

## SUPPORTING INFORMATION

### Facile synthesis of aminomethyl phosphinate esters as serine protease inhibitors with primed site interaction

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## **Abbreviations**

ABPP – Activity-based protein profiling

ACN - Acetonitrile

CatG – Cathepsin g

Cbz – Carboxybenzyl protecting group

ChT - Chymotrypsin

DCM - Dichloromethane

DIC – N,N'-Diisopropylcarbodiimide

DMAP – 4-Dimethylaminopyridine

DMSO – Dimethyl sulfoxide

DR – Diastereomeric ratio

ESI – Electrospray ionization

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HNE – Human neutrophil elastase

HPLC – High performance liquid chromatography

LC – Liquid chromatography

MS – Mass spectrometry

NE – Neutrophil elastase

NET – Neutrophil extracellular trap

NMR – Nuclear magnetic resonance

Phg – Phenyl glycine

PPE – Porcine pancreas elastase

PR3 – Proteinase 3

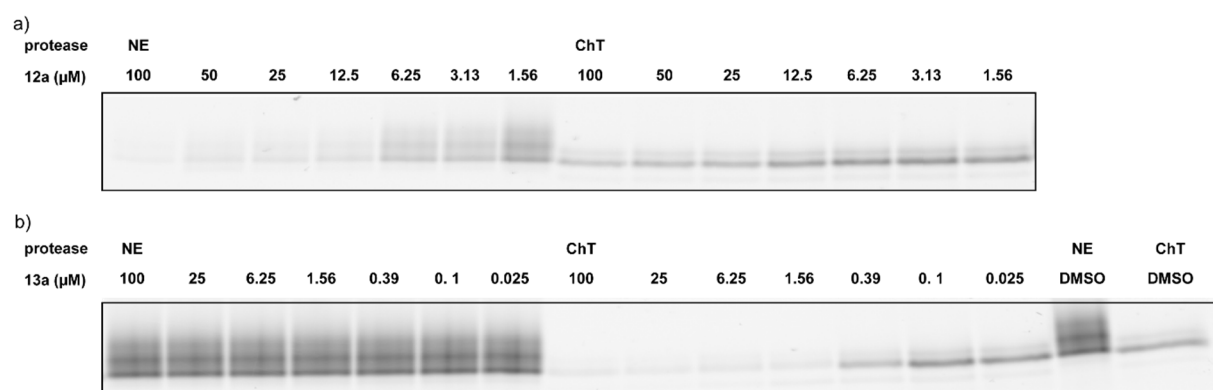
Rt – Retention time

SDS-PAGE – Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

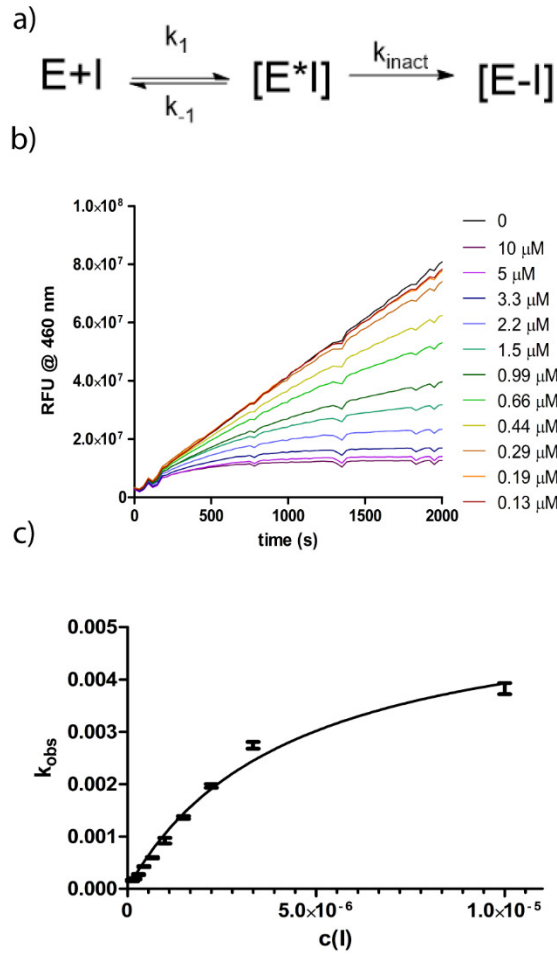
TLC – Thin layer chromatography

**Table S1: Energies of best-binding poses of phosphinates in *RR*- and *RS*-configuration, respectively**

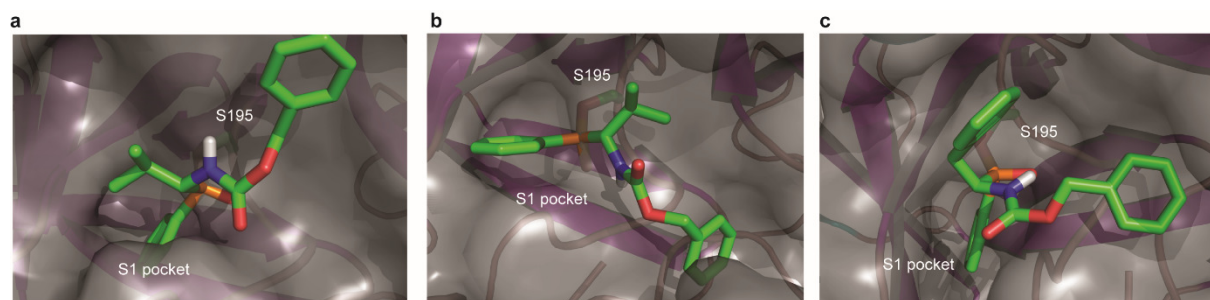
| <b>compound</b> | <b>enzyme</b> | <b>Energy of <i>RR</i> (kcal/mol)</b> | <b>Energy of <i>RS</i> (kcal/mol)</b> |
|-----------------|---------------|---------------------------------------|---------------------------------------|
| <b>12a</b>      | NE            | -7.40                                 | -8.37                                 |
| <b>12b</b>      | NE            | -7.49                                 | -7.56                                 |
| <b>12a</b>      | PR3           | -7.79                                 | -9.11                                 |
| <b>12b</b>      | PR3           | -7.24                                 | -8.17                                 |
| <b>10a</b>      | ChT           | -8.10                                 | -8.77                                 |
| <b>10b</b>      | ChT           | -7.18                                 | -7.31                                 |
| <b>11a</b>      | ChT           | -7.95                                 | -7.71                                 |
| <b>11b</b>      | ChT           | -6.92                                 | -7.47                                 |
| <b>13a</b>      | ChT           | -8.80                                 | -9.40                                 |



**Figure S1:** Inhibition of NE and ChT by different concentrations of **12a** (a) and **13a** as well as no inhibitor (b). Efficient inhibition of NE can still be seen with 12.5 μM of **12a** and good inhibition of ChT can still be seen with 1.56 μM of **13a**. Note that we originally performed an experiment titrating down **12a** or **13a** on a mixture of NE and ChT. Unfortunately, due to their similar molecular weight, these were not separable and overlapped on several types of gels. We therefore decided to perform the titration series separately.



**Figure S2: (a)** Two-step binding model of an irreversible covalent inhibitor. In the first step an enzyme-inhibitor-complex is formed by a reversible interaction. Subsequently, in an irreversible step the inhibitor reacts covalently with the enzyme. (b) formation of fluorescent product over time by ChT for different inhibitor concentrations. This is fitted to obtain  $k_{obs}$  values at different inhibitor concentrations (see 3.2 for formula). (c)  $k_{obs}/I$  plot can be fitted to retrieve  $k_{inact}$  and  $K_I^{app}$  values. After correcting for substrate competition using  $K_M$  the inhibition constant  $K_I$  is gained (see 3.2 for formula and  $K_M$  determination).



**Figure S3.** Pictures of unlikely binding poses of *RR*-configurations of phosphinates bound to the active site. (a) NE with compound **12a**; (b) PR3 with compound **12a**; (c) ChT with compound **13a**. Note that the group mimicking the side chain of the P1 amino acid (Val or Phe) does not interact with the S1 pocket and the Cbz group does not point towards the S2 site.

## 1. General

All starting materials and solvents were purchased from commercial vendors and used without further purification. Reactions were analyzed by Thin Layer Chromatography (TLC) on pre-coated 0.20 mm thick ALUGRAM® TLC sheets with fluorescent indicator and by liquid chromatography-mass spectrometry (LC-MS) performed on a Prominence Ultra-fast Liquid Chromatography system equipped with a 2x150 mm C18 analytical column (Waters X-Bridge) coupled to a MS-2020 single quadrupole mass analyzer (Shimadzu). A linear gradient of 5-90% acetonitrile in water (containing 0.1% formic acid) was used. Mass spectra shown are spectra of 10 mM stock solutions of diastereomeric mixtures used for biological assays. Diastereomeric ratios (DR) were calculated using peak areas of the 254 nm LC trace and the two peaks of the respective diastereomers were normalized to 100. DRs of the silica gel purified compounds and of the further HPLC-purified and DMSO dissolved compounds (stock solutions) are given and can be different from one another. Purity of compounds was assessed using the 215 nm HPLC trace and integrated using the i-PeakFinder algorithm (Shimadzu, Kyoto, Japan) with baseline type set to baseline length and threshold value set to 20. The injection peak and peaks determined to be baseline variations were removed manually and integrals for both diastereomers were summed up. Silica column chromatography was performed using 230-400 mesh silica (Kieselgel 60). High Pressure Liquid Chromatography (HPLC) purification was performed using a 10x150 mm C18 preparative column (X-Bridge). NMR spectra were recorded in deuterated solvents as indicated and measured using a Bruker UltraShield 300 MHz, 500MHz or 600MHz NMR Spectrometer. Chemical shifts are reported in ppm relative to the residual solvent peak and J-values are reported in Hertz. In cases with one major diastereomer the line list reports the major diastereomer. In cases of equal mixtures the peaks were integrated together and the integral normalized to whole integer values. Data analysis was done using MestReNova Version 14.1.2.25024 (Mestrelab Research, Spain). Infrared spectra were measured on a Vertex70 IR spectrometer from Bruker with a diamond ATR sampling accessory. All biological experiments (gel-based ABPP and kinetics) were done using the stock solutions in DMSO, containing a mixture of both diastereomers in different ratios.

## 2. Synthesis of compounds

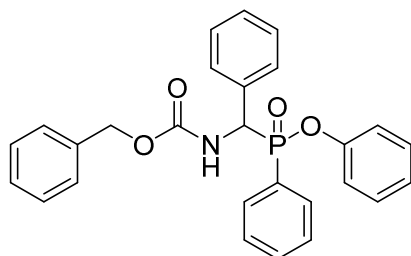
### 2.1 General procedure for the synthesis of phenyl phosphinates in two steps

Benzyl carbamate (1 eq.) was dissolved in dry ACN in an oven dried two-necked flask under argon atmosphere. The desired aldehyde (1.13 eq) and phosphine (1.3 eq.) were added and the reaction mixture is heated to reflux temperature. When full conversion of benzyl carbamate as determined by TLC was reached, the reaction solvent was evaporated, and the crude mixture dissolved in DCM. The crude product was extracted three times with saturated sodium

bicarbonate solution, the combined aqueous phases were acidified to pH 1 using concentrated hydrochloric acid and then extracted with DCM (3x). The combined organic phases were dried over magnesium sulfate and the solvent removed under vacuum, yielding the intermediate phosphinic acid which was used in the next step without further purification.

The phosphinic acid (1 eq.) was then dissolved in dry toluene in an oven dried two-necked flask under argon atmosphere. After addition of DIC (4 eq.), DMAP (4 mol%) and phenol (2 eq) the reaction mixture was heated to 80 °C and stirred for 4 hours. After full conversion of the starting material, the solvent was evaporated, and the crude product was dissolved in DCM. This mixture was washed with 1 M KHSO<sub>4</sub>, water, saturated sodium bicarbonate and brine. The organic phase was dried over magnesium sulfate and the solvent removed under vacuum. The crude product was purified by silica column chromatography.

### 2.2.1 phenyl (((benzyloxy)carbonyl)amino)(phenyl)methyl)(phenyl)phosphinate (10a)



#### *One step method*

Benzyl carbamate (76 mg, 0.5 mmol, 1.0 eq.) was dissolved in dry acetonitrile (5 mL) in an oven-dried two-necked flask under argon atmosphere. Benzaldehyde (62 mg, 0.6 mmol, 1.17 eq.) and dichlorophenylphosphine (116 mg, 0.65 mmol, 1.3 eq.) were added and the reaction mixture was heated to reflux for about 45 minutes. After the reaction mixture was cooled down to room temperature, phenol (94 mg, 1 mmol, 2 eq.) dissolved in dry acetonitrile (3 mL) was added dropwise and the solution was stirred for 15 minutes. Subsequently, triethylamine (0.29 mL, 2.0 mmol, 4 eq.) was added dropwise and the resulting mixture was stirred overnight at room temperature. The solvent was then removed under vacuum, the residue dissolved in DCM (25 mL) and the solution washed with saturated aqueous sodium bicarbonate solution (3x25 mL) and brine (3x25 mL). The organic phase was dried over magnesium sulfate, the solvent removed under vacuum and the crude product purified by silica column chromatography using a gradient of ethyl acetate in petroleum ether (5:95 to 60:40) yielding the product (30%). Diastereomeric ratio: 17:83

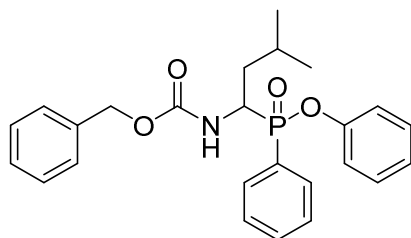
#### *Two step method*

The general procedure was followed using benzaldehyde (60 mg, 565 µmol) and dichlorophenylphosphine (116 mg, 90 µL, 648 µmol). The first step yielded the free acid



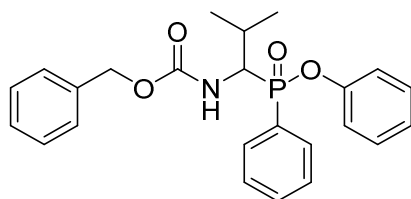
(88 mg, 0.23 mmol). The second step was performed using phenol (25 mg, 0.26 mmol, 1.2 eq), DIC (115 mg, 0.92 mmol, 4.2eq) and DMAP (1.5 mg, 0.015 mmol, 5 mol%). The title compound was obtained as a white powder (22 % over two steps). Diastereomeric ratio: 26:74  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.94 – 7.79 (m, 2H), 7.64 – 7.54 (m, 1H), 7.46 (dd,  $J$  = 7.5, 4.1 Hz, 4H), 7.39 – 7.27 (m, 6H), 7.13 (t,  $J$  = 7.7 Hz, 4H), 7.02 (d,  $J$  = 7.2 Hz, 1H), 6.86 (d,  $J$  = 8.0 Hz, 2H), 5.84 (s, 1H), 5.54 – 5.38 (m, 1H), 4.95 (q,  $J$  = 12.3 Hz, 2H).  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta$  150.4, 134.5, 133.2, 132.5, 132.4, 129.7, 129.5, 128.7, 128.6, 128.5, 128.4, 128.1, 127.9, 124.7, 120.3, 120.3, 67.3, 67.2, 55.2, 54.5, 29.7. FTIR ( $\text{cm}^{-1}$ ): 2928.70, 1695.5, 1459.4, 1202.69, 1118.32, 1022.99, 930.93, 750.51, 689.95. Molecular formula:  $\text{C}_{27}\text{H}_{24}\text{NO}_4\text{P}$ . ESI-MS  $[\text{M}+\text{H}]^+$ : calculated 458.15; found: 457.90. Retention factor  $R_f$  (1/1 EtOAc/PE)  $\cong$  0.46.  $R_t$  = 14.5 min. (diastereomer 1); 14.7 min. (diastereomer 2). Purity measured by LC-MS: 97.3 %.

### 2.3 phenyl (1-(((benzyloxy)carbonyl)amino)-3-methylbutyl)(phenyl)phosphinate (11a)



The general procedure was followed using isovaleraldehyde (50 mg, 581  $\mu\text{mol}$ ) and dichlorophenylphosphine (116 mg, 90  $\mu\text{L}$ , 648  $\mu\text{mol}$ ). The first step yielded the free acid (110 mg 0.3 mmol). The second step was performed using phenol (34 mg, 360  $\mu\text{mol}$ , 1.2 eq), DIC (146 mg, 1.26 mmol, 4.2 eq) and DMAP (1.5 mg, 15  $\mu\text{mol}$ , 5 mol%). The title compound was obtained as a white powder (28 % over two steps). Diastereomeric ratio: 7:93  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.90 – 7.78 (m, 2H), 7.62 – 7.36 (m, 3H), 7.31 (s, 3H), 7.19 (t,  $J$  = 7.9 Hz, 3H), 7.08 (q,  $J$  = 8.9, 7.9 Hz, 4H), 5.20 – 4.69 (m, 3H), 1.91 – 1.55 (m, 3H), 1.03 – 0.78 (m, 6H). Molecular formula:  $\text{C}_{25}\text{H}_{28}\text{NO}_4\text{P}$ . ESI-MS  $[\text{M}+\text{H}]^+$ : calculated: 438.18; found: 437.90. Retention factor  $R_f$  (1/1 EtOAc/PE)  $\cong$  0.45.  $R_t$  = 14.8 min. (diastereomer 1); 15.1 min. (diastereomer 2). Purity measured by LC-MS: >99.0 %.

### 2.4 phenyl (1-(((benzyloxy)carbonyl)amino)-2-methylpropyl)(phenyl)phosphinate (12a)



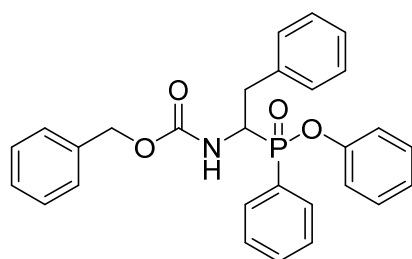
The general procedure was followed using isobutyraldehyde (41 mg, 568  $\mu\text{mol}$ ) and dichlorophenylphosphine (116 mg, 90  $\mu\text{L}$ , 648  $\mu\text{mol}$ ) but the reaction was carried out at 45  $^{\circ}\text{C}$ .

The first step yielded the free acid (82 mg, 0.24 mmol). The second step was performed using phenol (26 mg, 280  $\mu$ mol, 1.2 eq), DIC (116 mg, 1 mmol, 4.2 eq) and DMAP (1.5 mg, 15  $\mu$ mol, 5 mol%). The title compound was obtained as a white powder (14 % over two steps).

Diastereomeric ratio: 14:86

$^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  7.85 – 7.79 (m, 2H), 7.52 – 7.48 (m, 1H), 7.39 (td,  $J$  = 7.7, 3.6 Hz, 2H), 7.34 – 7.28 (m, 4H), 7.22 – 7.18 (m, 2H), 7.15 – 7.13 (m, 1H), 7.09 (dq,  $J$  = 7.9, 1.2 Hz, 2H), 7.07 – 7.03 (m, 1H), 5.19 (d,  $J$  = 11.0 Hz, 1H), 4.97 – 4.85 (m, 2H), 4.40 – 4.29 (m, 1H), 2.56 (hd,  $J$  = 6.8, 4.4 Hz, 1H), 1.17 – 1.12 (m, 6H).  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta$  156.1, 156.0, 150.7, 150.6, 136.2, 132.8, 132.8, 132.4, 132.3, 129.6, 128.5, 128.4, 128.0, 127.7, 124.6, 120.4, 120.4, 67.2, 66.9, 55.2, 54.4, 28.7, 28.2, 20.9, 20.8, 18.1, 18.1. FTIR ( $\text{cm}^{-1}$ ): 3257.32, 2928.70, 1710.10, 1534.98, 1484.92, 1215.07, 1195.11, 913.9, 689.55. Molecular formula:  $\text{C}_{24}\text{H}_{26}\text{NO}_4\text{P}$ . ESI-MS  $[\text{M}+\text{H}]^+$ : calculated: 424.16; found: 423.90. Retention factor  $R_f$  (1/1 EtOAc/PE)  $\cong$  0.47.  $R_t$  = 14.2 (diastereomer 1), 14.5 (diastereomer 2). Purity measured by LC-MS: 95.8 %.

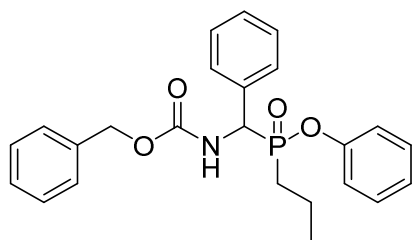
## 2.5 phenyl (1-(((benzyloxy)carbonyl)amino)-2-phenylethyl)(phenyl)phosphinate (13a)



The general procedure was followed using phenylacetaldehyde (70 mg, 883  $\mu$ mol) and dichlorophenylphosphine (116 mg, 90  $\mu$ L, 648  $\mu$ mol). The first step yielded the free acid (60 mg, 0.15 mmol). The second step was performed using phenol (20 mg, 0.5 mmol, 1.2 eq), DIC (73 mg, 0.63 mmol, 4.2 eq) and DMAP (1 mg, 7.5  $\mu$ mol, 5 mol%). The title compound was obtained as a white powder (8 % over two steps). Diastereomeric ratio: 39:61

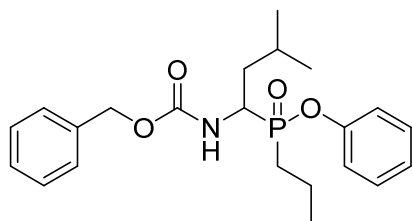
$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.91 – 7.82 (m, 2H), 7.57 – 7.47 (m, 1H), 7.40 (dp,  $J$  = 11.7, 3.9 Hz, 2H), 7.24 (dddd,  $J$  = 14.6, 12.0, 5.5, 3.2 Hz, 11H), 7.13 – 7.04 (m, 3H), 6.99 (dd,  $J$  = 6.7, 2.8 Hz, 1H), 5.52 – 5.28 (m, 1H), 4.94 (d,  $J$  = 1.4 Hz, 1H), 4.81 – 4.66 (m, 2H), 3.50 (dt,  $J$  = 14.4, 4.8 Hz, 1H), 3.21 – 2.88 (m, 1H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  133.2, 132.8, 132.6, 129.8, 129.4, 129.3, 128.6, 128.5, 128.0, 127.7, 126.9, 124.9, 120.6, 67.1, 66.8. Molecular formula:  $\text{C}_{28}\text{H}_{26}\text{NO}_4\text{P}$ . ESI-MS  $[\text{M}+\text{H}]^+$ : calculated: 472.16; found: 471.90. Retention factor  $R_f$  (1/1 EtOAc/PE)  $\cong$  0.54.  $R_t$  = 14.7 min. (diastereomer 1); 14.9 min. (diastereomer 2). Purity measured by LC-MS: >99.0 %.

## 2.6 phenyl (((benzyloxy)carbonyl)amino)(phenyl)methyl(propyl)phosphinate (10b)



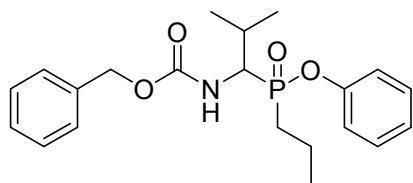
The general procedure was followed using benzaldehyde (62 mg, 584  $\mu$ mol) and dichloropropylphosphine (94 mg, 89  $\mu$ L, 648  $\mu$ mol). The first step yielded the free acid (100 mg, 300  $\mu$ mol). The second step was performed using phenol (132 mg, 340  $\mu$ mol, 1.2 eq.), DIC (158 mg, 1.26 mmol, 4.2 eq.) and DMAP (2 mg, 20  $\mu$ mol, 5 mol%). The title compound was obtained as a white powder (28 % over two steps). Diastereomeric ratio: 35:65.  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  7.43 (t,  $J$  = 8.5 Hz, 2H), 7.37 (t,  $J$  = 7.2 Hz, 2H), 7.35 – 7.26 (m, 6H), 7.25 – 7.22 (m, 1H), 7.16 – 7.08 (m, 3H), 7.01 (t,  $J$  = 7.4 Hz, 1H), 6.75 (d,  $J$  = 8.0 Hz, 1H), 6.14 – 5.88 (m, 1H), 5.31 – 5.18 (m, 1H), 5.18 – 4.92 (m, 2H), 1.77 – 1.64 (m, 3H), 0.99 (t,  $J$  = 7.4 Hz, 2H), 0.90 (td,  $J$  = 7.3, 1.5 Hz, 2H).  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta$ : 155.5, 151.0, 150.7, 135.9, 134.9, 134.4, 129.6, 129.4, 129.0, 128.7, 128.5, 128.5, 128.3, 128.2, 128.2, 128.1, 124.8, 124.5, 120.5, 120.5, 120.2, 120.1, 67.4, 67.3, 54.8, 54.6, 54.1, 53.9, 29.7, 29.1, 28.9, 28.3, 15.6, 15.4, 15.3, 15.1, 15.1. Molecular formula:  $\text{C}_{24}\text{H}_{26}\text{NO}_4\text{P}$ . ESI-MS  $[\text{M}+\text{H}]^+$ : calculated: 424.16; found: 423.90. Retention factor  $R_f$  (40/60 EtOAc/PE): 0.61.  $R_t$  = 14.4 min. (diastereomer 1); 14.6 min. (diastereomer 2). Purity measured by LC-MS: 91.9 %.

## 2.7 phenyl (1-(((benzyloxy)carbonyl)amino)-3-methylbutyl)(propyl)phosphinate (11b)



The general procedure was followed using isovaleraldehyde (51 mg, 581  $\mu$ mol) and dichloropropylphosphine (94 mg, 89  $\mu$ L, 648  $\mu$ mol). The first step yielded the free acid (180 mg, 0.55 mmol). The second step was performed using phenol (52 mg, 560  $\mu$ mol, 1.2 eq.), DIC (264 mg, 2.1 mmol, 4.2 eq.) and DMAP (3 mg, 25  $\mu$ mol, 5 mol%). The title compound was obtained as a white powder (27 % over two steps). Diastereomeric ratio: 28:72.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.41 – 7.27 (m, 7H), 7.15 (dd,  $J$  = 14.0, 7.4 Hz, 3H), 5.21 – 5.00 (m, 2H), 4.87 (d,  $J$  = 10.7 Hz, 1H), 4.29 (s, 1H), 1.88 – 1.70 (m, 4H), 1.58 (d,  $J$  = 1.9 Hz, 4H), 1.03 – 0.81 (m, 8H). FTIR ( $\text{cm}^{-1}$ ): 1712.72, 1549.2, 1491.3, 1252.55, 1205.23, 1025.93, 927.17, 758.74, 689.96. Molecular formula:  $\text{C}_{22}\text{H}_{30}\text{NO}_4\text{P}$ . ESI-MS  $[\text{M}+\text{H}]^+$ : calculated: 404.19; found: 403.95. Retention factor  $R_f$  (40/60 EtOAc/PE): 0.57.  $R_t$  = 14.6 min. (diastereomer 1); 15.0 min. (diastereomer 2). Purity measured by LC-MS: >99.0 %.

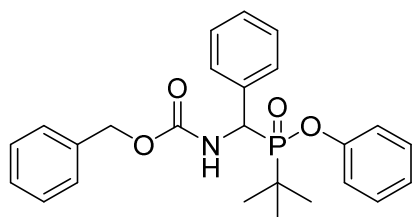
## 2.8 phenyl (1-(((benzyloxy)carbonyl)amino)-2-methylpropyl)(propyl)phosphinate (12b)



The general procedure was followed using isobutyraldehyde (41 mg, 569  $\mu$ mol) and dichloropropylphosphine (94 mg, 89  $\mu$ L, 648  $\mu$ mol). The first step yielded the free acid (120 mg 0.4 mmol). The second step was performed using phenol (42 mg, 480  $\mu$ mol, 1.2 eq.), DIC (201 mg, 1.6 mmol, 4.2 eq.) and DMAP (2.4 mg, 20  $\mu$ mol, 5 mol%). The title compound was obtained as a white powder (26 % over two steps). Diastereomeric ratio: 19:81

$^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  7.38 – 7.26 (m, 7H), 7.22 (dq,  $J$  = 7.7, 1.1 Hz, 1H), 7.19 – 7.11 (m, 2H), 5.34 (d,  $J$  = 10.7 Hz, 1H), 5.22 – 5.08 (m, 2H), 4.21 – 3.97 (m, 1H), 2.40 (dddd,  $J$  = 13.6, 6.9, 5.6, 2.6 Hz, 1H), 1.86 – 1.71 (m, 3H), 1.66 – 1.52 (m, 2H), 1.11 – 1.06 (m, 3H), 1.06 – 1.02 (m, 3H), 0.97 (td,  $J$  = 7.3, 1.6 Hz, 1H), 0.88 (td,  $J$  = 7.3, 1.6 Hz, 2H).  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta$  156.6, 156.5, 156.3, 151.1, 151.0, 150.8, 150.7, 136.2, 136.1, 129.8, 129.7, 128.6, 128.5, 128.3, 128.2, 128.1, 128.0, 124.7, 124.7, 120.5, 120.4, 120.3, 120.3, 67.6, 67.3, 67.3, 54.4, 54.3, 53.7, 53.6, 29.7, 29.1, 29.0, 28.4, 28.2, 28.1, 28.1, 20.9, 20.8, 20.7, 20.7, 17.7, 17.7, 17.5, 17.5, 15.6, 15.6, 15.6, 15.5, 15.5, 15.4. FTIR ( $\text{cm}^{-1}$ ): 2960, 1710.32, 1537.3, 1490.78, 1285.20, 1191.56, 1084.84, 1010.92, 921.81, 753.26, 692.9. Molecular formula:  $\text{C}_{21}\text{H}_{28}\text{NO}_4\text{P}$ . ESI-MS  $[\text{M}+\text{H}]^+$ : calculated: 390.18; found: 389.90. Retention factor  $R_f$  (40/60 EtOAc/PE): 0.56.  $R_t$  = 13.9 min. (diastereomer 1); 14.4 min. (diastereomer 2). Purity measured by LC-MS: >99.0 %.

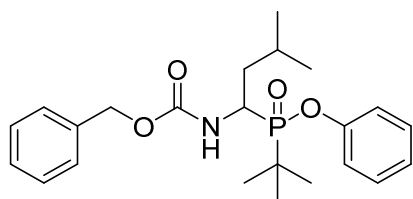
## 2.9 phenyl (((benzyloxy)carbonyl)amino)(phenyl)methyl)(*tert*-butyl)phosphinate (10c)



The general procedure was followed using benzaldehyde (62 mg, 584  $\mu$ mol) and *tert*-butyldichlorophosphine (103 mg, 648  $\mu$ mol). The first step yielded the free acid (90 mg, 250  $\mu$ mol). The second step was performed using phenol (30 mg, 310  $\mu$ mol, 1.2 eq.), DIC (141 mg, 1.12 mmol, 4.2 eq.) and DMAP (1.7 mg, 15  $\mu$ mol, 5 mol%). The title compound was obtained as a white powder (10 % over two steps). Diastereomeric ratio: 32:68

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.44 – 7.28 (m, 6H), 7.23 – 7.11 (m, 3H), 7.06 – 6.85 (m, 3H), 6.61 – 6.41 (m, 3H), 5.41 (t,  $J$  = 10.0 Hz, 1H), 5.22 – 4.95 (m, 3H), 1.33 (d,  $J$  = 16.0 Hz, 9H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  129.2, 128.9, 128.8, 128.3, 124.1, 120.0, 67.6, 24.9. FTIR ( $\text{cm}^{-1}$ ): 1712.96, 1529.26, 1489.21, 1239.26, 1200.51, 1025.39, 930.70, 808.47, 753.35, 686.92. Molecular formula:  $\text{C}_{25}\text{H}_{28}\text{NO}_4\text{P}$ . ESI-MS  $[\text{M}+\text{H}]^+$ : calculated: 438.18; found: 437.95. Retention factor  $R_f$  (60/40 EtOAc/PE): 0.47.  $R_t$  = 14.8 min. (diastereomer 1); 15.3 min. (diastereomer 2). Purity measured by LC-MS: 93.3 %.

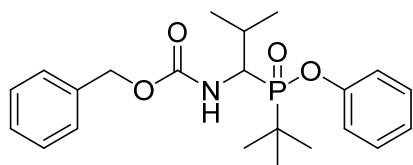
## 2.10 phenyl (1-(((benzyloxy)carbonyl)amino)-3-methylbutyl)(*tert*-butyl)phosphinate (11c)



The general procedure was followed using isovaleraldehyde (50 mg, 581  $\mu\text{mol}$ ) and *tert*-butyldichlorophosphine (103 mg, 648  $\mu\text{mol}$ ). The first step yielded the free acid (90 mg, 260  $\mu\text{mol}$ ). The second step was performed using phenol (26 mg, 280  $\mu\text{mol}$ , 1.2 eq.), DIC (138 mg, 1.2 mmol, 4.2 eq.) and DMAP (1.3 mg, 15  $\mu\text{mol}$ , 5 mol%). The title compound was obtained as a white powder (9 % over two steps). Diastereomeric ratio: 36:64

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.48 – 7.17 (m, 9H), 7.11 (t,  $J$  = 7.0 Hz, 1H), 5.30 (d,  $J$  = 10.4 Hz, 1H), 5.22 – 5.02 (m, 2H), 4.46 (dd,  $J$  = 10.8, 7.4 Hz, 1H), 1.68 (d,  $J$  = 13.3 Hz, 2H), 1.55 – 1.40 (m, 1H), 1.22 (d,  $J$  = 15.7 Hz, 9H), 0.91 (dd,  $J$  = 15.2, 6.5 Hz, 6H). Molecular formula:  $\text{C}_{23}\text{H}_{32}\text{NO}_4\text{P}$ . ESI-MS  $[\text{M}+\text{H}]^+$ : calculated: 418.21; found: 418.00. Retention factor  $R_f$  (60/40 EtOAc/PE): 0.53.  $R_t$  = 15.1 min. (diastereomer 1); 16.0 min. (diastereomer 2). Purity measured by LC-MS: 95.2 %.

## 2.11 phenyl (1-(((benzyloxy)carbonyl)amino)-2-methylpropyl)(*tert*-butyl)phosphinate (12c)



The general procedure was followed using benzyl carbamate (151 mg, 1 mmol) isobutyraldehyde (80 mg, 1.1 mmol) and *tert*-butyldichlorophosphine (206 mg, 648  $\mu\text{mol}$ ). The first step yielded the free acid (120 mg 366  $\mu\text{mol}$ ). The second step was performed using phenol (40 mg, 426  $\mu\text{mol}$ , 1.2 eq.), DIC (189 mg, 1.5 mmol, 4.2 eq.) and DMAP (2 mg,

16  $\mu\text{mol}$ , 5 mol%). The title compound was obtained as a white powder (4 % over two steps). Diastereomeric ratio: 100:0  
 $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  7.35 (d,  $J$  = 4.4 Hz, 4H), 7.33 – 7.27 (m, 5H), 7.14 – 7.09 (m, 1H), 5.47 (dd,  $J$  = 10.6, 3.3 Hz, 1H), 5.23 – 5.07 (m, 2H), 4.31 (ddd,  $J$  = 10.6, 8.9, 3.0 Hz, 1H), 2.32 (pdd,  $J$  = 6.8, 4.9, 2.9 Hz, 1H), 1.18 (d,  $J$  = 15.7 Hz, 9H), 1.02 – 0.91 (m, 6H).  $^{13}\text{C}$  NMR (151 MHz,  $\text{CDCl}_3$ )  $\delta$  156.3, 156.3, 152.7, 152.7, 136.3, 129.7, 128.6, 128.5, 128.1, 127.9, 124.1, 119.8, 119.7, 119.7, 67.2, 51.1, 50.5, 35.2, 34.6, 29.1, 24.7, 24.5, 20.7, 20.7, 17.2. FTIR ( $\text{cm}^{-1}$ ): 2960, 1707, 1543.49, 1487.88, 1241.56, 1207.52, 1161.00, 1003.27, 921.96, 770.49, 736.20. Molecular formula:  $\text{C}_{22}\text{H}_{30}\text{NO}_4\text{P}$ . ESI-MS  $[\text{M}+\text{H}]^+$ : calculated: 404.19; found: 403.95. Retention factor  $R_f$  (60/40 EtOAc/PE): 0.51.  $R_t$  = 15.4 min. (diastereomer 1). Purity measured by LC-MS: >99.0 %.

### 3. Gel-based competition experiments

#### 3.1 Inhibitor screening using FP-rhodamine

200 ng of the respective protease in 10  $\mu\text{L}$  of appropriate buffer (50 mM HEPES, 0.1 M NaCl, 0.1 % Triton-X pH 7.45 for NE and PR3, 0.1 M Tris/HCl, 0.5 M NaCl pH 7.5 for CatG, 0.1 M HEPES, 0.5 M NaCl pH 7.5 for Chymotrypsin and PBS containing 10 mM  $\text{Na}_2\text{HPO}_4$ , 1.3 mM  $\text{KH}_2\text{PO}_4$ , 137 mM NaCl and 2.68 mM KCl pH 7.5 for PPE) was diluted into 20  $\mu\text{L}$  of the inhibitors in the same buffer to reach a final inhibitor concentration of 100  $\mu\text{M}$  and incubated for 30 minutes at room temperature. For the control 1% of DMSO was used instead. After that FP-Rh was added to a final concentration of 1  $\mu\text{M}$  and incubated for another 30 minutes at room temperature. Subsequently 10  $\mu\text{L}$  of 4x Laemmli buffer was added to stop the reaction and after heating the mixture to 95  $^\circ\text{C}$  for 5 minutes it was resolved by 12 % SDS-PAGE. Gels were scanned on a Typhoon FLA 9500 and analyzed using Fiji.<sup>1</sup>

#### 3.2 Titration of 12a and 13a on ChT and NE

0.3  $\mu\text{L}$  of the respective inhibitor, 100x concentrated in DMSO, were added to 9.4  $\mu\text{L}$  of the required buffer (50 mM HEPES, 0.1 M NaCl, 0.1 % Triton-X pH 7.45 for NE and 0.1 M HEPES, 0.5 M NaCl pH 7.5 for Chymotrypsin). For control samples 0.3  $\mu\text{L}$  of DMSO were added. Subsequently, 200 ng of the respective protease in 20  $\mu\text{L}$  of buffer were added and the samples were incubated at room temperature for 30 minutes, after which 0.3  $\mu\text{L}$  FP-Rh were added to a final concentration of 1  $\mu\text{M}$  and the samples were incubated for another 30 minutes. Next, 10  $\mu\text{L}$  of 4x Laemmli buffer were added to stop the reaction and after heating the mixture to 95  $^\circ\text{C}$  for 5 minutes the samples were resolved by 12% SDS-PAGE. Gels were scanned on a Typhoon FLA 9500 and analyzed using Fiji.<sup>1</sup>

## 4. Kinetic experiments

### 4.1 $k_{obs}/I$ and $k_{inact}/K_I$ determination by kinetic experiments

Different inhibitors were diluted to 100x final concentration in DMSO and 1  $\mu$ L of these solutions put into the wells of a 96-well plate. Pure DMSO was used for the no-inhibitor control. The appropriate fluorogenic substrate (Elastase substrate V for NE, PPE and PR3, Chymotrypsin Substrate II for Chymotrypsin and CatG) 100x concentrated in DMSO was diluted to 4x concentration with the appropriate buffer (50 mM HEPES, 0.1 M NaCl, 0.1 % Triton-X pH 7.45 for NE and PR3, 0.1 M Tris/HCl, 10 mM  $\text{CaCl}_2$  pH 7.8 for Chymotrypsin and 0.2 M Tris pH 8 for PPE) and 25  $\mu$ L of this solution added to each well. After adding 24  $\mu$ L of buffer to each well the reaction was started by adding 50  $\mu$ L of 2x concentrated protease in the appropriate buffer and the generated fluorescence ( $\lambda_{ex}$  380 nm,  $\lambda_{em}$  460 nm) was measured over time at 30 °C for NE, PPE and ChT and at 37 °C for PR3 on a SpectraMax iD3 Multi Mode Microplate Reader (Molecular Devices). Final concentrations were 5 nM for NE, 86 nM for PR3, 50 nM for PPE and 1 nM for ChT. Final concentrations of inhibitors were varied for a dilution series. Final Volume was 100  $\mu$ L and final DMSO concentration was 2 % for each measurement. Every measurement was done in triplicates and the averaged background (appropriate substrate in buffer with a final concentration of 2 % DMSO) of three measurements was subtracted from each of the triplicates. For quantification data were only used from the linear part of the no inhibitor control and fitted to the equation

$RFU = \frac{v_i}{k_{obs}} [1 - e^{-k_{obs}t}]$  to calculate the  $k_{obs}$  values. The derived  $k_{obs}$  values were then plotted against their respective inhibitor concentrations and the curves fitted to the equation

$k_{obs} = \frac{k_{inact}}{K_I^{app} + [I]}$  to derive  $K_I^{app}$  and  $k_{inact}$  values. The obtained  $K_I^{app}$  values were then corrected for

substrate competition according to  $K_I^{app} = K_I * (1 + \frac{[S]}{K_M})$  using  $K_M$  values obtained in 3.2.

Fitting was done using GraphPad Prism 5.

### 4.2 Determination of substrate $K_M$

Dilution series of different substrates (fluorogenic elastase substrate V (MeOSuc-AAPV-AMC) for NE, PPE and PR3, fluorogenic chymotrypsin substrate 2 (Suc-AAPF-AMC) for ChT) were made to 100x the desired final concentration in DMSO and 1  $\mu$ L of these dilutions was put into the wells of a 96-well plate. A volume of 49  $\mu$ L of the appropriate buffers (50 mM HEPES, 0.1 M NaCl, 0.1 % Triton-X pH 7.5 for NE and PR3, 0.1 M Tris/HCl, 10 mM  $\text{CaCl}_2$  pH 7.8 for Chymotrypsin and 0.2 M Tris pH 8 for PPE) containing DMSO to reach a final DMSO concentration of 2 % was added to the wells. The reaction was started by adding 50  $\mu$ L of 2x concentrated protease in the appropriate buffer and the generated fluorescence ( $\lambda_{ex}$  380 nm,  $\lambda_{em}$  460 nm) over time was measured at 30 °C for NE, PPE and ChT and at 37 °C for PR3 on

a SpectraMax iD3 Multi Mode Microplate Reader (Molecular Devices). Final concentrations were 5 nM for NE, 86 nM for PR3, 50 nM for PPE and 1 nM for ChT. Final concentrations of substrate were varied for a dilution series. Final volume was 100  $\mu$ L and final DMSO concentration was 2 % for each measurement. Every measurement was done in triplicates and the averaged background (appropriate substrate at same concentration as in sample in buffer with a final concentration of 2 % DMSO) of three measurements was subtracted from each of the triplicates.

The initial reaction velocity was determined by calculating the slope of a linear fit to the progress curve during the first 15 minutes. The obtained initial reaction rates were then plotted against their respective substrate concentrations and the curves fitted to the formula

$v_i = \frac{V_{max}[S]}{K_M + [S]}$  to obtain  $K_M$ -values for the individual substrates and enzymes. Fitting was done using GraphPad Prism 5, and results are listed below

**$K_M$  values of commercial substrates used, measured under assay conditions.**

| Enzyme | Substrate                 | $K_M$ (mol/L)                                 |
|--------|---------------------------|---|
| NE     | Elastase Substrate V      | $6.24 \times 10^{-4} \pm 6.33 \times 10^{-5}$ |
| PPE    | Elastase Substrate V      | $2.10 \times 10^{-3} \pm 2.71 \times 10^{-4}$ |
| PR3    | Elastase Substrate V      | $4.53 \times 10^{-4} \pm 7.68 \times 10^{-5}$ |
| ChT    | Chymotrypsin Substrate II | $9.5 \times 10^{-5} \pm 6.32 \times 10^{-6}$  |

## 5. Computational experiments

### 5.1 Covalent docking NE

**12a**, **12b** and Val<sup>P</sup>(OPh)<sub>2</sub> (**1a**) were attached to the side chain of residue SER195 of chain E of NE (PDB: 1fuj) and docked according to the flexible side chain method.<sup>2</sup> The inhibitor was drawn in Chemdraw professional 15.1, converted to a pdb file using Chem3D 15.1 and converted into a PDBQT by Autodocktools 1.5.6. The connected complex was treated as a fully flexible side chain. The binding site was covered by preparing a 40x40x40 size grid box with grid spacing of 0.375 Å and the center at -46.069, 97.263, 0.130.

### 5.2 Covalent docking Pr3

**12a**, **12b** and Val<sup>P</sup>(OPh)<sub>2</sub> (**1a**) were attached to the side chain of residue SER195 of chain A of PR3 (PDB: 4wvp) and docked according to the flexible side chain method.<sup>2</sup> The inhibitor was drawn in Chemdraw professional 15.1, converted to a pdb file using Chem3D 15.1 and converted into a PDBQT by Autodocktools 1.5.6. The connected complex was treated as a



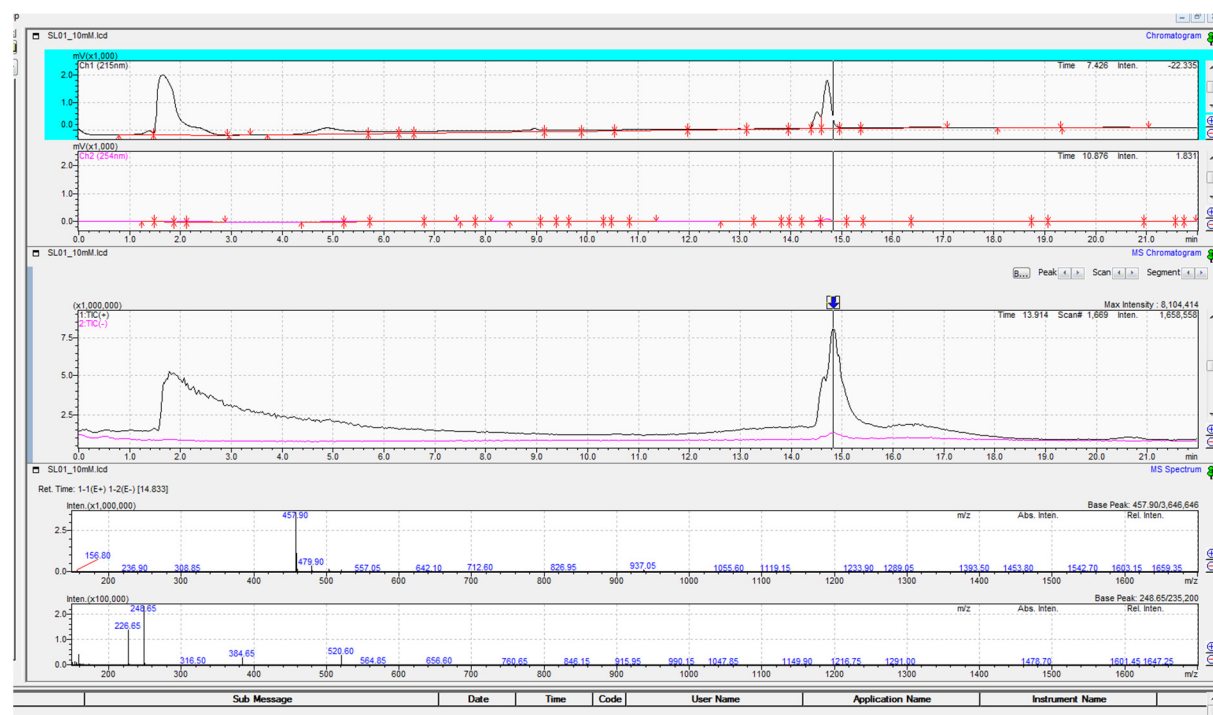
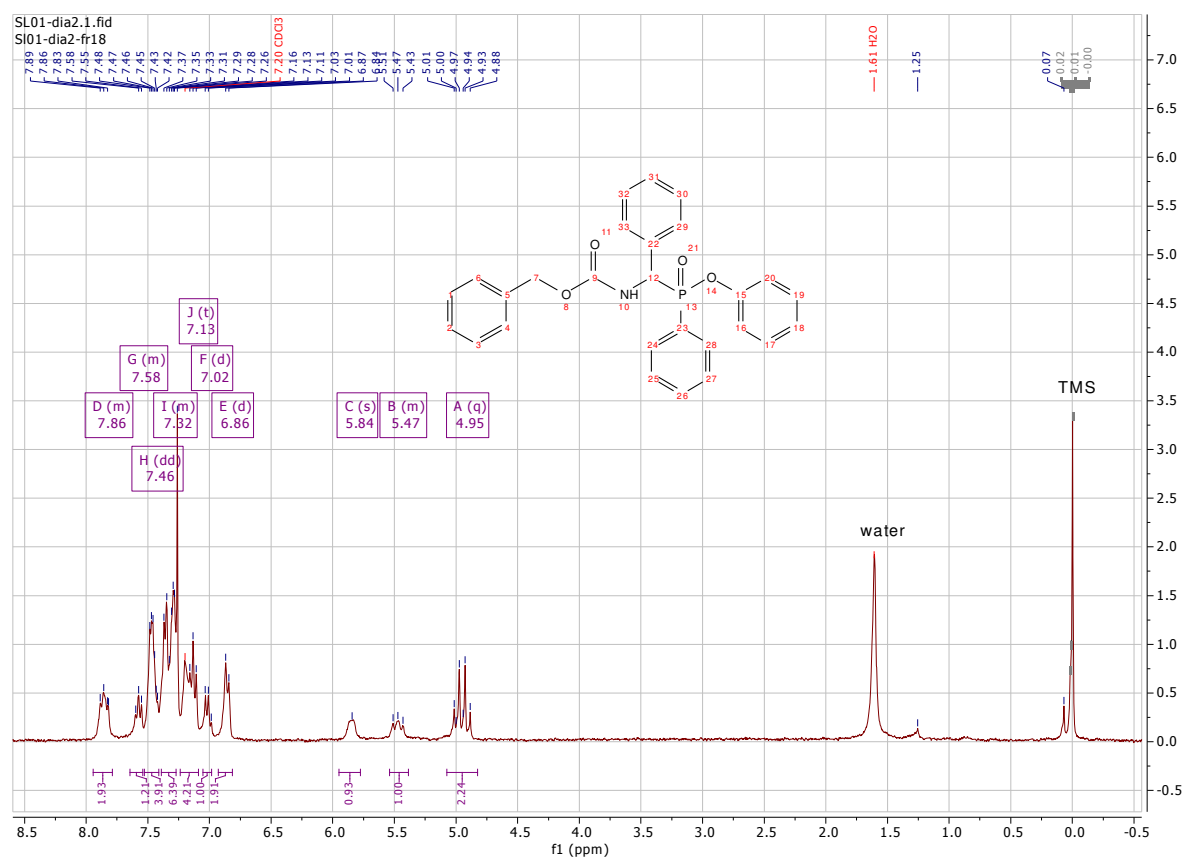
fully flexible side chain. The binding site was covered by preparing a 60x60x60 size grid box with grid spacing of 0.375 Å and the center at 25.408, 6.086, 33.533.

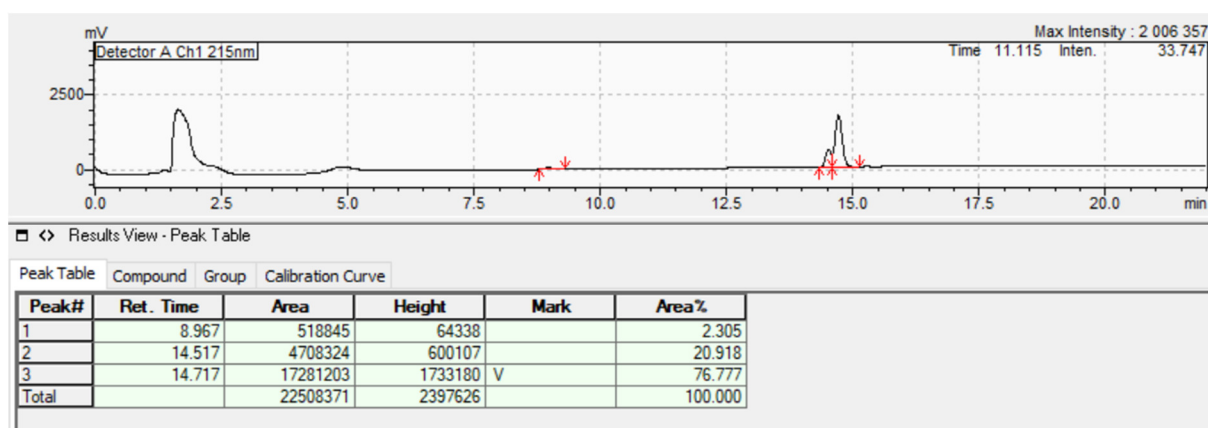
### 5.3 Covalent docking ChT

**10a, 11a, 13a, 10b, 11b** and  $\text{Phe}^{\text{P}}(\text{OPh})_2$  (**1b**) were attached to the side chain of residue SER195 of chain A of ChT (PDB: 4q2k) and docked according to the flexible side chain method.<sup>2</sup> The probes input file was drawn in Chemdraw professional 15.1, converted to a pdb file using Chem3D 15.1 and converted into a PDBQT by Autodocktools 1.5.6. The connected complex was treated as a fully flexible side chain. The binding site was covered by preparing a 60x60x60 size grid box with grid spacing of 0.375 Å and the center at 56.9627, -1.1535, 31.7927.

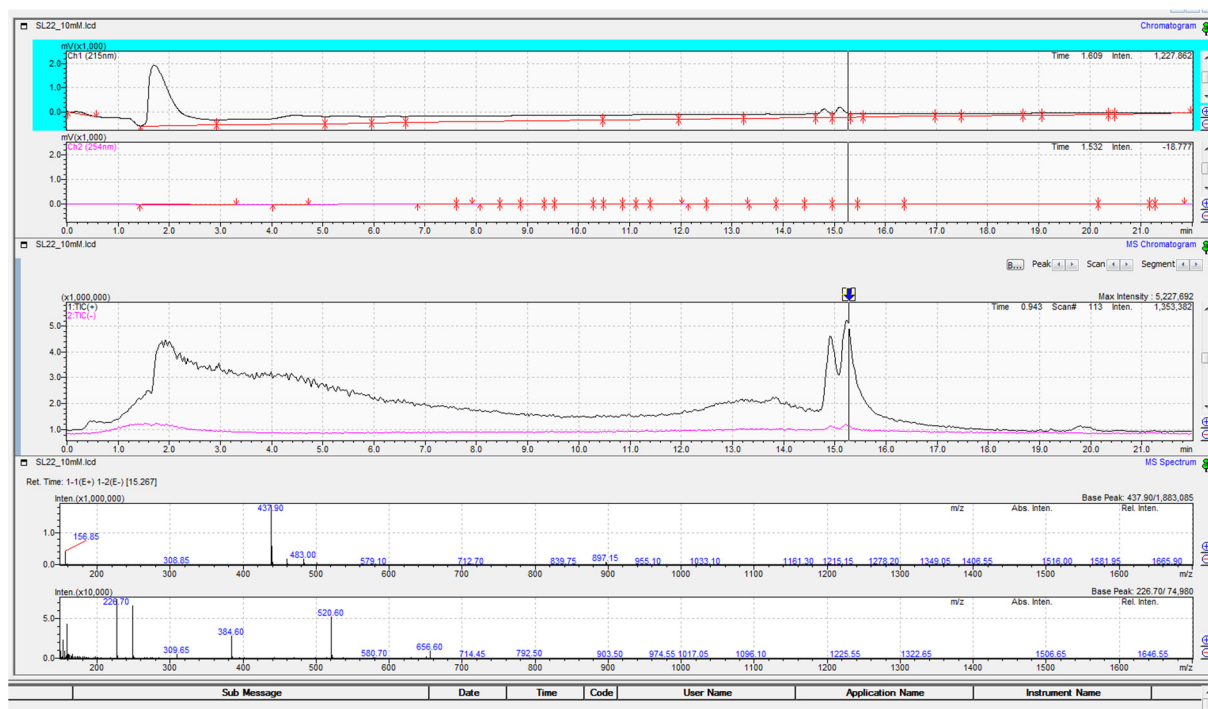
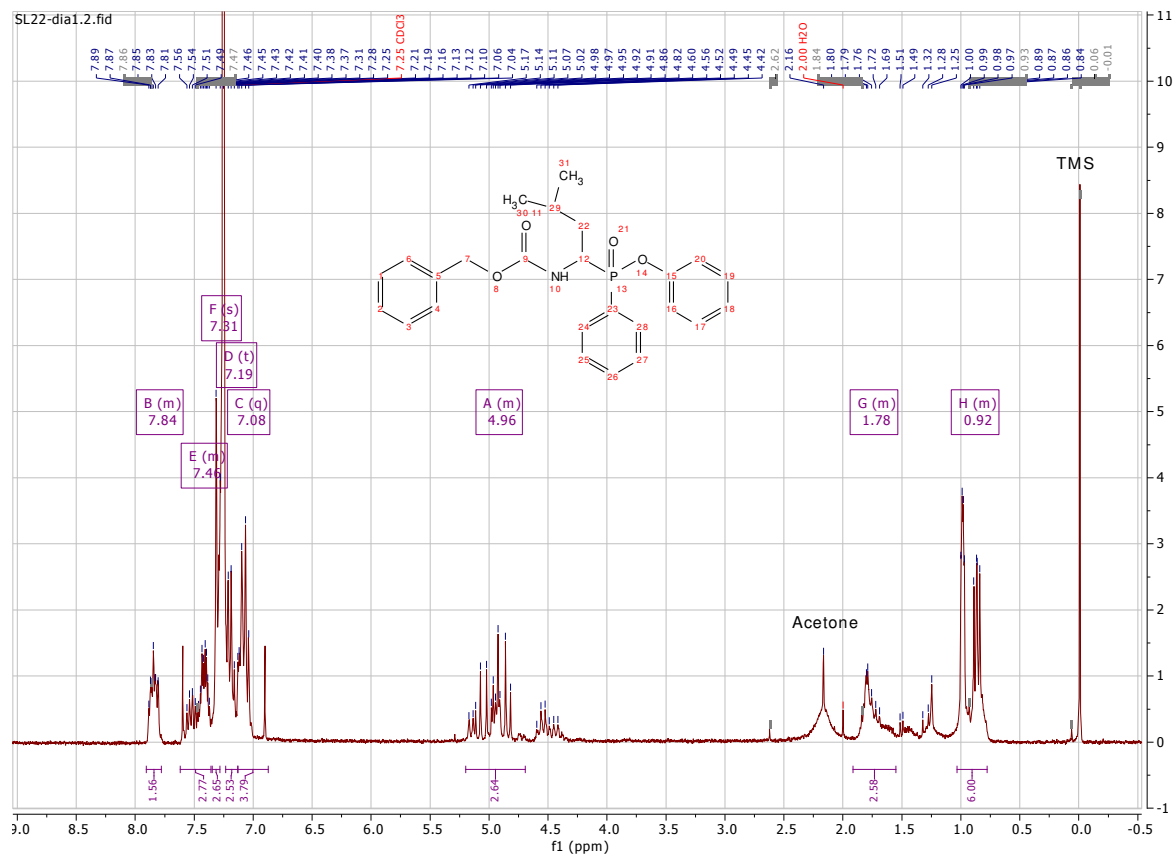
## 6. NMR spectra and LC-MS data

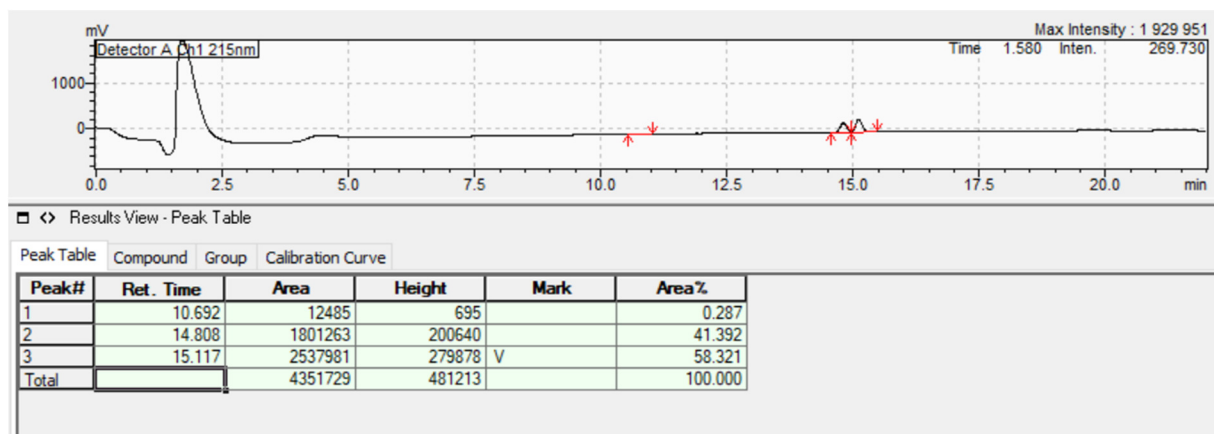
### 6.1 10a



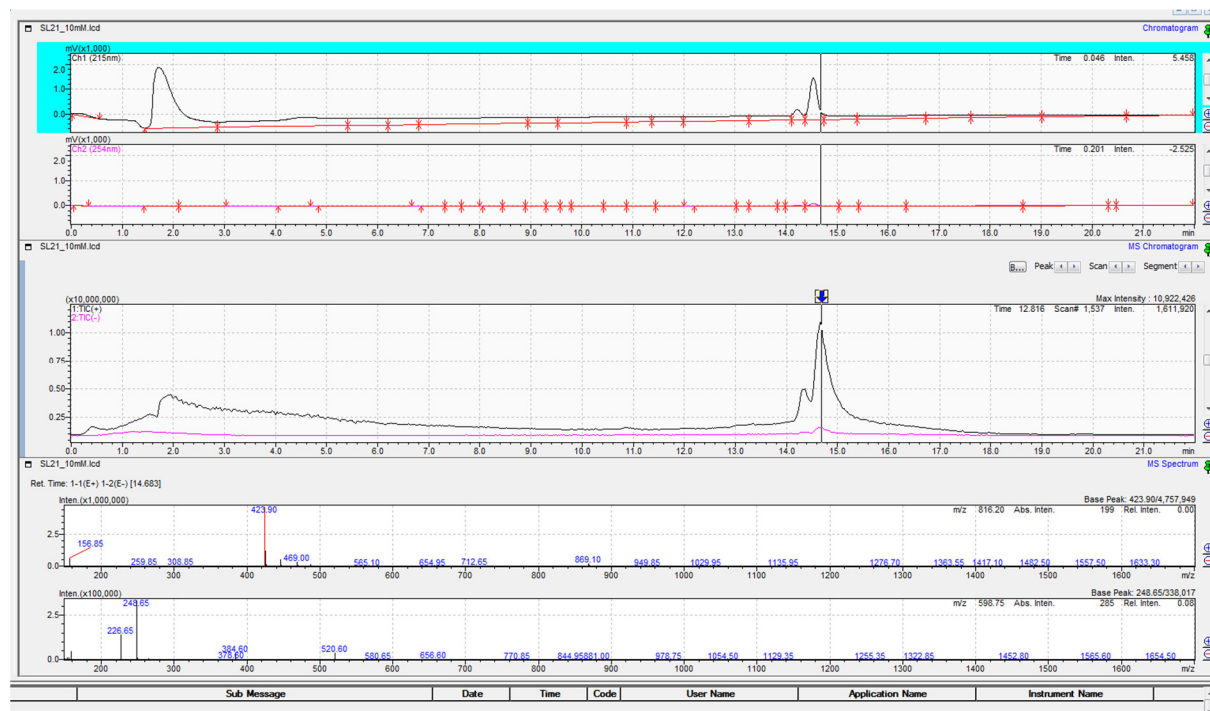
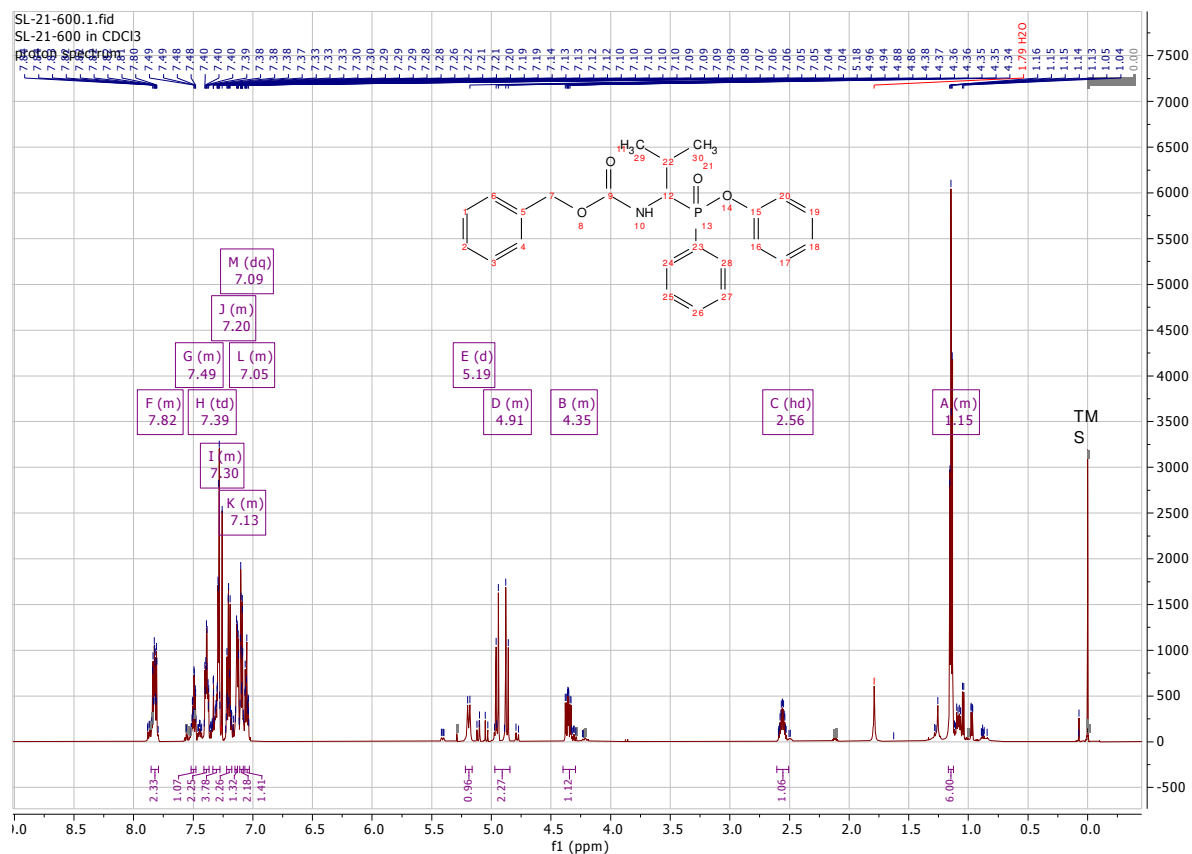


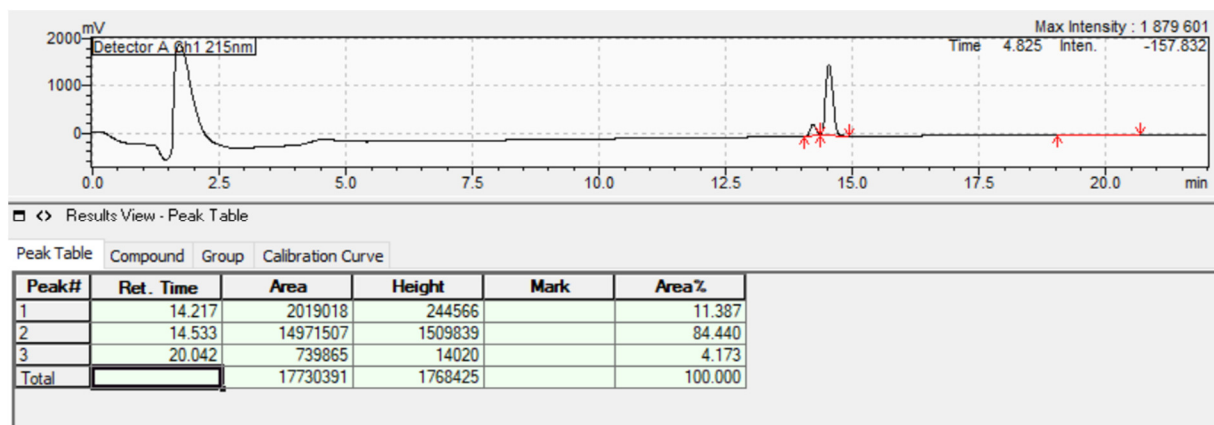
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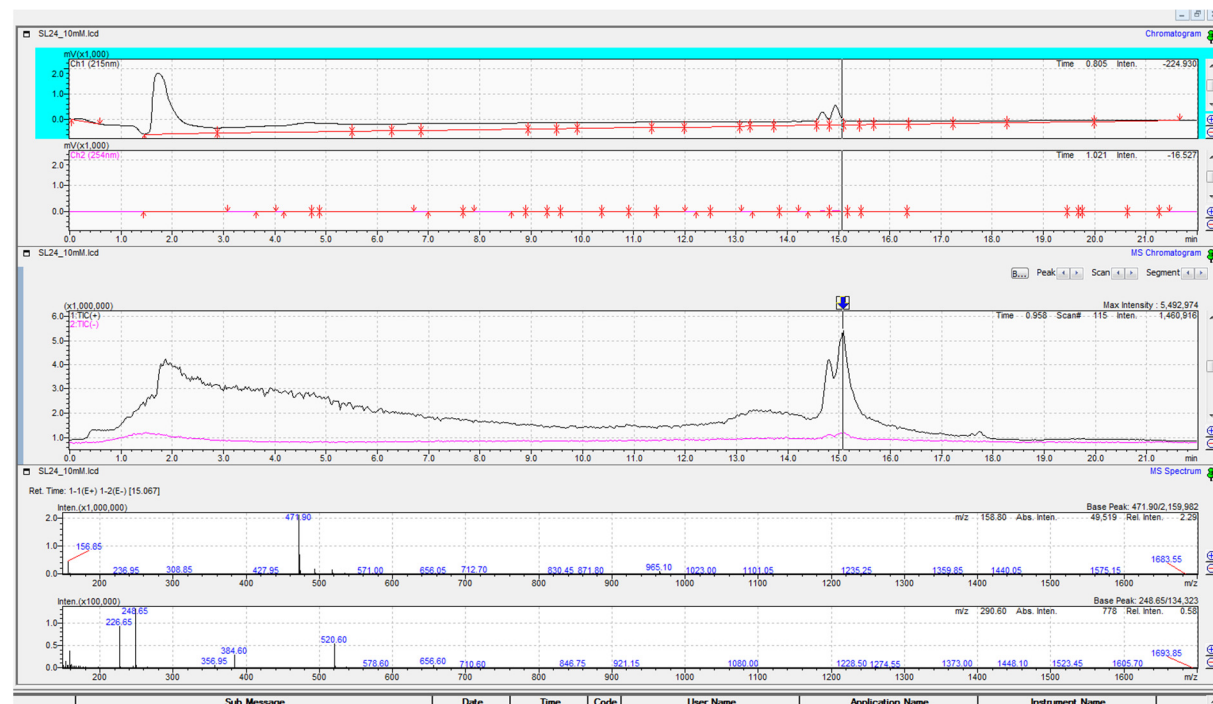
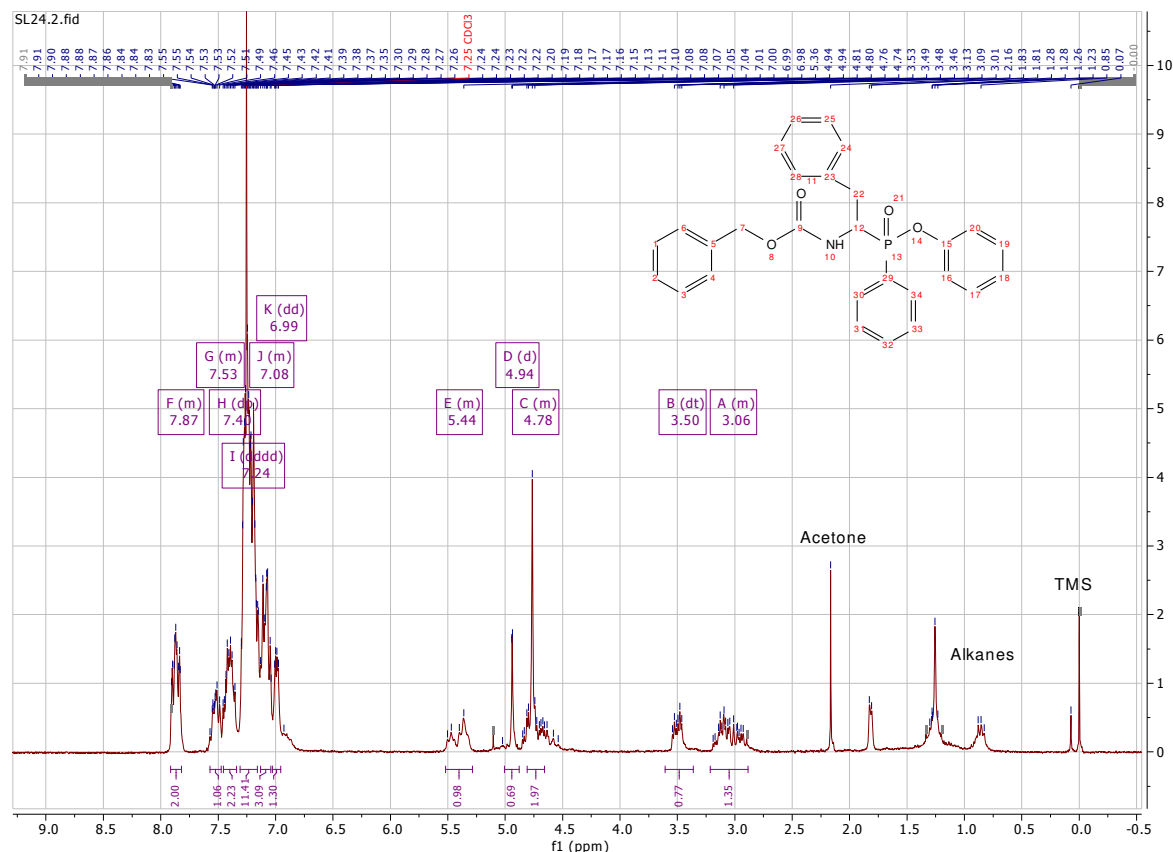


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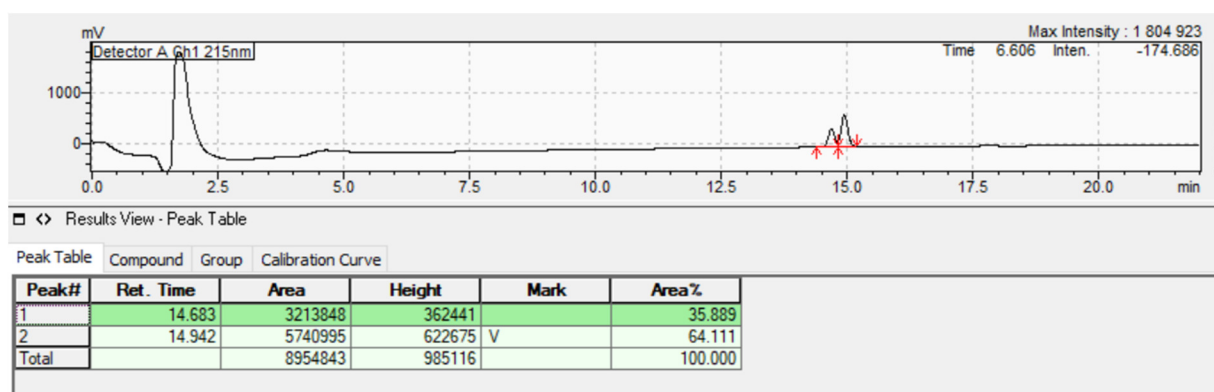




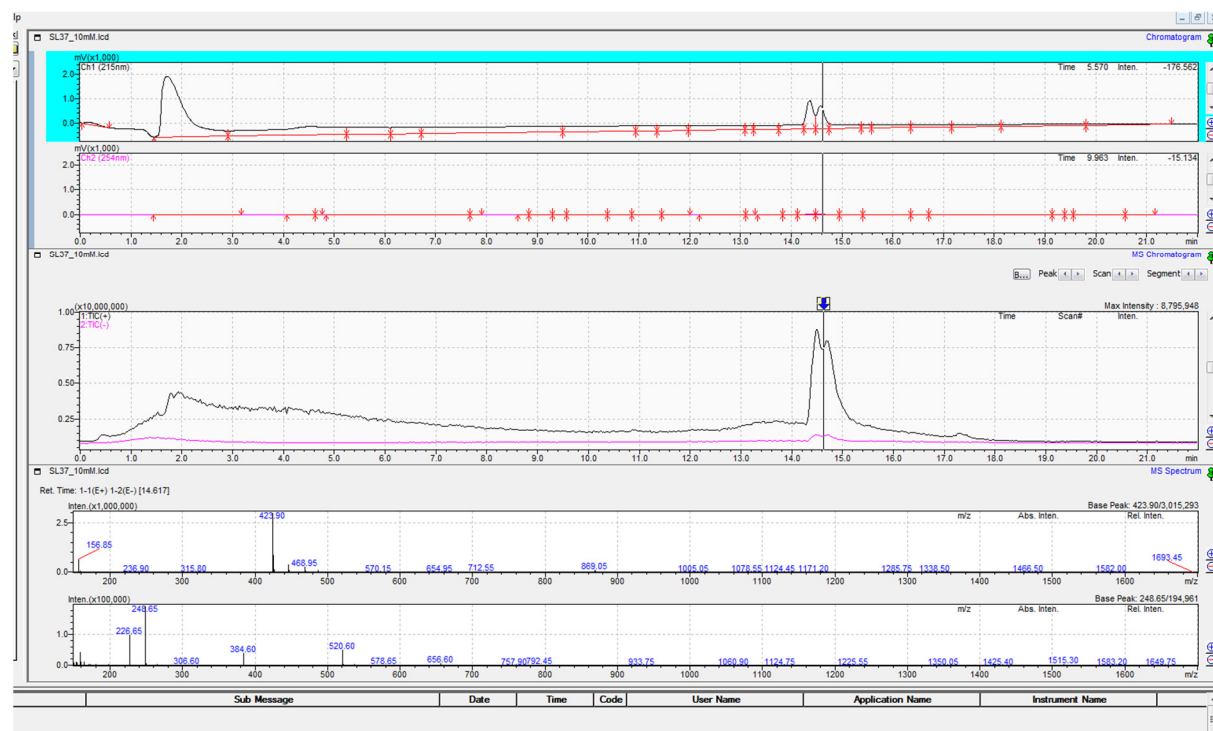
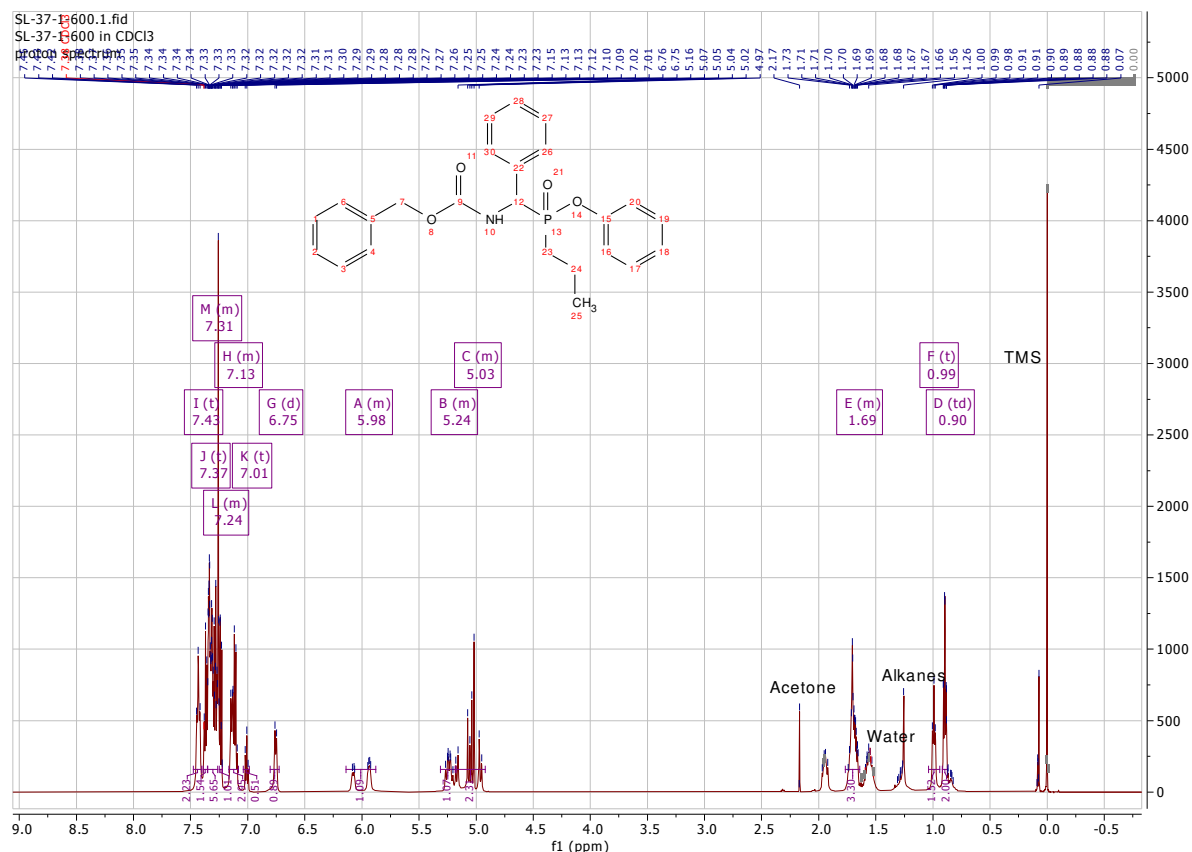
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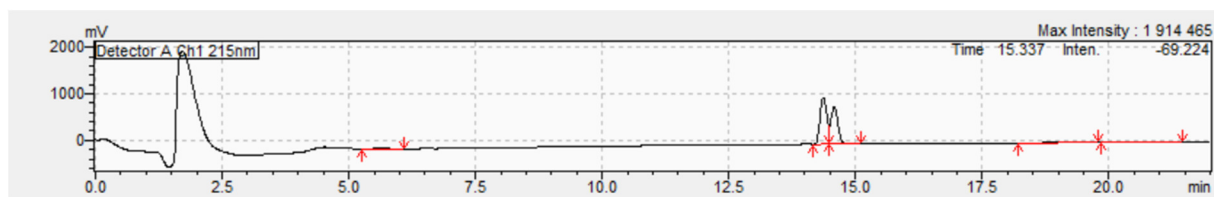






# 6.5 10b



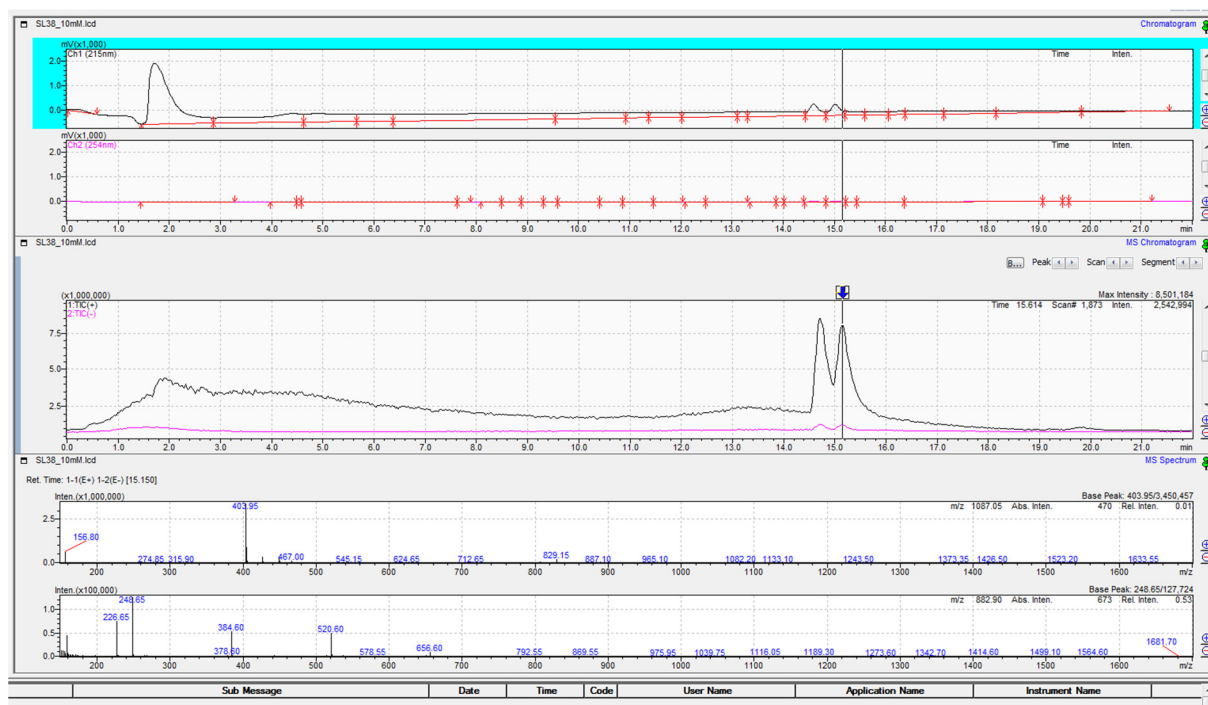
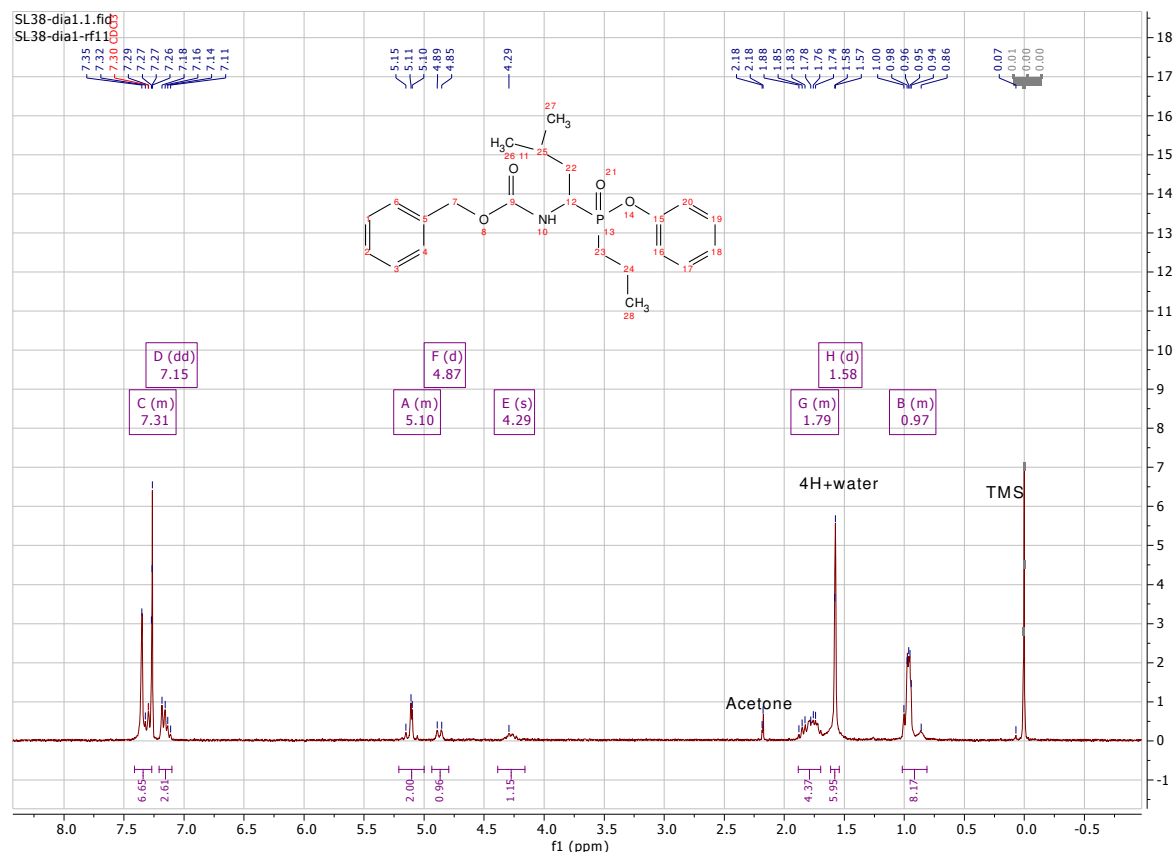


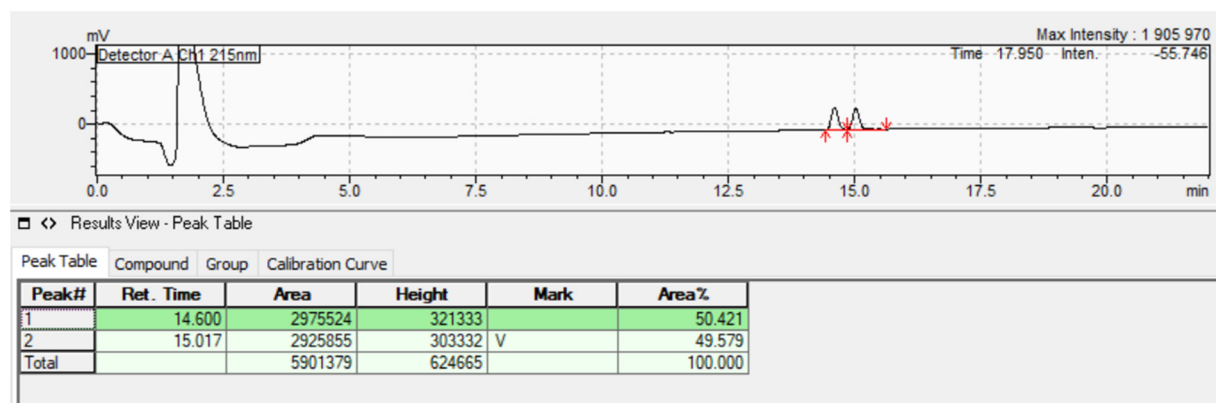
Results View - Peak Table

Peak Table Compound Group Calibration Curve

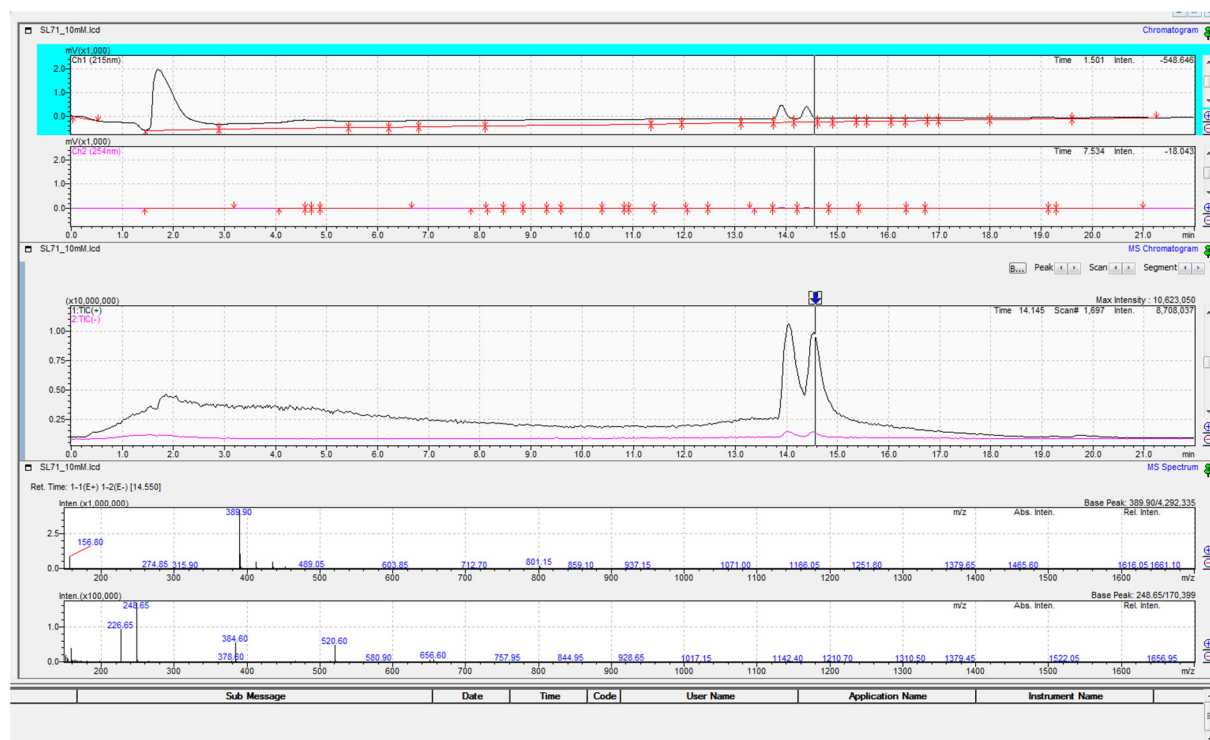
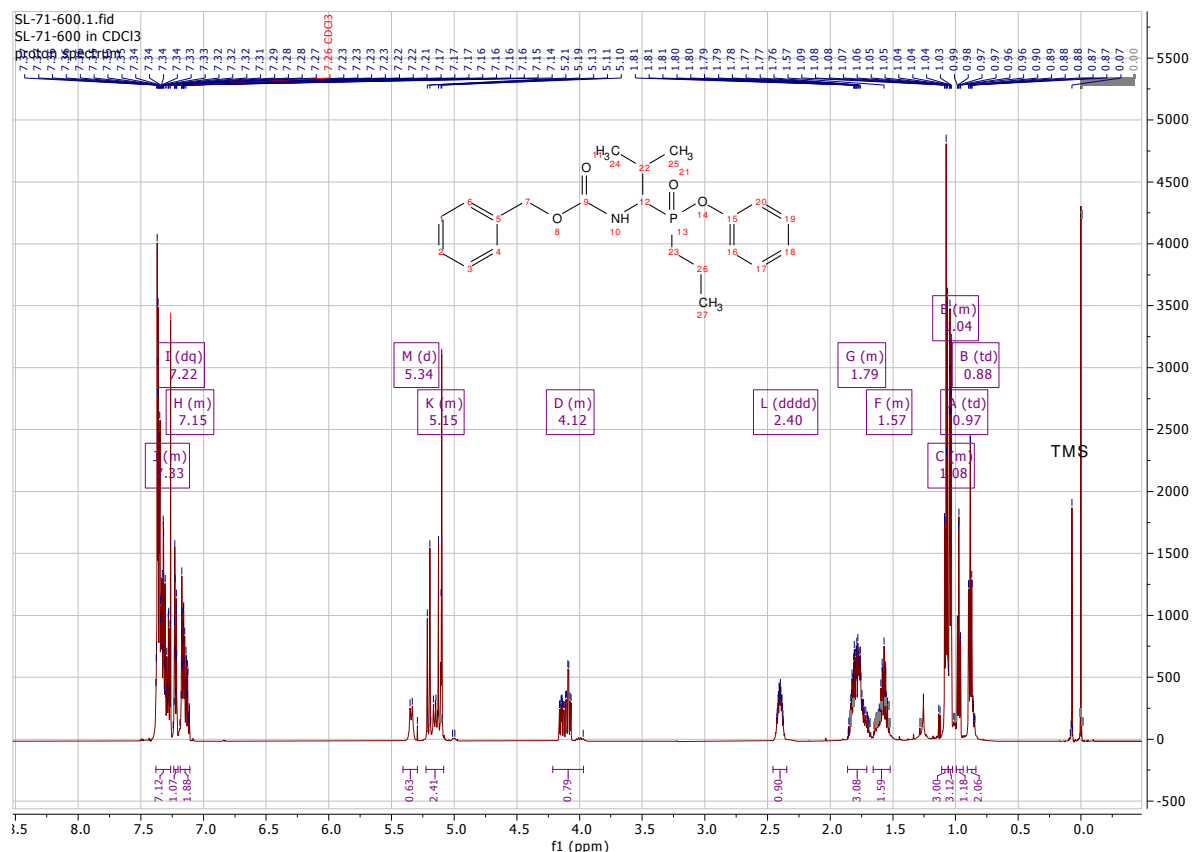
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|-------|-----------|----------|---------|------|---------|
| 1     | 5.633     | 227955   | 8707    |      | 1.234   |
| 2     | 14.367    | 9336579  | 1003071 |      | 50.549  |
| 3     | 14.583    | 7635680  | 795458  | V    | 41.340  |
| 4     | 19.158    | 581804   | 11410   |      | 3.150   |
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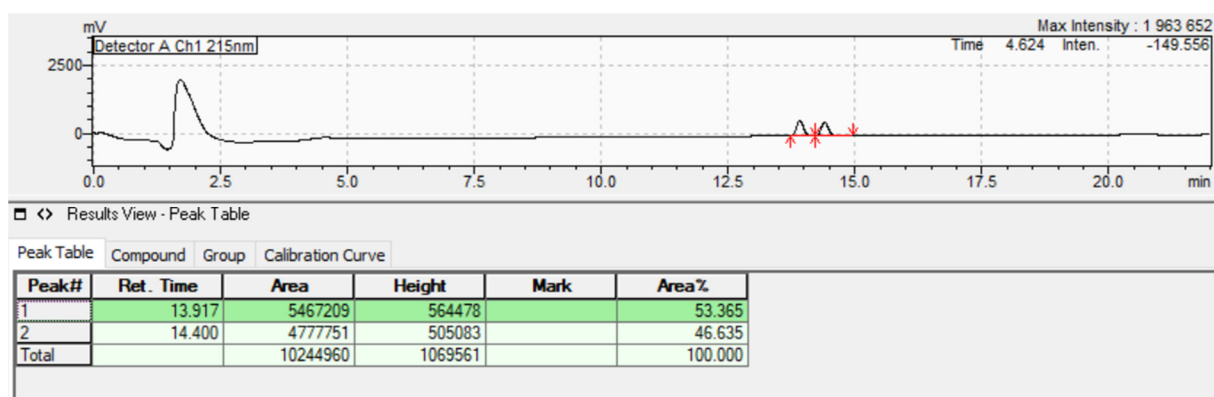
# 6.6 11b



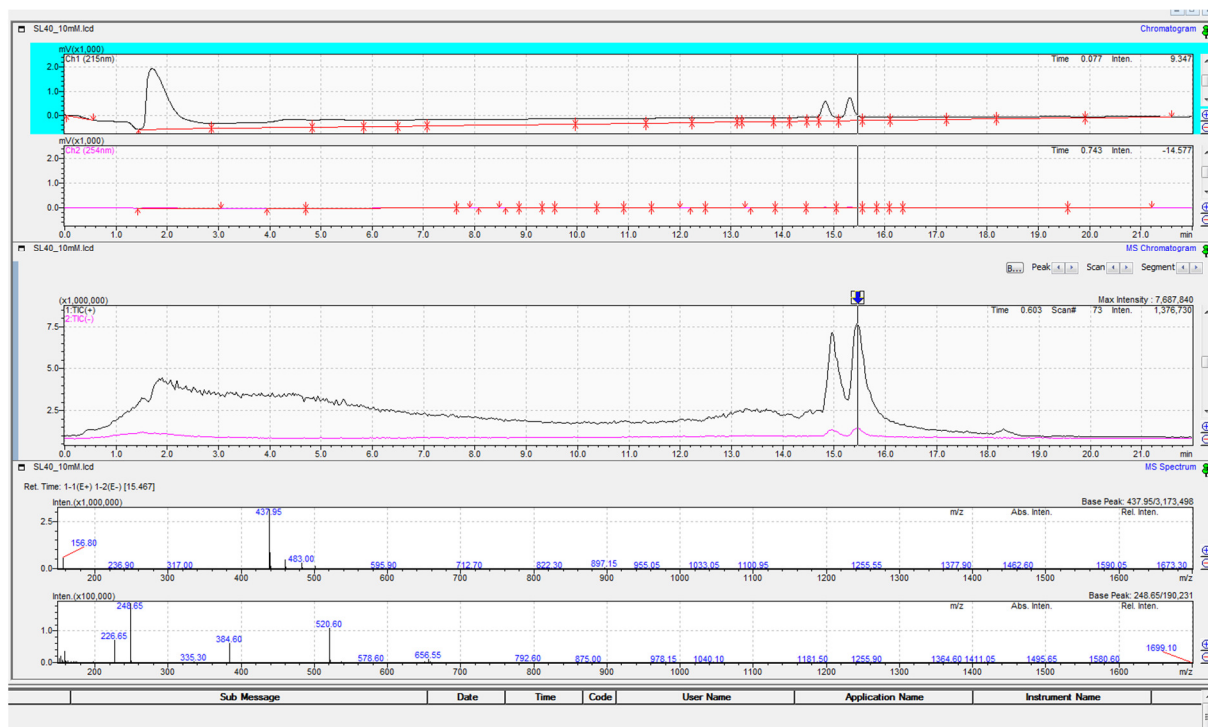
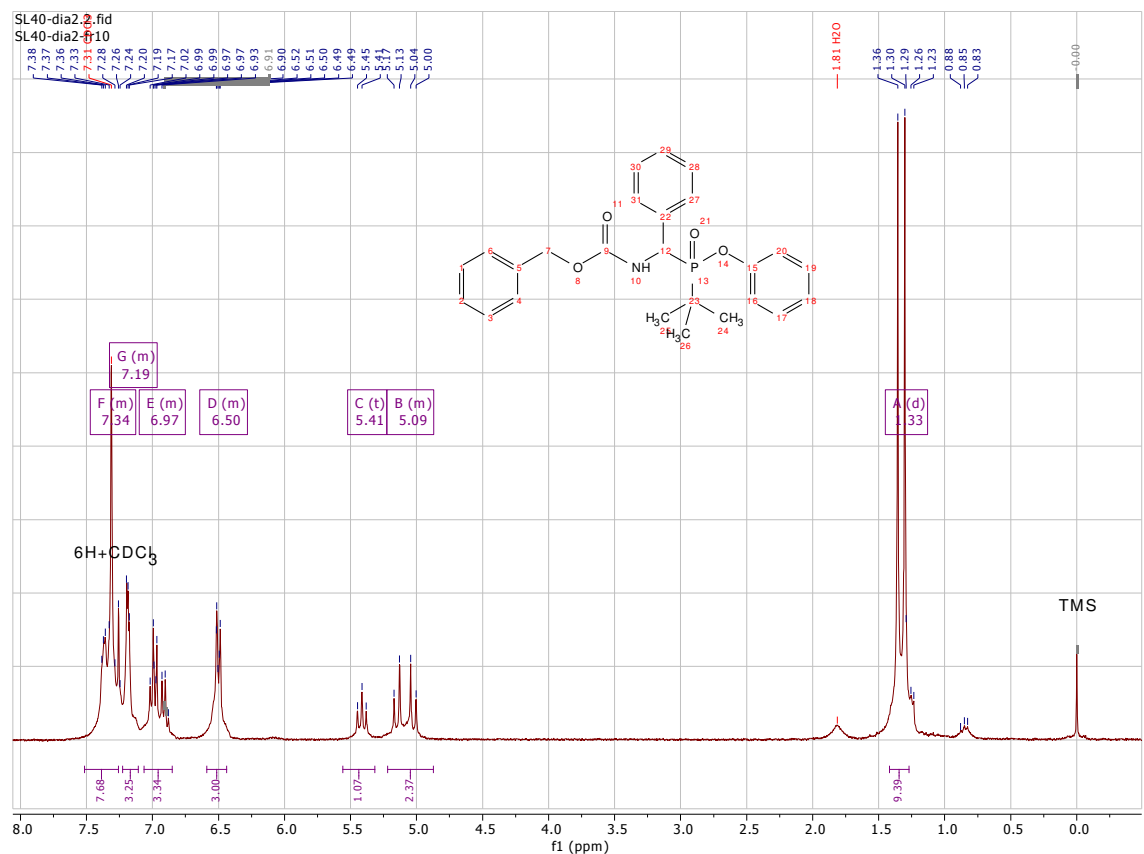


## 6.7 12b

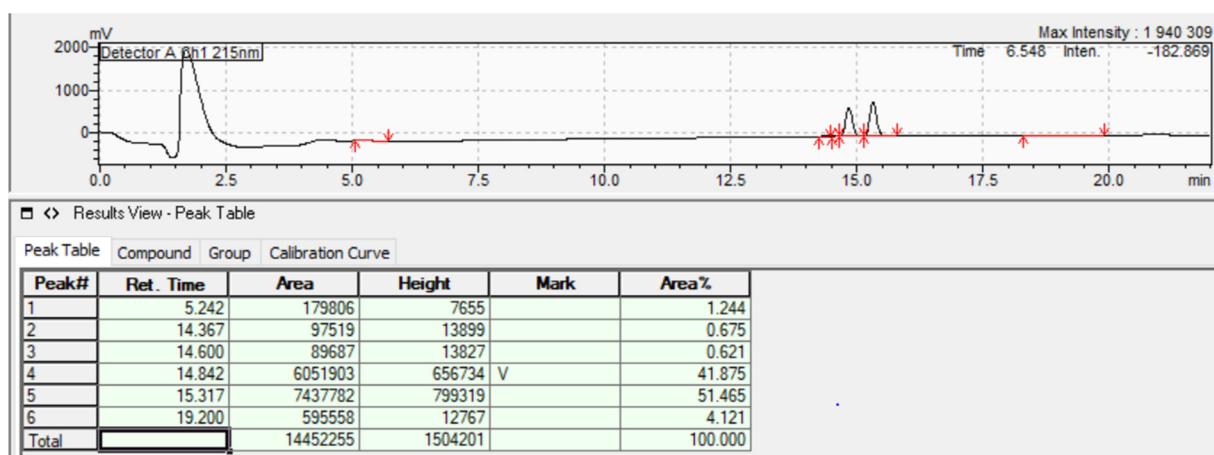




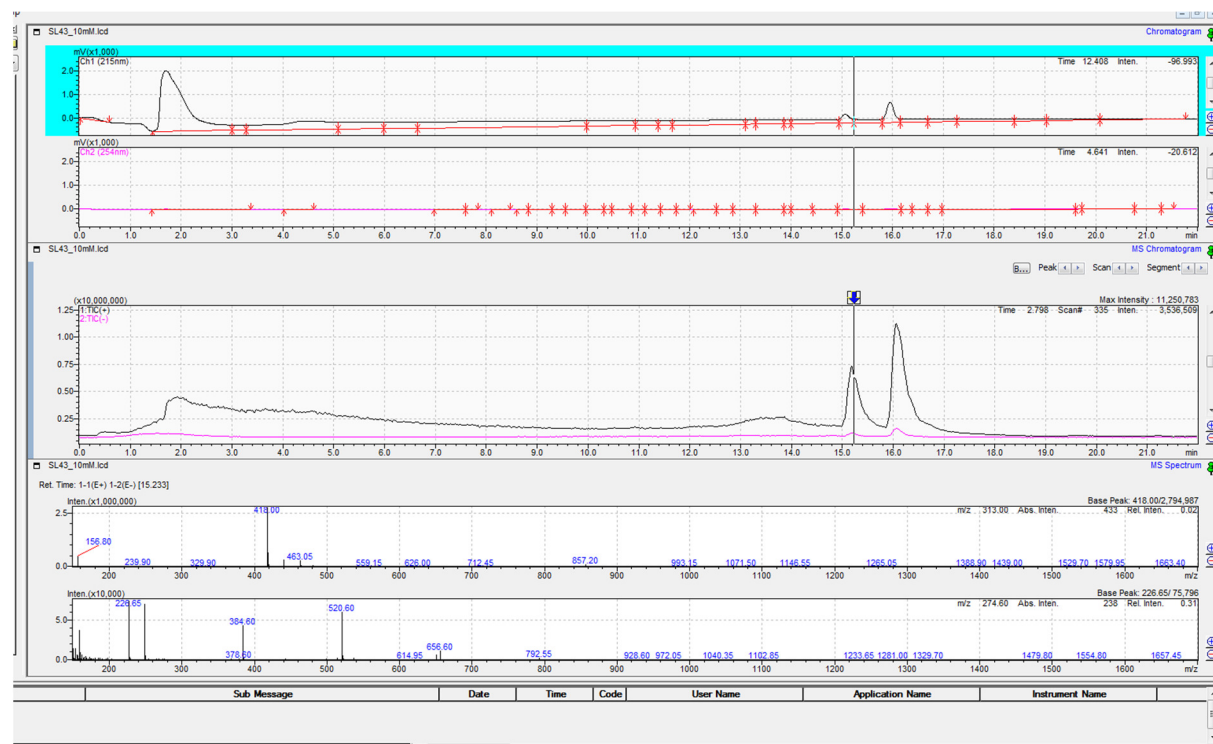
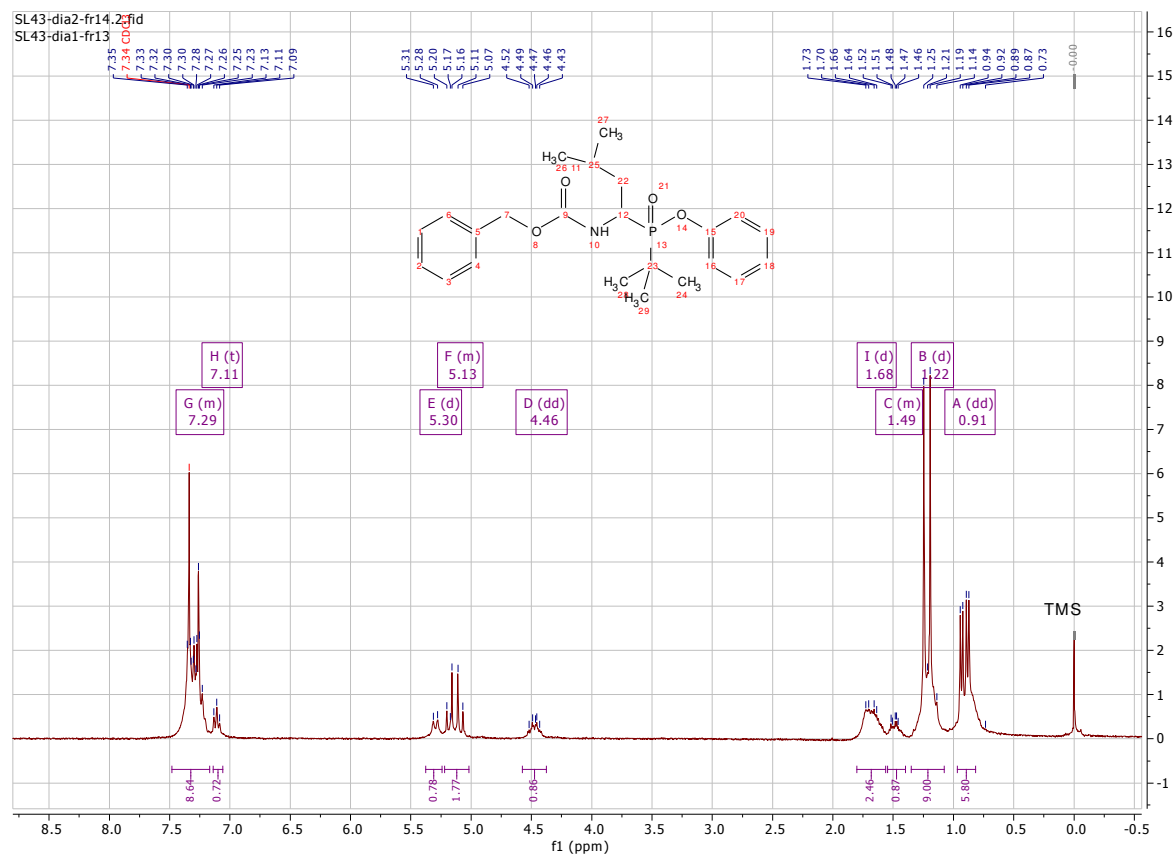
# 6.8 10c

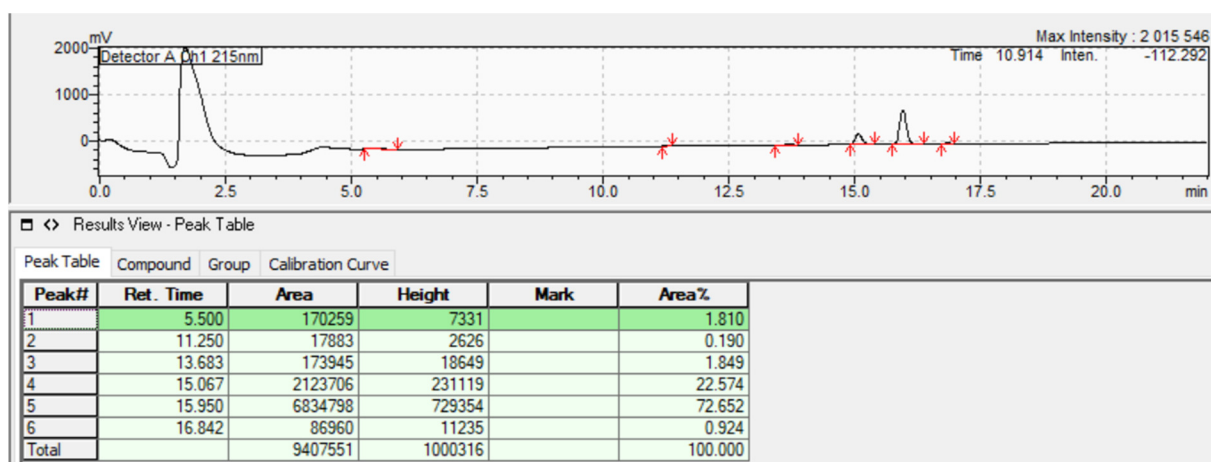




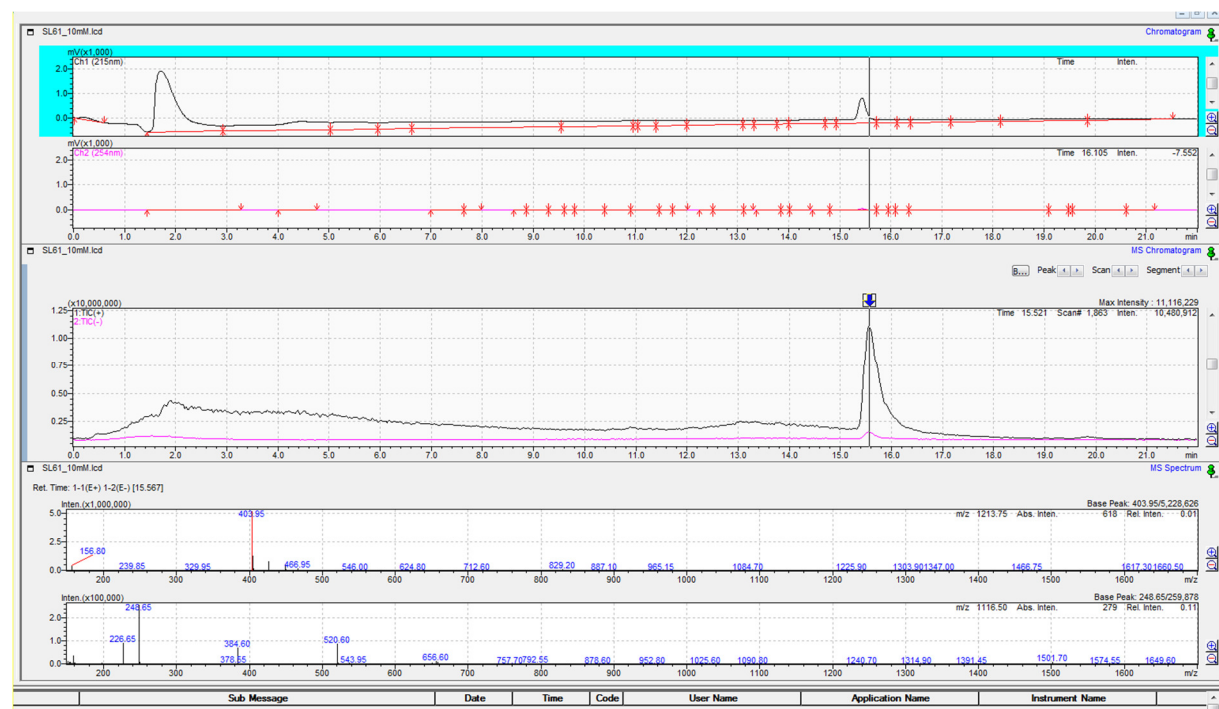
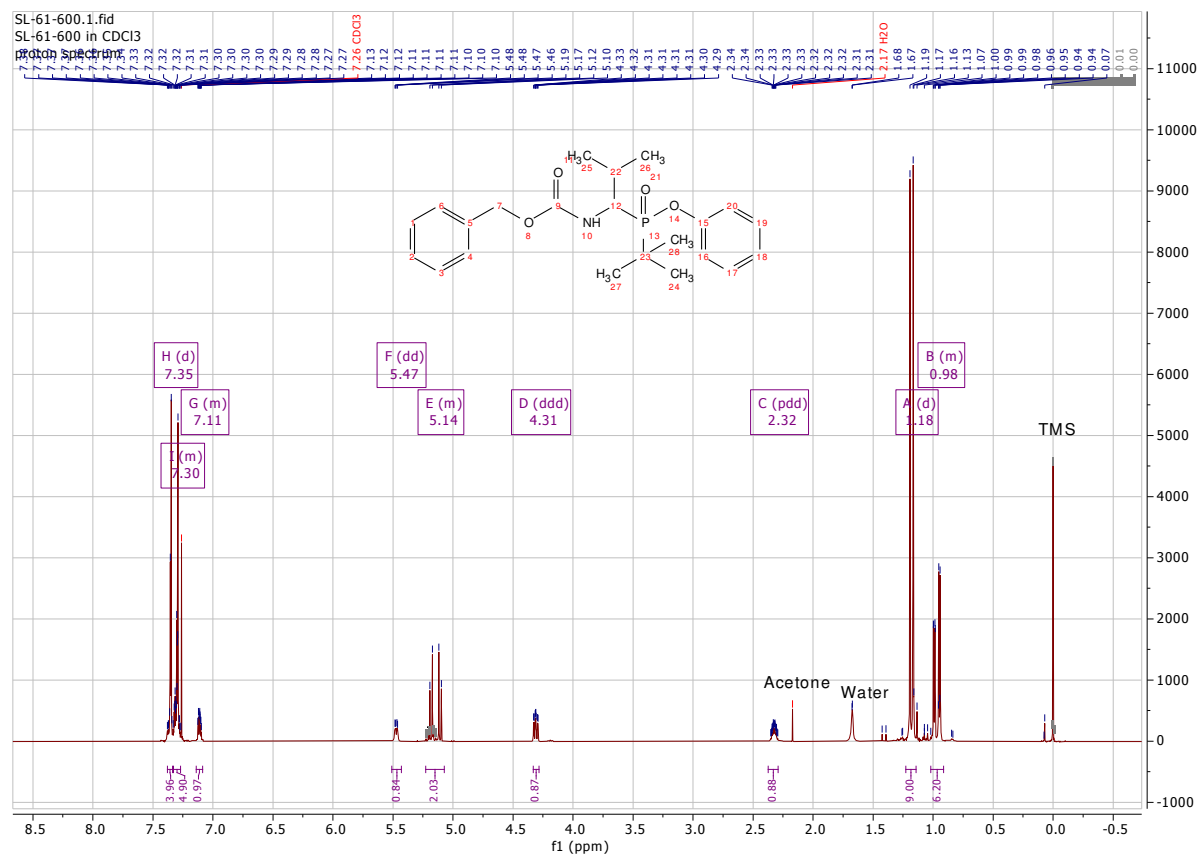


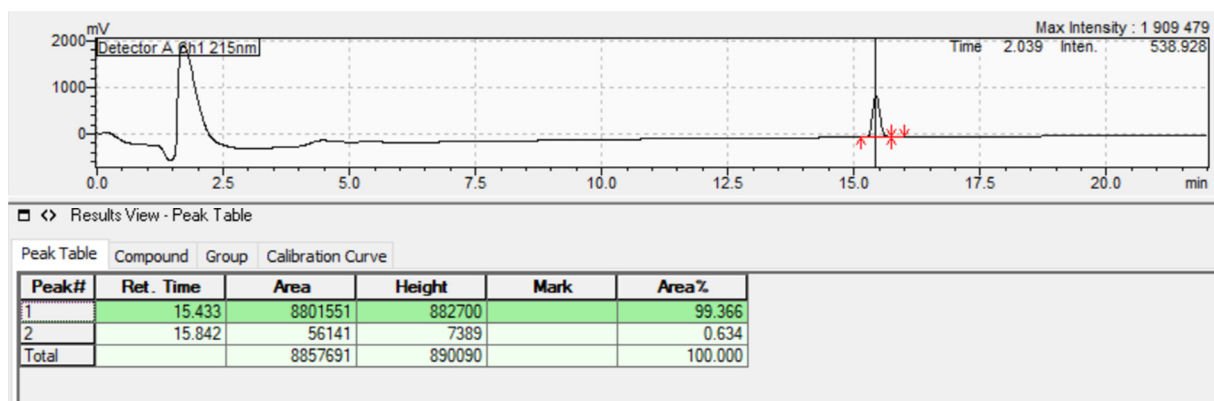
# 6.9 11c





## 6.10 12c





References:

1. Schindelin, J. *et al.* Fiji : an open-source platform for biological-image analysis. **9**, (2019).
2. Bianco, G., Forli, S., Goodsell, D. S. & Olson, A. J. Covalent docking using autodock : Two-point attractor and flexible side chain methods. **25**, 295–301 (2016).