Electronic Supplementary Material (ESI) for Chemical Science.

Vinylphosphonites for Staudinger-induced chemoselective peptide cyclization and functionalization

Marc-André Kasper[1,2†], Maria Glanz[1,2†], Andreas Oder[1], Peter Schmieder[1], Jens P. von Kries[1] and Christian P. R. Hackenberger[1,2,*]

[1]Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP), Chemical Biology Department, Robert-Rössle-Strasse 10, 13125 Berlin (Germany)

[2]Humboldt Universität zu Berlin, Department of Chemistry, Brook-Taylor-Str. 2, 12489 Berlin (Germany)

*e-mail: hackenbe@fmp-berlin.de

† authors contributed equally

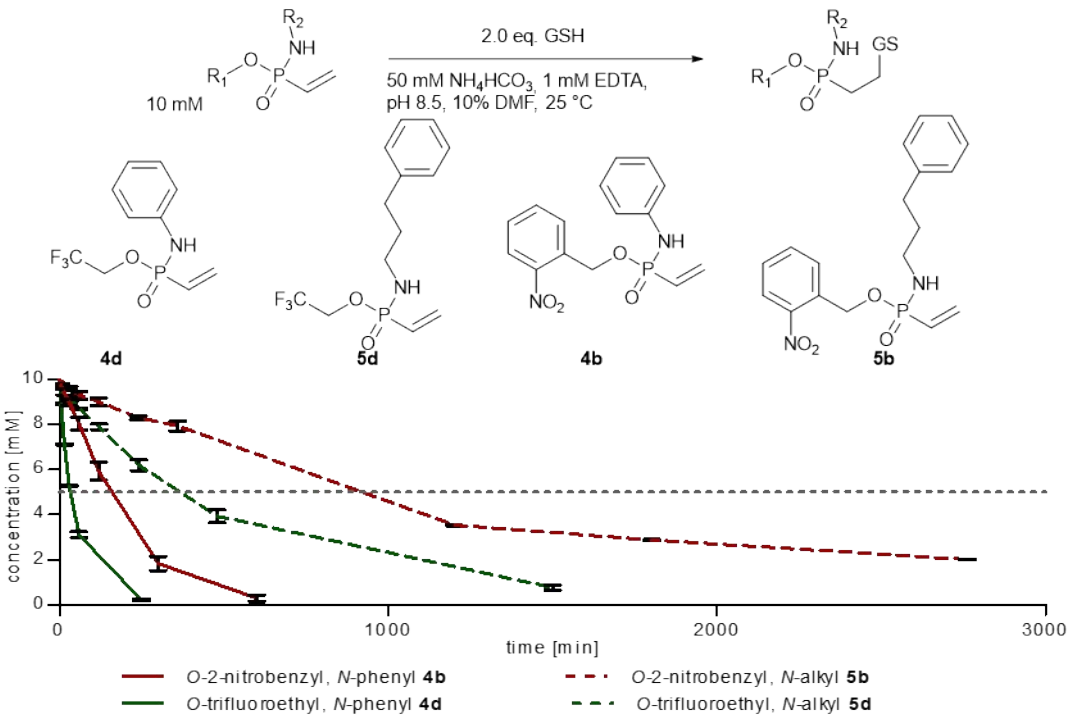
Supporting Information

1. Supplementary Figures

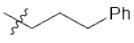
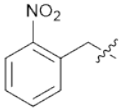
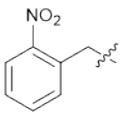
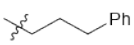
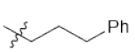
1.1. Figure S1

Glutathione addition to vinylphosphonamidates. (see chapter 3.1 for details) a) Concentration of starting materials **4b**, **4d**, **5b** and **5d** over time monitored by UPLC/MS (pH 8.5, 50 mM NH_4HCO_3, 1 mM EDTA). Values were calculated by integration of the peaks in relation to an internal standard (inosin). Sample were drawn from the reaction mixture and immediately diluted into 50 mM NaOAc buffer at pH 3.5 to stop the reaction and subjected to UPLC analysis. Peaks were assigned by MS. Shown are mean and error of three independent measurements (n=3). Solid line: *N*-phenyl substituents, dotted line: *N*-alkyl-substituents. b) Overview for all vinylphosphonamidates tested in this study in GSH addition under the same conditions. Stated is the time until 50% conversion is reached.

a)

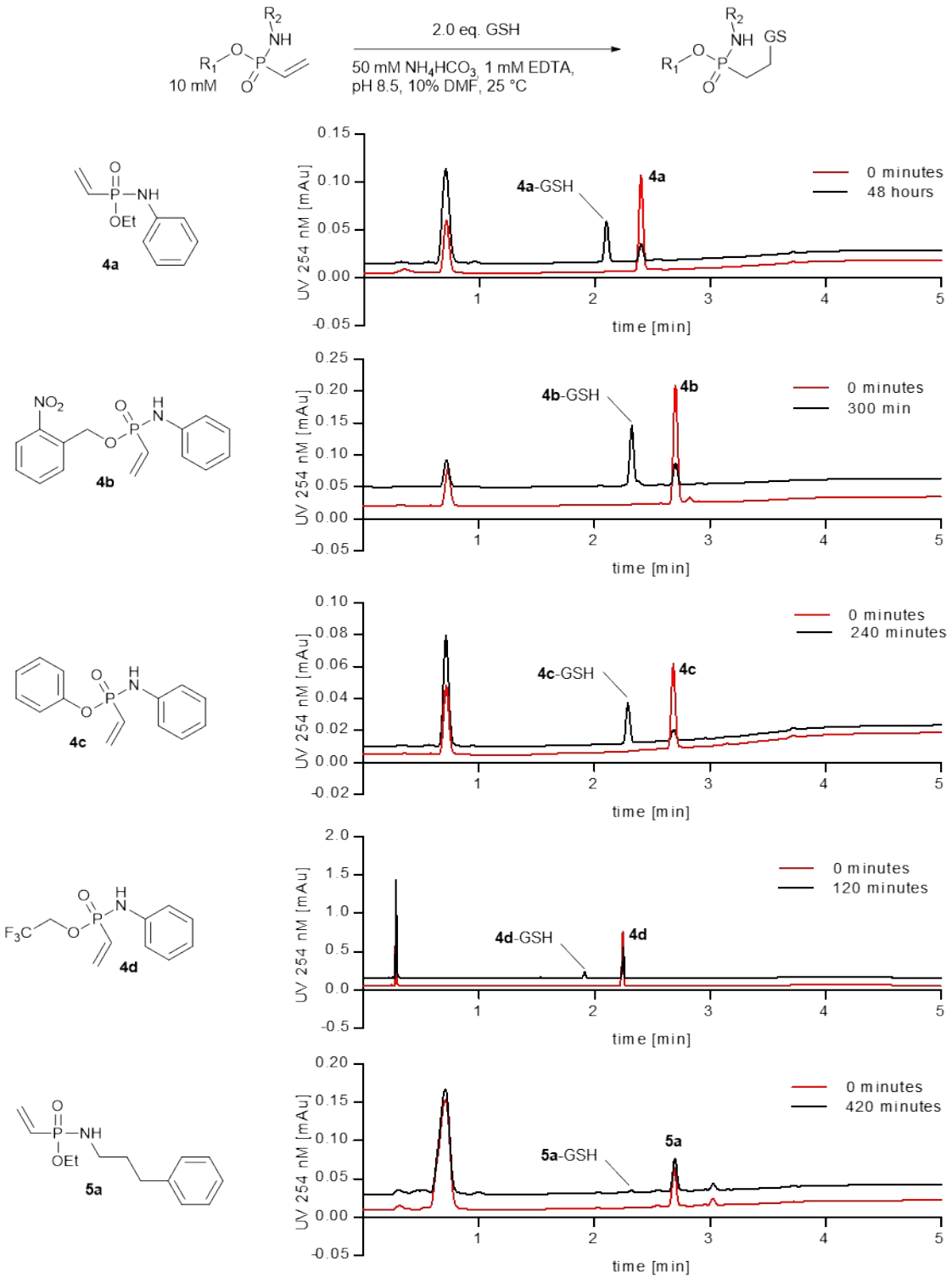


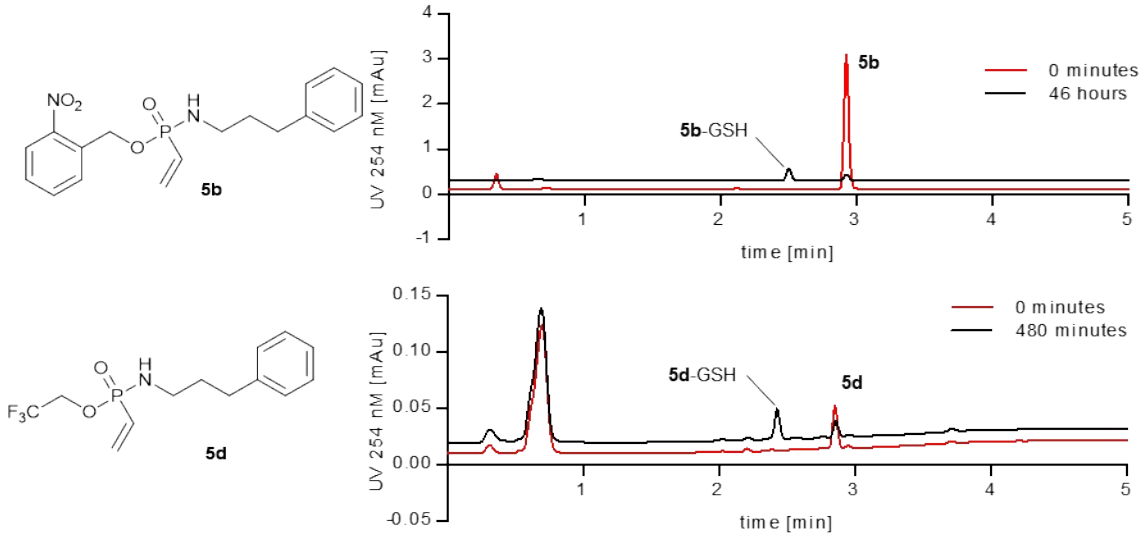
b)

entry	R_1	R_2	Comp No.	time until 50% conversion [min]
1			**4a**	650
2			**5a**	n.d.
3			**4b**	160
4			**5b**	900
5			**4c**	70
6			**4d**	30
7			**5d**	360

1.2. Figure S2

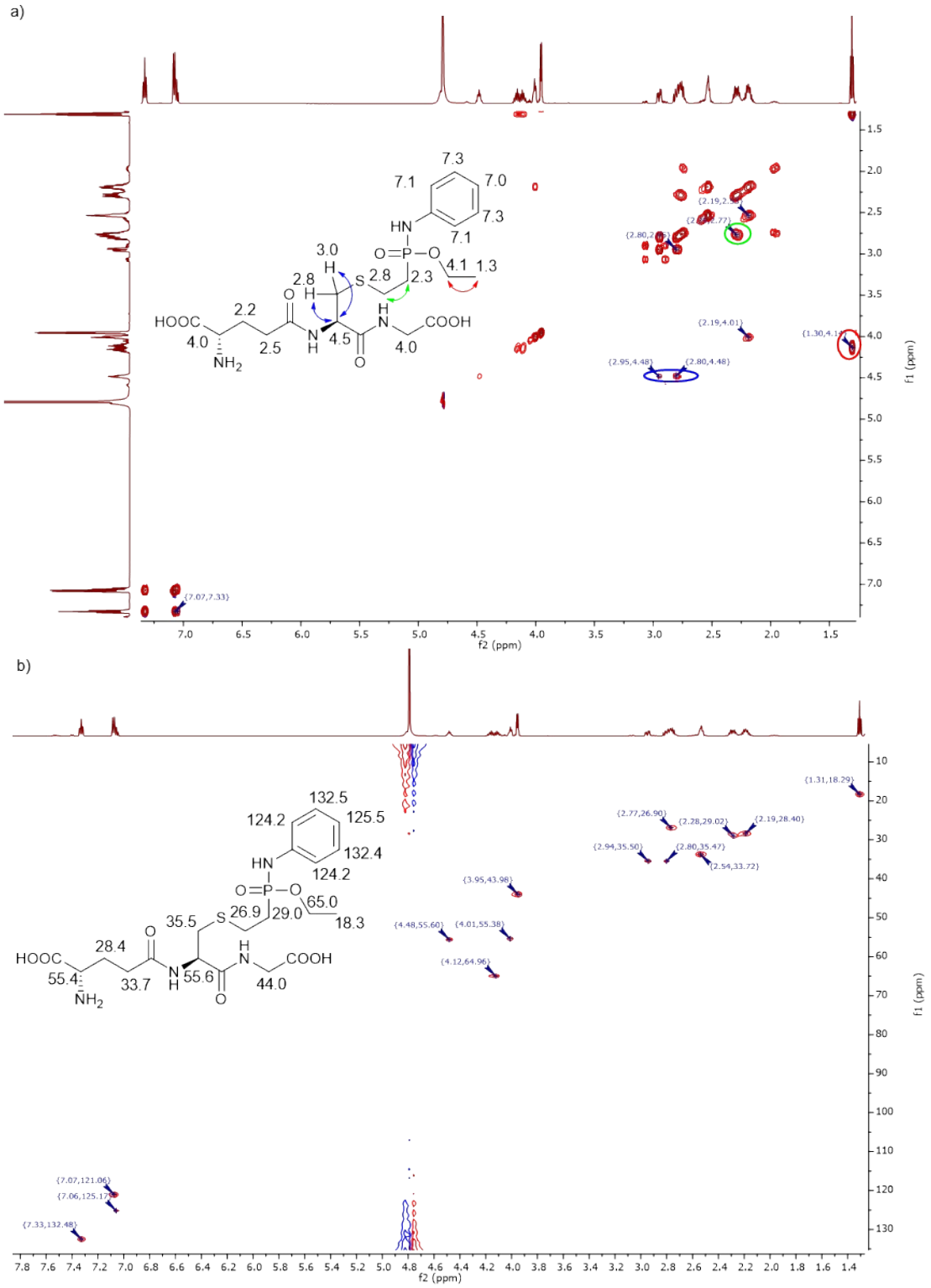
UPLC traces of the reaction of compounds **4a**, **4b**, **4c**, **4d**, **5a**, **5b** and **5d** before (red) and after the reaction (black) with two equivalents of glutathione, as stated in chapter 3.1. Shown are the traces of t=0 and the last time point of the measurements that have been performed for the time-course curves in Fig. 2 and S1. The appearance of a single product-peak for each of the compounds suggests a clean conversion to the desired cysteine adduct without side reactions. Peaks were assigned by MS.

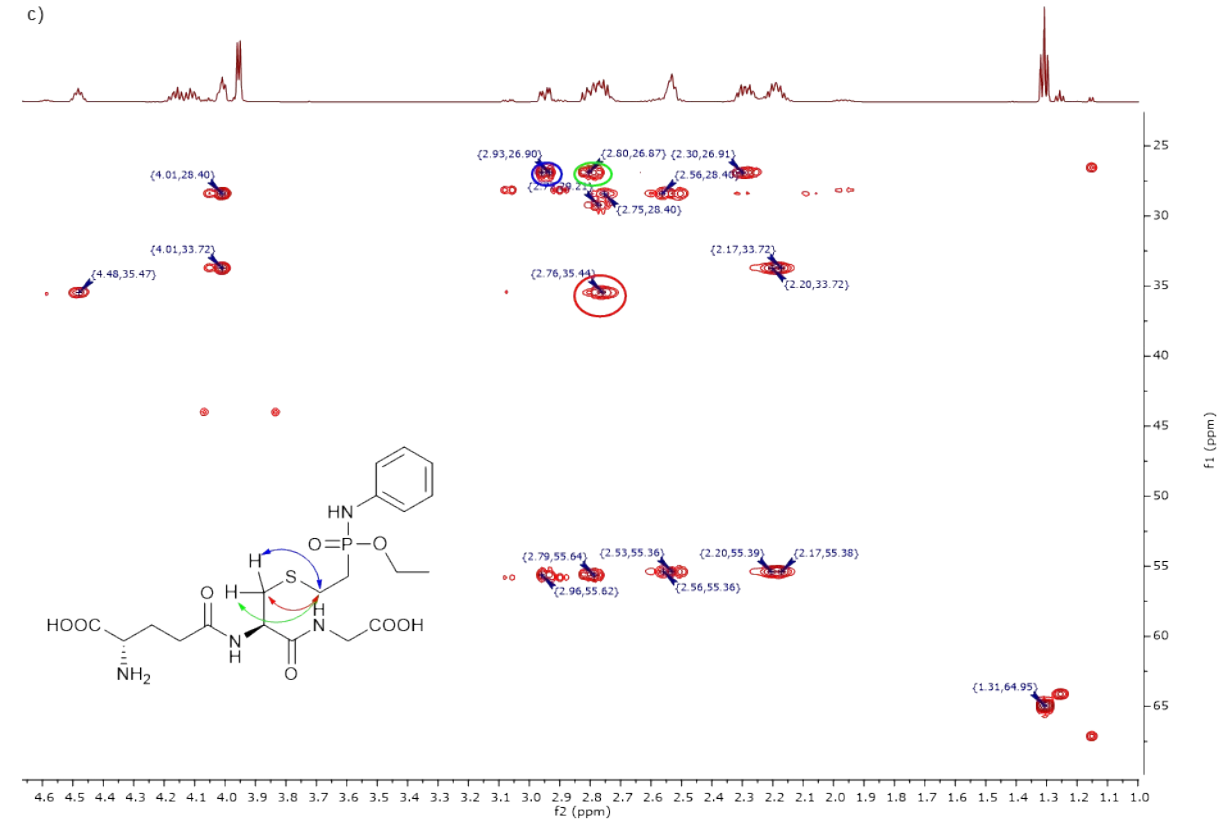


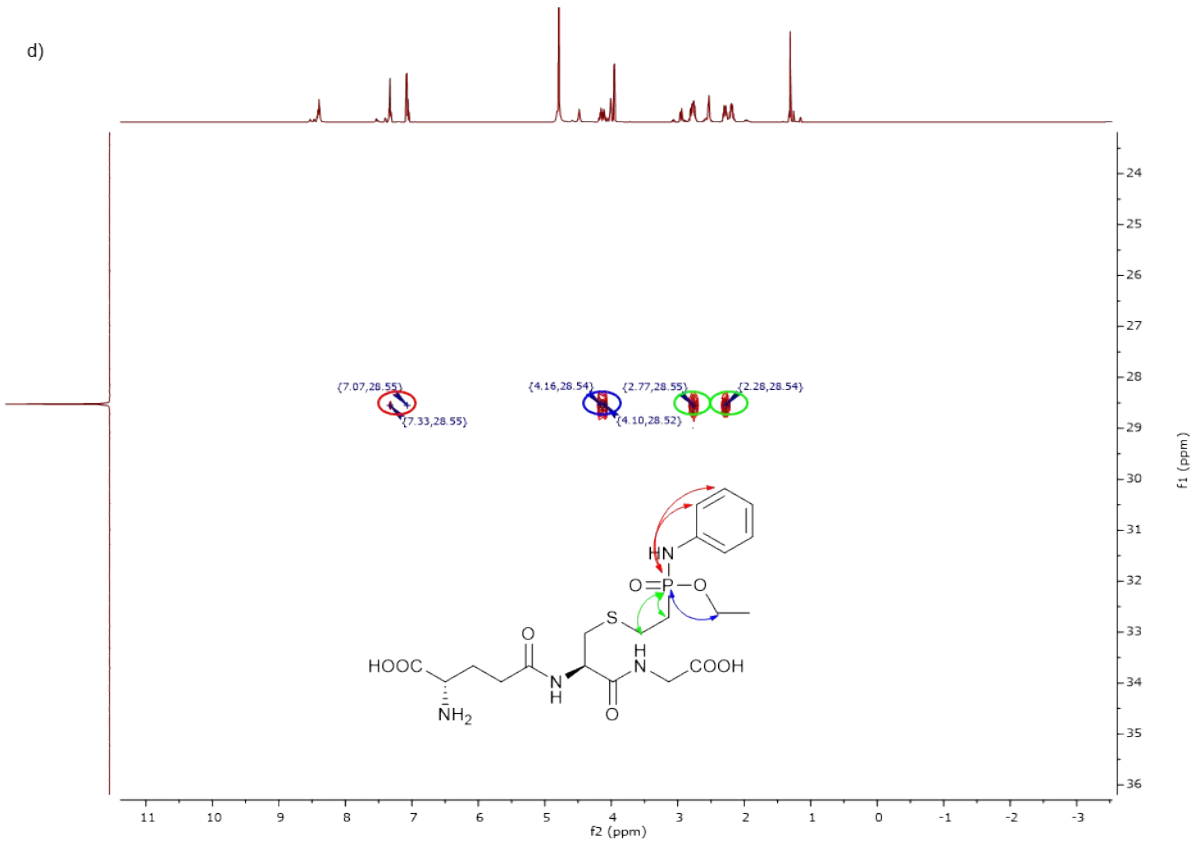

NO2
O
P
H
N
O
5b
UV 254 nM [mAu]
4
3
2
1
0
-1
5b
5b-GSH
0 minutes
46 hours
1
2
3
4
5
time [min]
F3C
O
P
O
H
N
5d
UV 254 nM [mAu]
0.15
0.10
0.05
0.00
-0.05
5d-GSH
5d
0 minutes
480 minutes
1
2
3
4
5
time [min]

1.3. Figure S3

Full assignment of the NMR-shifts of the gluthatione adduct to **4a** by two dimensional experiments. a) ^{1}H-^{1}H-COSY-spectrum with important highlighted correlations that underline the assignments of the ^{1}H-NMR-shifts. b) ^{1}H-^{13}C-HSQC-spectrum and assignment of the measured ^{13}C-NMR-shifts. c) ^{1}H-^{13}C-HMBC-spectrum. Three highlighted correlations demonstrate successful conjugation to the cysteine's sulfhydryl-group. d) ^{1}H-^{31}P-HMBC-spectrum. Four highlighted correlations underline that all three phosphorus substituents (EtO-, RCH_2- and PhNH-) are attached to the phosphorus core structure.



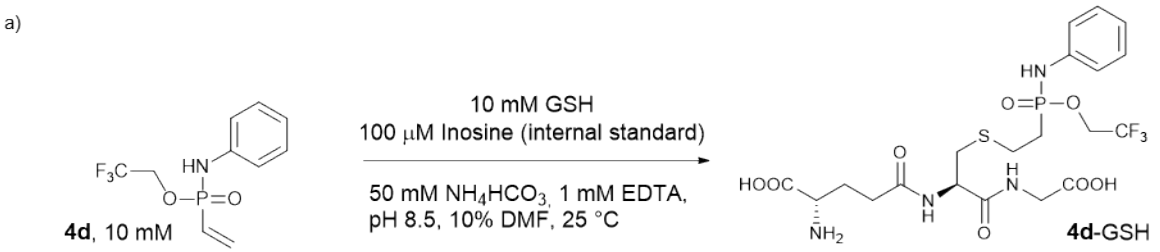

c)
{4.01,28.40}
{2.93,26.90}
{2.60,26.87}
{2.30,26.91}
{2.56,28.40}
{2.75,28.40}
{4.01,33.72}
{4.48,35.47}
{2.76,35.44}
{2.17,33.72}
{2.20,33.72}
{2.79,55.64}
{2.96,55.62}
{2.53,55.36}
{2.56,55.36}
{2.20,55.39}
{2.17,55.38}
{1.31,64.95}
HN
O=P–O
H
S
O
H
HOOC
N
H
NH2
H
N
COOH
O
f2 (ppm)
f1 (ppm)


d)
{7.07,28.55}
{7.33,28.55}
{4.16,28.54}
{2.77,28.55}
{2.28,28.54}
{4.10,28.52}
HN
O=P–O
S
O
H
HOOC
N
H
NH2
N
COOH
O
f2 (ppm)
f1 (ppm)

1.4. Figure S4

Determination of the second-order rate constant of the reaction between glutathione and **4d**. a) Reaction conditions. Reactions were performed as described in chapter 3.1 in a volume of 0.2 ml, as stated in chapter 3.1. The first sample (t=0) was drawn before the addition of glutathione. Samples were taken after 5, 15, 30, 60, and 120 min. Samples were drawn in a volume of 20 µl and immediately diluted into 80 µl of 50 mM NaOAc buffer at pH 3.6 to stop the reaction. b) Mathematic consideration for the determination of a second order rate constant with equal concentrations of the two reactants. c) Concentration of starting material over time. Calculated by integration of the peaks in relation to the internal standard (inosine). Shown are mean and error of three independent measurements. (n=3) d) Graph: 1/c over time and linear plot. Slope is the second order rate constant. Shown are mean and error of three independent measurements.

a)

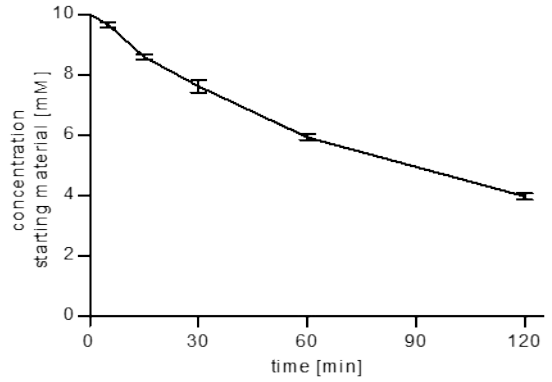


b)

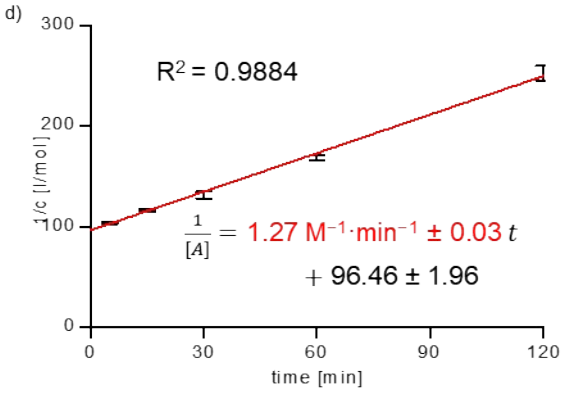
$$v = k \cdot [A] \cdot [B] \qquad \textbf{If } [\mathbf{A}] = [\boldsymbol{B}] \qquad \frac{d[A]}{[A]^2} = k\,dt$$

$$\frac{d[A]}{dt} = k \cdot [A] \cdot [B] \qquad \frac{d[A]}{dt} = k \cdot [A]^2 \qquad \frac{1}{[A]} = k\,t + \frac{1}{[A]_0}$$

c)

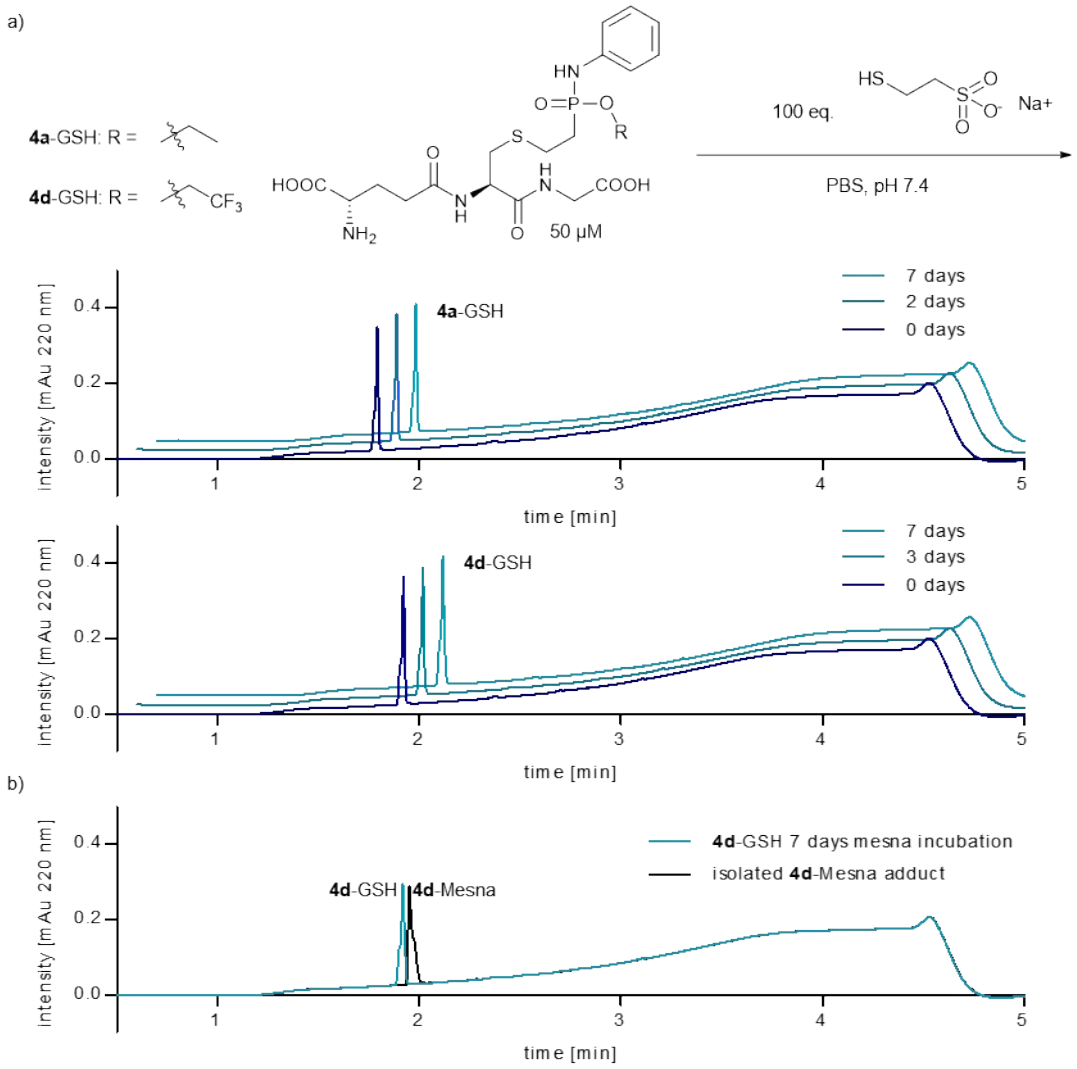


d)



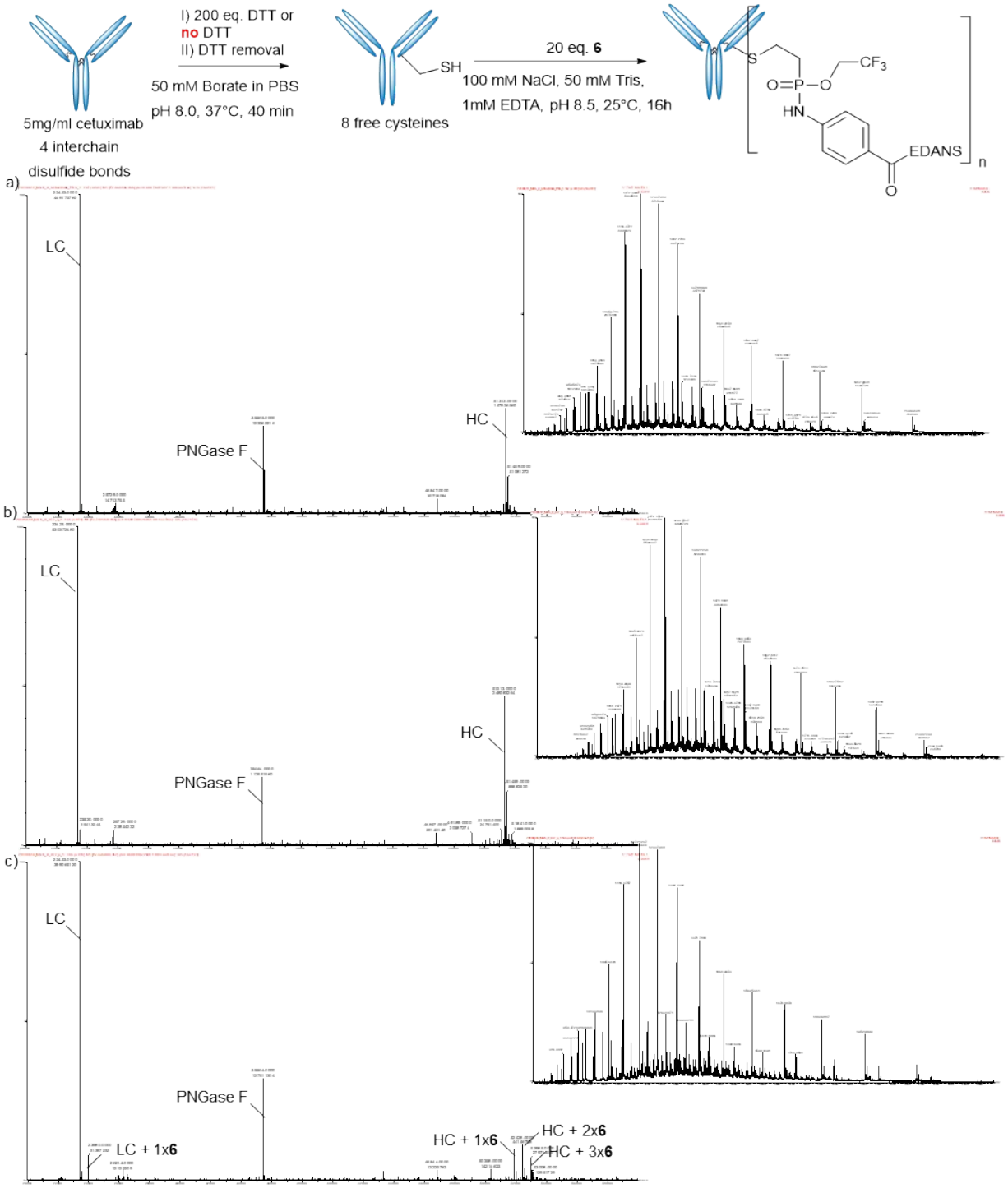
1.5. Figure S5

Incubation of the glutathione adducts of **4a** and **4d** with an excess of mesna to demonstrate stability towards thiol exchange. a) Conjugates were incubated at a concentration of 50 µM for 7 days at room temperature with 5 mM of mesna in PBS (100 eq.) and analyzed by UPLC/MS. Samples of 10 µl were analyzed after day 0, 2 and 7. No Signal was detected by MS over the whole chromatogramm for the **4a** and **4d**- Mesna adduct even after 7 days of incubation. b) Overlay of an isolated Mesna adduct to **4d** (black) and **4d**-GSH incubated with Mesna for 7 days. The difference in retention time underlines that the Mesna adduct is not formed by thiol exchange of **4d**-GSH.



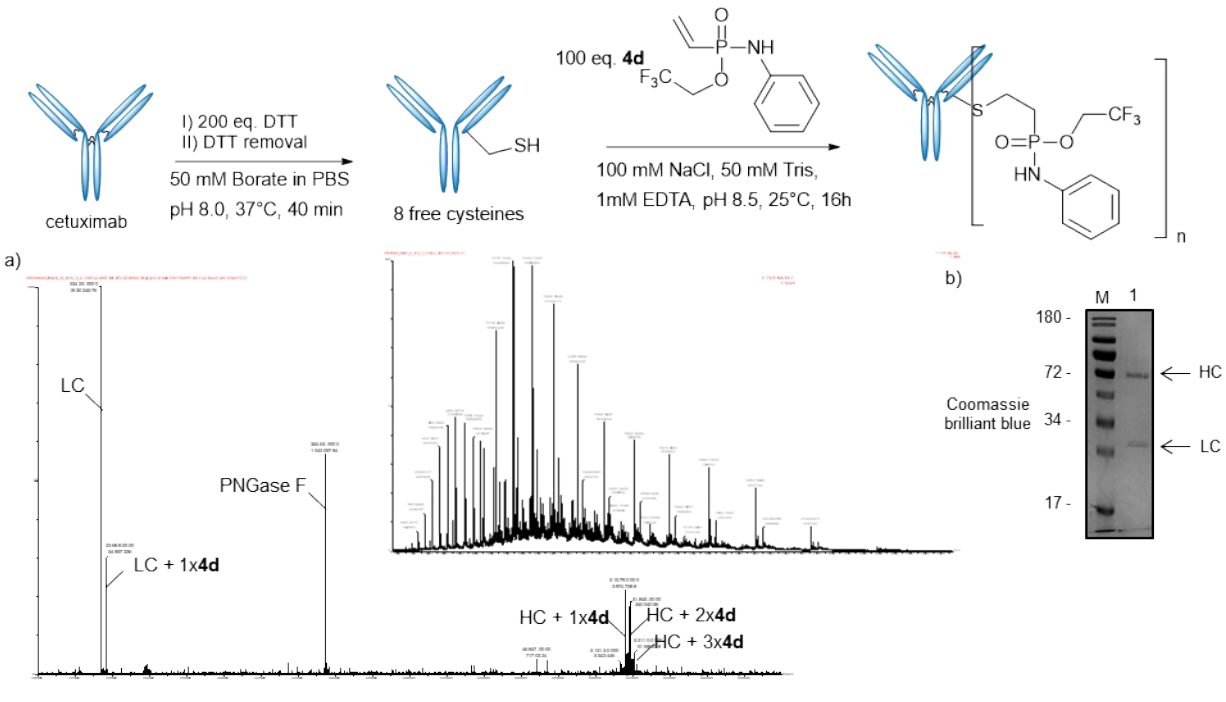
1.6. Figure S6

Modification of cetuximab with **6** and analysis with intact protein MS (See chapter 3.2 for synthesis details). Interchain reduction generates one free cysteine on the light chain and three on the heavy chain. MS-spectrum of cetuximab's heavy and light chain after deglycosylation with PNGase F and reduction with DTT as described in chapter 3.3. Shown are crude spectra before the deconvolution and Deconvoluted between 20000 and 60000 Da. Calculated delta mass for **6**: 557 Da. a) Unmodified cetuximab. b) cetuximab, incubated with 20 eq. **6** without prior reduction. No modification detected for heavy and light chain. c) cetuximab, incubated with 20 eq. **6** with prior reduction. Modification ratio calculated to 3.8 from the MS intensities as stated in chapter 3.3. HC: heavy chain, LC: light chain.



1.7. Figure S7

MS/MS experiments of cetuximab, modified with 100 eq. **4d** with prior reduction of the antibody as described in chapter 3.2. a) MS-spectrum of cetuximab's heavy and light chain after deglycosylation with PNGase F and reduction with DTT as described in chapter 3.3 to proof successful antibody modification. b) SDS Page analysis of the modified antibody. In-gel digestion for tandem-MS has been executed as described earlier.[1] Bands for HC and LC were excised and digested together. c) MS/MS results. Sequence coverage was determined using MASCOT. The phosphonamidate (delta-mass of **4d**) was searched on Y, S, T, C, K, H & R as variable modifications using maxquant. Aminoacids with a Probability of more than 0.7 are highlighted with a red M. (See chapter 2.9 for MS/MS details).



c)

Heavy Chain: Sequence coverage: 75%

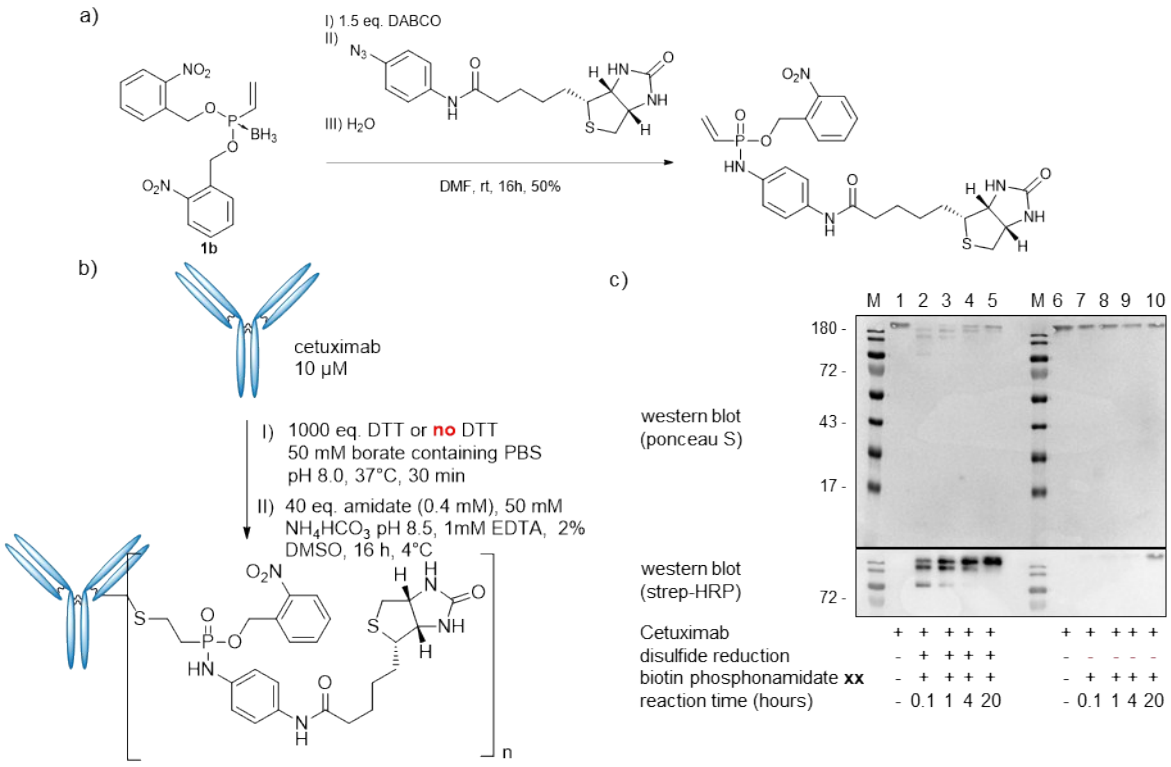
```
1   QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSGGNTDYNTPFTSRLSINKDNSKSQVFFKMNSLQSNDTAIYYCARALT
                                                  M  M
101 YYDYEFAYWGQGTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTY
                                 M
201 ICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS
                                    M
301 TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL

401 DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
```

Light chain: Sequence coverage: 89%

```
1   DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQNNNWPTTFGA
                                  M                M
101 GTKLELKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
                M
201 LSSPVTKSFNRGEC
```

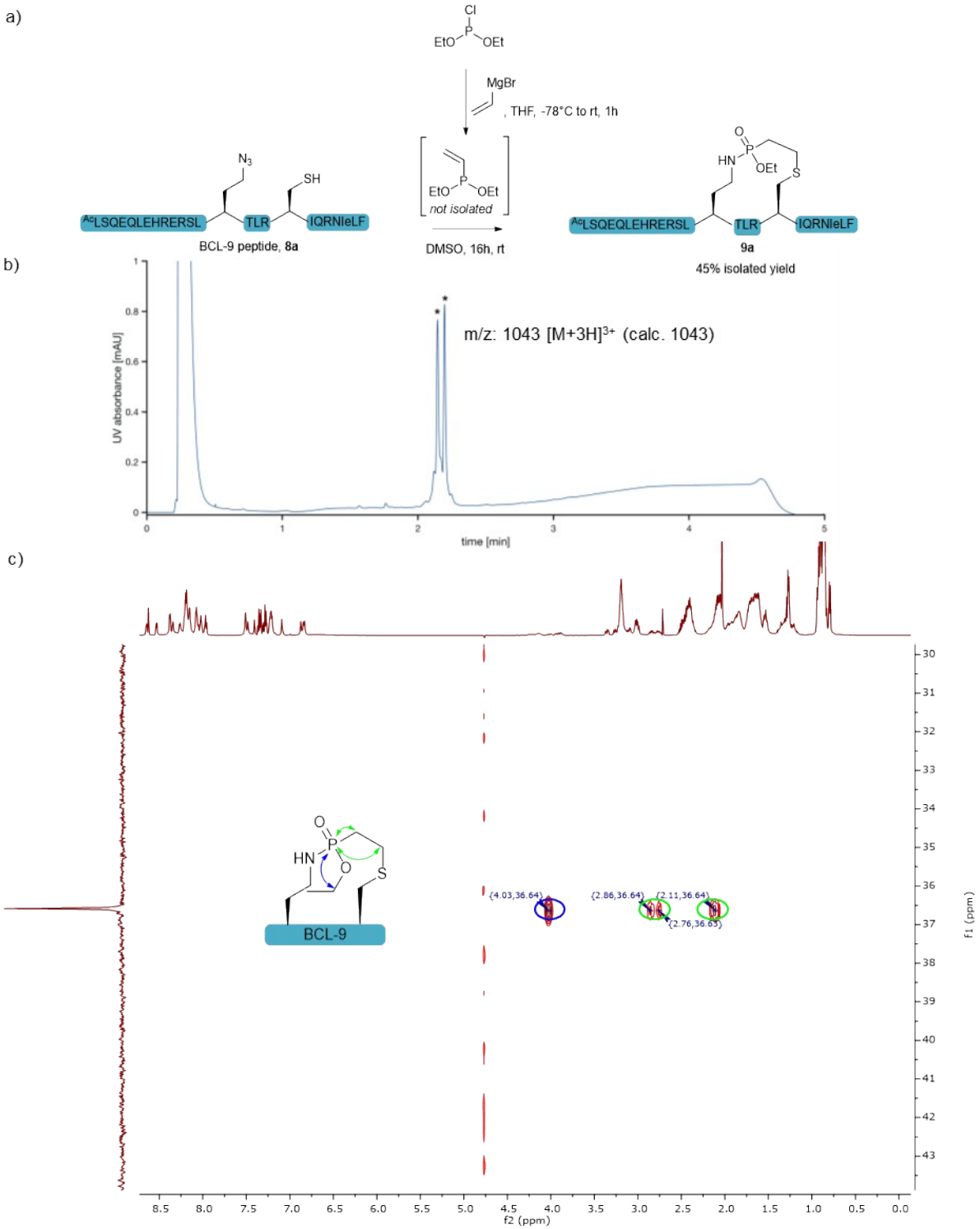
1.8. Figure S8

Antibody modifications with vinylphosphonamidates by reduction and alkylation of interchain disulfide bonds as described in chapter 3.4. a) Synthesis of a biotin modified O-2-nitrobenzyl vinylphophonamidate that can be detected by western blotting. b) Principle of the antibody modification procedure. Reaction was carried out either with or without prior disulfide reduction as previously described.[2] c) Western blot analysis: Lane 1 and 6: untreated antibody. Lane 2-5: prior DTT treatment. Lane 7-10: Control reactions without prior DTT treatment.



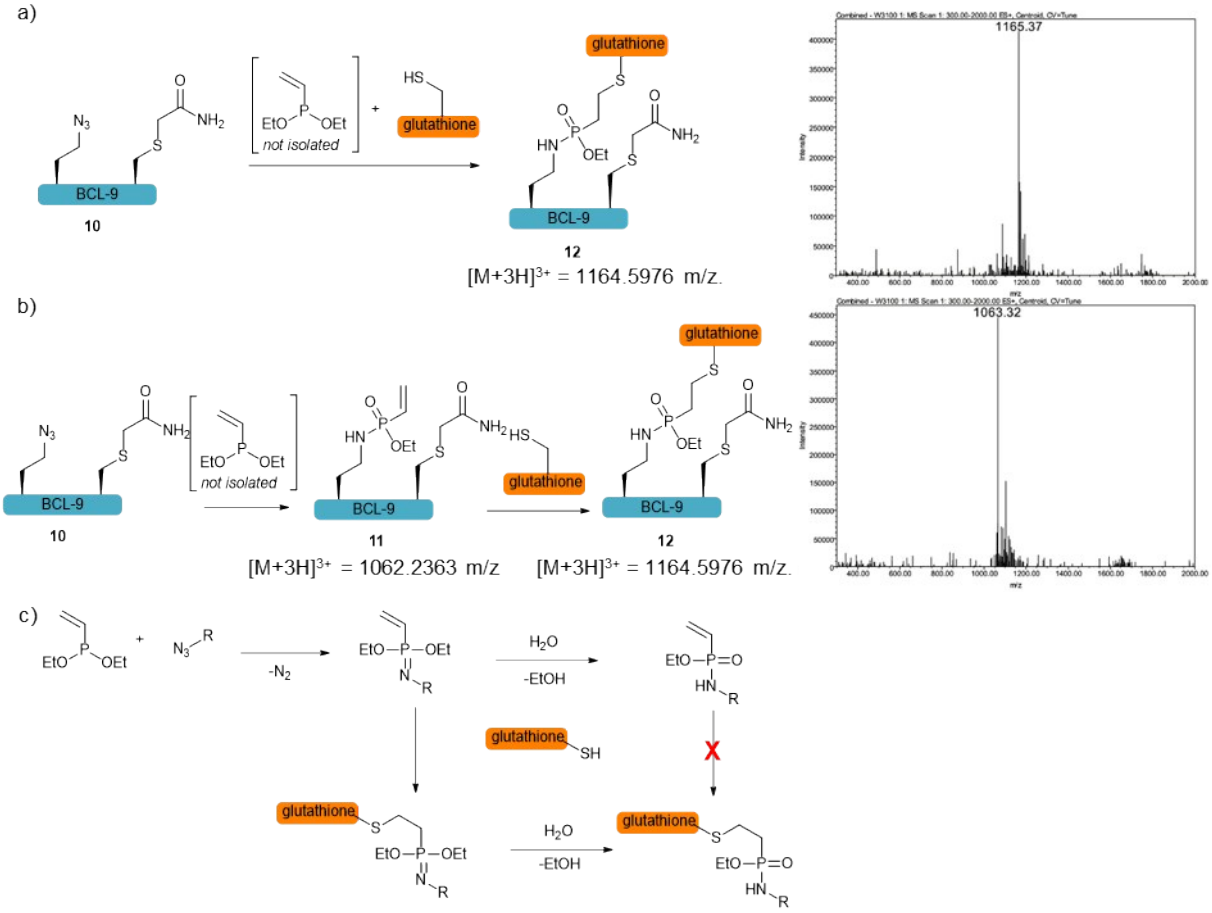
1.9. Figure S9

Reaction of azide and cysteine containing BCL-9 with a vinylphosphonite yields the cyclized product **9a**. a) Reaction scheme. b) UPLC-chromatogramm of the crude reaction mixture shows a clean conversion to two diastereoisomers (*racemic stereocenter at phosphorus). The mass fits the desired product. C) HMBC correlating ^{31}P-NMR and ^{1}H-NMR for the isolated product of the reaction. ^{31}P-shift matches the thiol addition product. In addition, ^{31}P-Correlations were assigned to the adjacent O-CH_2 and two CH_2 groups, which can only be present after thiol addition to the double bond of the vinylphosphonamidate. The correlation pattern matches the fully characterized and assigned NMR-Spectra of the GSH-thiol adduct to **4a** (See Figure S1).



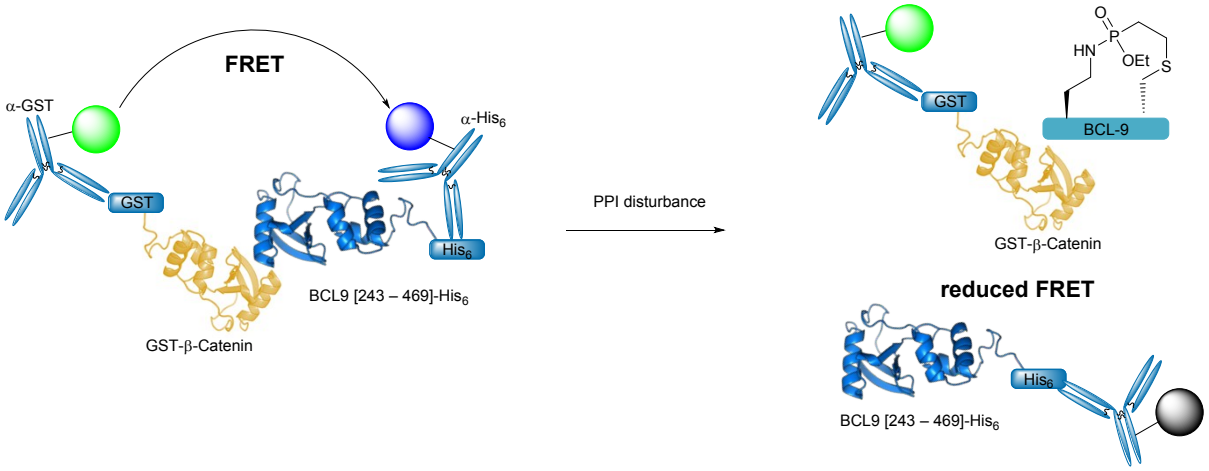
1.10. Figure S10

Thiol addition of unactivated vinylphosphonamidates proceeds with an intermediate of the SPhR. Depicted are mass-spectra after the respective reaction sequences. a) *In situ* addition of glutathione to the SPhR. b) Incubation of isolated phosphonamidate **11** with glutathione. c) Mechanistic assumption: Accelerated thiol addition to an intermediated of the SPhR such as the phosphonimidate. Mechanism adapted from Vallee et al.[3]



1.11. Figure S11

Principle of the HTRF assay. A FRET system is created between two fluorescently labeled antibodies recognizing a GST-tag on the β-Catenin and a His-tag on the BCL9 protein. If the proteins interact a FRET signal is generated upon antibody incubation that vanishes in case of disturbed interaction by a BCL-9 stapled peptide for instance. Protein expression and purification was performed as described in chapter 3.2 and 3.3. HTRF assay was performed as described in chapter 3.4.



2. General Information

2.1. Chemicals and solvents

Chemicals and solvents were purchased from Merck (Merck group, Germany), TCI (Tokyo chemical industry CO., LTD., Japan) and Acros Organics (Thermo Fisher scientific, USA) and used without further purification. Dry solvents were purchased from Acros Organics (Thermo Fisher scientific, USA). Amino acids and resins for SPPS were purchased from Novabiochem (Merck, USA) or Iris Biotech GmbH (Germany).

2.2. Flash- and thin layer chromatography

Flash column chromatography was performed, using NORMASIL 60® silica gel 40-63 µm (VWR international, USA). Glass TLC plates, silica gel 60 W coated with fluorescent indicator F254s were purchased from Merck (Merck Group, Germany). Spots were visualized by fluorescence depletion with a 254 nm lamp or manganese staining (10 g K_2CO_3, 1.5 g $KMnO_4$, 0.1 g NaOH in 200 ml H_2O), followed by heating.

2.3. Preparative HPLC

Preparative HPLC was performed on a Gilson PLC 2020 system (Gilson Inc, WI, Middleton, USA) using a VP 250/32 Macherey-Nagel Nucleodur C18 HTec Spum column (Macherey-Nagel GmbH & Co. Kg, Germany). The following gradient was used: A = H_2O + 0.1% TFA (trifluoroacetic acid), B = MeCN (acetonitrile) + 0.1% TFA, flow rate 30 ml/min, 5% B 0-5 min, 5-90% B 5-60 min, 90% B 60-65 min

2.4. NMR

NMR spectra were recorded with a Bruker Ultrashield 300 MHz spectrometer and a Bruker Avance III 600 MHz spectrometer (Bruker Corp., USA) at ambient temperature. Chemical shifts δ are reported in ppm relative to residual solvent peak ($CDCl_3$: 7.26 [ppm]; DMSO-d6: 2.50 [ppm]; acetone-d6: 2.05 [ppm]; CD_3CN 1.94 [ppm]; 4.79 D_2O [ppm] for ^1H-spectra and $CDCl_3$: 77.16 [ppm]; DMSO-d6: 39.52

[ppm]; acetone-d6: 29.84 [ppm]; CD3CN 1.32 [ppm]; for ^{13}C-spectra . Coupling constants *J* are stated in Hz. Signal multiplicities are abbreviated as follows: s: singlet; d: doublet; t: triplet; q: quartet; m: multiplet.

2.5. UPLC-UV/MS

UPLC-UV/MS traces were recorded on a Waters H-class instrument equipped with a quaternary solvent manager, a Waters autosampler, a Waters TUV detector and a Waters Acquity QDa detector with an Acquity UPLC BEH C18 1.7 µm, 2.1 x 50 mm RP column with a flow rate of 0.6 mL/min (Waters Corp., USA). The following gradient was used: A: 0.1% TFA in H_2O; B: 0.1% TFA in MeCN. 5% B 0 - 0.5 min, 5-95% B 0.5-3 min, 95% B 3-3.9 min, 5% B 3.9-5 min.

2.6. SPPS

SPPS was carried out manually or on a Tribute-UV peptide synthesizer (Protein technologies, USA) via standard Fmoc-based protocols.

2.7. HR-MS

High resolution ESI-MS spectra were recorded on a Waters H-class instrument equipped with a quaternary solvent manager, a Waters sample manager-FTN, a Waters PDA detector and a Waters column manager with an Acquity UPLC protein BEH C18 column (1.7 µm, 2.1 mm x 50 mm). Samples were eluted with a flow rate of 0.3 mL/min. The following gradient was used: A: 0.01% FA in H_2O; B: 0.01% FA in MeCN. 5% B: 0-1 min; 5 to 95% B: 1-7min; 95% B: 7 to 8.5 min. Mass analysis was conducted with a Waters XEVO G2-XS QTof analyzer.

2.8. Intact protein MS

Intact proteins were analyzed using a Waters H-class instrument equipped with a quaternary solvent manager, a Waters sample manager-FTN, a Waters PDA detector and a Waters column manager with an Acquity UPLC protein BEH C4 column (300 Å, 1.7 µm, 2.1 mm x 50 mm). Proteins were eluted with a flow rate of 0.3 mL/min. The following gradient was used: A: 0.01% FA in H_2O; B: 0.01% FA in MeCN. 5-95% B 0-6 min. Mass analysis was conducted with a Waters XEVO G2-XS QTof analyzer. Proteins were ionized in positive ion mode applying a cone voltage of 40 kV. Raw data was analyzed with MaxEnt 1.

2.9. Analytical HPLC-MS/MS

After in-gel digestion peptides were dissolved in water and analyzed by a reversed-phase capillary liquid chromatography system (Dionex Ultimate 3000 NCS-3500RS Nano, Thermo Scientific) connected to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, Germany). LC separations were performed with an in-house packed C18 column for reversed phase separation (column material: Poroshell 120 EC-C18, 2.7 µm (Agilent Technologies, USA) at an eluent flow rate of 300 µL/min using a gradient of 2-40% B in 38 min. Mobile phase A contained 0.1% formic acid in water, and mobile phase B 0.1% formic acid in acetonitrile. FT survey scans were acquired in a range of 350 to 1500 m/z with a resolution of 60000 (FMHM) and an AGC target value of 4e5. Precursor ions with charge states 2-4 were isolated with a mass selecting quadrupole (isolation window m/z 1.6). Precursor ions were fragmented in alternating mode using EThcD and HCD. HCD MS/MS spectra were acquired with 30% NCE. EThcD fragmentation was performed using charge dependent ETD parameters and the supplemental activation (sa) was set to 30%. For both fragmentation types the maximum injection time was set to 500 ms to collect 5e4 precursor ions. Fragment ion spectra were acquired with a resolution of 15000 (FWHM).

2.10. MALDI-TOF-MS

Spectra were measured on a Bruker LT microflex using a 2,5-DHAP matrix. Proteins were precipitated in cold acetone and resolved in 10 mM Ammoniumbicarbonate buffer at pH 7.4. Protein sample (0.8 µl) was mixed with 2% TFA in water (0.8 µl) and matrix (0.8 µl). After addition of matrix the solution was transferred a MSP 96 target (polished steel BC).

2.11. Protein concentration determination

Protein concentrations were determined by absorption spectroscopy measurements at 280 nm using the extinction coefficient and molecular weight of the protein on a NanoDrop ND-1000. In addition or as alternative concentrations were determined by BCA assay (Thermo Fisher Scientific, USA) according to the manufacturers protocol.

2.12. Protein purification

Protein purification was accomplished either with an ÄKTA FPLC or a BioRad NGC system as stated below.

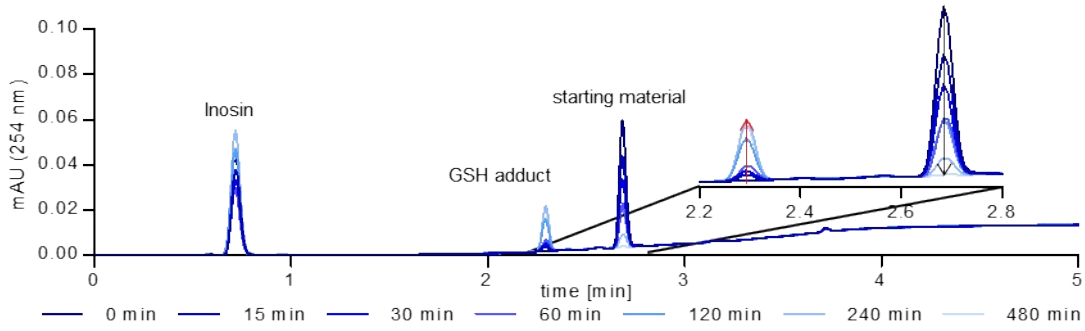
2.13. CD-spectroscopy

CD-spectroscopy was measured on a Jasco J-720 spectropolarimeter at 20°C and parameters set to: measured wavelength range 190 – 260 nm; data pitch of 0.1 nm; continuous scanning mode; 100 nm/min scanning speed; 1sec. response; 1.0 nm band width; 0.1 cm cell length; 5 accumulations.

3. Experimental procedures

3.1. Glutathione addition to differently substituted vinylphosphonamidates and analysis by UPLC/MS

Reaction buffer: 50 mM NH_4HCO_3, 1 mM EDTA pH 8.5. In an Eppendorf tube, 11 µl of a 200 mM phosphonamidate solution in DMF was mixed with 143 µl reaction buffer and 22 µl of a 10 mM solution of Inosin in a mixture of DMF and reaction buffer (1:1). A 16 µl sample was drawn for t=0. 40 µl of a solution containing 100 mM glutathione in reaction buffer (50 mM for rate constant determination), adjusted to pH 8.5 was added to the mixture to start the reaction. Sample were drawn from the reaction mixture after distinct time points and immediately diluted into 180 µl of a 50 mM NaOAc buffer at pH 3.5 to stop the reaction and subjected to UPLC analysis (10 µl per injection). Conversions were calculated by integration of the Peaks in relation to an internal standard (inosin). Peaks were assigned by MS. Exemplaric chromatograms of the reaction of glutathione with *O*-Phenyl-*N*-phenyl-*P*-vinyl-phosphonamidate (**4c**) after 0, 15, 30, 60, 120, 240 and 480 minutes are shown below.



3.2. Cetuximab modification with 4d or 6

5 µl of a 70 mM solution of DTT in 50 mM sodium borate in PBS (pH 8.0) was added to 50 µl of a cetuximan solution of 5.0 mg/ml in 50 mM sodium borate in PBS (pH 8.0) and the mixture was incubated at 37 °C for 40 min. Excess DTT removal and exchange to the conjugation buffer (50 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8.5 at 14°C) was conducted afterwards using 0.5 mL Zeba™ Spin Desalting Columns with 7K MWCO (Thermo Fisher Scientific, USA). 50 µl of the reduced antibody solution (4.55 mg/ml, 30.30 µM antibody) were mixed quickly afterwards with desired amount of **4d** or **6** dissolved in DMSO to give a final amount of 5% DMSO. The mixture was shaken at 850 rpm and 25 °C for 16 hours.
The crude antibody modification mixtures were purified by size-exclusion chromatography with a 5 ml HiTrap® desalting column and a flow of 1.5 ml/min eluting with PBS over four column volumes. The antibody containing fractions were pooled and concentrated to 50 µl by spin-filtration to 40 µl (MWCO: 10 kDa, 0.5 ml, Sartorius, Germany).

3.3. Antibody analyses

2 µl of the samples, that were prepared as described in 3.2 were subjected to SDS-PAGE analysis.

For MS analysis, 5 µl of the samples were diluted with 15 µl PBS and 1 µl PNGase-F solution (Pomega, Germany, Recombinant, cloned from Elizabethkingia miricola 10 u/µl) was added and the solution was incubated at 37 °C for at least 2 hours. Disulfide bridges were reduced by addition of 2 µl DTT solution (70 mM in H_2O) and incubation at 37°C for 30 min. 10 µl of the samples were diluted with 190 µl of pure water and subjected to intact protein MS (see chapter 2.8), injecting 3 µl for each sample.

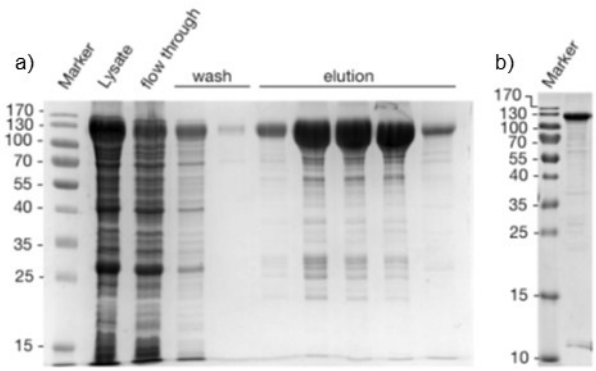
3.4. Cetuximab modification with biotin

Cetuximab modification was carried out by incubating the antibody (typical concentration c = 1.5 mg/ml, 10 µM) in a buffer containing 50 mM sodium borate and 10 mM DTT in PBS (pH 8.0) with a total volume of 80 µl at 37 °C for 30 min. Control reactions were carried out without DTT. Excess DTT removal and buffer exchange to a solution containing 50 mM NH_4HCO_3 and 1mM EDTA (pH 8.5) was conducted afterwards using 0.5 mL Zeba™ Spin Desalting Columns with 7K MWCO (Thermo Fisher Scientific, USA). 1.0 µl of a solution containing 20 mM vinyl phosphonamidate (40eq.) in DMSO was added quickly to 50 µl of the reduced antibody solution. 10 µl samples were drawn and deep frozen in liquid nitrogen to stop the reaction after 10 minutes, 1, 4 and 20 hours. 1.0 µl of the samples were subjected to SDS/PAGE and subsequent western-blot analysis.

3.5. Protein expression β-Catenin-GST

β-Catenin-GST (pGEX-Bcatfl was a gift from David Rimm (Addgene plasmid #24193)) was expressed in *E.coli* BL21 (DE3) with LB medium including 100 µg/ml Ampillicin. Cells were grown at 37°C at 180 rpm until OD_{600} reached 0.73 followed by induction with 0.2 mM IPTG and expression at 18°C at 180 rpm for 16 hours.

The cells were harvested by centrifugation at 4000 g for 15 minutes at 4°C. Cell lysis was carried out in Dulbecco's PBS with a sonicator at 30% for six minutes. Finally debris was centrifugation at 27000 g for 20 minutes. β-Catenin-GST was purified with a Bioscale™ Mini Profinity™ GST cartridge (5 ml) on a BioRad NGC system (Binding/Wash buffer: 50 mM Tris HCl pH 8.0/ 150 mM NaCl/ 0.1 mM EDTA; elution buffer: 50 mM Tris HCl pH 8.0/ 150 mM NaCl/ 0.1 mM EDTA/ 10 mM reduced glutathione). After elution the peak fractions were combined and dialyzed to Dulbecco's PBS. The protein was aliquoted at a concentration of 0.87 mg/ml, shock frozen and stored at -80°C. a) Purification of GST- β-Catenin. b) Pooled elution fractions rebuffered to PBS.



3.6. His_6-BCL9 (243 – 469) production

The cDNA for BCL9 was purchased from biocat (Gene ID 607) in a pCR-BluntII-TOPO vector.

PCR:

The Gene region of interest was amplified with BamHI and HindII restriction sites by PCR using complementary primer pairs (for conditions see table 8.4 and 8.5) (GTAGTGGATCCAACCAGGACCAGAATTCTTC and ATACGAAGCTTTTAC TGCTCGGGAGTCATATGGT) for cloning into the pET28a vector. Two PCRs were done in parallel.

Reaction mix of PCR:

ddH_2O	13.6 µl
Phu HF buffer (5x) (Thermo scientific, USA)	4 µl
template DNA (1 ng/µl)	1 µl
fwd primer mix (10 µM)	0.4 µl
rev primer mix (10 µM)	0.4 µl
dNTP mix (10 mM each) (Thermo scientific, USA)	0.4 µl
Phusion polymerase (Thermo scientific, USA)	0.2 µl

Cycle steps in mutagenesis PCR:

step	cycles	temp.[°C]	time
initial denaturation	1	98	30''
denaturation		98	10''
annealing	30	58	30''
extension		72	3'
final extension	1	72	10'
hold		4	∞

Digestion with restriction endonucleases

The PCR product as well as the target vector pET28a was digested with the restriction endonucleases BamHI and HindII for 70 minutes at 37°C. To the vector digestion, 1 µL of FastAP Themosensitive Alkaline Phosphatase (1 U/µl) was added and again incubated at 37°C for 15 minutes.

Reaction mix of restriction digestion:

	PCR product	vector
DNA	2 x 20 µl	16 µl (1 µg)
FastDigest™ buffer (10x)	5 µl	2
BamHI enzyme (10 U/µl)	1 µl	1
HindII enzyme (10U/µl)	1 µl	1
ddH_2O	3 µl	-

DNA purification

DNA fragments were purified by agarose gel electrophoresis (1% agarose in TAE buffer) at 120 V in 1x TAE buffer for 20 minutes. The samples were mixed with MidoriGreen Direct before loading, for visualization purposes. GeneRuler 1kb Plus DNA Ladder was used as size marker. The target DNA bands were excised from the agarose gel with a clean scalpel and purified with a GeneJET™ Gel Extraction Kit according to the manufacturer's protocol, but was eluted with 20µl ddH_2O contrary to the included elution buffer.

DNA ligation

The Ligation between vector and PCR product was done with T4 DNA ligase (Thermo Scientific USA) and the T4 Buffer (Thermo Scientific USA). The vector to insert ration was either 1:5 or 1:10 and was calculated according to length of both DNA parts and their respective concentration. The reation mixture was incubated at 16 °C for 16 hours and directly transformed into competent DH5α cells.

Reaction mix of Ligation:

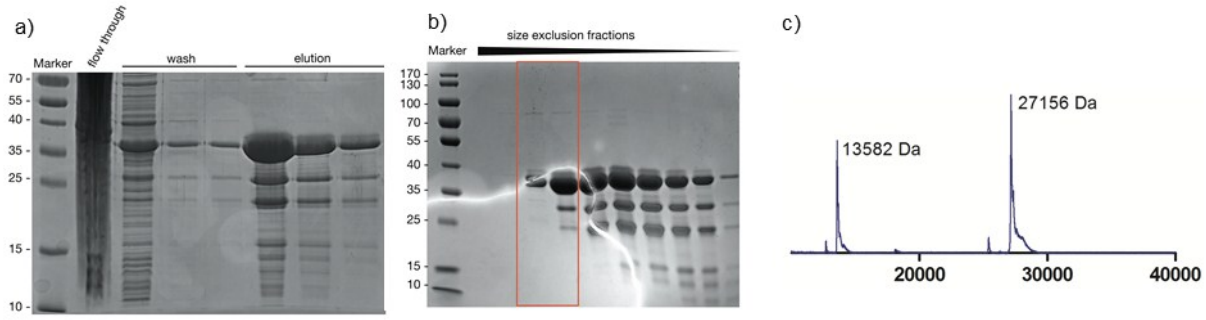
vector DNA	50 – 100 ng
insert DNA	variable
T4 buffer (10x) (Thermo scientific, USA)	2 µl
T4 DNA ligase (Thermo scientific USA)	1 µl
ddH_2O	adjust to 20 µl

Clones were picked and cultured over night in LB_{Kana} at 37°C; 180 rpm and DNA was isolated with a GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA) according to manufacturer's protocol, but again elution was done in 30 µl ddH_2O. DNA concentration was determined by Nanodrop and send to sequencing. DNA was stored at -20°C.

Protein expression and purification

Finally His-BCL9 (243 – 469) was expressed in *E.coli* BL21 (DE3) with LB medium including 100 µg/ml kanamycin. Cells were grown at 37°C at 180 rpm until OD_{600} reached 0.7 followed by induction with 0.5 mM IPTG and expression at 18°C at 180 rpm for 16 hours.

The cells were harvested by centrifugation at 4000 g for 15 minutes at 4°C. Cell lysis was carried out in Dulbecco's PBS with a sonicator at 30% for six minutes. Finally debris was centrifugation at 27000 g for 20 minutes. His-BCL9 (243 – 469) purified *via* 5 ml HisTrap™ FF column on a BioRad NGC system. Elution was performed in PBS with 500 mM Imidazole. Elution fractions containing the wanted protein were combined and further purified by size exclusion chromatography using a Superdex 75 10/300 GL column on a ÄKTA FPLC system. The product fractions were combined and concentrated with a Vivaspin 20 filter unit (MWCO 10 kDa) then shock frozen and stored at -80°C. a) Purification of BCL9-His6 *via* Ni-NTA beads. b) Further purification *via* SEC. D) MALDI-TOF spectra of pooled fractions.



3.7. Homogeneous Time Resolved Fluorescence (HTRF) energy transfer assay

Beta-catenin-GST (7,5 nM) was first mixed with peptides at different peptide concentrations in PBS with 0.05% Tween 20, in reaction volumes of 5 µl in 384-well microtiter plates (ProxyPlate, PerkinElmer), followed by addition of BCL9-His (125nM), Anti-GST-Terbium (CisBio; 1:100) and Anti-His-XL665 (CisBio; 1:100) in PBS with 0.05% Tween 20 at final reaction volumes of 10 µl. Final assay concentrations were 3,75 nM for beta-catenin, 62,5 nM for BCL-9 and contained 1:200 dilutions for each antibody in PBS with 0,05% Tween-20. After mixing on a plate shaker for 15 seconds at 1500 rpm the plate was incubated for one hour at room temperature. The plate was read in an Envision plate reader using excitation at 320 nm and emission reading at 620/665 nm wavelengths using the prism software for data plotting. Positive control wells represent a mixture of both proteins and both antibodies. Negative control wells did not contain beta-catenin.

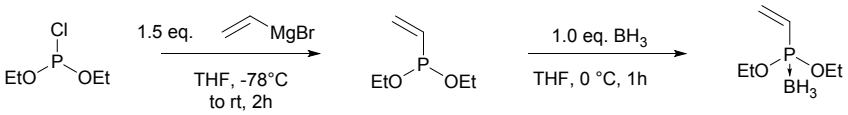
3.8. Cellular uptake experiments

All cell uptake experiments were carried out with HeLa (ATCC CCL-2) cells cultured in Dulbecco's MEM medium supplemented with 10% FBS and 1% Penicillin Streptomycin. 70 000 cells were seeded in an uncoated glass bottom 8-well µ-slide (Ibidi) 24 hours prior to treatment.
The cellular uptake with **FAM labeled peptides** was carried out by gently washing cells three times with DMEM without FBS. The FAM labeled peptides which were solved in DMSO (1 mM stock solution), were added to the medium after the final wash. The cells were incubated for at 37°C in a 5% CO_2 atmosphere for two hours. Then the cells were gently washed with 25 mM HEPES in Dulbecco's MEM supplemented with 10% FKS and cells were rested for 30 minutes at 37°C. The cell nucleus was stained with Hoechst 33342 and cells imaged with a Zeiss 710 confocal microscope.
The cellular uptake of **cR10-eGFP** was carried out by gently washing cells three times with HEPES buffer pH 7.5 (5 mM HEPES, 140 mM NaCl, 2.5 mM KCl, 5 mM glycine). The peptide-protein conjugate buffered in the same HEPES buffer was added to the cells in 200 µl at respective concentration and incubated for at 37°C in a 5% CO_2 atmosphere. After one hour cells were gently washed with 25 mM

HEPES in Dulbecco's MEM supplemented with 10% FKS and cells were rested for 30 minutes at 37°C. The cell nucleus was stained with Hoechst 33342 and cells imaged with a Zeiss 710 confocal microscope.
The percentage of cells that took up the GFP conjugate was determined by counting all cells showing nucleolar GFP relative to the total number of cells (all Hoechst stained nuclei). Each uptake experiment was repeated at least three times.

4. Organic and peptide synthesis

4.1. Diethyl vinylphosphonite borane (1a)

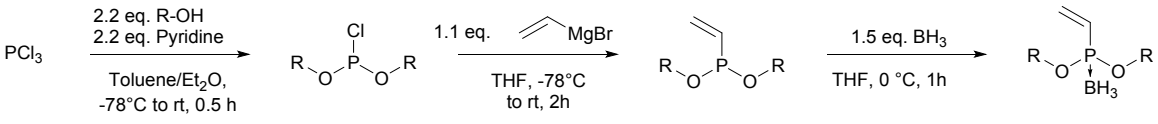


A 25-ml Schlenk flask was charged with 2.14 ml vinylmagnesium bromide (0.7 M in THF, 1.50 mmol, 1.5 eq.) under an argon atmosphere, cooled to -78 °C and 140 µl diethyl chlorophosphite (1.00 mmol, 1.0 eq.) were added drop wise. The yellowish solution was allowed to warm to 0 °C, stirred for another two hours and 1.00 ml of Borane (1.0 M in THF, 1.00 mmol, 1.0 eq.) were added and stirred for one more hour at 0°C. The organic solvents were removed under reduced pressure and the crude product was purified by flash column chromatography on silica gel (Hexane/EtOAc, 9:1) to yield the desired compound as colourless oil. (60 mg, 0.37 mmol, 37.0%)

^{1}H NMR (300 MHz, Chloroform-*d*) δ = 6.36 – 6.03 (m, 3H), 4.19 – 3.96 (m, 4H), 1.33 (t, *J*=7.1, 6H), 0.55 (ddd, *J*=190.3, 94.1, 16.6, 3H). ^{13}C NMR (75 MHz, Chloroform-*d*) δ = 134.62 (d, *J*=8.7), 130.12 (d, *J*=75.0), 63.16 (d, *J*=4.8), 16.59 (d, *J*=5.6). ^{31}P NMR (122 MHz, Chloroform-*d*) δ = 129.58 (dd, *J*=167.1, 82.6).

NMR data is in accordance with those reported in the literature.[4]

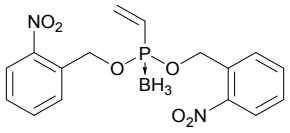
4.2. General procedure 1 for the synthesis of borane protected vinylphosphonites from phosphorous trichloride (route I)



A flame-dried Schlenkflask was charged with 1.00 mmol (1.0 eq.) of phosphorous trichloride in 15 ml of dry toluene and cooled to -78 °C. 2.2 mmol of pyridine (2.2 eq.) and a solution of 2.2 mmol of the alcohol (2.2 eq.) in 3 ml Et2O were added drop wise. The resulting suspension was allowed to warm to room temperature, stirred for another 30 min and cooled again to -78 °C. 1.10 mmol (1.1eq.) of vinyl magnesiumbromide (1.0 M in THF) was added and the reaction was stirred at room temperature for two hours. Finally, 1.50 mmol (1.5 eq.) of borane (1.0 M in THF) were added at 0 °C and the suspension was stirred for another hour. The crude product was dry packed on a silica column for purification.

It should be noted, that mass analysis of borane protected phosphonites failed for all of the tested compounds, possibly due to decomposition under ESI-conditions.

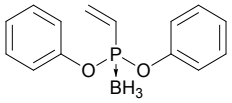
4.3. Di(2-nitrobenzyl) vinylphosphonite borane (1b)



The compound was synthesized according to the general procedure 1 from 260 µl PCl_3 (3.00 mmol). The crude borane protected phosphonite was purified by flash column chromatography (Hexane/EtOAc, 4:1) and obtained as a yellowish solid. (555 mg, 1.48 mmol, 49.2%)

^{1}H NMR (300 MHz, Chloroform-d) δ = 8.10 (d, J=8.2, 2H), 7.77 – 7.63 (m, 4H), 7.57 – 7.44 (m, 2H), 6.51 – 6.18 (m, 3H), 5.45 (qd, J=14.8, 7.5, 4H), 1.42 – -0.02 (m, 3H). ^{13}C NMR (75 MHz, Chloroform-d) δ = 146.80, 136.84 (d, J=10.2), 132.52 (d, J=6.8), 129.10, 129.05, 128.67, 128.61 (d, J=74.3), 125.09, 65.56 (d, J=3.6). ^{31}P NMR (122 MHz, Chloroform-d) δ = 136.23 (dd, J=151.3, 56.0).

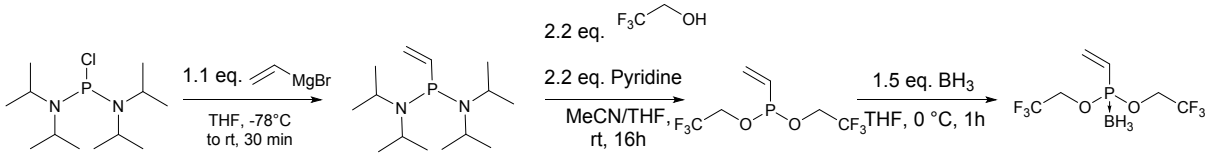
4.4. Diphenyl vinylphosphonite borane (1c)



The compound was synthesized according to the general procedure 1 from 393 µl PCl_3 (4.50 mmol). The crude borane protected phosphonite was purified by flash column chromatography (Hexane/EtOAc, 4:1) and obtained as a colourless oil. (700 mg, 2.71 mmol, 60.3%)

^{1}H NMR (300 MHz, Chloroform-d) δ = 7.39 (td, J=7.7, 5.5, 4H), 7.30 – 7.17 (m, 6H), 6.67 – 6.18 (m, 3H), 1.48 – 0.01 (m, 3H). 13C NMR (75 MHz, Chloroform-d) δ = 151.27 (d, J=8.7), 137.01 (d, J=12.5), 129.70 (d, J=1.0), 129.05 (d, J=71.1), 125.35 (d, J=1.3), 120.90 (d, J=4.2). ^{31}P NMR (122 MHz, Chloroform-d) δ = 134.08 – 130.87 (m).

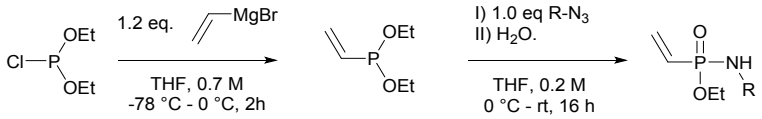
4.5. Bis(2,2,2-trifluoroethyl) vinylphosphonite borane (1d) (route II)



A flame-dried Schlenkflask was charged with 266 mg Bis(diisopropylamino)-chlorophosphine (1.0 mmol, 1.0 eq.), dissolved in 200 µl of dry THF and cooled to -78 °C. 1.1 ml of vinyl magnesiumbromide (1.0 M in THF, 1.10 mmol, 1.1eq.) were added and the reaction was stirred at room temperature for 30 minutes. A solution of 160 µl 2,2,2-Trifluoroethanol (2.20 mmol, 2.2 eq.) in 1 ml of MeCN and 4.9 ml tetrazole (0.45 M in MeCN, 2.20 mmol, 2.2 eq.) were added subsequently. The resulting suspension was stirred at room temperature overnight. Finally, 1.50 ml of borane (1.0 M in THF, 1.50 mmol, 1.5 eq.) were added at 0 °C and stirred for another hour. The crude product was dry packed on a silica column for purification (Hexane/CH2Cl2, 9:1 to 4:1) and obtained as a colourless liquid. (87 mg, 0.32 mmol, 32.2%)

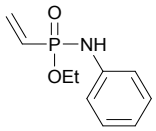
^{1}H NMR (300 MHz, Chloroform-d) δ = 6.52 – 6.11 (m, 3H), 4.36 (p, J=8.1, 4H), 0.62 (ddd, J=203.0, 103.5, 15.0, 3H).^{13}C NMR (75 MHz, Chloroform-d) δ = 137.57, 127.48 (d, J=79.1), 122.37 (qd, J=276.0, 7.5), 63.60 (qd, J=37.7, 2.5). ^{19}F NMR (282 MHz, Chloroform-d) δ = 2.13. ^{31}P NMR (122 MHz, Chloroform-d) δ = 145.49 (dd, J=135.6, 65.1).

4.6. General procedure 2 for the synthesis of vinylphosphonamidates from diethyl chlorophosphite (route III)



A 25-ml Schlenk flask was charged with 1.71 ml vinylmagnesium bromide (0.7 M in THF, 1.20 mmol, 1.2 eq.) under an argon atmosphere, cooled to -78 °C and 140 µl diethyl chlorophosphite (1.00 mmol, 1.0 eq.) were added drop wise. The yellowish solution was allowed to warm to 0 °C, stirred for another two hours and 1.00 mmol of azide (1.0 eq.) dissolved in 3.2 ml of THF was added and stirred over night at room temperature. 5 ml of water were added and stirred for another 24 h. The solvents were removed under reduced pressure and the crude product was purified by flash column chromatography on silica gel.

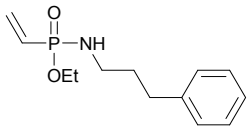
4.7. Ethyl-*N*-phenyl-*P*-vinylphosphonamidate (4a)



The compound was synthesized according to the general procedure 2 from 1.15 ml diethyl chlorophosphite (8 mmol). The crude phosphonamidate was purified by flash column chromatography (EtOAc) and obtained as a white solid. (675 mg, 3.20 mmol, 40.0%)

^{1}H NMR (600 MHz, Chloroform-d) δ = 7.24 (dd, J=8.5, 7.3, 2H), 7.05 – 7.01 (m, 2H), 6.99 (d, J=5.8, 1H), 6.94 (tt, J=7.3, 1.1, 1H), 6.33 – 6.23 (m, 2H), 6.10 (ddd, J=50.3, 9.6, 5.1, 1H), 4.29 – 4.04 (m, 2H), 1.35 (t, J=7.1, 3H). ^{13}C NMR (151 MHz, Chloroform-d) δ = 140.43, 134.44, 129.28, 127.51 (d, J=172.7), 121.26, 117.31 (d, J=6.6), 60.44 (d, J=6.2), 16.22 (d, J=7.0). ^{31}P NMR (122 MHz, Chloroform-d) δ = 15.68. HRMS for $C_{10}H_{15}NO_2P^+$ $[M+H]^+$ calcd.: 212.0835, found: 212.0839.

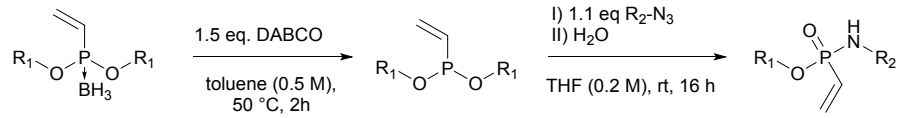
4.8. Ethyl-*N*-(3-phenyl-propyl)-*P*-vinyl-phosphonamidate (5a)



The compound was synthesized according to the general the general procedure 2 from 290 µl diethyl chlorophosphite (2 mmol). The crude phosphonamidate was purified by flash column chromatography (EtOAc) and obtained as a colourless oil. (165 mg, 0.65 mmol, 32.5%)

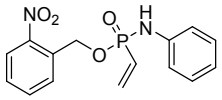
^{1}H NMR (300 MHz, Chloroform-d) δ = 7.28 (dd, J=8.1, 6.2, 2H), 7.23 – 7.11 (m, 3H), 6.28 – 5.89 (m, 3H), 4.04 (qt, J=10.2, 7.2, 2H), 2.92 (dq, J=9.1, 7.0, 2H), 2.84 – 2.70 (m, 1H), 2.70 – 2.60 (m, 2H), 1.82 (p, J=7.3, 2H), 1.31 (t, J=7.1, 3H). ^{13}C NMR (75 MHz, Chloroform-d) δ = 141.28, 132.98 (d, J=1.5), 128.42 (d, J=169.0), 128.34, 128.24, 125.88, 59.95 (d, J=5.7), 40.23, 33.53 (d, J=5.6), 32.86, 16.32 (d, J=6.7). ^{31}P NMR (122 MHz, Chloroform-d) δ = 20.82. HRMS for $C_{13}H_{21}NO_2P^+$ calcd.: 254.1304, found: 254.1312.

4.9. General procedure 3 for the synthesis of vinylphosphonamidates from borane protected vinylphosphonites (route IV)



A flame-dried Schlenkflask was charged with 1.0 mmol (1.0 eq.) of the borane protected phosphonite under an argon atmosphere and dissolved in 2 ml of dry toluene. In an argon stream, DABCO (1.50 mmol, 1.5 eq.) was added and the solution was heated to 50 °C for 2 hours. The mixture was allowed to cool to room temperature and a solution of 1.1 mmol of the azide (1.1 eq.) in 3 ml dry THF or dry DMF was added. The resulting solution was stirred at room temperature overnight. Finally 1 ml of water was added and stirred for another two hours. The crude product was purified by column chromatographie.

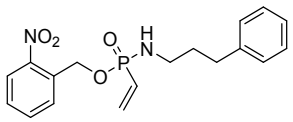
4.10. 2-Nitrobenzyl-*N*-phenyl-*P*-vinyl-phosphonamidate (4b)



The compound was synthesized according to the general procedure 3 from 100 mg Di(2-nitrobenzyl) vinylphosphonite borane (**1b**) (0.266 mmol). The crude phosphonamidate was purified by flash column chromatography (30% hexane in EtOAc) and obtained as a colourless solid. (38 mg, 0.119 mmol, 44.7%)

^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.12 (dd, *J* = 8.2, 1.3 Hz, 1H), 7.83 (dd, *J* = 7.9, 1.3 Hz, 1H), 7.68 (td, *J* = 7.6, 1.3 Hz, 1H), 7.50 (ddd, *J* = 8.1, 7.4, 1.5 Hz, 1H), 7.21 (dd, *J* = 8.5, 7.2 Hz, 2H), 7.09 – 6.82 (m, 3H), 6.61 – 6.00 (m, 4H), 5.57 (ddd, *J* = 67.1, 14.9, 7.6 Hz, 2H). ^{13}C NMR (75 MHz, Chloroform-*d*) δ 146.89, 139.47, 135.77, 135.75, 134.02, 132.72 (d, *J* = 7.6 Hz), 129.44, 128.80 (d, *J* = 3.3 Hz), 126.71 (d, *J* = 172.1 Hz), 125.01, 121.96, 117.63 (d, *J* = 6.5 Hz), 62.75 (d, *J* = 4.8 Hz). ^{31}P NMR (122 MHz, $CDCl_3$) δ 16.83. HRMS for $C_{15}H_{16}N_2O_4P^+$ $[M+H]^+$ calcd.: 319.0842, found: 319.0825.

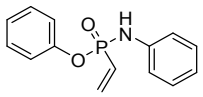
4.11. 2-Nitrobenzyl-*N*-(3-phenyl-propyl)-*P*-vinyl-phosphonamidate (5b)



The compound was synthesized according to the general procedure 3 from 100 mg Di(2-nitrobenzyl) vinylphosphonite borane (**1b**) (0.266 mmol). The crude phosphonamidate was purified by flash column chromatography (20% hexane in EtOAc) and obtained as a yellowish solid. (55 mg, 0.153 mmol, 57.5%)

^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.12 (d, *J* = 8.2 Hz, 1H), 7.77 (d, *J* = 7.8 Hz, 1H), 7.73 – 7.60 (m, 1H), 7.56 – 7.43 (m, 1H), 7.27 (t, *J* = 7.2 Hz, 2H), 7.23 – 7.10 (m, 3H), 6.55 – 5.88 (m, 3H), 5.43 (h, *J* = 7.4 Hz, 2H), 2.98 (dd, *J* = 9.4, 5.1 Hz, 3H), 2.65 (t, *J* = 7.7 Hz, 2H), 1.84 (p, *J* = 7.1 Hz, 2H). ^{13}C NMR (75 MHz, Chloroform-*d*) δ 141.14, 133.98 (d, *J* = 1.5 Hz), 133.88, 133.21 (d, *J* = 7.7 Hz), 128.75, 128.60, 128.53, 128.36, 128.24, 126.50, 125.91, 124.84, 62.30 (d, *J* = 4.4 Hz), 40.27, 33.45 (d, *J* = 5.5 Hz), 32.83. ^{31}P NMR (122 MHz, $CDCl_3$) δ 21.87. HRMS for $C_{18}H_{22}N_2O_4P^+$ $[M+H]^+$ calcd.: 361.1312, found: 361.1304.

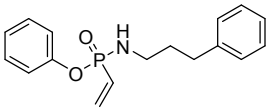
4.12. Phenyl-*N*-phenyl-*P*-vinyl-phosphonamidate (4c)



The compound was synthesized according to the general procedure 3 from 134 mg Diphenyl vinylphosphonite borane (**1c**) (0.519 mmol). The crude phosphonamidate was purified by flash column chromatography (50% hexane in EtOAc) and obtained as a yellowish oil. (34 mg, 0.131 mmol, 25.3%)

^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.32 – 7.25 (m, 5H), 7.24 – 7.20 (m, 2H), 7.16 (t, *J* = 7.3 Hz, 1H), 7.10 – 6.96 (m, 3H), 6.56 – 6.12 (m, 3H), 5.64 (d, *J* = 5.7 Hz, 1H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 149.87 (d, *J* = 8.1 Hz), 139.26, 135.98, 129.71, 129.41, 126.99 (d, *J* = 173.3 Hz), 125.08, 122.19, 120.72 (d, *J* = 4.6 Hz), 118.06 (d, *J* = 6.5 Hz). ^{31}P NMR (243 MHz, $CDCl_3$) δ 12.74. HRMS for $C_{14}H_{15}NO_2P^+$ $[M+H]^+$ calcd.: 260.0835, found: 260.0836.

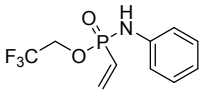
4.13. Phenyl-*N*-(3-phenyl-propyl)-*P*-vinyl-phosphonamidate (5c)



The compound was synthesized according to the general procedure 3 from 150 mg Diphenyl vinylphosphonite borane (**1c**) (0.581 mmol). The crude phosphonamidate was purified by flash column chromatography (30% EtOAc in hexane) and obtained as a white solid. (100 mg, 0.332 mmol, 57.1%)

^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.35 – 7.28 (m, 4H), 7.26 – 7.19 (m, 3H), 7.19 – 7.13 (m, 3H), 6.41 – 6.01 (m, 3H), 3.20 – 2.92 (m, 3H), 2.63 (t, *J* = 7.7 Hz, 2H), 1.80 (pd, *J* = 7.1, 1.6 Hz, 2H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 150.63 (d, *J* = 7.9 Hz), 141.30, 134.03, 129.64, 128.43, 128.35, 127.98 (d, *J* = 172.0 Hz), 125.99, 124.55, 120.58 (d, *J* = 4.9 Hz), 40.48, 33.38 (d, *J* = 5.6 Hz), 32.87. ^{31}P NMR (243 MHz, $CDCl_3$) δ 18.79. HRMS for $C_{17}H_{21}NO_2P^+$ $[M+H]^+$ calcd.: 302.1304, found: 302.1315.

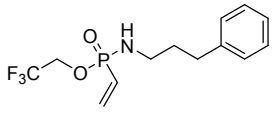
4.14. 2,2,2-trifluoroethyl-*N*-phenyl-*P*-vinyl-phosphonamidate (4d)



The compound was synthesized according to the general procedure 3 from 90 mg Bis(2,2,2-trifluoroethyl) vinylphosphonite borane (**1d**) (0.333 mmol). The crude phosphonamidate was purified by flash column chromatography (60 to 70% EtOAc in hexane) and obtained as a white solid. (27 mg, 0.102 mmol, 30.6%)

^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.27 (dd, *J* = 9.3, 6.3 Hz, 2H), 7.13 – 6.86 (m, 3H), 6.65 (d, *J* = 6.2 Hz, 1H), 6.53 – 6.01 (m, 3H), 4.70 – 4.06 (m, 2H). ^{13}C NMR (75 MHz, Chloroform-*d*) δ 138.93, 136.50 (d, *J* = 1.7 Hz), 129.43, 125.70 (d, *J* = 172.5 Hz), 122.23, 117.68 (d, *J* = 6.7 Hz), 60.42 (qd, *J* = 37.7, 5.1 Hz), 51.28. ^{31}P NMR (122 MHz, $CDCl_3$) δ 17.93. ^{19}F NMR (282 MHz, $CDCl_3$) δ 2.69. HRMS for $C_{10}H_{12}F_3NO_2P^+$ $[M+H]^+$ calcd.: 266.0552, found: 266.0552.

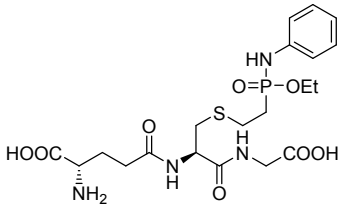
4.15. 2,2,2-trifluoroethyl-*N*-(3-phenyl-propyl)-phenyl-*P*-vinyl-phosphonamidate (5d)



The compound was synthesized according to the general procedure 3 from 80 mg Bis(2,2,2-trifluoroethyl) vinylphosphonite borane (**1d**) (0.296 mmol). The crude phosphonamidate was purified by flash column chromatography (70 to 80% EtOAc in hexane) and obtained as a colourless oil. (18 mg, 0.059 mmol, 19.8%)

^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.36 – 7.12 (m, 6H), 6.54 – 5.94 (m, 3H), 4.29 (dqt, *J* = 12.2, 8.4, 4.1 Hz, 2H), 2.96 (dt, *J* = 9.6, 7.1 Hz, 2H), 2.82 (d, *J* = 8.4 Hz, 1H), 2.66 (t, *J* = 7.7 Hz, 2H), 1.85 (p, *J* = 7.3 Hz, 2H). ^{13}C NMR (75 MHz, Chloroform-*d*) δ 141.00, 135.06 (d, *J* = 1.5 Hz), 128.41, 128.22, 126.69 (d, *J* = 170.2 Hz), 126.00, 60.48 (qd, *J* = 37.2, 4.6 Hz), 40.08, 33.40, 33.32, 32.79. ^{31}P NMR (122 MHz, $CDCl_3$) δ 22.94. ^{19}F NMR (282 MHz, $CDCl_3$) δ 2.40. HRMS for $C_{13}H_{18}F_3NO_2P^+$ $[M+H]^+$ calcd.: 308.1022, found: 308.1017.

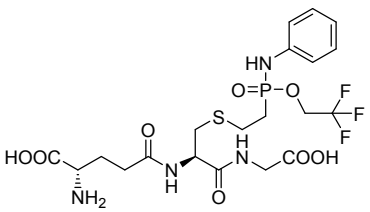
4.16. Ethyl-*N*-phenyl-*P*-(2-(glutathionyl-thio)ethyl)-phosphonamidate



A 5-ml round-bottom-flask was charged with 12.2 mg Ethyl-*N*-phenyl-*P*-vinyl-phosphonamidate (**4a**) (0.058 mmol, 1.00 eq.) and 150 µl DMF. A solution containing 21.4 mg glutathione (0.070 mmol, 1.20 eq.) in 150 µl H_2O was adjusted to with potassium carbonate to pH 9.0 and added to the reaction mixture, which was stirred for 24h at room temperature. All volatiles were removed under reduced pressure and the crude product was purified by preparative HPLC and obtained as a colourless solid after lyophilization as a mixture of diastereoisomers (18 mg, 0.035 mmol, 60.3%).

^{1}H NMR (600 MHz, Deuterium Oxide) δ 7.53 – 7.22 (m, 2H), 7.15 – 6.89 (m, 3H), 4.44 (tt, J = 9.1, 4.5 Hz, 1H), 4.26 – 3.96 (m, 2H), 3.89 – 3.51 (m, 3H), 3.04 – 2.90 (m, 1H), 2.86 – 2.63 (m, 3H), 2.54 – 2.39 (m, 2H), 2.37 – 2.19 (m, 2H), 2.19 – 2.03 (m, 2H), 1.37 – 1.23 (m, 3H). ^{13}C NMR (151 MHz, Deuterium Oxide) δ 176.03, 174.84, 173.90, 171.74 (d, *J* = 3.7 Hz), 139.43, 129.79, 122.48, 118.34 (d, *J* = 6.1 Hz), 62.27 (d, *J* = 6.9 Hz), 54.15, 52.80 (d, *J* = 14.3 Hz), 43.33, 32.82 (d, *J* = 12.9 Hz), 31.45, 26.22, 26.08 (d, *J* = 124.0 Hz), 24.10 (d, *J* = 10.6 Hz), 15.54 (d, *J* = 6.4 Hz). ^{31}P NMR (243 MHz, D_2O) δ 31.68. HRMS for $C_{20}H_{32}N_4O_8PS^+$ $[M+H]^+$ calcd.: 519.1673, found: 519.1675.

4.17. 2,2,2-trifluoroethyl-*N*-phenyl-*P*-(2-(glutathionyl-thio)ethyl)-phosphonamidate

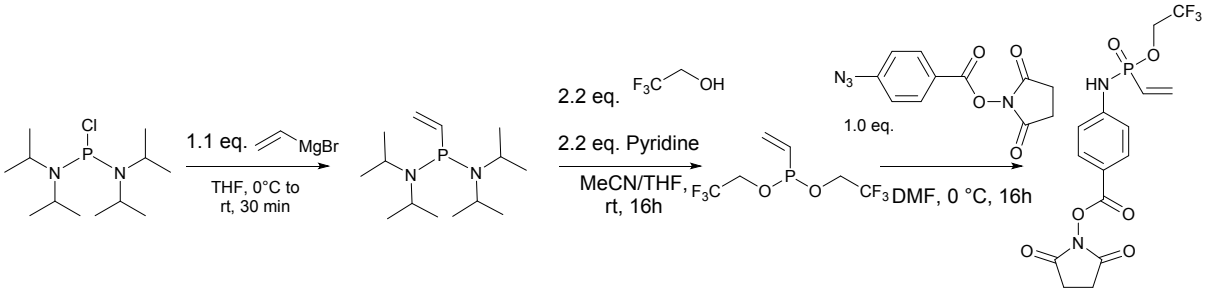


A 5-ml round-bottom-flask was charged with 17.2 mg 2,2,2-trifluoroethyl-*N*-phenyl-*P*-vinyl-phosphonamidate (**4d**) (0.065 mmol, 1.00 eq.) and 150 µl DMF. A solution containing 21.85 mg glutathione (0.072 mmol, 1.10 eq.) in 150 µl H_2O was adjusted to with potassium carbonate to pH 9.0

and added to the reaction mixture, which was stirred for 24h at room temperature. All volatiles were removed under reduced pressure and the crude product was purified by preparative HPLC and obtained as a colourless solid after lyophilization as a mixture of diastereoisomers (33 mg, 0.058 mmol, 89.0%).

^{1}H NMR (600 MHz, Deuterium Oxide) δ 7.27 (dd, *J* = 8.5, 7.3 Hz, 2H), 7.05 – 7.00 (m, 3H), 4.54 – 4.36 (m, 3H), 4.00 (td, *J* = 6.6, 3.4 Hz, 1H), 2.89 (dd, *J* = 14.1, 5.4 Hz, 1H), 2.80 – 2.66 (m, 3H), 2.49 (ddt, *J* = 9.7, 7.4, 3.1 Hz, 2H), 2.40 – 2.25 (m, 2H), 2.22 – 2.06 (m, 2H). ^{13}C NMR (151 MHz, Deuterium Oxide) δ 174.15, 172.76, 172.51 – 172.47 (m), 171.42, 138.58, 129.78, 123.02, 118.82 (d, *J* = 5.1 Hz), 60.93 (qd, *J* = 37.6, 5.1 Hz), 52.76 (d, *J* = 9.6 Hz), 52.17, 41.04, 32.70 (d, *J* = 6.8 Hz), 30.87, 26.41, 25.59, 25.41, 23.94. ^{31}P NMR (243 MHz, D_2O) δ 33.70. ^{19}F NMR (282 MHz, D_2O) δ 2.60, 1.91. HRMS for $C_{20}H_{29}F_3N_4O_8PS^+$ $[M+H]^+$ calcd.: 573.1390, found: 573.1391.

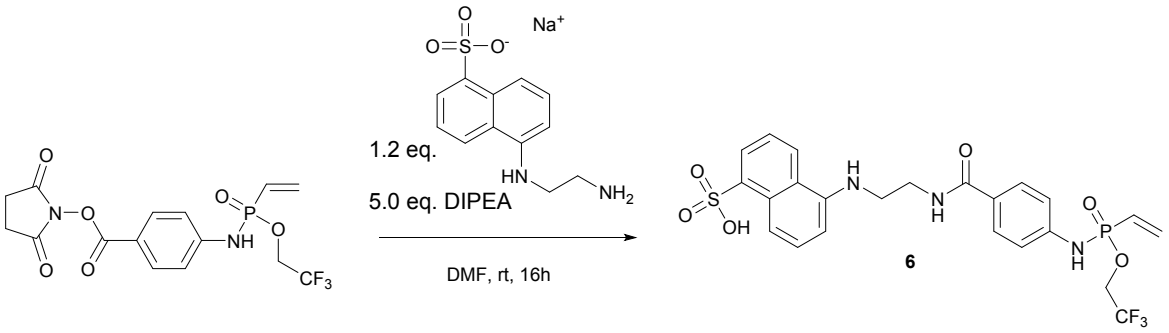
4.18. Trifluorethyl-*N*-(4-(2,5-dioxo-1-pyrrolidinyl)oxy-carbonyl-phenyl)-*P*-vinylphosphonamidate



A flame-dried Schlenkflask was charged with 266 mg Bis(diisopropylamino)-chlorophosphine (1.0 mmol, 1.0 eq.), dissolved in 200 µl of dry THF and cooled to 0 °C. 1.1 ml of vinyl magnesiumbromide (1.0 M in THF, 1.10 mmol, 1.1eq.) were added and the reaction was stirred at room temperature for 30 minutes. A solution of 160 µl 2,2,2-Trifluoroethanol (2.20 mmol, 2.2 eq.) in 1 ml of MeCN and 4.9 ml tetrazole (0.45 M in MeCN, 2.20 mmol, 2.2 eq.) were added subsequently. The resulting suspension was stirred at room temperature overnight. Afterwards, 260 mg 4-azidobenzoic-acid-*N*-hydroxysuccinimide ester (1.0 mmol, 1.0 eq.), dissolved in 1 ml of dry DMF was added and the suspension was stirred overnight. All volatiles were removed under reduced pressure and the crude product was purified by silica column (EtOAc/n-hexane, 4:1) and obtained as a colourless solid. (223 mg, 0.55 mmol, 55.0%)

^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.93 (d, *J* = 8.8 Hz, 3H), 7.06 (d, *J* = 8.8 Hz, 2H), 6.54 – 5.96 (m, 3H), 4.58 – 4.15 (m, 2H), 2.84 (s, 4H). ^{13}C NMR (75 MHz, Chloroform-*d*) δ 169.79, 161.35, 146.14, 137.44, 132.37, 126.35, 124.07, 117.83, 117.24 (d, *J* = 7.1 Hz), 60.76 (qd, *J* = 37.8, 5.1 Hz), 25.63. ^{31}P NMR (122 MHz, $CDCl_3$) δ 17.53. ^{19}F NMR (282 MHz, $CDCl_3$) δ 2.88. HRMS for $C_{15}H_{15}F_3N_2O_6P^+$ $[M+H]^+$ calcd.: 407.0614, found: 407.0614.

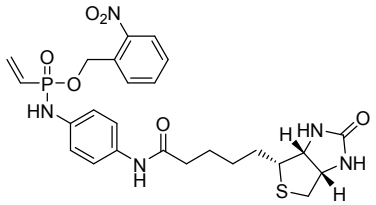
4.19. 5-((2-(*O*-trifluorethyl-*P*-vinyll-phosphonamidato-*N*-benzoyl)ethyl)amino)naphthalene-1-sulfonic acid (6)



A 10-ml round-bottom-flask was charged with 50 mg Trifluorethyl-N-(4-(2,5-dioxo-1-pyrrolidinyl)oxy-carbonyl-phenyl)-P-vinylphosphonamidate (0.123 mmol, 1.0 eq.), 43 mg EDANS sodium salt (0.147 mmol, 1.2 eq.) and 1.2 ml DMF. 107 µl DIPEA (0.615 mmol, 5.0 eq.) were added and the solution was stirred overnight. The crude product was purified by preparative HPLC and obtained as a colourless solid after lyophilization (60.360,3 mg, 0.108 mmol, 88.0%). HRMS for $C_{23}H_{24}F_3N_3O_6P^+$ $[M+H]^+$ calcd.: 558.1070, found: 558.1071.

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.55 (t, *J* = 8.7 Hz, 2H), 8.41 (s, 1H), 8.11 (d, *J* = 8.5 Hz, 1H), 8.00 (d, *J* = 7.1 Hz, 1H), 7.78 (d, *J* = 8.4 Hz, 2H), 7.42 (dt, *J* = 29.4, 7.9 Hz, 2H), 7.13 (d, *J* = 8.4 Hz, 2H), 6.99 (s, 1H), 6.44 – 6.16 (m, 3H), 4.76 – 4.46 (m, 2H), 3.61 (q, *J* = 6.3 Hz, 2H), 3.47 (t, *J* = 6.3 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 166.99, 153.70, 144.71, 144.02, 136.66, 130.59, 128.98, 128.24, 127.34, 127.15, 126.39, 125.19, 124.88, 124.78, 124.07 (d, *J* = 6.3 Hz), 123.10, 117.39, 117.34, 61.90 – 59.65 (m), 45.88, 38.01. ^{31}P NMR (243 MHz, DMSO) δ 17.26. ^{19}F NMR (282 MHz, DMSO) δ 4.13.

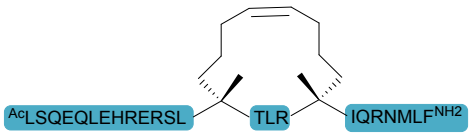
4.20. 2-Nitrobenzyl-*N*-(4-biotinamido-phenyl)-*P*-vinyl phosphonamidate



N-(4-azidophenyl) biotinamide was synthesized as previously described.[2] The above stated compound was synthesized according to the general procedure 3 from 302 mg Di(2-nitrobenzyl) vinylphosphonite borane (**1b**) (0.803 mmol, 1.50 eq.) in DMF. The crude phosphonamidate was purified by flash column chromatography (12-15% MeOH in CH_2Cl_2) and obtained as yellowish solid. (150 mg, 0.268 mmol, 50.1%)

^{1}H NMR (300 MHz, DMSO-d6) δ 9.76 (s, 1H), 8.13 (d, J = 8.1 Hz, 1H), 8.02 (d, J = 8.4 Hz, 1H), 7.85 – 7.73 (m, 2H), 7.69 – 7.54 (m, 1H), 7.40 (d, J = 8.8 Hz, 2H), 6.97 (d, J = 8.8 Hz, 2H), 6.61 – 5.99 (m, 5H), 5.37 (qd, J = 14.8, 7.4 Hz, 2H), 4.30 (dd, J = 7.8, 4.9 Hz, 1H), 4.22 – 4.00 (m, 1H), 3.15 – 3.01 (m, 1H), 2.82 (dd, J = 12.4, 5.1 Hz, 1H), 2.58 (d, J = 12.4 Hz, 1H), 2.25 (t, J = 7.3 Hz, 2H), 1.75 – 1.26 (m, 6H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 171.02, 163.10, 147.09, 136.39, 135.17, 134.63, 133.42, 133.00 (d, *J* = 7.7 Hz), 129.84, 129.44, 128.97, 125.18, 120.54, 118.24 (d, *J* = 6.6 Hz), 62.45 (d, *J* = 4.1 Hz), 61.41, 59.56, 55.78, 48.97, 45.79, 36.44, 28.54 (d, *J* = 12.6 Hz), 25.56. ^{31}P NMR (122 MHz, DMSO) δ 16.30. HRMS for $C_{25}H_{31}N_5O_6PS^+$ $[M+H]^+$ calcd.: 560.1727, found: 560.1725.

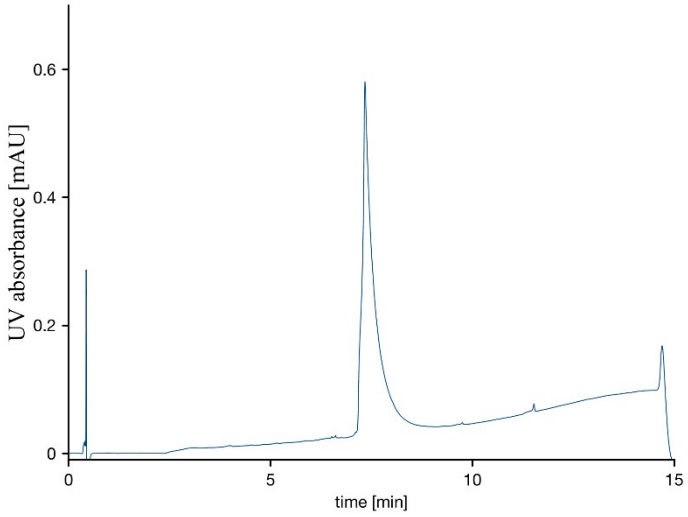
4.21. Synthesis of RCM-BCL9-Nle peptide 7



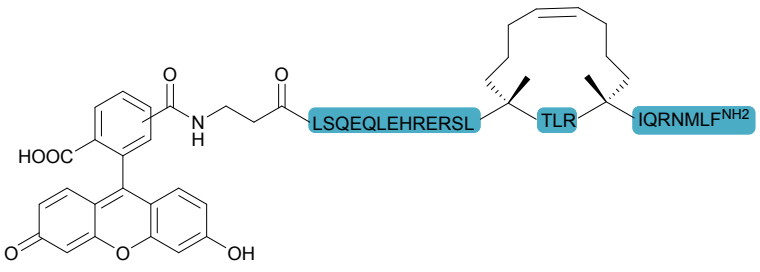
The RCM-BCL9 peptide was synthesized in a 0.05 mmol scale on a Rink Amide Resin with a loading of 0.78 mmol/g. Until the first olefinic amino acid the synthesis was carried out on a PTI synthesizer with single couplings (5 eq. amino acid for 40 minutes). Olefinic amino acids (2 eq.) were coupled manually with HATU (2eq.) and DIPEA (4 eq.) for two hours and full conversion was checked by trial cleavage. The three amino acids in between the olefinic ones were coupled manually (5 eg. for 40 minutes) all amino acids following the olefinic ones were coupled in double coupling steps with five equivalents for 40 minutes. After the second olefinic amino acids the synthesis was resumed by automated SPPS on the PTI synthesizer. The *N*-terminus was acetylated by treatment with a lutidine:acetic anhydride:DMF (5:6:89;v:v:v) for 10 minutes. To generate the hydrocarbon staple the resin was incubated with a 10 mM solution of bis(tricyclohexylphosphine)-benzylidene ruthenium (IV) (1st generation Grubb's catalyst) in 1,2-dichloroethane for one hour twice.

The final cleavage from resin was achieved by incubation with a mixture of TFA:TIS:H_2O (95:2.5:2.5;v:v:v) for two hours followed by precipitation in cold diethylether. The crude peptide was purified by preparative reverse phase C18 HPLC and the product was gained as white powder (14 mg, 3.9 µmol, 7.8% yield).

HRMS: m/z: 1019.5471 $[M+3H]^{3+}$ (calc. 1019.5881 m/z).



4.22. Synthesis of FAM RCM-BCL9-Nle peptide 7-CF

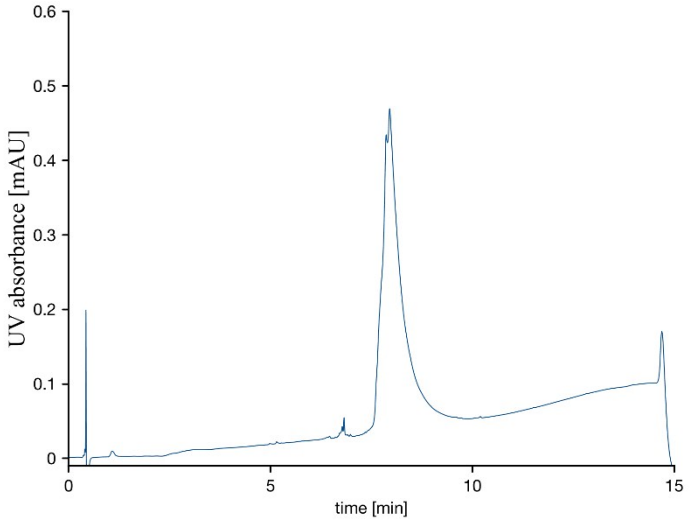


The FAM-RCM-BCL9 peptide was synthesized in a 0.05 mmol scale on a Rink Amide Resin with a loading of 0.78 mmol/g. Until the first olefinic amino acid the synthesis was carried out on a PTI synthesizer

with single couplings (5 eq. amino acid for 40 minutes). Olefinic amino acids (2 eq.) were coupled manually with HATU (2eq.) and DIPEA (4 eq.) for two hours and full conversion was checked by trial cleavage. The three amino acids in between the olefinic ones were coupled manually (5 eg. for 40 minutes) all amino acids following the olefinic ones were coupled in double coupling steps with five equivalents for 40 minutes. After the second olefinic amino acids the synthesis was resumed by automated SPPS on the PTI synthesizer and Fmoc-β-Ala was coupled *N*-terminally. To generate the hydrocarbon staple the resin was incubated with a 10 mM solution of bis(tricyclohexylphosphine)-benzylidene ruthenium (IV) (1st generation Grubb's catalyst) in 1,2-dichloroethane for one hour twice. Finally the peptide was fluorescein labeled by coupling 5-(6)-carboxyfluorescein succinimidyl ester (2 eq.) with DIPEA (3 eq.) as activating base for two hours.

The final cleavage from resin was achieved by incubation with a mixture of TFA:TIS:H_2O (95:2.5:2.5;v:v:v) for two hours followed by precipitation in cold diethylether. The crude peptide was purified by preparative reverse phase C18 HPLC and the product was gained as yellow powder (16 mg, 4.02 µmol, 8.0% yield).

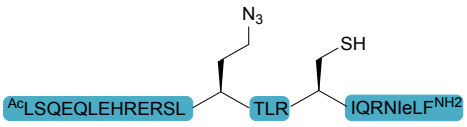
HRMS: m/z: 1148.5795 $[M+3H]^{3+}$ (calc. 1148.6129 m/z); 861.6882 $[M+4H]^{4+}$ (calc. 861.7117 m/z).



4.23. General procedure 4 for synthesis of BCL9-peptides

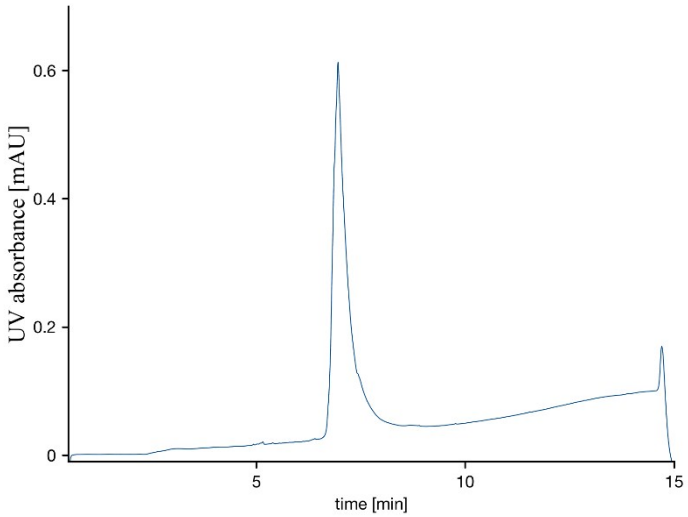
The BCL9 peptide was synthesized in a 0.1 mmol scale on a Rink Amide Resin with a loading of 0.78 mmol/g. The synthesis was carried out on a PTI synthesizer with a single coupling of each standard amino acid (5 eq. amino acid for 40 min). Fmoc-Azidohomoalanin (2 eq.) was coupled with HATU (2 eq.) for four hours. Fmoc deprotection was accomplished by treating the peptide with 20% piperidine in DMF for 3 minutes two to three times according to UV read out. N-terminal acetylation was done by incubation with a mixture of lutidine:acetic acid:DMF (6%:5%:89%;v;v;v) for 10 minutes. Final cleavage from solid support was carried out by incubating the resin with 4 ml of a TFA:TIS:H2O (95:2.5:2.5) mixture for two hours and precipitated in cold diethylether.

4.24. Synthesis of BCL9-Nle-Aha-LCys peptide 8a

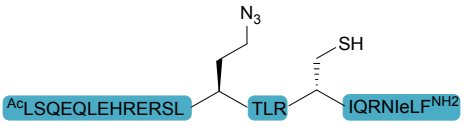


The Peptide was synthesized according to general procedure 4. The crude peptide was purified by preparative reverse phase C18 HPLC (0-5 min 95/5, water (0.1%TFA)/MeCN (0.1%TFA); 5-60 min 10/90, water (0.1%TFA)/MeCN (0.1%TFA)). The product **8a** was gained as white powder (35 mg as TFA-salt, 9.7 µmol, 9.7 % yield).

HRMS: m/z: 1012.5317 $[M+3H]^{3+}$ (calc. 1012.5532 m/z).

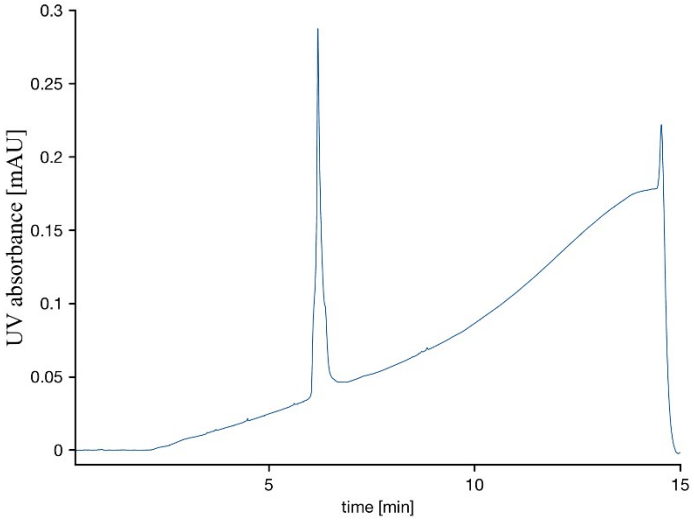


4.25. Synthesis of BCL9-Nle-Aha-DCys 8b

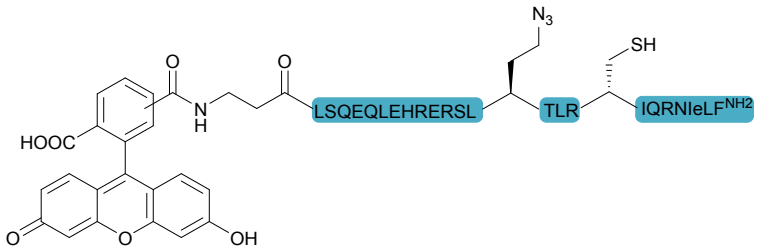


The Peptide was synthesized according to general procedure 4. The crude peptide was purified by preparative reverse phase C18 HPLC (0-5 min 95/5, water (0.1%TFA)/MeCN (0.1%TFA); 5-60 min 10/90, water (0.1%TFA)/MeCN (0.1%TFA)). The product was gained as white powder (50.5 mg as TFA-salt, 14.0 µmol, 14 % yield).

HRMS: m/z: 1012.5383 $[M+3H]^{3+}$ (calc. 1012.5532 m/z).

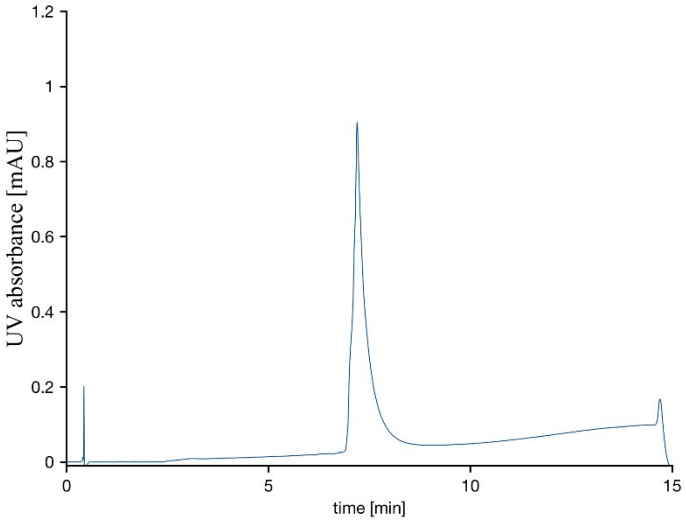


4.26. Synthesis of FAM-BCL9-Nle-Aha-DCys peptide (8b-CF)



The Peptide was synthesized according to general procedure 4. After the final regular amino acid coupling and Fmoc-deprotection, Fmoc-β-Alanine (5 eq.) was coupled manually with HATU (5 eq.) and DIPEA (10 eq.) as activating base for one hour. After Fmoc-deprotection 5-(6)-carboxyfluorescein succinimidyl ester (2 eq.) was coupled with DIPEA (3 eq.) as activating base for four hours. Final cleavage from solid support was carried out by incubating the resin with 4 ml of a TFA:TIS:H2O (95:2.5:2.5) mixture for two hours and precipitated in cold diethylether. The crude peptide was purified by preparative reverse phase C18 HPLC (0-5 min 95/5, water (0.1%TFA)/MeCN (0.1%TFA); 5-60 min 10/90, water (0.1%TFA)/MeCN (0.1%TFA)). The product was gained as yellow powder (42.0 mg as TFA-salt, 10.2 µmol, 10.2 % yield).

HRMS: m/z: 1141.5472 [M+3H]3+ (calc. 1141.5780 m/z); 856.4244 [M+4H]4+ (calc. 856.4355 m/z).

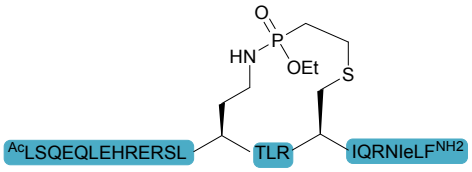


4.27. General procedure 5 for the Staudinger induced peptide cyclization

Vinyl magnesium bromide in THF (1 M, 2 ml, 2 mmol, 1 eq.) was cooled to -78°C in a flame dried schlenk flask and diethylchlorophosphite (0.286 ml, 2 mmol, 1 eq.) was added. The solution was stirred for 10 minutes at -78°C and let warm to room temperature and stirred for another 90 minutes. The full consumption of starting material was confirmed by 31P-NMR and used as crude in the following Staudinger reaction with an azido-peptide.

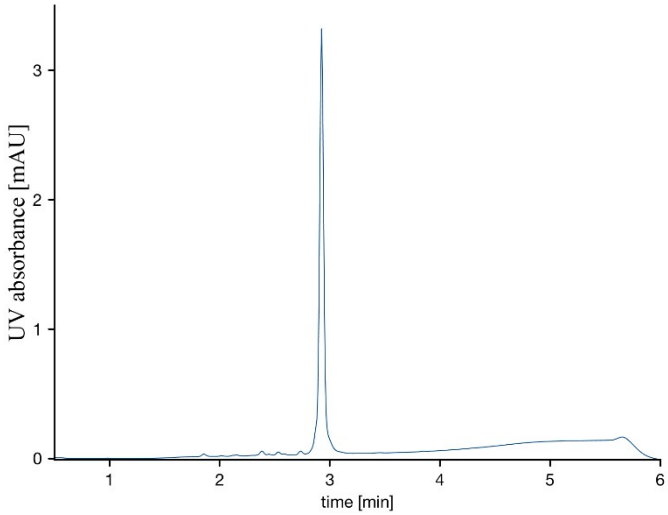
Purified azido-peptide was dissolved in DMSO at a 3 mM concentration and dried in a flame-dried flask for one hour prior to adding five equivalents of bisethoxyalkene-phosphonite (volume according to percentage of product determined by NMR). After the reaction mixture was stirred over night at room temperature and full conversion was observed by UPLC-UV/MS, water was added and the reaction mixture directly lyophilized. The crude product was purified by preparative HPLC.

4.28. Synthesis of cyclic-BCL9-Nle-Aha-LCys peptide 9a

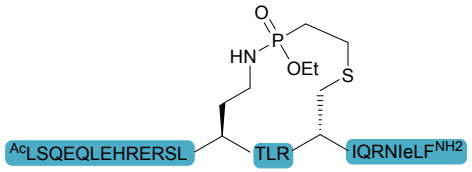


Peptide 8a (15 mg, 4.16 µmol, 1eq.) was reacted according to the general procedure 5. The crude peptide was purified by preparative reverse phase C18 HPLC (0-5 min 95/5, water (0.1%TFA)/MeCN (0.1%TFA); 5-60 min 10/90, water (0.1%TFA)/MeCN (0.1%TFA)). The product 7a was gained as white powder (7 mg as TFA-salt, 1.89 µmol, 45.4 % yield).

HRMS: m/z: 1043.1953 [M+3H]3+ (calc. 1043.2292 m/z).

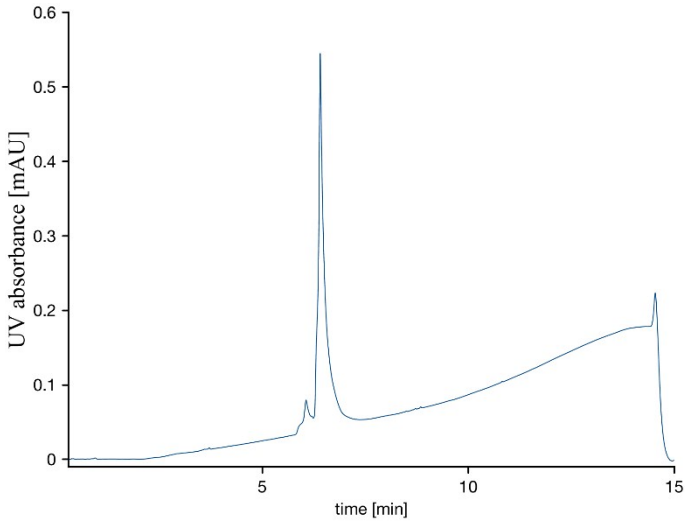


4.29. Synthesis of Cyclic-BCL9-Nle-Aha-DCys peptide synthesis 9b

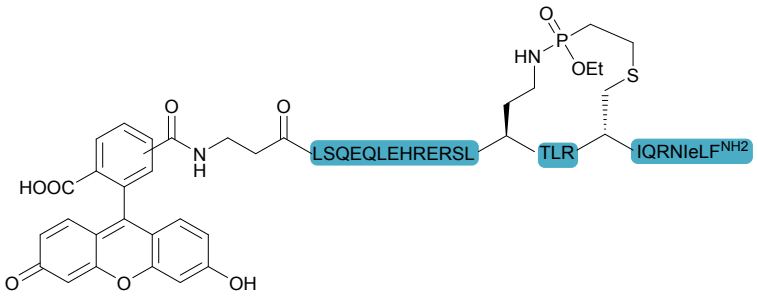


Peptide 8b (34 mg, 9.43 µmol, 1eq.) was reacted according to the general procedure 5. The crude peptide was purified by preparative reverse phase C18 HPLC (0-5 min 95/5, water (0.1%TFA)/MeCN (0.1%TFA); 5-60 min 10/90, water (0.1%TFA)/MeCN (0.1%TFA)). The product 8b was gained as white powder (15 mg as TFA-salt, 4.06 µmol, 43.1 % yield).

HRMS: m/z: 1043.2085 [M+3H]3+ (calc. 1043.2292 m/z).

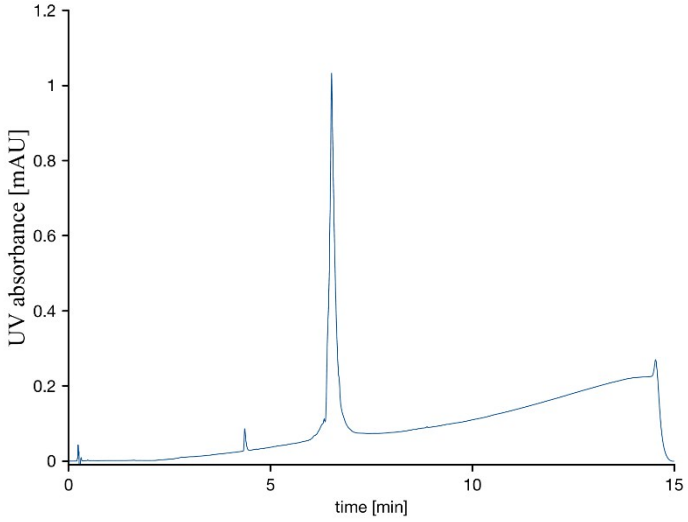


4.30. FAM labeled cyclic-BCL9-Nle-Aha-DCys peptide synthesis (9b-CF)

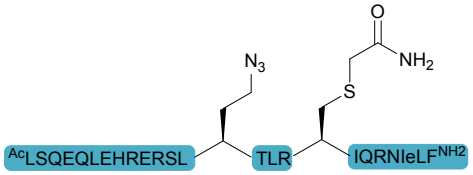


Peptide 8b-CF(21 mg, 5.1 µmol, 1eq.) was reacted according to the general procedure 5. The crude peptide was purified by preparative reverse phase C18 HPLC (0-5 min 95/5, water (0.1%TFA)/MeCN (0.1%TFA); 5-60 min 10/90, water (0.1%TFA)/MeCN (0.1%TFA)). The product 8b-CF was gained as yellow powder (12 mg as TFA-salt, 2.9 µmol, 56.9 % yield).

HRMS: m/z: 1172.1996 [M+3H]3+ (calc. 1172.2539 m/z); 879.4191 [M+4H]4+ (calc. 879.4424 m/z).

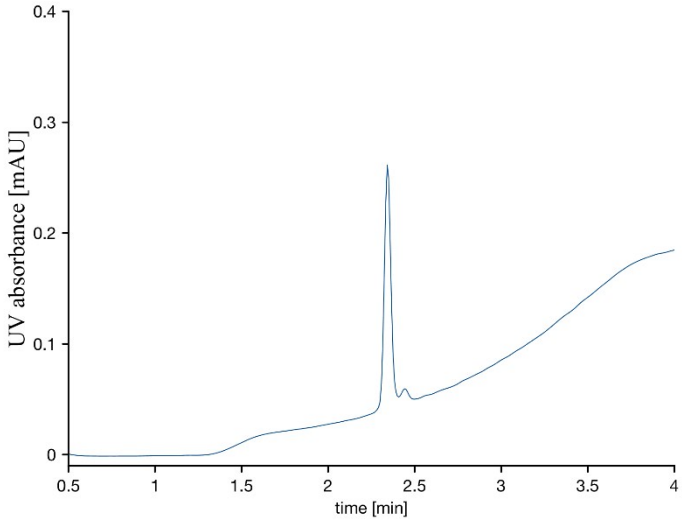


4.31. Alkylated-BCL9-Nle-Aha-LCys peptide synthesis 10

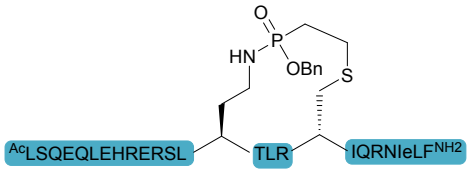


Peptide 8a (43.2 mg, 12.0 µmol) was incubated with iodoacetamide (22.2 mg, 120 µmol, 10eq.) in a mixture of ammoniumbicarbonate buffer and acetonitrile (6:2) with a final pH of 8.5. After one hour the mixture was purified by preparative HPLC 0-5 min 95/5, water (0.1%TFA)/MeCN (0.1%TFA); 5-60 min 10/90, water (0.1%TFA)/MeCN (0.1%TFA)). The product was gained as white powder (25 mg as TFA-salt, 6.8 µmol, 57 % yield).

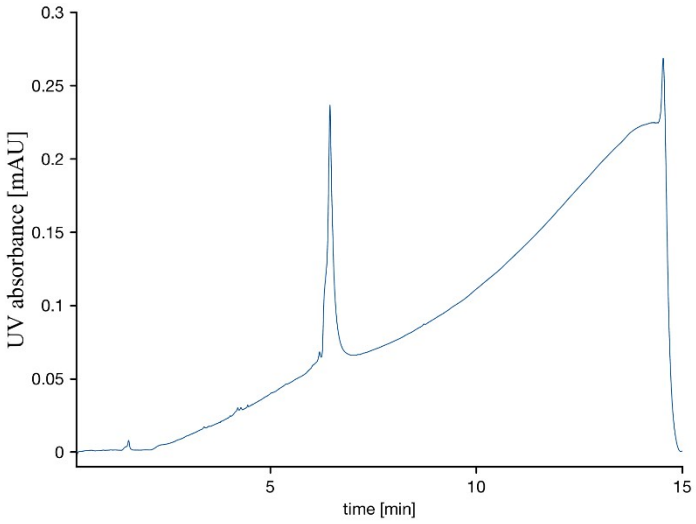
HRMS: m/z: 1031.5342 [M+3H]3+ (calc. 1031.5604 m/z).



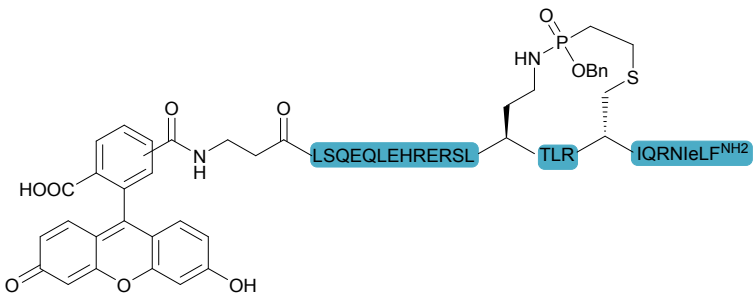
4.32. Synthesis of cyclic-benzylphosphonamidate-BCL9-Nle-Aha-DCys peptide 13



Bis(diisopropylamino) chlorophosphine (267 mg, 1 mmol, 1 eq.) was given into a flame dried schlenk flask and dried under vacuo for 30 minutes. Vinyl magnesium bromide in THF (1 M, 1.1 ml, 1.1 mmol, 1.1 eq.) was added at -78°C and the reaction mixture was stirred for 10 minutes. Then the reaction was let to warm up to room temperature and stirred for another 30 minutes. First tetrazole in acetonitrile (0.45 M, 5.56 ml, 2.5 mmol, 2.5 eq.) was added to the reaction mixture followed by benzylalcohol (0.26 ml, 2.5 mmol, 2.5 eq.). The reaction was stirred for 16 hours and used further as crude after confirmation of product formation *via* ^{31}P-NMR (product at 164.2 ppm). Peptide **8b** (15 mg, 4.04 µmol, 1eq.) was solubilized in DMSO and reacted with the crude phosphonite (24.2 µmol, 6 eq.) in a final concentration of 10 mM. The reaction mixture was stirred for 16 hours at 40°C and the crude peptide was purified by preparative reverse phase C18 HPLC (0-5 min 95/5, water (0.1%TFA)/MeCN (0.1%TFA); 5-60 min 10/90, water (0.1%TFA)/MeCN (0.1%TFA)). The product **13** was gained as white powder (5 mg as TFA-salt, 1.33 µmol, 32.9 % yield). HRMS: m/z: 1063.8917 $[M+3H]^{3+}$ (calc. 1063.9010 m/z).

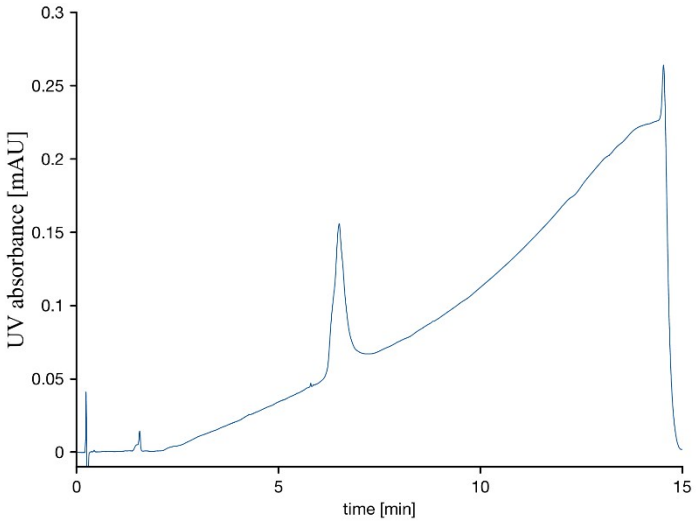


4.33. Synthesis of FAM labeled cyclic- benzylphosphonamidate-BCL9-Nle-Aha-DCys peptide (13-CF)

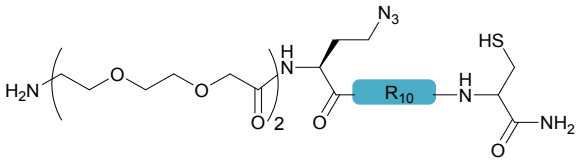


Bis(diisopropylamino) chlorophosphine (267 mg, 1 mmol, 1 eq.) was given into a flame dried schlenk flask and dried under vacuo for 30 minutes. Vinyl magnesium bromide in THF (1 M, 1.1 ml, 1.1 mmol, 1.1 eq.) was added at -78°C and the reaction mixture was stirred for 10 minutes. Then the reaction was let to warm up to room temperature and stirred for another 30 minutes. First tetrazole in acetonitrile (0.45 M, 5.56 ml, 2.5 mmol, 2.5 eq.) was added to the reaction mixture followed by benzylalcohol (0.26 ml, 2.5 mmol, 2.5 eq.). The reaction was stirred for 16 hours and used further as crude after confirmation of product formation *via* ^{31}P-NMR (product at 164.2 ppm). Peptide **8b-CF** (22 mg, 5.93 µmol, 1eq.) was solubilized in DMSO and reacted with the crude phosphonite (35.6 µmol, 6 eq.) in a final concentration of 10 mM. The reaction mixture was stirred for 16 hours at 40°C and the crude peptide was purified by preparative reverse phase C18 HPLC (0-5 min 95/5, water (0.1%TFA)/MeCN (0.1%TFA); 5-60 min 10/90, water (0.1%TFA)/MeCN (0.1%TFA)). The product **13-CF** was gained as yellow powder (3.1 mg as TFA-salt, 0.75 µmol, 12.6 % yield).

LRMS: m/z: 1193.11 $[M+3H]^{3+}$ (calc. 1192.9258 m/z).

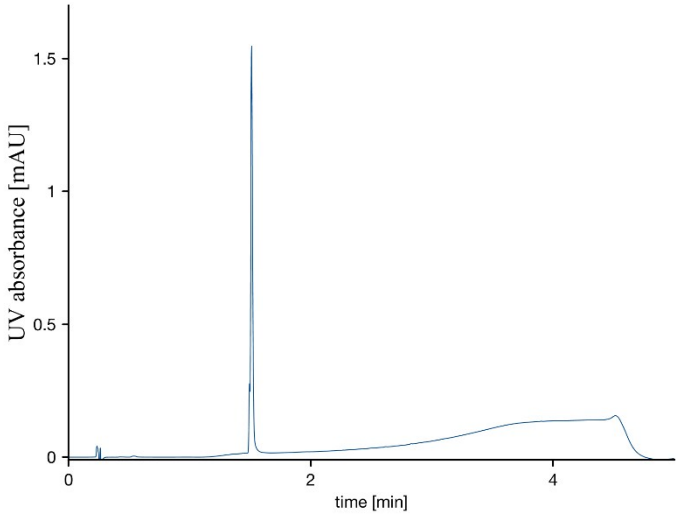


4.34. Linear-(R_{10})-Aha-Cys 15

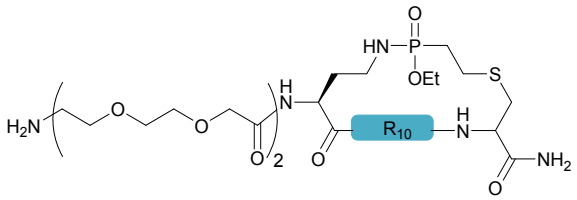


The cyclic-(R10)-azido peptide was synthesized in a 0.1 mmol scale on a Rink Amide Resin with a loading of 0.78 mmol/g. The synthesis was carried out on a PTI synthesizer with double couplings of each amino acid (5 eq. amino acid for 40 min) in DMF apart from Fmoc-azidohomoalanin, which (2eq.) was coupled with HATU (2 eq.) for four hours in presence of NMM (4 eq.) as activating base. SPPS was ending with an Fmoc deprotection step to yield the free *N*-terminal amine. Final cleavage from resin was accomplished in two cleavage steps. First the peptide was cleaved from resin with a mixture of TFA:TIS:H_2O (92:4:4;v:v:v) for 2.5 hours, followed by a second cleavage of not fully cleaved protecting groups with TFA:TIS:DTT:Thioanisole (88:2:2:8;v:v:v:v) for one hour. The crude peptide was purified by preparative reverse phase C18 HPLC (0-5 min 95/5, water (0.1%TFA)/MeCN (0.1%TFA); 5-60 min 10/90, water (0.1%TFA)/MeCN (0.1%TFA)). The product **15** was gained as white powder (78 mg as TFA-salt, 24.1 µmol, 24.1 % yield).

HRMS: m/z: 700.0462 $[M+3H]^{3+}$ (calc. m/z: 700.0908).

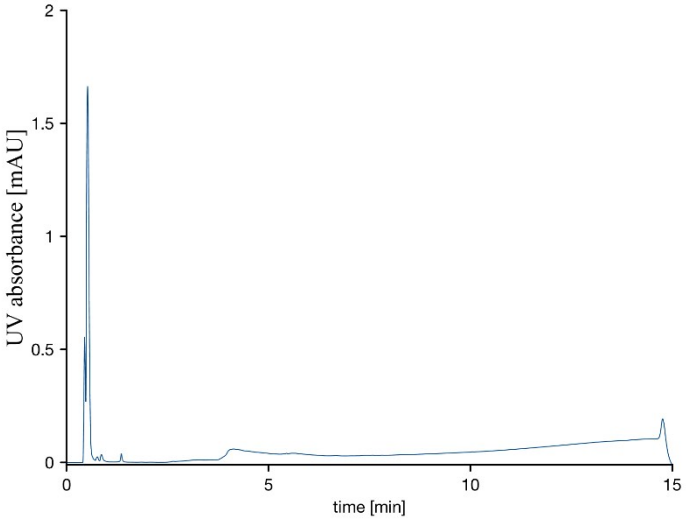


4.35. Cyclic-R10-Staudinger-Macrocycle 16

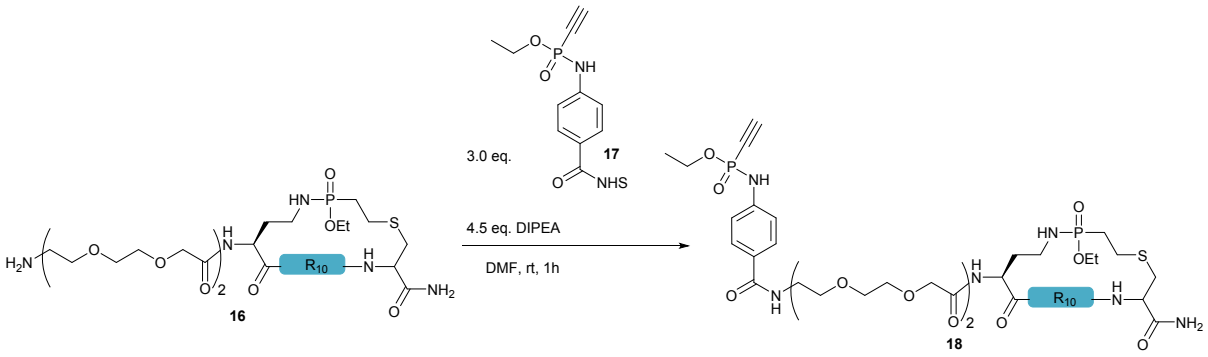


Peptide **15** was reacted according to general procedure 5 with the deviation that the reaction was carried out at 50°C instead of room temperature. The crude peptide was purified by preparative reverse phase C18 HPLC (0-5 min 95/5, water (0.1%TFA)/MeCN (0.1%TFA); 5-60 min 10/90, water (0.1%TFA)/MeCN (0.1%TFA)). The product **16** was gained as white powder (10.7 mg as TFA-salt, 3.2 µmol, 26.8 % yield).

HRMS: m/z: 730.7141 $[M+3H]^{3+}$ (calc. 730.7668 m/z).

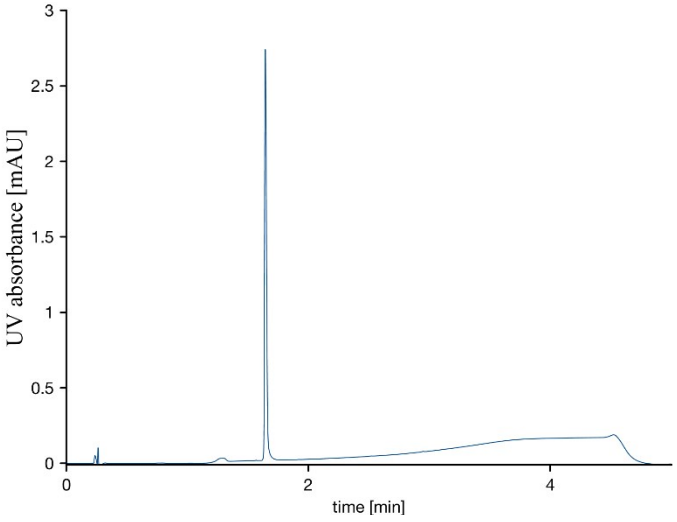


4.36. Alkyne-Cyclic-R_{10}-Staudinger-Macrocycle 18



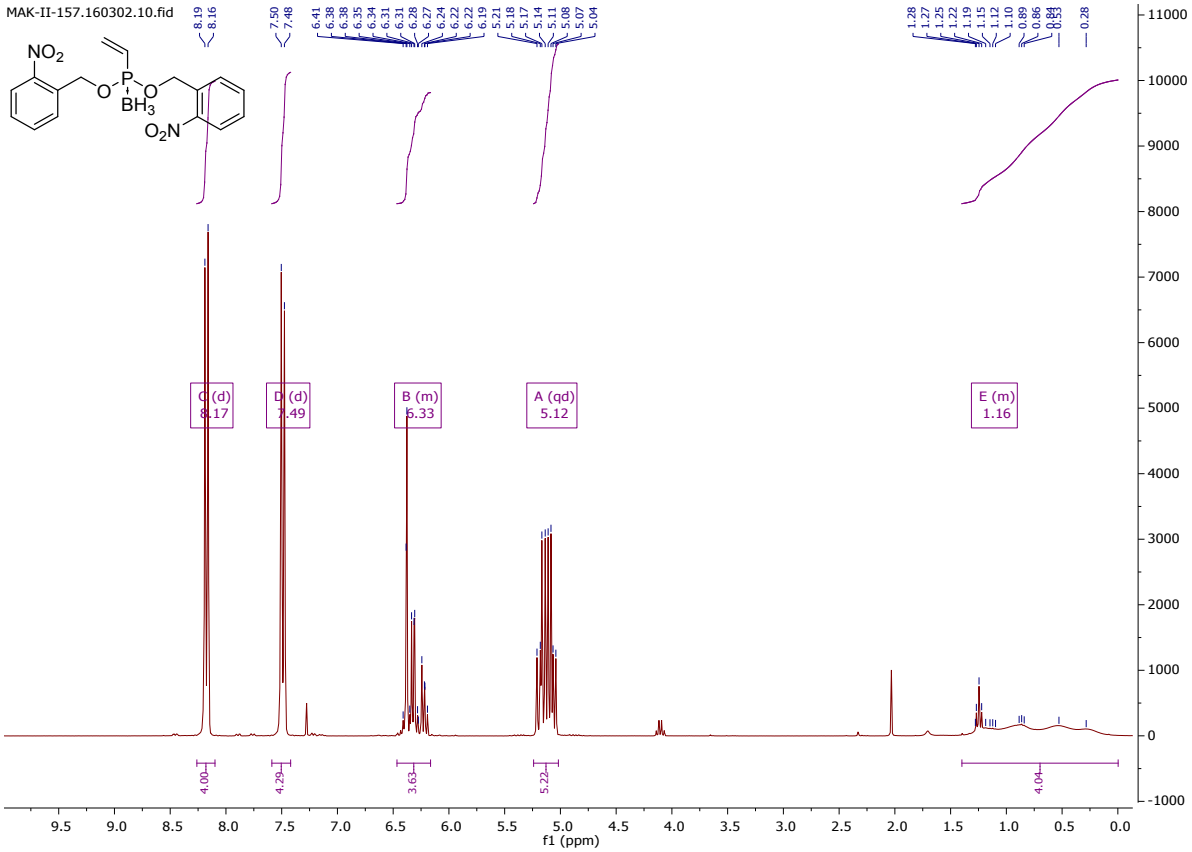
Peptide **16** (20 mg, 6 µmol, 1 eq.) solubilized in DMF (400 µl, c = 15 mM) was incubated with NHS-phosphonamidate alkyne **17** (6.3 mg, 18.0 µmol, 3eq.) in presence of DIPEA (4.68 µl, 26.9 µmol, 4.5 eq.) for one hour. After confirmation of product formation by LC-MS, the crude was diluted with water and purified by preparative HPLC. The product **18** was gained as white powder (9.8 mg as TFA salt, 2.8 µmol, 46 %yield).

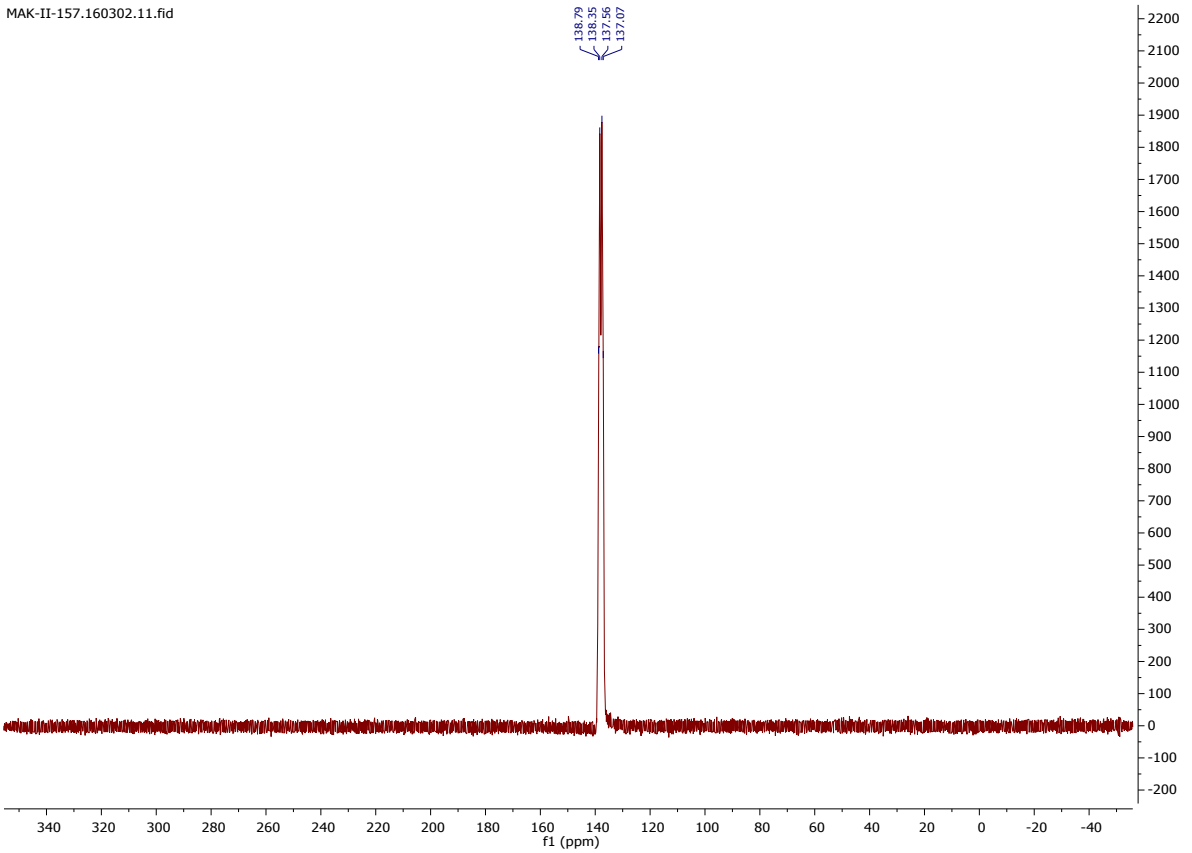
LRMS: m/z: 809.0552 $[M+3H]^{3+}$ (calc. 809.1134 m/z).

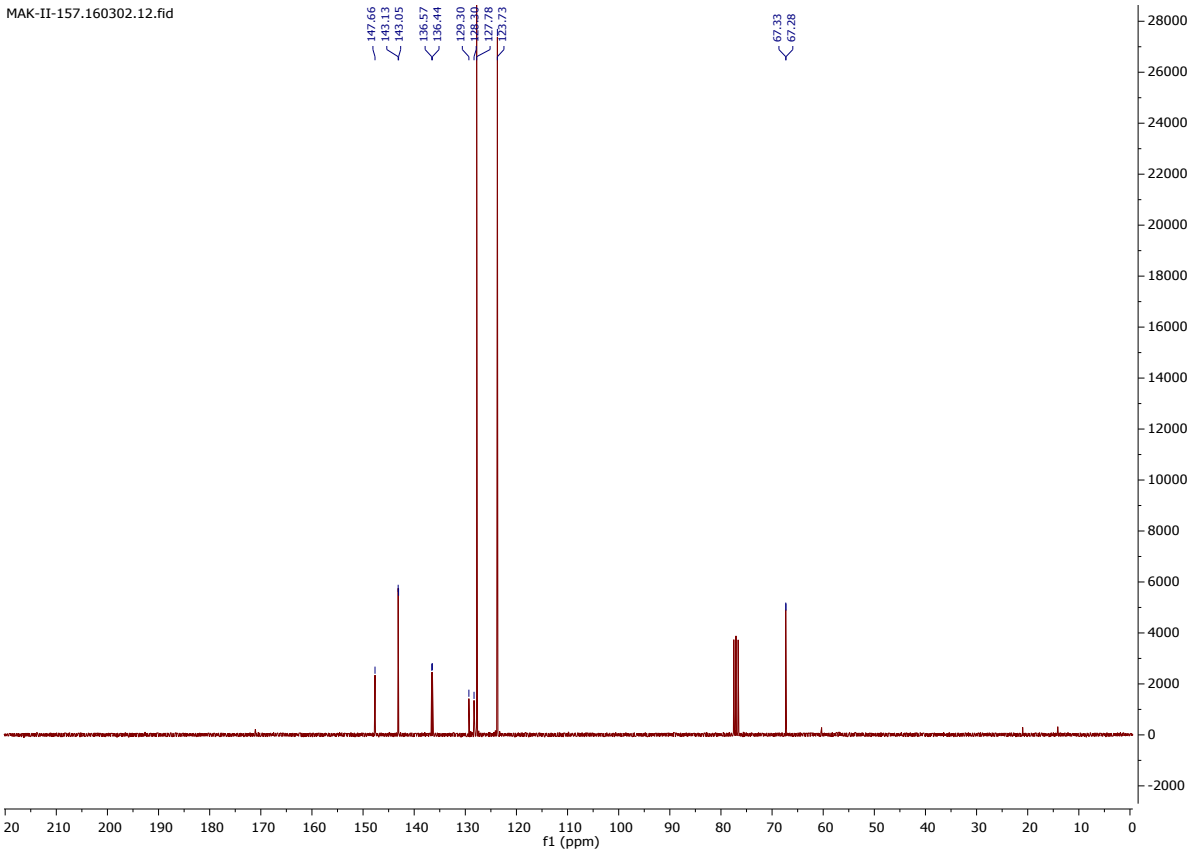


5. NMR spectra

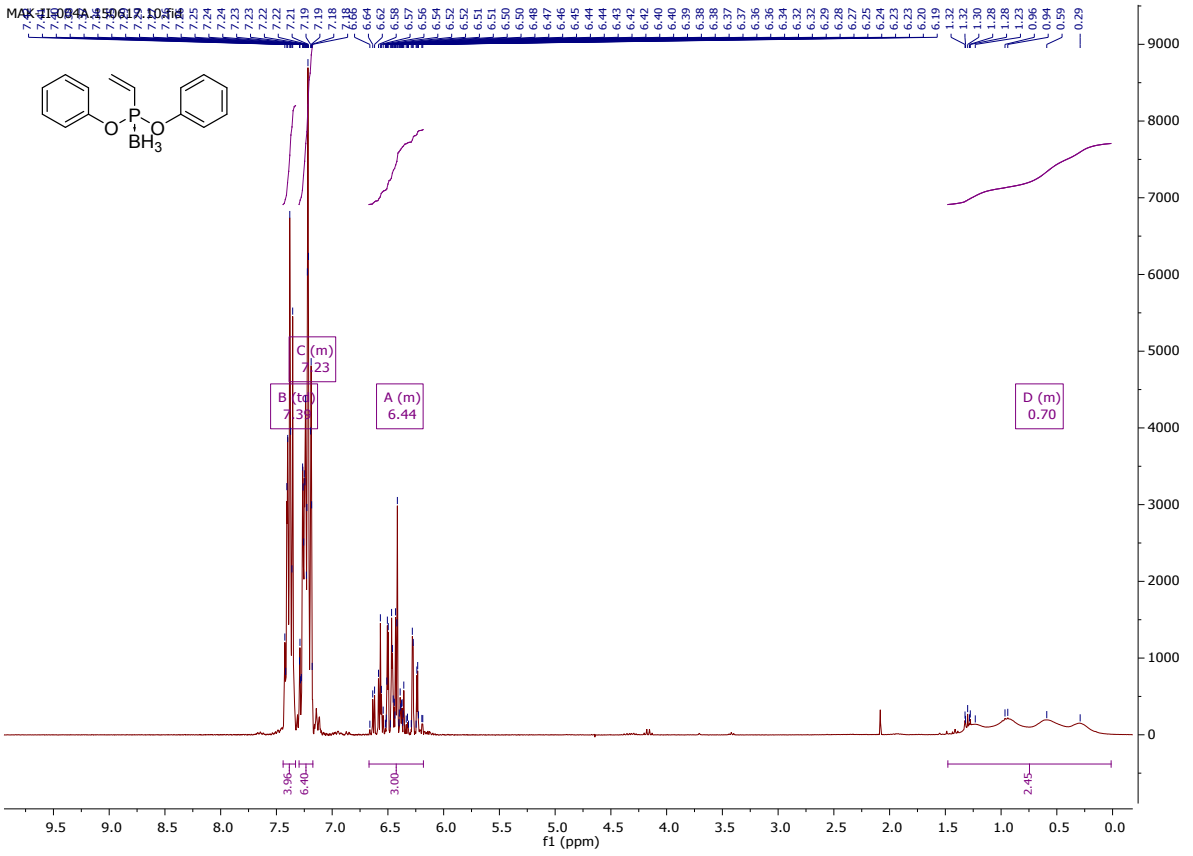
Di(2-nitrobenzyl) vinylphosphonite borane (**1b**)

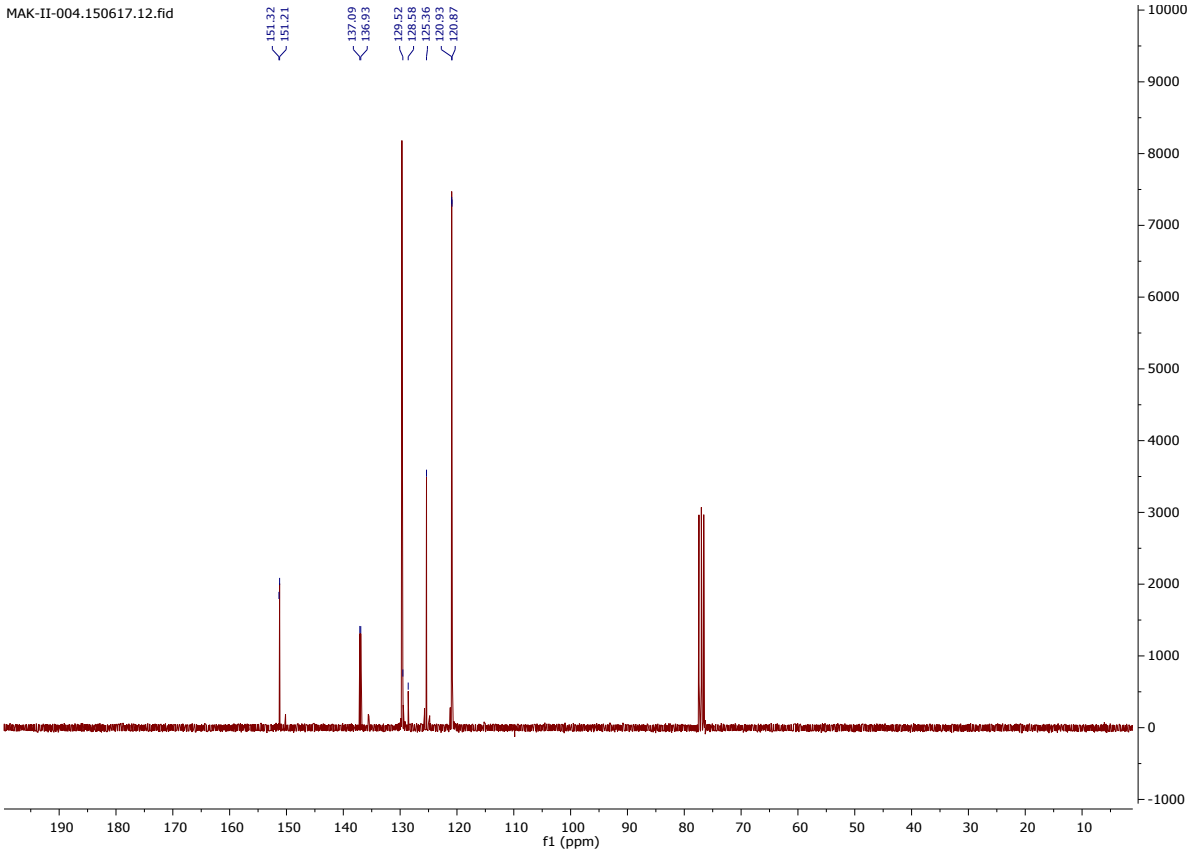


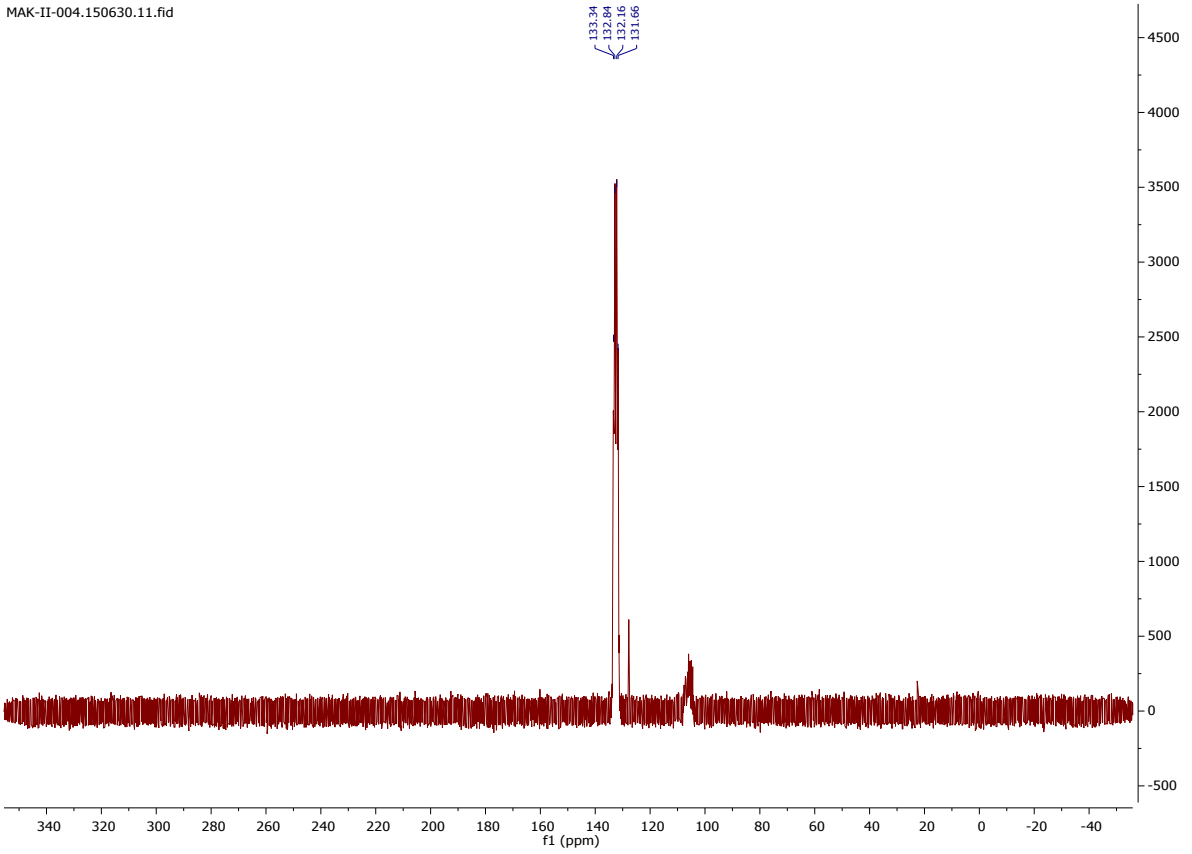




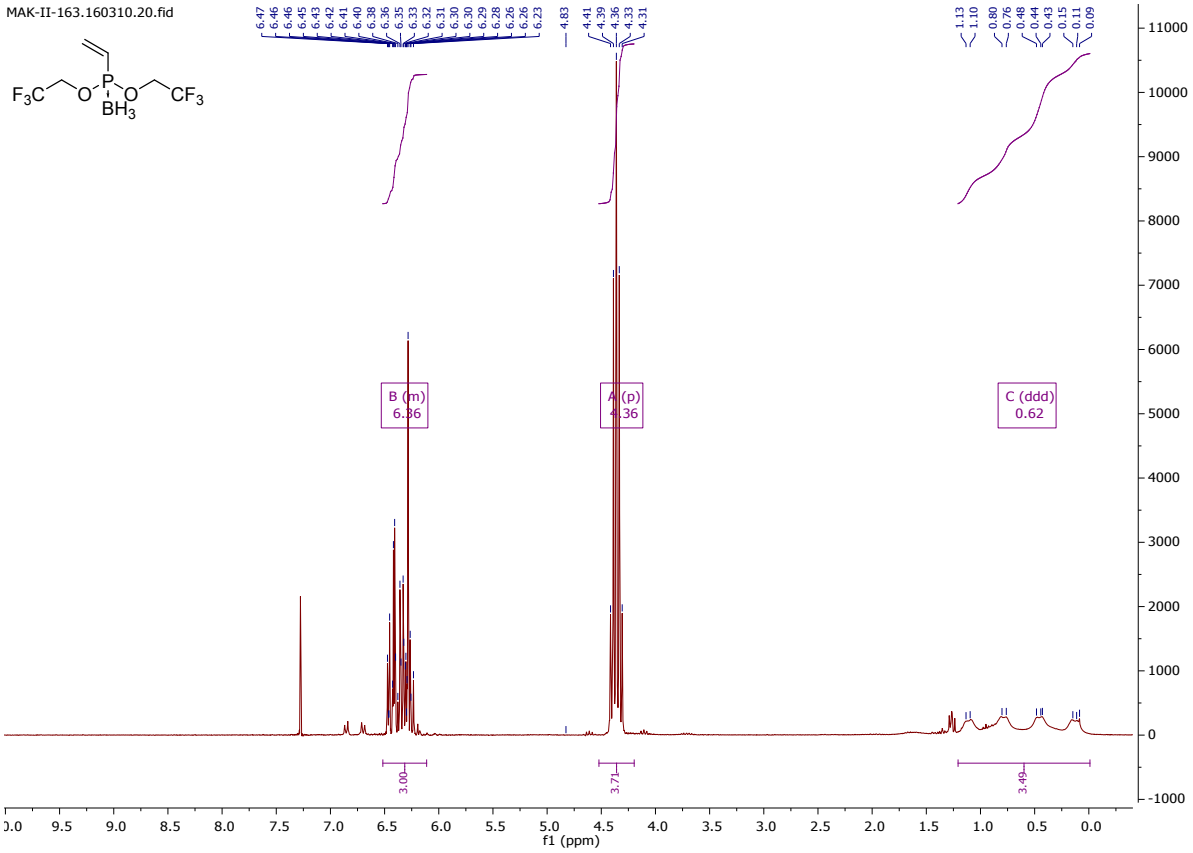
Diphenyl vinylphosphonite borane (**1c**)

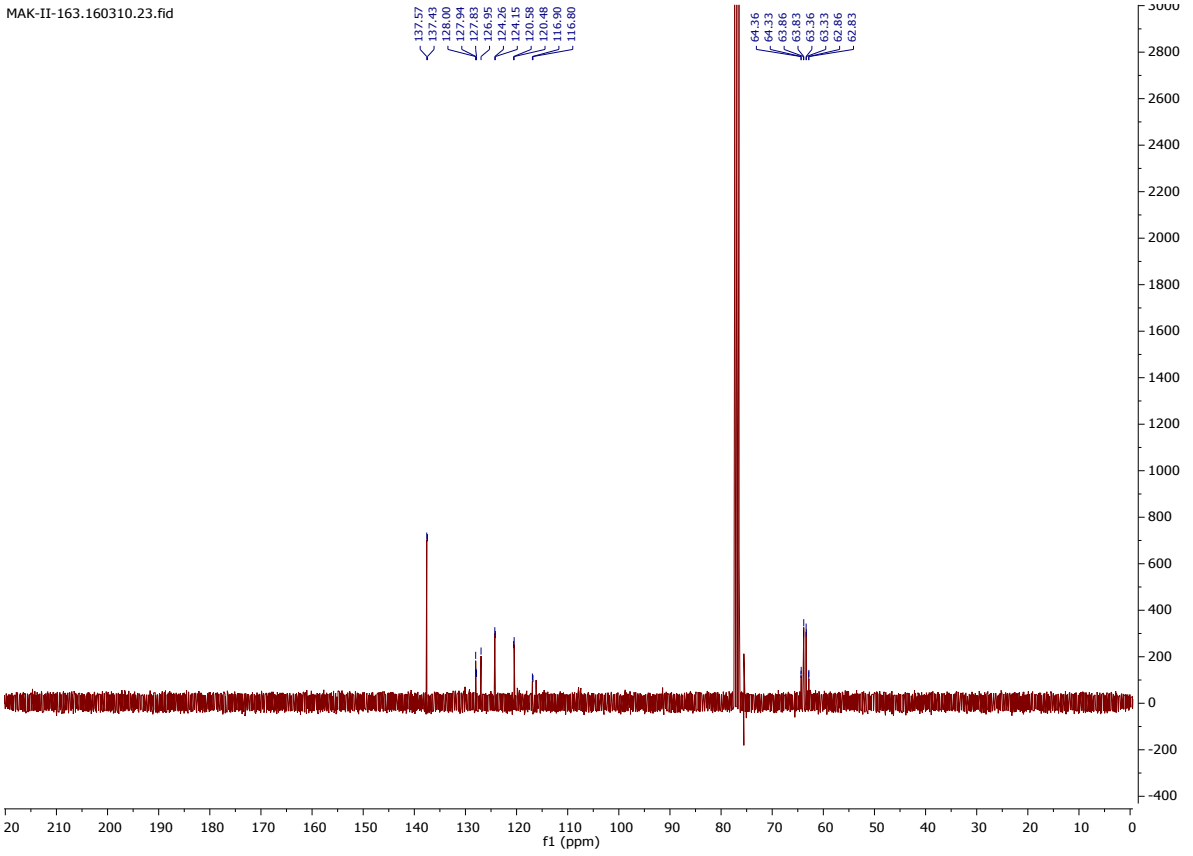


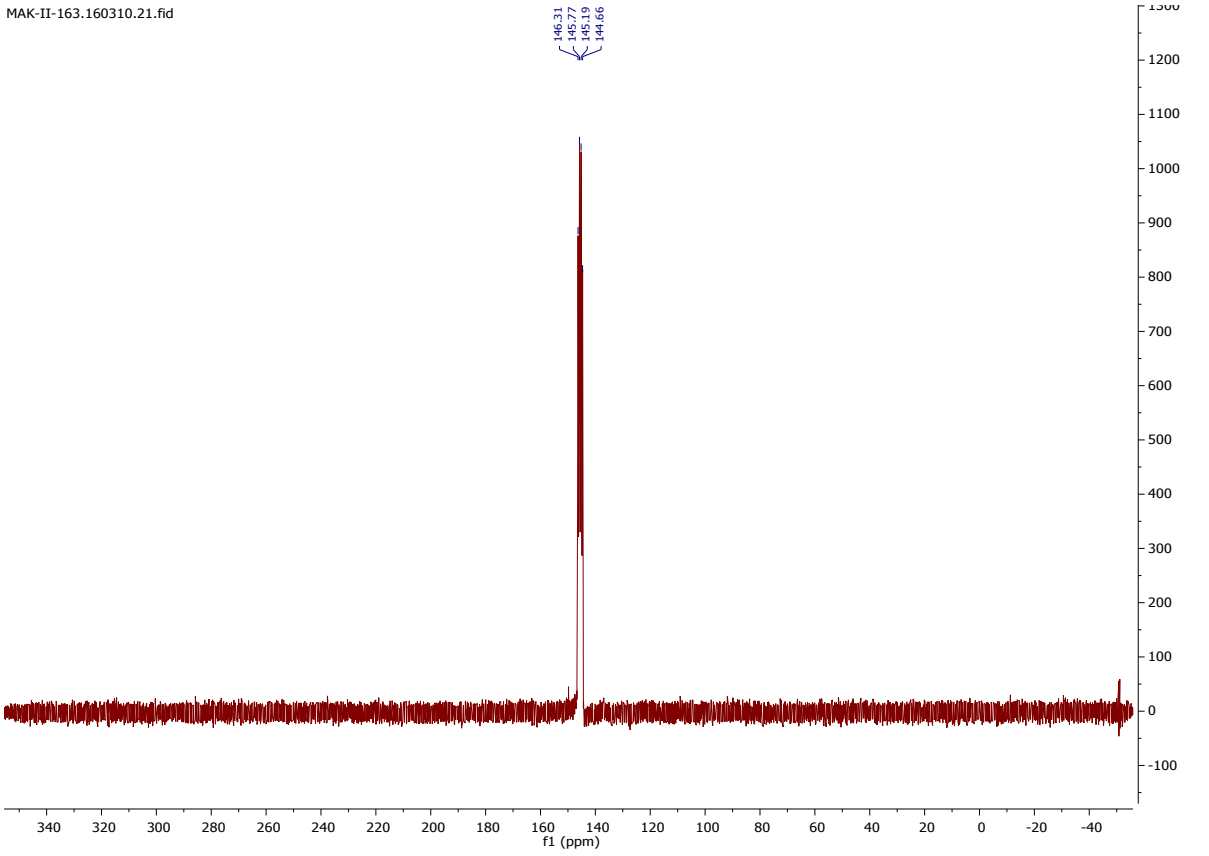

MAK-II-004.150617.12.fid
151.32
151.21
137.09
136.93
129.52
128.58
125.36
120.93
120.87
f1 (ppm)

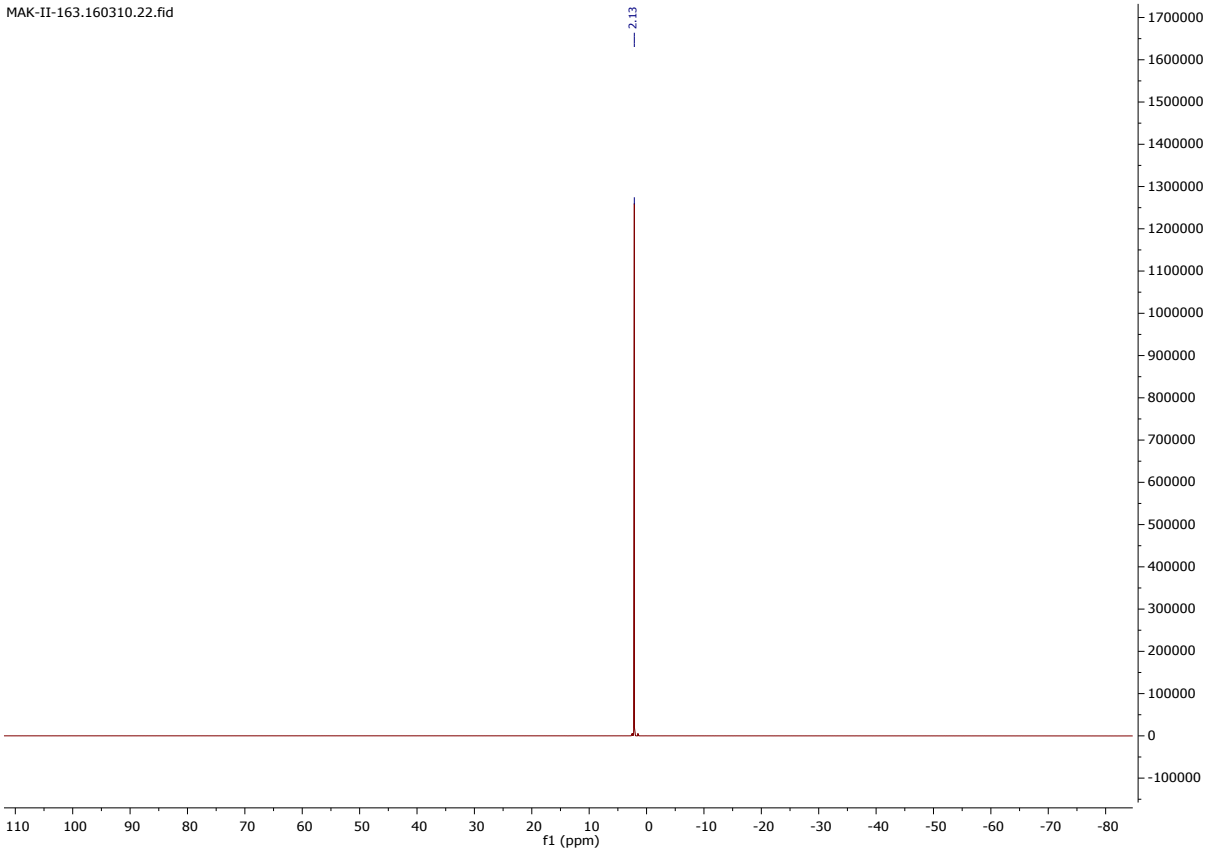

MAK-II-004.150630.11.fid
133.34
132.84
132.16
131.66
f1 (ppm)

Bis(2,2,2-trifluoroethyl) vinylphosphonite borane (**1d**)

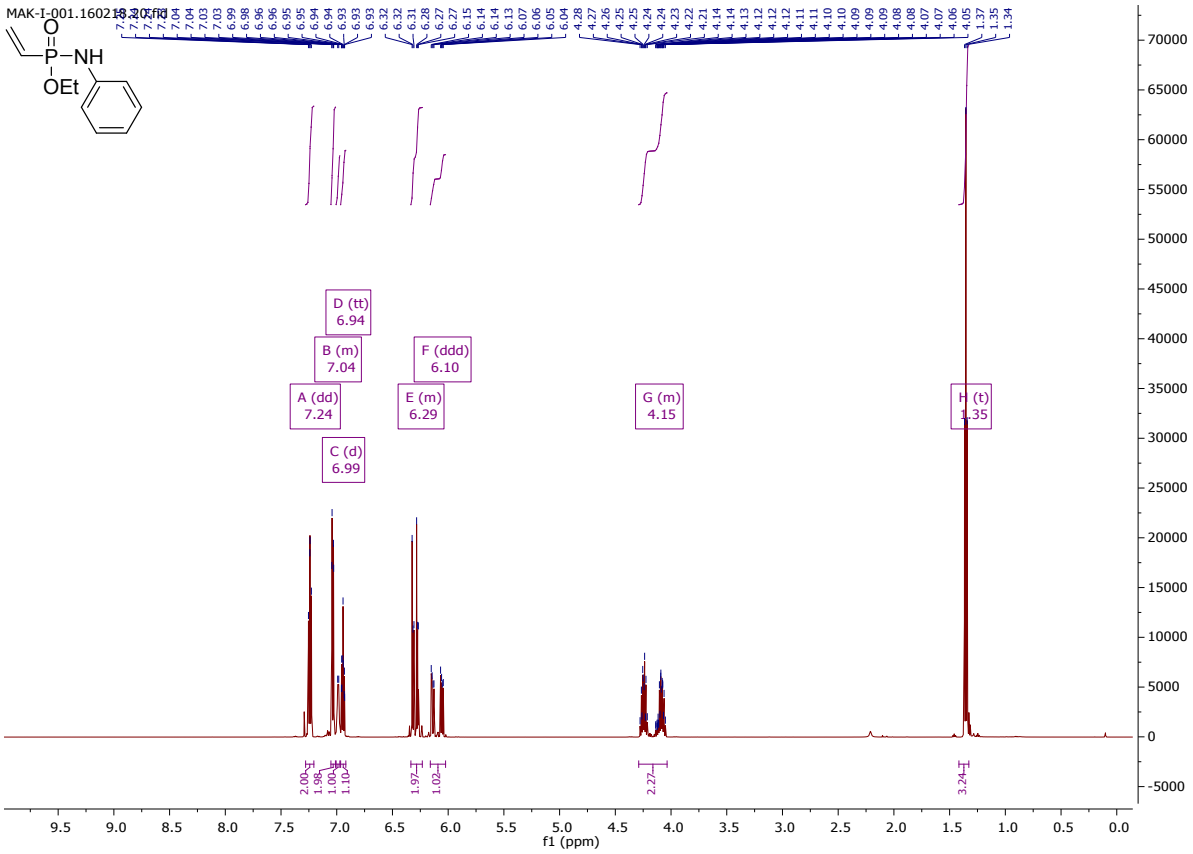


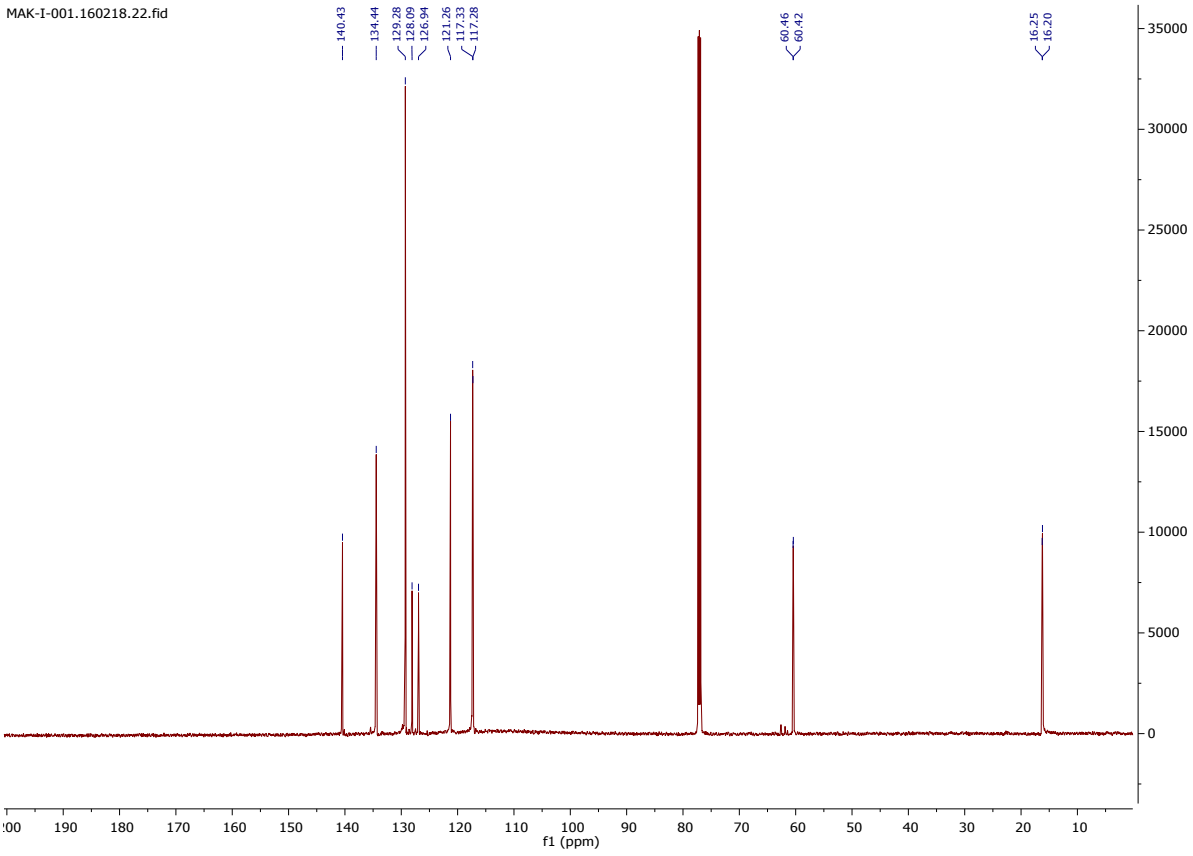


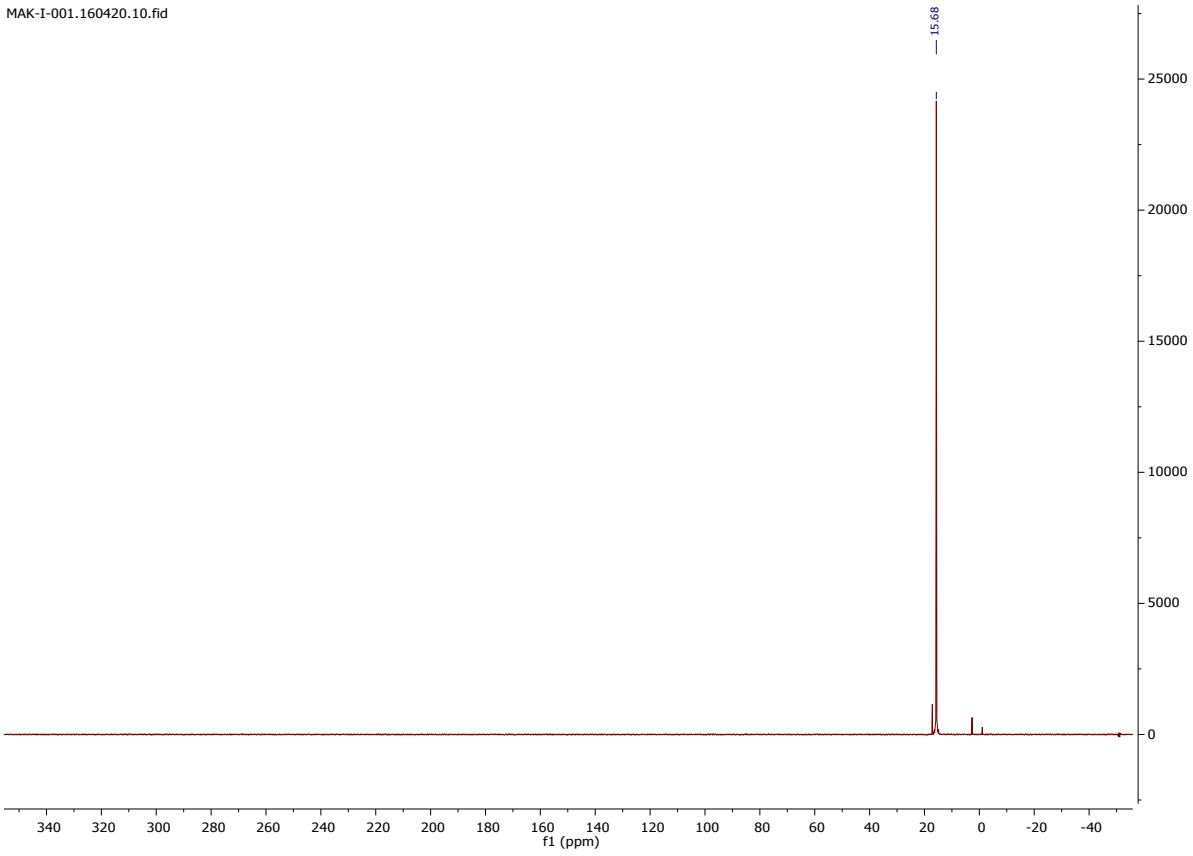

MAK-II-163.160310.21.fid
146.31
145.77
145.19
144.66
f1 (ppm)


MAK-II-163.160310.22.fid
2.13
f1 (ppm)

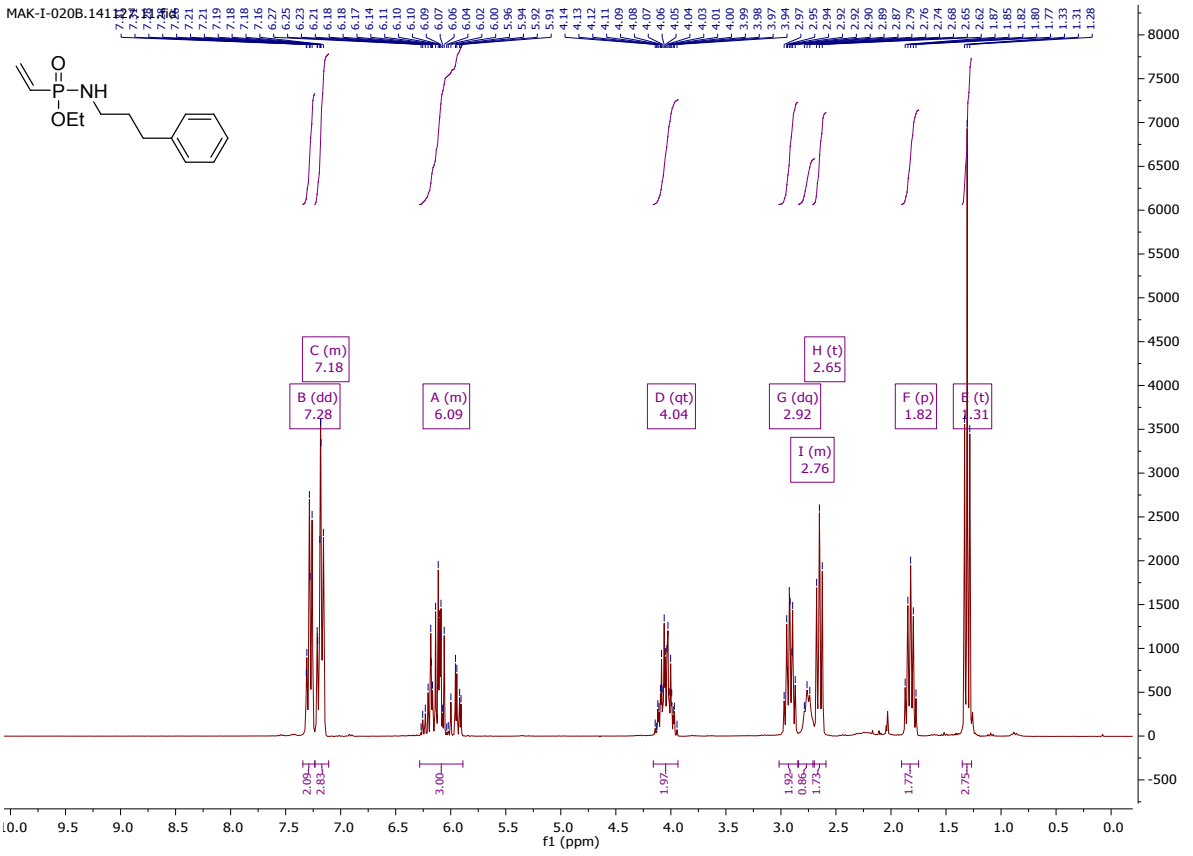
Ethyl-N-phenyl-P-vinylphosphonamidate (**4a**)

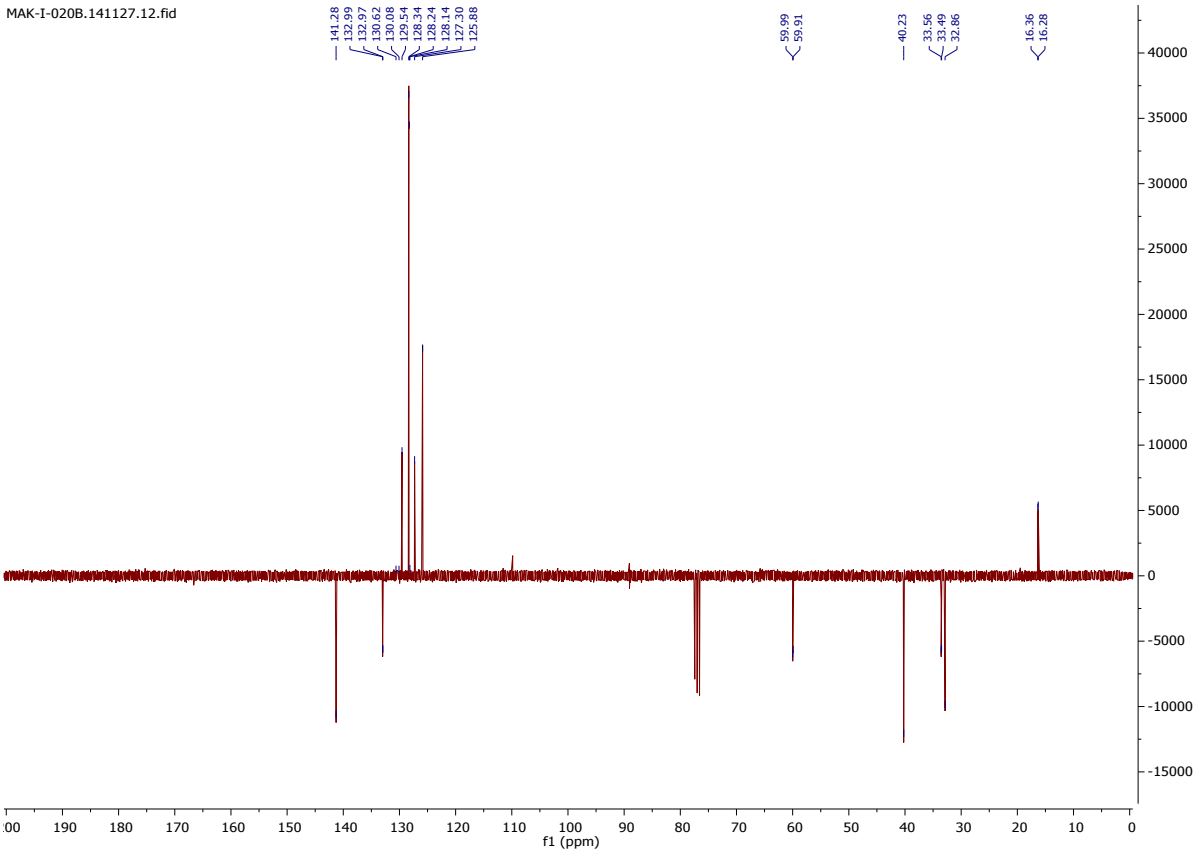


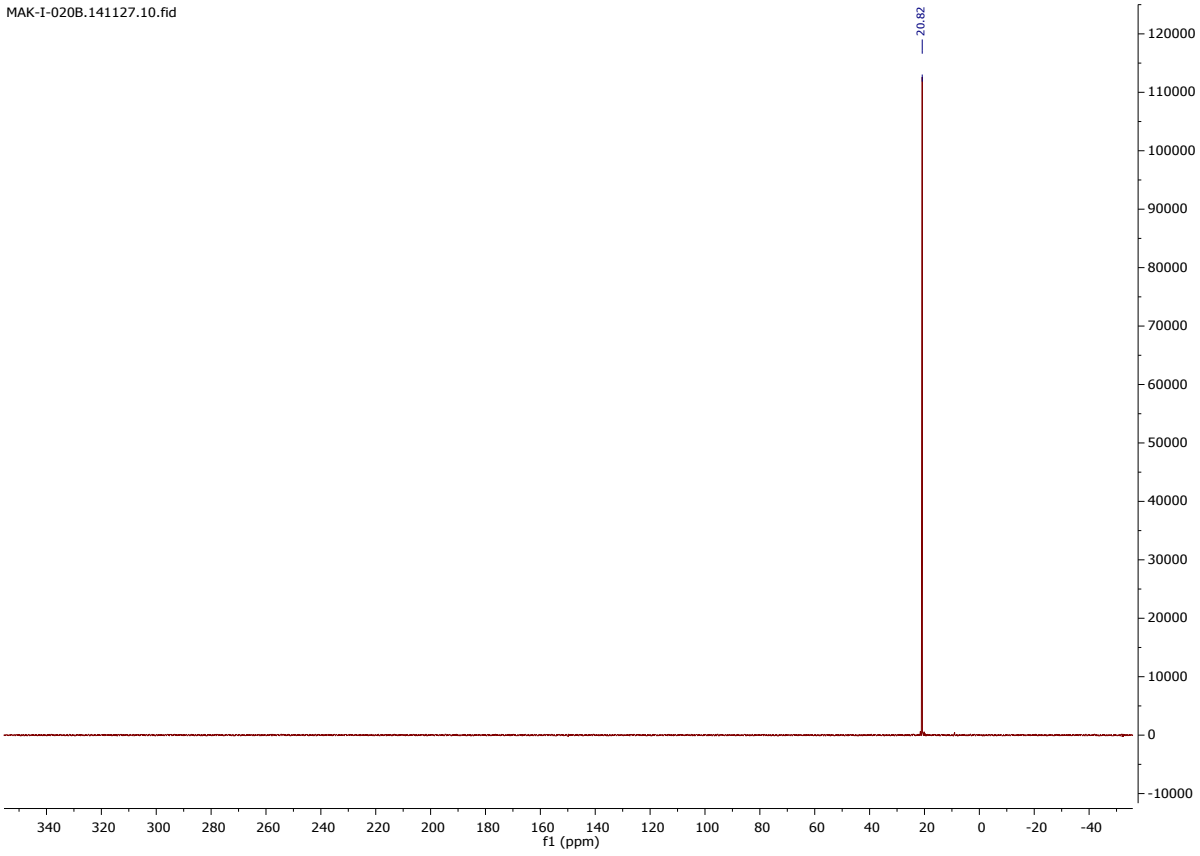




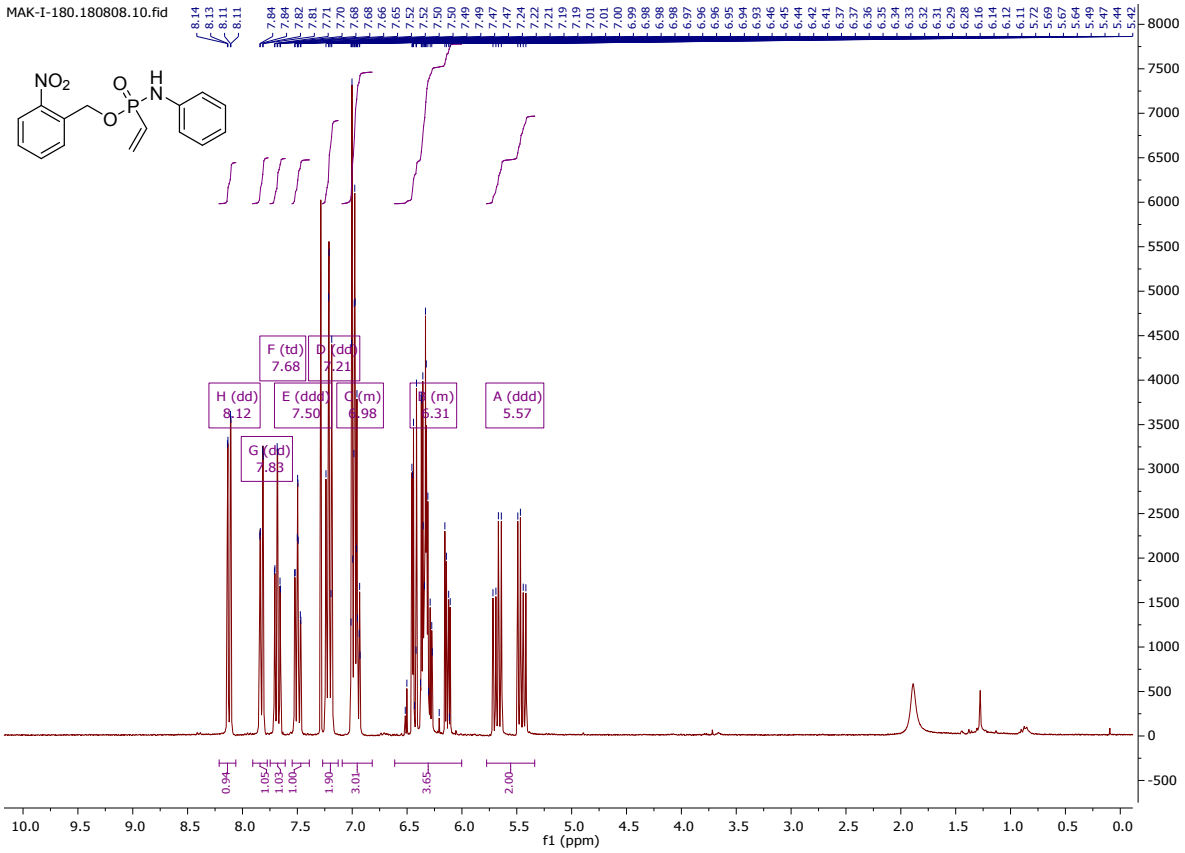
Ethyl-N-(3-phenyl-propyl)-P-vinyl-phosphonamidate (**5a**)

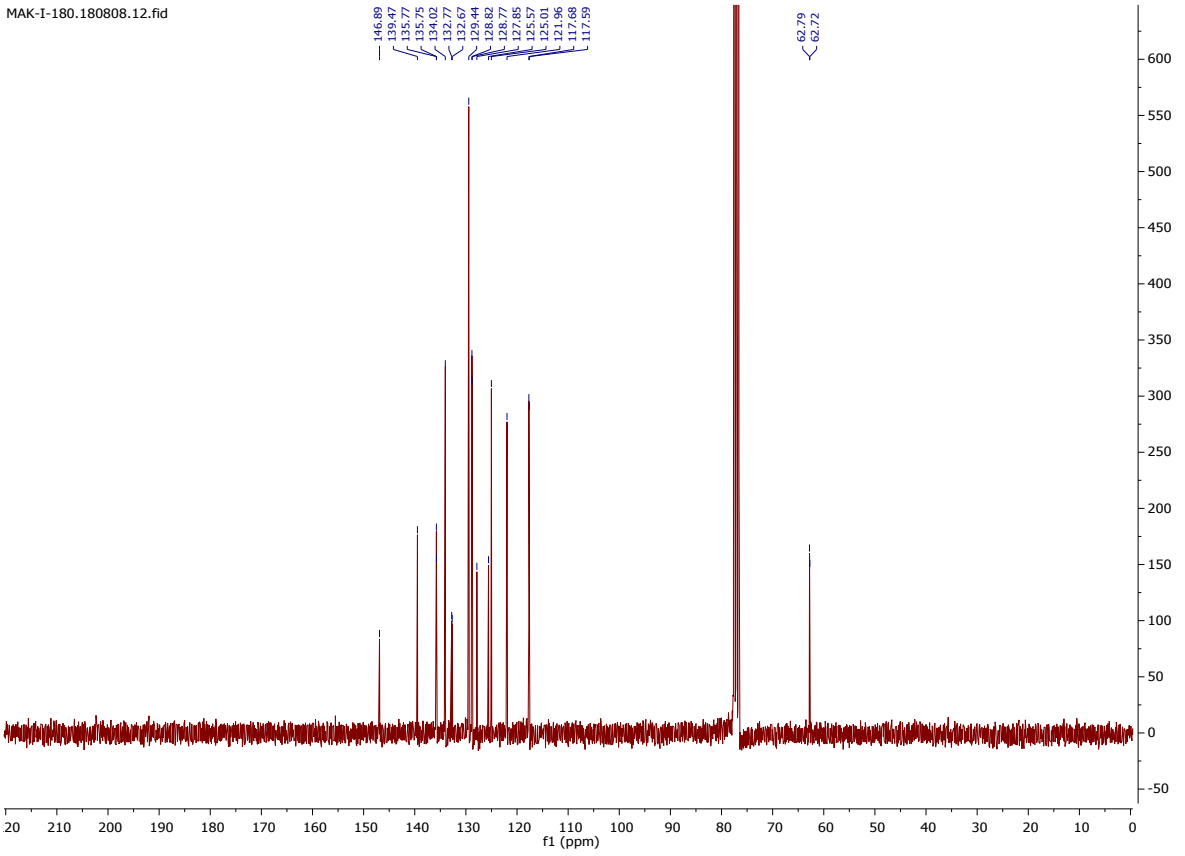


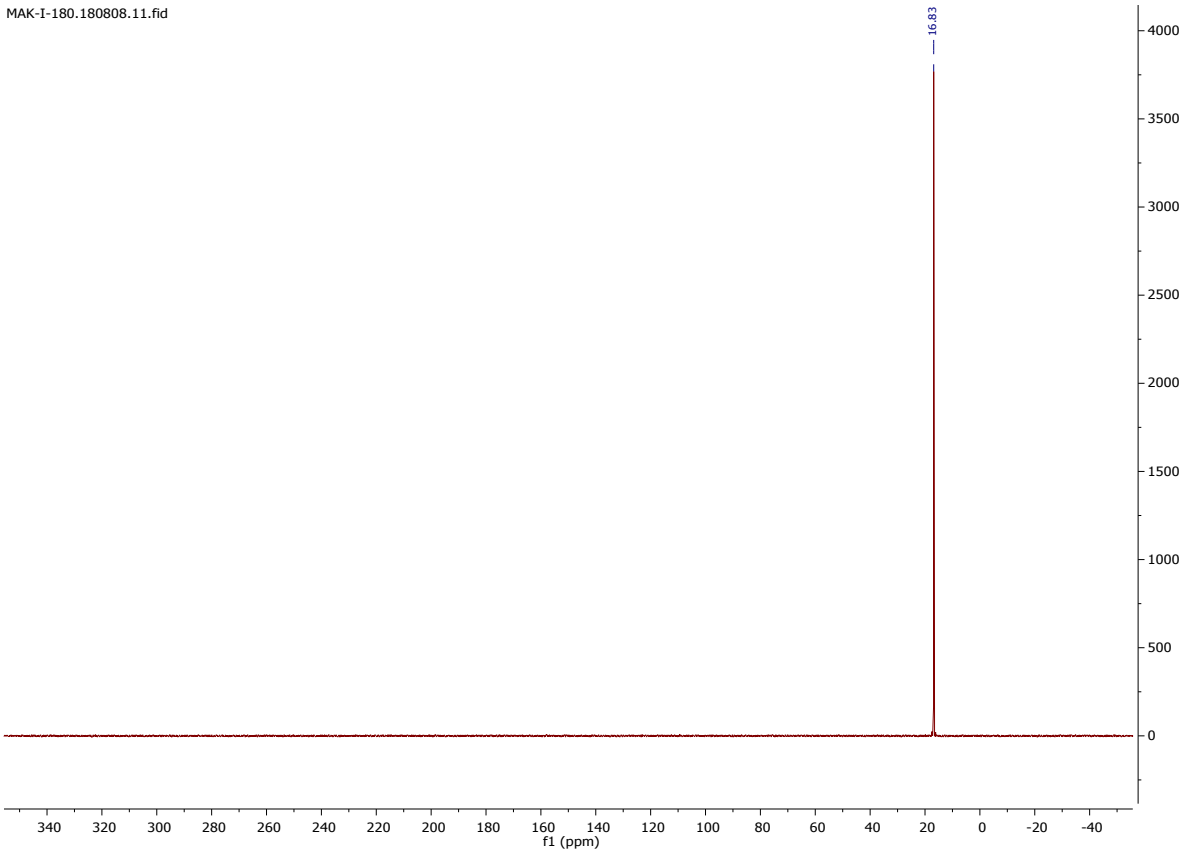

MAK-I-020B.141127.12.fid
141.28
132.99
132.97
130.62
130.08
129.54
128.34
128.24
128.14
127.30
125.88
59.99
59.91
40.23
33.56
33.49
32.86
16.36
16.28
f1 (ppm)


MAK-I-020B.141127.10.fid
20.82
f1 (ppm)

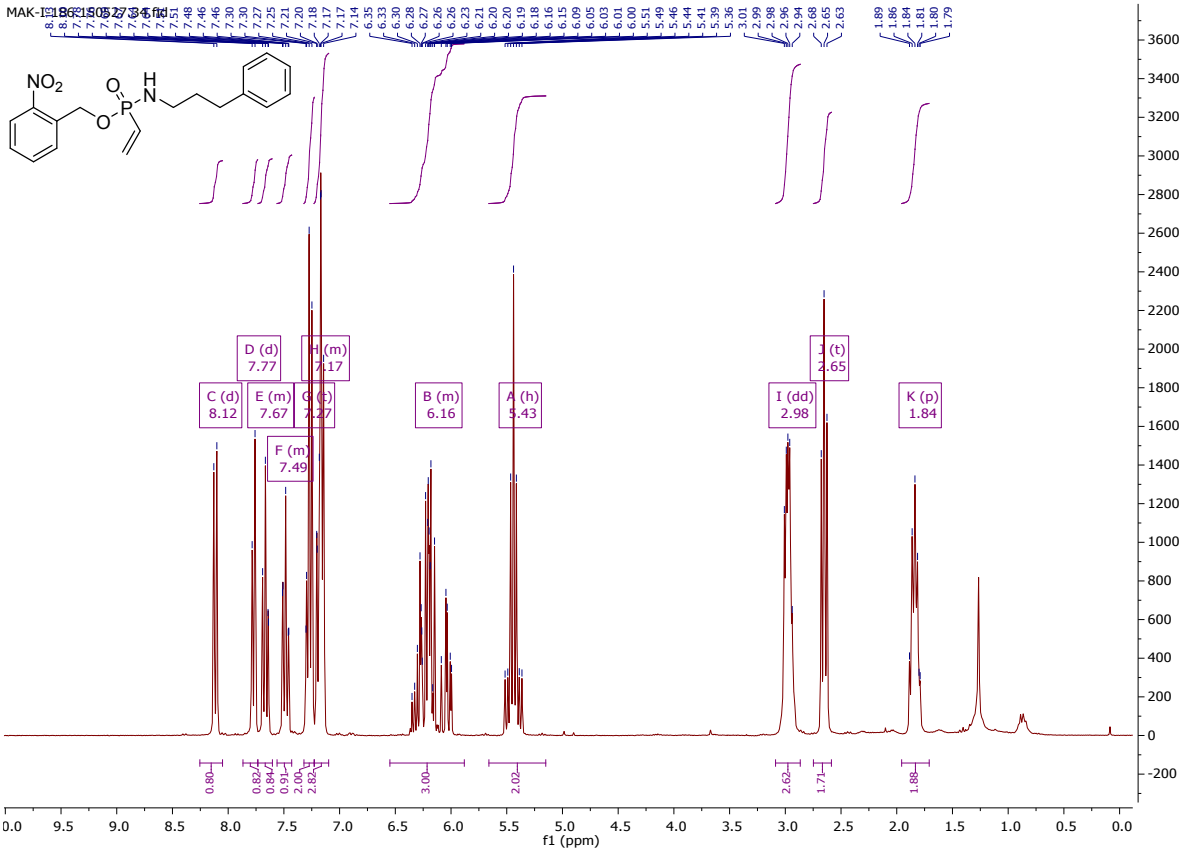
2-Nitrobenzyl-*N*-phenyl-*P*-vinylphosphonamidate (**4b**)

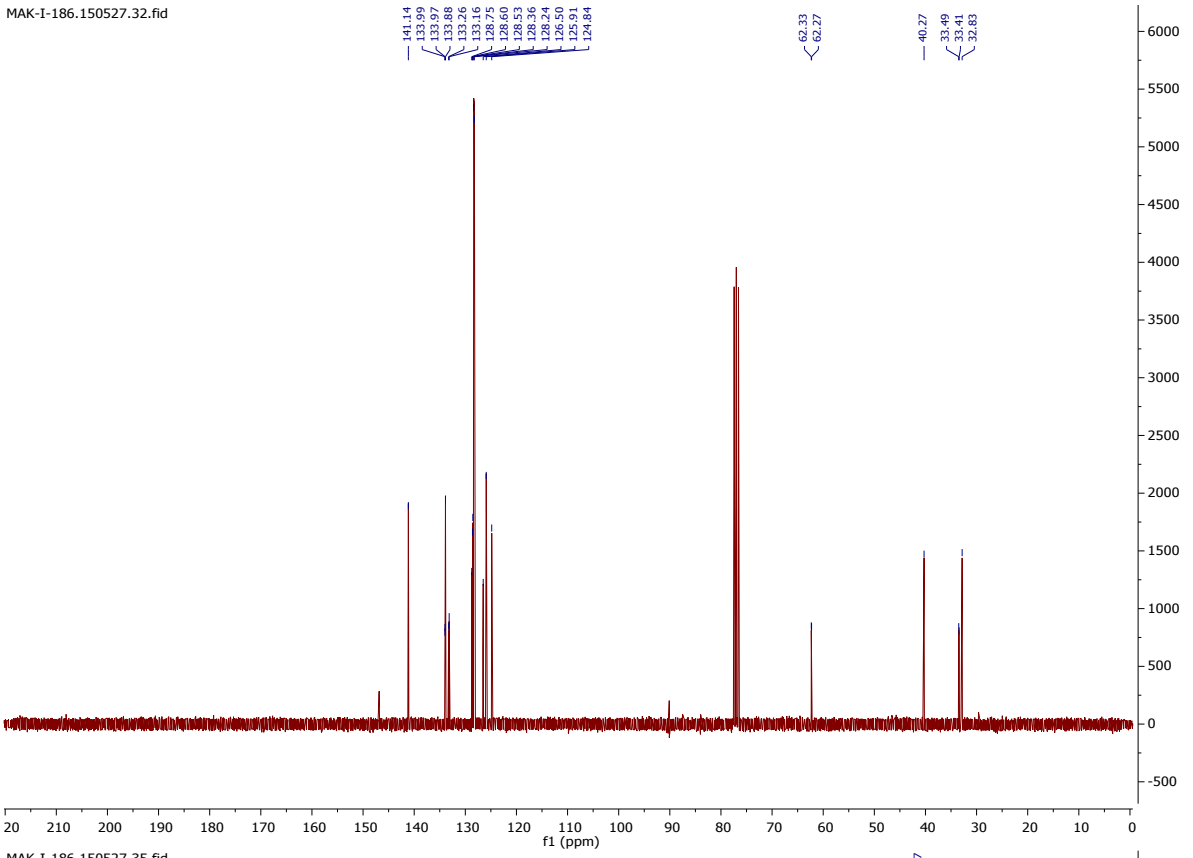


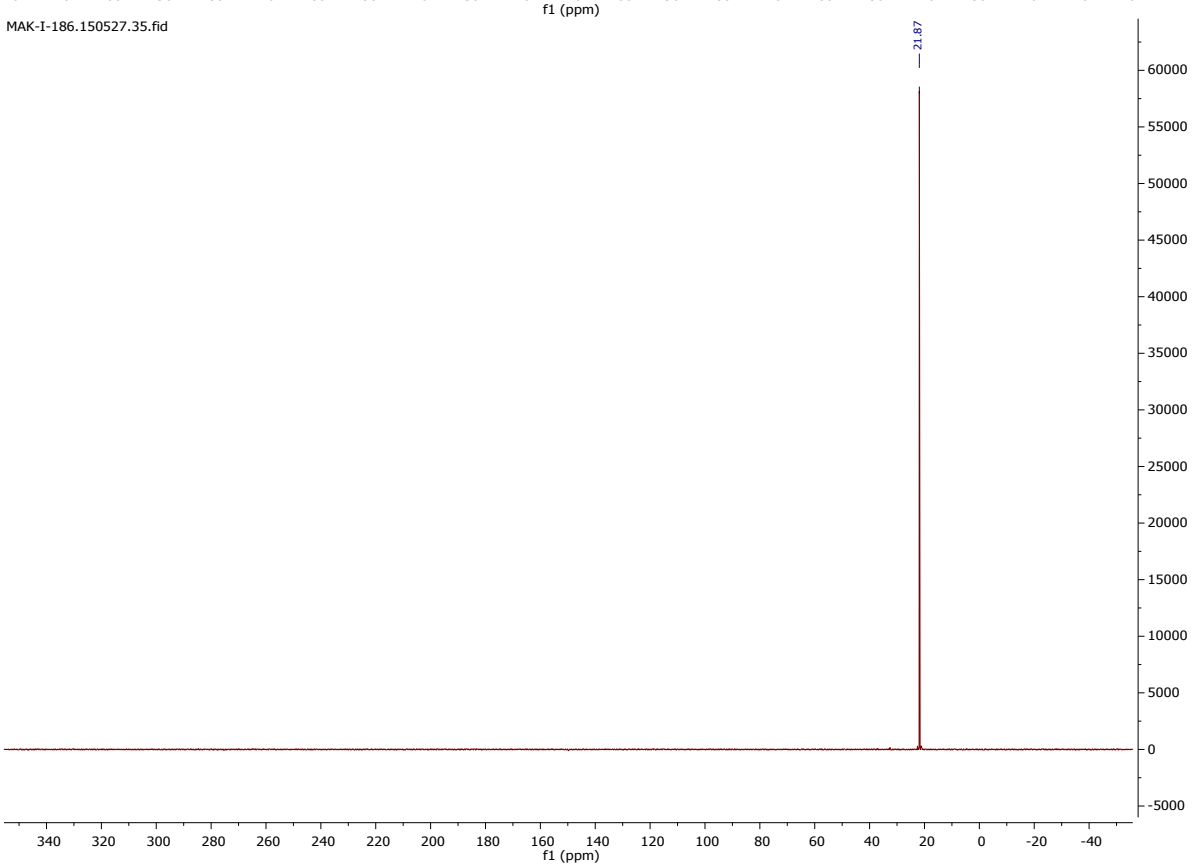




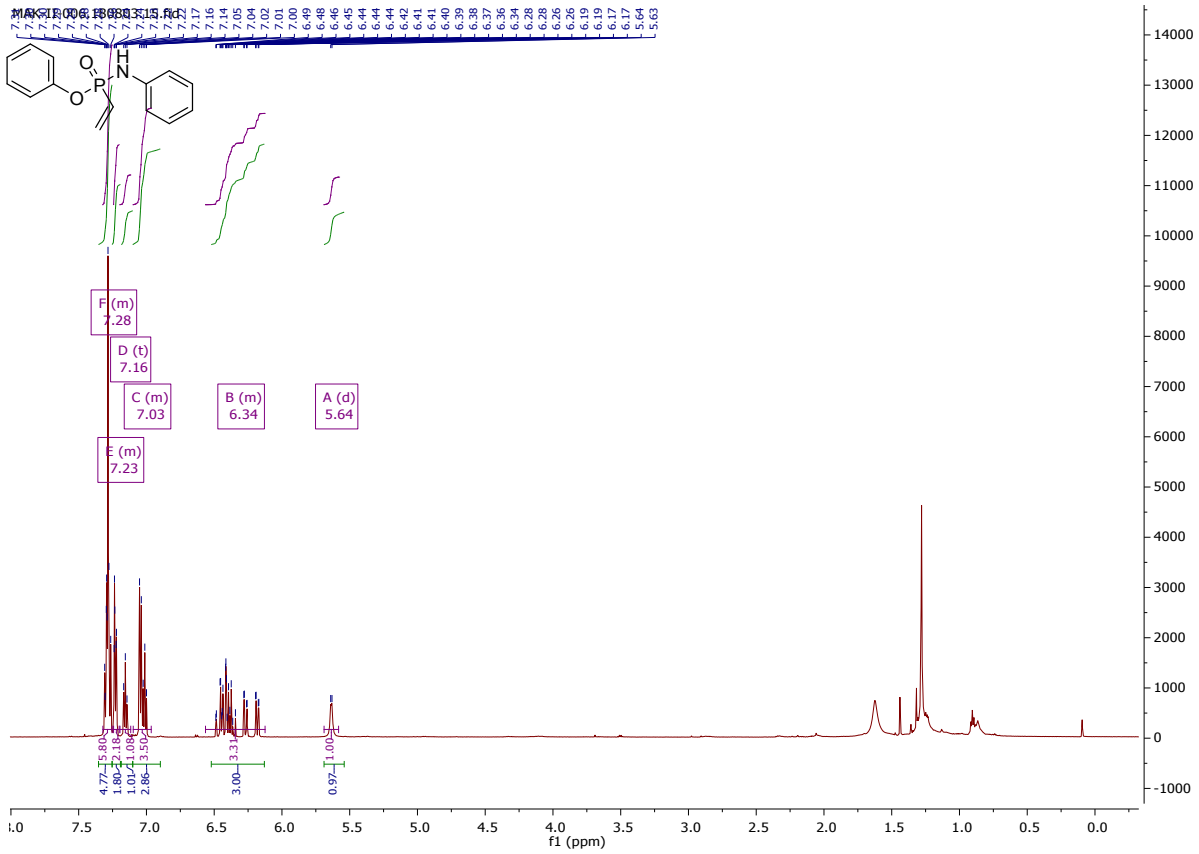
2-Nitrobenzyl-*N*-(3-phenyl-propyl)-*P*-vinylphosphonamidate (**5b**)

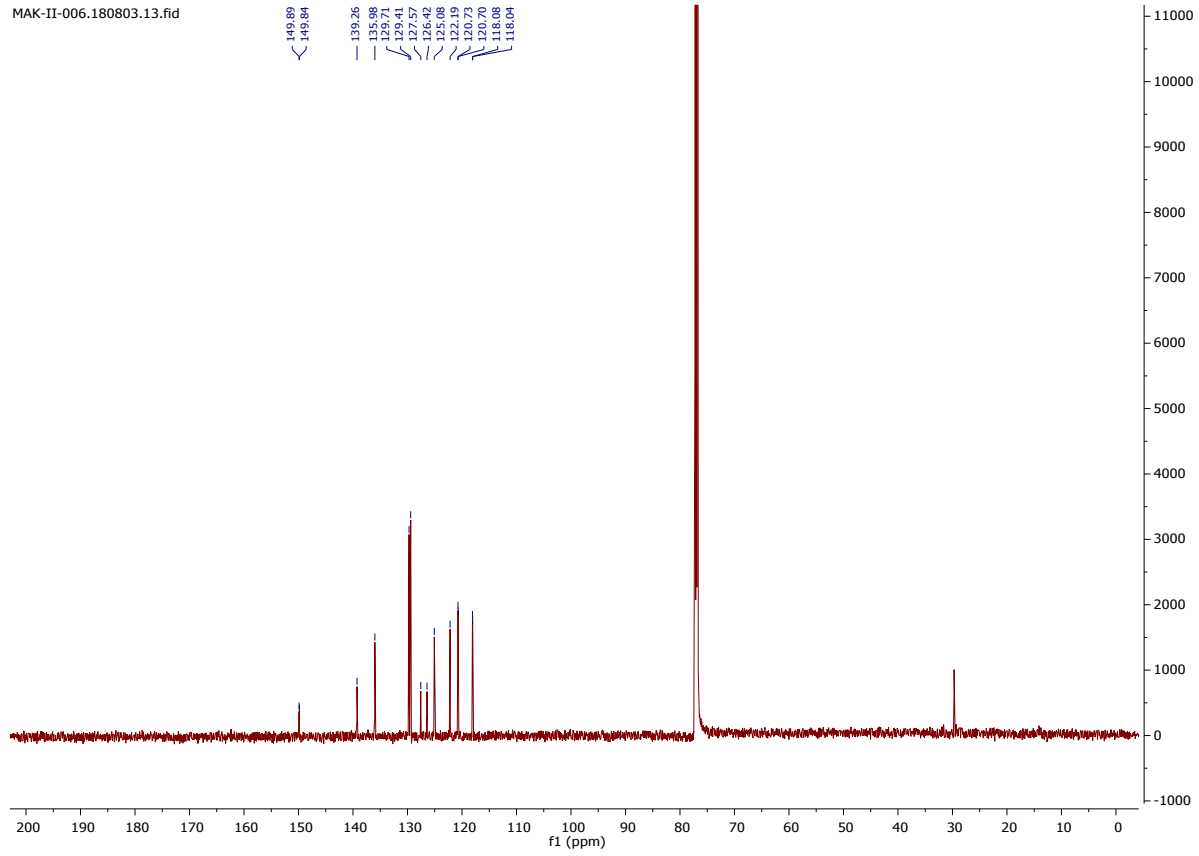


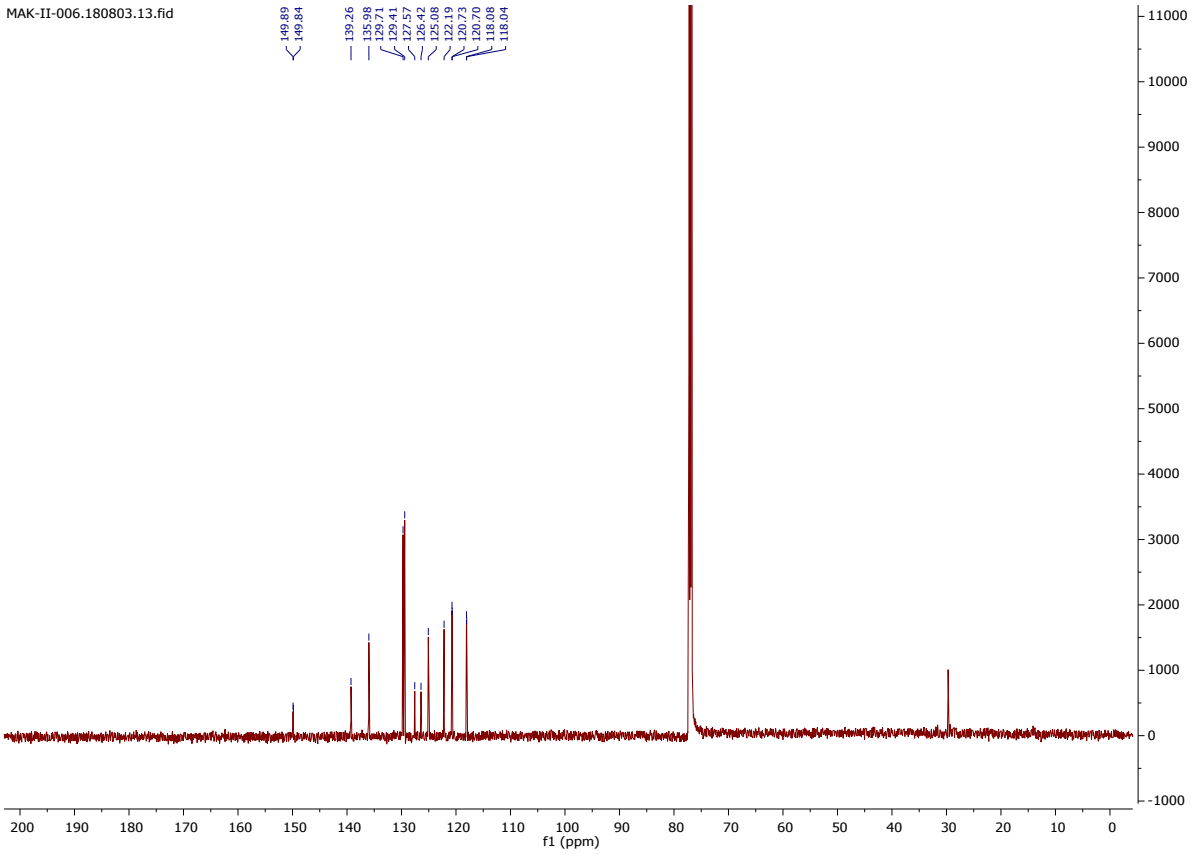

MAK-I-186.150527.32.fid
141.14
133.99
133.97
133.88
133.26
133.16
128.75
128.60
128.53
128.36
128.24
126.50
125.91
124.84
62.33
62.27
40.27
33.49
33.41
32.83
f1 (ppm)


MAK-I-186.150527.35.fid
21.87
f1 (ppm)

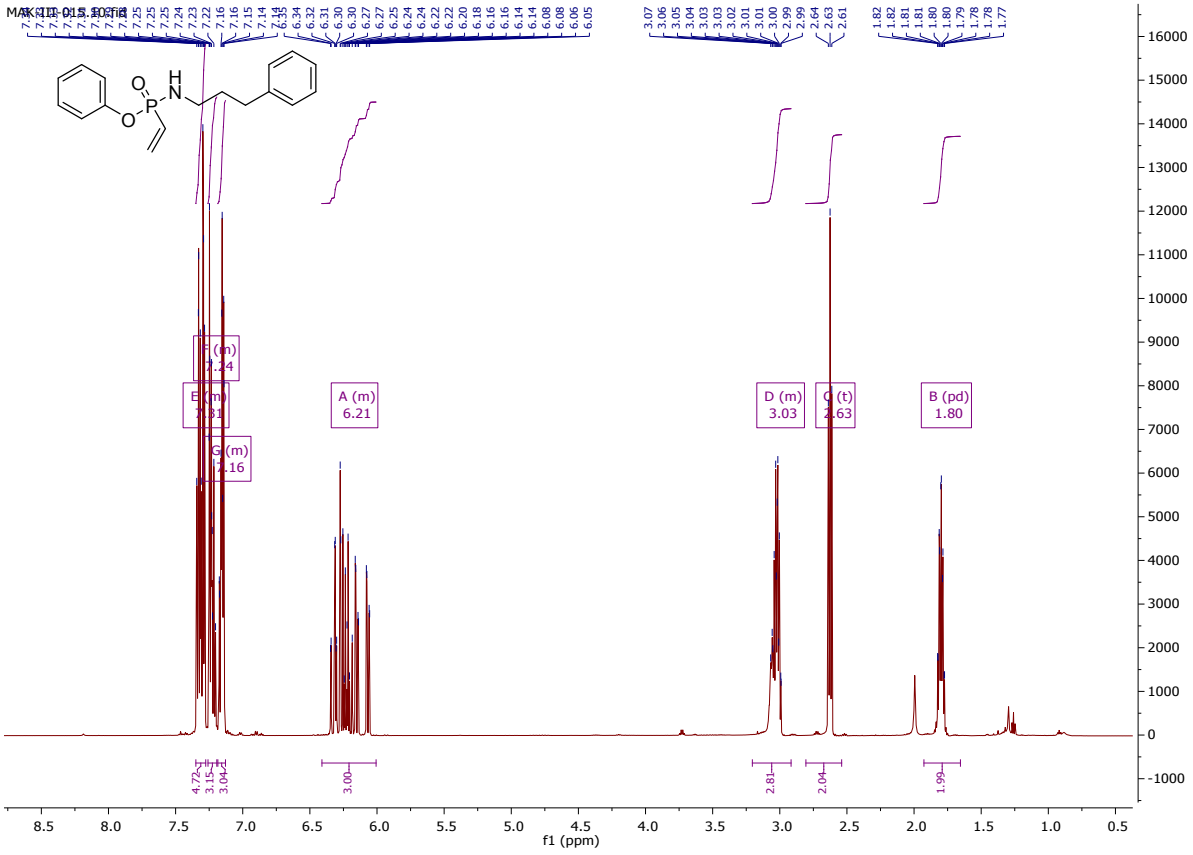
phenyl-*N*-phenyl-*P*-vinylphosphonamidate (**4c**)

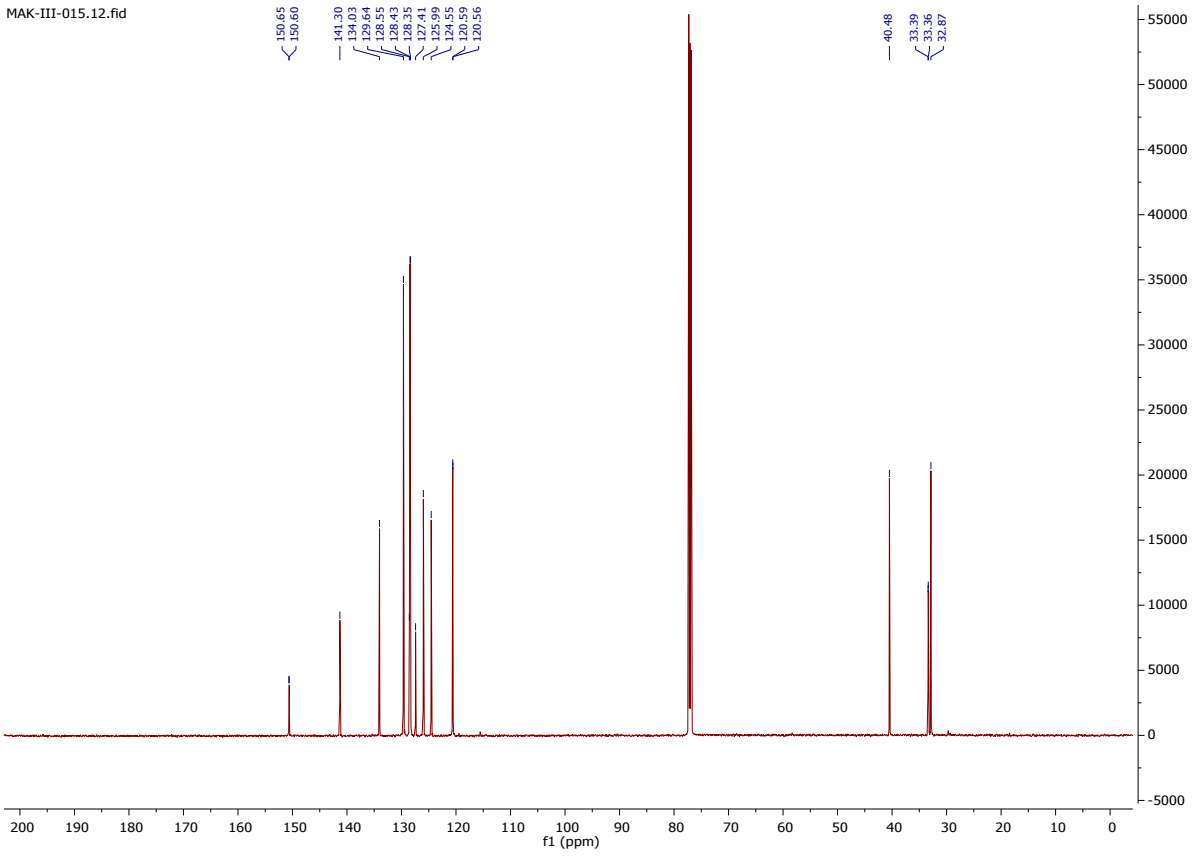


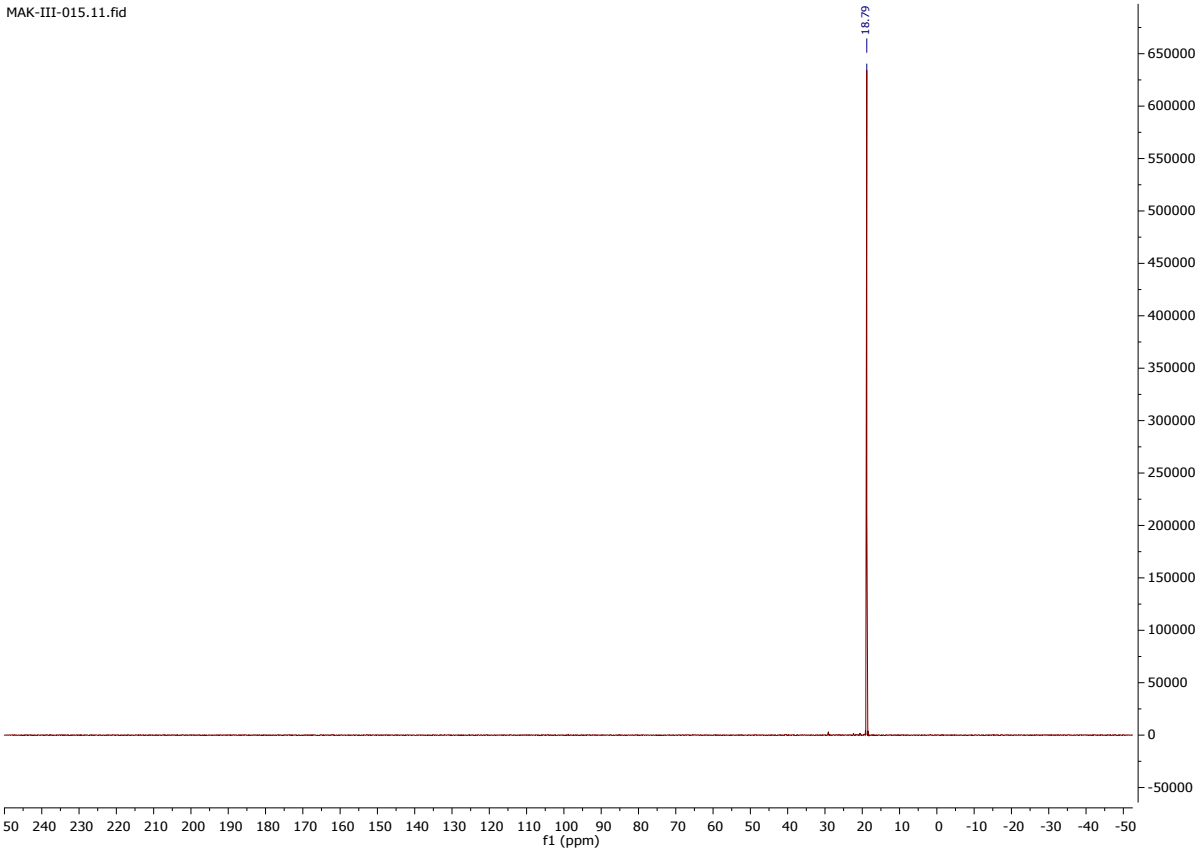




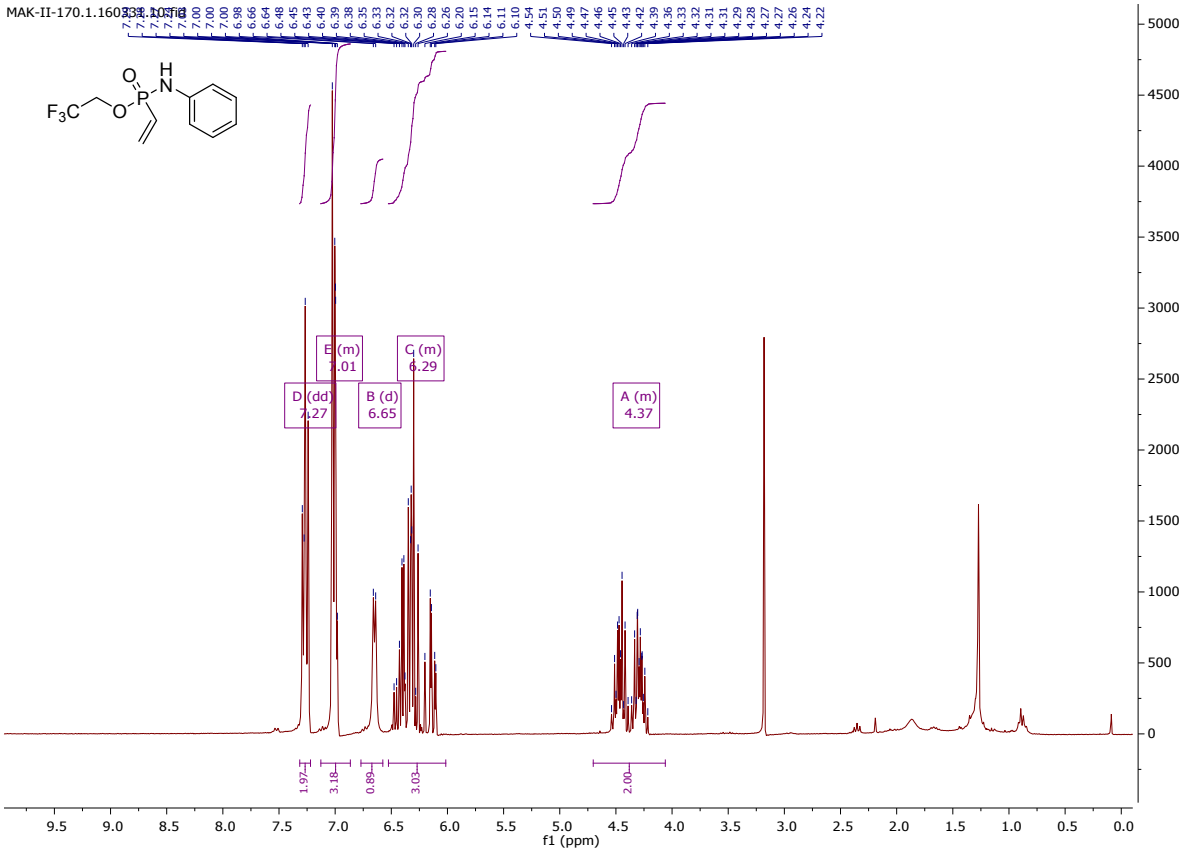
Phenyl-*N*-(3-phenyl-propyl)-*P*-vinylphosphonamidate (**5c**)

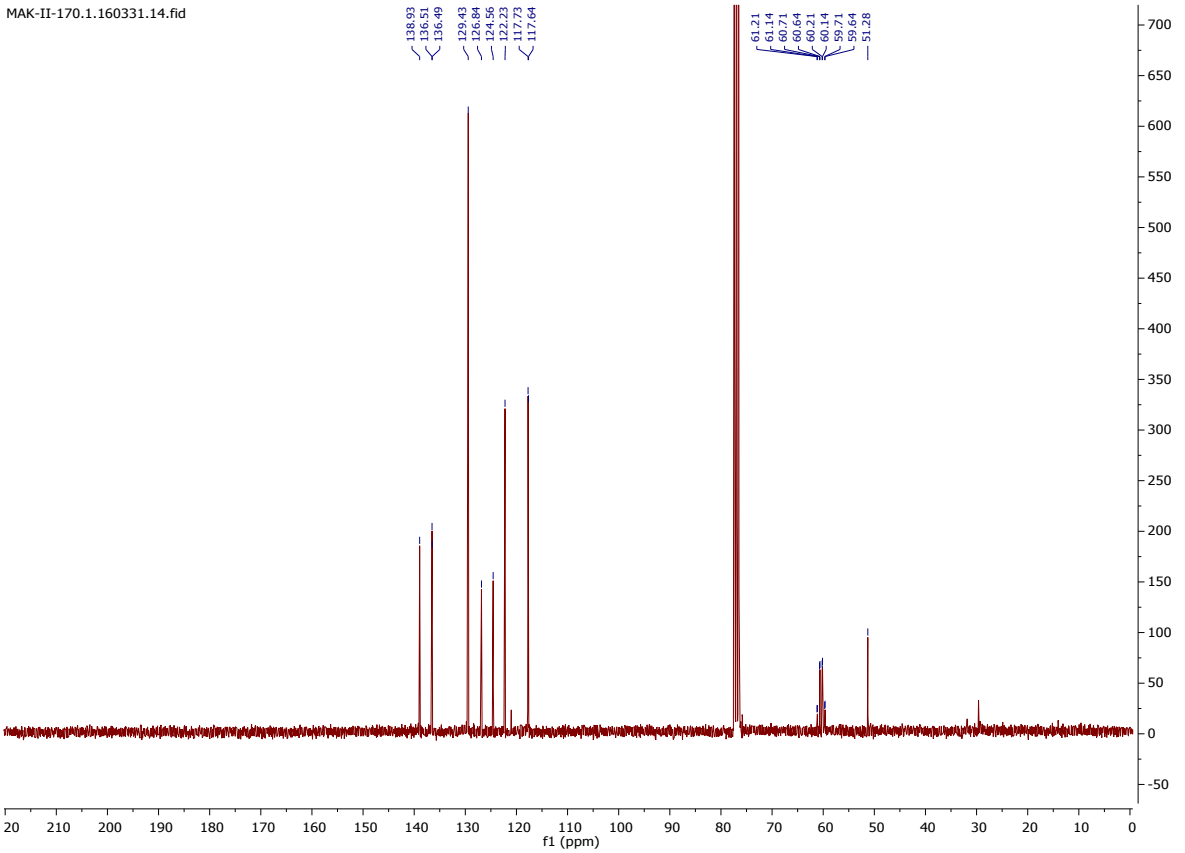


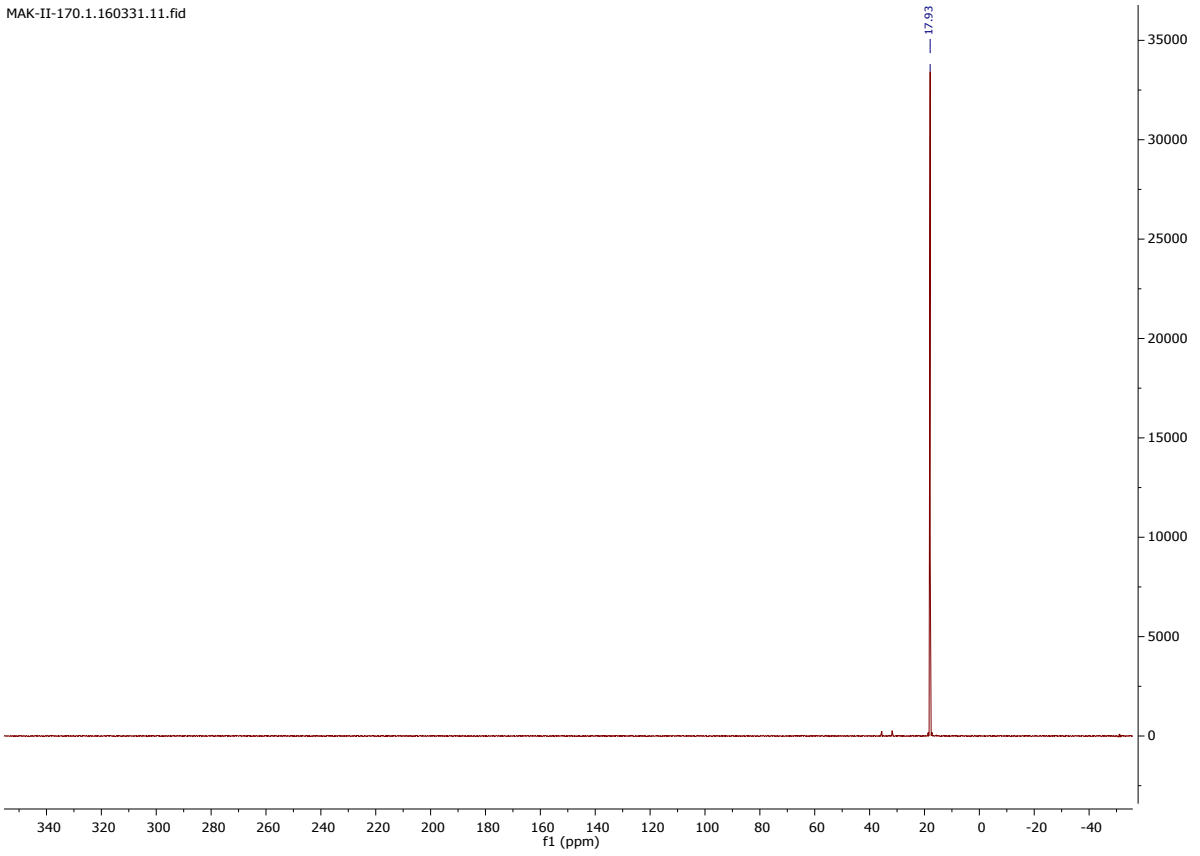

MAK-III-015.12.fid
150.65
150.60
141.30
134.03
129.64
128.55
128.43
128.35
127.41
125.99
124.55
120.59
120.56
40.48
33.39
33.36
32.87
f1 (ppm)

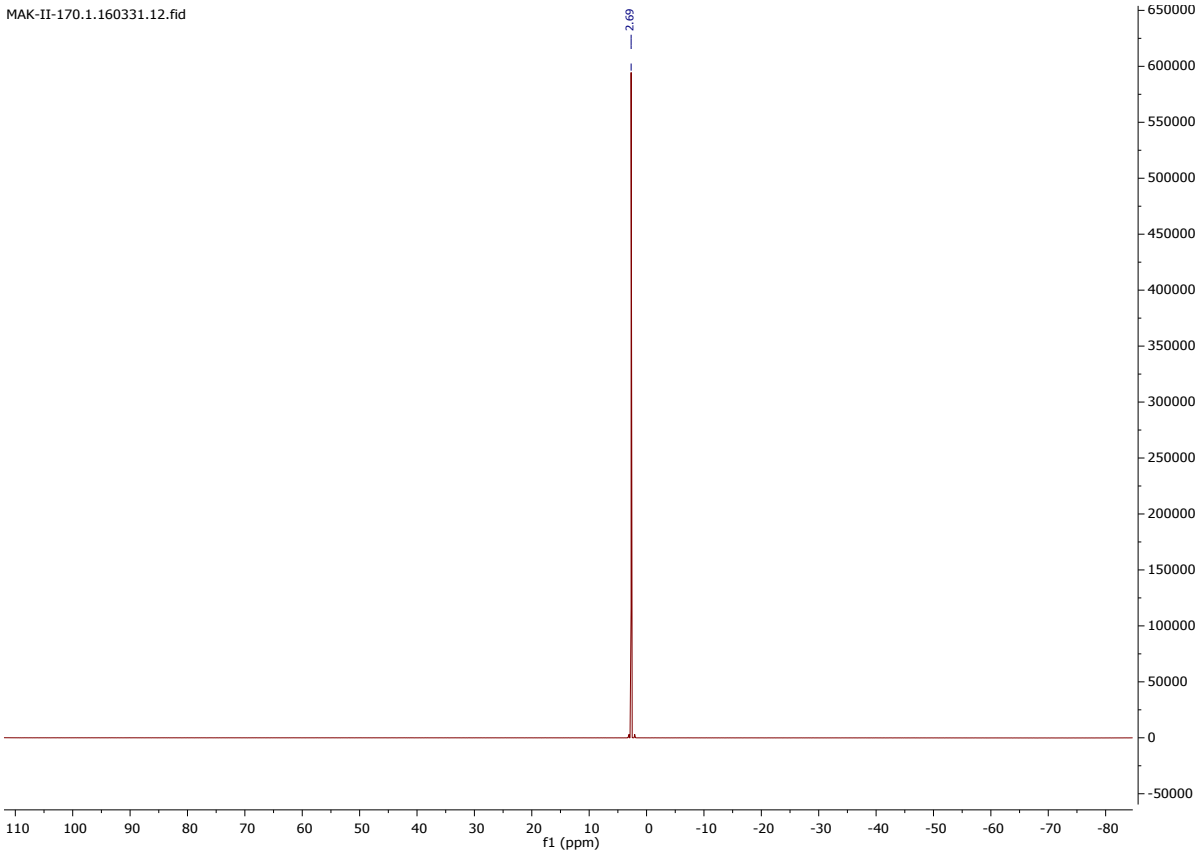

MAK-III-015.11.fid
18.79
f1 (ppm)

2,2,2-trifluoroethyl-*N*-phenyl-*P*-vinylphosphonamidate (**4d**)

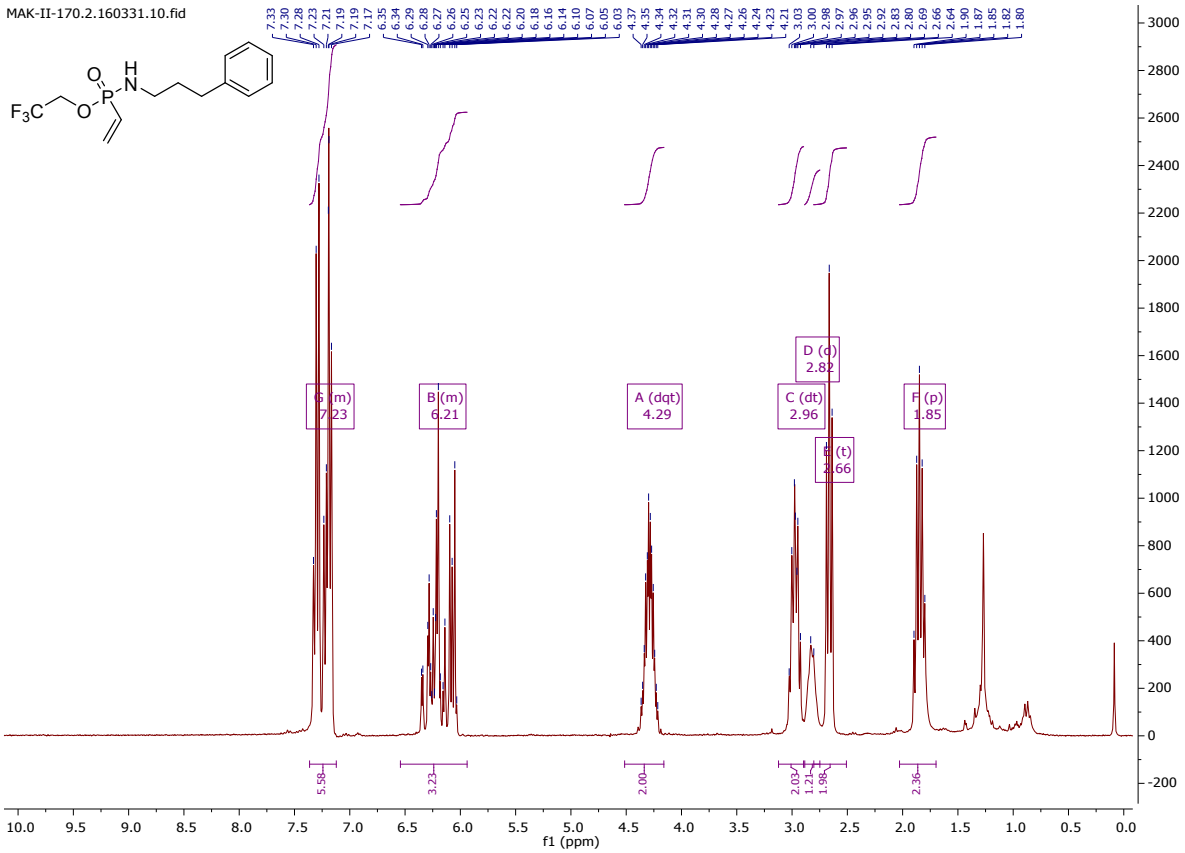


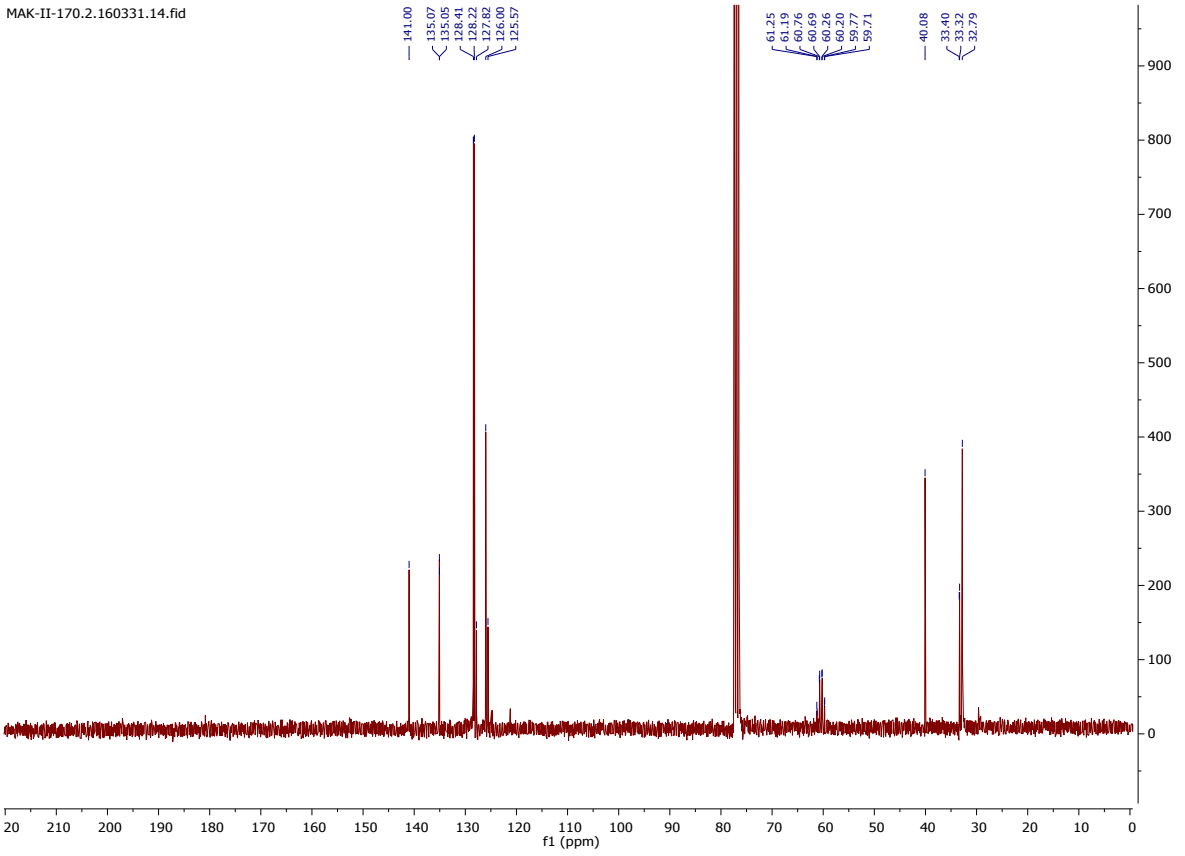


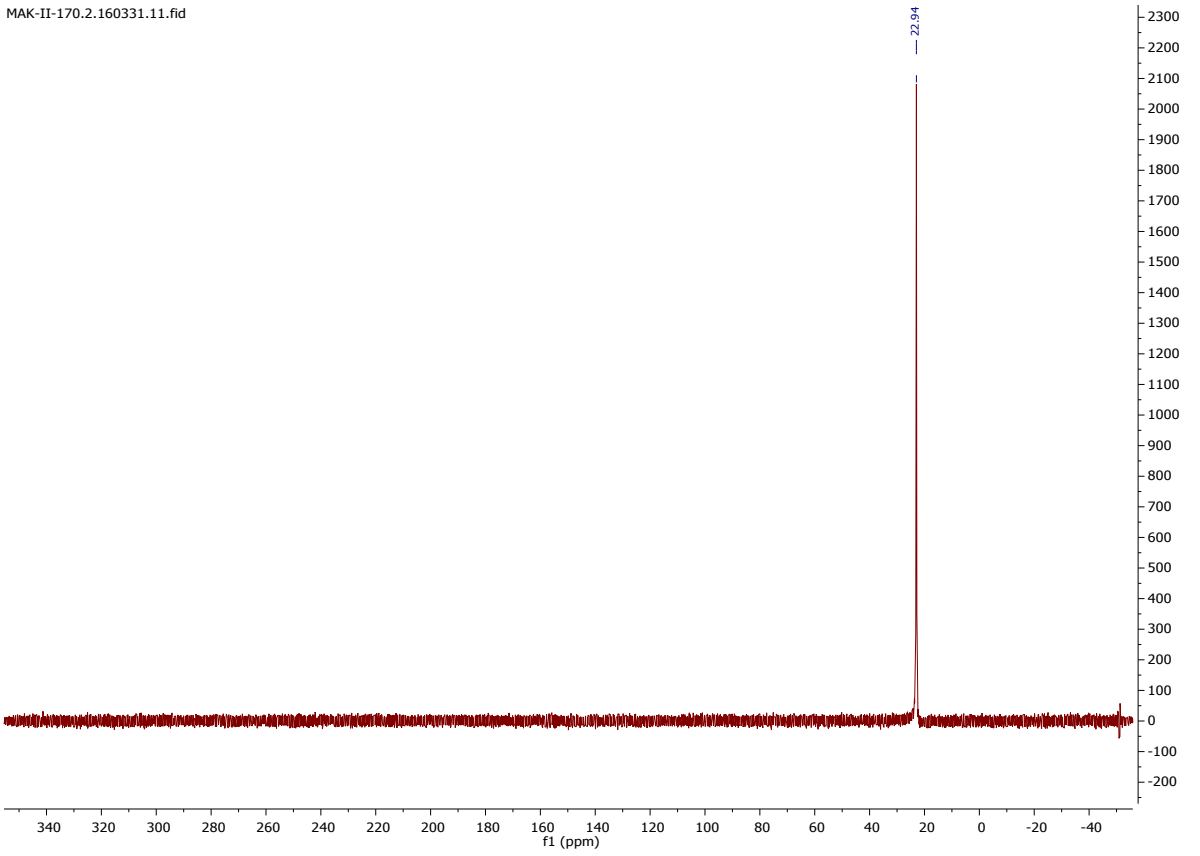

MAK-II-170.1.160331.11.fid
17.93
35000
30000
25000
20000
15000
10000
5000
0
340 320 300 280 260 240 220 200 180 160 140 120 100 80 60 40 20 0 -20 -40
f1 (ppm)

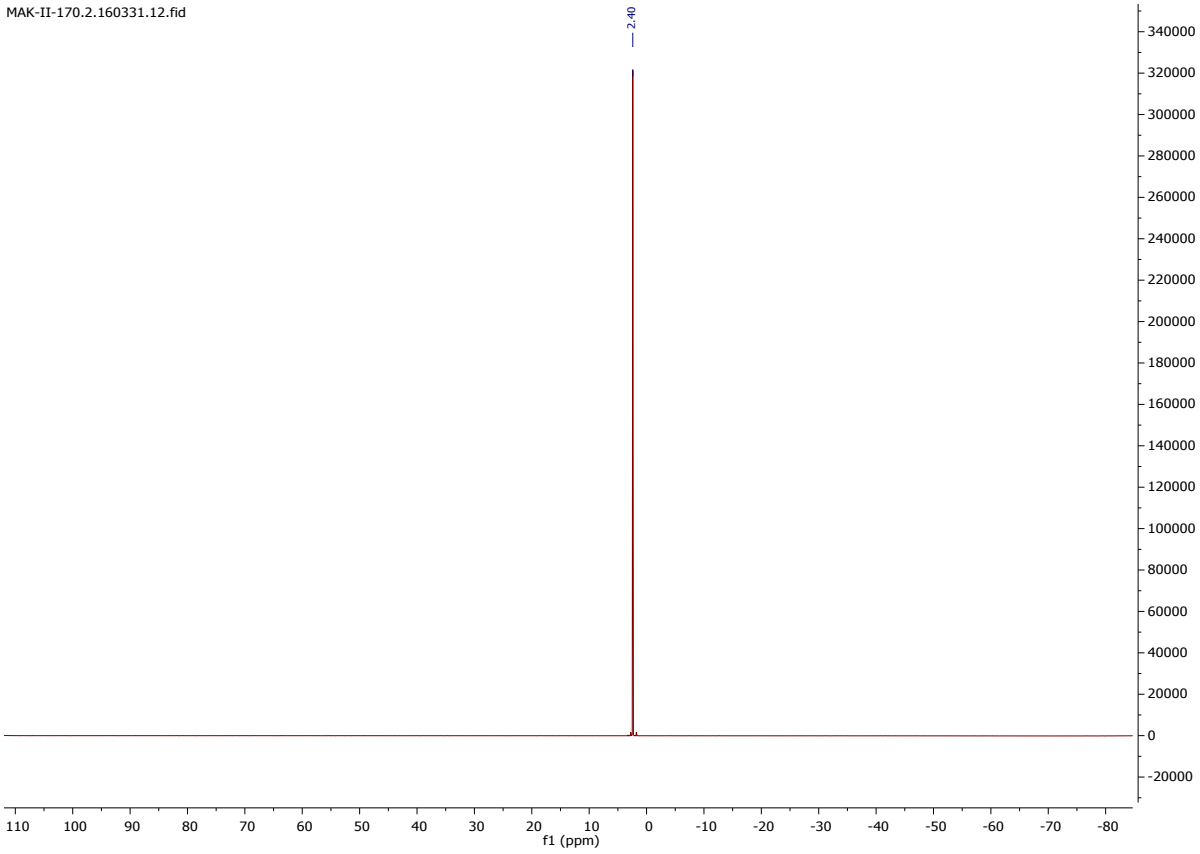

MAK-II-170.1.160331.12.fid
2.69
650000
600000
550000
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
-50000
110 100 90 80 70 60 50 40 30 20 10 0 -10 -20 -30 -40 -50 -60 -70 -80
f1 (ppm)

2,2,2-trifluoroethyl-*N*-(3-phenyl-propyl)-phenyl-*P*-vinyl-phosphonamidate (**5d**)

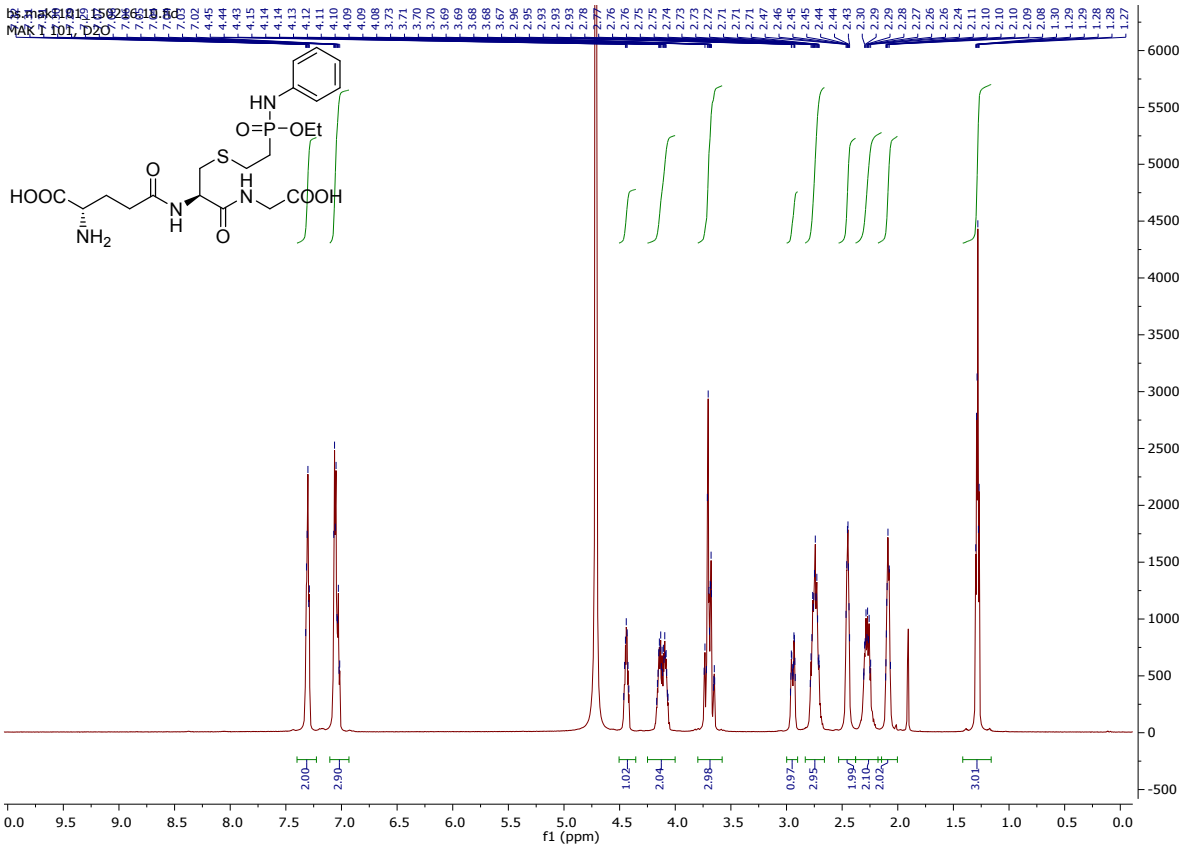


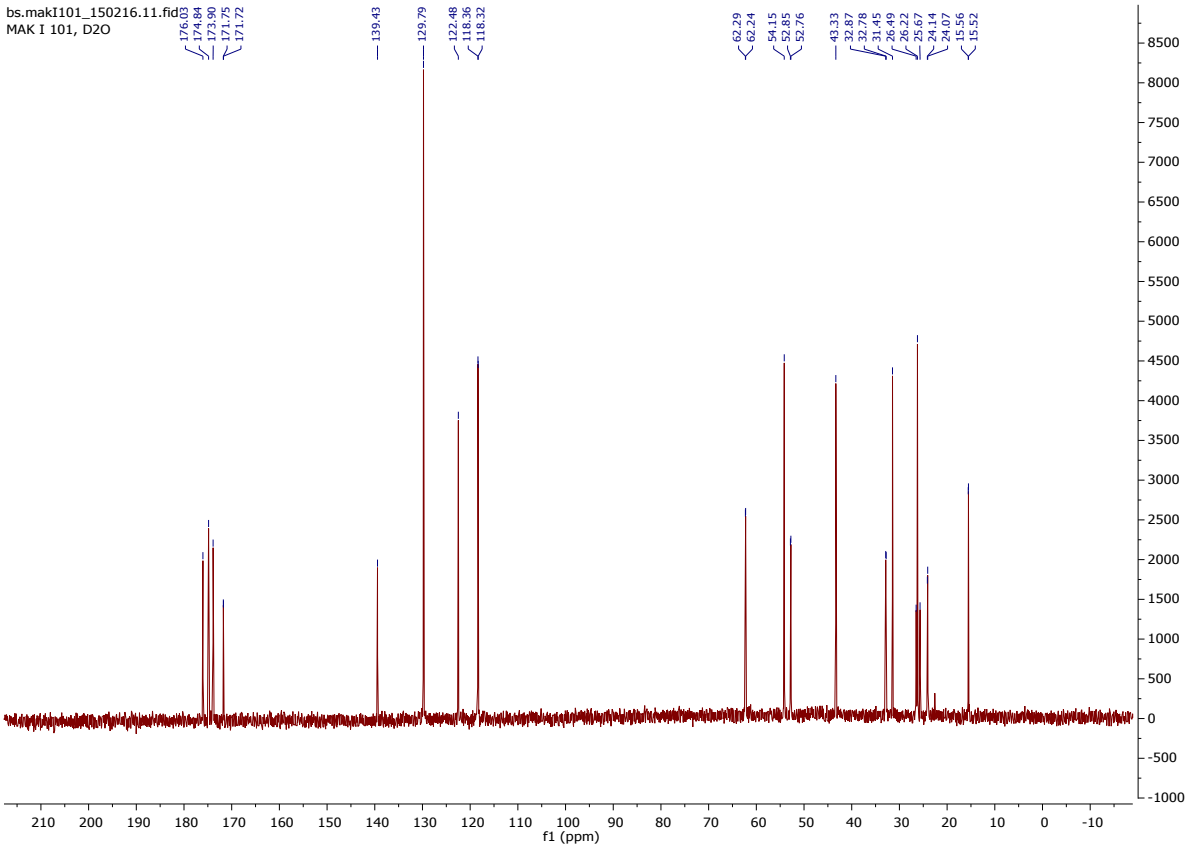


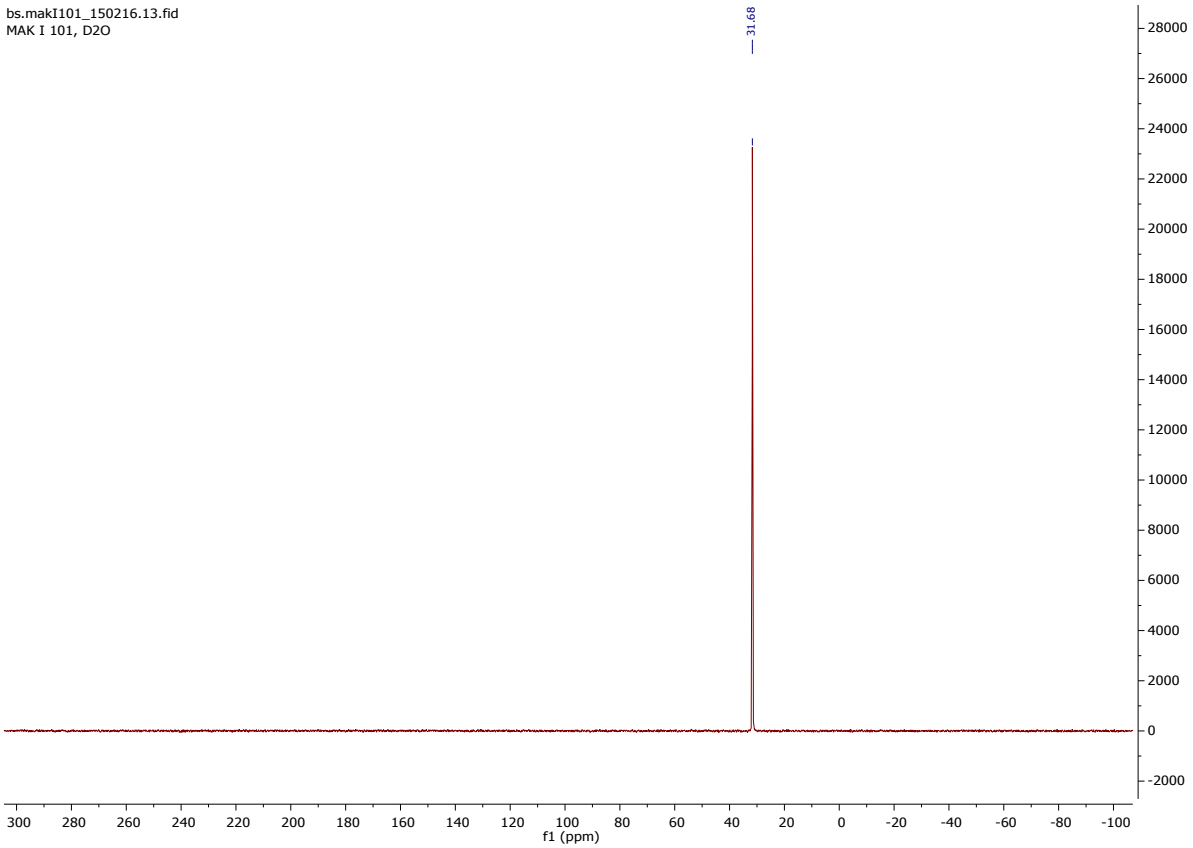

MAK-II-170.2.160331.11.fid
22.94
f1 (ppm)


MAK-II-170.2.160331.12.fid
2.40
f1 (ppm)

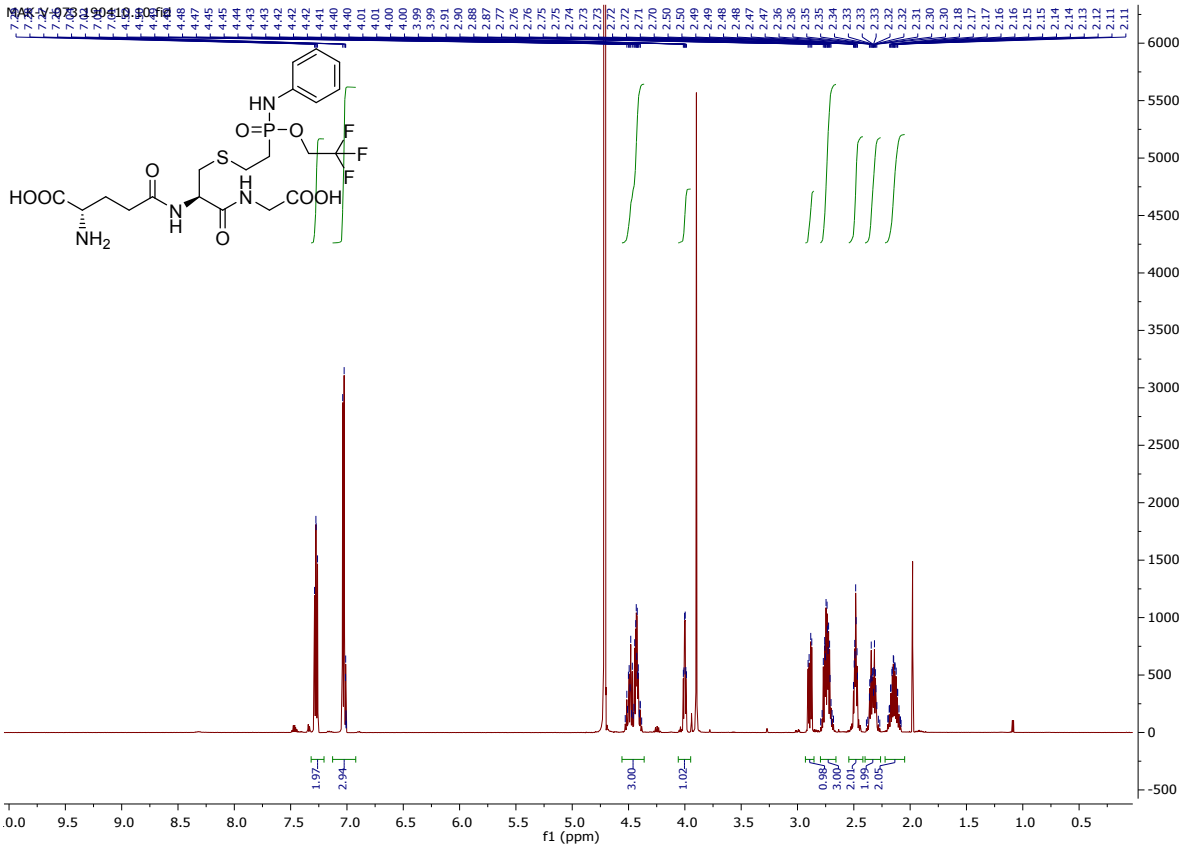
Ethyl-*N*-phenyl-*P*-(2-(glutathionyl-thio)ethyl)-phosphonamidate

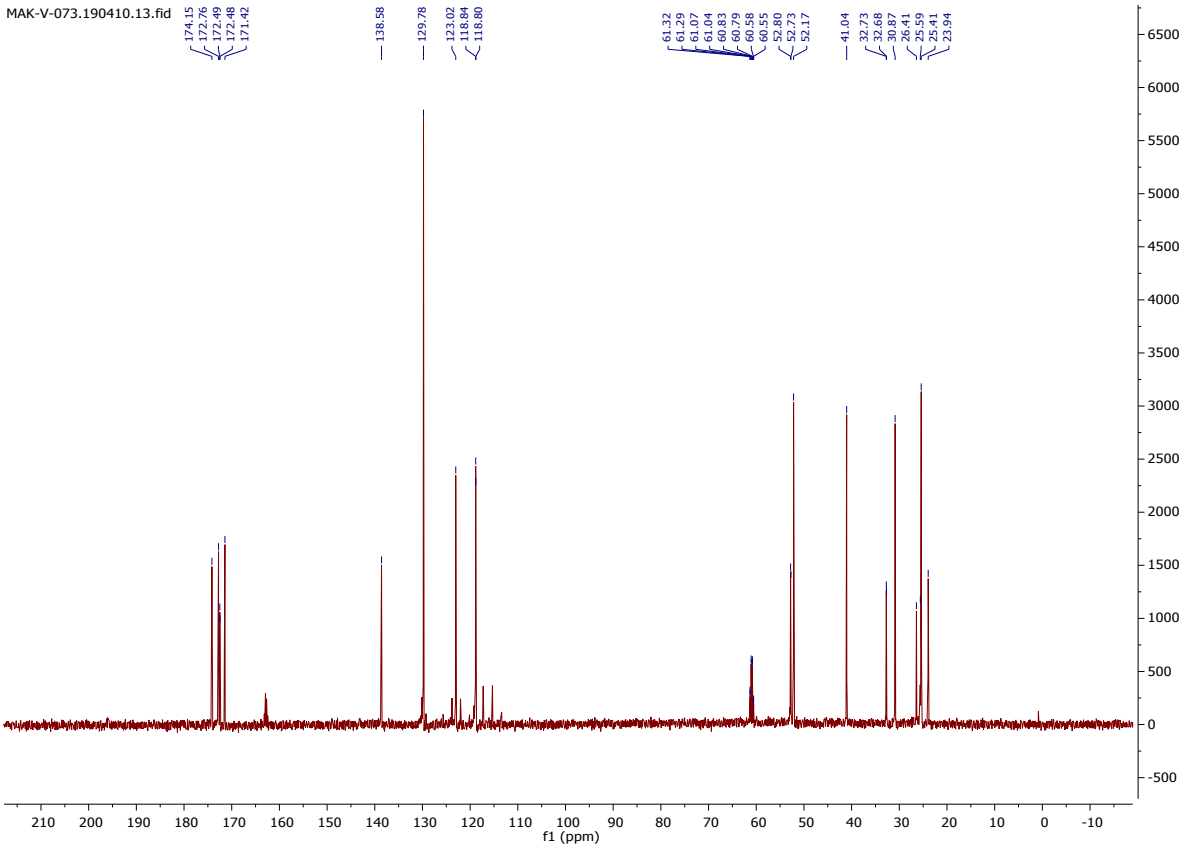


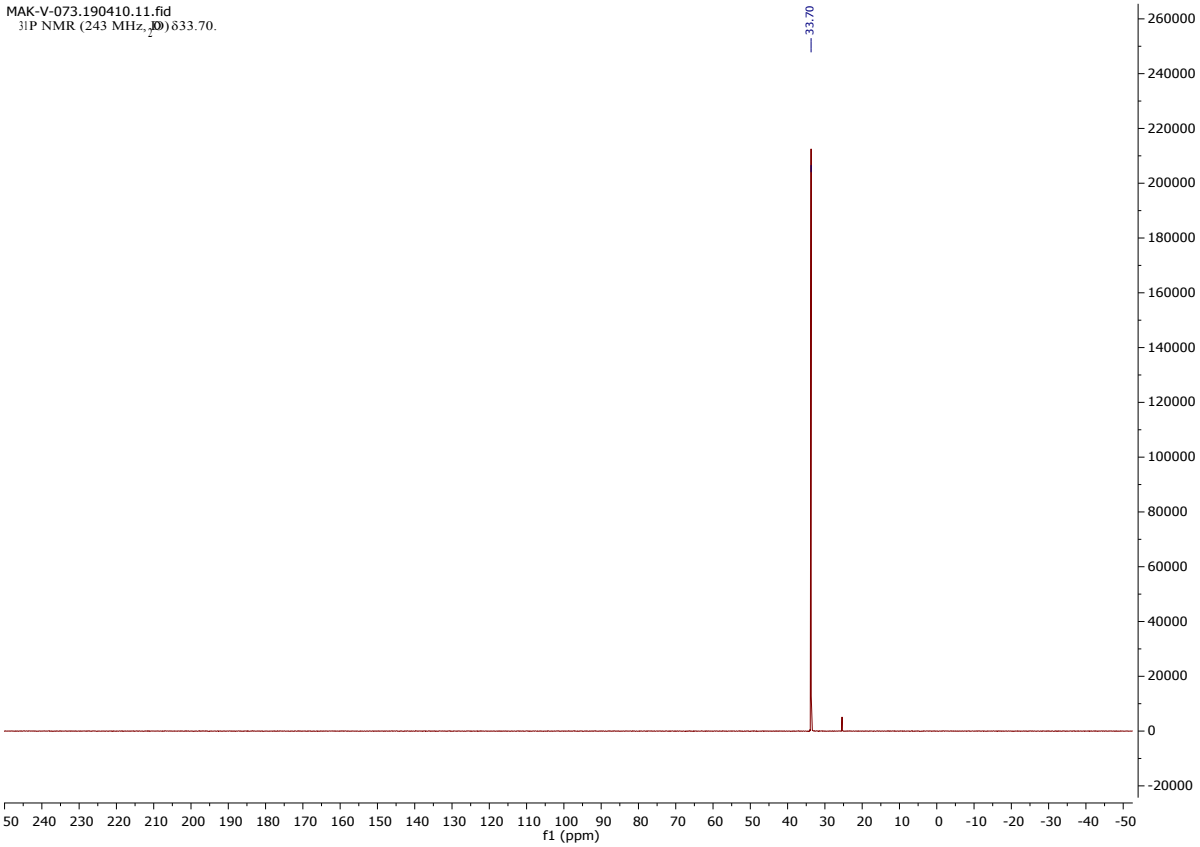


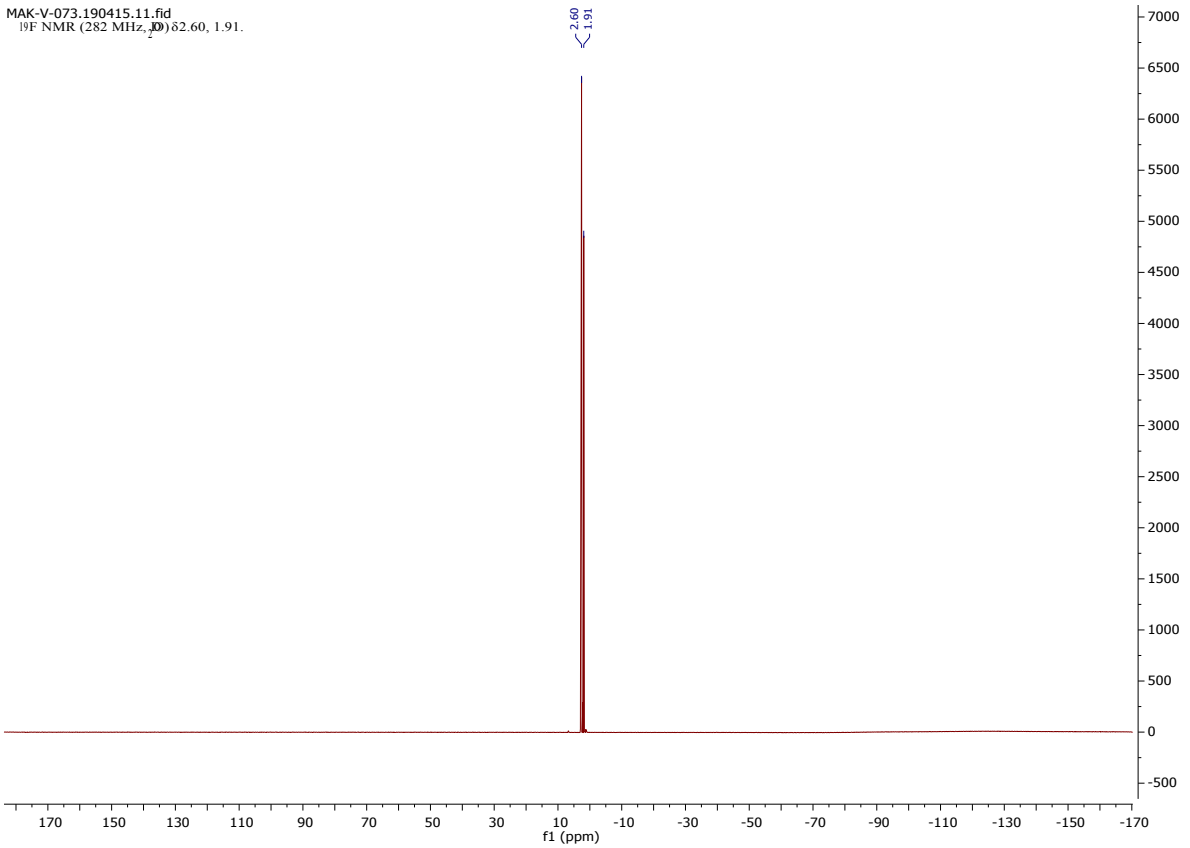


2,2,2-trifluoroethyl-*N*-phenyl-*P*-(2-(glutathionyl-thio)ethyl)-phosphonamidate

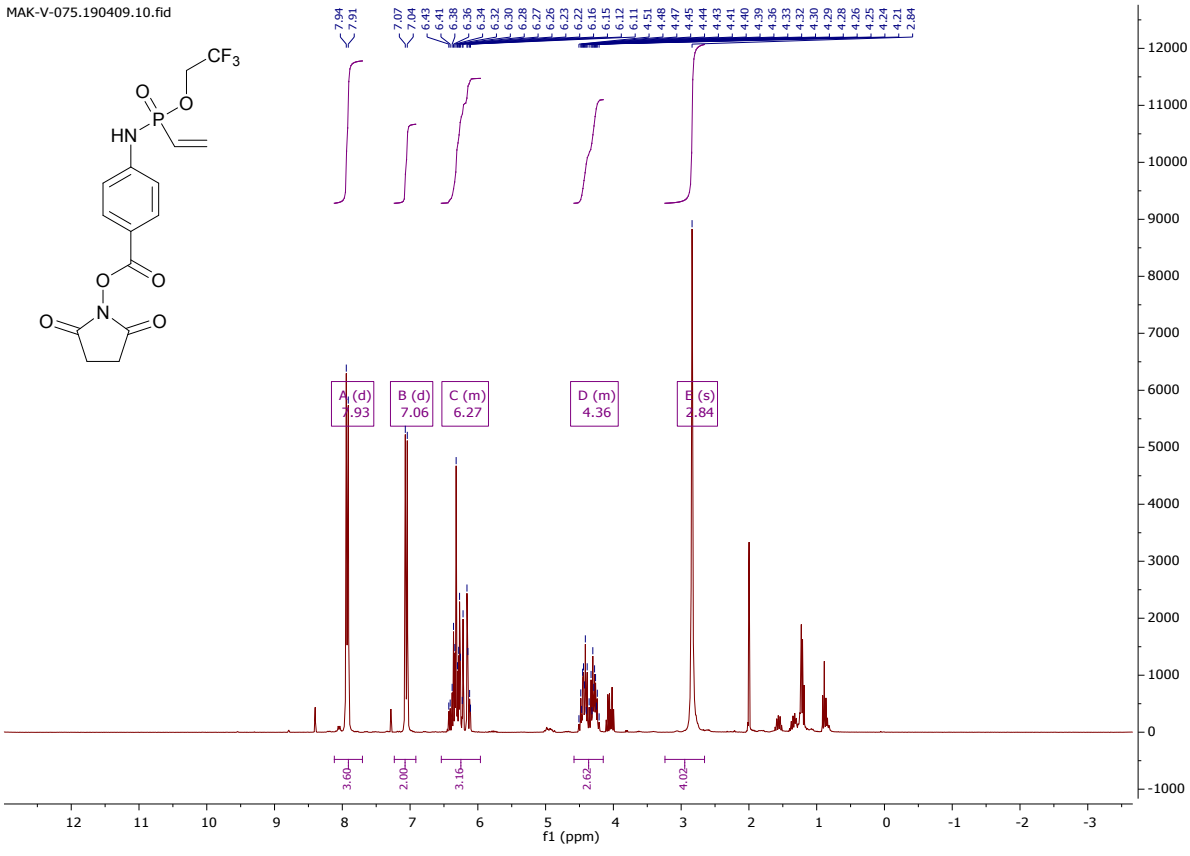


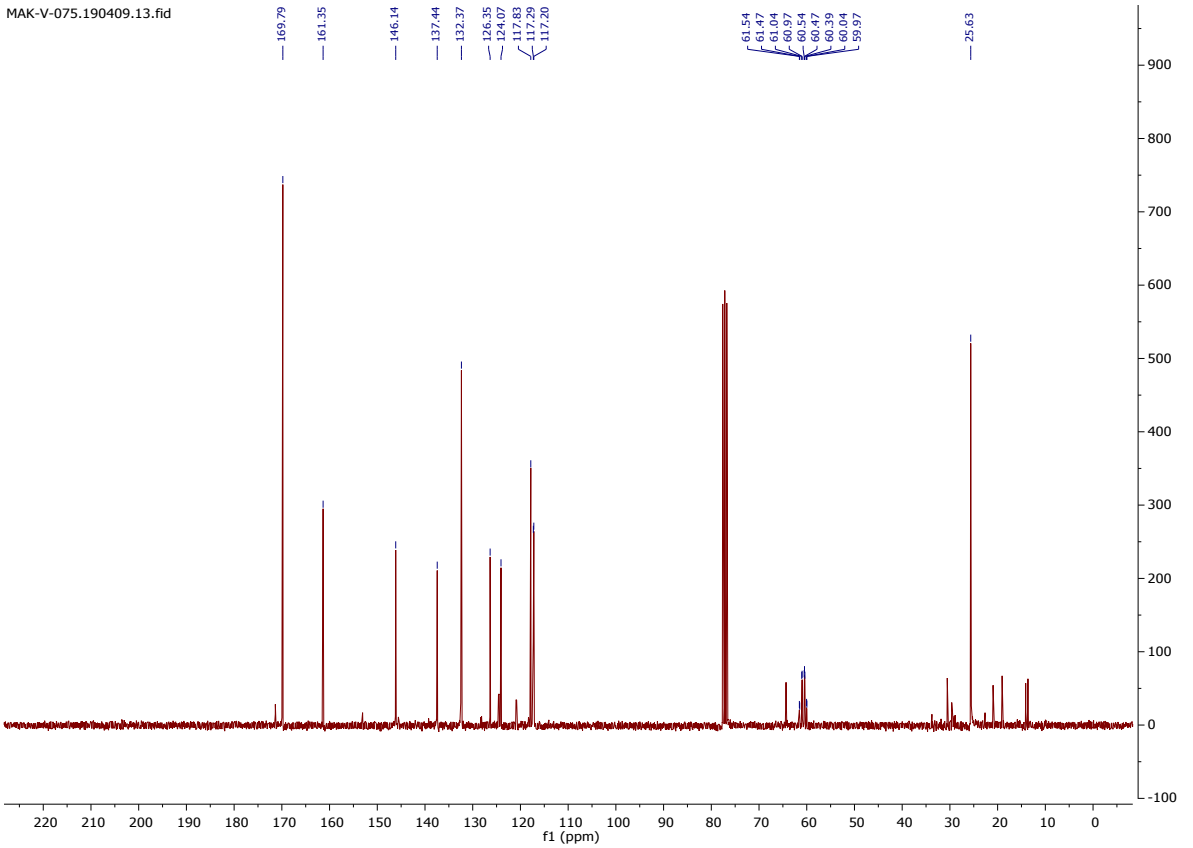

MAK-V-073.190410.13.fid
174.15
172.76
172.49
172.48
171.42
138.58
129.78
123.02
118.84
118.80
61.32
61.29
61.07
61.04
60.83
60.79
60.58
60.55
52.80
52.73
52.17
41.04
32.73
32.68
30.87
26.41
25.59
25.41
23.94
f1 (ppm)

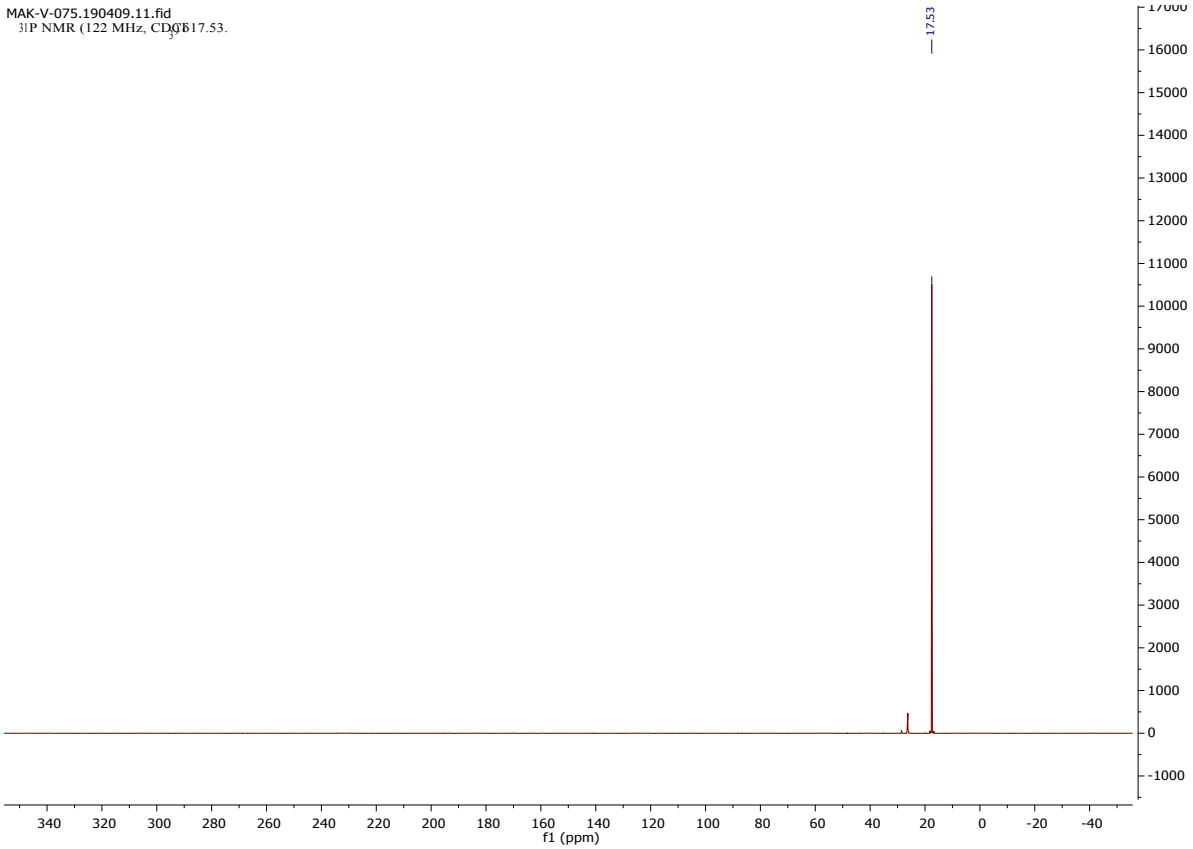

MAK-V-073.190410.11.fid
31P NMR (243 MHz, D2O) δ33.70.
33.70
f1 (ppm)

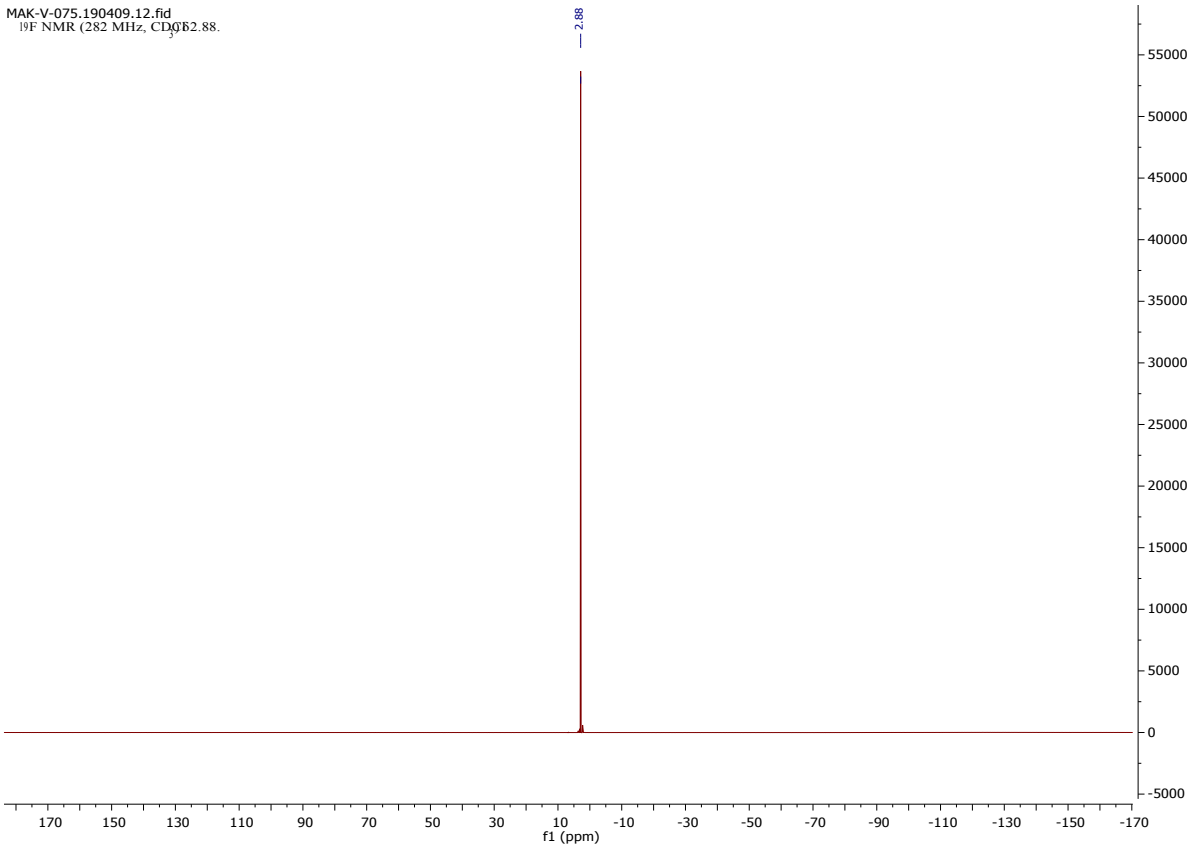


Trifluorethyl-N-(4-(2,5-dioxo-1-pyrrolidinyl)oxy-carbonyl-phenyl)-P-vinylphosphonamidate

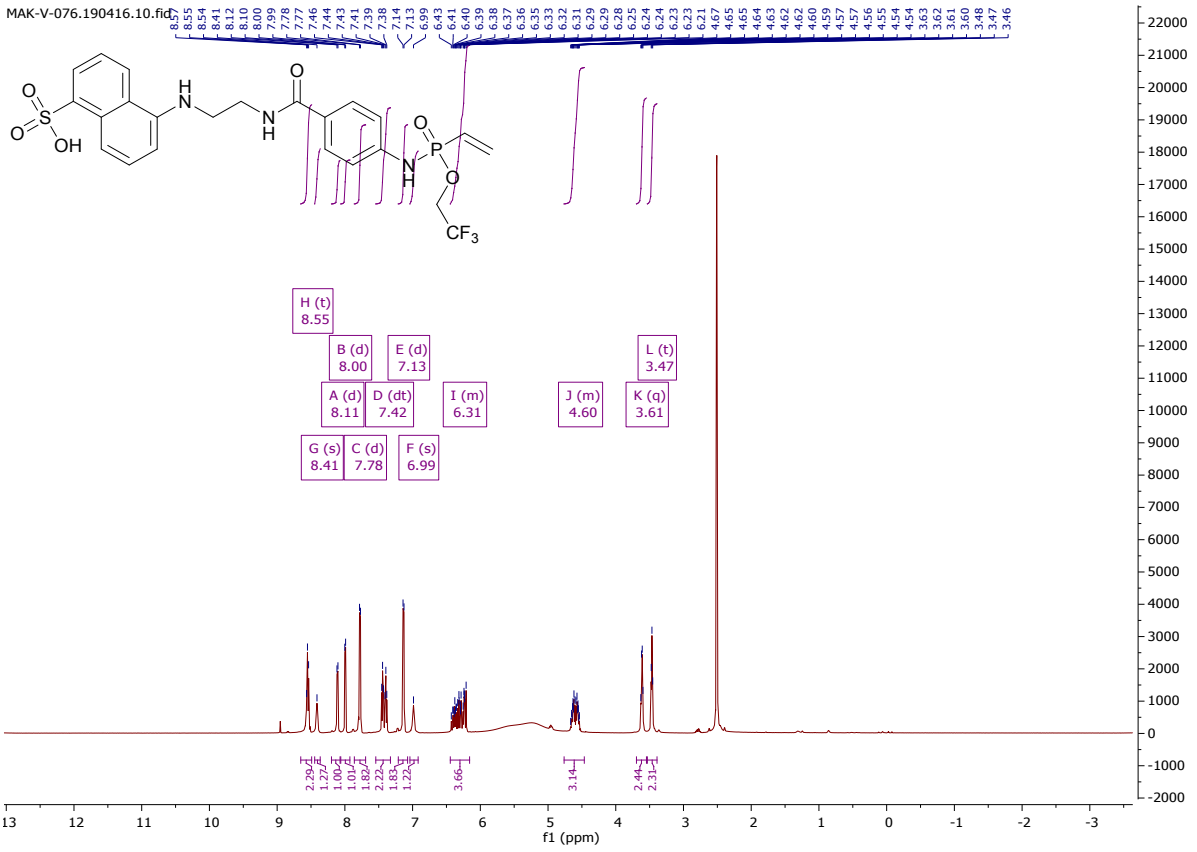


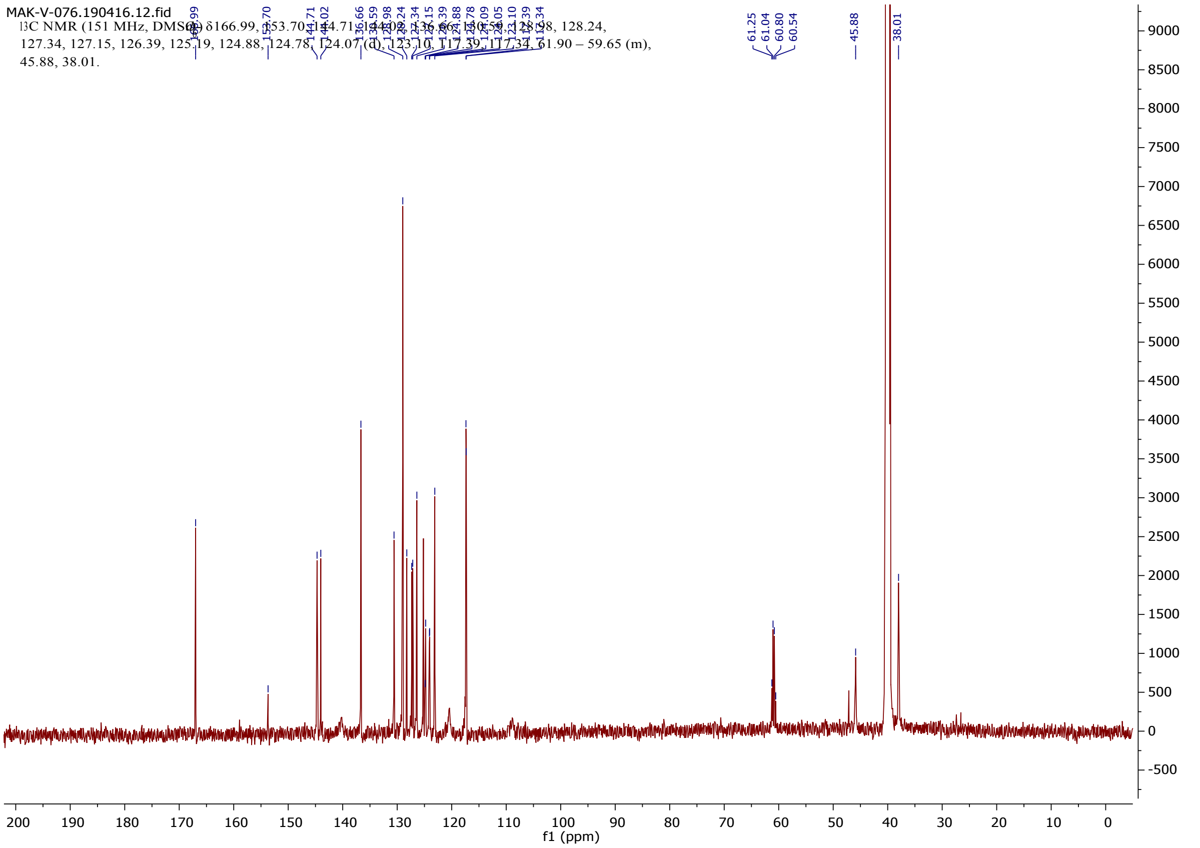

MAK-V-075.190409.13.fid
169.79
161.35
146.14
137.44
132.37
126.35
124.07
117.83
117.29
117.20
61.54
61.47
61.04
60.97
60.54
60.47
60.39
60.04
59.97
25.63
f1 (ppm)

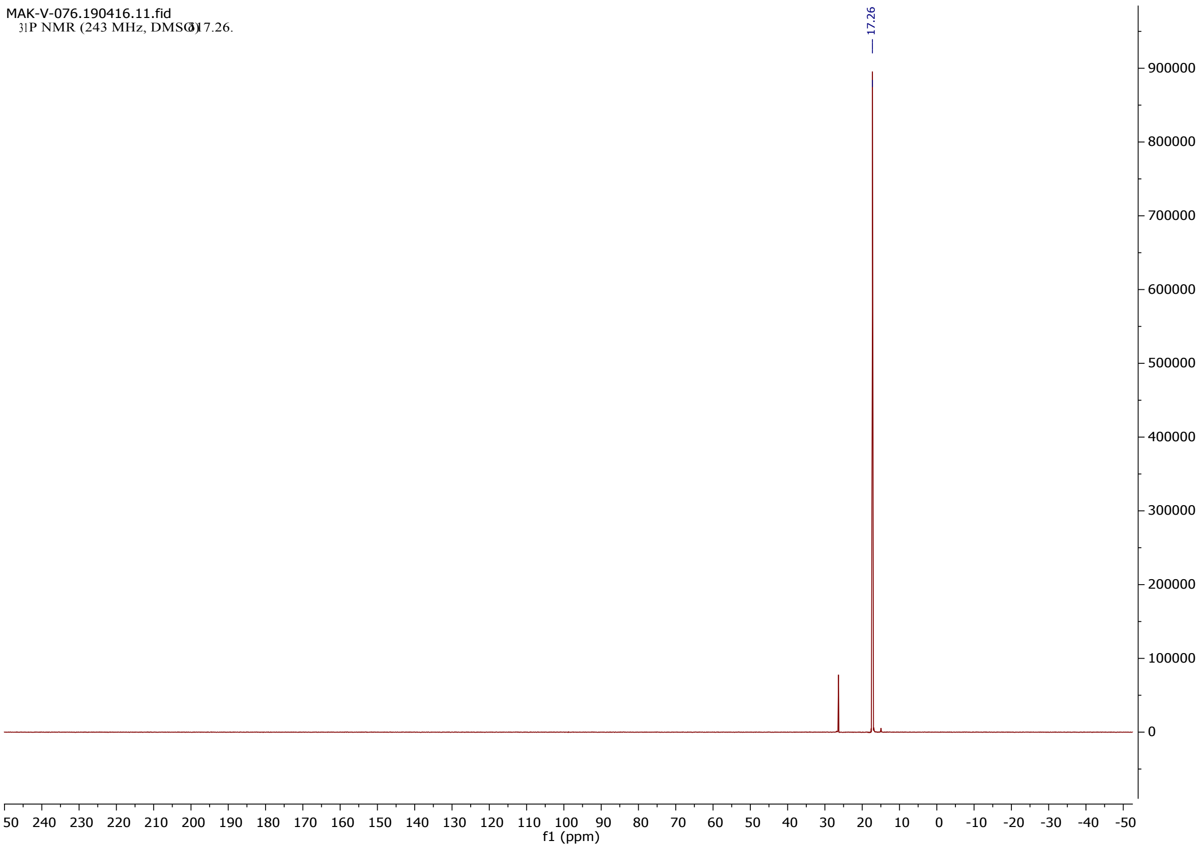

MAK-V-075.190409.11.fid
31P NMR (122 MHz, CDCl3) δ 17.53.
17.53
f1 (ppm)

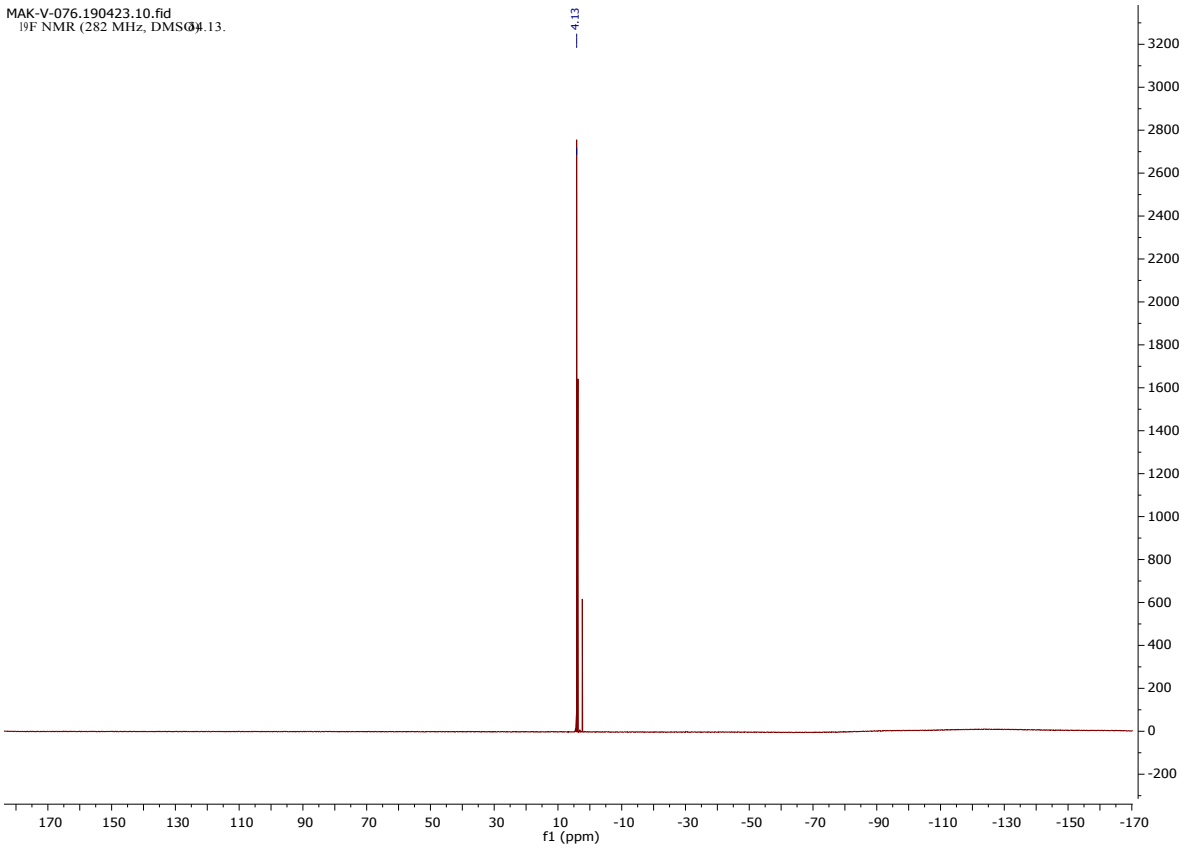


5-((2-(O-trifluorethyl-P-vinyll-phosphonamidato-N-benzoyl)ethyl)amino)naphthalene-1-sulfonic acid (**6**)

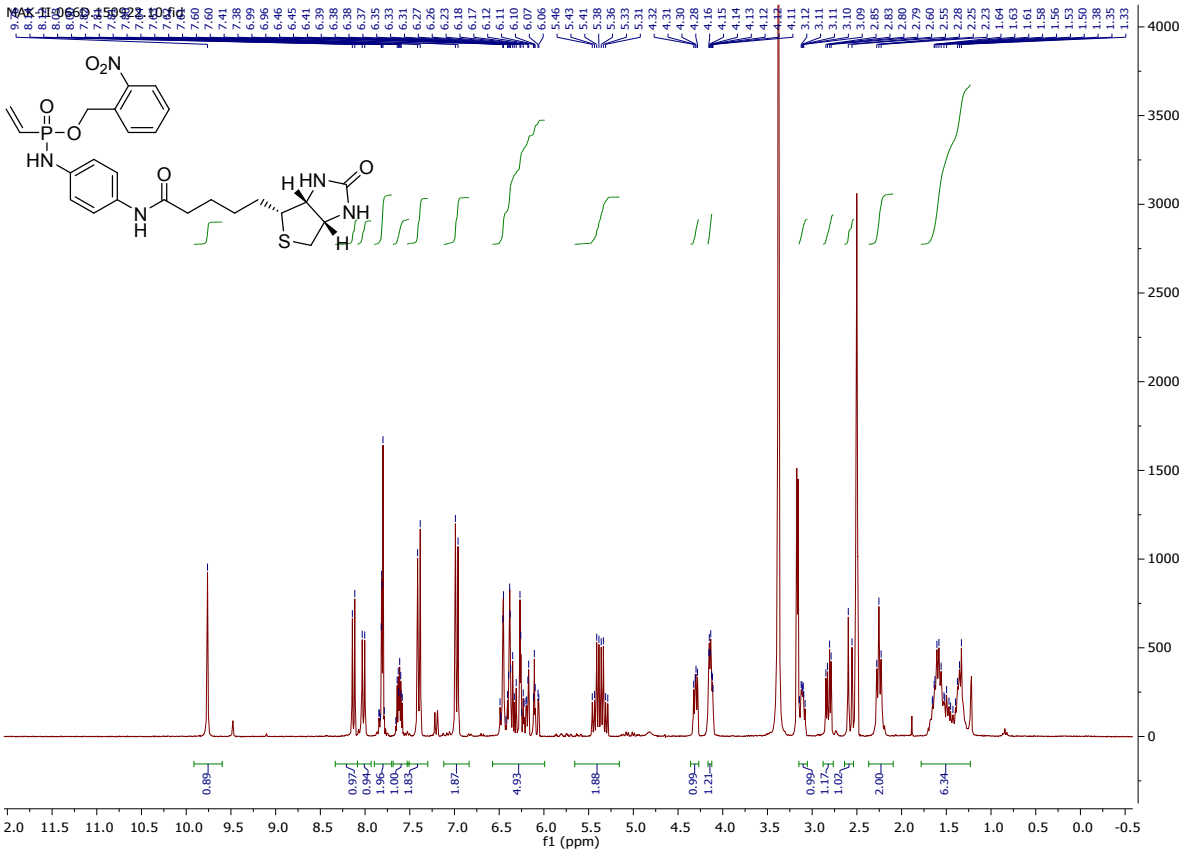


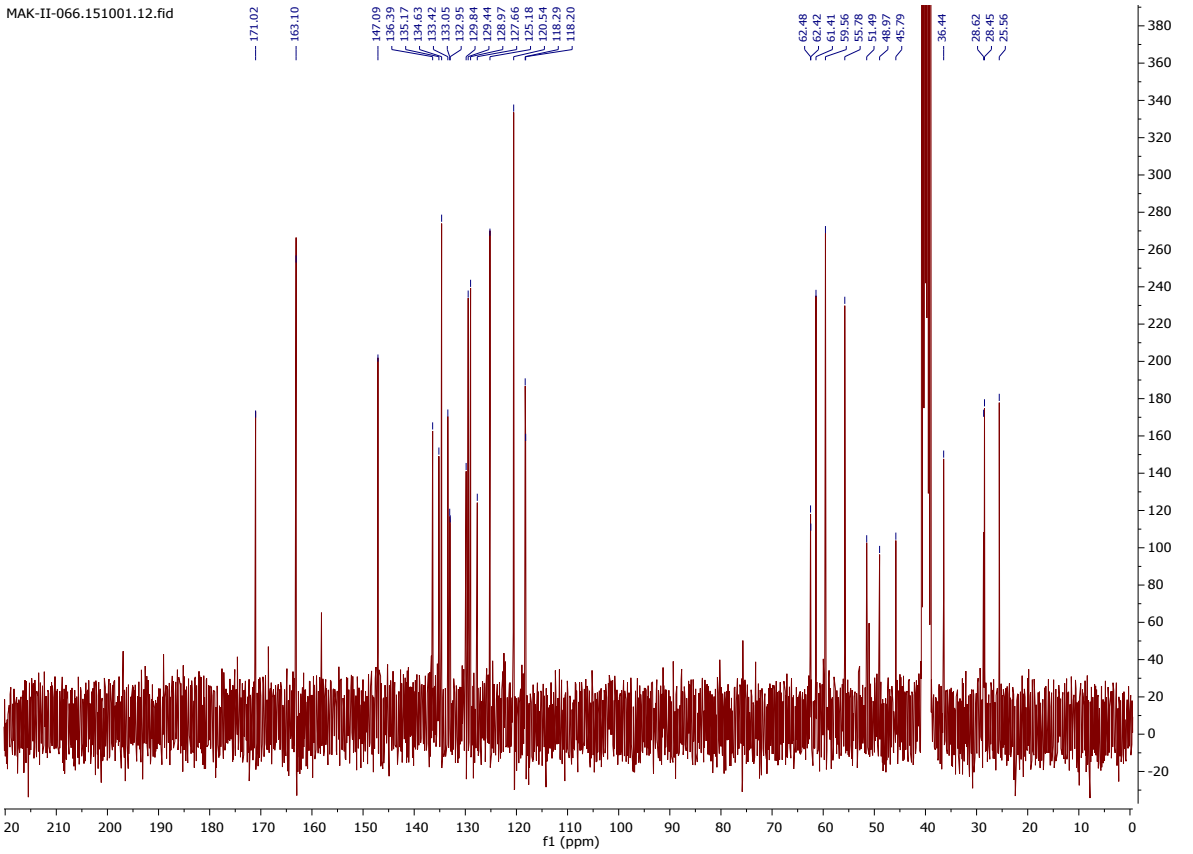

MAK-V-076.190416.12.fid
13C NMR (151 MHz, DMSO) δ 166.99, 153.70, 144.71, 144.02, 136.66, 130.59, 128.98, 128.24, 127.34, 127.15, 126.39, 125.19, 124.88, 124.78, 124.07 (d), 123.10, 117.39, 117.34, 61.90 – 59.65 (m), 45.88, 38.01.
61.25
61.04
60.80
60.54
45.88
38.01
f1 (ppm)

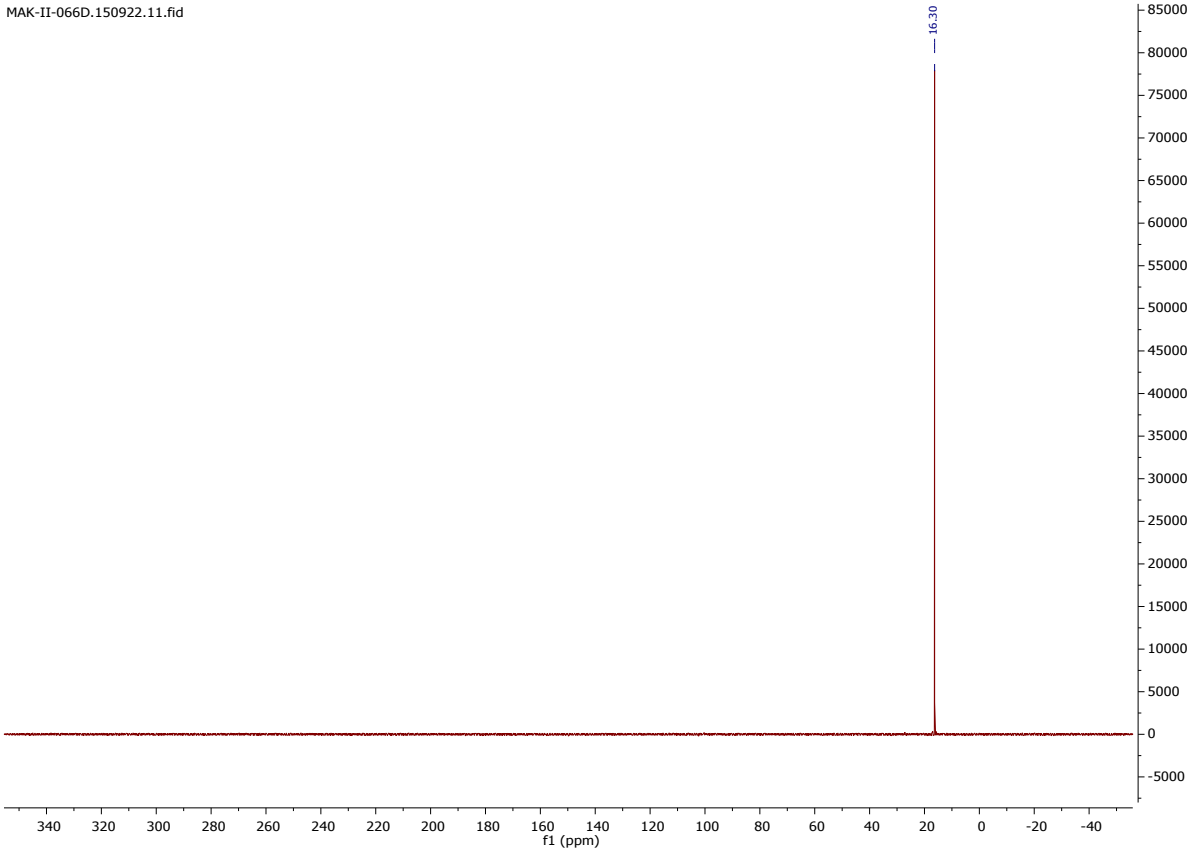

MAK-V-076.190416.11.fid
31P NMR (243 MHz, DMSO) δ 17.26.
17.26
f1 (ppm)



2-Nitrobenzyl-*N*-(4-biotinamido-phenyl)-*P*-vinyl phosphonamidate




MAK-II-066.151001.12.fid
171.02
163.10
147.09
136.39
135.17
134.63
133.42
133.05
132.95
129.84
129.44
128.97
127.66
125.18
120.54
119.29
118.20
62.48
62.42
61.41
59.56
55.78
51.49
48.97
45.79
36.44
28.62
28.45
25.56
f1 (ppm)


MAK-II-066D.150922.11.fid
16.30
f1 (ppm)

6. References

1. A. Shevchenko, H. Tomas, J. Havli, J. V. Olsen and M. Mann, *Nat. Protoc.*, 2007, **1**, 2856.
2. M.-A. Kasper, M. Glanz, A. Stengl, M. Penkert, S. Klenk, T. Sauer, D. Schumacher, J. Helma, E. Krause, M. C. Cardoso, H. Leonhardt and C. Hackenberger, *Angew. Chem. Int. Ed.*, in press doi:10.1002/anie.201814715.
3. M. R. J. Vallée, L. M. Artner, J. Dernedde and C. P. R. Hackenberger, *Angew. Chem. Int. Ed.*, 2013, **52**, 9504-9508.
4. S. p. Ortial and J.-L. Montchamp, *Org. Lett.*, 2011, **13**, 3134-3137.