Protease-Triggered Self-Immolative Acyl Phosphates for Controlled Phosphate Release

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Supporting Information

General Methods

All chemicals were obtained from commercial suppliers and used without further purification unless otherwise noted. Reactions were conducted in oven-dried glassware equipped with magnetic stirring under an inert atmosphere of nitrogen or argon. Purification of products was performed by flash column chromatography on a Biotage Isolera system using SEPAFLASH Ruby silica cartridges or SEPAFLASH C18 spherical cartridges for reverse-phase separations. Reaction progress was monitored by thin-layer chromatography (TLC) on silica gel plates (Merck), visualized under UV light (254 nm) and/or by staining with $KMnO_4$ or *p*-anisaldehyde. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker Avance (400 MHz) and Agilent DD2 (500, 600, and 700 MHz) spectrometers. ^{1}H NMR spectra (400, 500, 600 or 700 MHz), ^{31}P NMR spectra (162, 202, or 243 MHz), and ^{13}C NMR spectra (126 or 151 MHz) were acquired in $CDCl_3$, MeOD, or CD_3CN with chemical shifts (δ) reported in parts per million (ppm) relative to tetramethylsilane (TMS, δ = 0.00 ppm) or residual solvent signals ($CDCl_3$: δH 7.26, δC 77.16; MeOD: δH 3.31, δC 49.00; CD_3CN: δH 1.94, δC 1.32). Coupling constants (J) are reported in hertz (Hz). Multiplicities are designated as: s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet. High-resolution mass spectrometry (HRMS) was performed on an Agilent 6538 UHD instrument equipped with an electrospray ionization (ESI) source or JOEL AccuTOF equipped with direct analysis in real time (DART). Reported masses correspond to the most abundant isotopomer. Chymotrypsin inhibition assays were conducted using a BMG Labtech CLARIOstar microplate reader. Optical rotations were measured using a Rudolph Research Analytical Autopol IV polarimeter.

Acyl Phosphate (1c-f) Aminolysis Assay

Acyl phosphate stability was evaluated under pseudo–first-order conditions using excess lysine as a representative nucleophile and monitored by ^{31}P NMR spectroscopy. Reactions were conducted at room temperature in 5 mm NMR tubes with a total volume of 600 µL. Previously prepared aliquots of compounds **1c–f** were dissolved in 480 µL of H_2O containing 100 mM MOPS buffer (pH 7.0). A stock solution of methylphosphonic acid (100 mM in 100

mM MOPS, pH 7.0) was prepared as an internal standard, and 60 µL was added to the acyl phosphate solution. A lysine stock solution (1 M in 1 M MOPS, pH 7.0) was then added (60 µL) to initiate the reaction. The reaction mixture was homogenized by gentle pipette mixing and immediately subjected to NMR acquisition.

Experiments were acquired as an array using the *zgig2d* pulse program with the following parameters: relaxation delay (d1) = 10.97 s, interscan delay (d20) = 60 s, acquisition time (aq) = 9 s, number of scans (NS) = 3 per time point, pulse width (pw) = 90°, and array size = 183. Data was processed using MestReNova (Mnova). Post-acquisition averaging of four consecutive FIDs (N = 4) was performed to improve signal-to-noise as previously described.[1] Acyl phosphate concentrations were determined by integration and normalized to the internal standard. Concentration–time profiles were constructed, and pseudo–first-order rate constants were obtained from linear fits of ln([acyl phosphate]) versus time.

Chymotrypsin Mediated Hydrolysis Studies

Enzymatic hydrolysis studies were performed under physiologically relevant conditions and monitored by NMR spectroscopy. Previously prepared aliquots of **8a** or **8b** were dissolved in 288 µL of D_2O containing 100 mM HEPES buffer (pH 8.0). Maleic acid (1 M stock solution in D_2O, 6 µL) was used as an internal standard, and calcium chloride (1 M stock solution in D_2O, 6 µL) was added to enhance enzyme stability and activity. A freshly prepared stock solution of α-chymotrypsin (200 µM in 100 mM HEPES buffer, pH 8.0) was then added (300 µL) to initiate the reaction. The mixture was gently homogenized by pipette mixing, transferred to a 5 mm NMR tube, and placed in the spectrometer.

The probe temperature was ramped to 37 °C and allowed to equilibrate with the NMR tube for 10 min prior to acquisition. Experiments were acquired as a PAD (pseudo-2D) array with the following parameters: relaxation delay (d1) = 1 s, acquisition time (aq) = 4.5 s, pulse width (pw) = 45°, receiver gain = 14, and number of scans (NS) = 64 per time point. The PAD array was configured to record one fid every 30 min. Data was processed using MestReNova (Mnova). Acyl phosphate concentrations were determined by integration and normalized to the internal standard. Concentration–time profiles were constructed, and initial rates were obtained from linear fits of the early, linear region of the concentration versus time plot.

Chymotrypsin mediated hydrolysis assessment using reverse-phase HPLC.

In a 1.5 mL Eppendorf tube, previously prepared aliquots of **8a** were dissolved in 288 µL of H_2O containing 100 mM HEPES buffer (pH 8.0) (Final substrate concentration 10 mM). Calcium chloride (1 M stock solution in H2O, 6 µL) was added to enhance enzyme stability and activity. A freshly prepared stock solution of α-chymotrypsin (200 µM in 100 mM HEPES buffer, pH 8.0) was then added (300 µL) to initiate the reaction. The reaction mixture was

incubated at 37 °C for 6 hours at which time, 10 µL of this mixture was diluted 10-fold with HPLC dilution buffer (50 mM ammonium acetate pH 5.0, 10 mM EDTA, 90 µL). 50 µL of the resulting solution was injected into the HPLC. All standards were prepared in the same buffer solution.

Gradient HPLC analysis method is as follows: Waters Symmetry C18 column (4.6 mm × 250 mm, 5 micron), solvent A = 50 mM ammonium acetate pH 5.0, solvent B = acetonitrile, flow rate = 1.0 mL/min, detection wavelength = 260 nm, 25 °C.

Chymotrypsin Inhibition Assay

Chymotrypsin inhibition assays were performed in 96-well plates under physiologically relevant conditions (50 mM HEPES buffer, pH 8.0, 5 mM $CaCl_2$, 37 °C). Reactions contained 10 µM enzyme (E) and 2 mM chromogenic substrate (Suc-Phe-pNA, S) in a final volume of 200 µL per well. 2x stock solutions of chymotrypsin and inhibitor were prepared in 50 mM HEPES buffer (pH 8.0) containing 5 mM $CaCl_2$. Separately, a 2x substrate stock solution (4 mM) was prepared in the same buffer (50 mM HEPES pH 8.0, 5 mM $CaCl_2$). All stock solutions were equilibrated at 37 °C for 10 min prior to initiation of the reaction. Reactions were initiated by adding 100 µL of the pre-equilibrated substrate solution to 100 µL of the enzyme–inhibitor mixture in each well, affording final concentrations of 10 µM enzyme and 2 mM substrate. Plates were immediately transferred to a microplate reader, and formation of p-nitroaniline (pNA) was monitored by measuring absorbance at 410 nm. The plate reader was programmed with the following parameters: absorbance wavelength = 410 nm; flashes per well = 30; volume correction = 200 µL; total cycles = 120; cycle time = 30 s. Inhibitor concentrations tested were 0.75, 1.5, and 3 mM for both **8a** and **8b** and all experiments were performed in triplicate. Enzyme activity was determined from the slopes of absorbance versus time plots following correction for background substrate hydrolysis (negative control). Absorbance values were converted to concentration using a calibration curve for p-nitroaniline under identical buffer conditions.

Phenyl dihydrogen phosphate (1a)

To a stirred solution of NaOH (1.6 g, 40 mmol, 4.0 equiv) in H_2O (20 mL) at 0 °C was added phenyl dichlorophosphate (1.5 mL, 10 mmol, 1.0 equiv). The mixture was stirred at 0 °C for 30 min, then allowed to warm to room temperature and stirred overnight. The reaction was acidified to pH 1 with 6 M HCl and transferred to a separatory funnel. The product was extracted with EtOAc (3 × 30 mL), and the combined organic extracts were filtered over anhydrous $MgSO_4$, and dried under reduced pressure to afford phenyl dihydrogen phosphate as an amber oil (1.56 g, 90%). ^{1}H-, ^{31}P-, and ^{13}C- NMR characterizations are consistent with literature.[2]

Methyl dihydrogen phosphate (1b)

To a stirred solution of NaOH (1.2 g, 30 mmol, 3.0 equiv) in H_2O (20 mL) at 0 °C was added methyl dichlorophosphate (1.0 mL, 10 mmol, 1.0 equiv). The mixture was stirred at 0 °C for 30 min, then allowed to warm to room temperature and stirred overnight. The reaction was acidified to pH 2 with Dowex 50WX8 hydrogen form ion exchange resin, filtered, and lyophilized to afford methyl dihydrogen phosphate as a white amorphous semisolid (690 mg, 62%). ^{1}H-, ^{31}P-, and ^{13}C- NMR characterizations are consistent with literature.[3]

General Methods for Acylphosphate 1c-f

Phenyl dihydrogen phosphate (1 mmol, 1.0 equiv.) was added to a round-bottom flask equipped with a magnetic stir bar containing carboxylic anhydride (1.5 mmol, 1.5 equiv.) in 3 mL of anhydrous acetonitrile. Triethylamine (2 mmol, 2.0 equiv.) was added dropwise to the solution, and the reaction mixture was stirred vigorously overnight at ambient temperature. Reaction progress was monitored by thin-layer chromatography (TLC). Upon completion, the solvent was evaporated under reduced pressure, and the resultant oil was resuspended in acetonitrile-water (1:19 v/v). The crude product was purified by reverse-phase column chromatography (C18, acetonitrile/water, 5–95% gradient) to afford the desired acylphosphate as the triethylammonium salt in good to excellent yield.

(phenyl phosphoric) propionic anhydride (1c)

^{1}H NMR (500 MHz, D_2O) δ 7.46 – 7.37 (m, 1H), 7.29 – 7.17 (m, 1H), 3.20 (q, J = 7.3 Hz, 2H), 2.50 (qd, J = 7.5, 0.6 Hz, 1H), 1.28 (t, J = 7.3 Hz, 3H), 1.11 (t, J = 7.5 Hz, 1H); ^{31}P NMR (202 MHz, CD_3CN) δ -14.88; ^{13}C NMR (126 MHz, d2o) δ 173.02, 172.94, 151.17, 151.11, 129.77, 129.76, 129.65, 129.65, 124.86, 124.85, 124.16, 124.15, 120.37, 120.36, 120.34, 120.32, 46.59, 28.37, 28.32, 8.14, 7.99; HRMS (ESI-): m/z $[M-H]^-$ calcd for $C_9H_{10}O_5P$, 229.0271; found 229.0281.

Isobutyric (phenyl phosphoric) anhydride (1b)

^{1}H NMR (500 MHz, CD_3OD) δ 7.35 – 7.28 (m, 2H), 7.20 (d, *J* = 7.7 Hz, 2H), 7.12 (t, *J* = 7.4 Hz, 1H), 3.16 (q, *J* = 7.3 Hz, 6H), 2.57 (hept, *J* = 7.0 Hz, 1H), 1.28 (t, *J* = 7.3 Hz, 8H), 1.15 (d, *J* = 7.7 Hz, 6H); ^{31}P NMR (202 MHz, cd_3od) δ -13.53; ^{13}C NMR (126 MHz, CD_3OD) δ 174.23, 174.16, 153.75, 153.69, 130.35, 125.05, 121.58, 121.54, 47.57, 36.28, 36.23, 18.97, 9.09; HRMS (ESI-): m/z $[M-H]^-$ calcd for $C_{10}H_{12}O_5P$, 243.0428; found 243.0428.

(phenyl phosphoric) pivalic anhydride (1c)

^{1}H NMR (400 MHz, CD_3CN) δ 7.36 – 7.27 (m, 2H), 7.23 – 7.16 (m, 2H), 7.11 (td, *J* = 7.4, 1.1 Hz, 1H), 3.01 (qd, *J* = 7.3, 4.7 Hz, 5H), 1.20 (t, *J* = 7.3 Hz, 8H), 1.14 (s, 9H); ^{31}P NMR (162 MHz, CD_3CN) δ -14.87; ^{13}C NMR (126 MHz, CD_3CN) δ 175.38, 175.30, 153.70, 153.70, 153.64,

130.28, 130.27, 124.75, 124.74, 121.54, 121.51, 49.89, 49.89, 49.87, 49.86, 46.88, 40.40, 40.36, 27.15, 9.00; HRMS (ESI-): m/z $[M-H]^-$ calcd for $C_{11}H_{14}O_5P$, 257.0584; found 257.0594.

Benzoic (phenyl phosphoric) anhydride (1d)[4]

^1H NMR (600 MHz, CD_3CN) δ 8.04 – 7.99 (m, 1H), 7.67 – 7.61 (m, 0H), 7.53 – 7.47 (m, 1H), 7.37 – 7.28 (m, 2H), 7.11 (ddt, *J* = 8.3, 7.1, 1.4 Hz, 0H), 3.03 (qd, *J* = 7.3, 4.4 Hz, 3H), 1.22 (t, *J* = 7.3 Hz, 4H); ^{31}P NMR (243 MHz, CD_3CN) δ -14.63; ^{13}C NMR (126 MHz, cd_3cn) δ 163.94, 163.88, 154.16, 154.10, 134.51, 131.52, 131.47, 130.75, 130.23, 130.22, 129.67, 124.41, 124.40, 121.53, 121.49, 118.33, 46.79, 8.96; HRMS (ESI-): m/z $[M-H]^-$ calcd for $C_{13}H_{10}O_5P$, 277.0217; found 277.0279.

Ethyl 5-bromo-2,2-dimethylpentanoate (2)[5]

To an oven-dried round-bottom flask (150 mL) equipped with a magnetic stir bar was added anhydrous THF (30 mL) under N_2. The solution was cooled to –78 °C (dry ice/acetone bath), and to it was added lithium diisopropylamide (2.0 M in THF/heptane/ethylbenzene, 7.5 mL, 15.0 mmol, 1.5 equiv). Ethyl isobutyrate (1.3 mL, 10.0 mmol, 1.0 equiv) was then added dropwise over 5 min, and the resulting solution was stirred vigorously for 45 min at –78 °C. 1,3-Dibromopropane (1.8 mL, 15.0 mmol, 1.5 equiv) was then added neat, and the reaction mixture was stirred for an additional 2 h at –78 °C before being allowed to warm to room temperature where it stirred for a further 2 h. The reaction was quenched by addition of saturated aqueous NH_4Cl (30 mL) and extracted with EtOAc (3 × 30 mL). The combined organic layers were washed with brine (30 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (2-propanol/hexanes, 0-15%) to afford ethyl 5-bromo-2,2-dimethylpentanoate as a colorless oil (2.01 g, 79%). NMR characterizations are consistent with literature; ^1H NMR (400 MHz, $CDCl_3$) δ 4.04 (q, *J* = 7.1 Hz, 2H), 3.30 (t, *J* = 6.6 Hz, 2H), 1.79 – 1.67 (m, 2H), 1.61 – 1.53 (m, 1H), 1.17 (t, *J* = 7.1 Hz, 3H), 1.10 (s, 6H). ^{13}C NMR (126 MHz, cdcl3) δ 177.56, 60.48, 41.85, 39.18, 33.95, 28.60, 25.23, 14.31.

Ethyl 5-azido-2,2-dimethylpentanoate (3)[5]

Ethyl 5-bromo-2,2-dimethylpentanoate (474 mg, 2 mmol, 1.0 equiv) was dissolved in DMSO (20 mL) in a 50 mL round-bottom flask equipped with a magnetic stir bar. Sodium azide (650 mg, 10 mmol, 5 equiv) was added in one portion, and the mixture was stirred at 50 °C for 18 h. The reaction was then cooled to room temperature, transferred to a separatory funnel, and diluted with EtOAc (100 mL). The organic layer was washed with brine (5 × 20 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure to afford ethyl 5-azido-2,2-dimethylpentanoate as a pale yellow oil (382 mg, 96%), which was used directly without further purification; ^1H NMR (500 MHz, $CDCl_3$) δ 4.10 (q, *J* = 7.1 Hz, 2H), 3.22 (t, *J* = 6.5 Hz,

2H), 1.60 – 1.48 (m, 4H), 1.22 (t, *J* = 7.1 Hz, 3H), 1.16 (s, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 177.50, 60.44, 51.76, 41.92, 37.61, 25.16, 24.63, 14.24.

5-Azido-2,2-dimethylpentanoic acid (4)[5]

Ethyl 5-azido-2,2-dimethylpentanoate (400 mg, 2 mmol, 1.0 equiv) was dissolved in EtOH (4 mL) and combined with 2 M NaOH (4 mL, 8 mmol, 4.0 equiv) in a 25 mL round-bottom flask equipped with a magnetic stir bar. The reaction mixture was heated to reflux and stirred for 3 h. After cooling to room temperature, the solution was adjusted to pH 2 with 2 M HCl and extracted with EtOAc (3 × 30 mL). The combined organic layers were washed with brine (30 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (2-propanol/hexanes, 0-15%) to afford 5-azido-2,2-dimethylpentanoic acid as a white powder (294 mg, 86%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.28 – 3.22 (m, 3H), 1.63 – 1.54 (m, 4H), 1.19 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 184.71, 51.71, 41.93, 37.35, 24.95, 24.58.

5-Azido-2,2-dimethylpentanoic (phenyl phosphoric) anhydride (5a)

5-Azido-2,2-dimethylpentanoic acid (257 mg, 1.5 mmol, 1.5 equiv) was dissolved in anhydrous DCM (6 mL) in an oven-dried scintillation vial equipped with a magnetic stir bar under an argon atmosphere. The solution was cooled in an ice bath (0 °C), and oxalyl chloride (150 uL, 1.75 mmol, 1.75 equiv) was added dropwise over 3 min. A catalytic amount of DMF (1–2 drops) was then added, and the reaction mixture was allowed to warm to room temperature and stirred until cessation of gas evolution (~2 h). Without isolation of the intermediate acyl chloride, a solution of phenyl dihydrogen phosphate (174 mg, 1 mmol, 1.0 equiv) in anhydrous DCM/pyridine (1:3, 4 mL) was added dropwise, and the mixture was stirred overnight. The solvents were removed under pressure, and the crude residue was purified by reverse-phase flash chromatography ($MeCN/H_2O$ + 0.1% TEA, 2–100%) to afford 5-azido-2,2-dimethylpentanoic (phenyl phosphoric) anhydride as a colorless oil (185 mg, 59%) of the triethylammonium salt. ^{1}H NMR (500 MHz, CD_3CN) δ 7.35 – 7.27 (m, 2H), 7.22 – 7.17 (m, 2H), 7.13 – 7.06 (m, 1H), 3.18 (t, J = 6.3 Hz, 2H), 3.02 (qd, J = 7.3, 4.6 Hz, 5H), 1.53 – 1.40 (m, 4H), 1.22 (t, J = 7.3 Hz, 8H), 1.11 (s, 6H); ^{31}P NMR (162 MHz, CD_3CN) δ -14.43; ^{13}C NMR (126 MHz, CD_3CN) δ 174.85, 174.77, 153.92, 153.86, 130.19, 124.52, 124.51, 121.53, 121.49, 52.35, 46.79, 43.70, 43.65, 38.04, 25.14, 25.11, 8.93; HRMS (DART-) m/z $[M-H]^-$ calcd for $C_{13}H_{17}N_3O_5P$ 326.09113; found 326.09249.

5-Azido-2,2-dimethylpentanoic (methyl phosphoric) anhydride (5b)

5-Azido-2,2-dimethylpentanoic acid (257 g, 1.5 mmol, 1.0 equiv) was dissolved in anhydrous DCM (6 mL) in an oven-dried scintillation vial equipped with a magnetic stir bar under an argon atmosphere. The solution was cooled in an ice bath (0 °C), and oxalyl chloride (150 uL,

1.75 mmol, 1.75 equiv) was added dropwise over 3 min. A catalytic amount of DMF (1–2 drops) was then added, and the reaction mixture was allowed to warm to room temperature and stirred until cessation of gas evolution (~2 h). Without isolation of the intermediate acyl chloride, a solution of methyl dihydrogen phosphate (112 mg, 1 mmol, 1.0 equiv) in anhydrous DCM/pyridine (1:3, 4 mL) was added dropwise, and the mixture was stirred overnight. The solvents were removed under pressure, and the crude residue was purified by reverse-phase flash chromatography (MeCN/H_2O + 0.1% TEA, 2–100%) to afford 5-azido-2,2-dimethylpentanoic (phenyl phosphoric) anhydride as a colorless oil (128 mg, 51%) of the triethylammonium salt. ^{1}H NMR (500 MHz, CD_3OD) δ 3.66 (d, J = 11.5 Hz, 3H), 3.20 (q, J = 7.3 Hz, 6H), 1.67 – 1.54 (m, 5H), 1.31 (t, J = 7.3 Hz, 9H), 1.22 (s, 6H); ^{31}P NMR (202 MHz, CD_3OD) δ -6.01.

2-(Diphenylphosphanyl)phenyl)methanol (6)[6]

To a stirred solution of 2-(diphenylphosphanyl)benzoic acid (612 mg, 2 mmol, 1.0 equiv) in anhydrous THF (20 mL) at 0 °C was added lithium aluminum hydride (2.0 M in THF, 6 mmol, 3 equiv) dropwise over 10 min. The mixture was allowed to warm to room temperature and stirred for 2 h, during which reaction progress was monitored by TLC. Upon completion, the reaction was cooled to 0 °C, and excess $LiAlH_4$ was quenched by the slow, dropwise addition of EtOAc (5 mL). The solvents were removed under reduced pressure, redissolved in 100 mL EtOAc, and transferred to a separatory funnel where it was washed with 3x20mL of brine. The organic layer was then dried over anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude residue was purified by flash column chromatography (EtOAc/hexanes, 0–50%) to afford 2-(diphenylphosphanyl)methanol as a colorless oil (444 mg, 76%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.52 (ddd, J = 7.8, 4.4, 1.2 Hz, 1H), 7.41 – 7.17 (m, 13H), 6.91 (ddd, J = 7.7, 4.7, 1.3 Hz, 1H), 2.04 (s, 2H); ^{31}P NMR (202 MHz, $CDCl_3$) δ -16.22; ^{13}C NMR (126 MHz, cdcl3) δ 144.97, 144.79, 135.98, 135.91, 133.95, 133.80, 133.44, 129.30, 128.95, 128.69, 128.63, 128.05, 128.01, 127.88, 127.87, 63.95, 63.77.

2-(diphenylphosphaneyl)benzyl acetyl-*L*-phenylalaninate (7)

To a stirred solution of DCC (226 mg, 1.1 mmol, 1.1 equiv) in anhydrous DCM (2.5 mL) under argon was added a solution of N-acetyl-L-phenylalanine (414 g, 2 mmol, 1.0 equiv) and DMAP (12 mg, 0.1 mmol, 0.1 equiv) in anhydrous DCM (2.5 mL). The mixture was stirred vigorously at room temperature for 10 min, after which a solution of 2-(diphenylphosphanyl)methanol (350 mg, 1.2 mmol, 1.2 equiv) in anhydrous DCM (5 mL) was added dropwise. The reaction mixture was stirred overnight and filtered through a pad of Celite. The filtrate was concentrated under reduced pressure, and the crude residue was purified by flash column chromatography (EtOAc/hexanes, 0–50%) to afford 2-(diphenylphosphanyl)benzyl acetyl-L-phenylalaninate as a colorless oil (235 mg, 49%); ^{1}H

NMR (400 MHz, $CDCl_3$) δ 7.43 – 7.16 (m, 16H), 7.02 – 6.91 (m, 3H), 5.66 (d, *J* = 7.9 Hz, 1H), 5.49 (dd, *J* = 12.3, 1.1 Hz, 1H), 5.39 (dd, *J* = 12.3, 1.3 Hz, 1H), 4.59 (dt, *J* = 7.9, 5.6 Hz, 1H), 2.90 (dd, *J* = 13.9, 5.7 Hz, 1H), 2.72 (dd, *J* = 13.9, 5.5 Hz, 1H), 1.90 (s, 3H), 1.56 (s, 2H); ^{31}P NMR (162 MHz, $CDCl_3$) δ -16.50; ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.27, 169.52, 139.60, 139.40, 137.19, 137.06, 136.51, 136.43, 136.41, 136.33, 135.96, 134.35, 134.35, 134.17, 134.01, 133.95, 133.80, 130.11, 130.07, 129.50, 129.40, 129.15, 129.10, 129.01, 128.83, 128.82, 128.80, 128.77, 128.77, 128.74, 128.58, 127.10, 66.23, 66.04, 53.07, 37.44, 23.28; HRMS (DART+) m/z $[M+H]^+$ calcd for $C_{30}H_{29}NO_3P$ 482.18796; found 482.18783.

(*S*)-5-(2-acetamido-3-phenylpropanamido)-2,2-dimethylpentanoic (phenyl phosphoric) anhydride (8a)

2-(diphenylphosphanyl)benzyl acetyl-L-phenylalaninate (241 mg, 0.5 mmol, 1.0 equiv) was dissolved in anhydrous DMF (3 mL) and transferred to an oven dried scintillation vial under inert atmosphere with magnetic stir bar. Separately, a solution of 5-azido-2,2-dimethylpentanoic (phenyl phosphoric) anhydride (188 mg, 0.6 mmol, 1.2 equiv) and triethylamine (150 uL, 1.08 mmol, 2.16 equiv) in anhydrous DMF (2 mL) was prepared and added to the reaction vessel. The mixture was stirred at rt overnight, then quenched with H_2O (1 mL) and stirred an additional 30 min. The crude reaction mixture was purified directly by reverse-phase flash chromatography ($MeCN/H_2O$ + 0.1% TEA, gradient 2–60%) to afford (S)-5-(2-acetamido-3-phenylpropanamido)-2,2-dimethylpentanoic (phenyl phosphoric) anhydride as a colorless oil (169 mg, 71%); ^1H NMR (600 MHz, D_2O) δ 7.45 – 7.40 (m, 2H), 7.40 – 7.35 (m, 2H), 7.35 – 7.30 (m, 1H), 7.28 – 7.23 (m, 3H), 7.21 (dq, J = 7.7, 1.2 Hz, 2H), 4.48 (t, J = 7.8 Hz, 1H), 3.22 (q, J = 7.3 Hz, 5H), 3.08 – 2.93 (m, 4H), 2.74 (s, 1H), 1.98 (s, 3H), 1.35 (ddd, J = 10.0, 7.0, 3.3 Hz, 2H), 1.30 (t, J = 7.3 Hz, 7H), 1.26 – 1.17 (m, 2H), 1.14 (d, J = 5.3 Hz, 6H); 31P NMR (243 MHz, cd3cn) δ -13.93; ^{13}C NMR (126 MHz, D_2O) δ 175.92, 175.84, 173.78, 172.73, 151.24, 151.18, 136.25, 129.71, 129.70, 129.06, 128.68, 127.17, 124.80, 124.79, 120.37, 120.34, 55.43, 46.59, 42.88, 42.84, 39.33, 37.24, 36.45, 23.96, 23.79, 23.74, 21.53, 8.14; HRMS (ESI-) m/z $[M-H]^-$ calcd for $C_{24}H_{30}N_2O_7P$ 489.1796; found 489.1800.

(*S*)-5-(2-acetamido-3-phenylpropanamido)-2,2-dimethylpentanoic (methyl phosphoric) anhydride (8b)

2-(diphenylphosphanyl)benzyl acetyl-L-phenylalaninate (241 mg, 0.5 mmol, 1.0 equiv) was dissolved in anhydrous DMF (3 mL) and transferred to an oven dried scintillation vial under inert atmosphere with magnetic stir bar. Separately, a solution of 5-azido-2,2-dimethylpentanoic (methyl phosphoric) anhydride (150 mg, 0.6 mmol, 1.2 equiv) and triethylamine (150 uL, 1.08 mmol, 2.16 equiv) in anhydrous DMF (2 mL) was prepared and added to the reaction vessel. The mixture was stirred at rt overnight, then quenched with

H_2O (1 mL) and stirred an additional 30 min. The crude reaction mixture was purified directly by reverse-phase flash chromatography (MeCN/H_2O + 0.1% TEA, gradient 2–60%) to afford (S)-5-(2-acetamido-3-phenylpropanamido)-2,2-dimethylpentanoic (methyl phosphoric) anhydride as a colorless oil (132 mg, 64%); ^{1}H NMR (600 MHz,CD_3CN) δ 7.84 (d, *J* = 8.6 Hz, 1H), 7.27 (d, *J* = 5.2 Hz, 4H), 7.20 (ddd, *J* = 6.8, 4.6, 3.0 Hz, 1H), 7.13 (s, 1H), 4.45 (ddd, *J* = 9.8, 8.5, 4.9 Hz, 1H), 3.60 (d, *J* = 11.3 Hz, 3H), 3.29 – 3.19 (m, 1H), 3.12 (dd, *J* = 13.8, 4.9 Hz, 1H), 3.07 – 2.94 (m, 7H), 2.88 (dd, *J* = 13.8, 9.8 Hz, 1H), 2.41 (s, 4H), 1.83 (s, 3H), 1.57 – 1.35 (m, 4H), 1.23 (t, *J* = 7.3 Hz, 9H), 1.14 (d, *J* = 9.7 Hz, 6H); ^{31}P NMR (243 MHz, CD_3CN) δ -7.81 (q, *J* = 11.5 Hz); ^{13}C NMR (126 MHz, CD_3CN) δ 175.42, 175.33, 172.63, 170.93, 139.47, 130.29, 129.17, 127.32, 56.08, 53.89, 53.84, 46.73, 44.05, 44.01, 39.58, 38.72, 37.87, 25.71, 25.51, 25.00, 23.00, 8.93; HRMS (ESI-) m/z $[M-H]^-$ calcd for $C_{19}H_{28}N_2O_7P$ 427.1640; found 427.1638.

Supplemental Figures

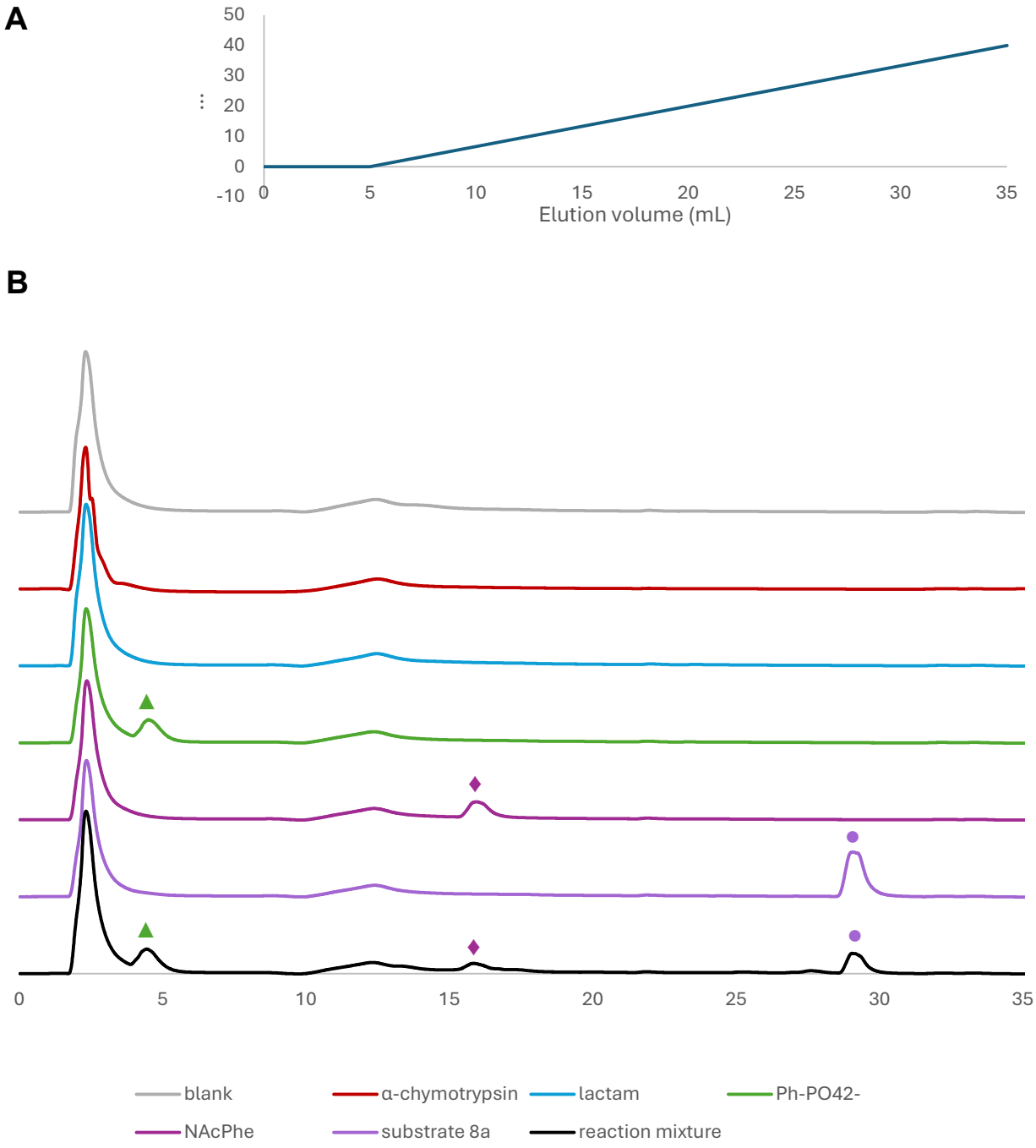


Figure S1. HPLC traces of the enzymatic reaction components

A) HPLC solvent gradient; change in % solvent B over elution volume. Solvent A: 50 mM ammonium acetate pH 5.0, solvent B: acetonitrile. B) Chromatogram of blank, α-chymotrypsin, lactam, phenylphosphate (▲, V_R = 4.5 mL), NAcPhe (♦, V_R = 16.1 mL), substrate 8a (●, V_R = 29.3 mL), and enzymatic reaction mixture. V_R : retention volume. A_{260} vs retention time in mL.

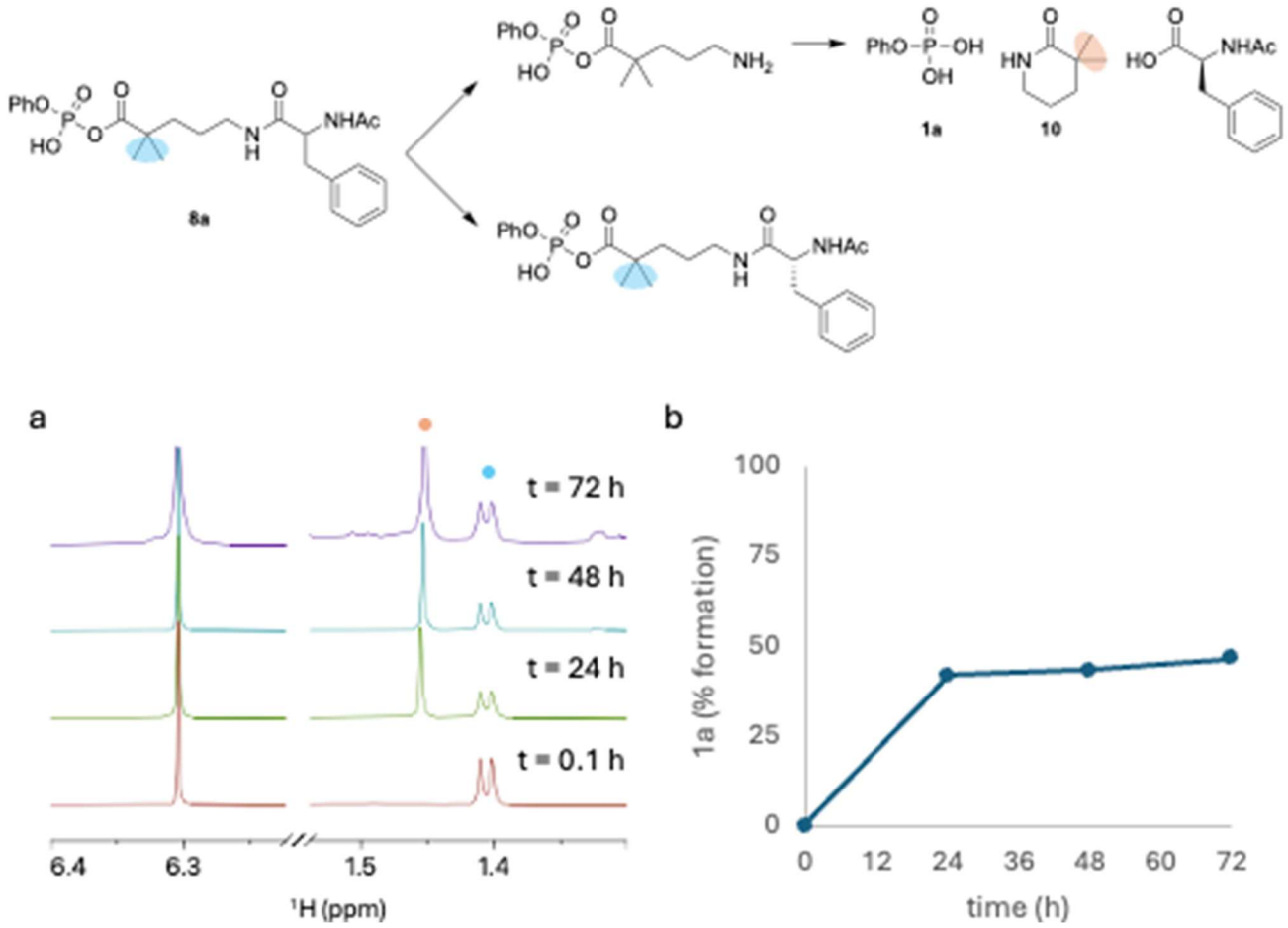


*Figure S2: (a) ^{1}H NMR spectra of CT + **8a** (1M HEPES pH 8, 100 μM CT, 10 mM **8a**, 37 °C, 10 mM 10 mM $CaCl_2$); highlighted peaks correspond to the protons of the matching colour in the scheme above. Enzyme was incubated with **8a** for 48h prior to second addition of enzyme. (b) Dependence of the concentration of **1b** as a function of time.*

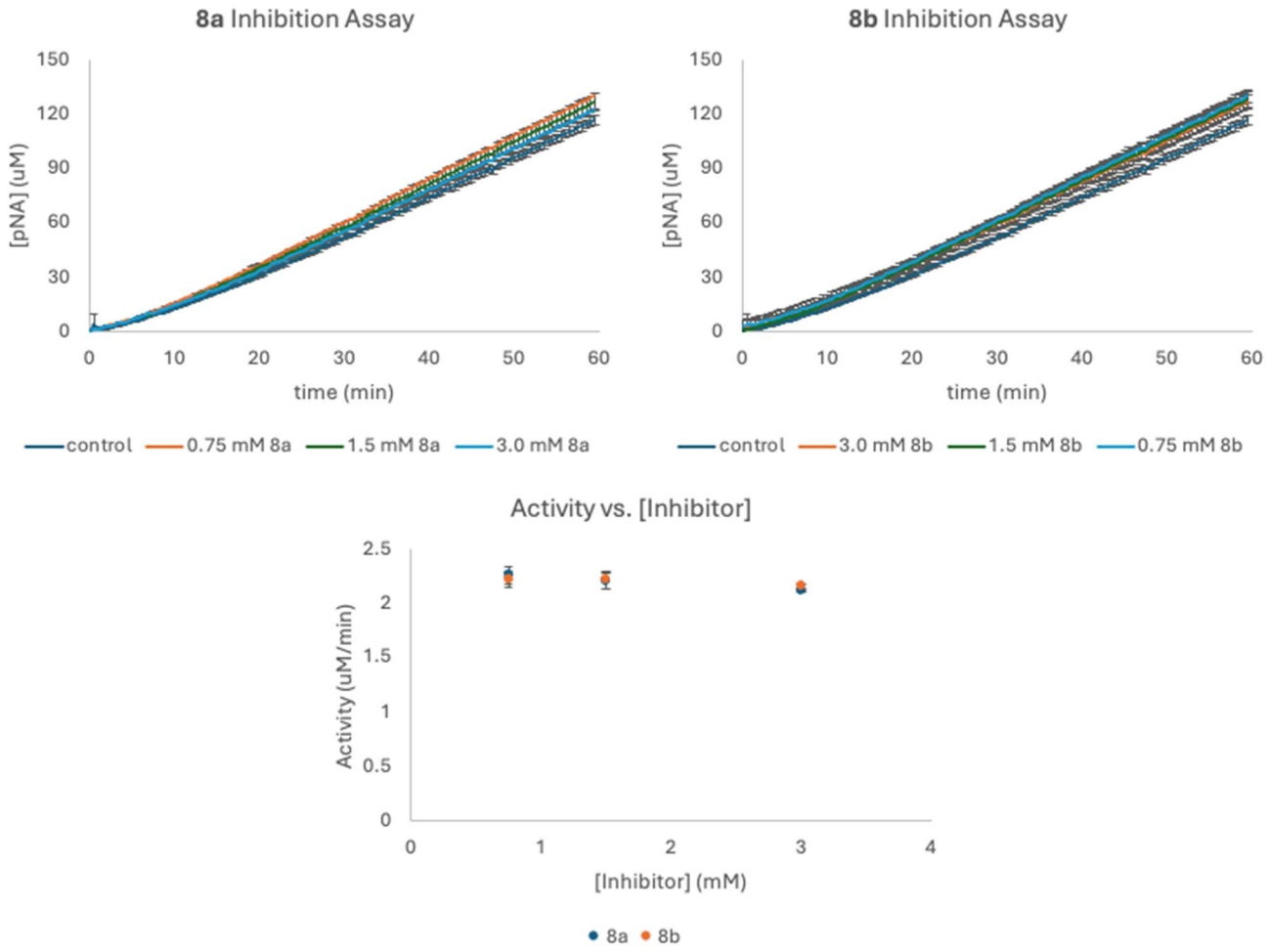


*Figure S3: Chymotrypsin inhibition assay for compounds **8a** and **8b**. Enzymatic activity was measured in 50 mM HEPES buffer (pH 8.0) containing 5 mM $CaCl_2$ at 37 °C using Suc-Phe-pNA (2 mM) as the chromogenic substrate. Reactions were performed in 96-well plates with a final enzyme concentration of 10 µM and inhibitor concentrations of 0.75, 1.5, and 3 mM. Formation of p-nitroaniline (pNA) was monitored at 410 nm over time. Enzyme activity was determined from the slopes of absorbance versus time plots after correction for background substrate hydrolysis and conversion to concentration using a pNA calibration curve. Data represent the mean of three independent experiments (± SD).*

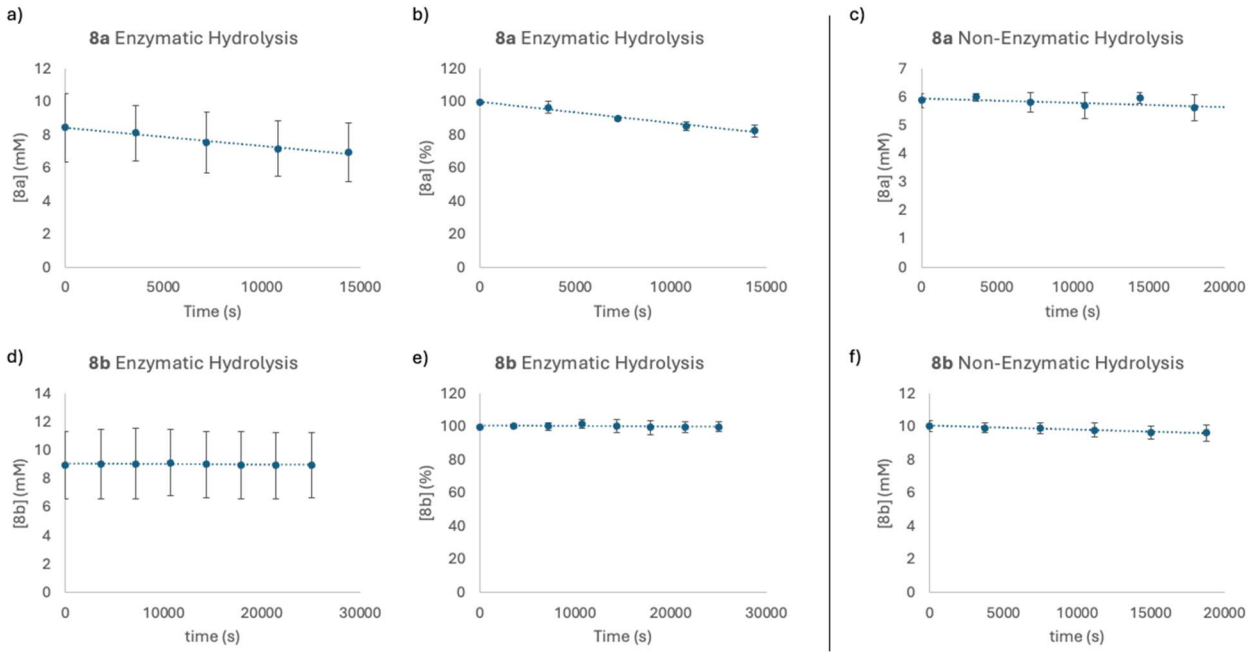
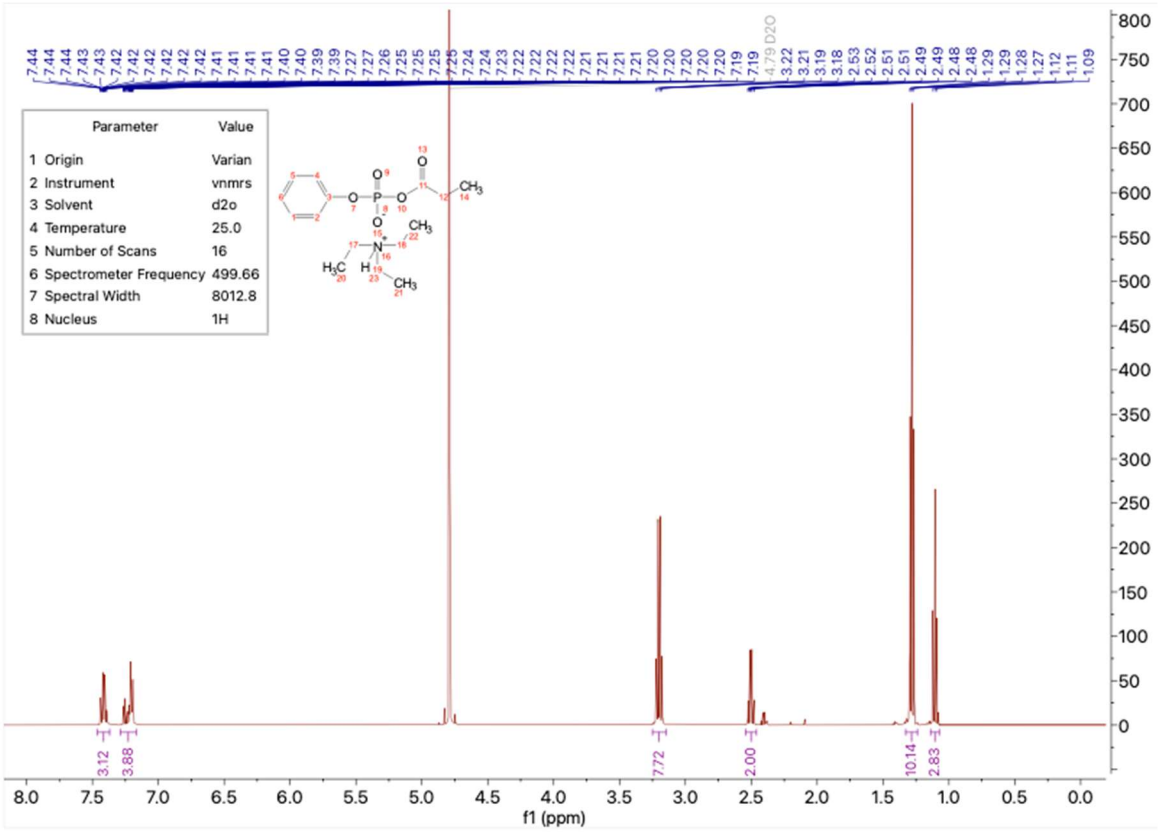
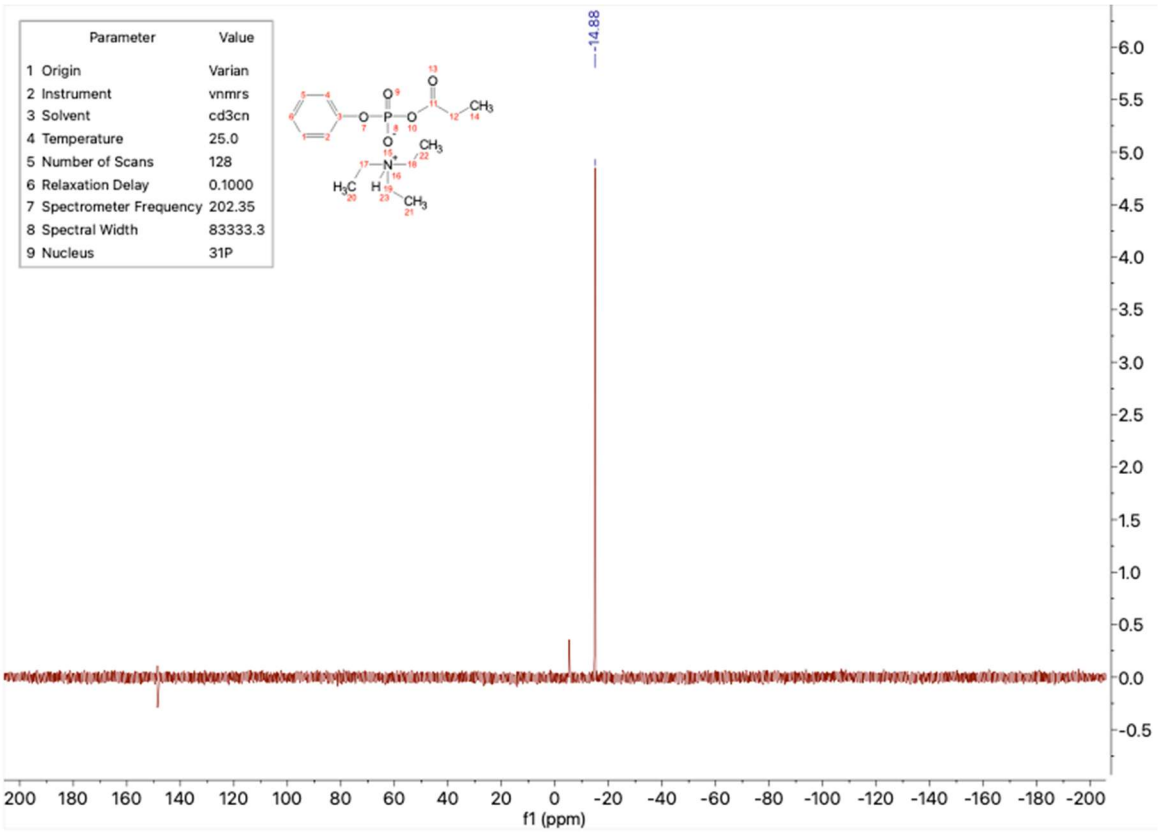


*Figure S4: Hydrolysis of compounds **8a** and **8b** monitored by ^{1}H and ^{31}P NMR under enzymatic and non-enzymatic conditions. Reactions were performed at 37 °C in 100 mM HEPES (pH 8.0) containing 10 mM $CaCl_2$ and enzyme (100 µM) where indicated. (a, d) Gem-dimethyl ^{1}H NMR signal integration for **8a** and **8b** versus time (+ enzyme). (b, e) Acylphosphate ^{31}P NMR signal integration for **8a** and **8b** versus time (+ enzyme). (c, f) Acylphosphate ^{31}P NMR signal integration for **8a** and **8b** versus time (– enzyme).*

Characterizations of New Compounds

(phenyl phosphoric) propionic anhydride **(1c)**

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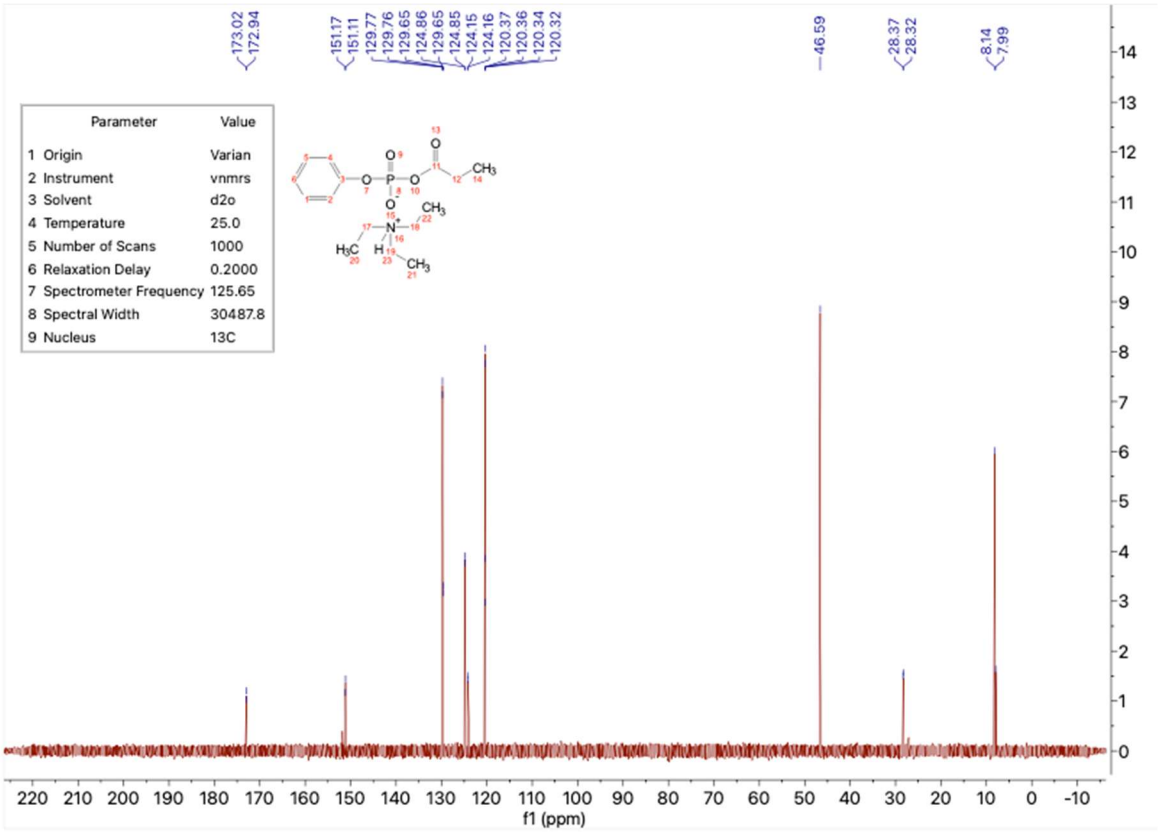
3 Solvent cd3cn
4 Temperature 25.0
5 Number of Scans 128
6 Relaxation Delay 0.1000
7 Spectrometer Frequency 202.35
8 Spectral Width 83333.3
9 Nucleus 31P
-14.88
f1 (ppm)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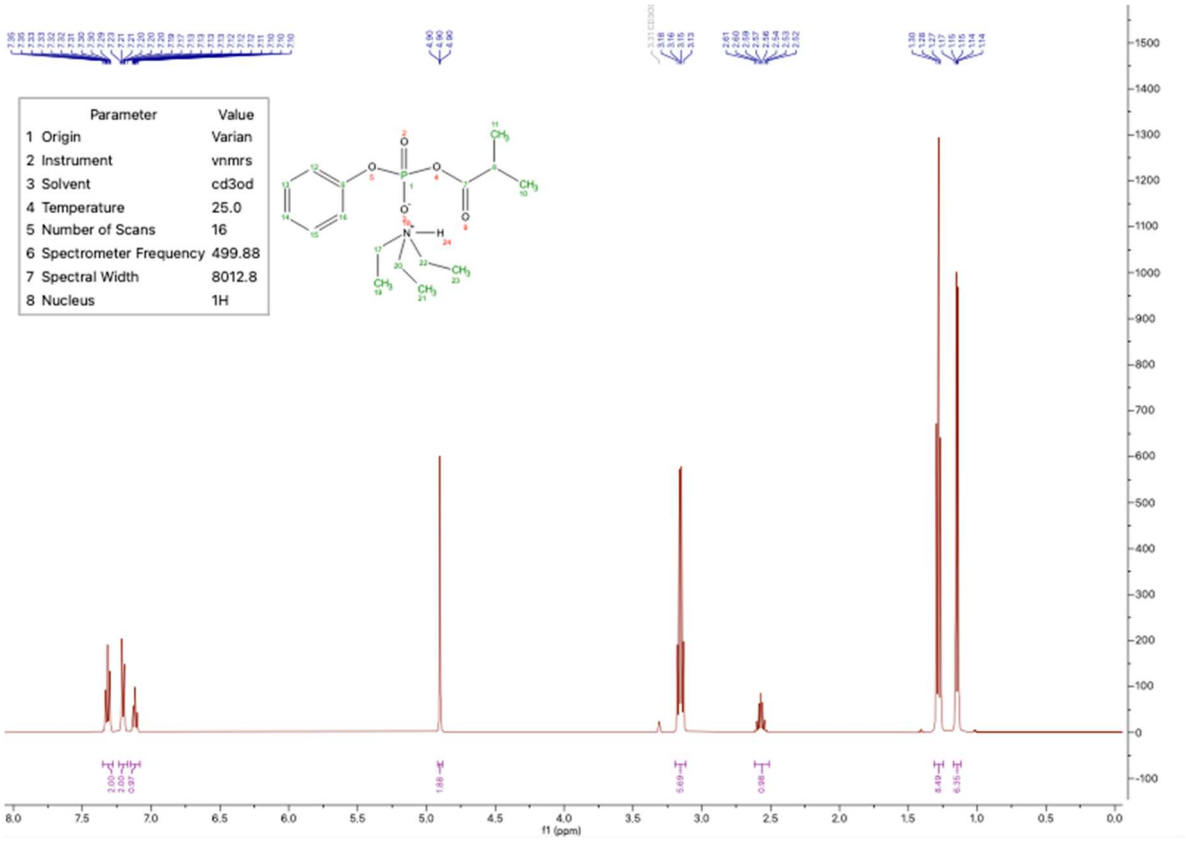
2 Instrument vnmrs
3 Solvent d2o
4 Temperature 25.0
5 Number of Scans 1000
6 Relaxation Delay 0.2000
7 Spectrometer Frequency 125.65
8 Spectral Width 30487.8
9 Nucleus 13C
173.02
172.94
151.17
151.11
129.77
129.76
129.65
124.86
129.65
124.85
124.15
124.16
120.37
120.36
120.34
120.32
46.59
28.37
28.32
8.14
7.99
f1 (ppm)

Isobutyric (phenyl phosphoric) anhydride **(1d)**



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2 Instrument	vnmrs
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4 Temperature	25.0
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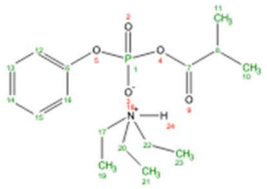
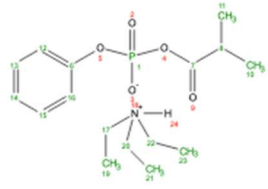
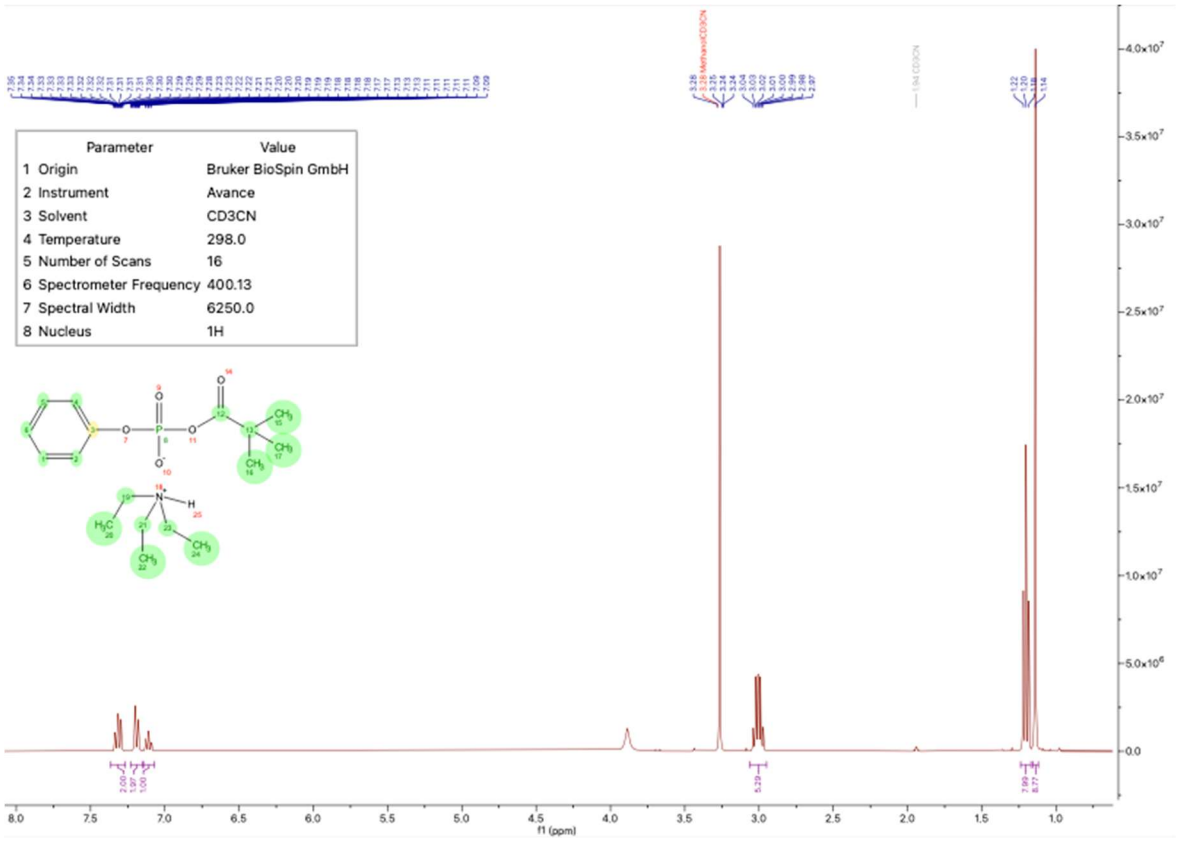
8 Nucleus	31P



Parameter	Value
1 Origin	Varian
2 Instrument	vnmrs
3 Solvent	cd3od
4 Temperature	25.0
5 Number of Scans	1000
6 Spectrometer Frequency	125.65
7 Spectral Width	30487.8
8 Nucleus	13C



(phenyl phosphoric) pivalic anhydride **(1e)**



Parameter	Value
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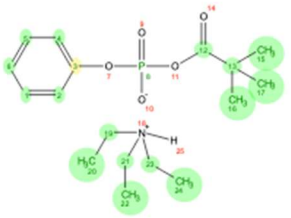
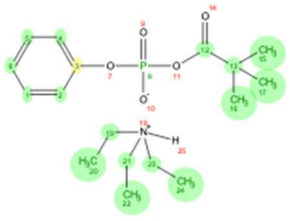
2 Instrument	Avance
3 Solvent	CD3CN
4 Temperature	298.0
5 Number of Scans	256
6 Spectrometer Frequency	161.98
7 Spectral Width	96153.8
8 Nucleus	31P

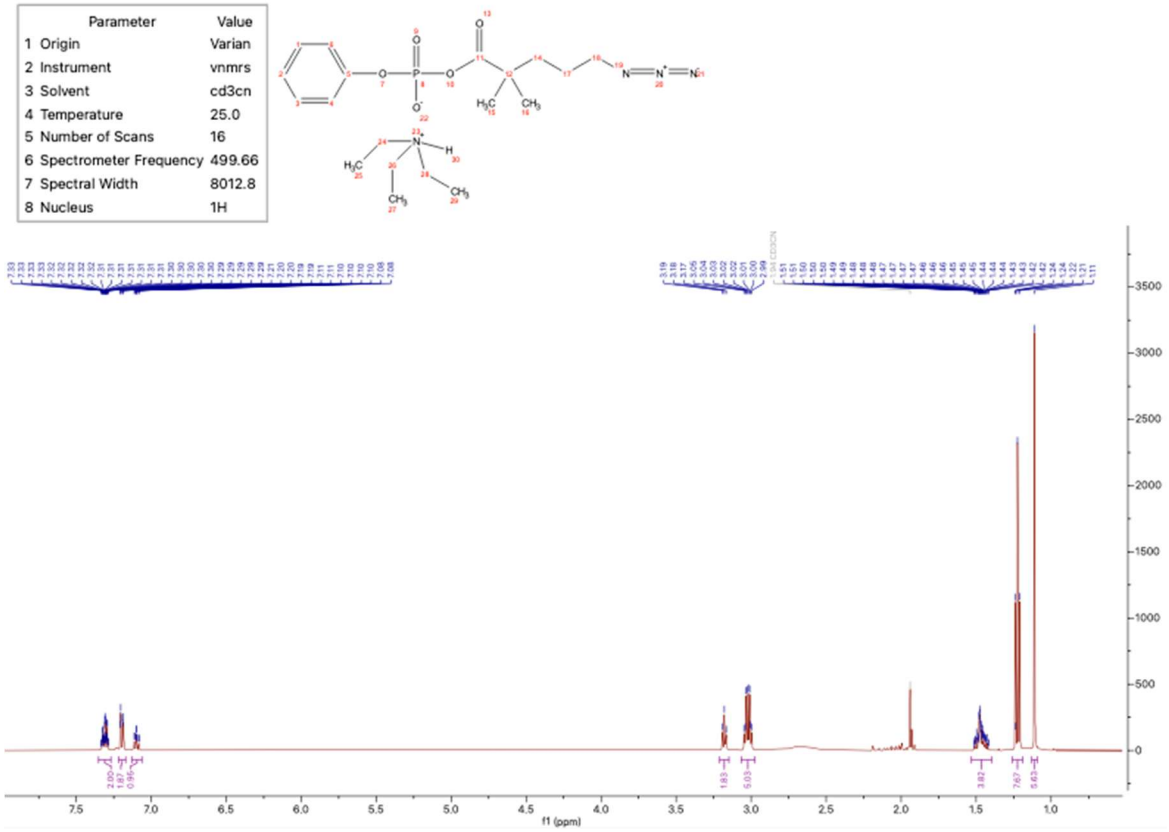


Parameter	Value
1 Origin	Varian
2 Instrument	vnmrs
3 Solvent	cd3cn
4 Temperature	25.0
5 Number of Scans	1000
6 Spectrometer Frequency	125.65
7 Spectral Width	30487.8
8 Nucleus	13C

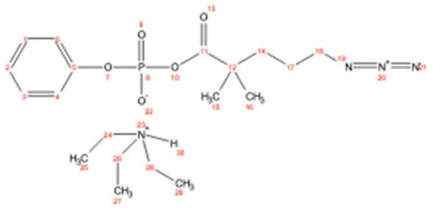


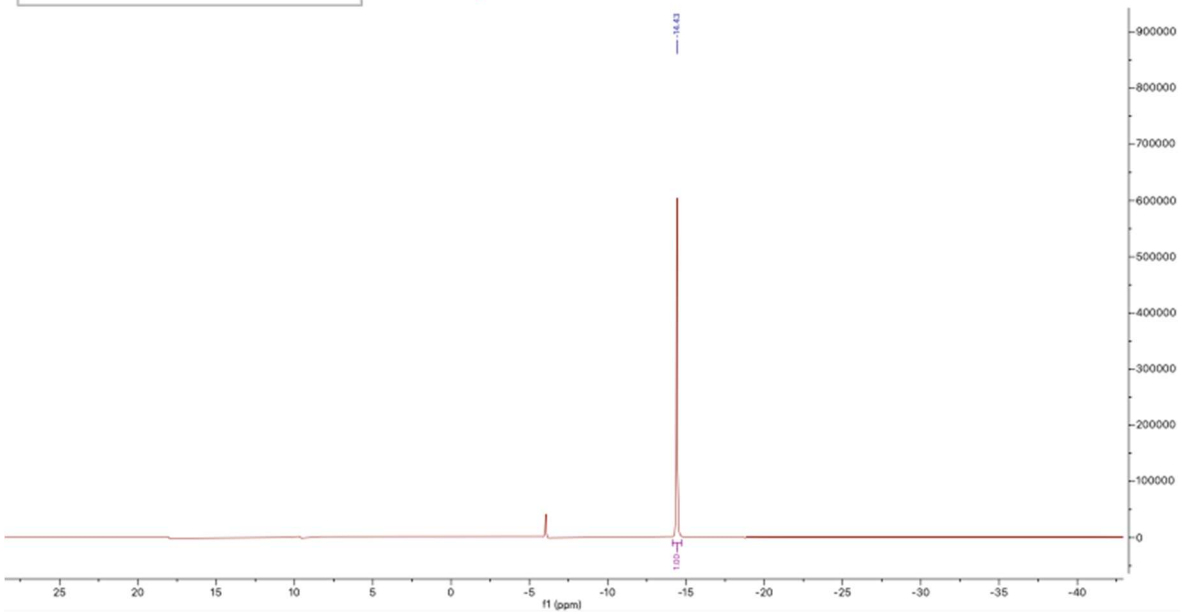
5-azido-2,2-dimethylpentanoic (phenyl phosphoric) anhydride **(5a)**

Parameter	Value
1 Origin	Varian
2 Instrument	vnmrs
3 Solvent	cd3cn
4 Temperature	25.0
5 Number of Scans	16
6 Spectrometer Frequency	499.66
7 Spectral Width	8012.8
8 Nucleus	1H

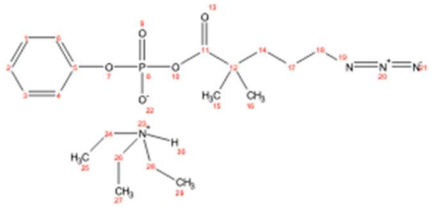


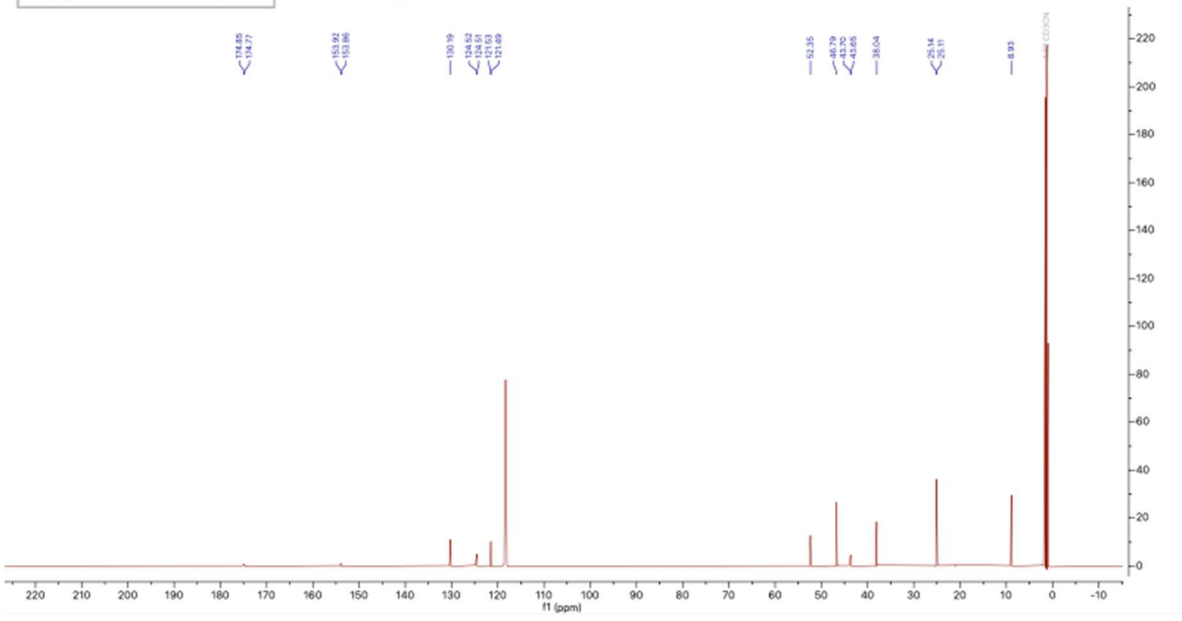
Parameter	Value
1 Origin	Bruker BioSpin GmbH
2 Instrument	Avance
3 Solvent	CD3CN
4 Temperature	298.0
5 Number of Scans	256
6 Spectrometer Frequency	161.98
7 Spectral Width	96153.8
8 Nucleus	31P





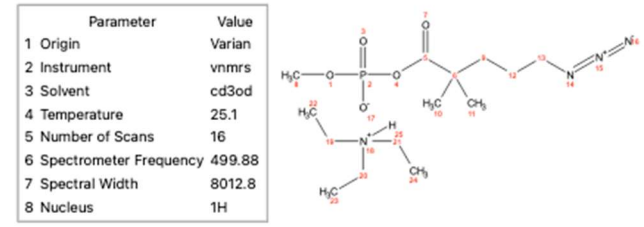
Parameter	Value
1 Origin	Varian
2 Instrument	vnmrs
3 Solvent	cd3cn
4 Temperature	25.0
5 Number of Scans	1000
6 Spectrometer Frequency	125.65
7 Spectral Width	30487.8
8 Nucleus	13C

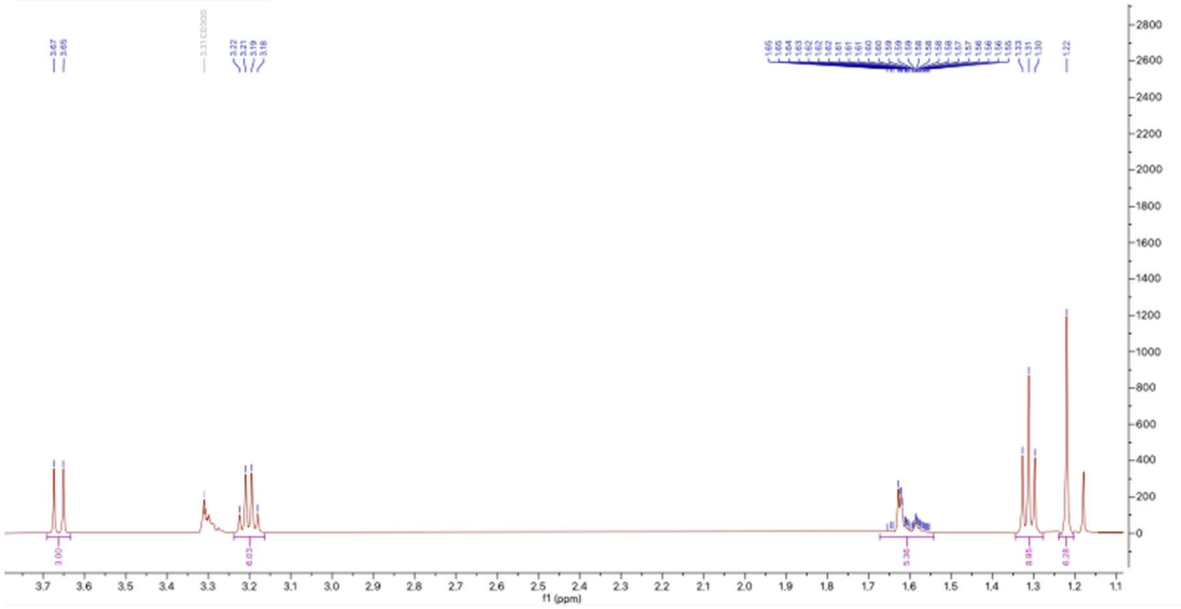




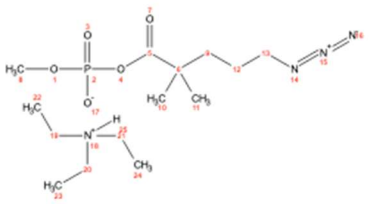
5-azido-2,2-dimethylpentanoic (methyl phosphoric) anhydride ***(5b)***

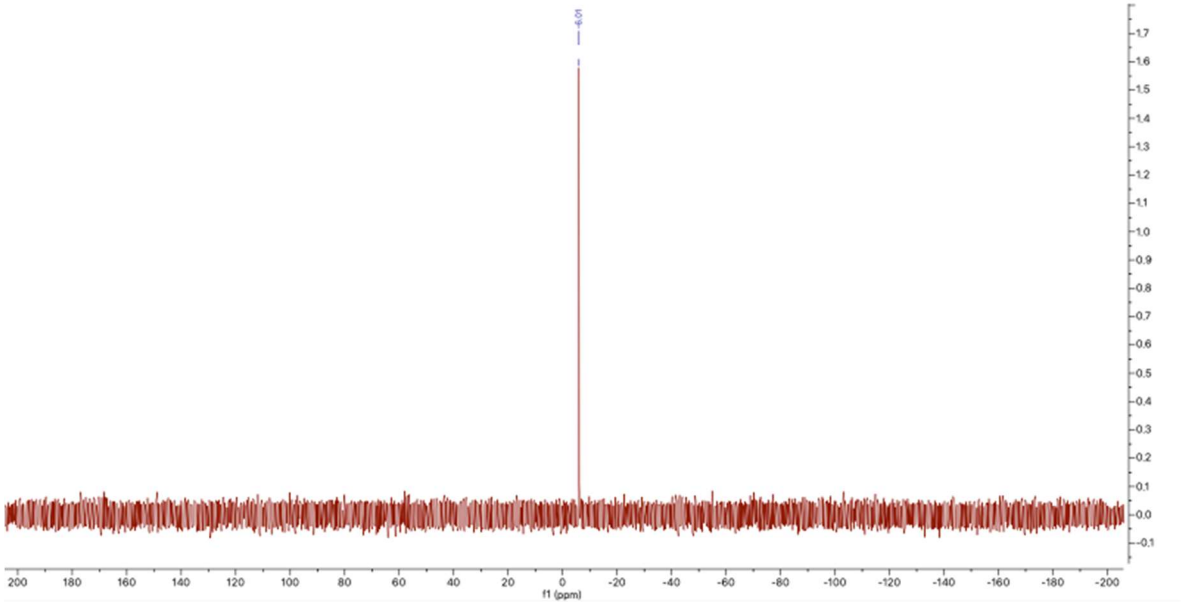
	Parameter	Value
1	Origin	Varian
2	Instrument	vnmrs
3	Solvent	cd3od
4	Temperature	25.1
5	Number of Scans	16
6	Spectrometer Frequency	499.88
7	Spectral Width	8012.8
8	Nucleus	1H





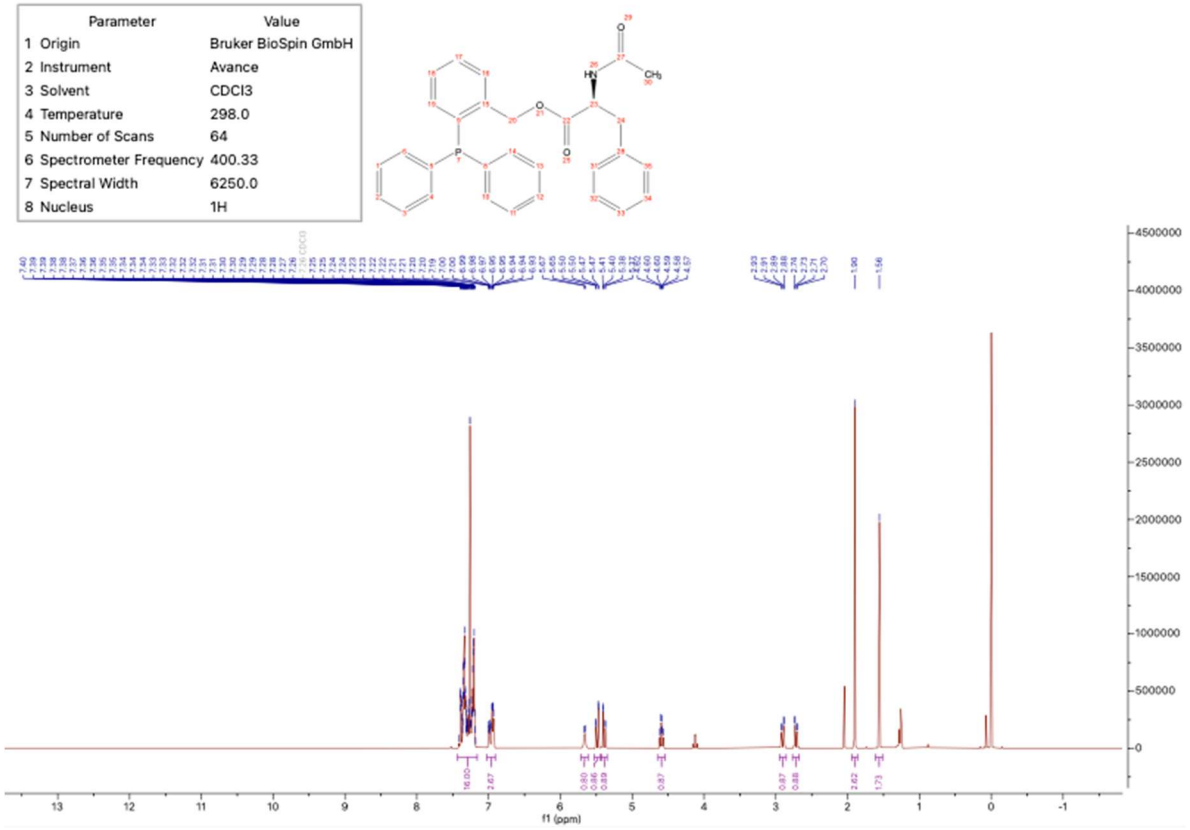
	Parameter	Value
1	Origin	Varian
2	Instrument	vnmrs
3	Solvent	cd3od
4	Temperature	25.1
5	Number of Scans	128
6	Spectrometer Frequency	202.35
7	Spectral Width	83333.3
8	Nucleus	31P



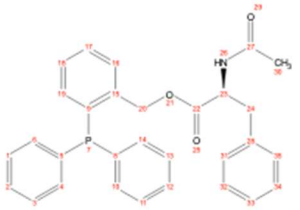


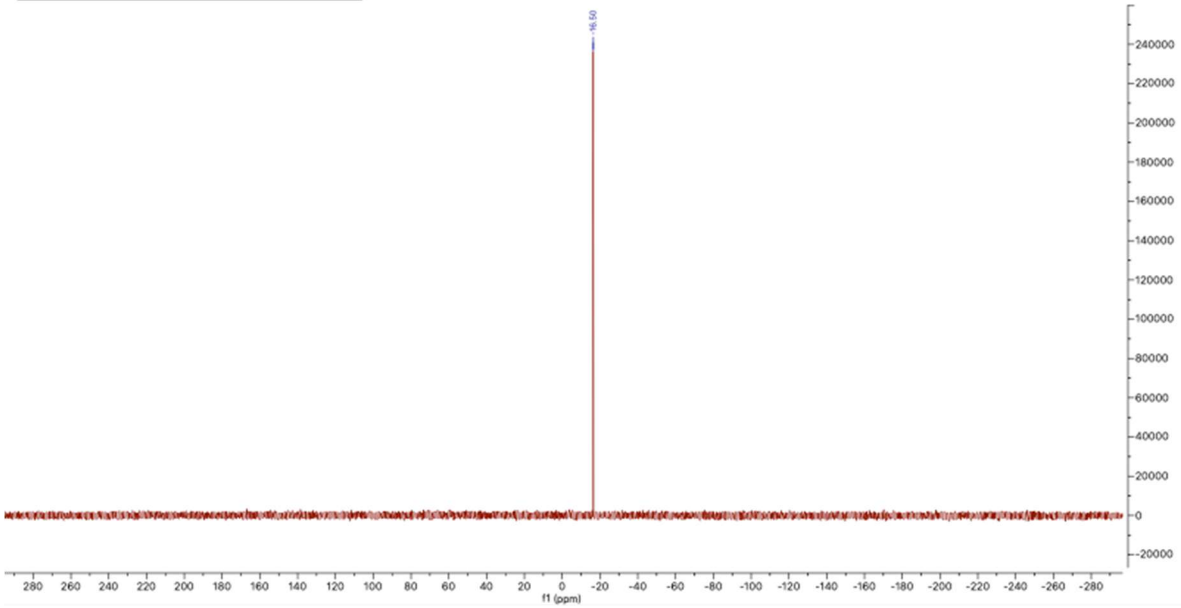
2-(diphenylphosphaneyl)benzyl acetyl-L-phenylalaninate **(7)**

Parameter	Value
1 Origin	Bruker BioSpin GmbH
2 Instrument	Avance
3 Solvent	CDCl3
4 Temperature	298.0
5 Number of Scans	64
6 Spectrometer Frequency	400.33
7 Spectral Width	6250.0
8 Nucleus	1H

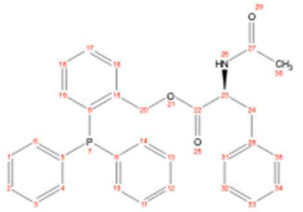


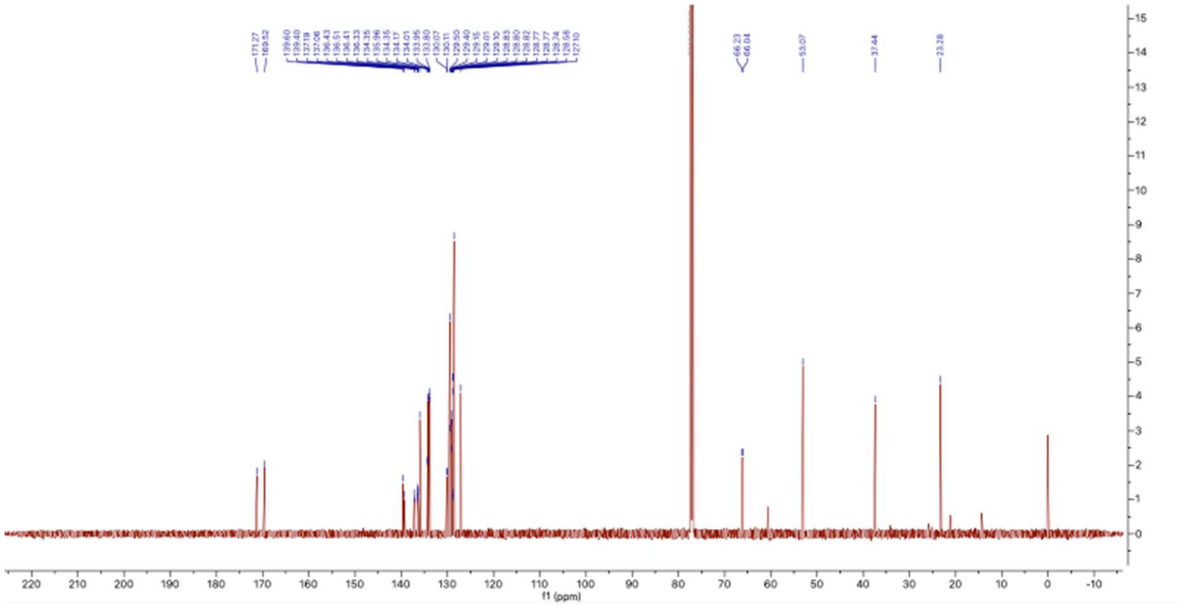
Parameter	Value
1 Origin	Bruker BioSpin GmbH
2 Instrument	Avance
3 Solvent	CDCl3
4 Temperature	298.0
5 Number of Scans	256
6 Spectrometer Frequency	162.06
7 Spectral Width	96153.8
8 Nucleus	31P





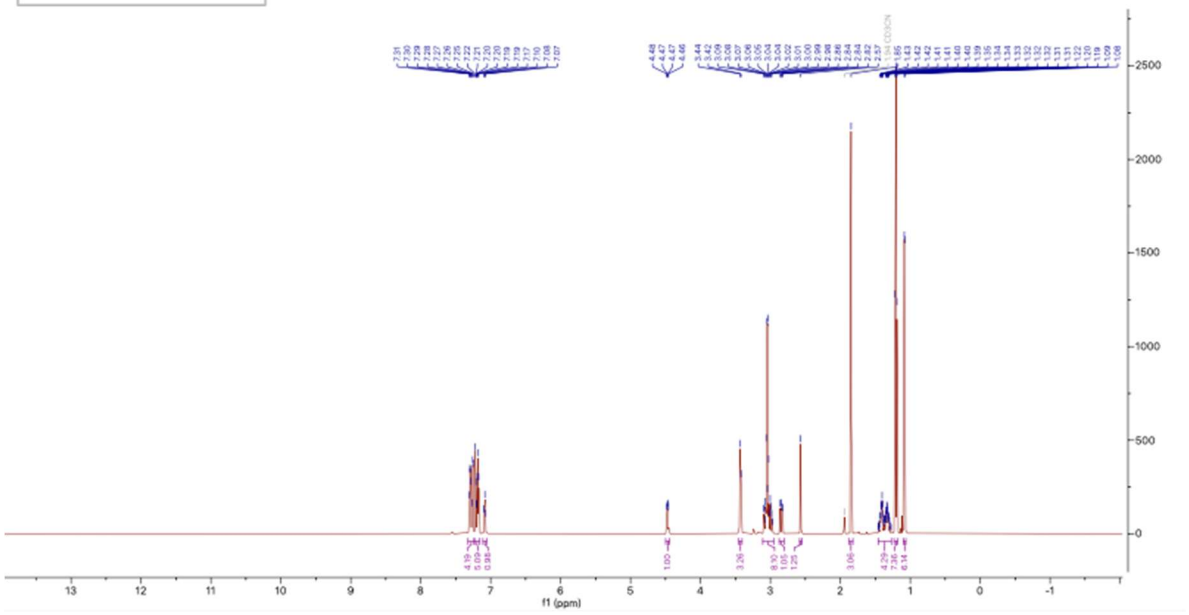
Parameter	Value
1 Origin	Varian
2 Instrument	vnmrs
3 Solvent	cdcl3
4 Temperature	25.0
5 Number of Scans	1000
6 Spectrometer Frequency	125.65
7 Spectral Width	30487.8
8 Nucleus	13C



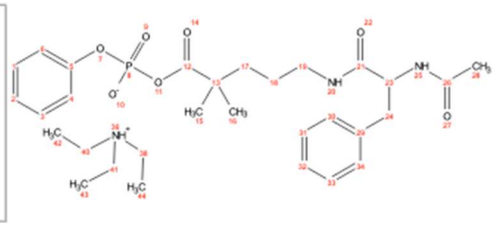


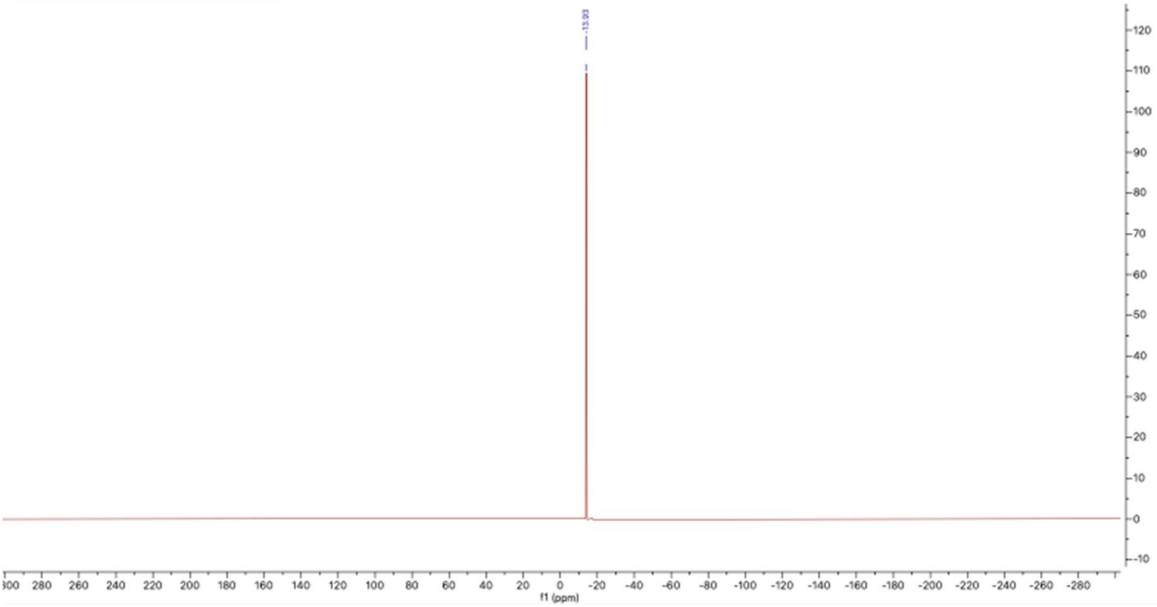
(S)-5-(2-acetamido-3-phenylpropanamido)-2,2-dimethylpentanoic (phenyl phosphoric) anhydride **(8a)**

Parameter	Value
1 Origin	Varian
2 Instrument	vnmrs
3 Solvent	cd3cn
4 Temperature	25.4
5 Number of Scans	32
6 Spectrometer Frequency	599.81
7 Spectral Width	9615.4
8 Nucleus	1H



Parameter	Value
1 Origin	Varian
2 Instrument	vnmrs
3 Solvent	cd3cn
4 Temperature	25.4
5 Number of Scans	256
6 Spectrometer Frequency	242.80
7 Spectral Width	147058.8
8 Nucleus	31P





Parameter	Value
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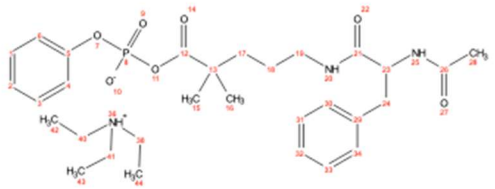
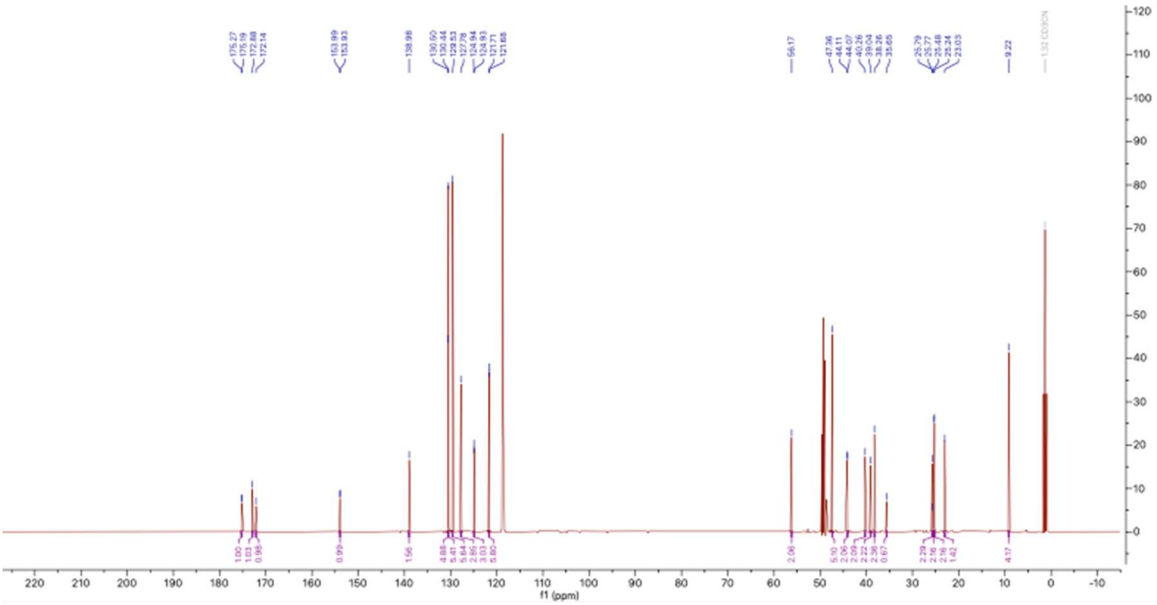
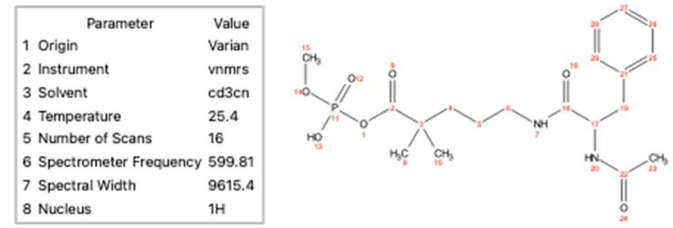
2 Instrument	vnmrs
3 Solvent	cd3cn
4 Temperature	25.0
5 Number of Scans	1000
6 Spectrometer Frequency	125.65
7 Spectral Width	30487.8
8 Nucleus	13C

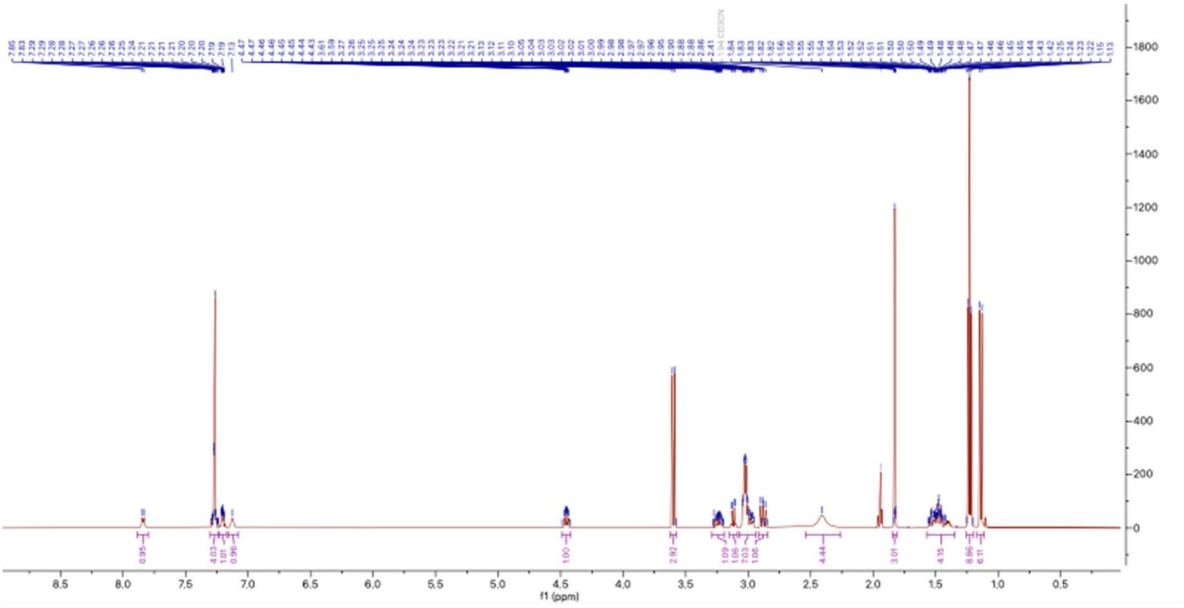




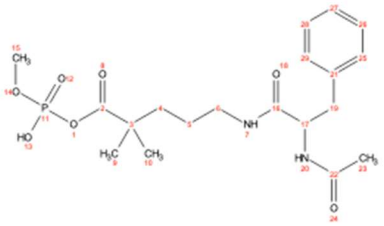
(S)-5-(2-acetamido-3-phenylpropanamido)-2,2-dimethylpentanoic (methyl phosphoric) anhydride **(8b)**

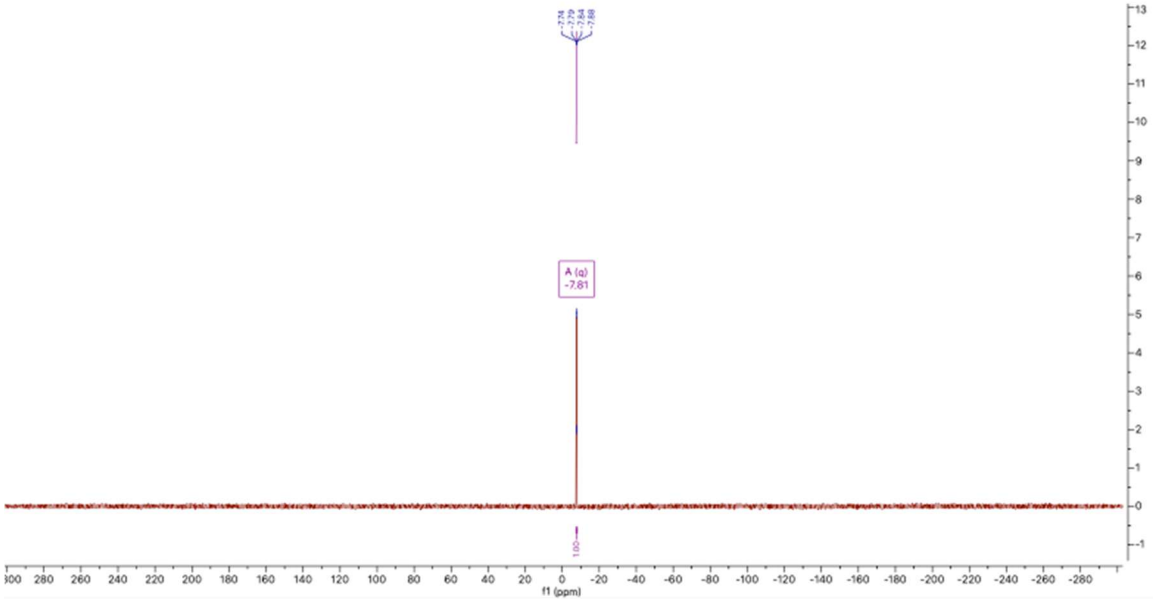
Parameter	Value
1 Origin	Varian
2 Instrument	vnmrs
3 Solvent	cd3cn
4 Temperature	25.4
5 Number of Scans	16
6 Spectrometer Frequency	599.81
7 Spectral Width	9615.4
8 Nucleus	1H



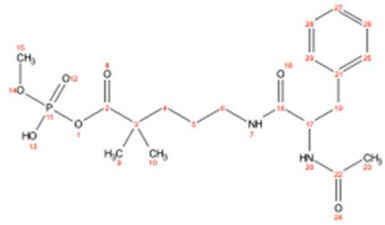


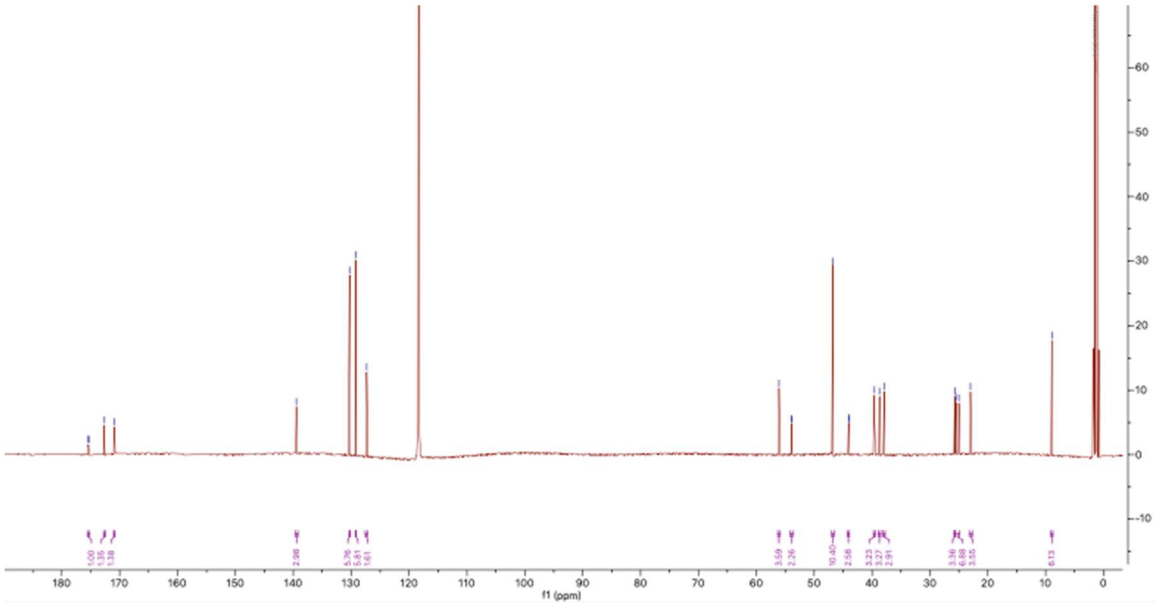
Parameter	Value
1 Origin	Varian
2 Instrument	vnmrs
3 Solvent	cd3cn
4 Temperature	25.4
5 Number of Scans	128
6 Spectrometer Frequency	242.80
7 Spectral Width	147058.8
8 Nucleus	31P





Parameter	Value
1 Origin	Varian
2 Instrument	vnmrs
3 Solvent	cd3cn
4 Temperature	25.0
5 Number of Scans	1000
6 Spectrometer Frequency	125.65
7 Spectral Width	30487.8
8 Nucleus	13C





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