

SUPPORTING INFORMATION

Synthesis and Biological Evaluation of (*E*)-4-Hydroxy-3-Methylbut-2-enyl Phosphate (HMBP) Aryloxy Triester Phosphoramidate Prodrugs as Activators of V γ 9/V δ 2 T-Cells Immune Response

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CF10 3NB, UK.

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I. T-cell isolation, culture and activation

PBMCs were isolated from heparinised venous blood from consented healthy donors (approved by the NRES Committee West Midlands ethical board; REC reference 14/WM/1254). Blood was layered over lymphoprep[®] (Stem Cell Technologies) and PBMC isolated according to manufacturers instructions. The cell culture medium used throughout was RPMI-1640 media supplemented with 2 mM L-glutamine, 25 mM HEPES, 1% sodium pyruvate, 50 µg/ml penicillin/streptomycin (Invitrogen) and 10% fetal calf serum (Sigma). For activation assays, 5×10^5 PBMC were cultured for 20 h in the presence of medium alone or the indicated concentrations of HMB-PP (Sigma), Zoledronate (Sigma), **5a**, **5b**, **5c** and **5d** (as described above). Cultured PBMC were labelled with Zombie[®] aqua viability dye (Biolegend) and cells were stained for surface antigens by antibodies directed against CD3 (UCHT1; 1:100), CD8 (SK1; 1:200), CD25 (2A3; 1:200); all Biolegend, CD69 (TP1.55.3; 1:20) and TCR V γ 9 (IMMU360; 1:400); Beckman Coulter, and TCR V δ 2 (123R3; 1:200); Miltenyi. For killing assays, V γ 9/V δ 2 T cells were expanded from 2×10^5 /ml PBMC cultured with 10 µM zoledronate for 14 days and supplemented with 100 U/ml IL-2 into the media every 2-3 days, yielding 83 – 91% V γ 9/V δ 2 T cells. T24 (ATCC HTB4) cell line were labelled with 0.1µM CFSE and incubated for 4 hours with 10 µM zoledronate or 10 nM ProPAgens (**5a - d**), before being washed five-times in medium and co-cultured with expanded V γ 9/V δ 2 T cells for 18 hours. All cells were then labelled with Zombie[®] aqua viability dye and CFSE⁺ Zombie[®] aqua⁺ cells measured. All data were acquired on an LSR II (Beckton Dickinson) and data analysed with FlowJo V10.1 (TreeStar). Tabulated data were analysed in Graphpad PRISM 7 (Graphpad Software Inc).

Supporting Figure S1.

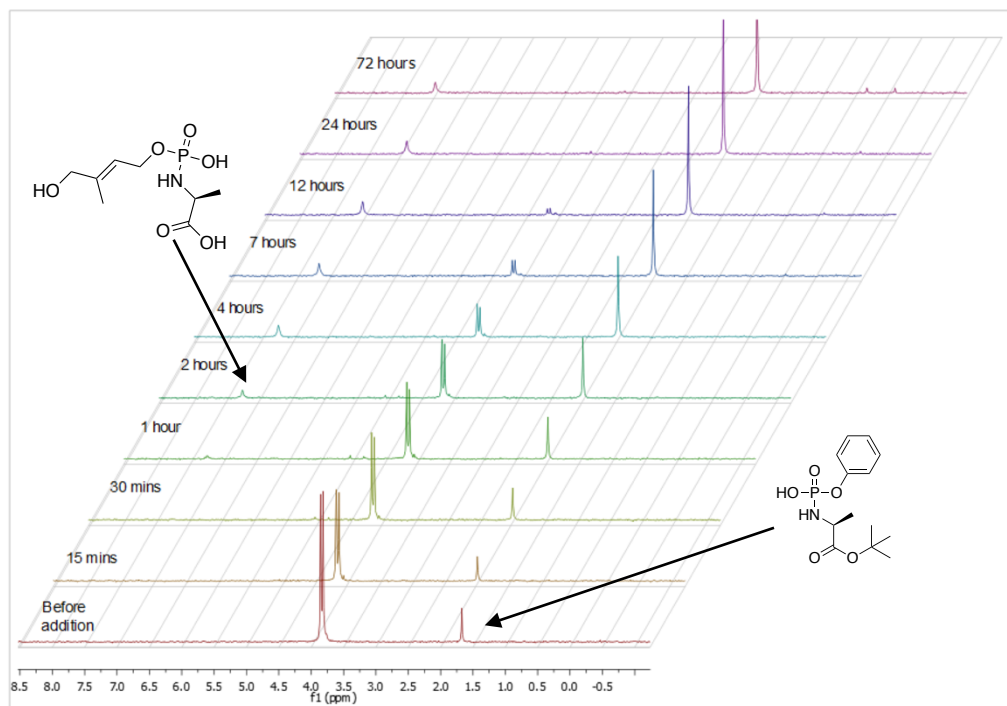


Figure S1: ^{31}P NMR of HMBP ProPAGEN 6d incubated with the carboxypeptidase cathepsin A. The two singlets at 4.02 ppm corresponds to the two diastereoisomers of ProPAGEN 6d. The singlet at 6.87 ppm corresponds to the amino acyl monoester as shown. The singlet at 1.98 ppm corresponds to the degradation product shown in the figure and confirmed by mass spec (see below). The presence of the undesired metabolite that has a ^{31}P NMR is a reflection of the low stability of these compounds. The experiment was carried out as reported previously by Osgerby et al. 2017, 60 (8), 3518–3524.

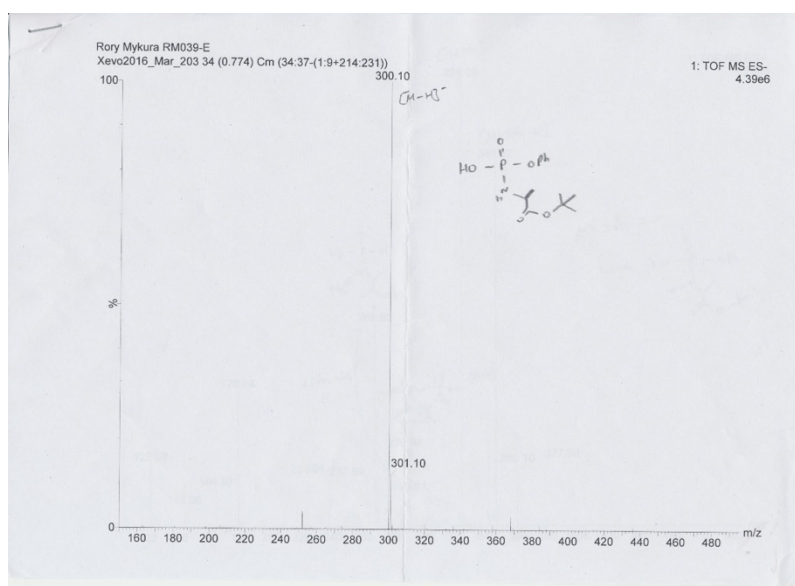
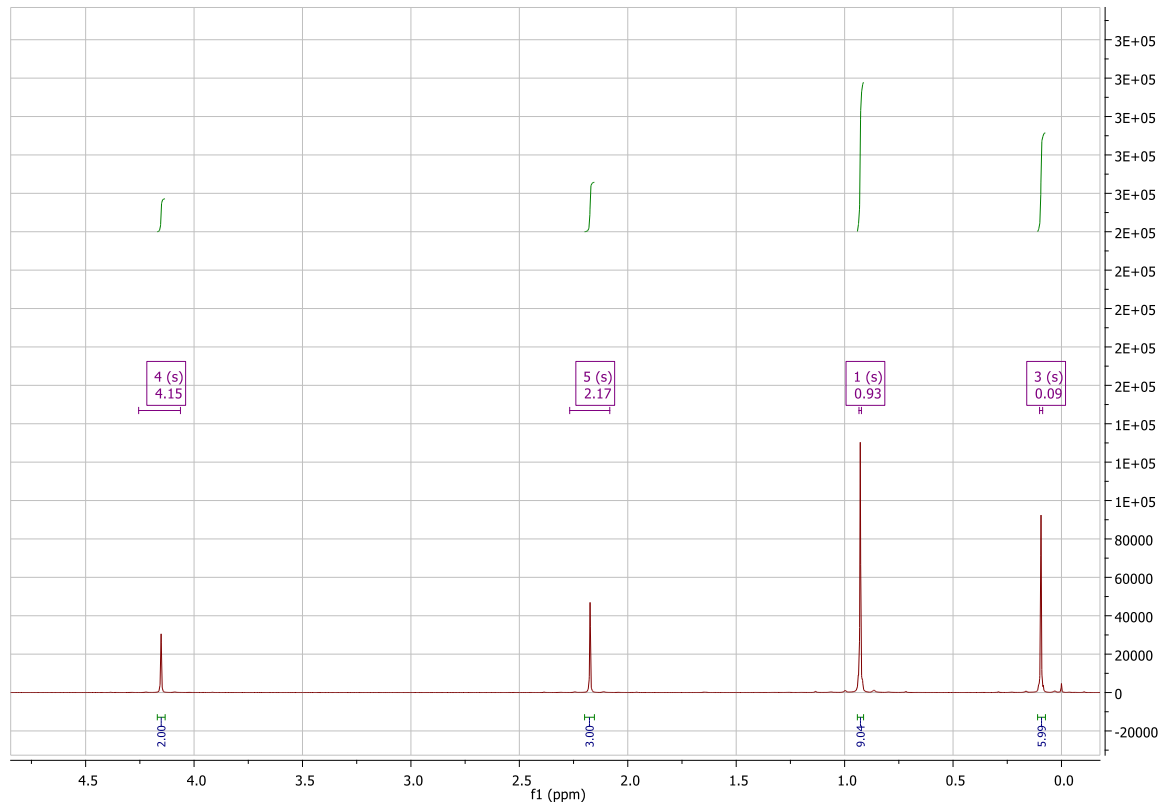


Figure S2. Negative ion spectrum for HMBP ProPAGEN 6d following incubation with cathepsin A for 72 hours.

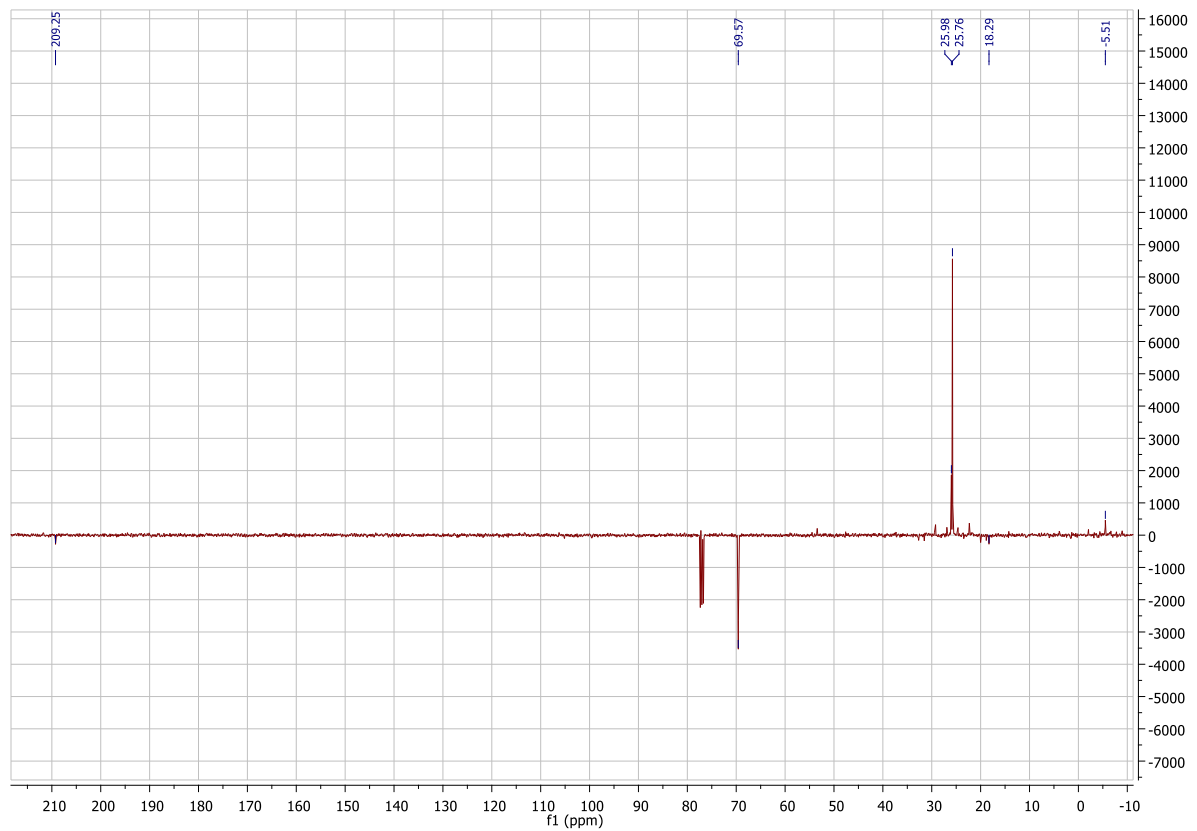
II. NMR spectra

1-((tert-butyldimethylsilyl)oxy)propan-2-one, 2:

^1H NMR

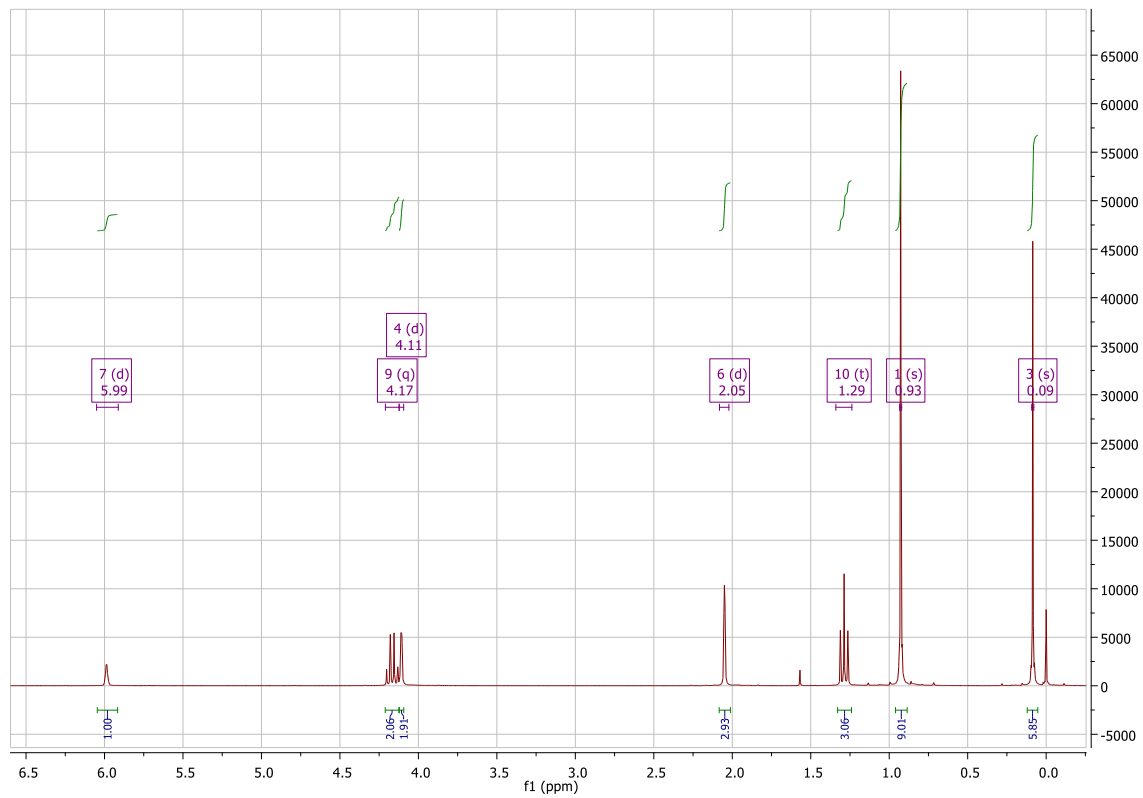


^{13}C NMR

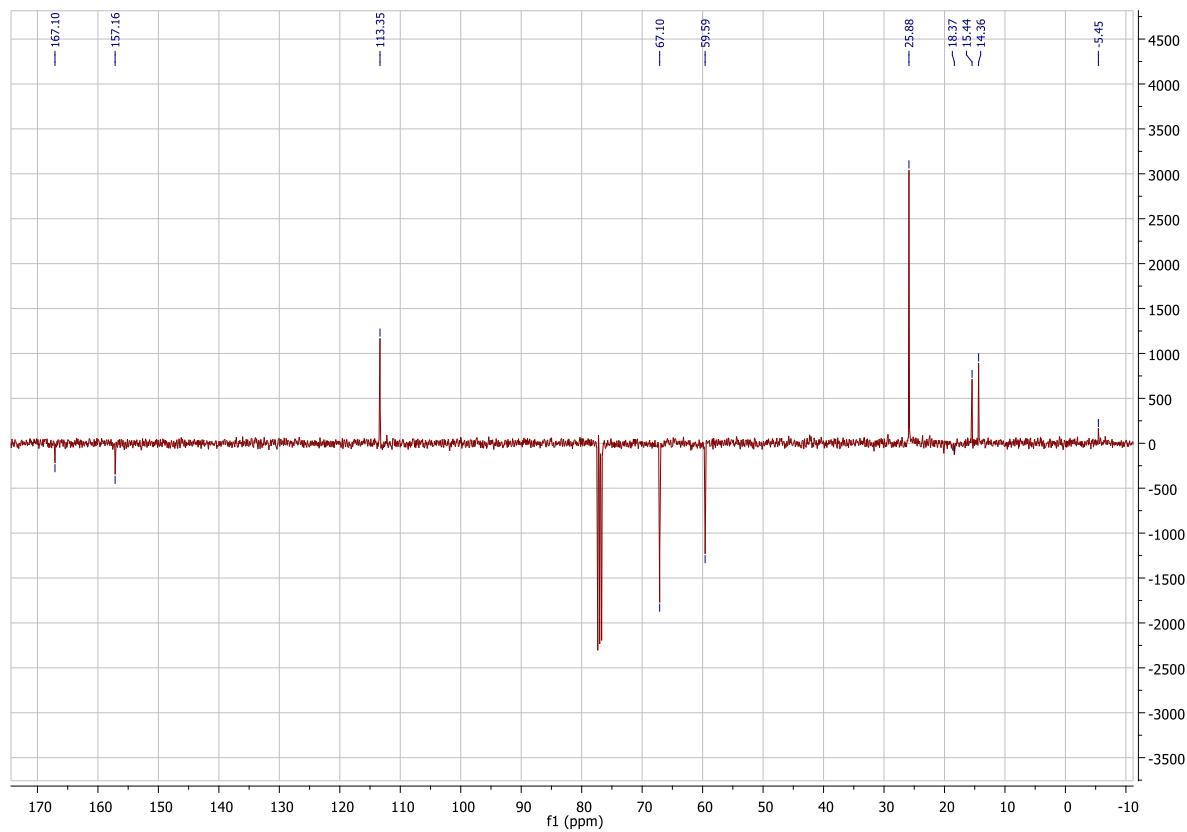


Ethyl (E)-4-((tert-butyl dimethylsilyl)oxy)-3-methylbut-2-enoate, 3:

¹H NMR

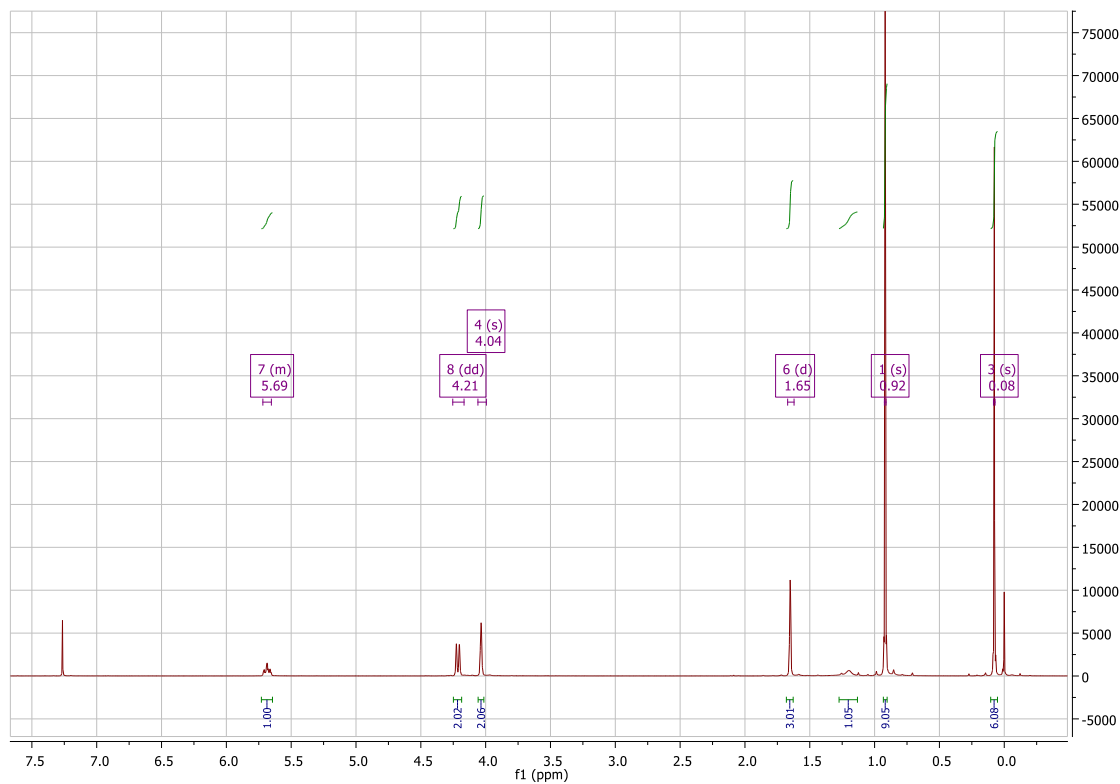


¹³C NMR

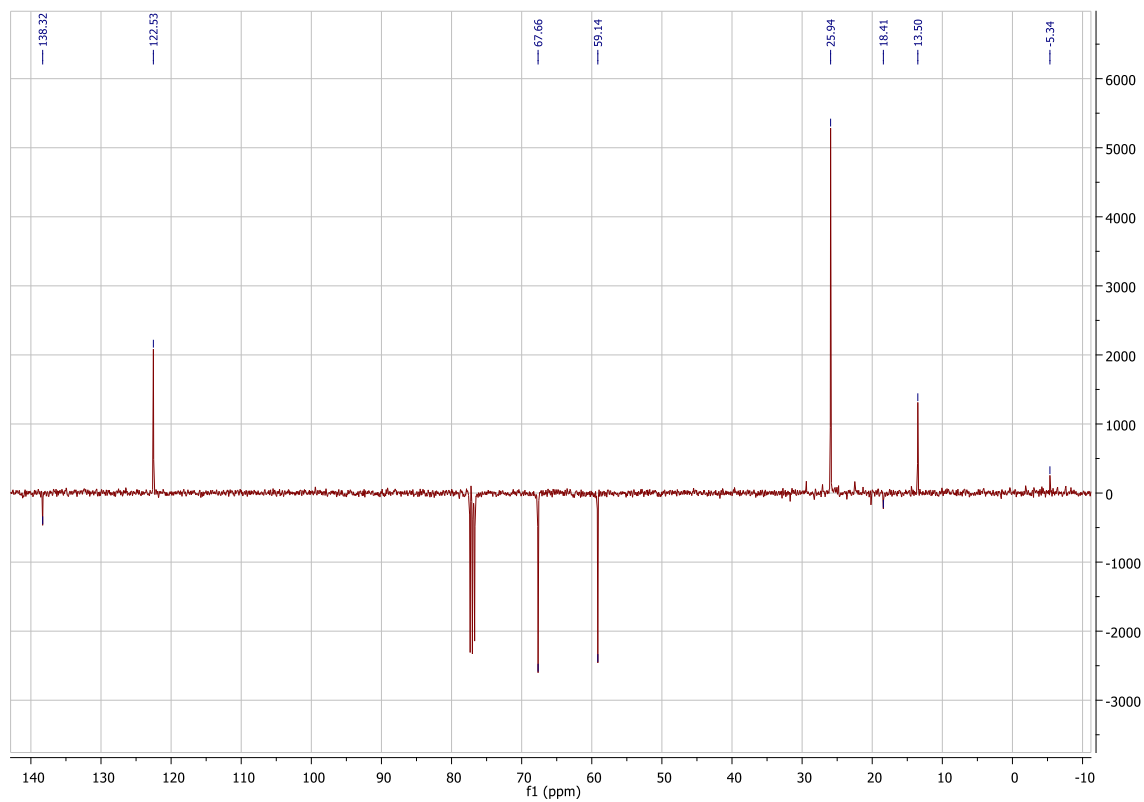


(E)-4-((tert-butyldimethylsilyl)oxy)-3-methylbut-2-en-1-ol, 4:

¹H NMR

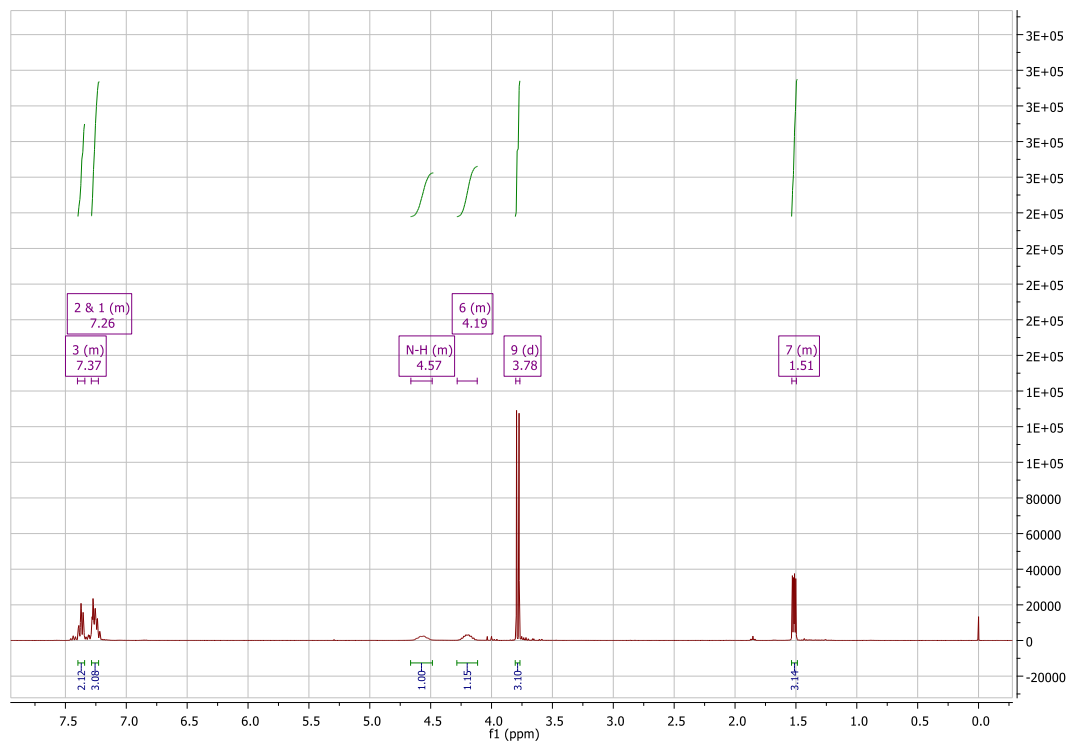


¹³C NMR

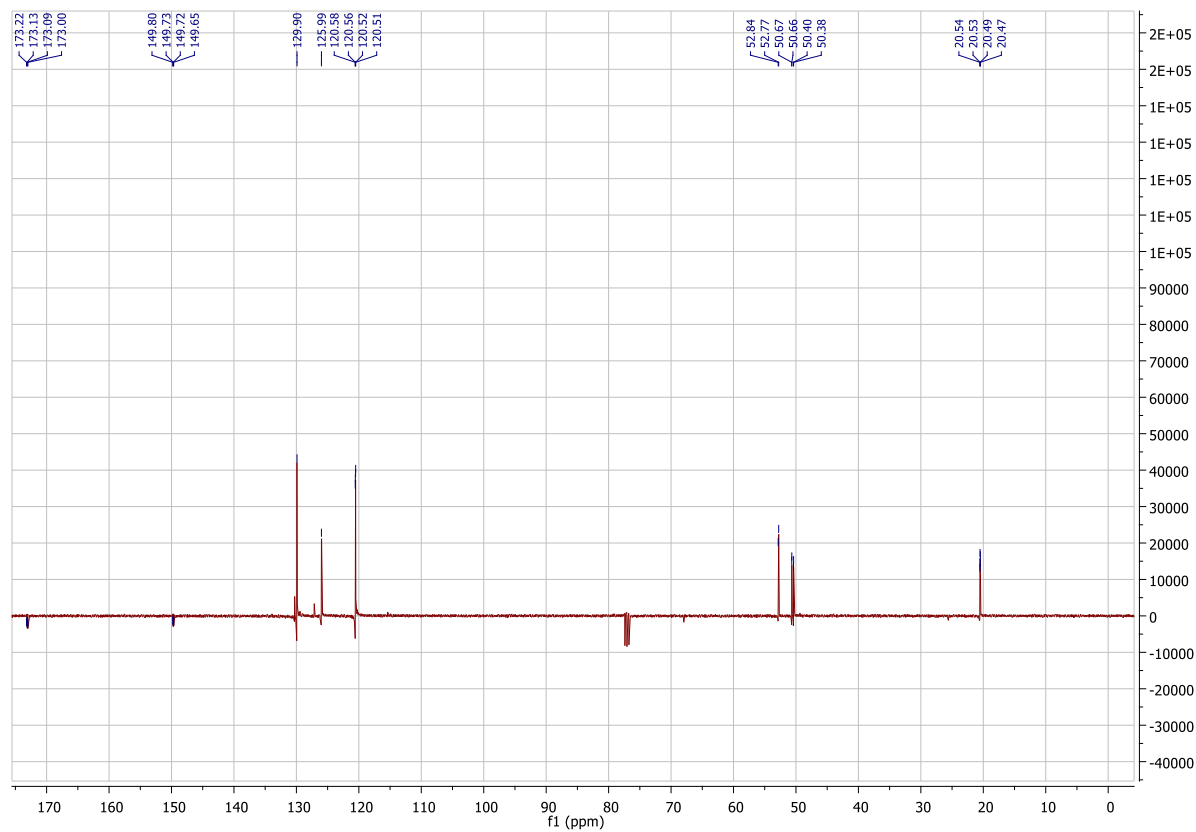


Methyl (chloro(phenoxy)phosphoryl)-L-alaninate, 9a:

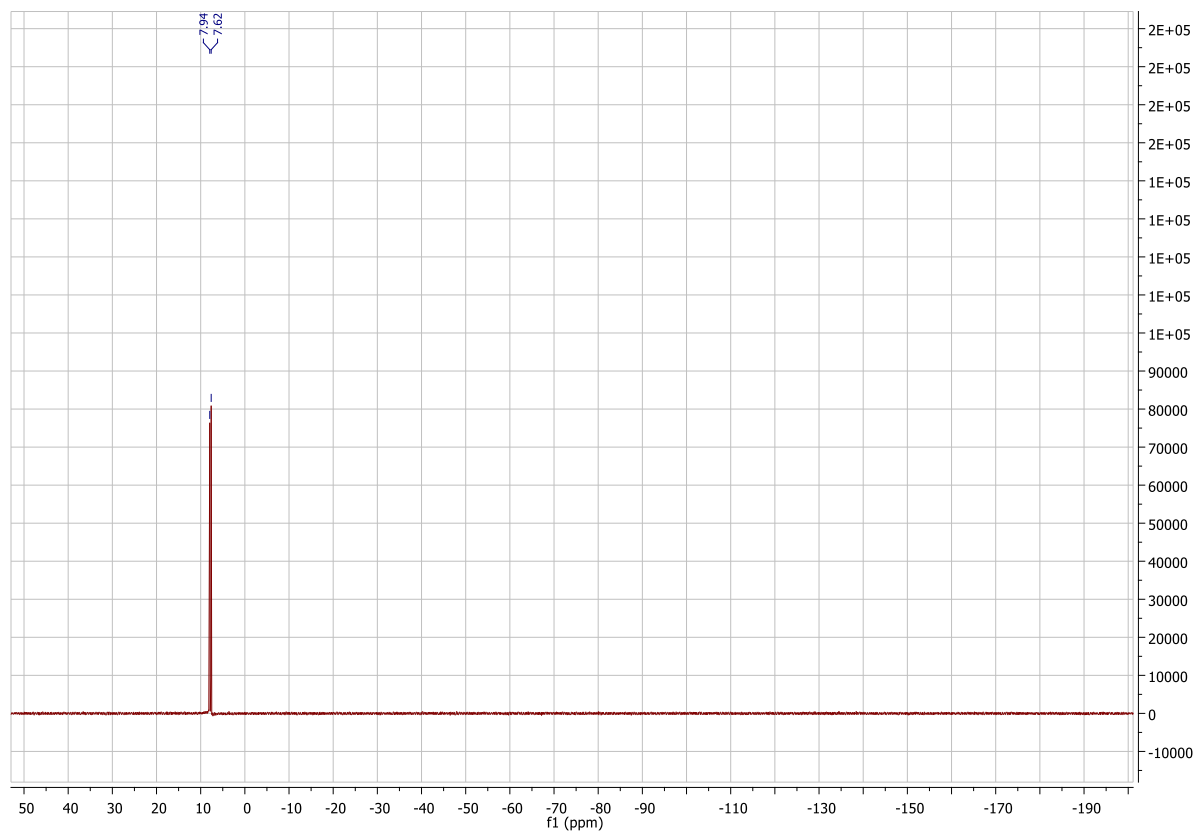
^1H NMR



^{13}C NMR

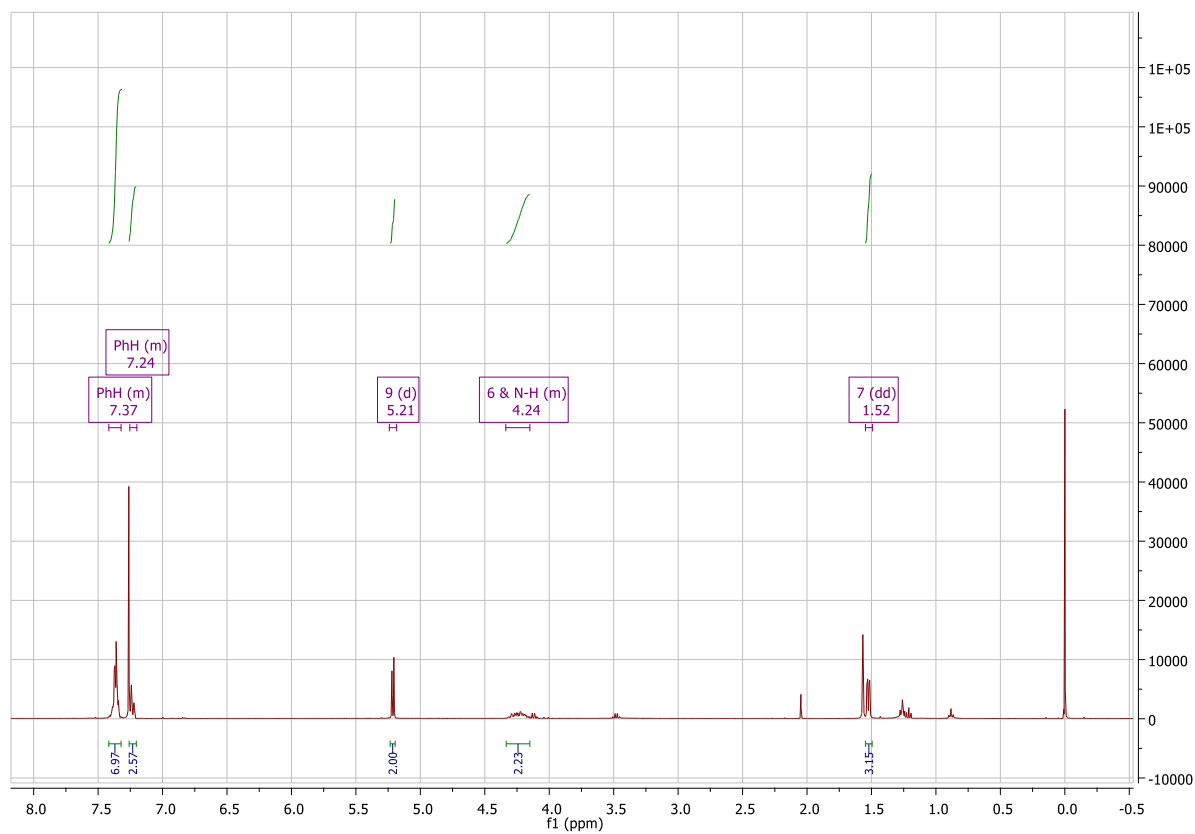


^{31}P NMR

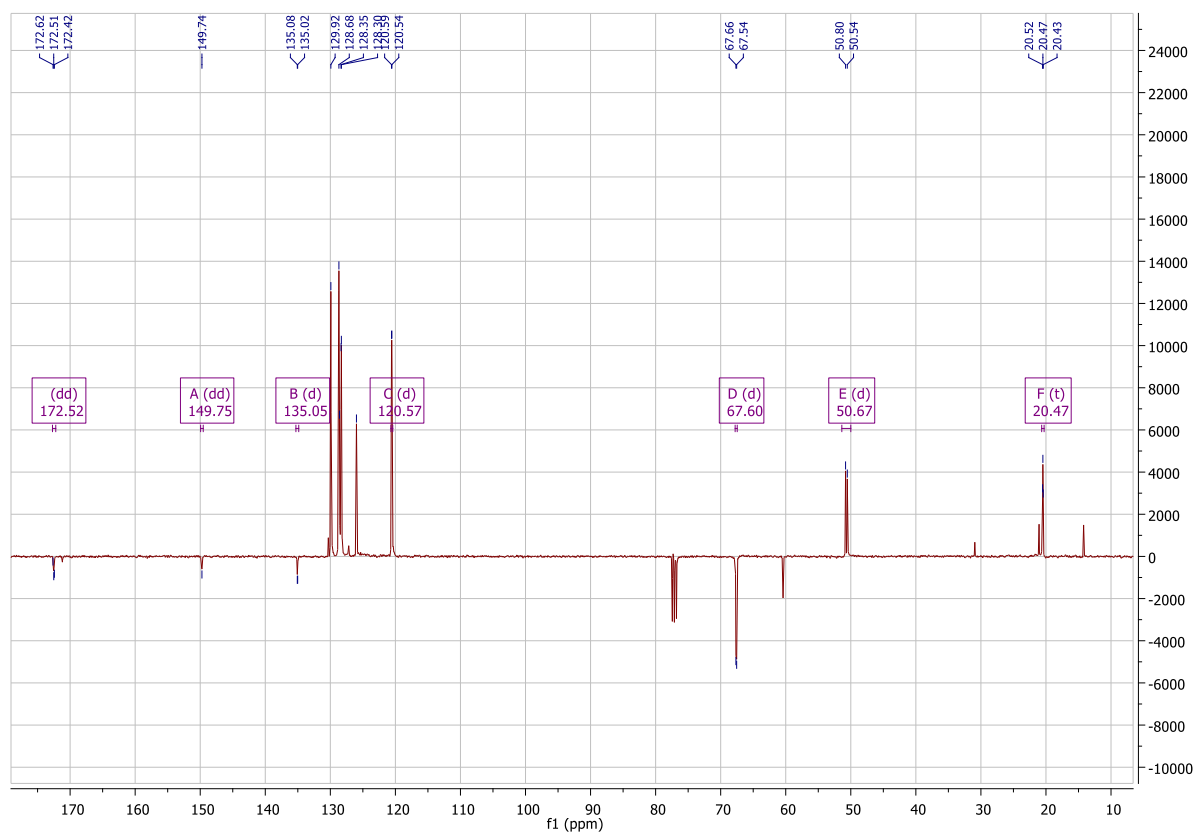


Benzyl (chloro(phenoxy)phosphoryl)-L-alaninate, 9b:

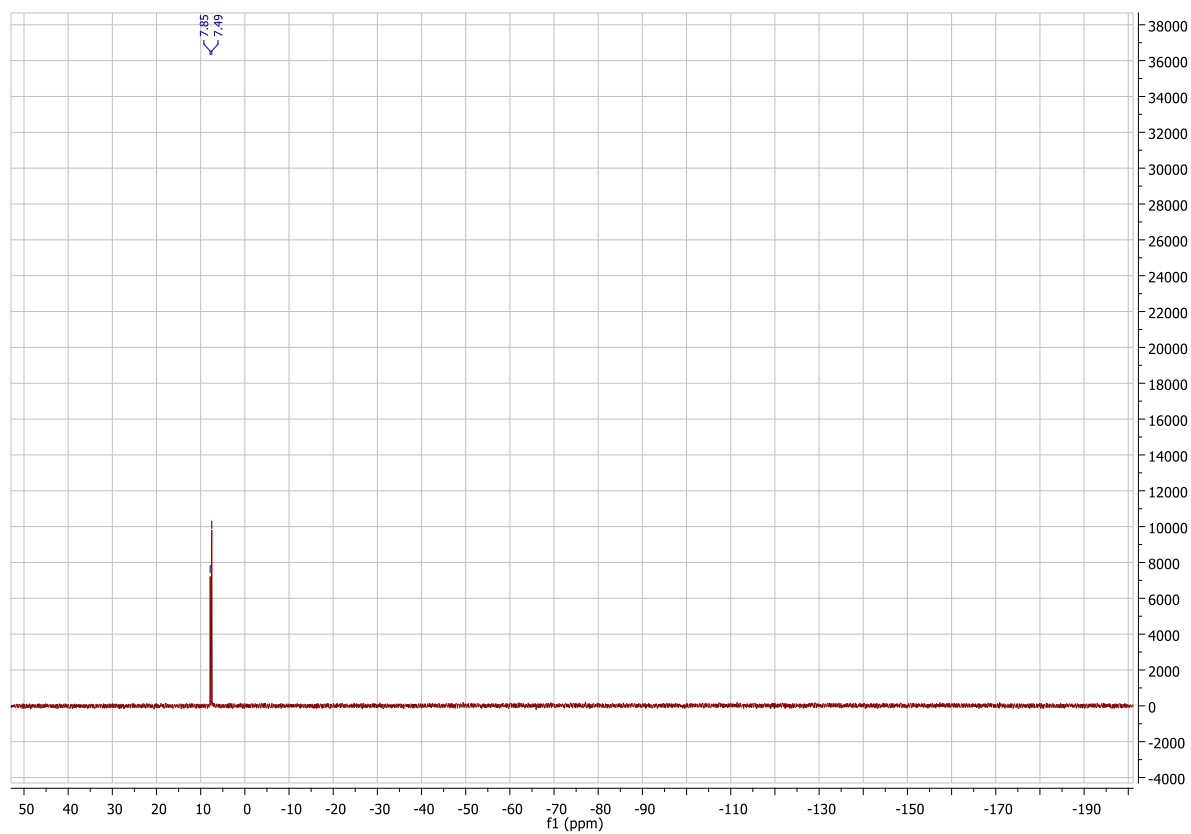
^1H NMR



¹³C NMR

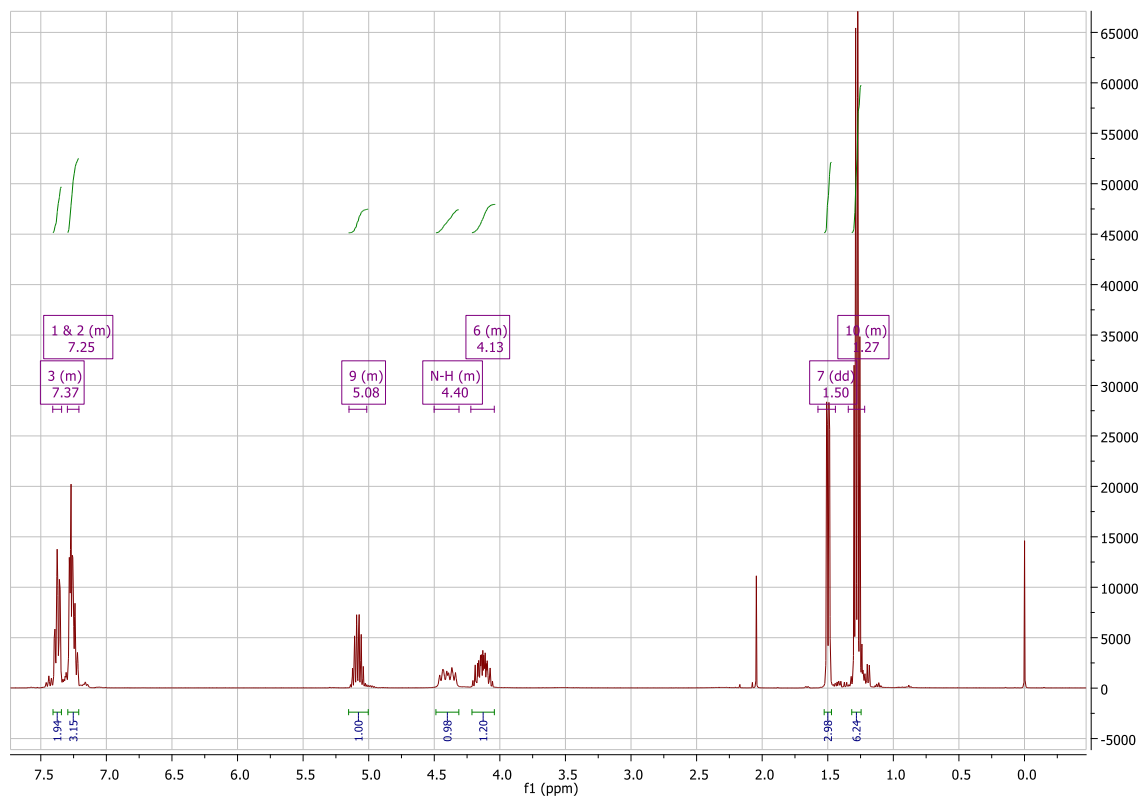


³¹P NMR

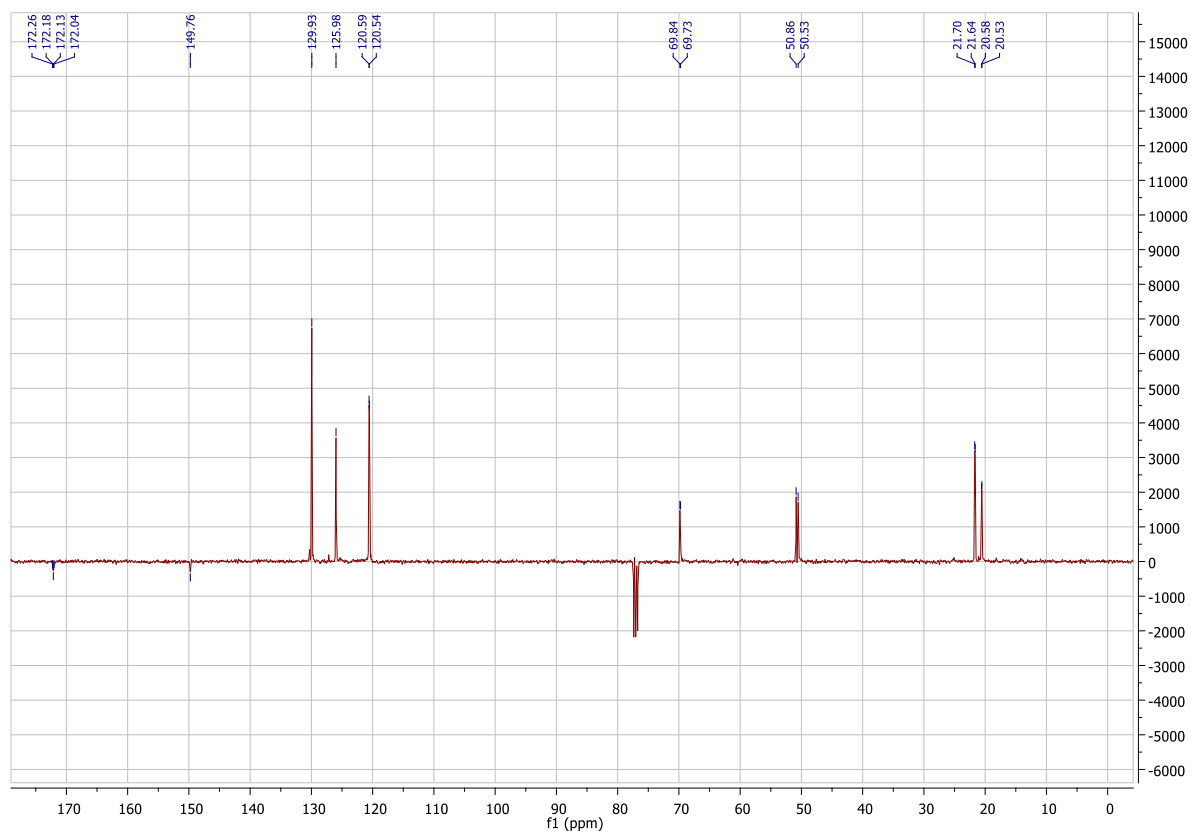


Isopropyl (chloro(phenoxy)phosphoryl)-L-alaninate, 9c:

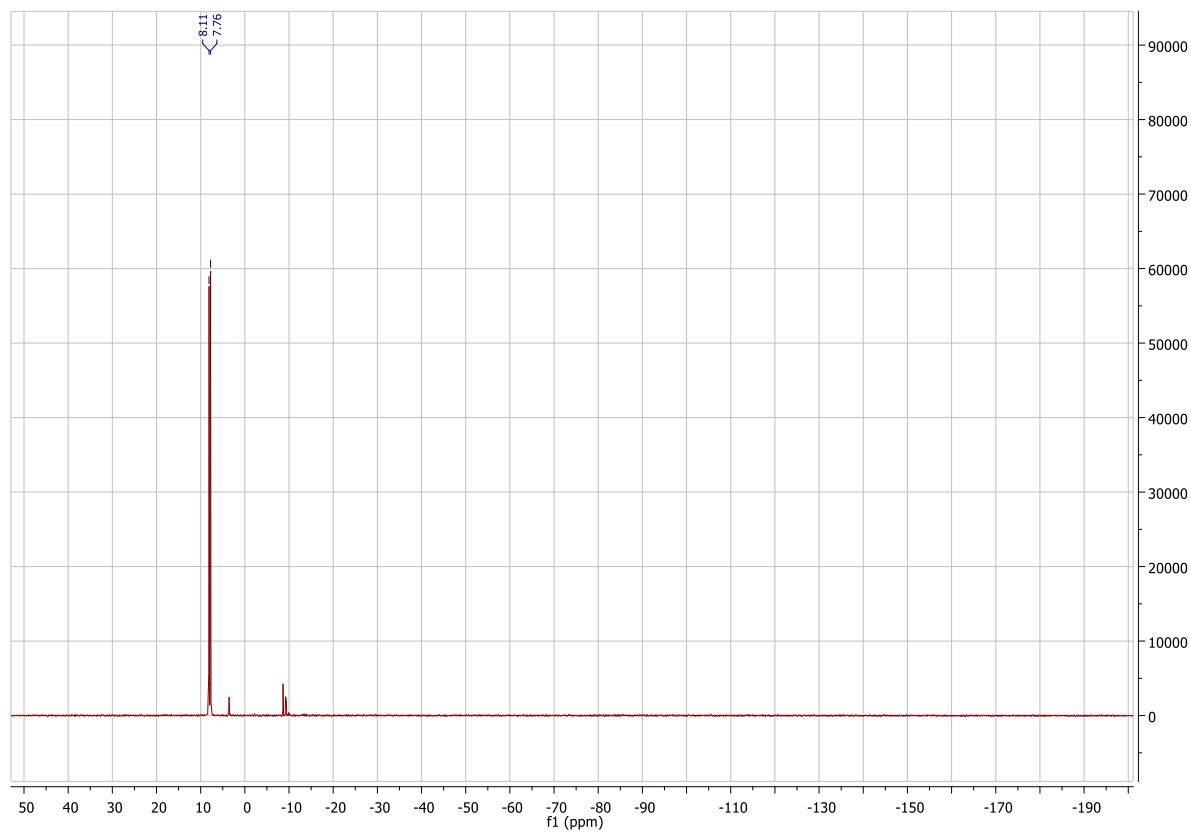
¹H NMR



¹³C NMR

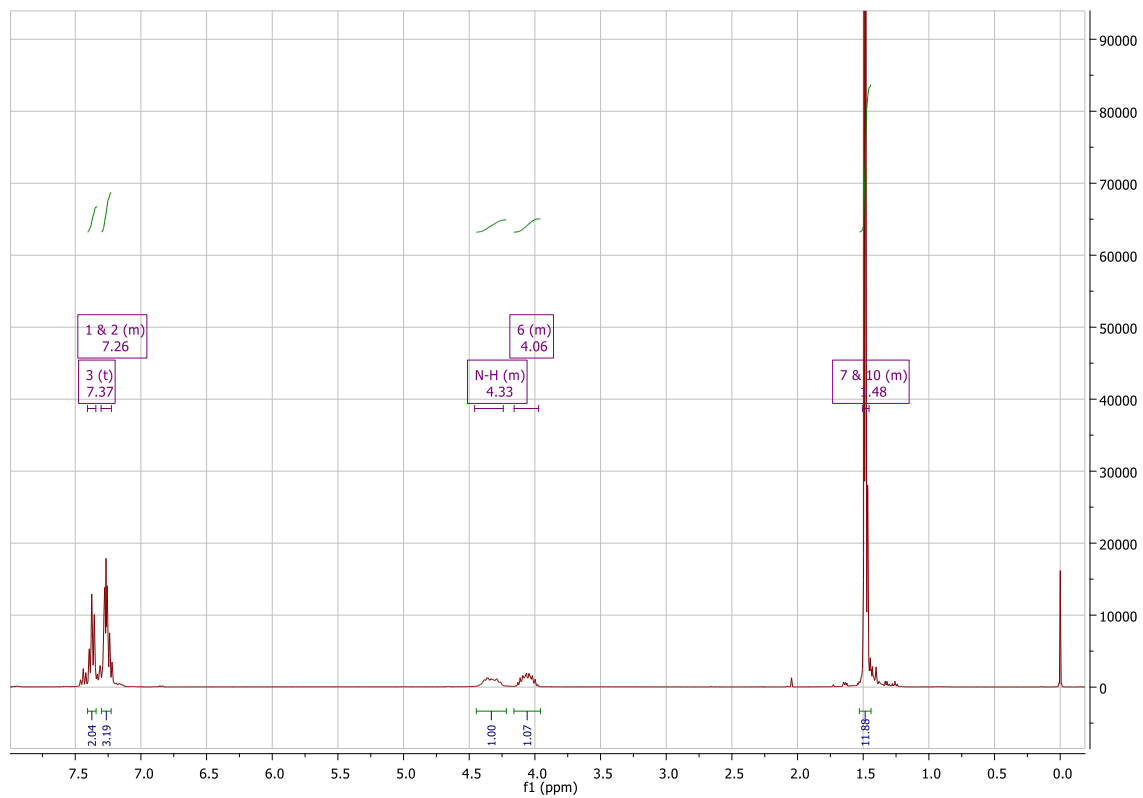


^{31}P NMR

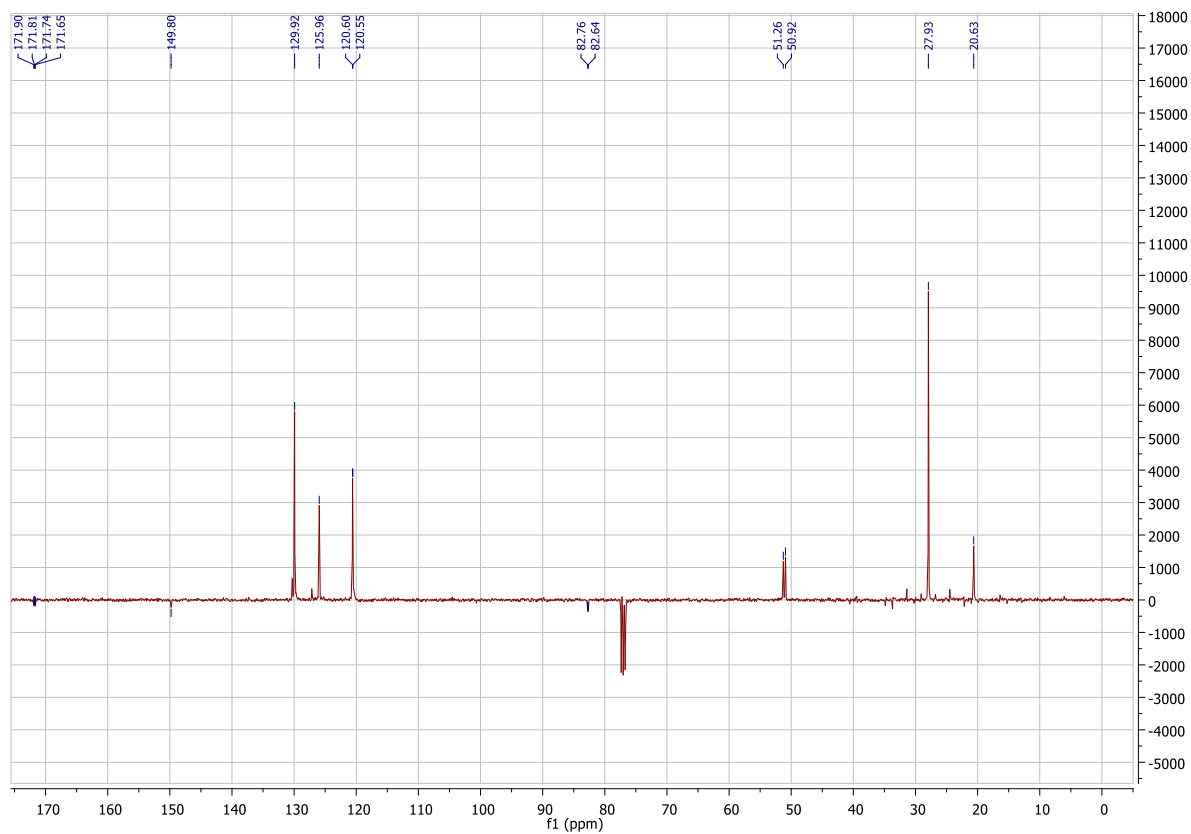


Tert-butyl (chloro(phenoxy)phosphoryl)-L-alaninate, 9d:

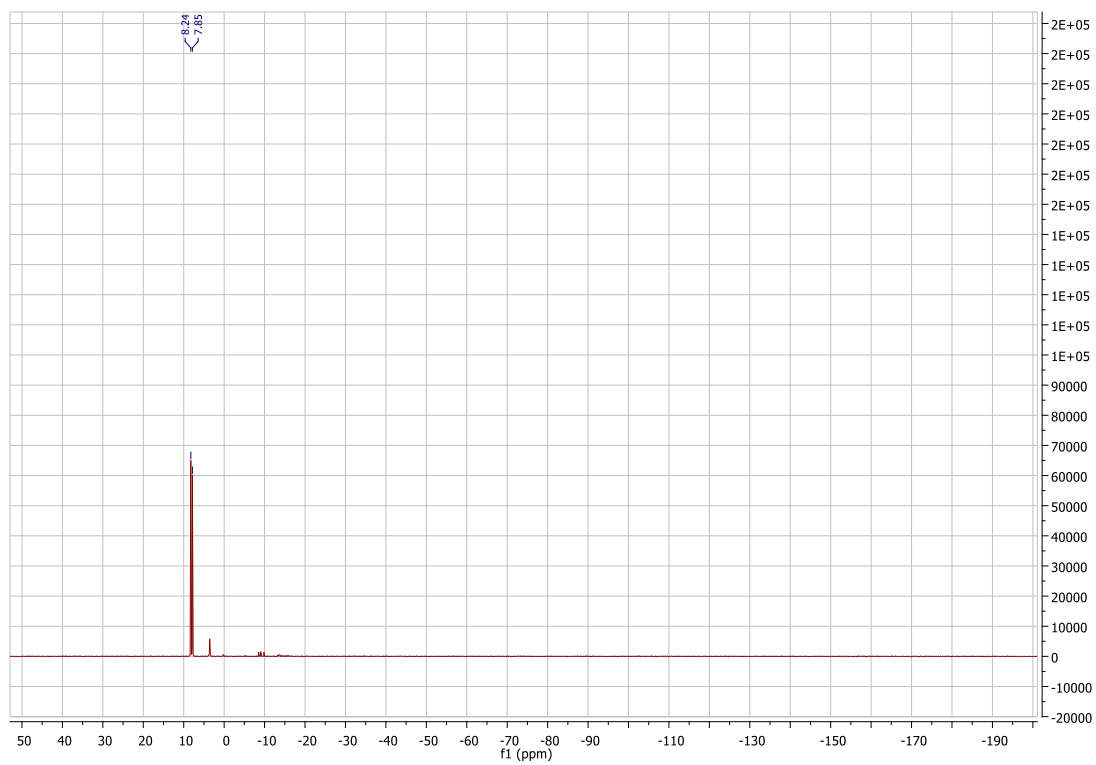
^1H NMR



^{13}C NMR

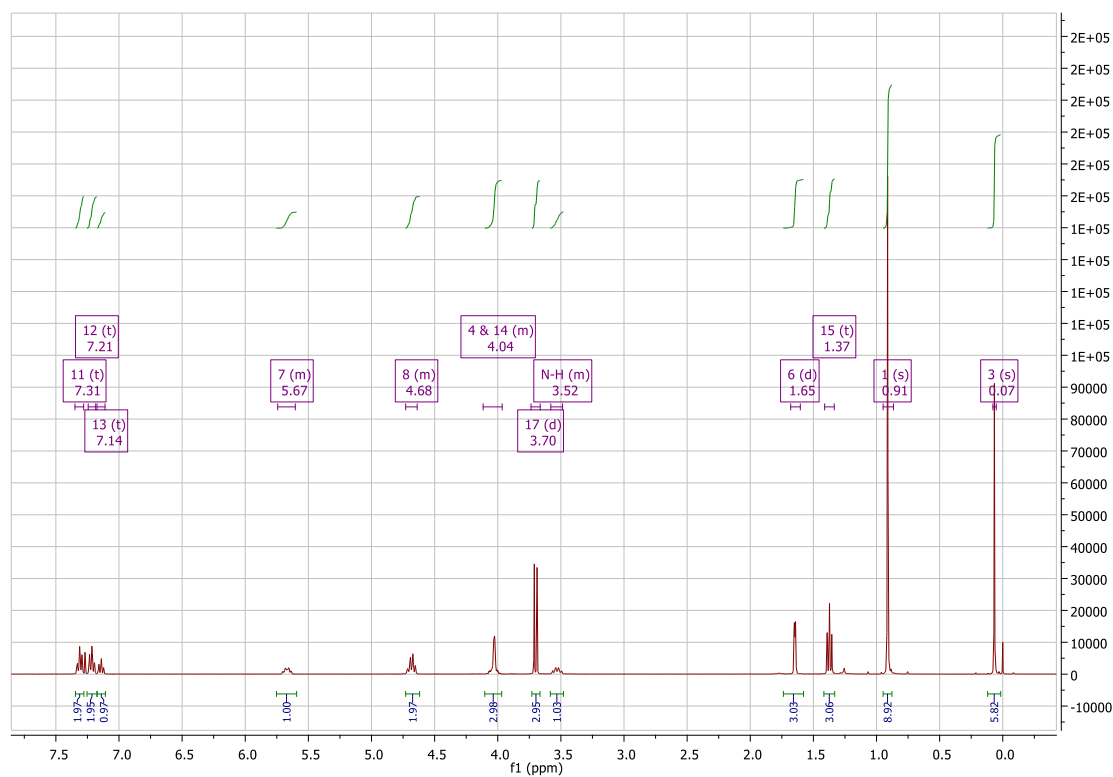


^{31}P NMR

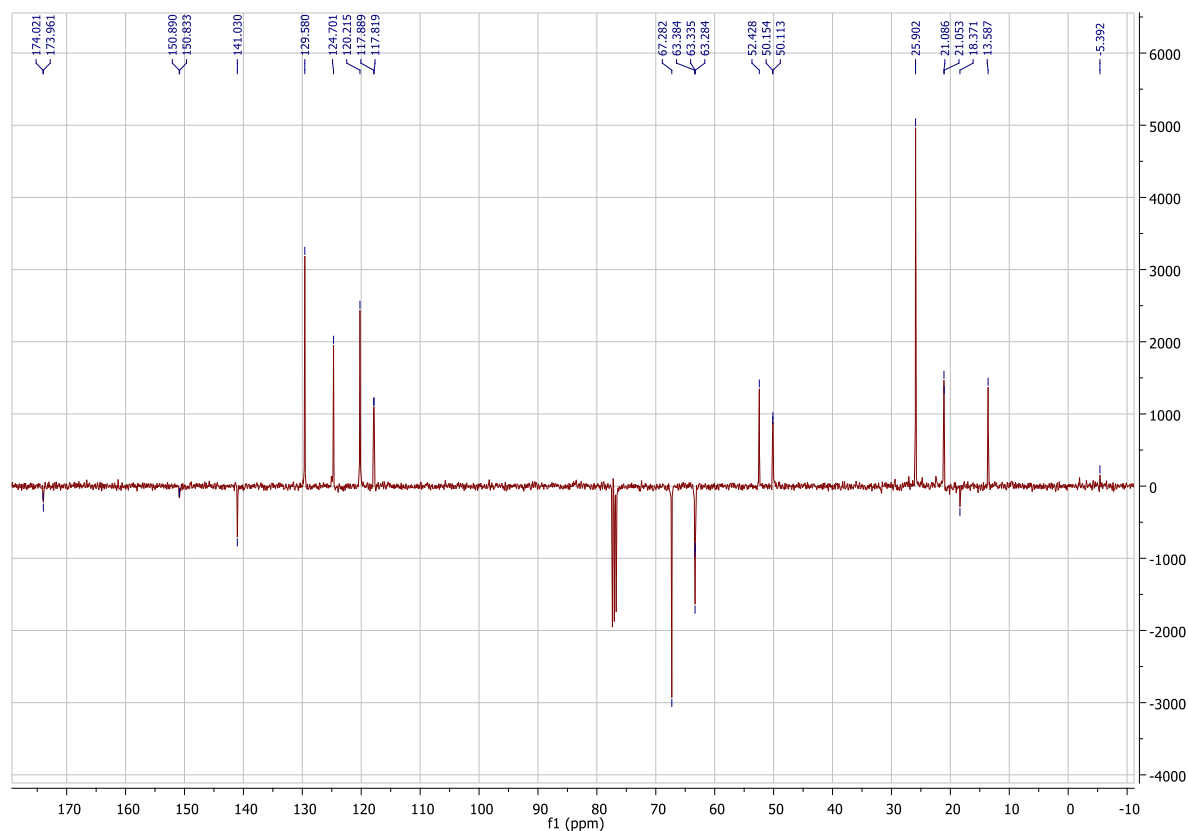


Methyl (((E)-4-((tert-butyldimethylsilyl)oxy)-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 5a:

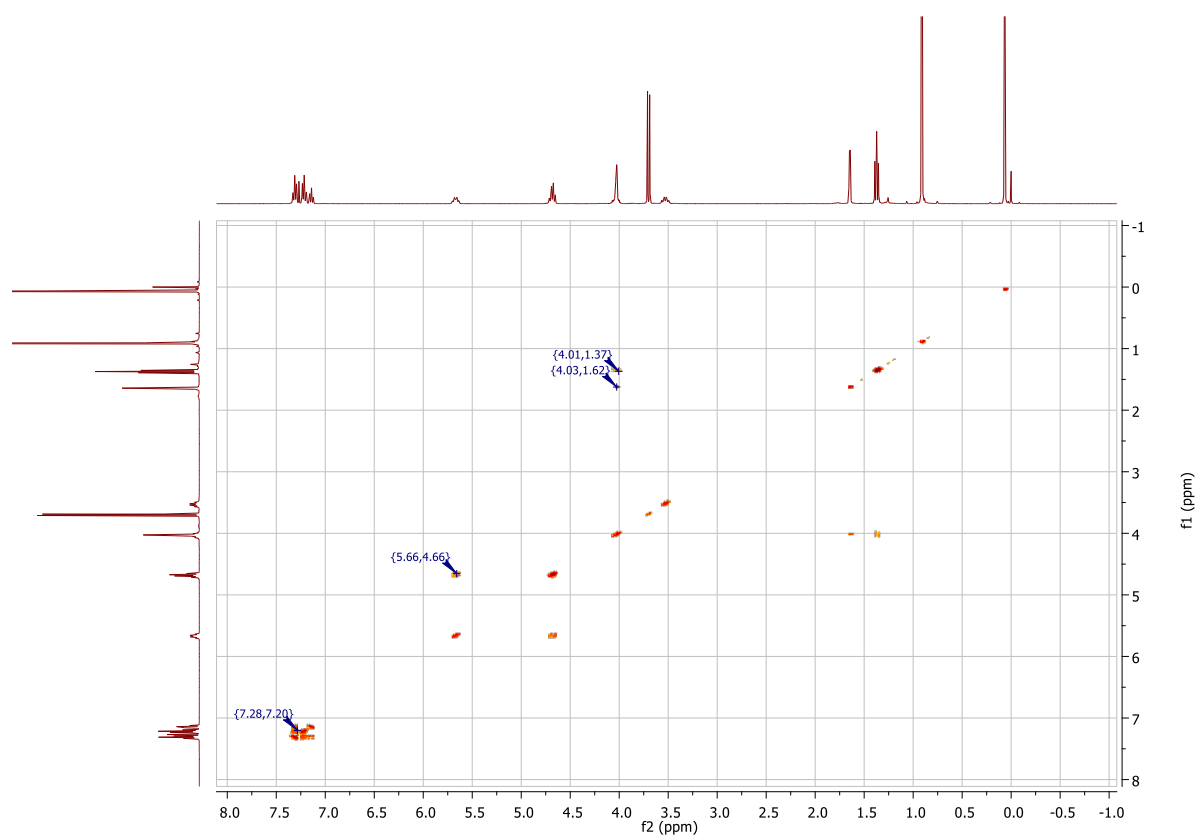
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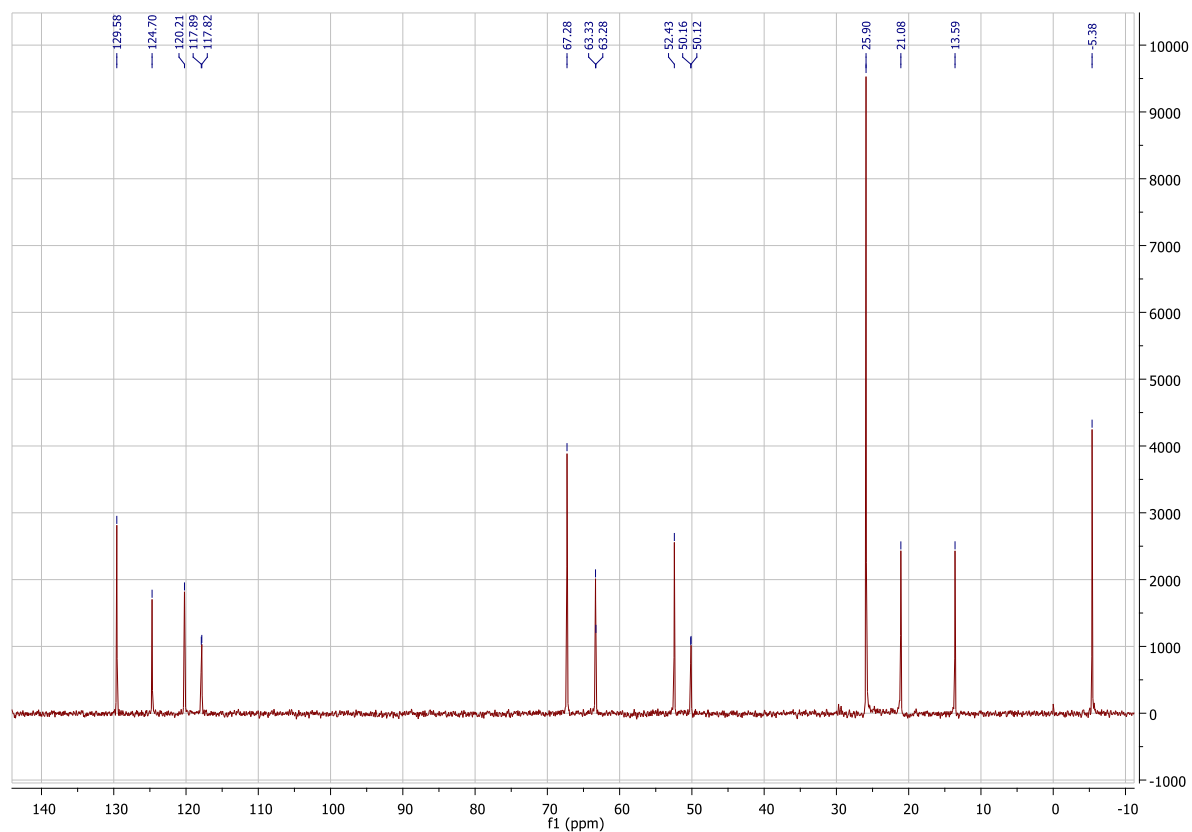
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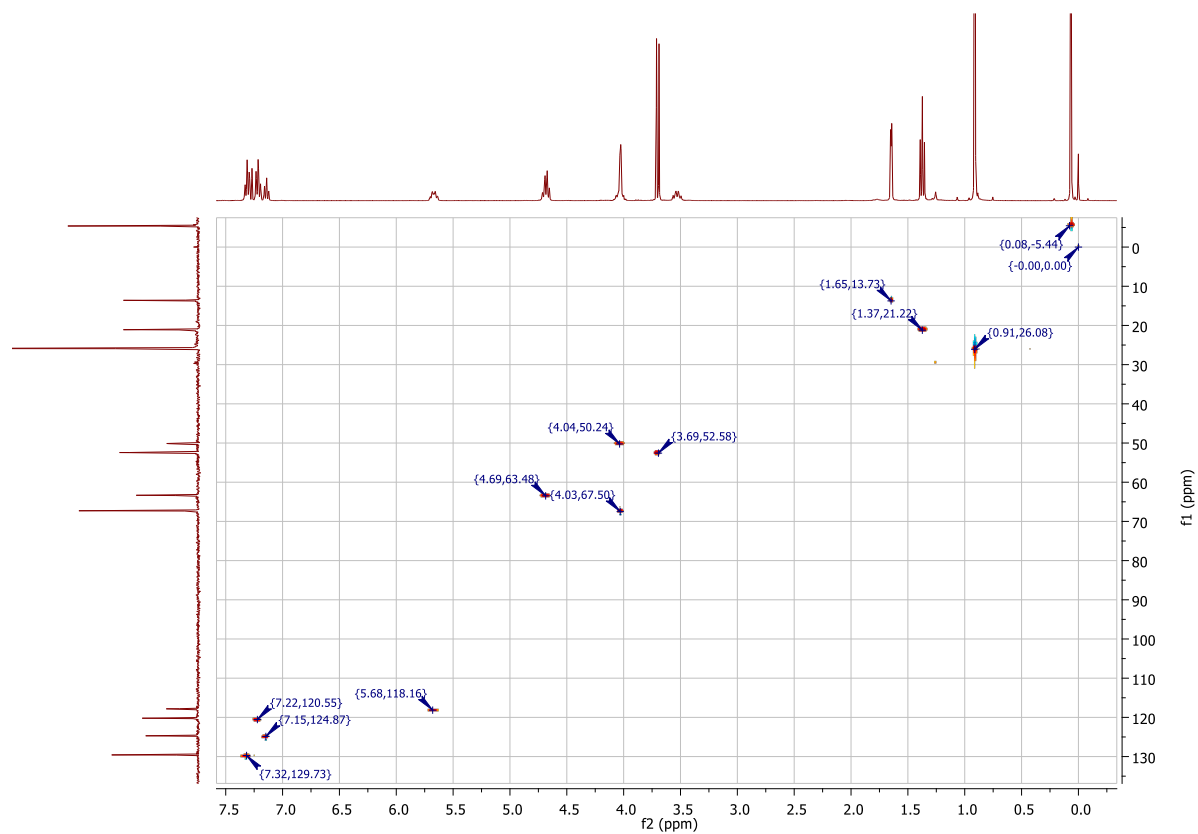
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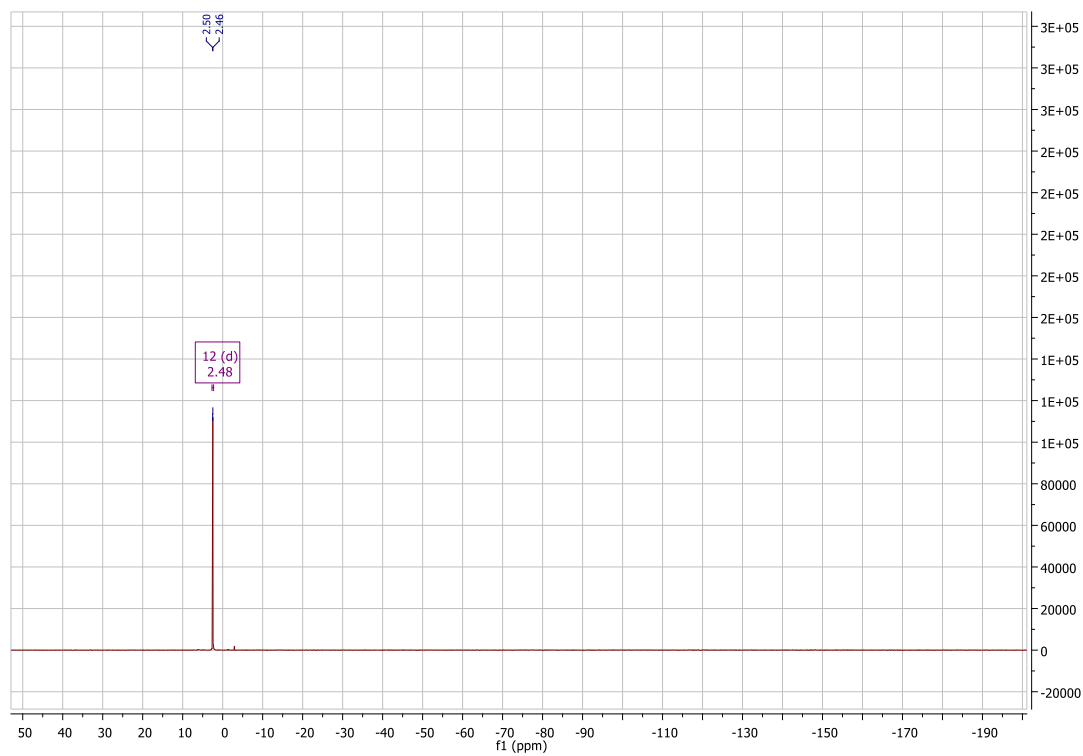
^{13}C DEPT-45



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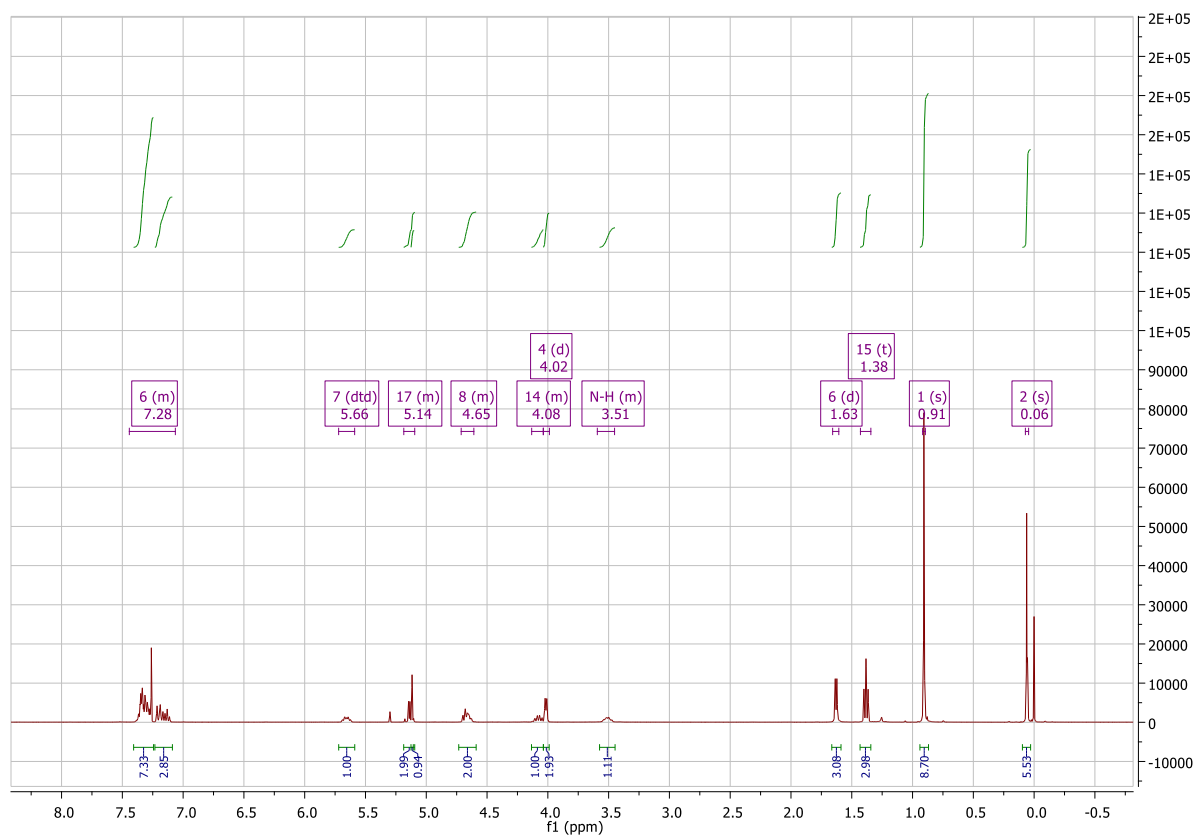


^{31}P NMR

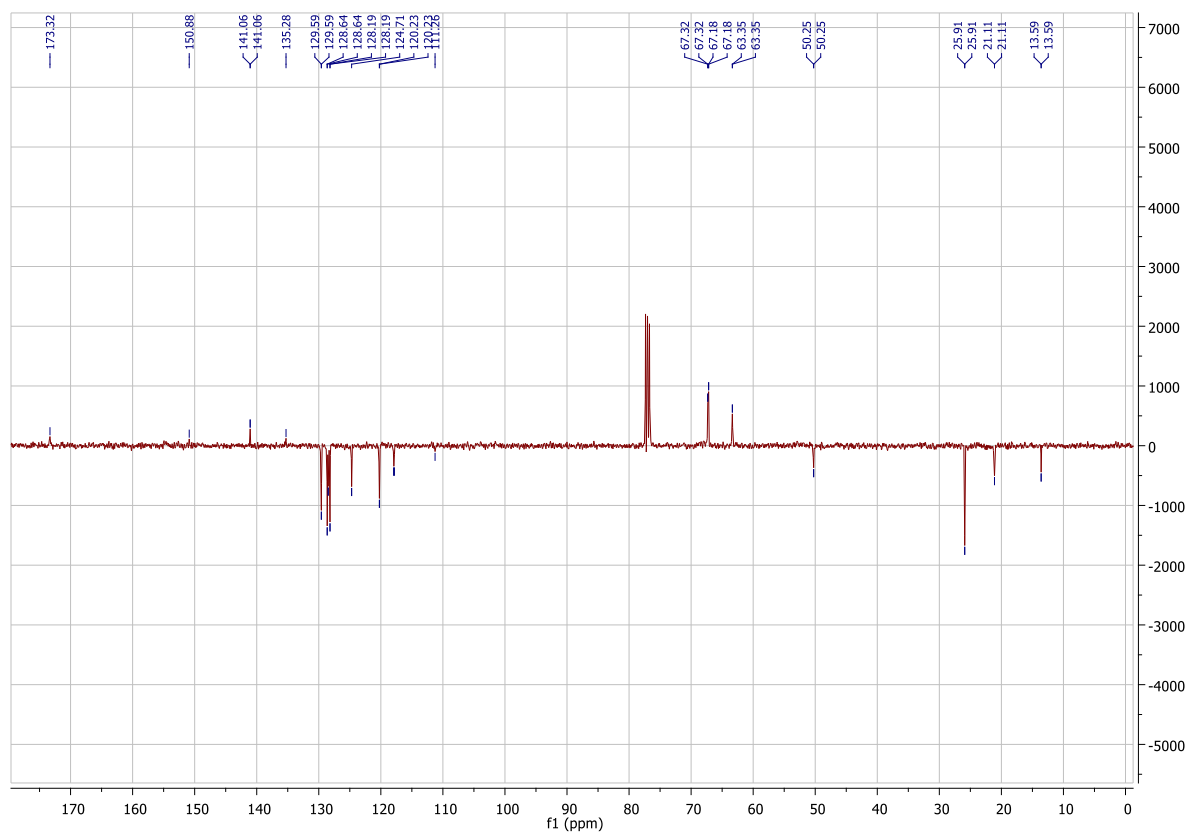


Benzyl (((E)-4-((tert-butyldimethylsilyloxy)-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 5b:

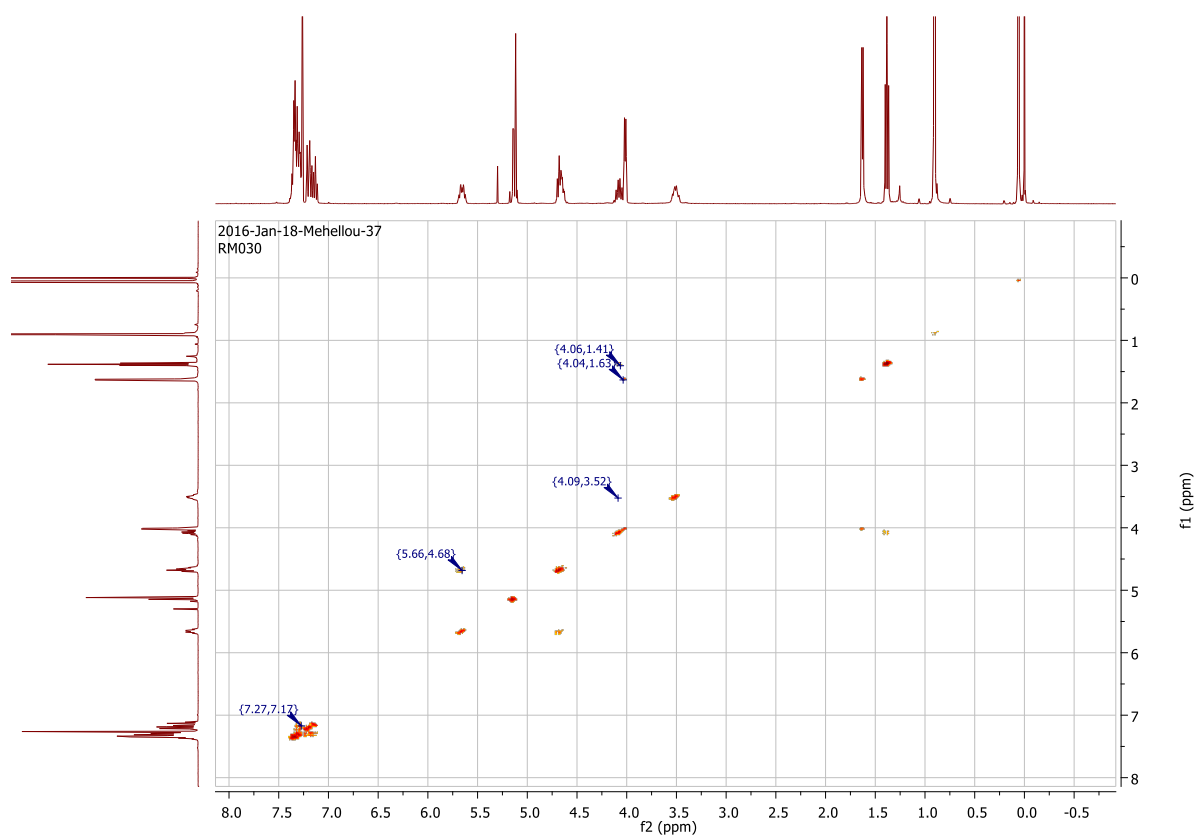
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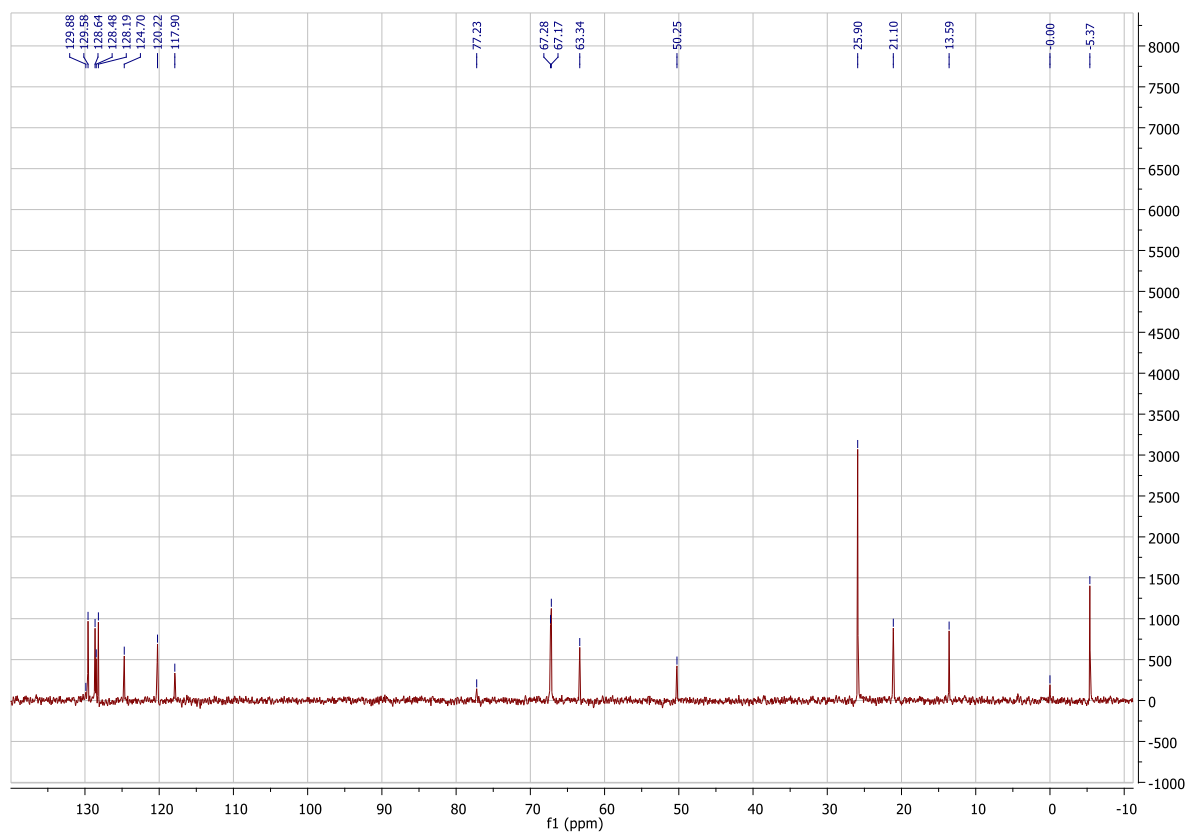
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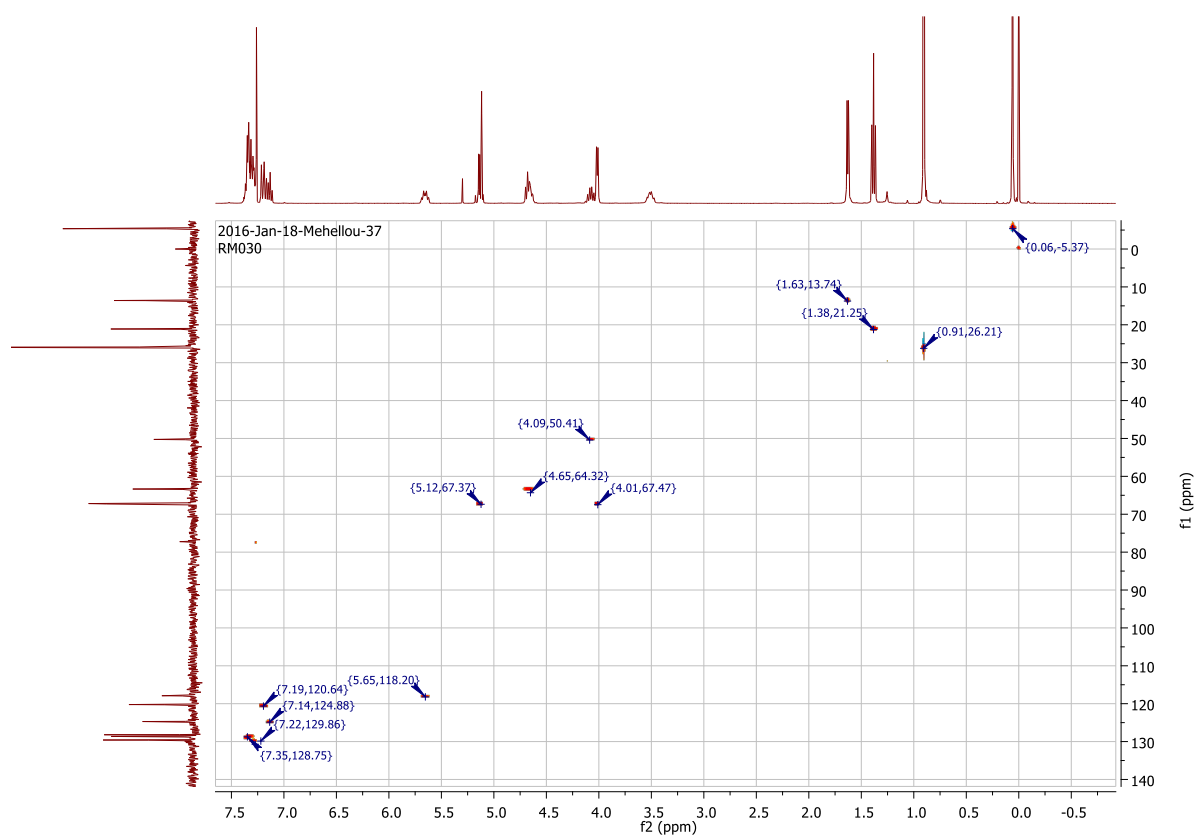
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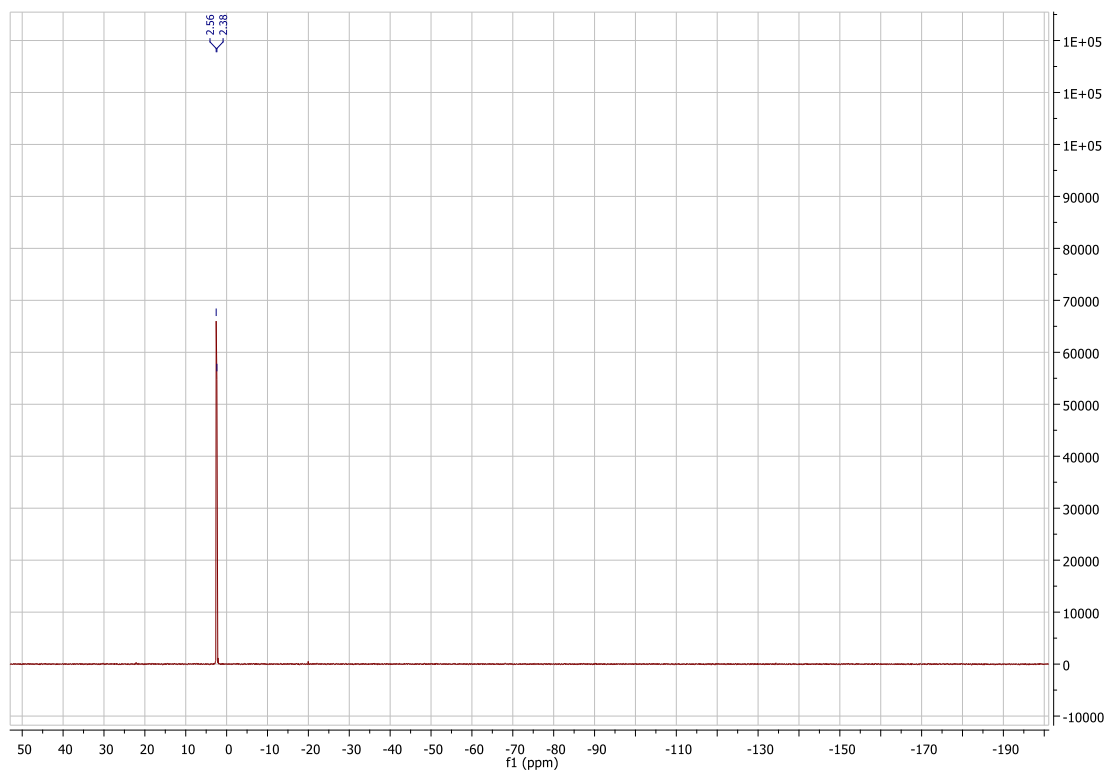
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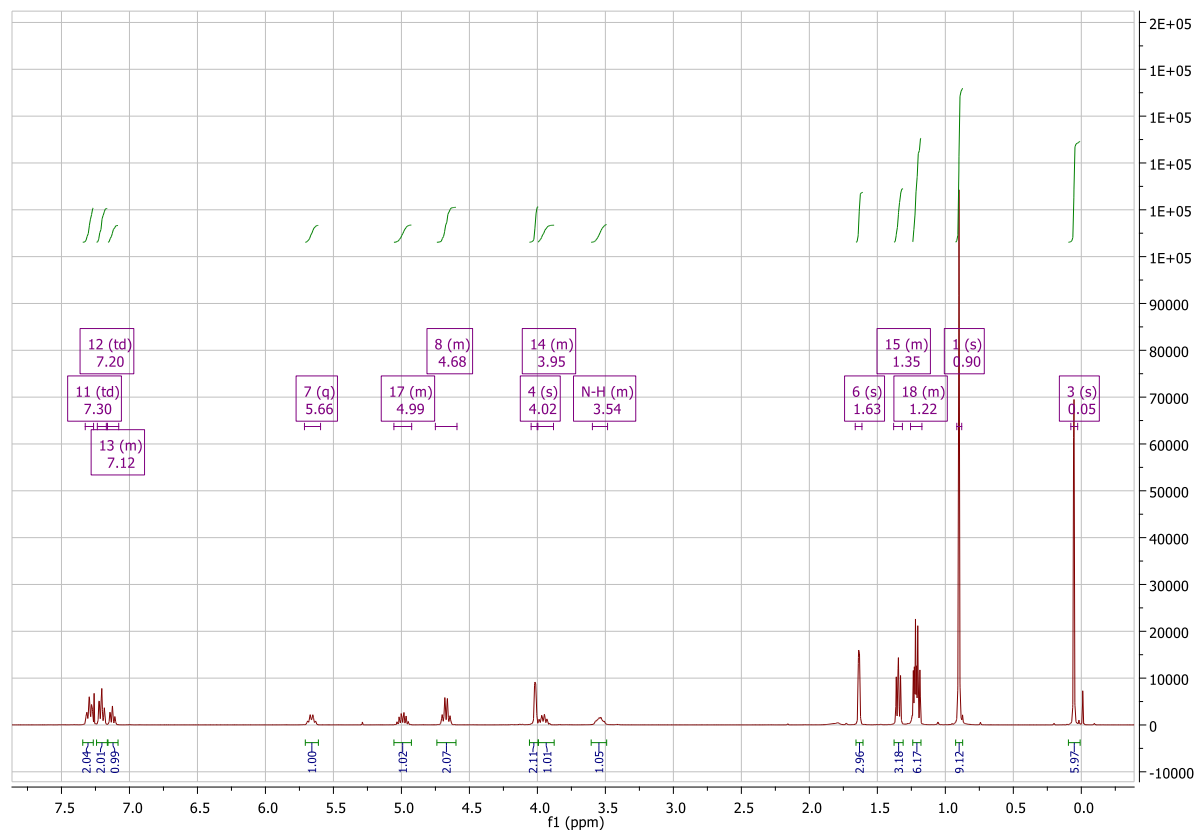


³¹P NMR

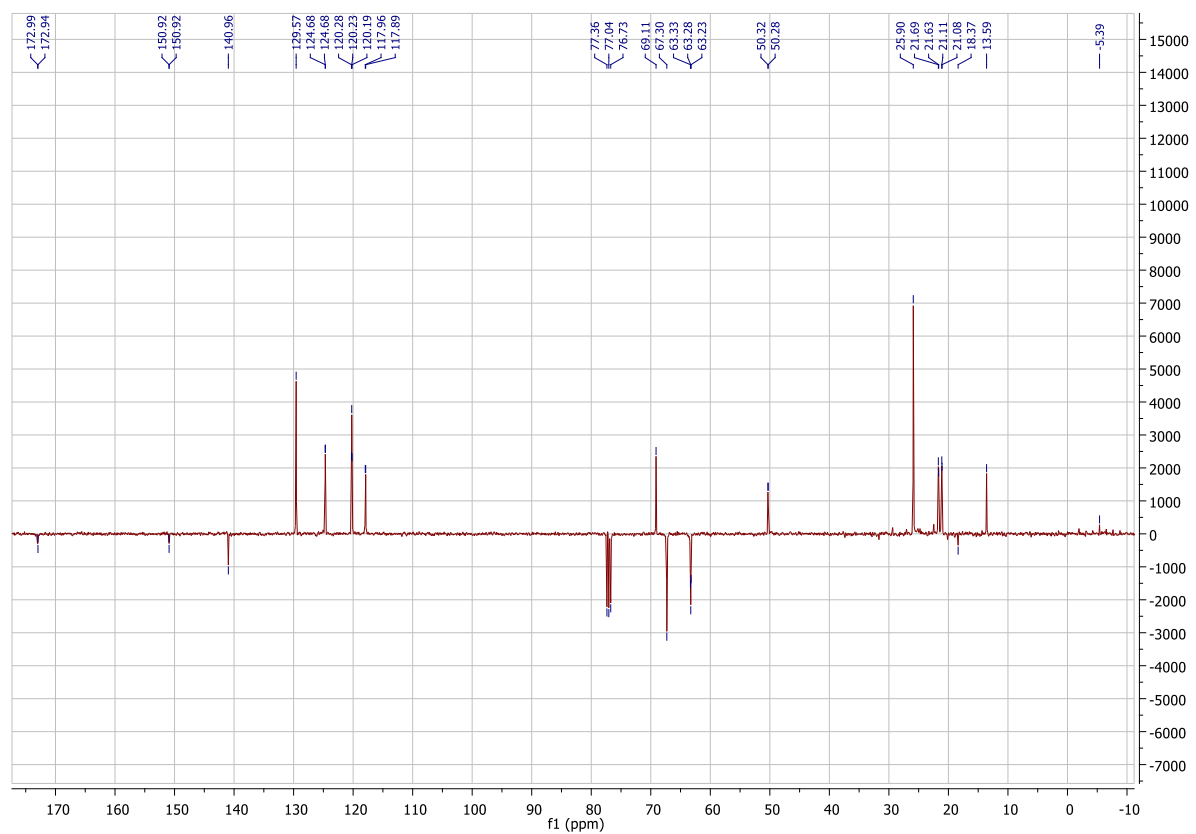


Isopropyl (((E)-4-((tert-butyldimethylsilyl)oxy)-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 5c:

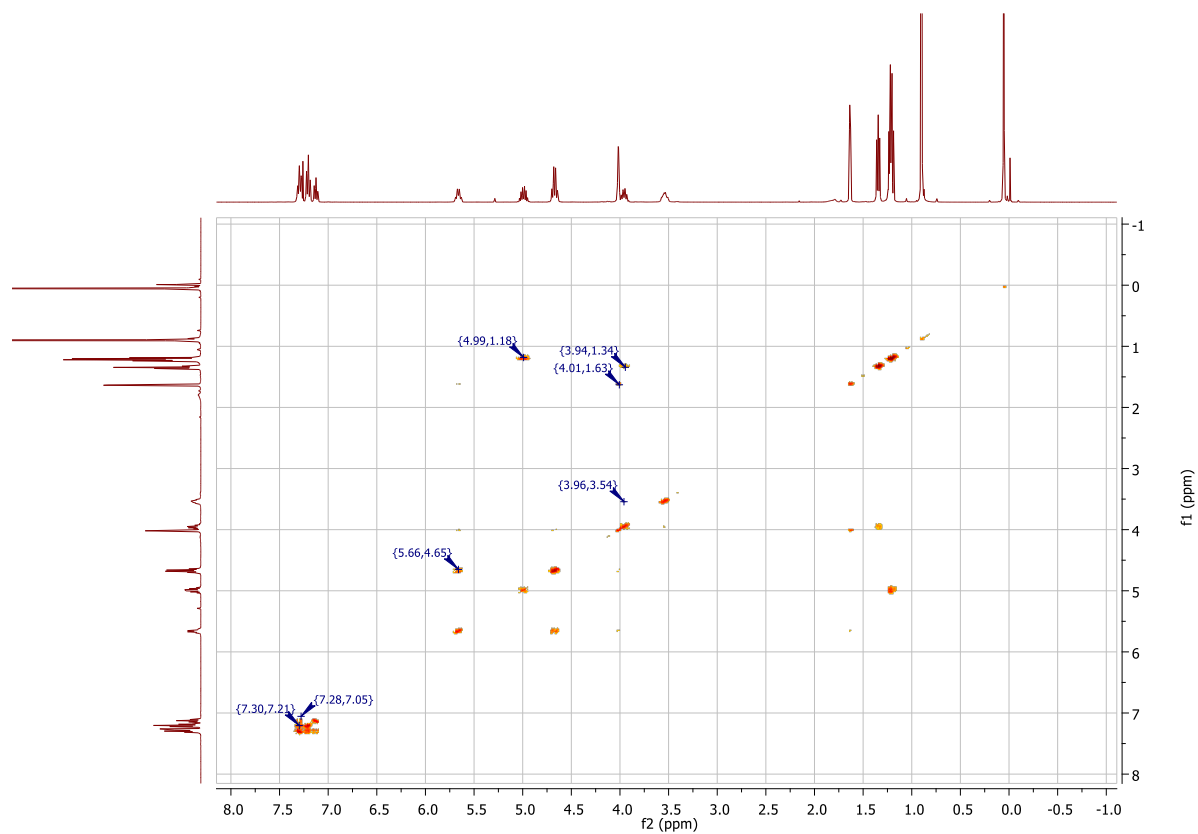
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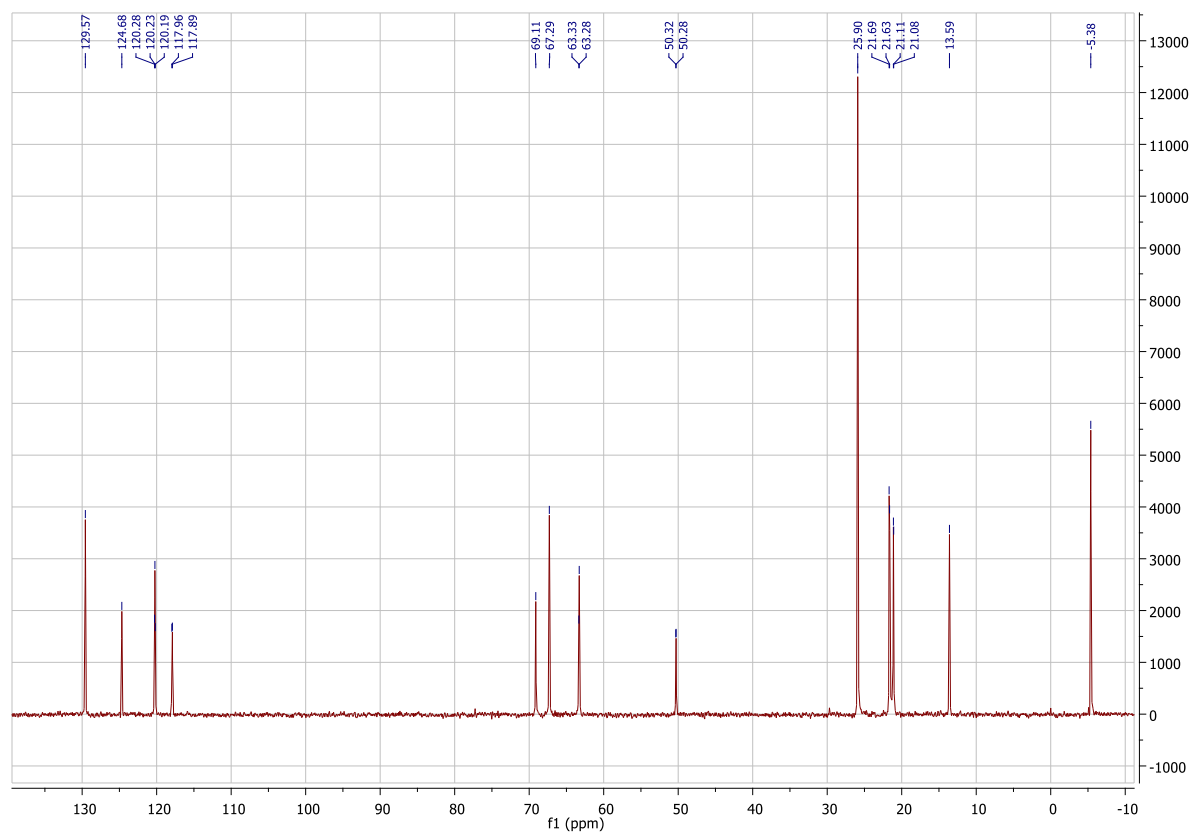
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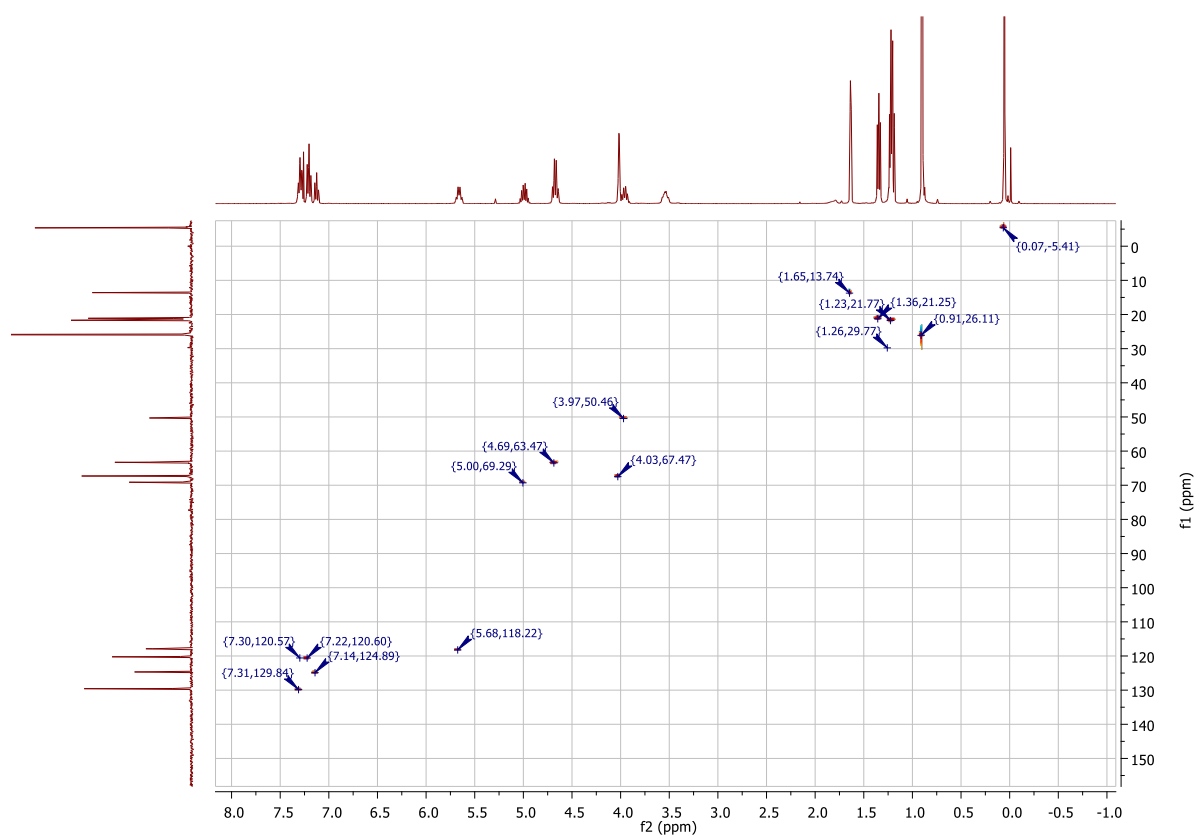
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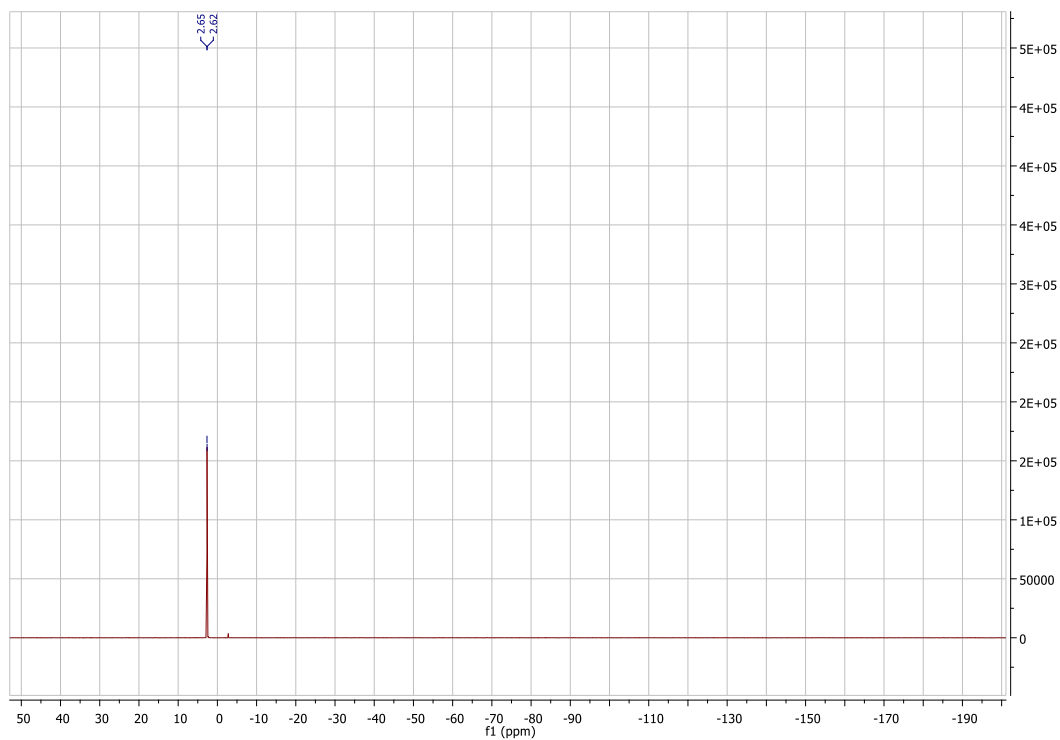
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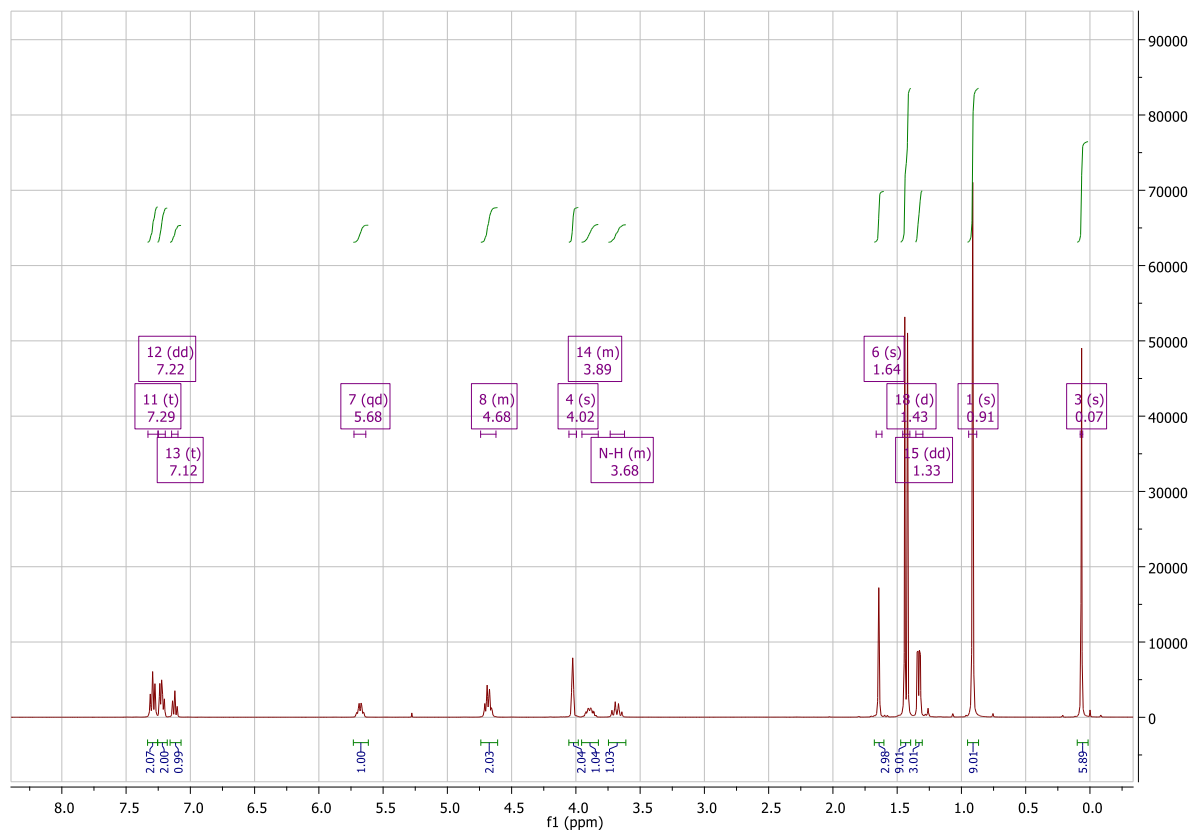
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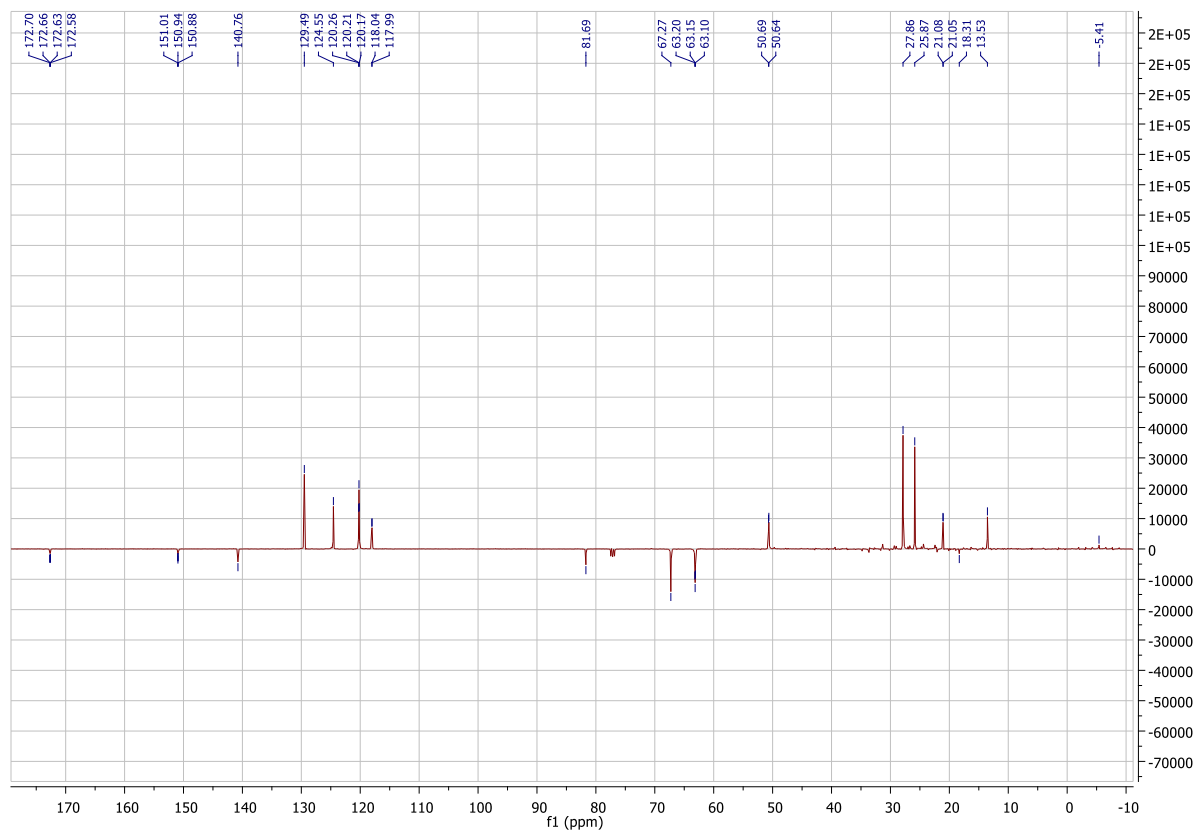
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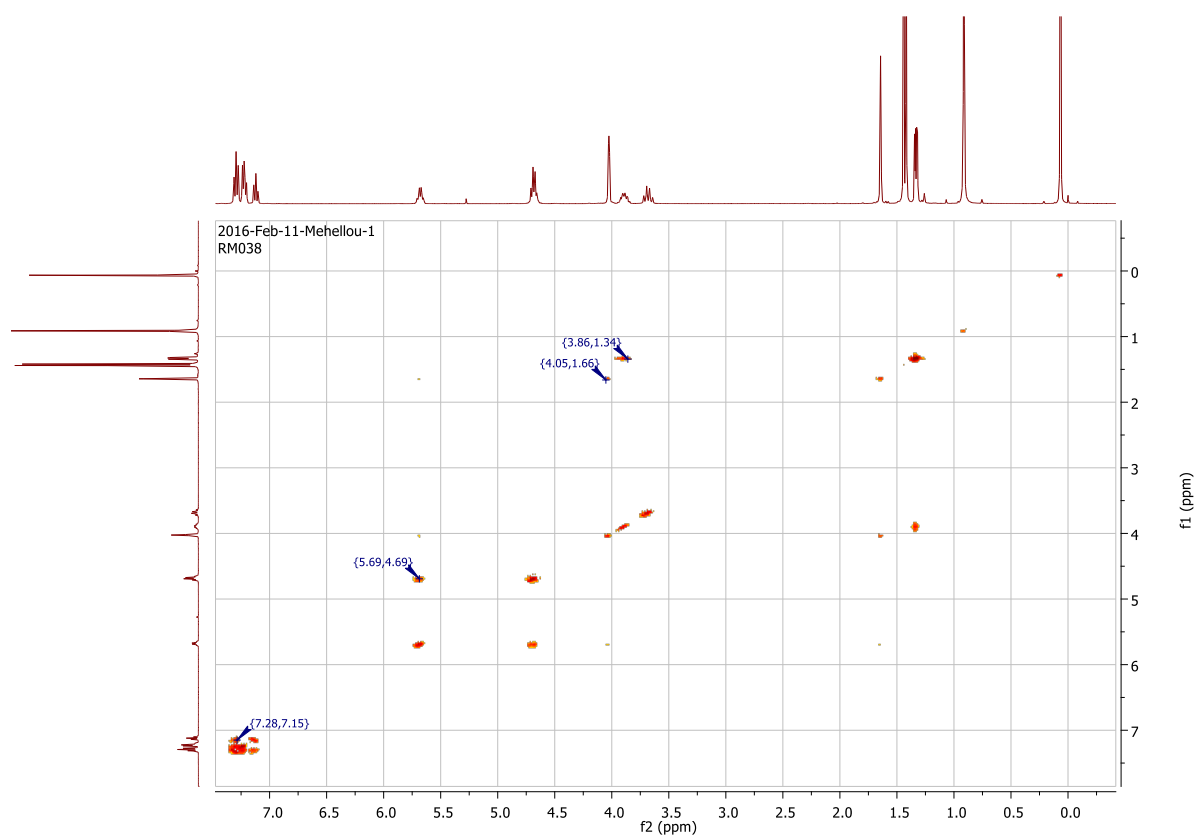
Tert-butyl (((E)-4-((tert-butyldimethylsilyl)oxy)-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 5d:
¹H NMR



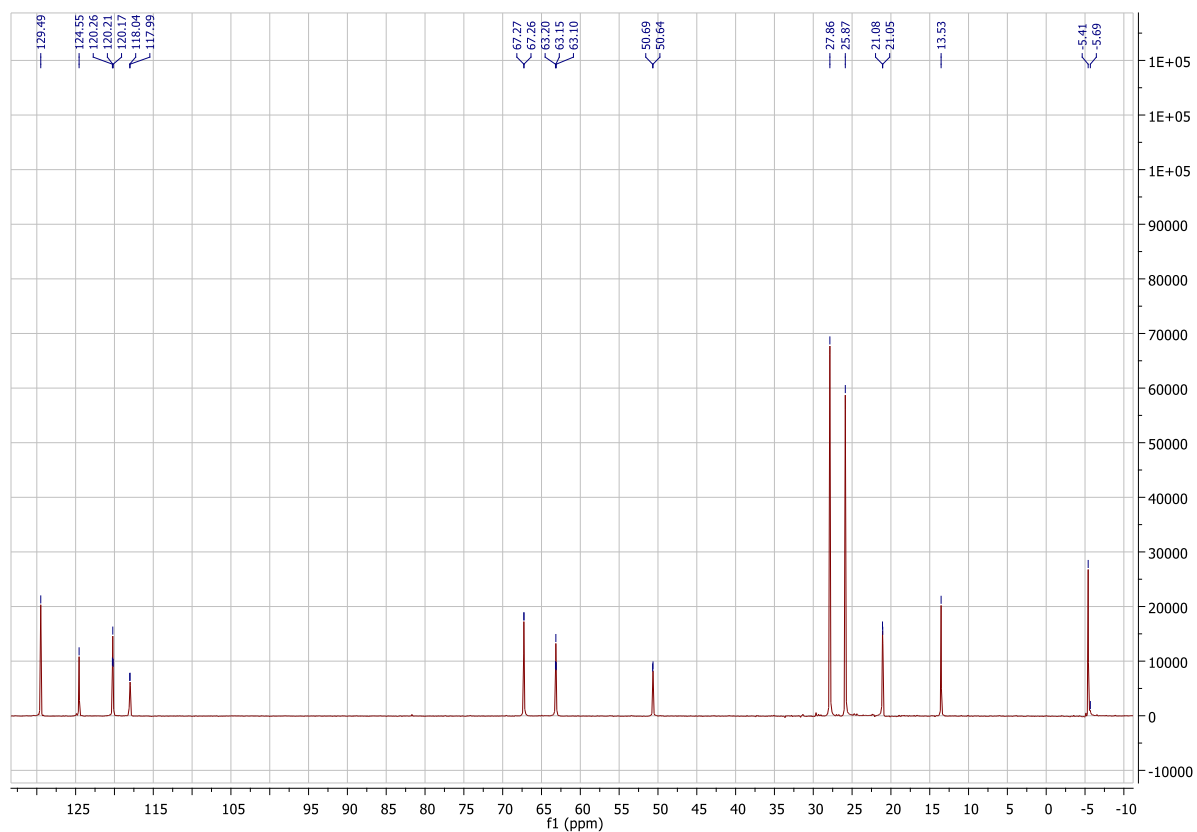
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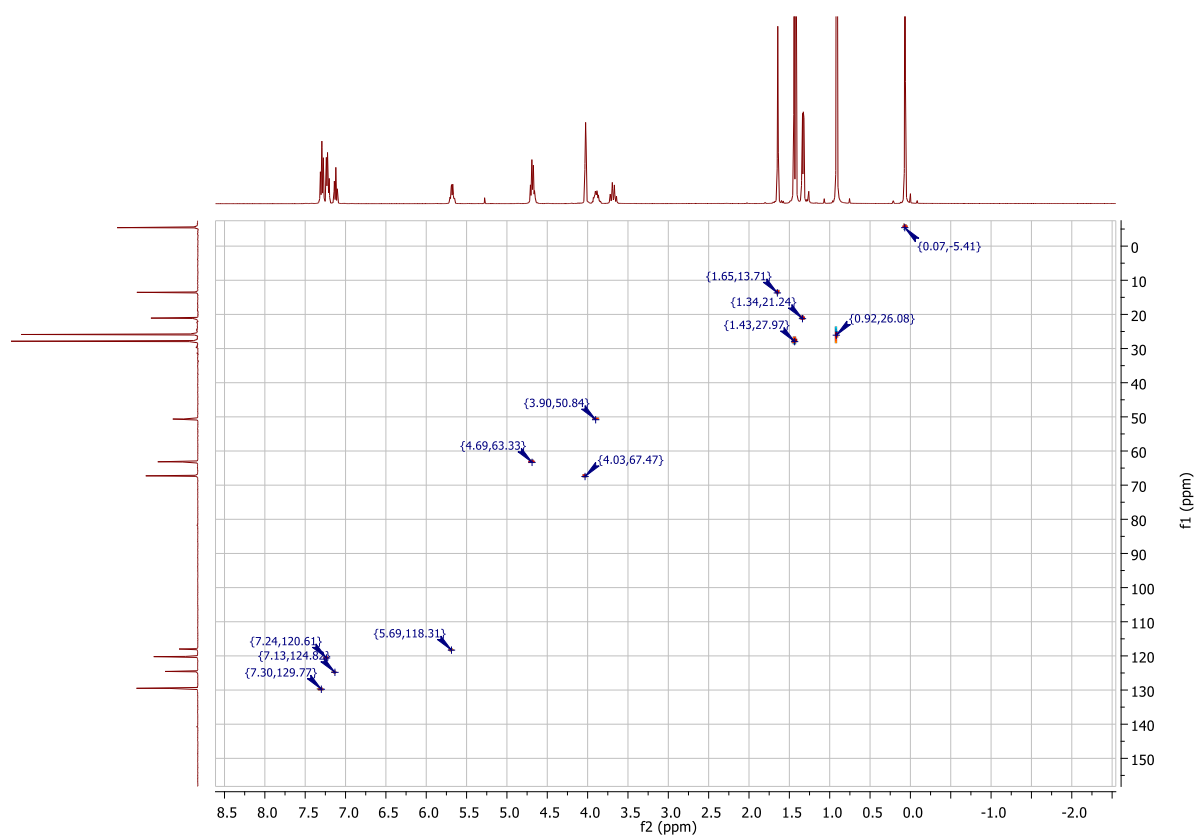
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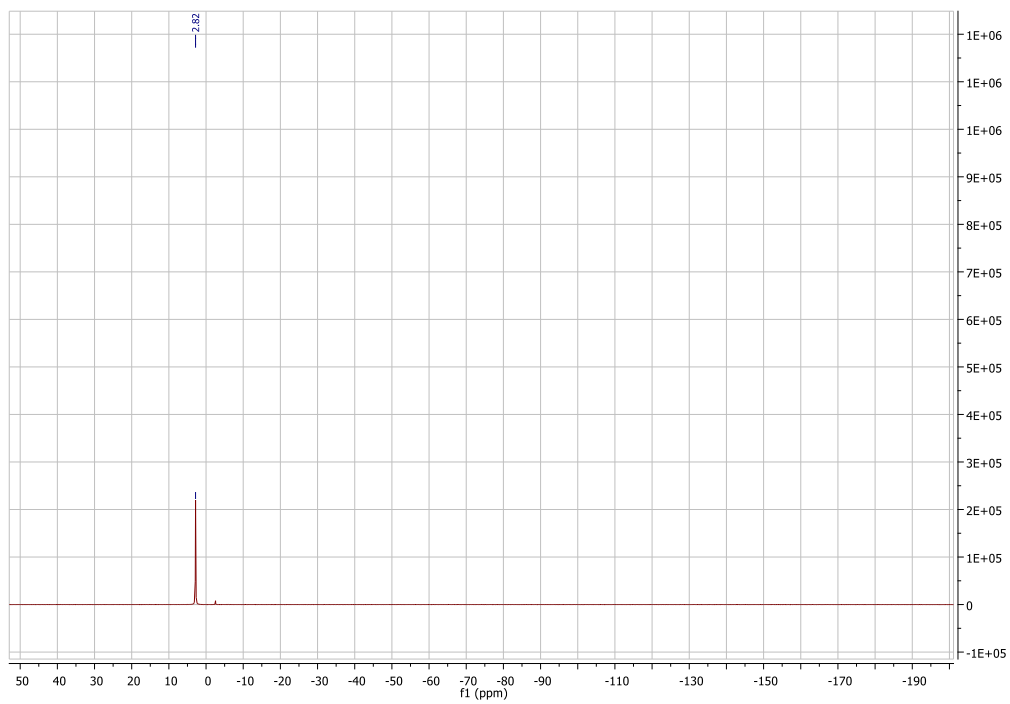
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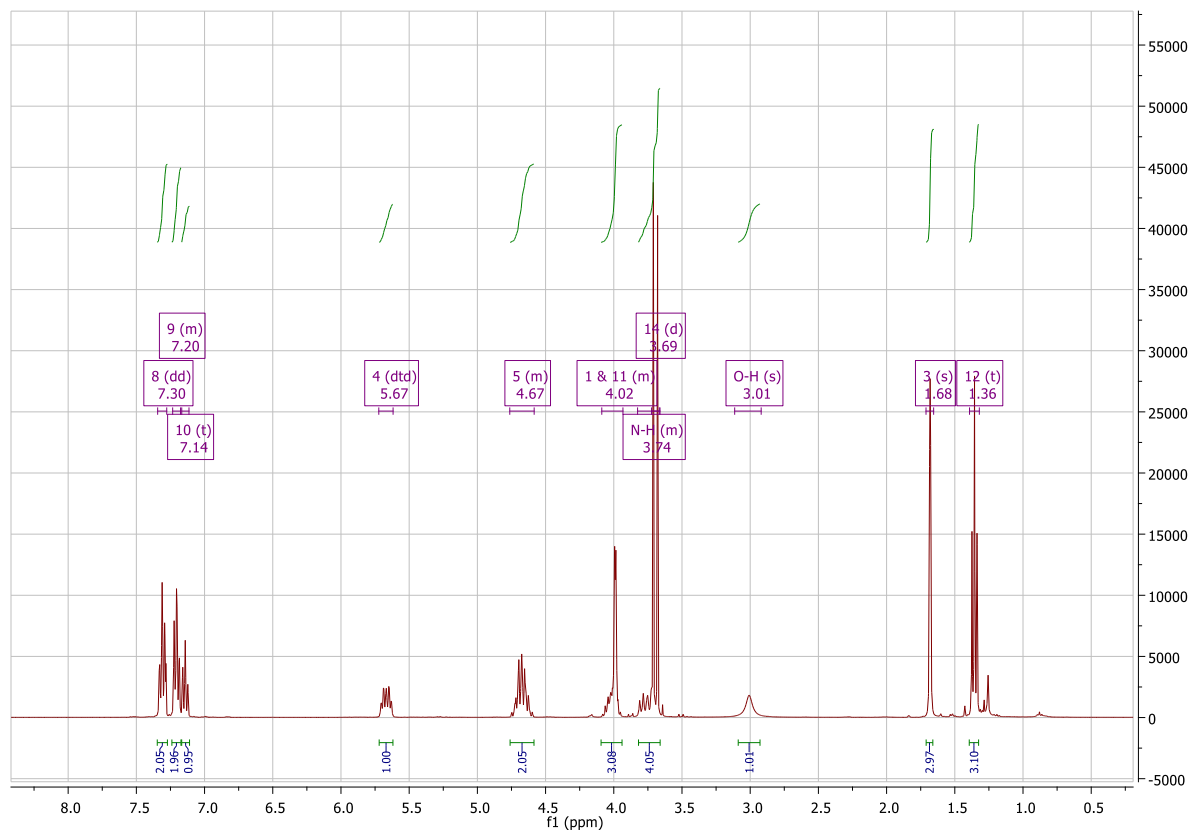
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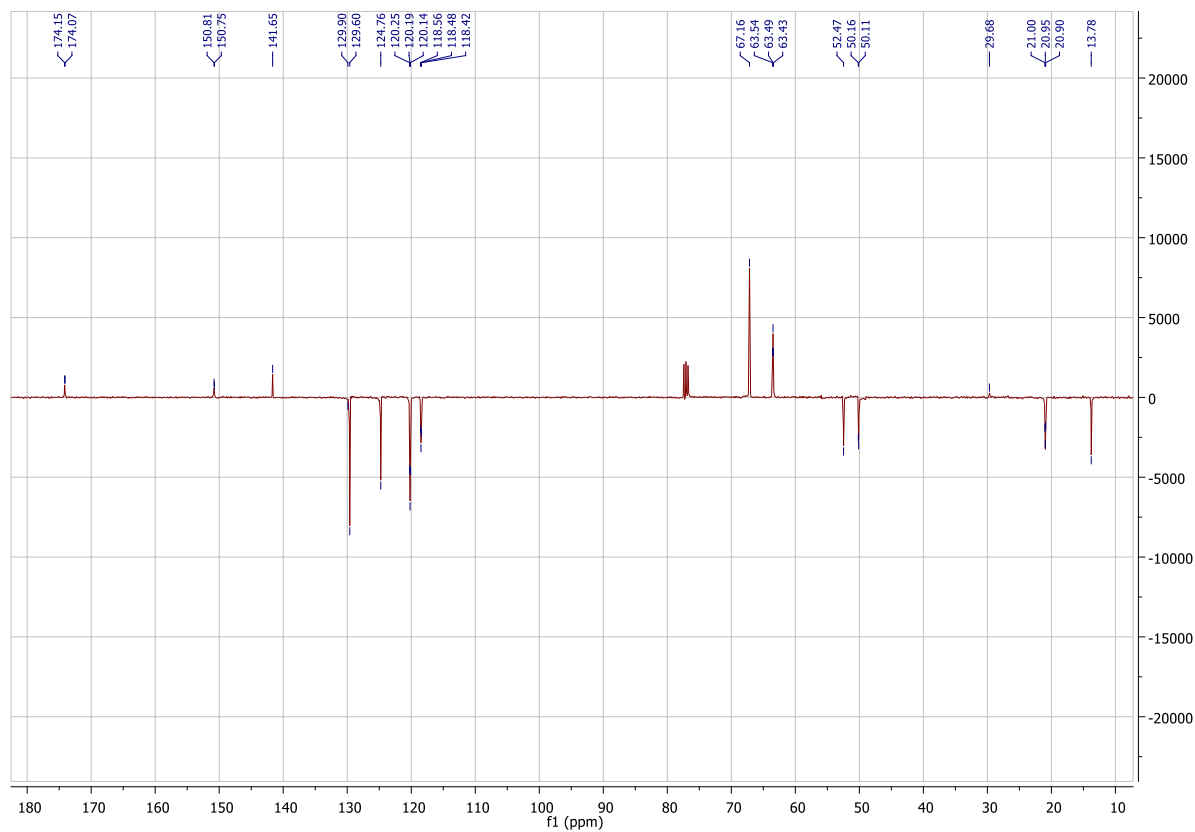
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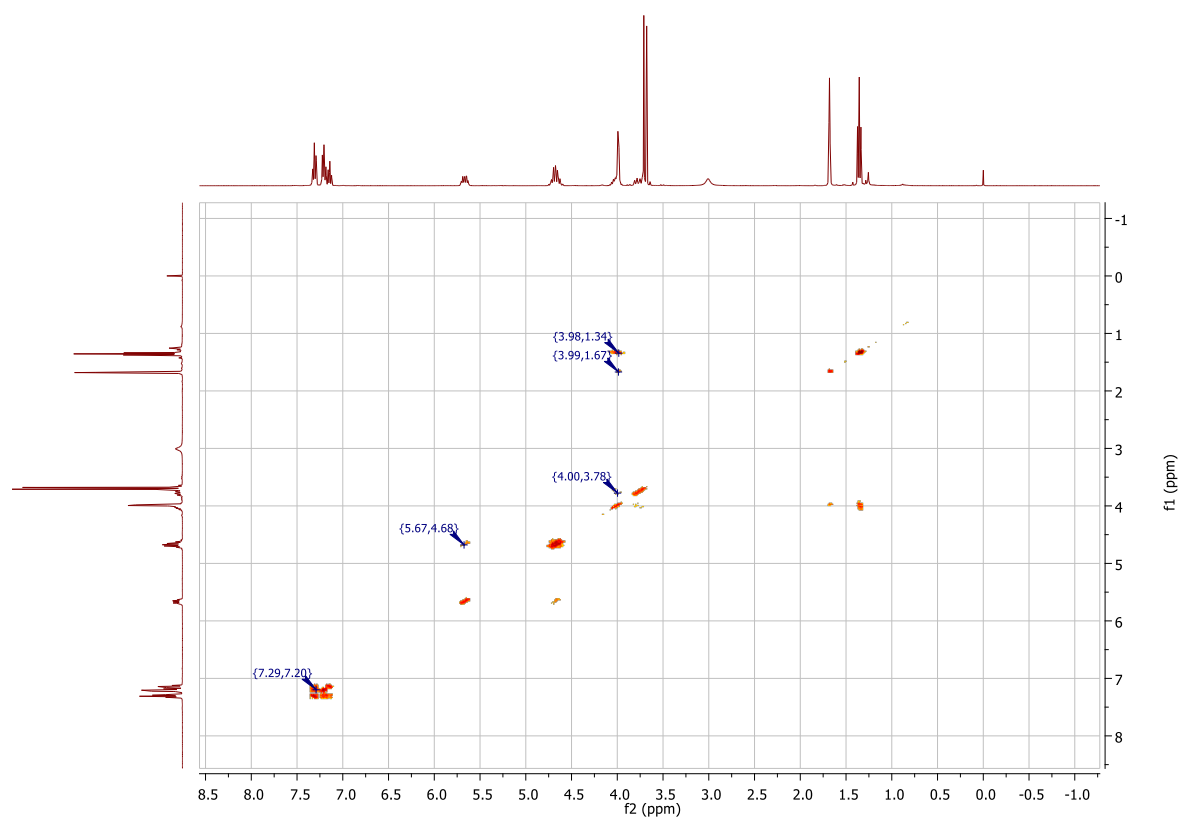
Methyl (((E)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 6a:
¹H NMR



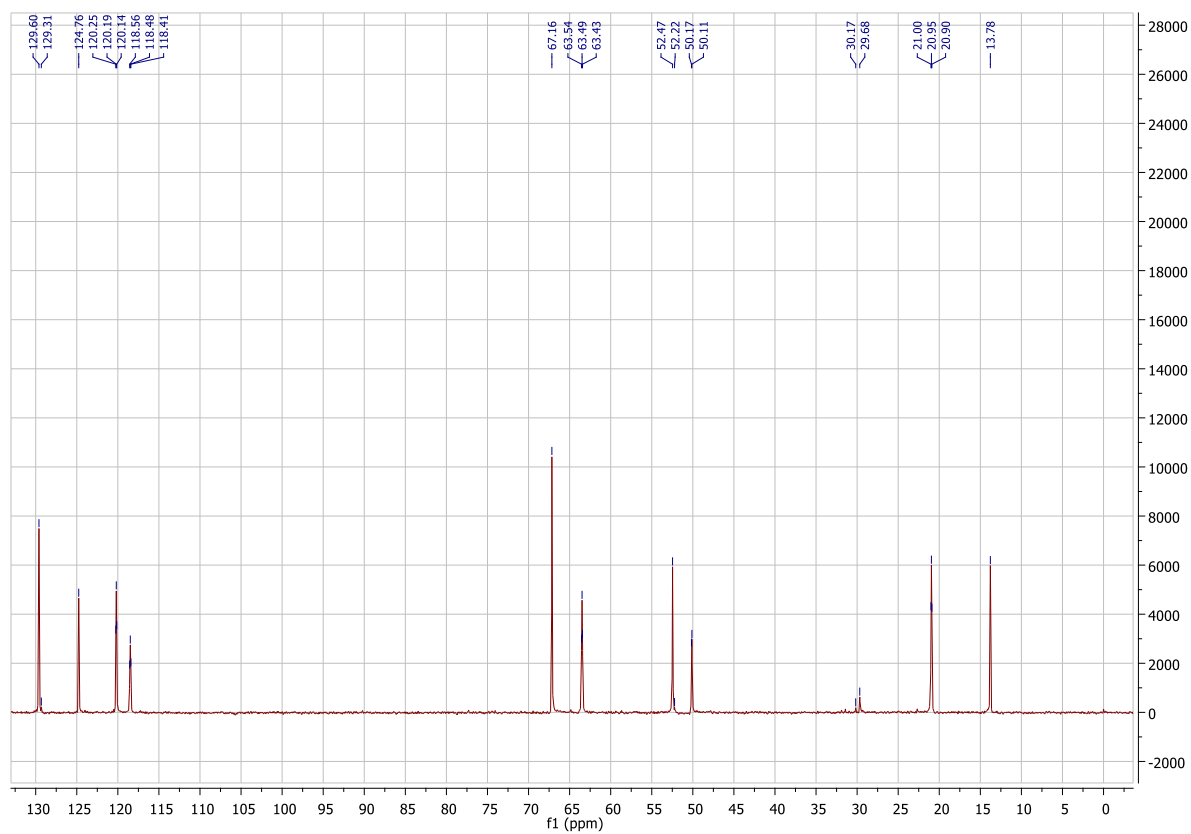
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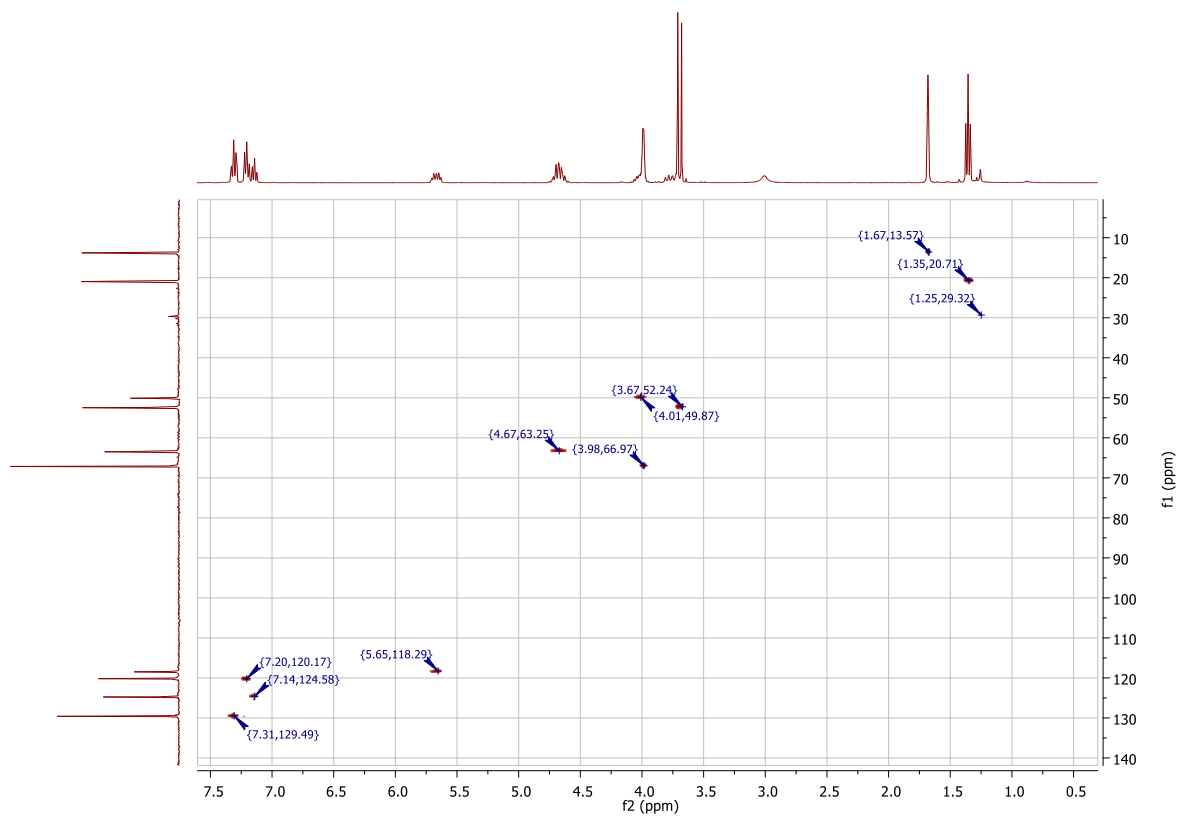
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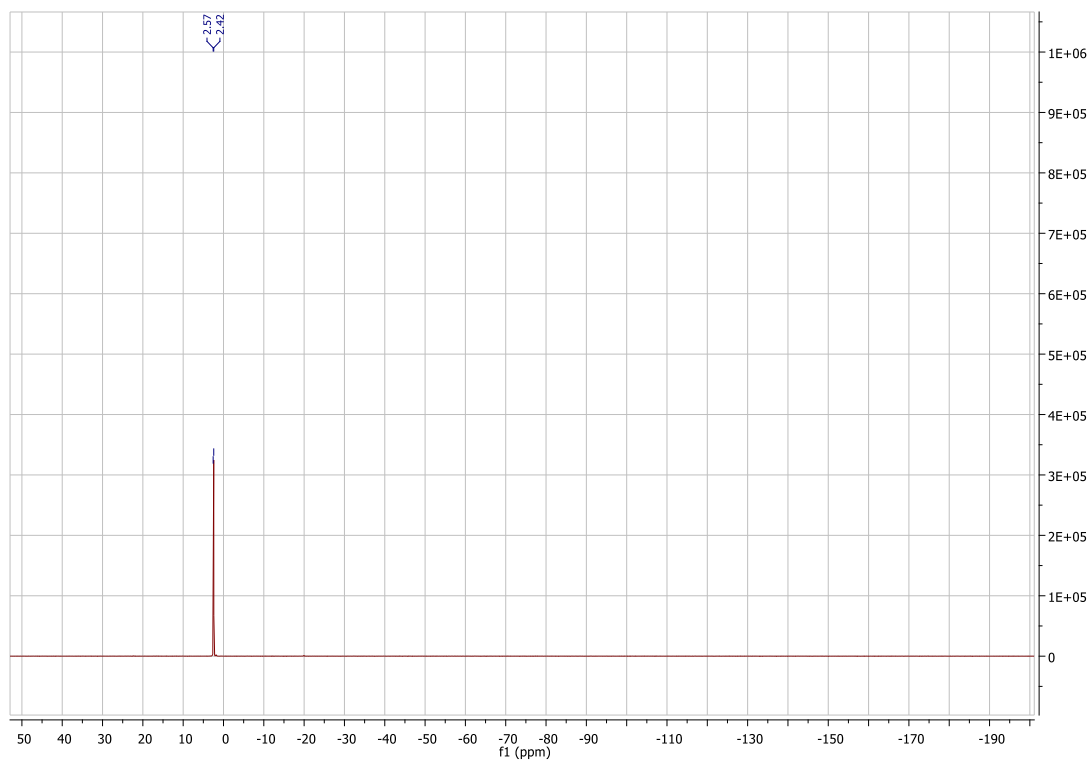
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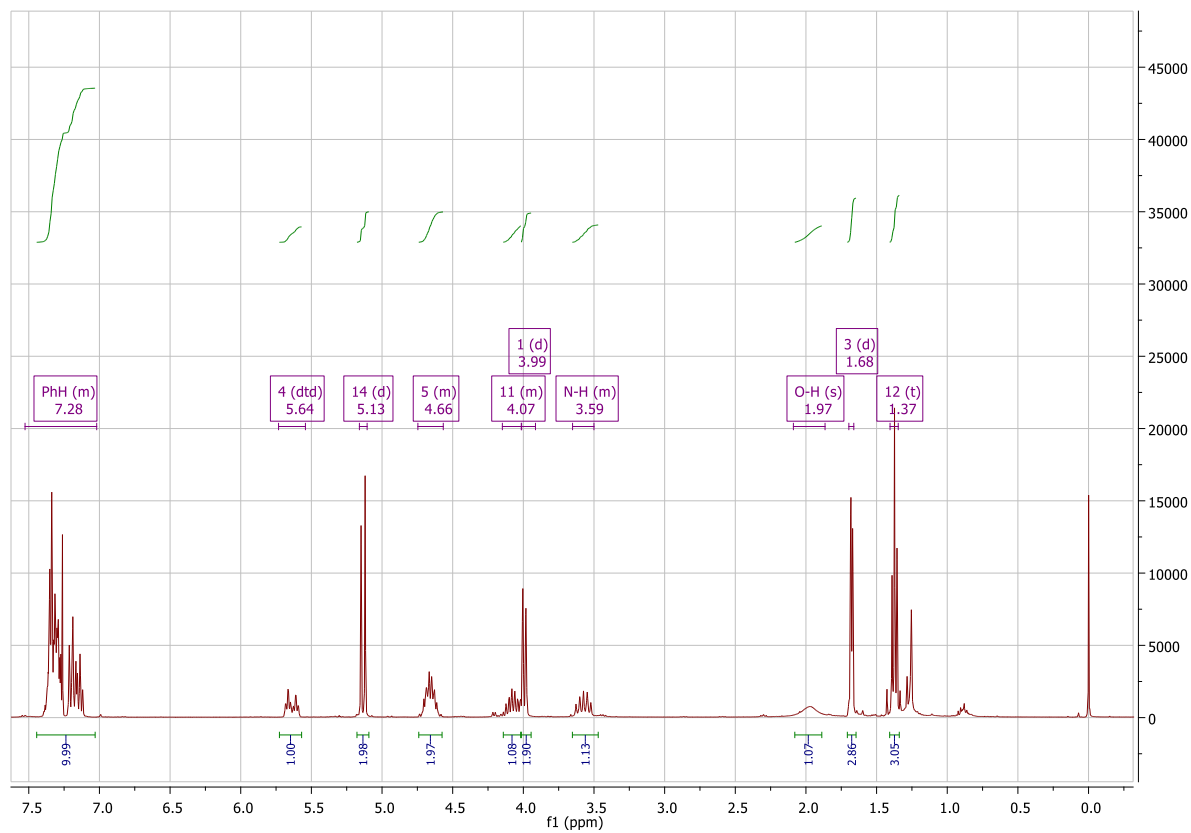
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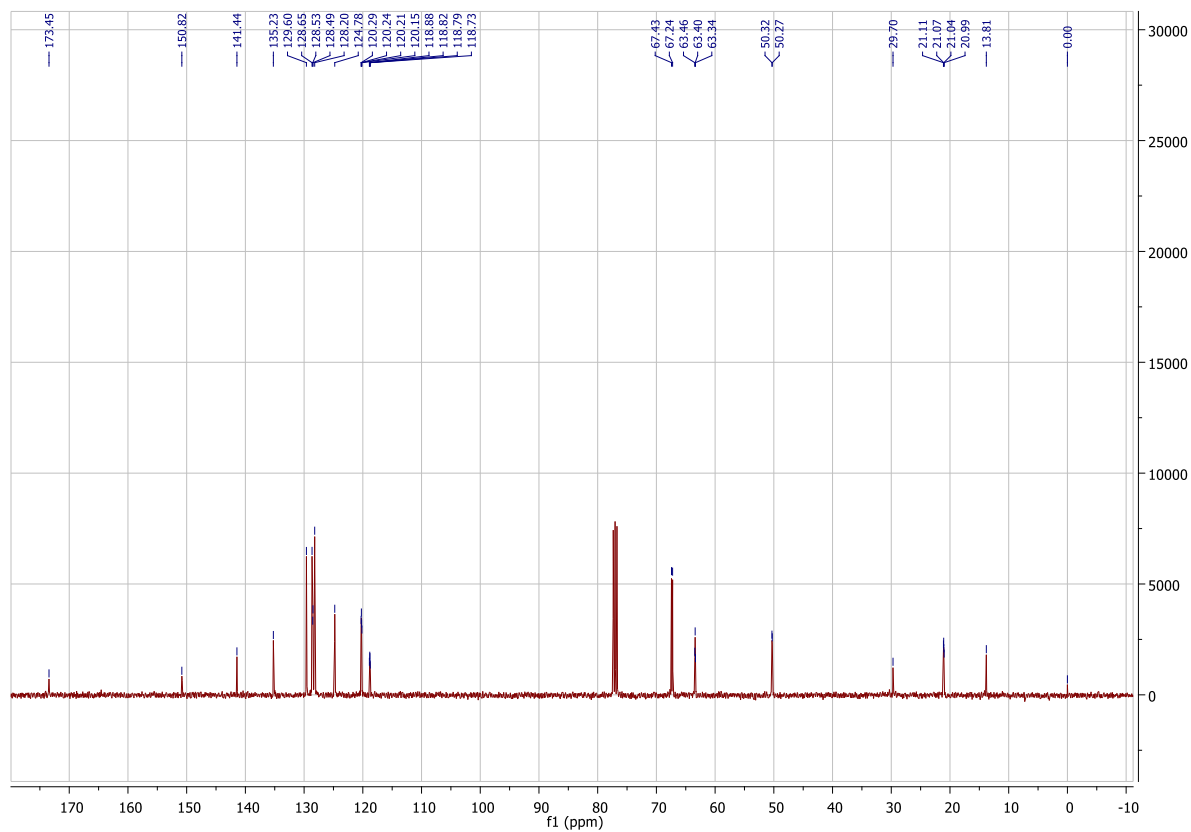
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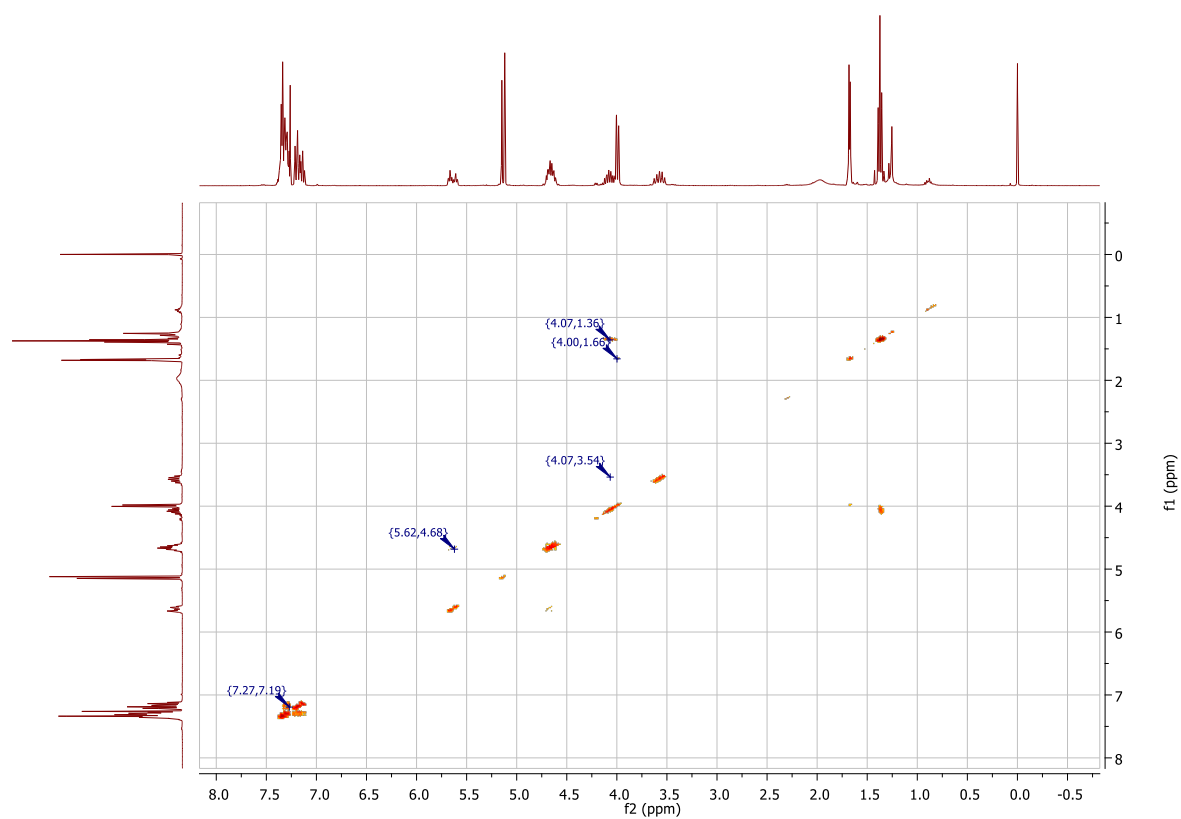
Benzyl (((E)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 6b:
¹H NMR



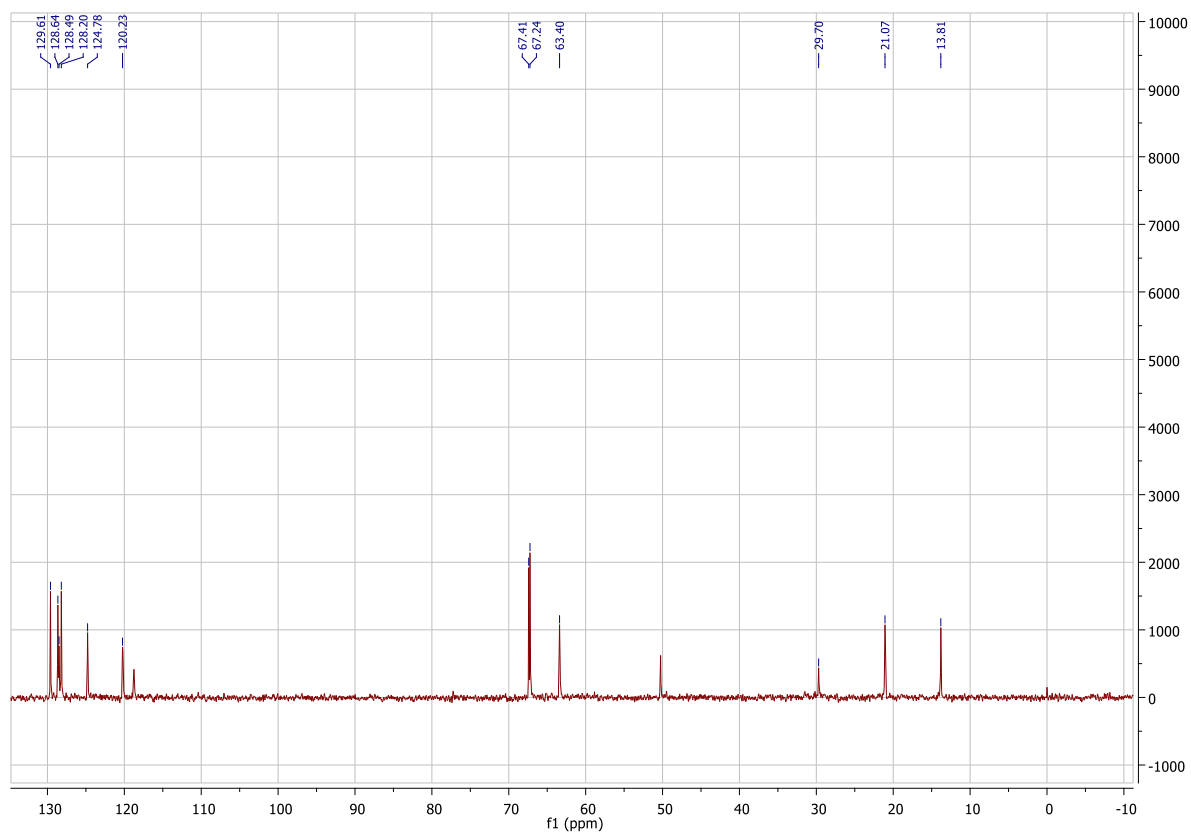
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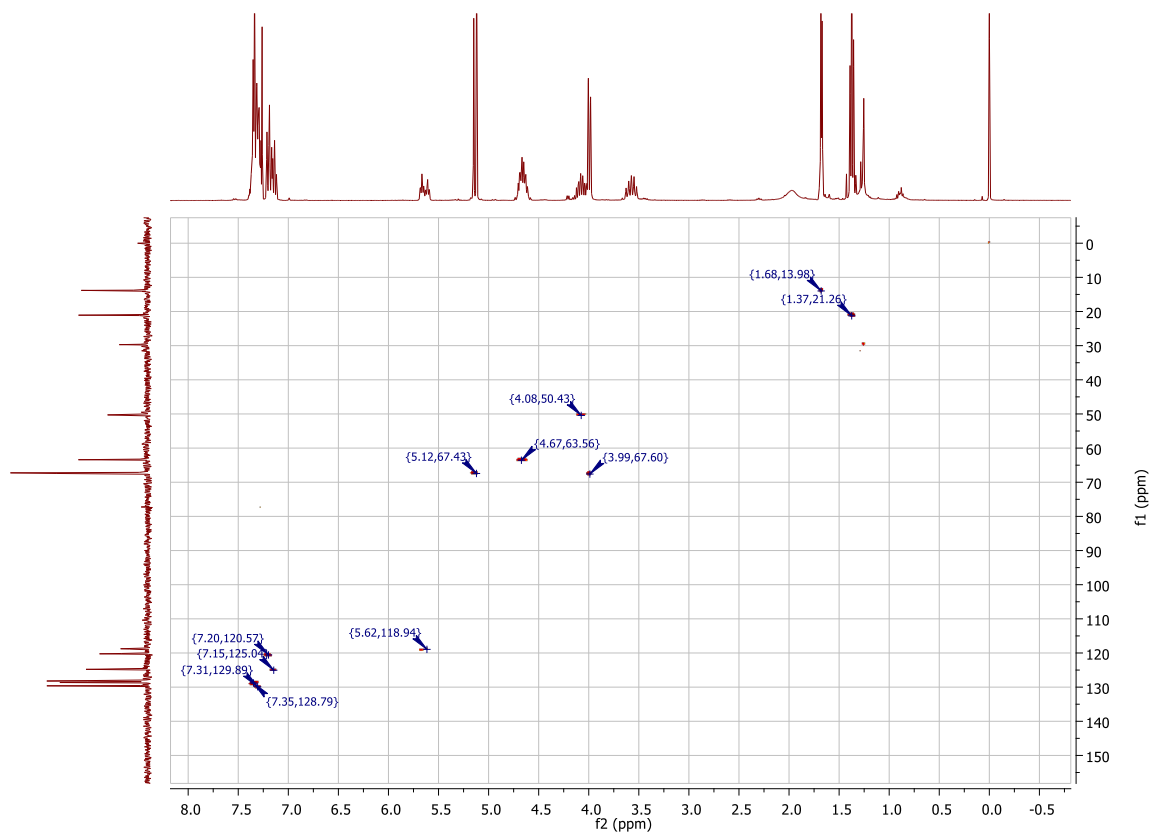
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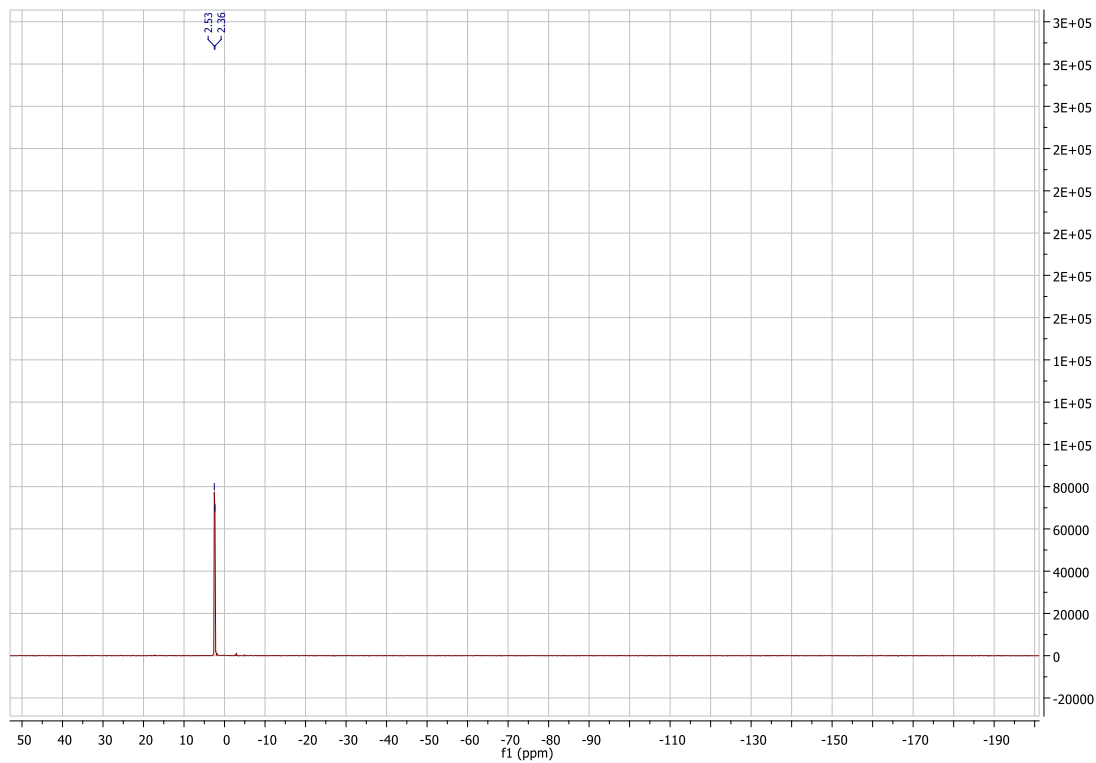
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HSQC

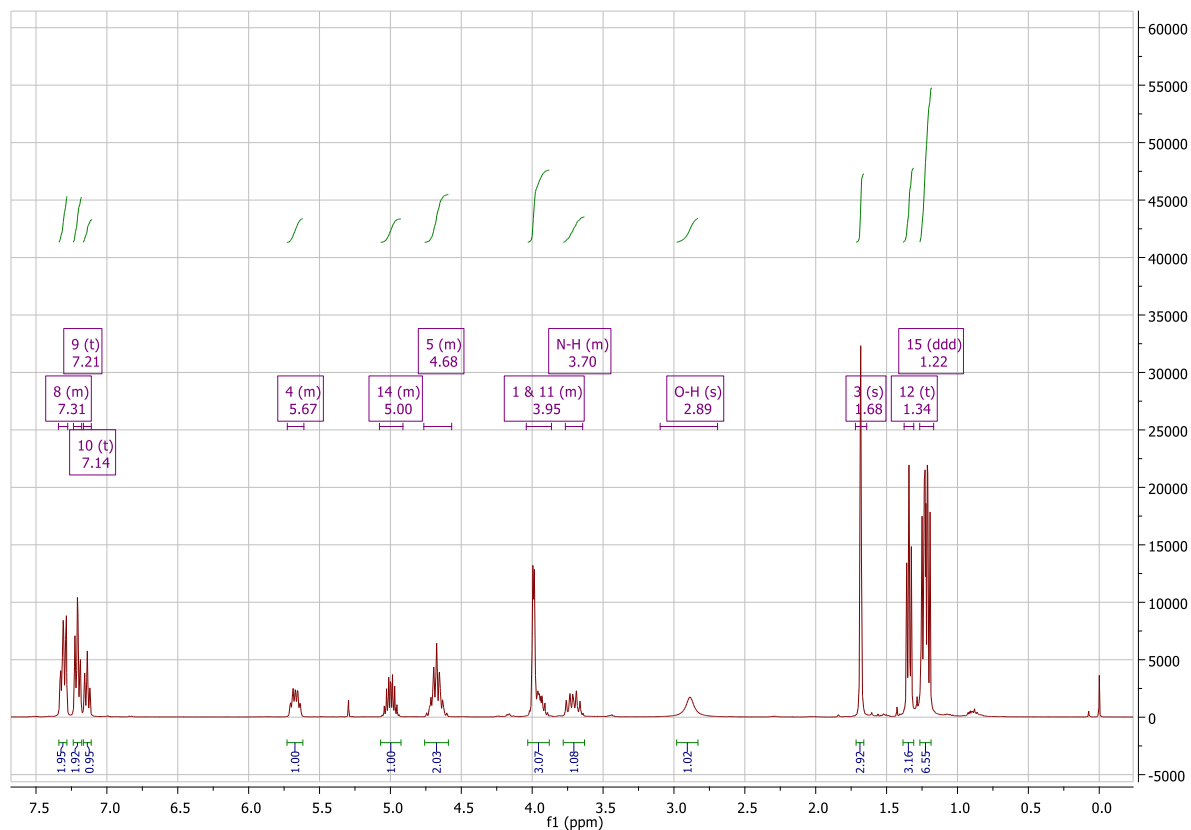


^{31}P NMR

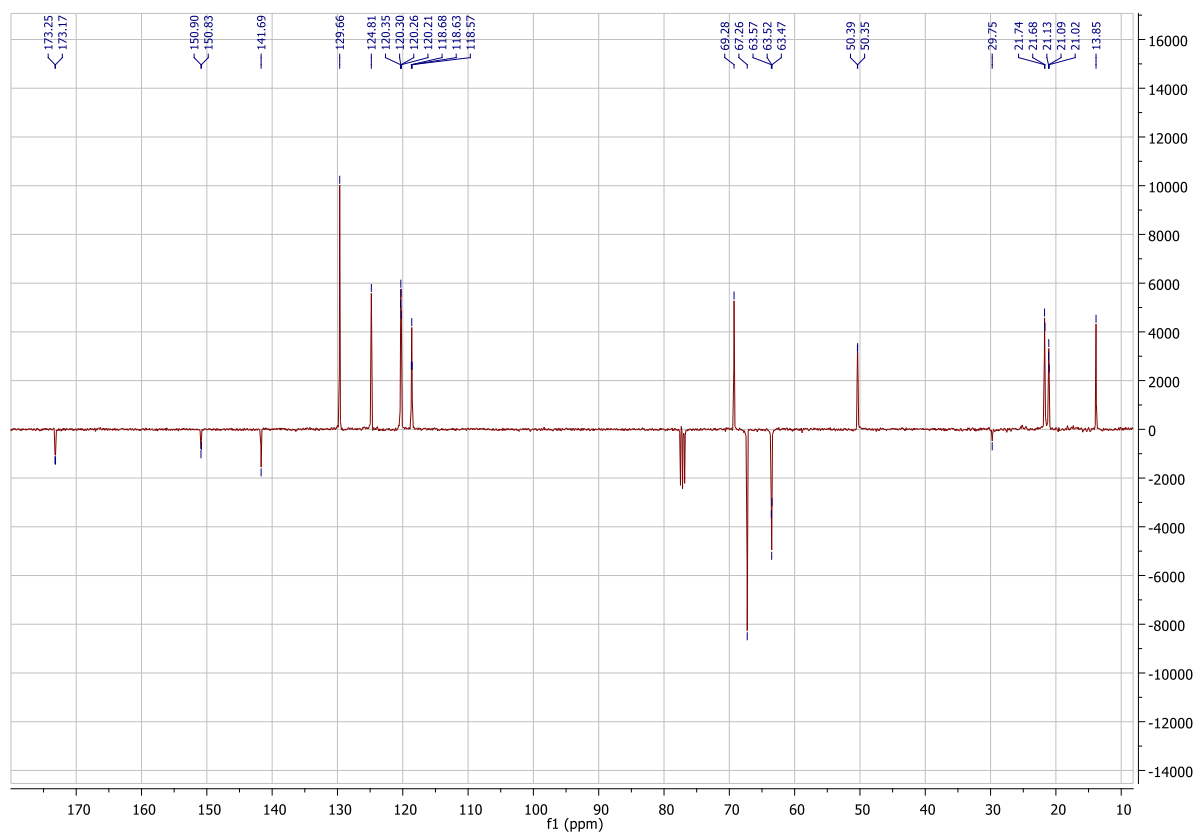


Isopropyl (((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 6c:

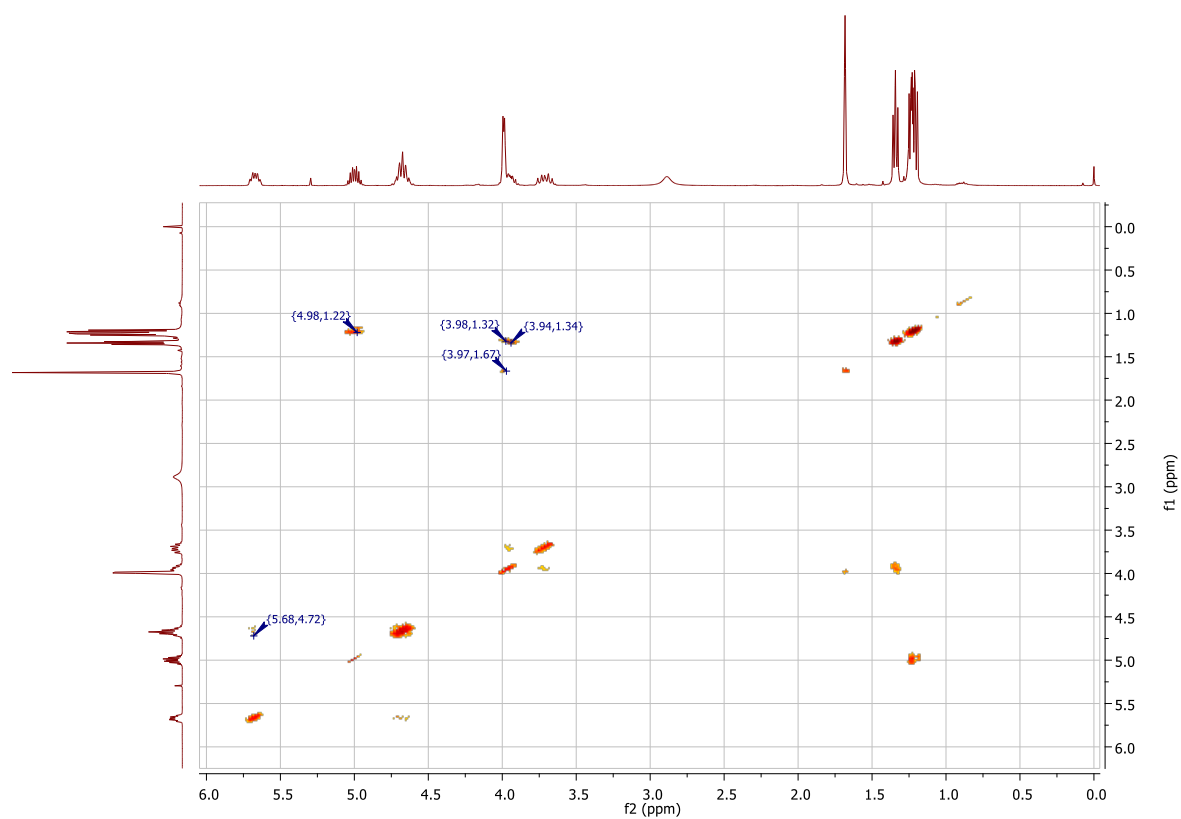
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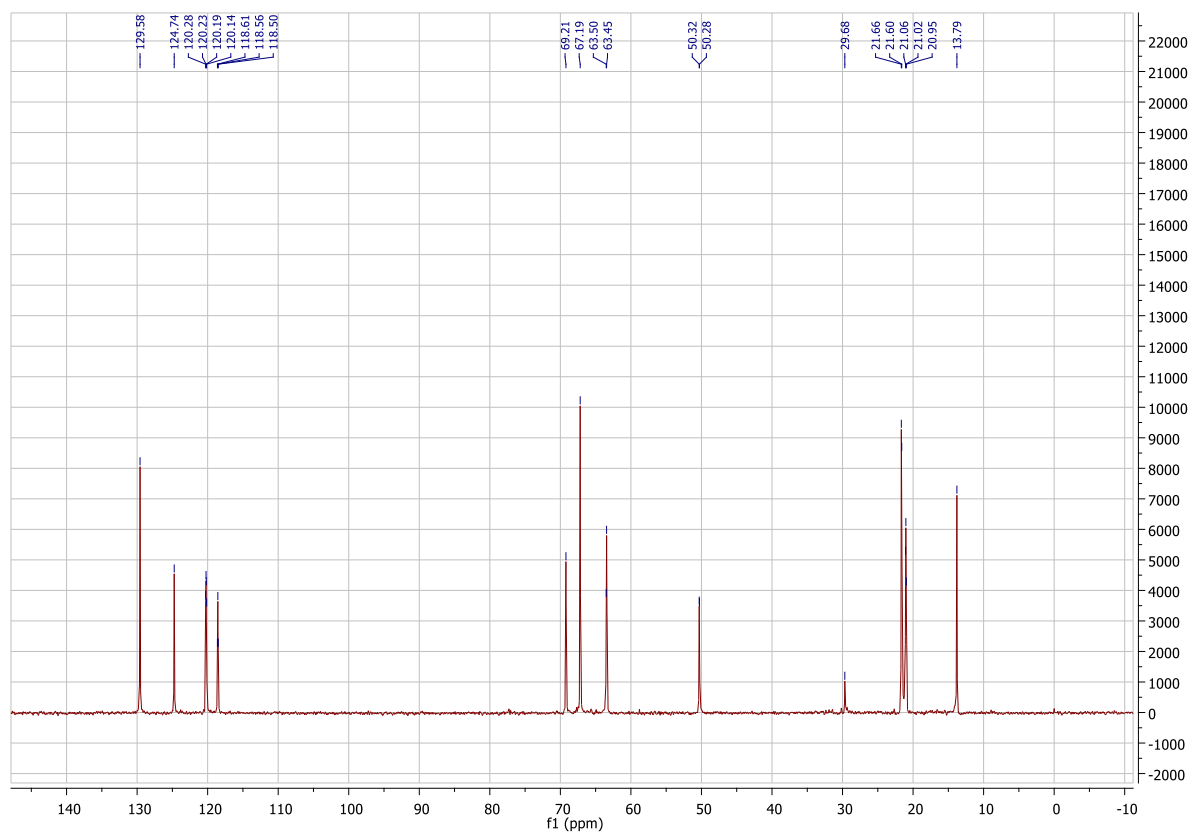
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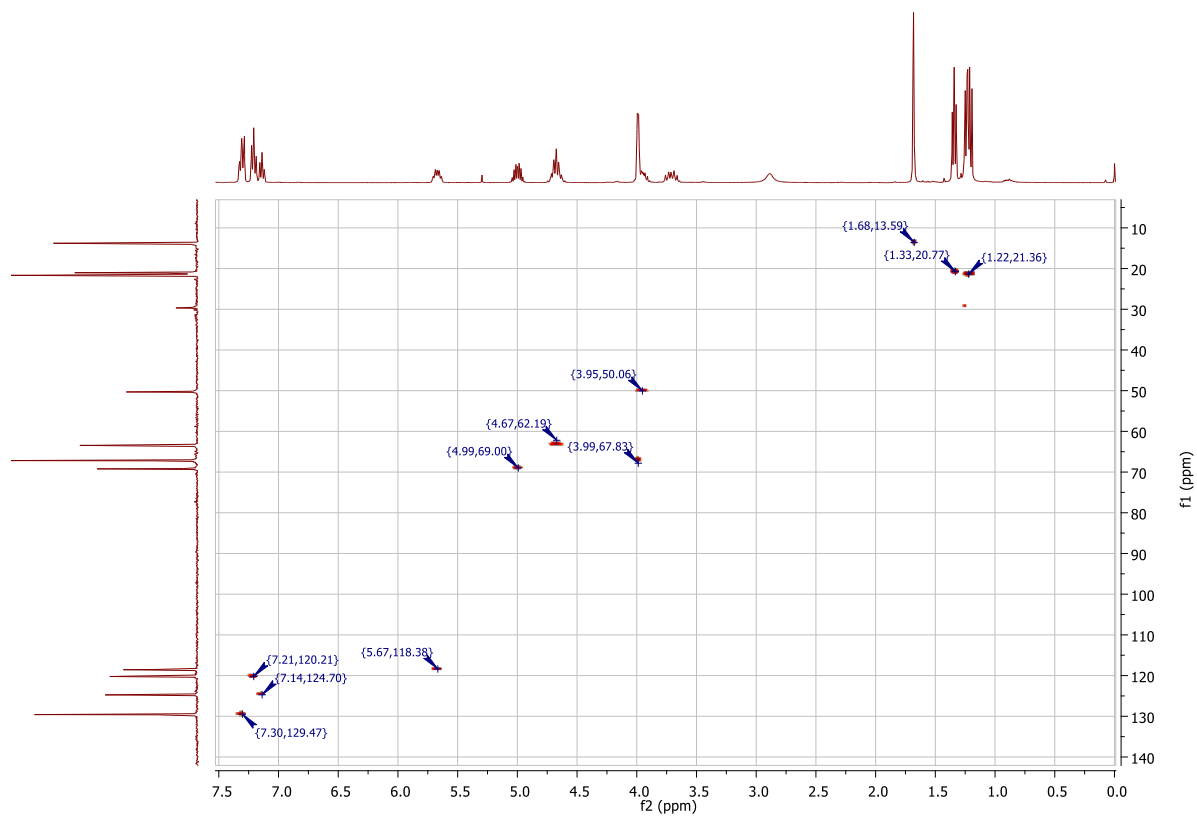
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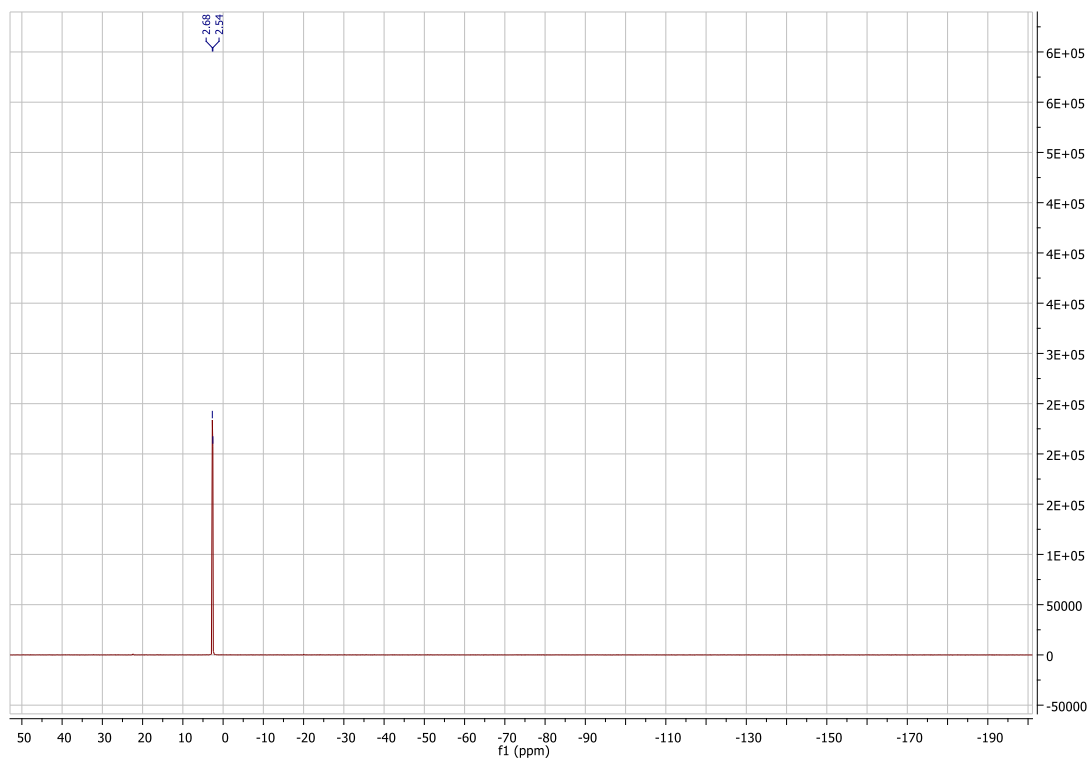
¹³C DEPT-45



HSQC

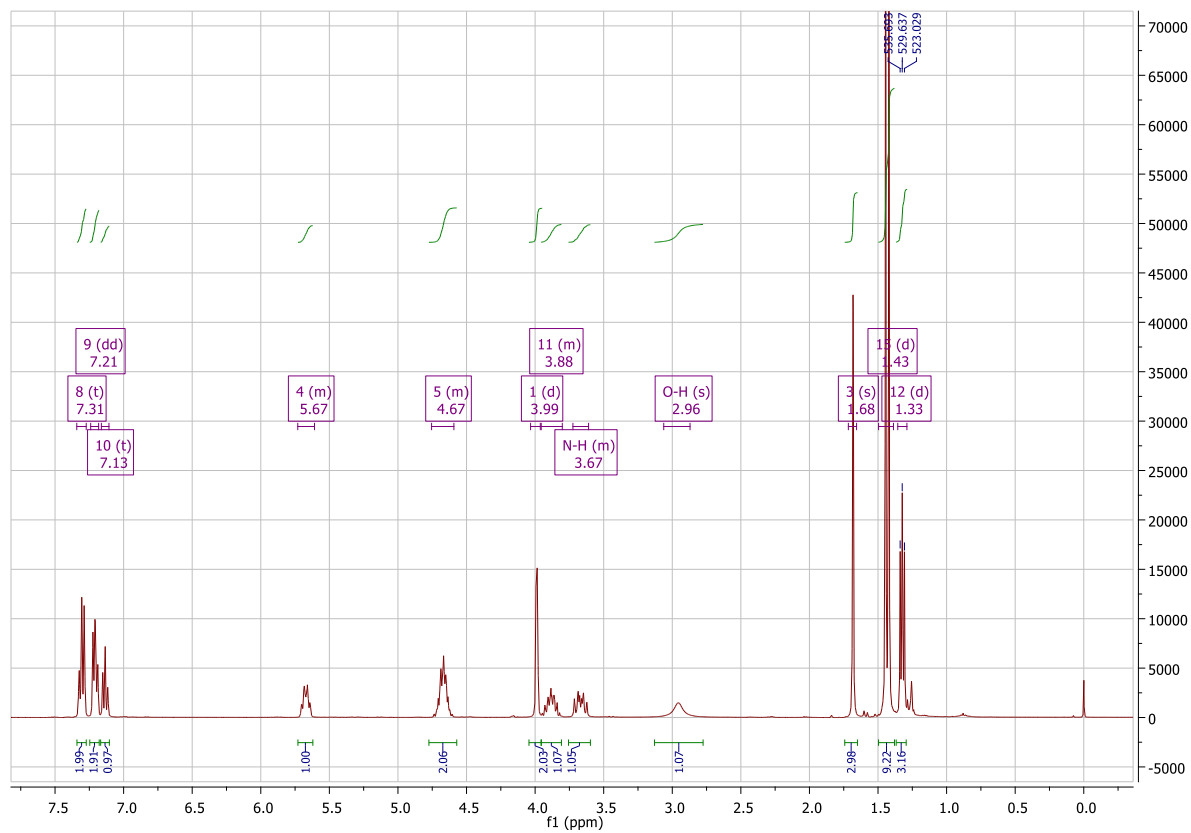


^{31}P NMR

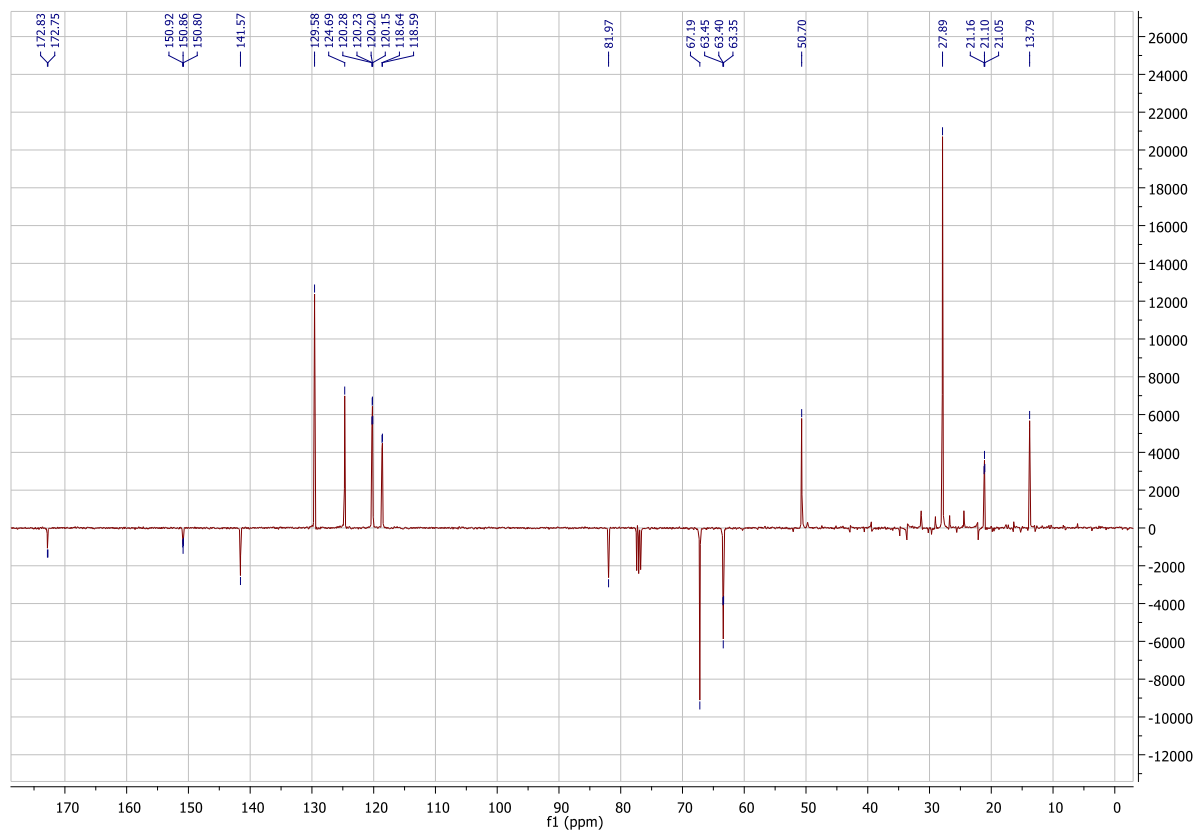


Tert-butyl (((E)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 6d:

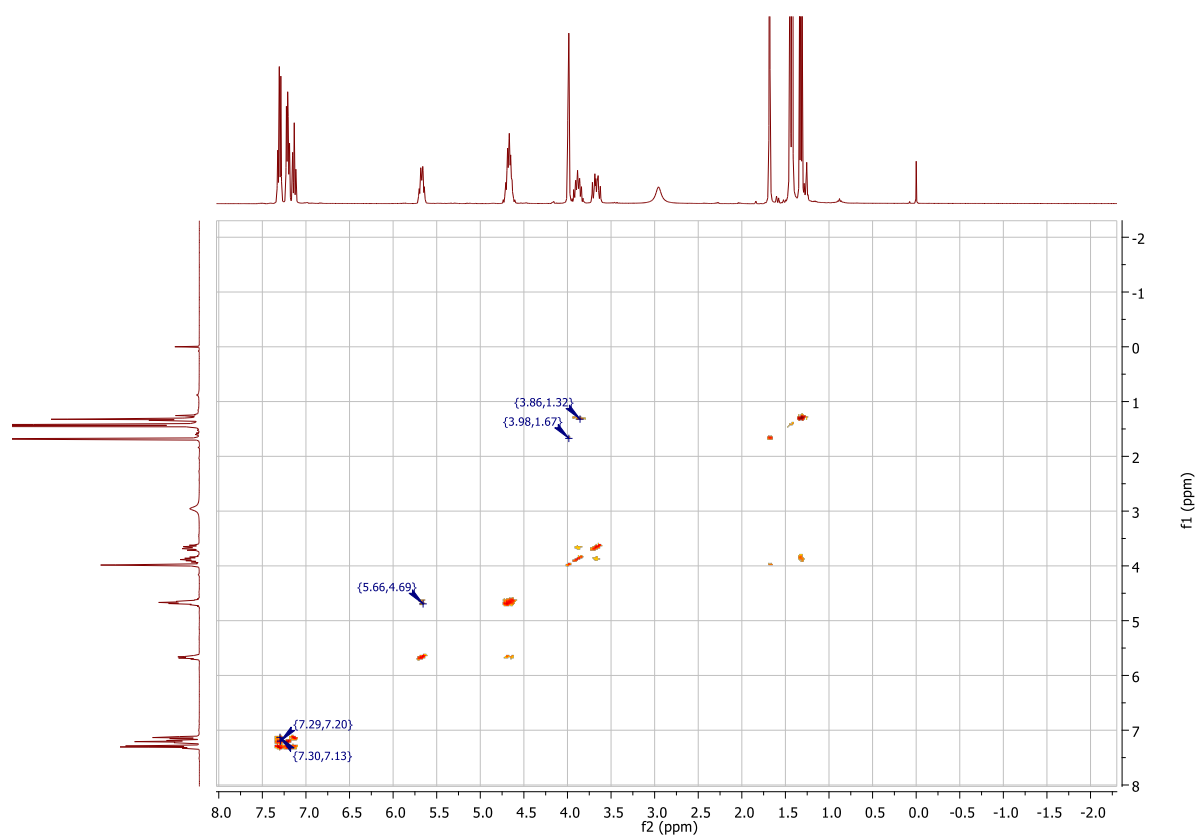
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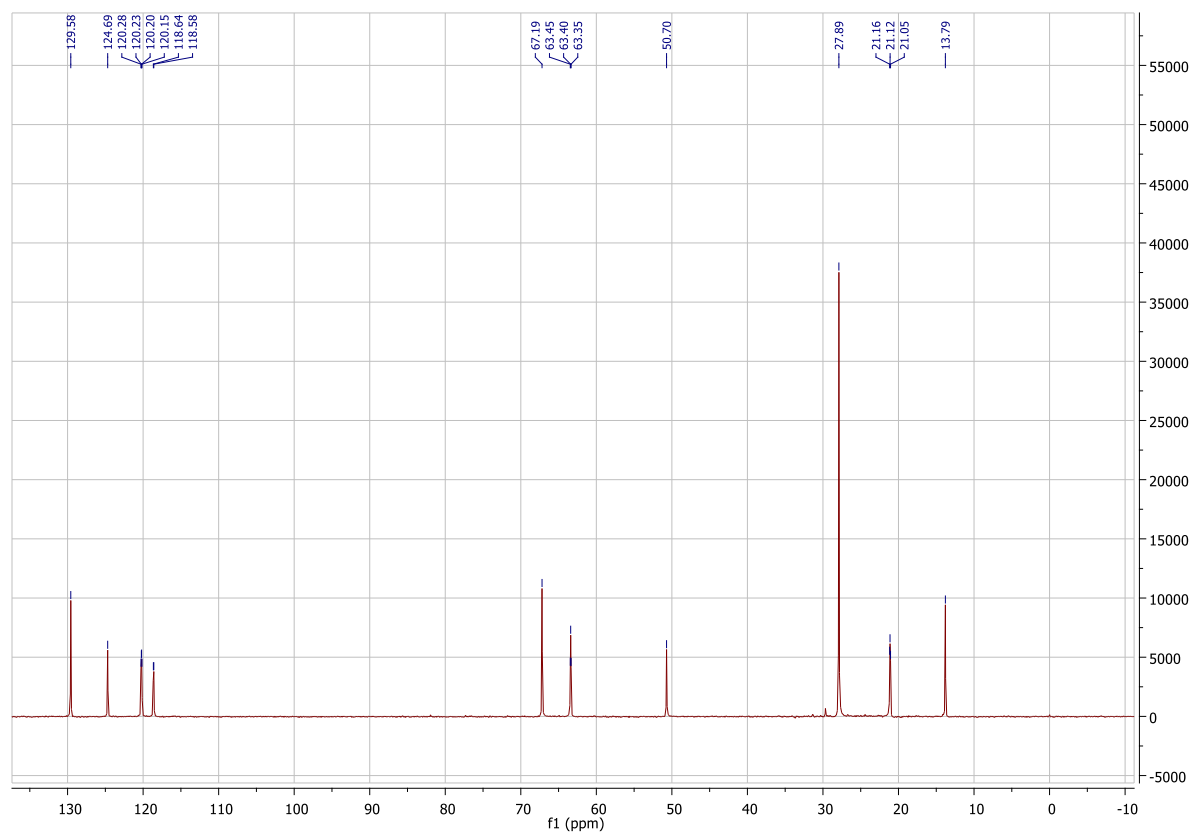
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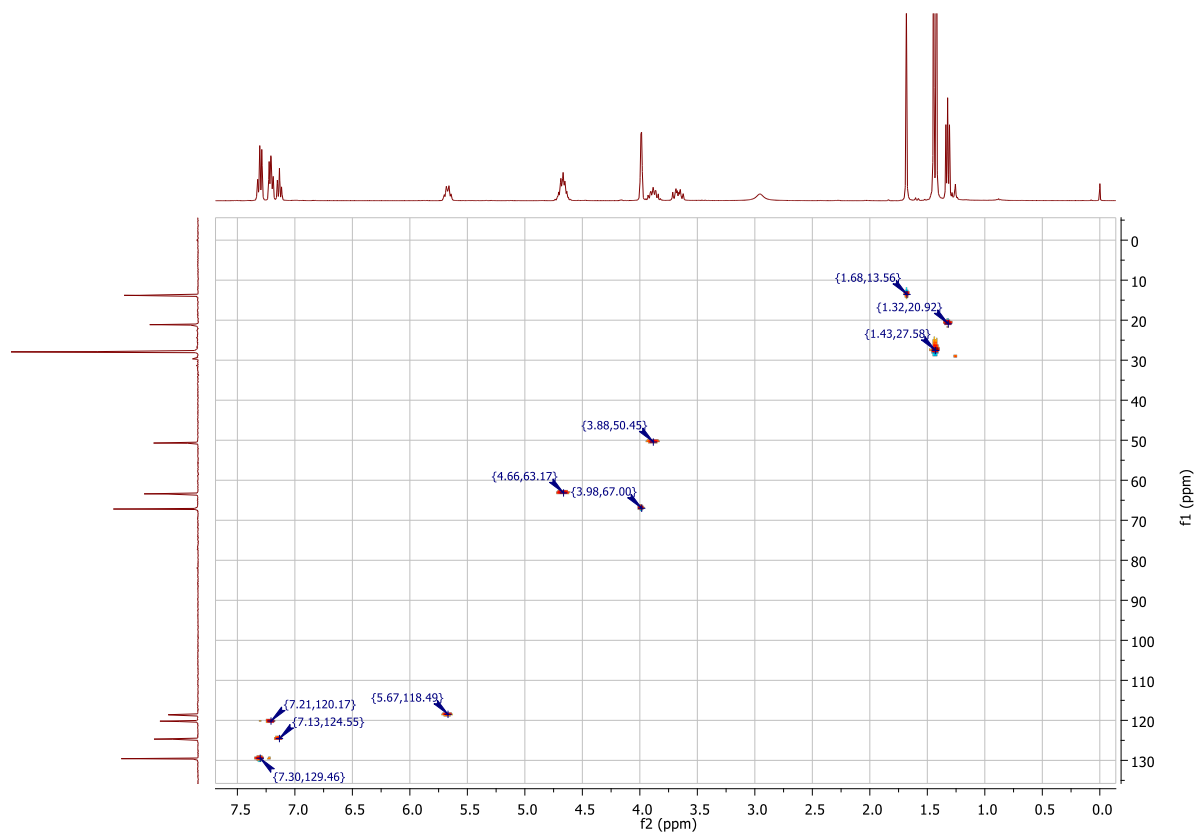
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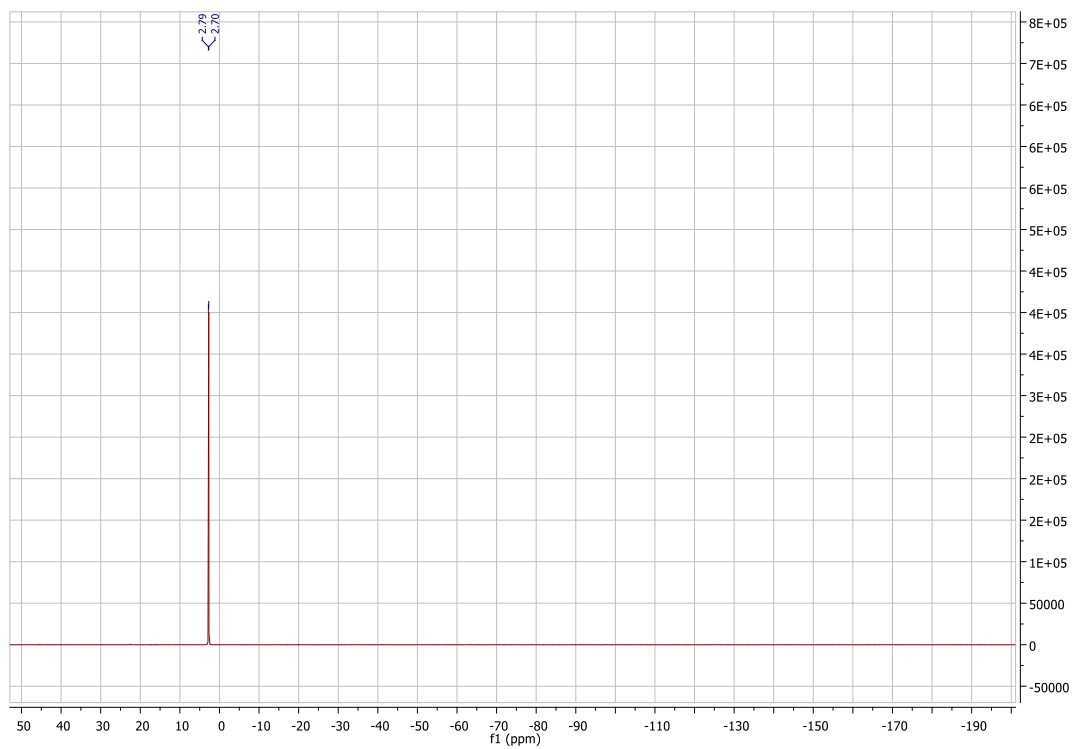
¹³C DEPT-45



HSQC

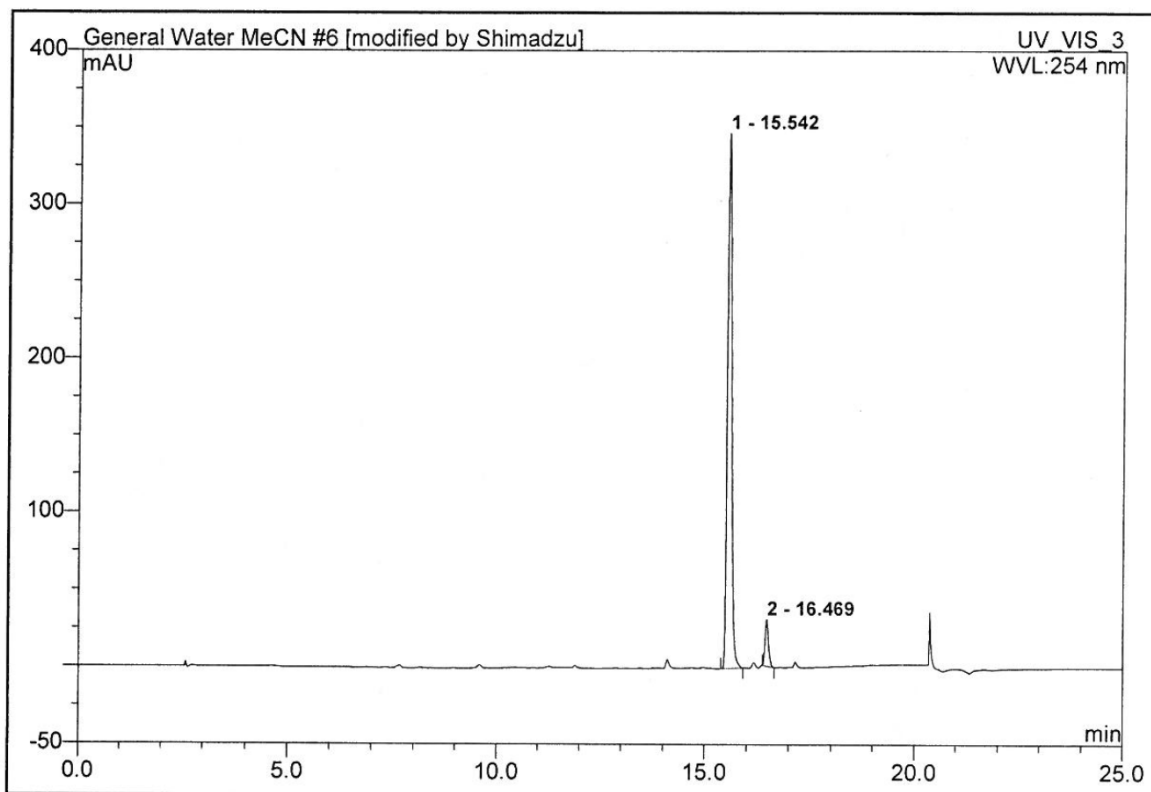


^{31}P NMR



III. HPLC spectra

6 RM27 Compound 5a			
Sample Name:	RM27	Injection Volume:	20.0
Vial Number:	1_4	Channel:	UV_VIS_3
Sample Type:	unknown	Wavelength:	254
Control Program:	MeCN 25min gradient	Bandwidth:	8
Quantif. Method:	General Method	Dilution Factor:	1.0000
Recording Time:	24/2/2017 13:05	Sample Weight:	1.0000
Run Time (min):	25.00	Sample Amount:	1.0000

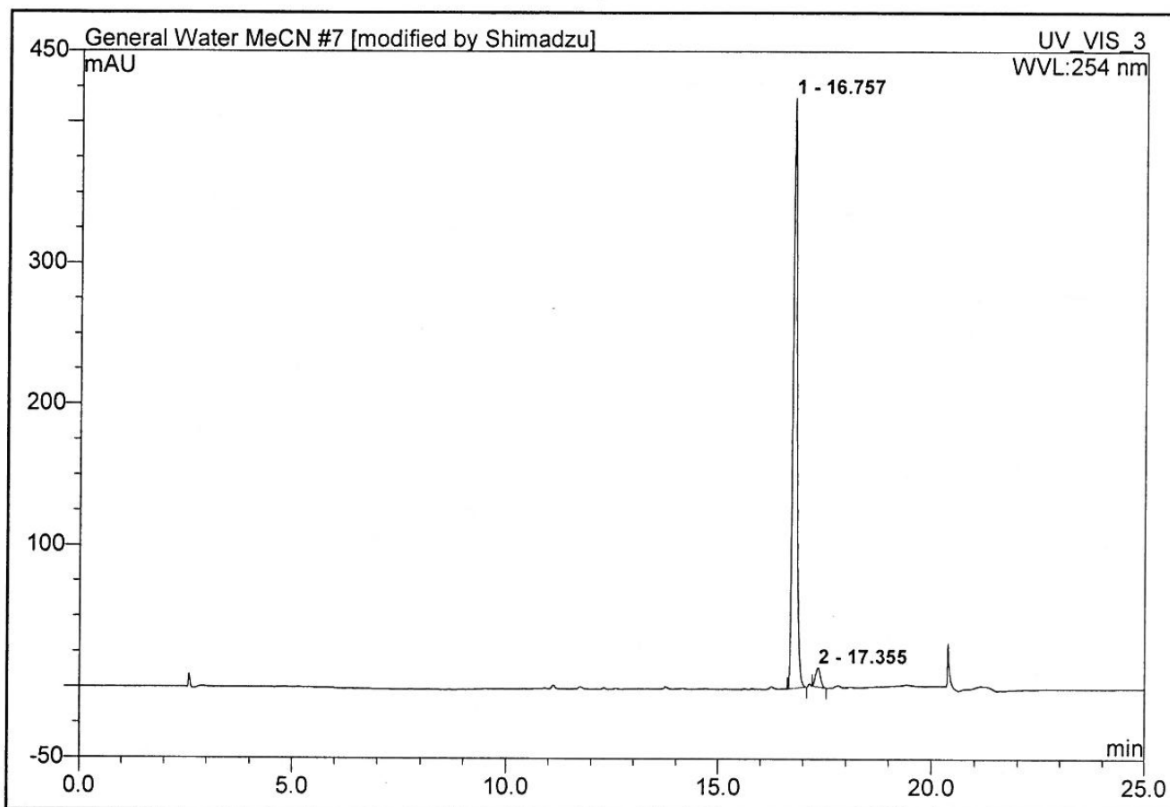


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	15.54	n.a.	347.176	42.070	94.04	n.a.	BMB
2	16.47	n.a.	30.133	2.667	5.96	n.a.	BMB
Total:			377.309	44.737	100.00	0.000	

7 RM29

Compound 5b

Sample Name:	RM29	Injection Volume:	20.0
Vial Number:	1_5	Channel:	UV_VIS_3
Sample Type:	unknown	Wavelength:	254
Control Program:	MeCN 25min gradient	Bandwidth:	8
Quantif. Method:	General Method	Dilution Factor:	1.0000
Recording Time:	24/2/2017 13:30	Sample Weight:	1.0000
Run Time (min):	25.00	Sample Amount:	1.0000

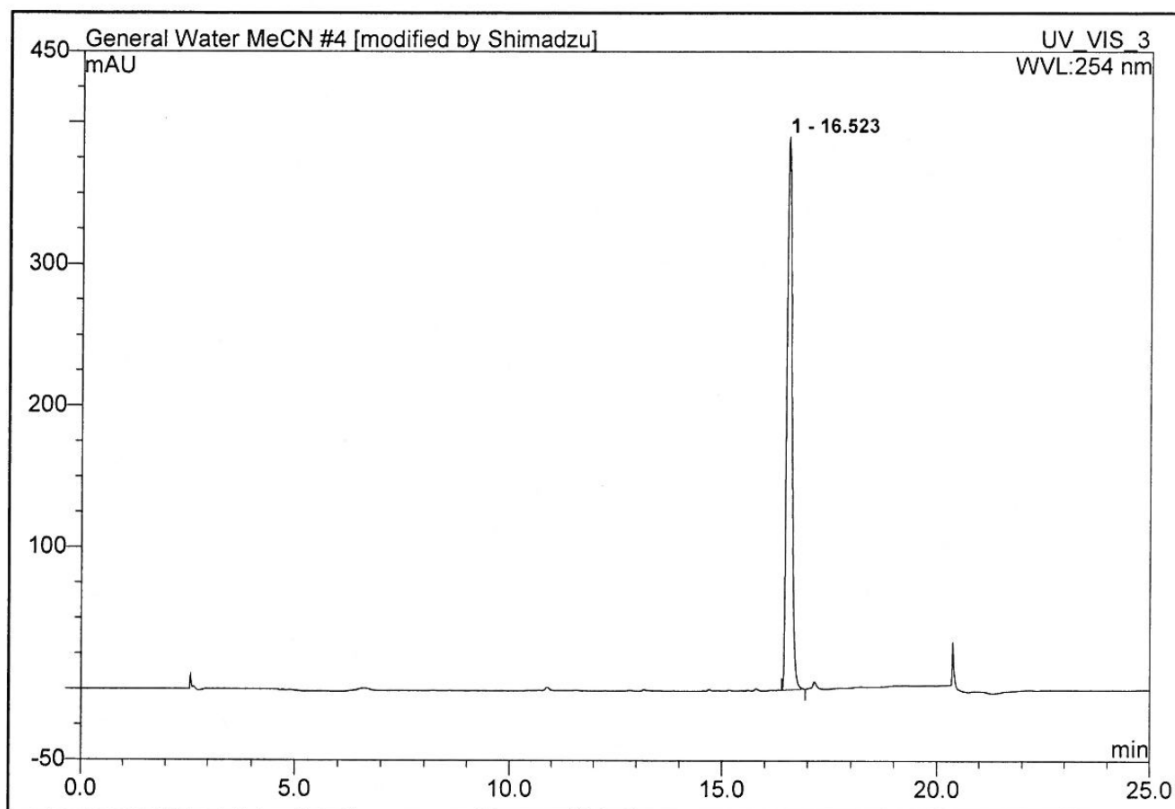


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	16.76	n.a.	417.330	47.534	96.47	n.a.	BMB
2	17.35	n.a.	13.383	1.742	3.53	n.a.	BMB
Total:			430.713	49.275	100.00	0.000	

4 **RM20**

Compound 5c

Sample Name:	RM20	Injection Volume:	20.0
Vial Number:	1_2	Channel:	UV_VIS_3
Sample Type:	unknown	Wavelength:	254
Control Program:	MeCN 25min gradient	Bandwidth:	8
Quantif. Method:	General Method	Dilution Factor:	1.0000
Recording Time:	24/2/2017 12:14	Sample Weight:	1.0000
Run Time (min):	25.00	Sample Amount:	1.0000

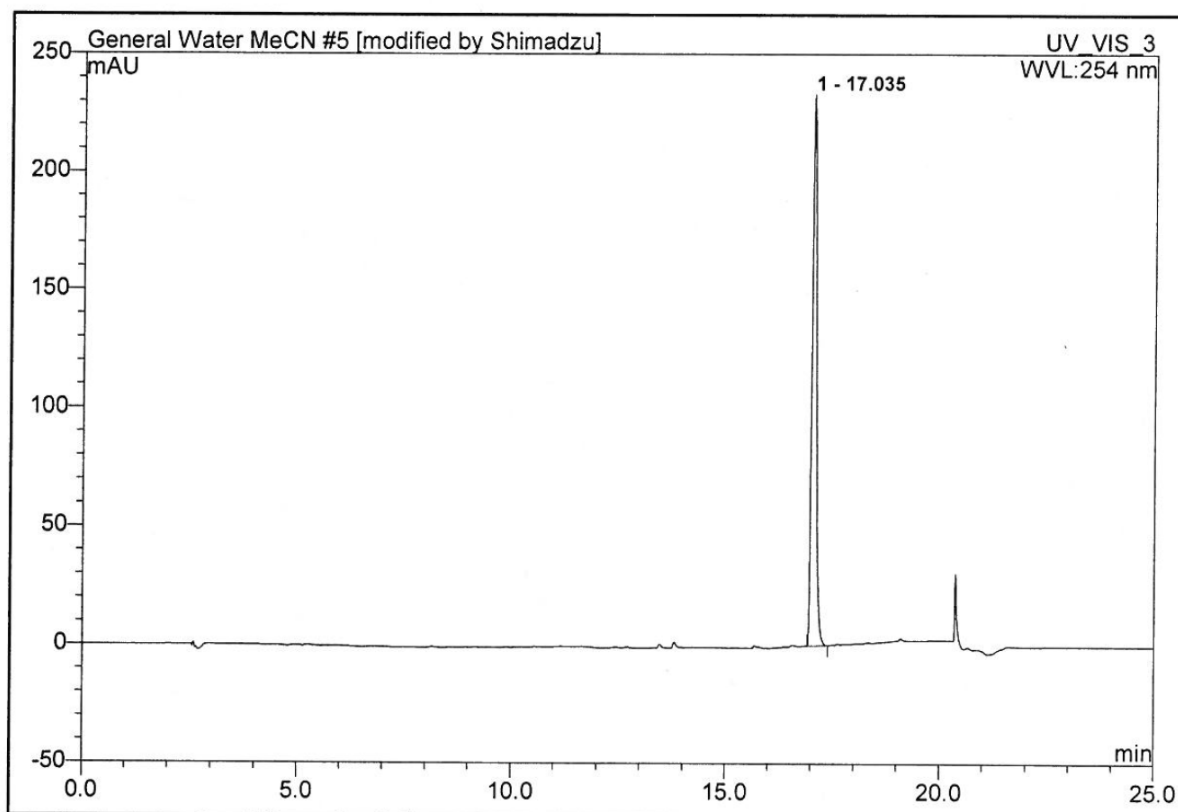


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	16.52	n.a.	389.805	54.383	100.00	n.a.	BMB
Total:			389.805	54.383	100.00	0.000	

5 RM25

Compound 5d

Sample Name:	RM25	Injection Volume:	20.0
Vial Number:	1_3	Channel:	UV_VIS_3
Sample Type:	unknown	Wavelength:	254
Control Program:	MeCN 25min gradient	Bandwidth:	8
Quantif. Method:	General Method	Dilution Factor:	1.0000
Recording Time:	24/2/2017 12:39	Sample Weight:	1.0000
Run Time (min):	25.00	Sample Amount:	1.0000



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	17.03	n.a.	232.935	27.980	100.00	n.a.	BMB
Total:			232.935	27.980	100.00	0.000	

IV. Mass spec data

protected Me ester ProTide 8a

ROSHNI MALDE
1224846

Elemental Composition Report **Roshni Malde RM27** **Compound 5a**

Single Mass Analysis

Tolerance = 2.0 PPM / DBE: min = -1.5, max = 1000.0

Element prediction: Off

Monoisotopic Mass, Even Electron Ions

416 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 0-100 H: 0-100 N: 0-4 O: 0-7 Na: 0-1 Si: 1-1 P: 1-1

Minimum: -1.5

Maximum: 5.0 2.0 1000.0

Mass	Calc. Mass	mDa	PPM	DBE	Formula
480.1953	480.1947	0.6	1.2	5.5	C21 H36 N O6 Na Si P

protected Bn ester ProTide 8b

ROSHNI MALDE
1224846

Elemental Composition Report **Roshni Malde RM29** **Compound 5b**

Single Mass Analysis

Tolerance = 2.0 PPM / DBE: min = -1.5, max = 1000.0

Element prediction: Off

Monoisotopic Mass, Even Electron Ions

489 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 0-100 H: 0-100 N: 0-4 O: 0-7 P: 1-1 Si: 1-1 Na: 0-1

Minimum: -1.5

Maximum: 5.0 2.0 1000.0

Mass	Calc. Mass	mDa	PPM	DBE	Formula
556.2267	556.2260	0.7	1.3	9.5	C27 H40 N O6 P Si Na

protected ipr ester ProTide 8c

ROSHNI MALDE
1224846

Elemental Composition Report Roshni Malde (RM20)

Compound 5c

Single Mass Analysis

Tolerance = 2.0 PPM / DBE: min = -1.5, max = 1000.0

Element prediction: Off

Monoisotopic Mass, Even Electron Ions

444 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 0-100 H: 0-100 N: 0-4 O: 0-7 Na: 0-1 P: 1-1 Si: 1-1

Minimum:					-1.5
Maximum:	5.0	2.0	1000.0		
Mass	Calc. Mass	mDa	PPM	DBE	Formula
508.2259	508.2260	-0.1	-0.2	5.5	C23 H40 N O6 Na P Si

protected tBu ester ProTide 8d

ROSHNI MALDE
1224846

Elemental Composition Report Roshni Malde (RM25)

Compound 5d

Single Mass Analysis

Tolerance = 2.0 PPM / DBE: min = -1.5, max = 1000.0

Element prediction: Off

Monoisotopic Mass, Even Electron Ions

460 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 0-100 H: 0-100 N: 0-4 O: 0-7 Na: 0-1 Si: 1-1 P: 1-1

Minimum:					-1.5
Maximum:	5.0	2.0	1000.0		
Mass	Calc. Mass	mDa	PPM	DBE	Formula
522.2423	522.2417	0.6	1.1	5.5	C24 H42 N O6 Na Si P