F3 P
	408.0436	-1.2	-2.9	5.5	437.8	5.9	C13 H15 N O7 F3 Na P
	408.0462	-3.8	-9.3	17.5	437.8	6.0	C19 H9 N5 O2 F2 P
	408.0377	4.7	11.5	14.5	437.8	6.0	C20 H11 N O2 F3 Na P

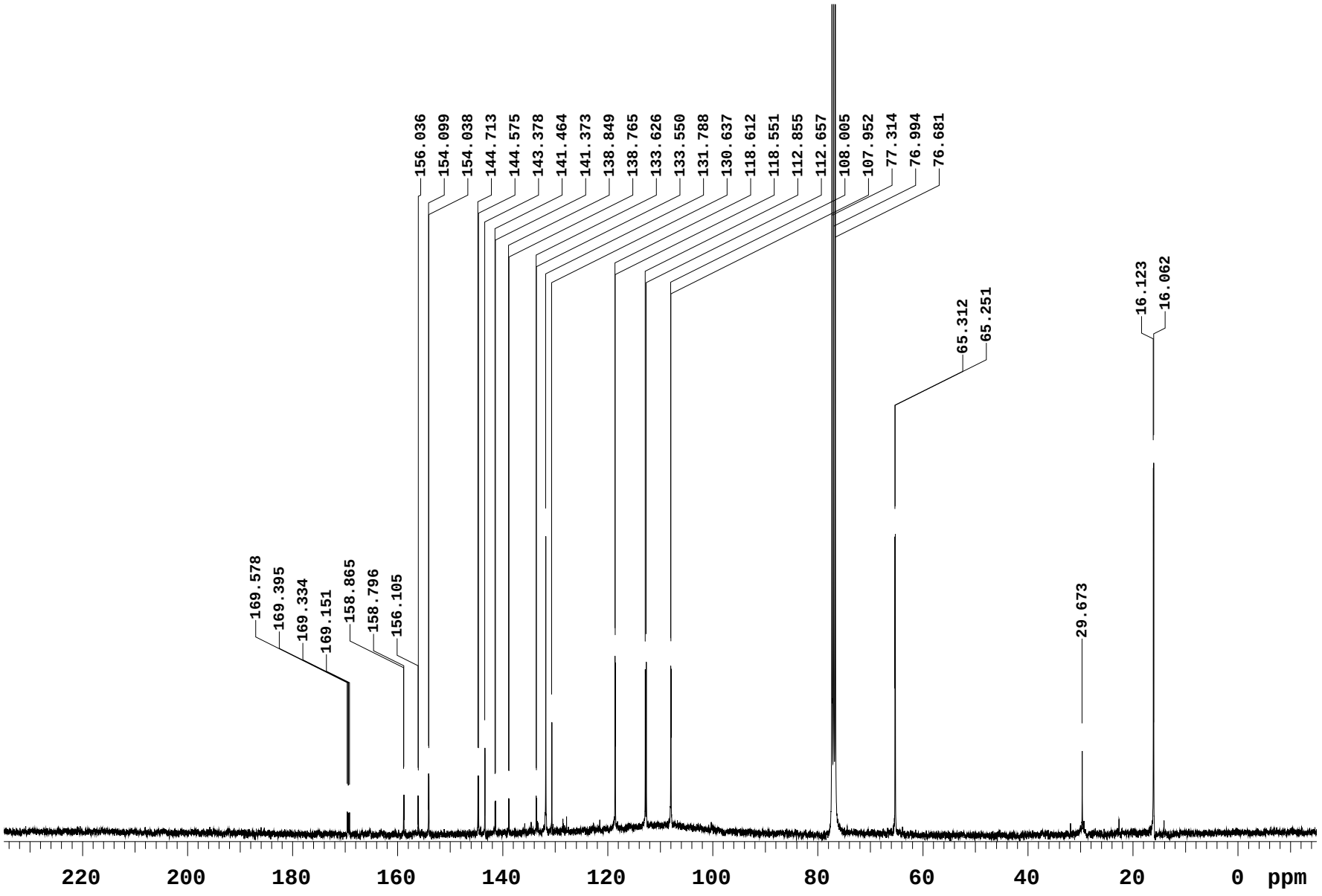
Pg-F2pRes-f

Sample Name **Pg-F2pRes-f**	Pulse sequence **PROTON**	Temperature **25**	Study owner **suvendu**
Date collected **2016-06-13**	Solvent **cdcl3**	Spectrometer **druidarch-mercury400**	Operator **suvendu**



13C-Pg-F2pRes-Final

Sample Name	**13C-Pg-F2pRes-Final**	Pulse sequence	**CARBON**	Temperature	**25**	Study owner	**suvendu**
Date collected	**2016-06-13**	Solvent	**cdcl3**	Spectrometer	**druidarch-mercury400**	Operator	**suvendu**

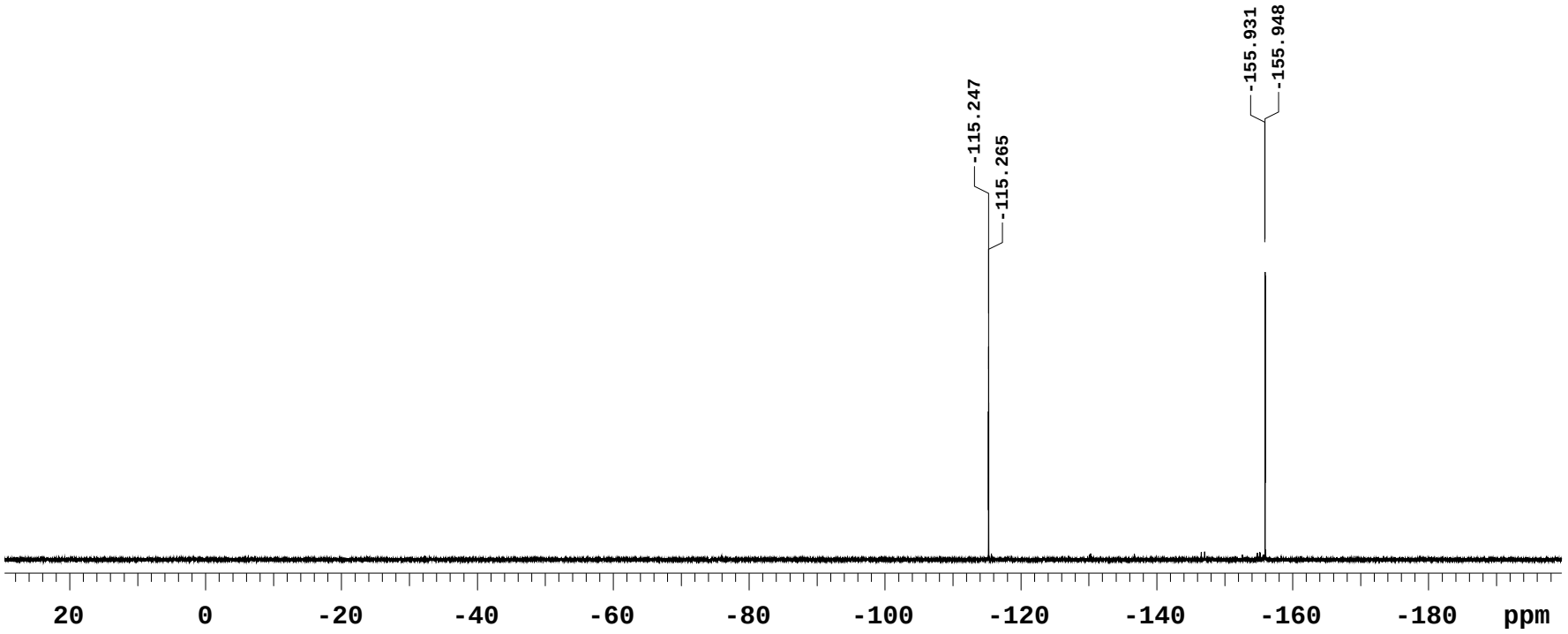


Data file /home/suvendu/vnmrsys/data/13C-Pg-F2pRes-Final_20160613_01/CARBON_01.fid

Plot date 2016-06-14

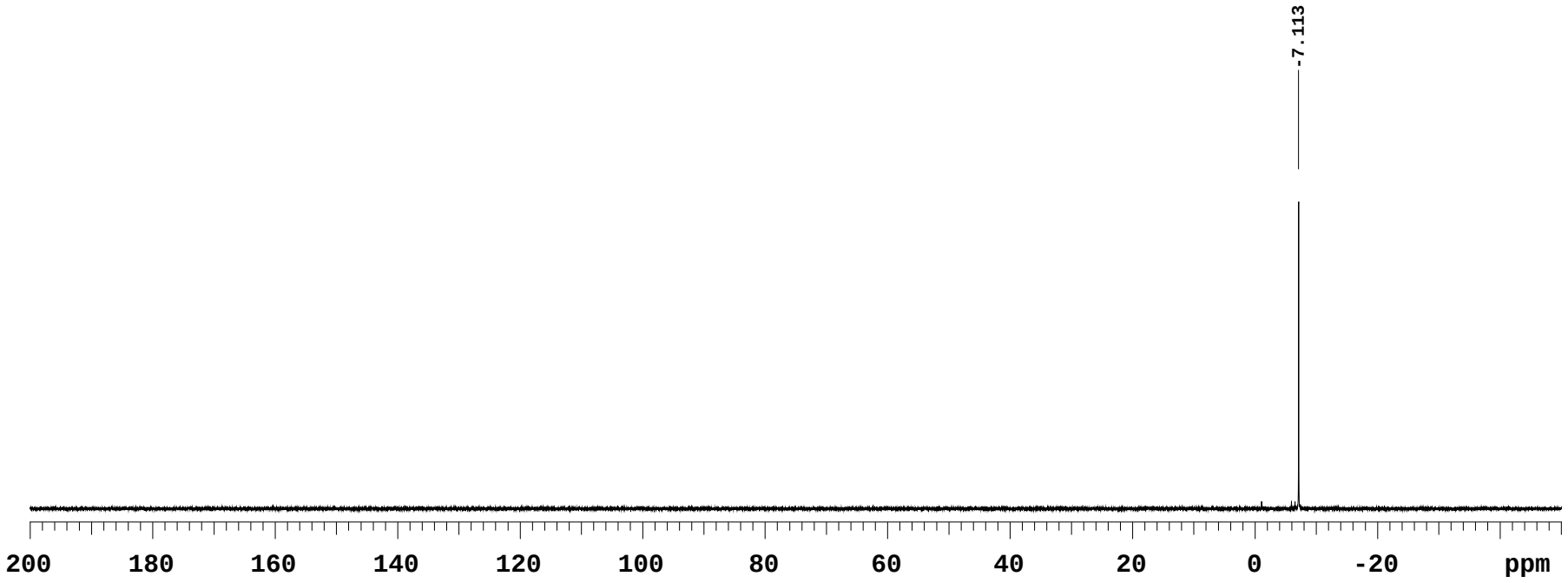
19F-Pg-F2pRes-final

Sample Name	**19F-Pg-F2pRes-final**	Pulse sequence	**FLUORINE**	Temperature	**25**	Study owner	**suvendu**
Date collected	**2016-06-13**	Solvent	**cdcl3**	Spectrometer	**druidarch-mercury400**	Operator	**suvendu**



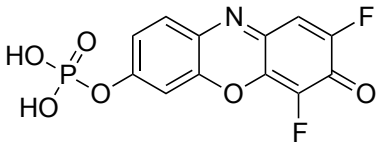
31P-Pg-F2pRes-final

Sample Name	**31P-Pg-F2pRes-final**	Pulse sequence	**PHOSPHORUS**	Temperature	**25**	Study owner	**suvendu**
Date collected	**2016-06-13**	Solvent	**cdcl3**	Spectrometer	**druidarch-mercury400**	Operator	**suvendu**



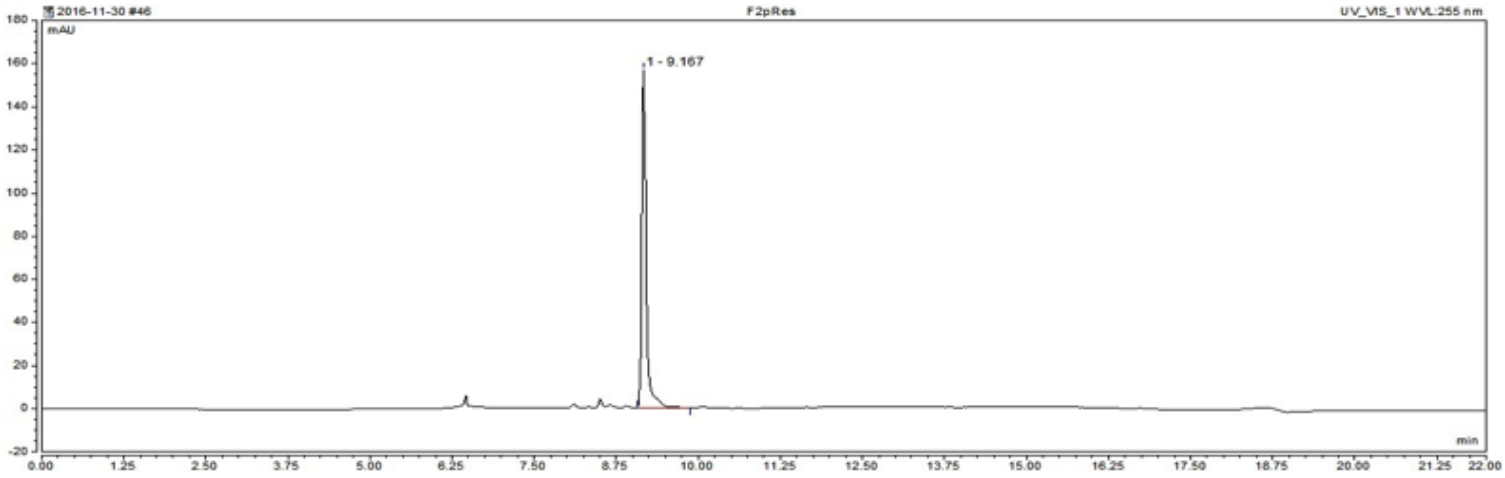
Spectral Data for Compound 8:

1. HRMS
2. ^{1}H NMR
3. ^{19}F NMR
4. ^{31}P NMR



Compound 8

Chemical Formula: $C_{12}H_6F_2NO_6P$
Exact Mass: 328.99
Molecular Weight: 329.15



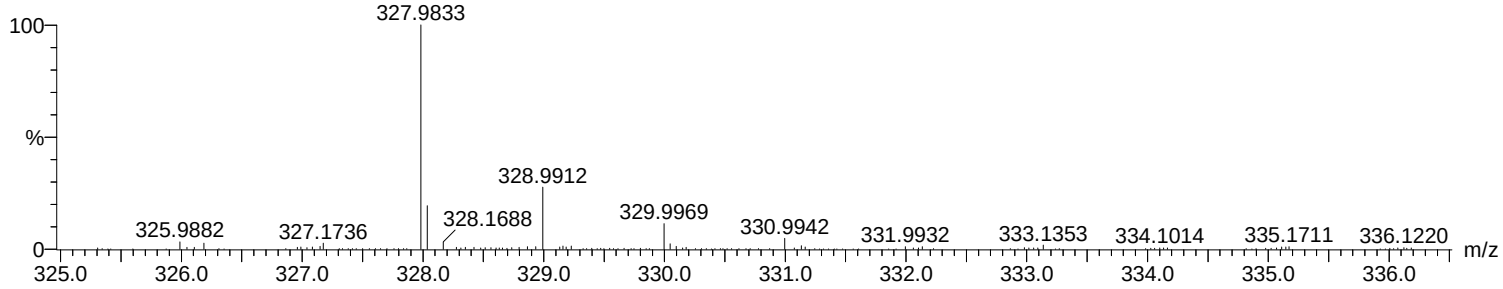
HPLC chromatogram of compound 8

Single Mass Analysis

Tolerance = 5.0 mDa / DBE: min = -100.0, max = 400.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
1547 formula(e) evaluated with 10 results within limits (up to 20 best isotopic matches for each mass)
Elements Used:
C: 2-200 H: 0-120 N: 0-10 O: 0-20 F: 2-2 Na: 0-1 P: 1-1

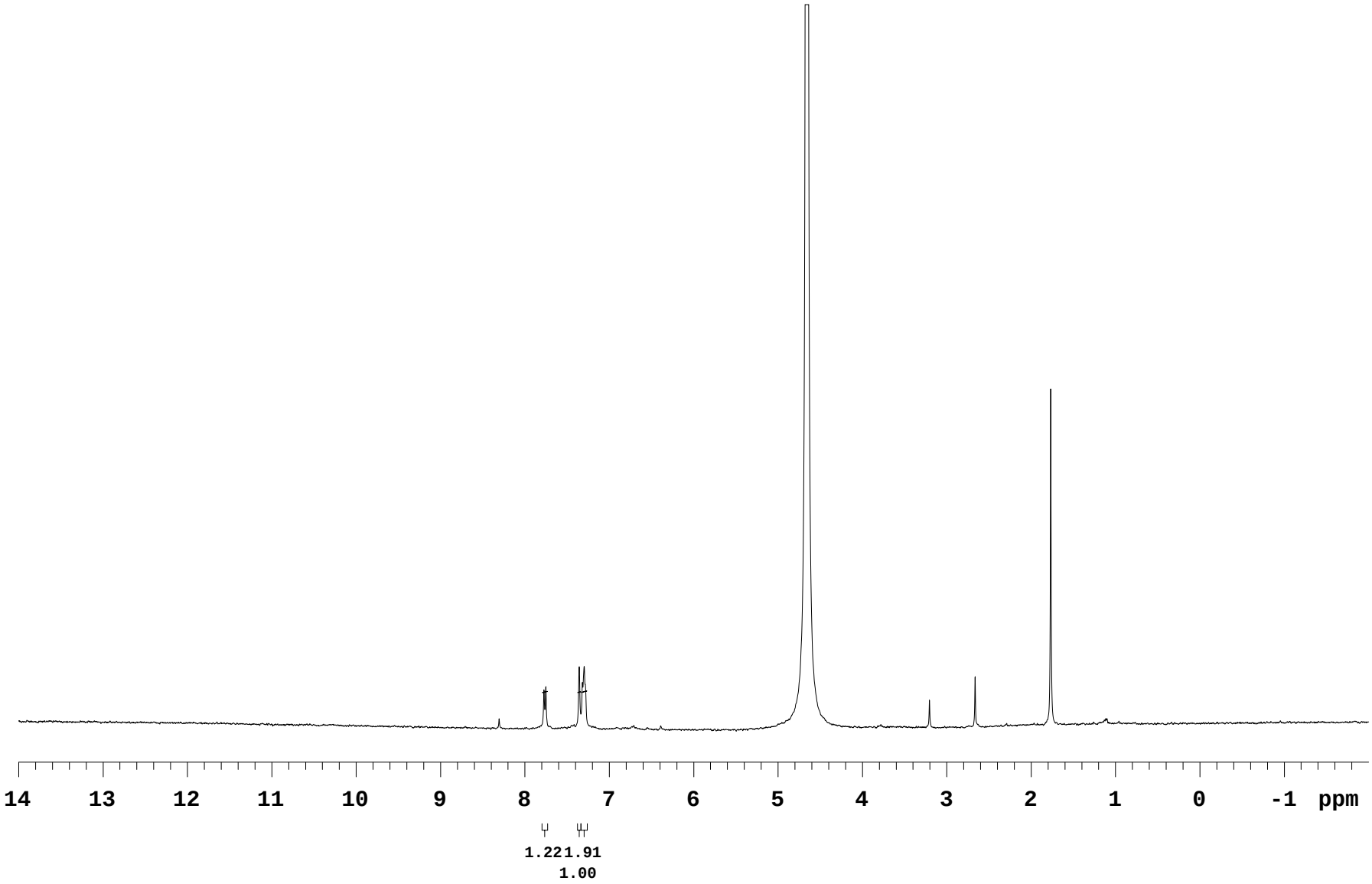
DRS-3_HRMS KE319
LCT XE
15Ba1022_LCT_5 54 (1.166) AM (Cen,4, 85.00, Ar,12000.0,390.90,0.70,LS 3); Sm (SG, 2x3.00); Sb (5,40.00); Cm (43:129) 1: TOF MS ES-
6.45e+004



Minimum:				-100.0			
Maximum:		5.0	10.0	400.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
327.9833	327.9836	-0.3	-0.9	15.5	551.0	2.2	C13 H N5 O2 F2 P
	327.9823	1.0	3.0	10.5	551.1	2.3	C12 H5 N O6 F2 P
	327.9852	-1.9	-5.8	16.5	550.2	1.5	C16 H2 N3 F2 Na P
	327.9812	2.1	6.4	12.5	551.6	2.8	C11 H2 N5 O2 F2 Na P
	327.9857	-2.4	-7.3	-1.5	552.9	4.1	C3 H10 N O11 F2 Na P
	327.9799	3.4	10.4	7.5	551.6	2.9	C10 H6 N O6 F2 Na P
	327.9796	3.7	11.3	11.5	552.3	3.5	C8 H N7 O4 F2 P
	327.9871	-3.8	-11.6	3.5	552.8	4.1	C4 H6 N5 O7 F2 Na P
	327.9876	-4.3	-13.1	19.5	549.8	1.0	C18 H N3 F2 P
	327.9881	-4.8	-14.6	1.5	552.5	3.7	C5 H9 N O11 F2 P

F2pRes

Sample Name **F2pRes**	Pulse sequence **PROTON**	Temperature **22**	Study owner **suvendu**
Date collected **2015-10-07**	Solvent **d2o**	Spectrometer **druidarch-mercury400**	Operator **suvendu**

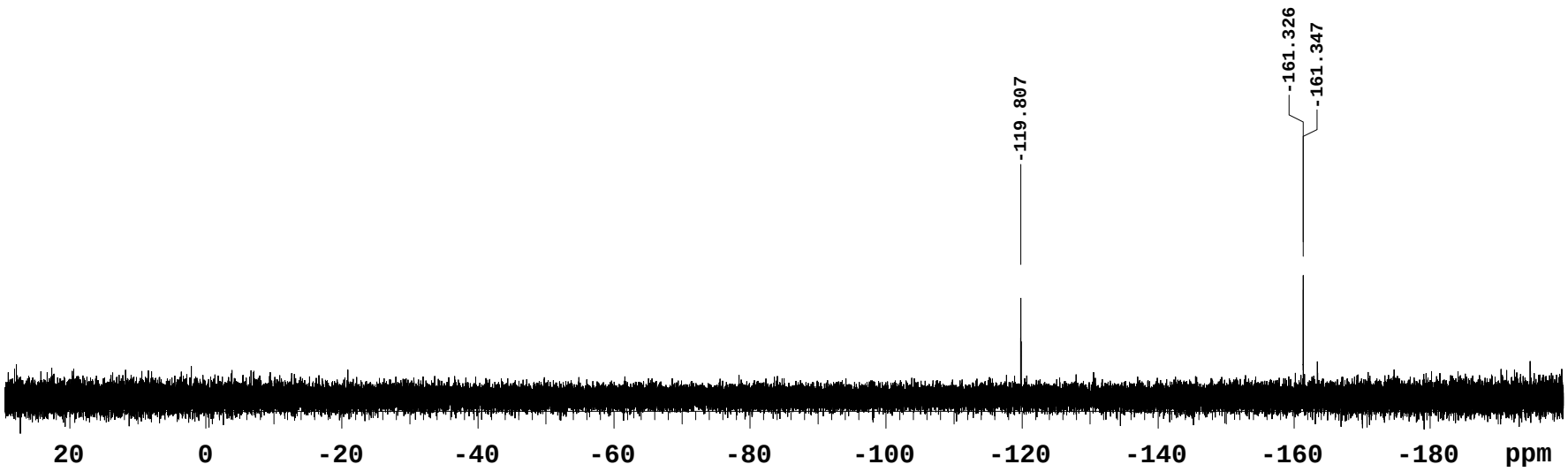


Data file /home/suvendu/vnmrsys/data/F2pRes_20151007_01/PROTON_01.fid

Plot date 2016-02-02

5-19F-F2pRes

Sample Name **5-19F-F2pRes**	Pulse sequence **FLUORINE**	Temperature **22**	Study owner **suvendu**
Date collected **2015-11-24**	Solvent **d2o**	Spectrometer **druidarch-mercury400**	Operator **suvendu**



5-31P-F2pRes

Sample Name **5-31P-F2pRes**	Pulse sequence **PHOSPHORUS**	Temperature **22**	Study owner **suvendu**
Date collected **2015-11-25**	Solvent **d2o**	Spectrometer **druidarch-mercury400**	Operator **suvendu**