Electronic Supplementary Material (ESI) for RSC Chemical Biology.

/Supporting Information

Phosphinate esters as novel warheads for activity-based probes targeting serine proteases

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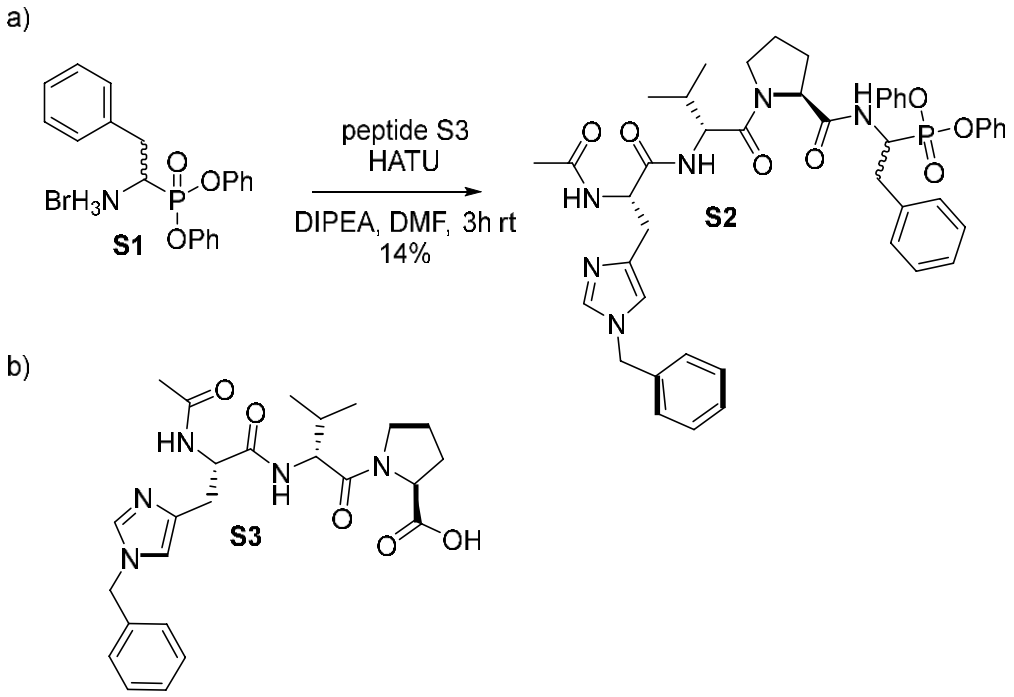
Abbreviations

BCA	Bicinchoninic acid assay
CatG	Cathepsin G
DAPI	4′,6-diamidino-2-phenylindole
DCI	3,4-dichloroisocoumarin
DCM	dichloromethane
DIPEA	N'N-diisopropylethylamine
DMF	dimethyformamide
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
EA	ethyl acetate
ESI	electron spray ionization
HATU	(1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
HBSS	Hank's balanced salt solution
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
HSA	human serum albumin
LC-MS	liquid chromatography – mass spectrometry
NE	neutrophil elastase
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PR3	Proteinase 3
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAMRA	5-Carboxytetramethylrhodamine
THPTA	Tris(3-hydroxypropyltriazolylmethyl)amine
TLC	thin layer chromatography



Scheme S1: (a) Synthesis of peptide CatG inhibitor used to compete labelling in neutrophil cells. (b) acetyl-capped peptide used for the synthesis of S2.

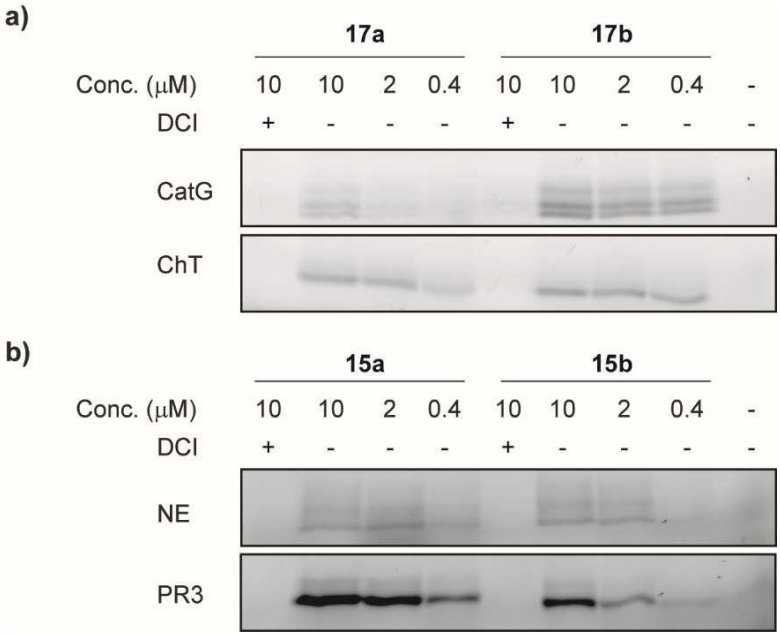


*Figure S1: Labelling of purified enzymes by a serial dilution of the different diastereomers of phosphinate ABPs **15** and **17**. (a) Labelling of CatG and ChT by probes **17a** and **17b**. (b) Labelling of NE and PR3 by probes **15a** and **15b**. In all cases active site labelling is demonstrated by competition with DCI prior to probe incubation. Note that a direct comparison between panel a and panel b cannot be made due to different photomultiplier settings of the scanner when acquiring images. Labeling intensity reveals that diastereomers **17b** and **15a** are the most potent ones.*

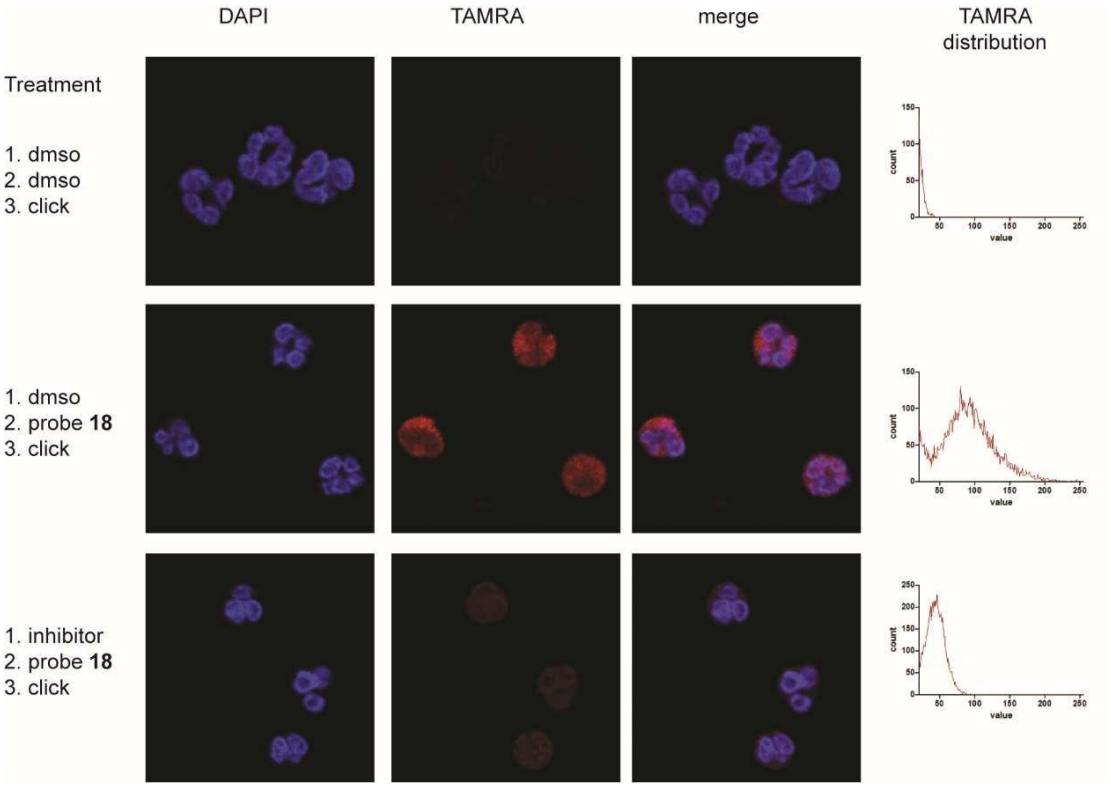


*Figure S2: A second set of images from fluorescent microscopy experiments after labelling of neutrophils with peptidic Phe^{PhP} ABP **18** and subsequent 'click'-reaction with TAMRA. Depicted are representative images showing three neutrophil cells. Clear labelling can be seen in the probe-treated cells (middle panels) in comparison with the vehicle-treated ones (upper panels). Additionally, treatment of neutrophils with a CatG inhibitor prior to probe treatment diminishes labelling by ABP **18** (lower panels). Histogram showing distribution of brightness in the TAMRA channel reveal a clear difference between probe treated samples and controls. Brightness of DAPI channel images was adjusted for better representation.*

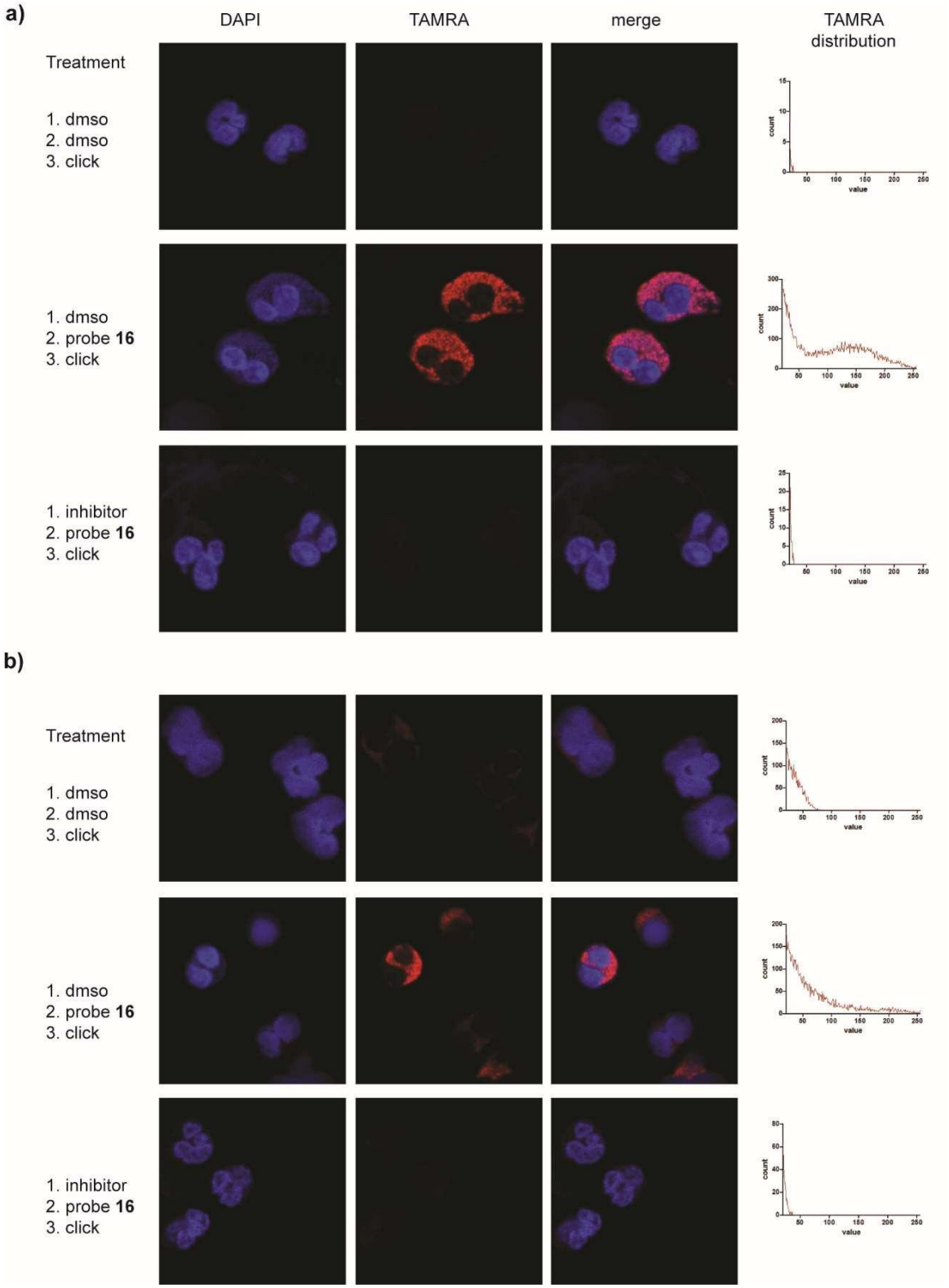


*Figure S3: A first (Fig. S3a) and second (Fig. S3b) set of images from fluorescent microscopy experiments after labelling of neutrophils with peptidic Val^{PhP} ABP **16** and subsequent 'click'-reaction with TAMRA. Depicted are representative images showing two (Fig. S3a) or three (Fig. S3b) neutrophil cells. Clear labelling can be seen in the probe-treated cells (middle panels) in comparison with the vehicle-treated ones (upper panels). Additionally, treatment of neutrophils with an NE inhibitor prior to probe treatment diminishes labelling by ABP **16** (lower panels). Histograms showing distribution of brightness in the TAMRA channel also reveal the difference between probe treated cells and control samples. Brightness of DAPI channel images was adjusted for better representation.*

Materials and Methods

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 5-80% acetonitrile in water (containing 0.1% formic acid) was used. High resolution mass spectra shown are spectra of 10 mM stock solutions used for biological assays. 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 25. The injection peak and peaks determined to be baseline variations were removed manually and integrals for different stereoisomers were summed up to give a total percentage of compound where applicable. 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. Data analysis was done using MestReNova Version 14.1.2.25024 (Mestrelab Research, Spain). All biological experiments (gel-based ABPP and kinetics) were done using the stock solutions in DMSO, containing either a pure diastereomer or a mixture of stereoisomers (as mentioned). Carboxybenzyl-protected phosphinate starting materials were synthesized as described elsewhere.[1]

HL-60 cell line was obtained from Merck. Experiments involving the use of human neutrophils were reviewed and approved by the UZ/KU Leuven ethics committee before being performed (study number S62598). Healthy volunteers were informed about the risks of their participation in the study and consented before blood being taken. Blood was taken by trained MDs.

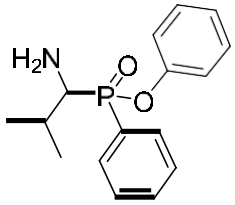
2. Synthesis of compounds

2.1 General procedure of deprotection of phosphinates

A solution of 33% hydrobromic acid in acetic acid was added to the Cbz-protected phosphinates and the solution was stirred at room temperature for 1h after which complete Cbz deprotection was seen by LC-MS. The reaction was then quenched with water and extracted with DCM (2x). Next, the pH of the aqueous phase was adjusted to approximately 9 and extracted with DCM (3x). The combined organic phases of the second extraction step

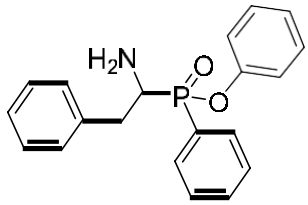
were dried over magnesium sulfate and the solvent evaporated under reduced pressure, yielding the product in sufficient purity for the next step.

2.1.1 Synthesis phenyl (1-amino-2-methylpropyl)(phenyl)phosphinate, 13



The reaction was performed according to the general procedure using phenyl (1-(((benzyloxy)carbonyl)amino)-2-methylpropyl)(phenyl)phosphinate (Cbz-Val$^{PhP;}$ 19.1 mg, 45 µmol, 1 eq.) and 0.4 mL of HBr/AcOH, yielding the title product as a yellow oil (7.5 mg, 26 µmol, 58%) that was used in the next step without purification. Molecular formula: $C_{16}H_{20}NO_2P$. ESI-MS $[M+H]^+$: calculated 290.12, found 289.90.

2.1.2 Synthesis of phenyl (1-amino-2-phenylethyl)(phenyl)phosphinate,14



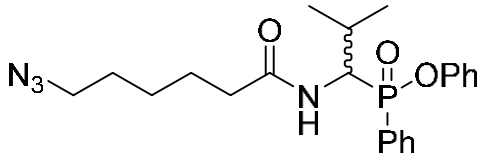
The reaction was performed according to the general procedure using phenyl (1-(((benzyloxy)carbonyl)amino)-2-phenylethyl)(phenyl)phosphinate (Cbz-Phe^{PhP}; 14.6 mg, 31 µmol, 1 eq.) and 0.4 mL of HBr/AcOH, yielding the title product as a yellow oil (8.6 mg, 25.5 µmol, 82%) that was used in the next step without purification. Molecular formula: $C_{20}H_{20}NO_2P$. ESI-MS $[M+H]^+$ calculated 338.12, found 337.85.

2.2 General procedure for the coupling of deprotected phosphinates with the linker

To the respective linker (peptide or azido hexanoic acid) in DMF (final concentration approximately 0.12 M) was added HATU (1 eq.) and DIPEA (4-5 eq.) and the solution was stirred at room temperature for 30 minutes. Next, this solution was added to a solution of the deprotected phosphinates in DMF (approx. 0.06 M) and the reaction was stirred at rt. After

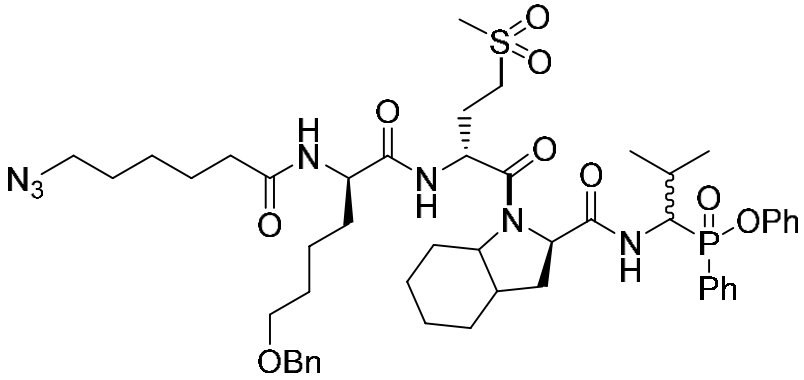
completion of the reaction as assessed by LC-MS the solvent was evaporated under reduced pressure and the crude product was purified by HPLC.

2.2.1 Synthesis of phenyl (1-(6-azidohexanamido)-2-methylpropyl)(phenyl)phosphinate, 15a/b



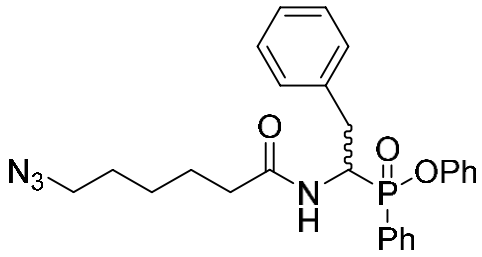
The reaction was performed according to the general procedure using compound **13** (11 mg, 38 µmol, 1 eq.), HATU (14 mg, 38 µmol, 1 eq.), 6-azidohexanoic acid (6 mg, 38 µmol, 1 eq.) and DIPEA (25 mg, 190 µmol, 5 eq.) stirring overnight in DMF. Both diastereomeric products were separated by HPLC, yielding the two products as powder (First peak 0.39 mg, 0.9 µmol, 2.4 %; second peak 1.144 mg, 2.7 µmol, 7 %). Molecular formula $C_{22}H_{29}N_4O_3P$. HRMS $[M+H]^+$: calculated 429.20499; found 429.2049 (diastereomer 1); 429.2047 (diastereomer 2). Purity as measured by LC-MS 98.9% (diastereomer 1), 91.3% (diastereomer 2).

2.2.2 Synthesis of phenyl (1-((2R)-1-((R)-2-((R)-2-(6-azidohexanamido)-6-(benzyloxy)hexanamido)-4-(methylsulfonyl)butanoyl)octahydro-1H-indole-2-carboxamido)-2-methylpropyl)(phenyl)phosphinate, 16



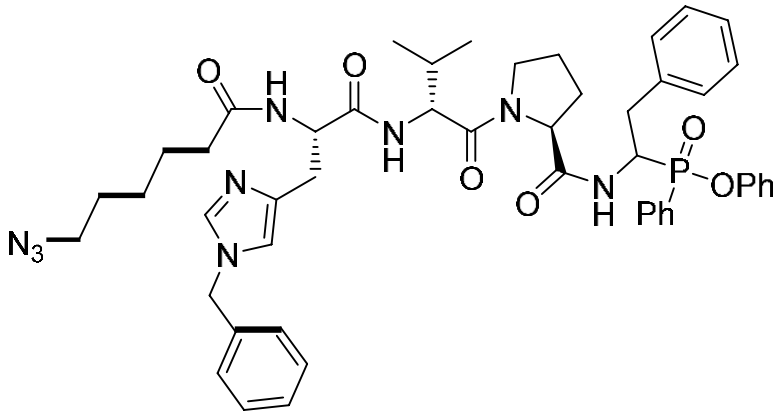
The reaction was performed according to the general procedure using compound **13** (9 mg, 31 µmol, 1 eq.), HATU (12 mg, 31 µmol, 1 eq.), the linker **19** (21.5 mg, 31 µmol, 1 eq.) and DIPEA (21 mg, 162 µmol, 5 eq.) stirring overnight in DMF. After HPLC purification the product was obtained as a powder (0.7 mg, 0.73 µmol, 2.3 %). Molecular formula $C_{49}H_{68}N_7O_9PS$. HRMS $[M+H]^+$ calculated 962.4609; found 962.4613. Purity as measured by LC-MS 96.4%.

2.2.3 Synthesis of phenyl (1-(6-azidohexanamido)-2-phenylethyl)(phenyl)phosphinate, 17a/b



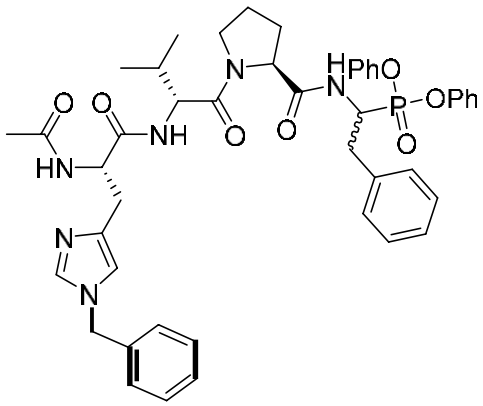
The reaction was performed according to the general procedure using compound **14** (4.3 mg, 12.7 µmol, 1 eq.), HATU (4.9 mg, 12.7 µmol, 1 eq.), 6-azidohexanoic acid (2.0 mg, 12.7 µmol, 1 eq.) and DIPEA (6.6 mg, 51 µmol, 4 eq.) stirring for 3h in DMF. Both diastereomeric products were separated by HPLC, yielding the two products as powder (First peak 0.59 mg, 1.24 µmol, 10 %; second peak 0.78 mg, 1.64 µmol, 13 %). Molecular formula $C_{26}H_{29}N_4O_3P$. HR-MS $[M+H]^+$ calculated 477.2050; found 477.2049 (diastereomer 1), 477.2049 (diastereomer 2). Purity as measured by LC-MS >99% (diastereomer 1), 95.4% (diastereomer 2).

2.2.4 Synthesis of phenyl (1-((S)-1-(Na-(6-azidohexanoyl)-Nt-benzyl-L-histidyl-D-valyl)pyrrolidine-2-carboxamido)-2-phenylethyl)(phenyl)phosphinate, 18



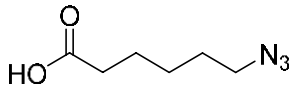
The reaction was performed according to the general procedure using compound **14** (4.3 mg, 12.7 µmol, 1 eq.), HATU (4.9 mg, 12.7 µmol, 1 eq.), the linker **20** (7.4 mg, 12.7 µmol, 1 eq.) and DIPEA (6.6 mg, 51 µmol, 4 eq.) stirring overnight in DMF. After HPLC purification the product was obtained as a powder (1.34 mg, 1.5 µmol, 12 %). Molecular formula $C_{49}H_{58}N_9O_6P$. HR-MS $[M+H]^+$ calculated: 900.4320; found: 900.4331. Purity as measured by LC-MS >99%.

2.2.5 Synthesis of diphenyl (1-((S)-1-(Na-acetyl-Nt-benzyl-L-histidyl-D-valyl)pyrrolidine-2-carboxamido)-2-phenylethyl)phosphonate, S2



The reaction was performed according to the general procedure using compound the diphenylphosphonate analog of phenylalanine (5.1 mg, 14.5 µmol, 1 eq.), HATU (5.5 mg, 14.5 µmol, 1 eq.), the peptide **S3** (7.0 mg, 14.5 µmol, 1 eq.) and DIPEA (9.4 mg, 72.5 µmol, 5 eq.) stirring 3h in DMF. After HPLC purification the product was obtained as a powder (1.34 mg, 1.5 µmol, 12 %). Molecular formula $C_{45}H_{51}N_6O_7P$. HR-MS $[M+H]^+$ calculated: 819.3629 found: 819.3621. Purity as measured by LC-MS 98%.

2.3 Synthesis of azido hexanoic acid (N_3-Hx-OH)



6-Bromohexanoic (488 mg, 2.5 mmol, 1.0 eq.) acid was dissolved in 5 mL of DMSO. Next sodium azide (195 mg, 3.0 mmol, 1.2 eq.) and TBAI (catalytical amount) were added. The solution was stirred for 4 hours at room temperature after which 150 mL of water was added. The aqueous phase was then extracted with EA (3x75 mL) and the combined organic phases were washed with water (2x100 mL), dried and concentrated under reduced pressure to yield the product as a yellow oil (347 mg, 2.2 mmol, 88 %). Molecular formula $C_6H_{11}N_3O_2$. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.29 (t, *J* = 6.8 Hz, 2H), 2.38 (t, *J* = 7.4 Hz, 2H), 1.65 (tt, *J* = 14.0, 7.1 Hz, 4H), 1.45 (qd, *J* = 7.8, 7.0, 4.7 Hz, 2H).^{13}C NMR (75 MHz, $CDCl_3$) δ 180.2, 51.5, 34.1, 28.8, 26.4, 24.4.

2.4 Synthesis of peptide linkers

2.4.1 general procedure

Peptides were synthesized on chlorotrityl resin as solid support using Fmoc as the N-terminal protection group. The resin was loaded shaking the first amino acid (3 eq.) and DIPEA (6 eq.) with the resin(1 eq.) in dry DCM overnight. After blocking the resin with DCM/MeOH/DIPEA for one hour, the protecting group was removed with 20% piperidine in DMF for 30 minutes. The next amino acids were then coupled in DMF and the N-terminal protecting group removed. Eventually the peptides were capped with 6-azidohexanoic acid, cleaved from the resin using TFA in DCM and purified by HPLC.

N_3Hx-Nle(6-OBz)-Met(O_2)-Oic-OH, 19: MS $[M+H]^+$ calculated for $C_{33}H_{50}N_6O_8S$ 691.34, found 691.15

N_3Hx-His(Bzl)-Val-Pro-OH, 20: MS $[M+H]^+$ calculated for $C_{29}H_{40}N_8O_5$ 581.31, found 581.10

Ac-His(Bzl)-Val-Pro-OH, S3: MS $[M+H]^+$ calculated for $C_{25}H_{33}N_5O_5$ 484.25, found 484.35

3. Neutrophil isolation and lysis

3.1 Neutrophil isolation

Neutrophils were isolated according to a reported procedure.[2] Briefly, 10 mL of blood was drawn from a healthy volunteer and aliquots of 5 mL were layered over 5 mL of neutrophil isolation media (Cedarlane Laboratories, Canada). The blood was then centrifuged at room temperature for 35 minutes at 500 rcf. The neutrophil layer was then separated from the rest of the blood, diluted with HBSS (without Ca and Mg) and centrifuged for 10 minutes at 350 rcf. Subsequently, the pellet was suspended in 2 mL of red blood cell lysis buffer, the red blood cells lysed and the suspension centrifuged for 5 min at 250 rcf. After that, cells were washed again in HBSS (without Ca and Mg), centrifuged for 5 min at 250 rcf and then suspended in HBSS (with Ca and Mg) containing 2% human serum albumin. Cells were then counted and diluted to the appropriate density for the desired experiments.

3.2 Neutrophil cell lysis

Isolated neutrophils in HBSS/HSA were washed twice with HBSS without HSA and then lysed for 30 minutes on ice using twice the pellet volume of lysis buffer (0.1 M HEPES, 0.5 M NaCl, 0.5% Triton-X, pH 7.5). Cell debris was then pelleted by centrifugation for 15 minutes at 4 °C and 17.000 rcf. The supernatant was transferred to a new vial and shock-frozen in liquid nitrogen. The total protein concentration was determined by BCA using bovine serum albumin as standard.

4. Labeling experiments

4.1 Labeling of purified proteases

The purified protease (200 ng in 10 µL of buffer) was added to 19.4 µL of buffer (100 mM HEPES, 0.5 M NaCl, pH 7.5). Then 0.3 µL of DCI (final concentration of 100 µM) or DMSO were added to the sample and the sample was incubated for 30 minutes. Subsequently 0.3 µL of the probes in 100x concentration or DMSO were added and incubated with the samples for another 30 minutes. When titrating the P1 probes, no DCI incubation was done and the samples were thus directly incubated with the probes or DMSO. Probes were then 'click'-reacted with TAMRA-alkyne (see 4.4 for general procedure) for one hour before 10 µL of 4x sample buffer were added, the sample heated to 95 °C for 5 minutes and analyzed by SDS-PAGE using a 12% acrylamide gel. In the case of labeling with FP-rhodamine, no click reaction was performed and the samples were directly subjected to sample buffer and heating.

4.2 Labeling of HL60-cell lysates or neutrophil lysates

The respective lysate was added to the buffer (100 mM HEPES, 0.5 M NaCl, pH 7.5) to a final concentration of 1 mg/mL and 0.3 µL of the respective probe in 100x concentration was added. If DCI incubation prior to probe labelling was desired, 0.3 µL of DCI in 100x concentration (final concentration 100 µM) or 0.3 µL of DMSO was added and the sample incubated for 30 minutes before probes were added. In the cases when purified protease was spiked, 200 ng of purified protease in 10 µL of buffer was added before probe addition. In all cases the final sample volume was 30 µL. The samples were then incubated with the probes for 30 minutes before being 'click'-reacted with TAMRA-alkyne (see 4.4 for general procedure). Subsequently, 10 µL of 4x sample buffer were added, the sample heated to 95 °C for 5 minutes and analyzed by SDS-PAGE using a 12% acrylamide gel. In the case of labeling with FP-rhodamine, no click reaction was performed and the samples were directly subjected to sample buffer and heating.

4.3. Labeling of live neutrophils with subsequent lysis

Neutrophils were isolated (see 3.1) and adjusted to a density of $1x10^6$ cells/mL in HBSS with Ca and Mg containing 4% HSA. For each experiment 0.5 µL of probes (1000x concentrated) were added to a volume of 0.5 mL of cell suspension ($0.5x10^6$ cells) and the probes incubated with the cells for one hour. The cells were then washed three times by centrifuging and re-suspending them in HBSS containing Ca and Mg and eventually lysed for 30 minutes on ice

using lysis buffer (0.1 M HEPES, 0.5 M NaCl, pH 7.5 containing 0.5% NP40). The lysed cell suspension was then centrifuged for 15 minutes at 4 °C, the supernatant taken off and 'click'-reacted with TAMRA-alkyne (see 4.4) for one hour. The reaction was stopped by addition of sample buffer, the samples heated to 95 °C for 5 minutes and then analyzed by SDS-PAGE using a 12% acrylamide gel. In the case of labeling with FP-rhodamine, the cells were incubated with DMSO instead of the probes and the supernatant incubated with FP-rhodamine after lysis. No click reaction was performed and the samples were directly subjected to sample buffer and heating after incubation with FP-rhodamine.

4.4 'click-reaction' of probes with TAMRA-alkyne on purified protease and lysates

To 30 µL of the samples were added 0.3 µL of TAMRA-alkyne (2.5 mM in DMSO, final concentration 25 µM), 0.3 µL of THPTA (5 mM in DMSO, final concentration 50 µM), 0.6 µL of $CuSO_4$ (freshly prepared 50 mM solution in water, final concentration 100 µM) and 0.6 µL of sodium ascorbate (freshly prepared 50 mM solution in water, final concentration 100 µM). The samples were then incubated for one hour at room temperature before being treated as indicated in the individual experiment.

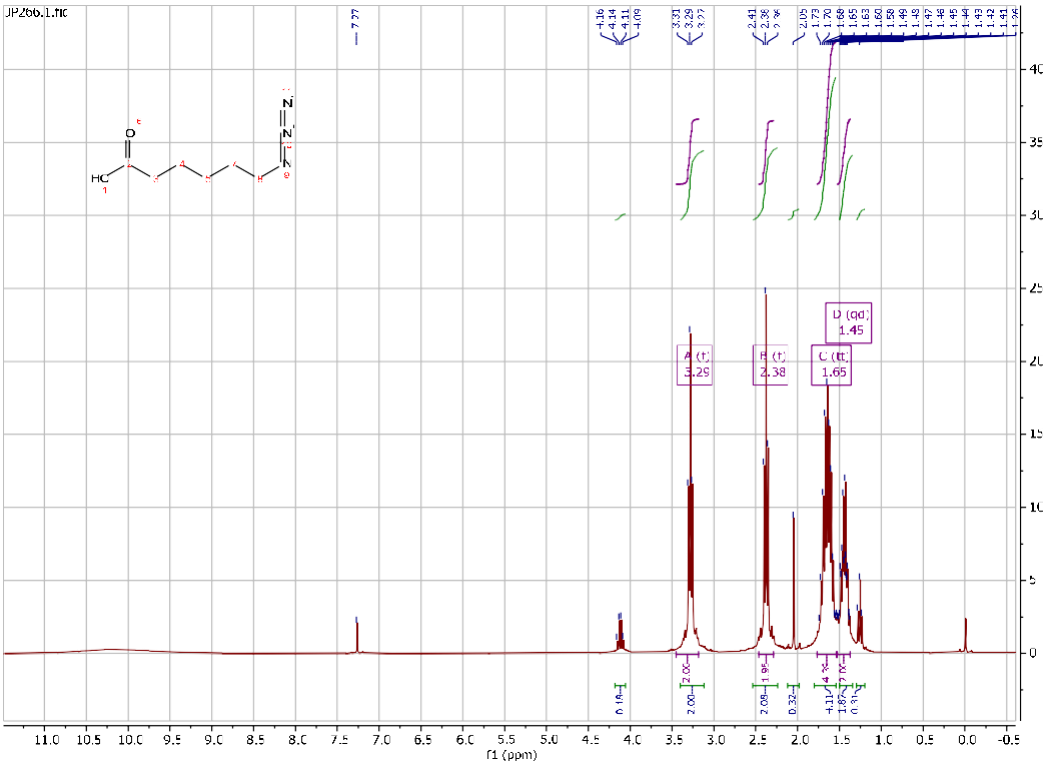
5. Microscopy on neutrophil cells using the probes

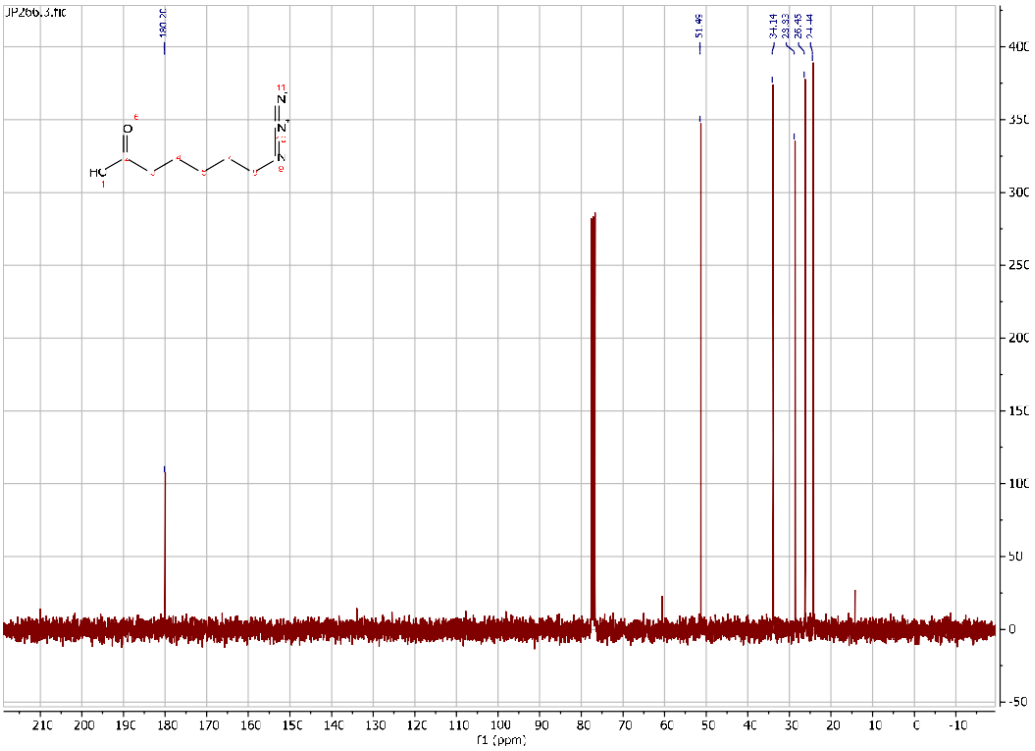
Neutrophil cells were isolated as described in section 3.1 and adjusted to a density of $0.8x10^6$ cells/mL in HBSS with Ca and Mg containing 2% HSA. 50 µL ($40x10^3$ cells) of the neutrophil suspension were then added to individual wells of a 96-well flat glass-bottom plate (Cellvis) coated with poly-d-lysine (gibco) and the cells let adhere to the plate bottom for 45 minutes at 37 °C. Next, 50 µL of respective inhibitor or DMSO in HBSS/HSA (2x solution, final DMSO conc. 0.1%, final buffer volume 100 µL) were added and the cells incubated for 1.5 h at 37 °C. The buffer was then removed and the respective probes or DMSO in HBSS/HSA were added (final DMSO conc. 0.1%, final buffer volume 100 µL) and incubated with the cells for 1.5 h at 37 °C. Subsequently, the buffer was removed, the cells washed with DPBS and fixed in 10% formalin (3.7% formaldehyde) for 15 minutes at room temperature, the cells washed with DPBS again and permeabilized using 0.05% Triton-x in DPBS for 1 minute at room temperature after which the cells were washed three times with DPBS. 'Click'-reaction was then performed on the cells by adding 1 µL of each: 2 mM TAMRA-alkyne in DMSO, 20 mM THPTA in DMSO, 0.1 M $CuSO_4$ in water and 1 M sodium ascorbate in water to 96 µL of 0.1 M HEPES, 0.5 M NaCl, pH 7.5. Final concentrations were 20 µM TAMRA-alkyne, 200 µM THPTA, 1 mM $CuSO_4$ and 10 mM sodium ascorbate and final DMSO content was 2%. The

reaction was performed for one hour at room temperature, the mixture removed, the cells washed with DPBS and incubated with 300 nM DAPI for 5 minutes. Eventually the cells were washed three times with DPBS, shaking the cells for 10 minutes in DPBS each time to allow solubilization of un-reacted click-compounds. Cells were kept in DPBS at 4 °C until imaging was performed. Images were acquired on a Zeiss LSM 880 confocal Airyscan microscope and processed using Fiji.[3] Histograms were created using GraphPadPrism Version 5.02 (GraphPad Software Inc.).

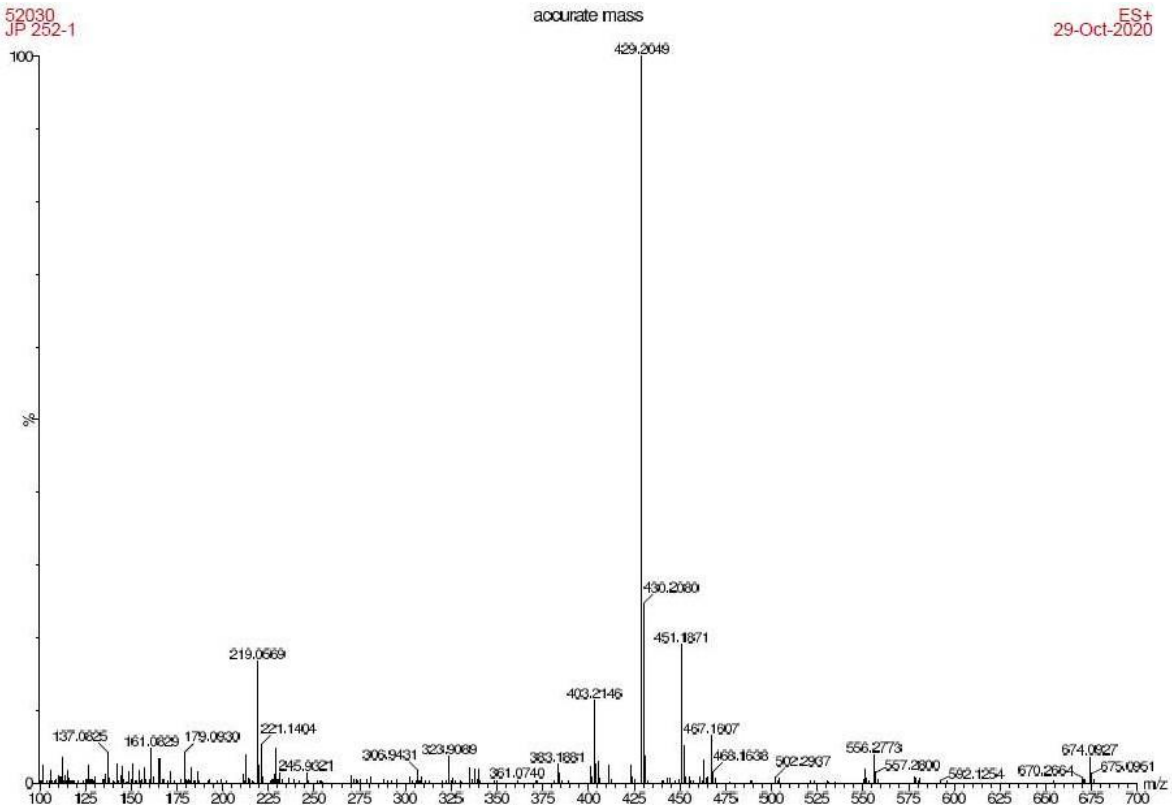
Spectra

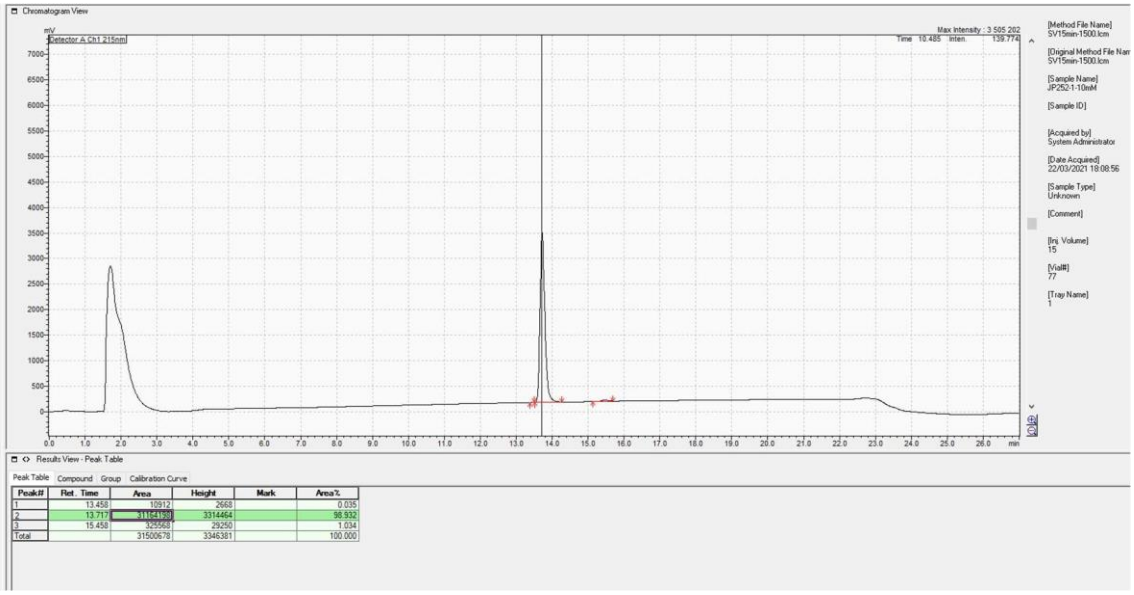
1 6-azidohexanoic acid

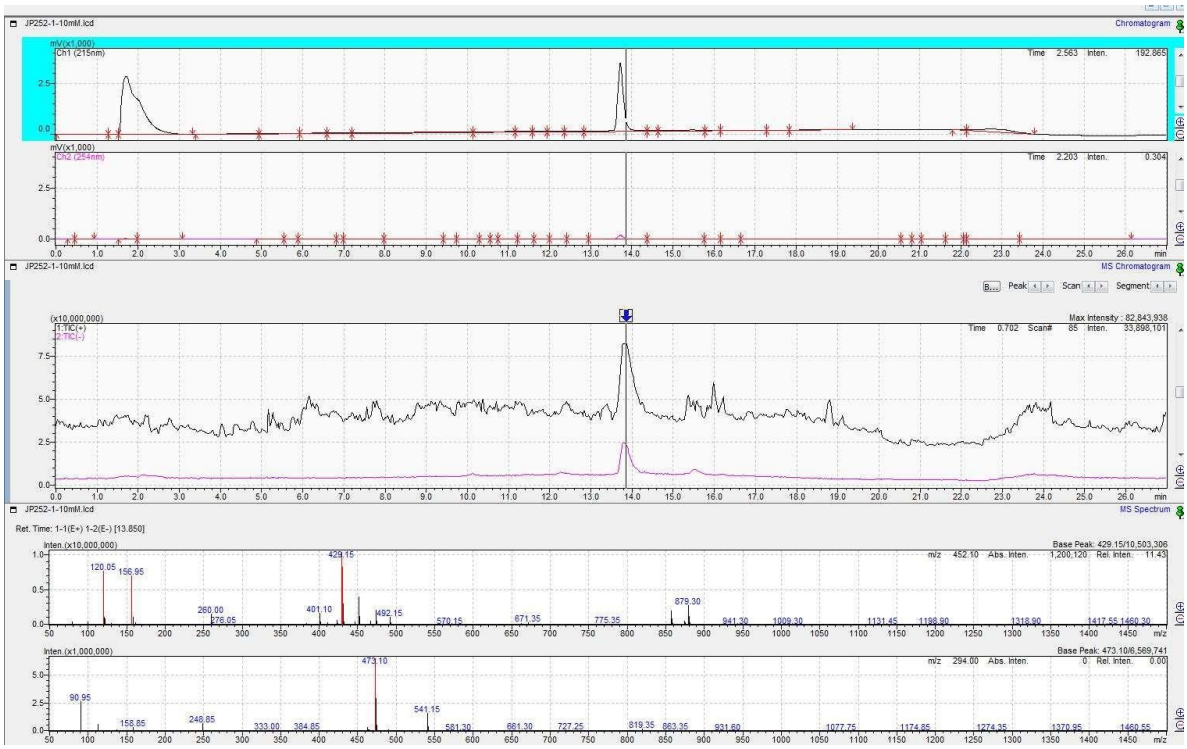




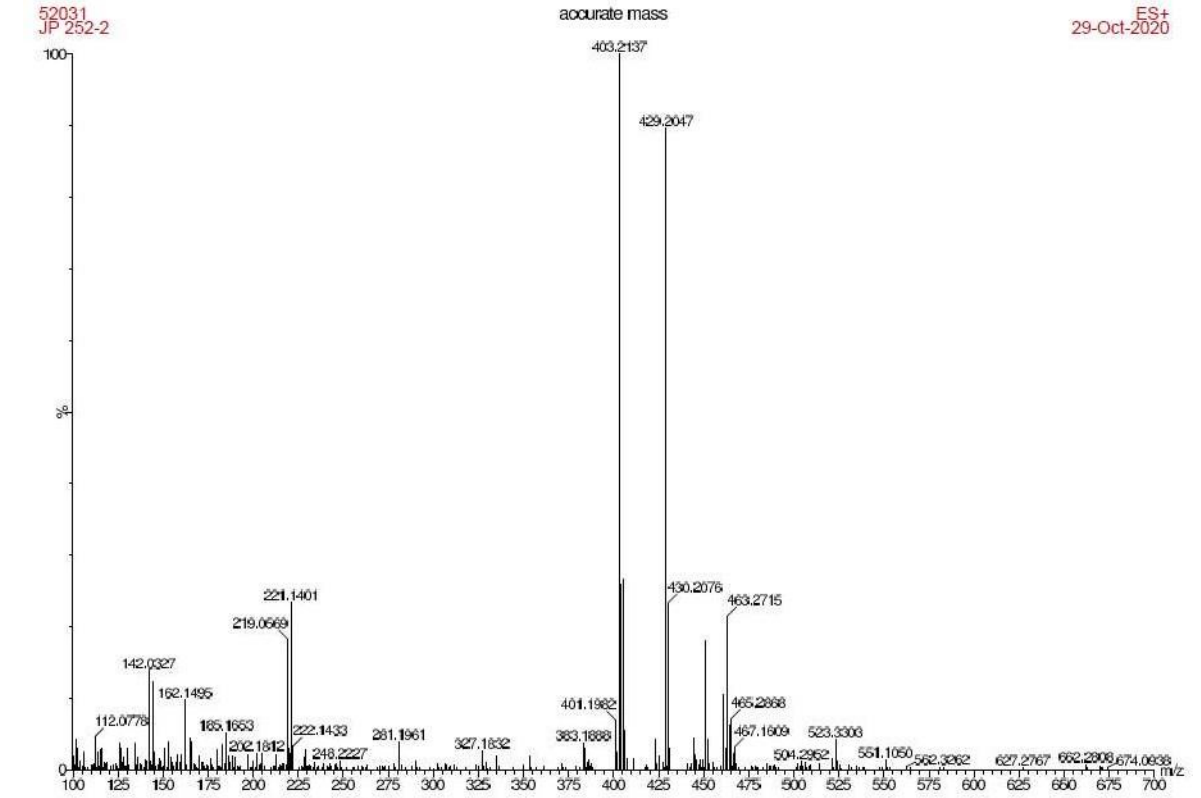
2 Compound 15 – Diastereomer 1

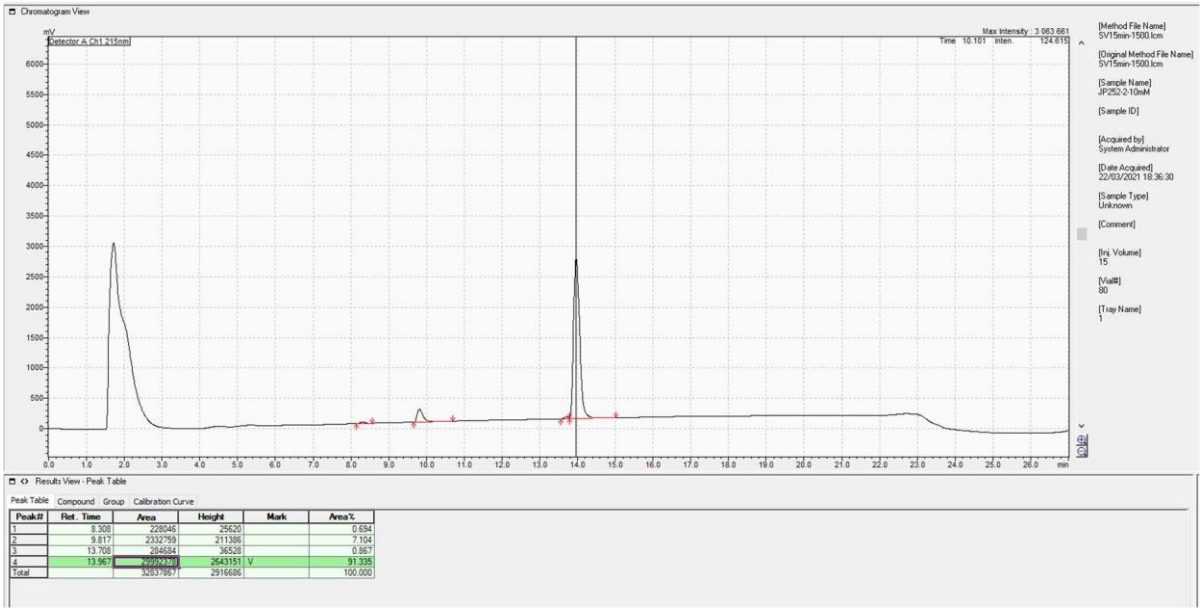


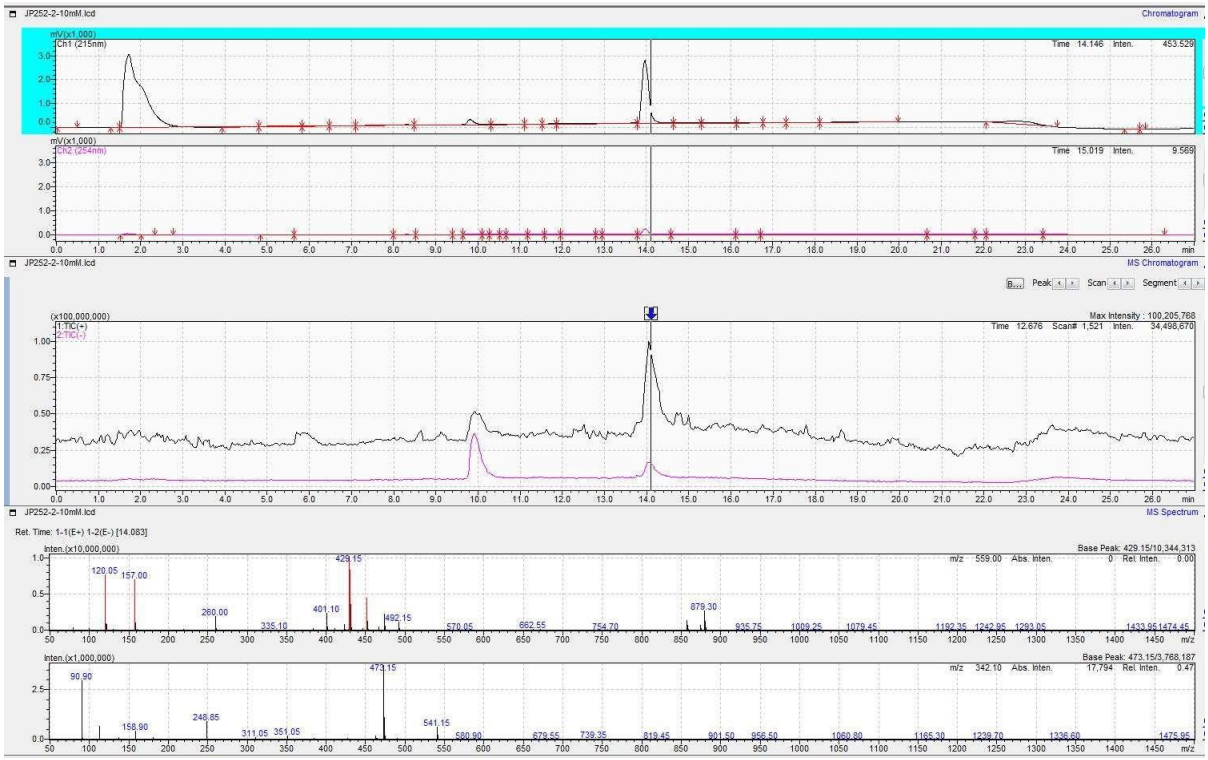



JP252-1-10mM.lcd
Chromatogram
mV(x1,000)
Ch1 (215nm)
Time 2.563 Inten. 192,865
mV(x1,000)
Ch2 (254nm)
Time 2.203 Inten. 0.304
min
JP252-1-10mM.lcd
MS Chromatogram
Peak
Scan
Segment
(x10,000,000)
1:TIC(+)
2:TIC(-)
Max Intensity : 82,843,938
Time 0.702 Scan# 85 Inten. 33,898,101
min
JP252-1-10mM.lcd
MS Spectrum
Ret. Time: 1-1(E+) 1-2(E-) [13.850]
Inten.(x10,000,000)
Base Peak: 429.15/10,503,306
m/z 452.10 Abs. Inten. 1,200,120 Rel. Inten. 11.43
120.05
156.95
260.00
276.05
401.10
429.15
492.15
570.15
671.35
775.35
879.30
941.30
1009.30
1131.45
1188.90
1318.90
1417.55
1460.30
m/z
Inten.(x1,000,000)
Base Peak: 473.10/6,569,741
m/z 294.00 Abs. Inten. 0 Rel. Inten. 0.00
90.95
158.85
248.85
333.00
384.85
473.10
541.15
581.30
681.30
727.25
819.35
863.35
931.60
1077.75
1174.85
1274.35
1370.95
1460.55
m/z

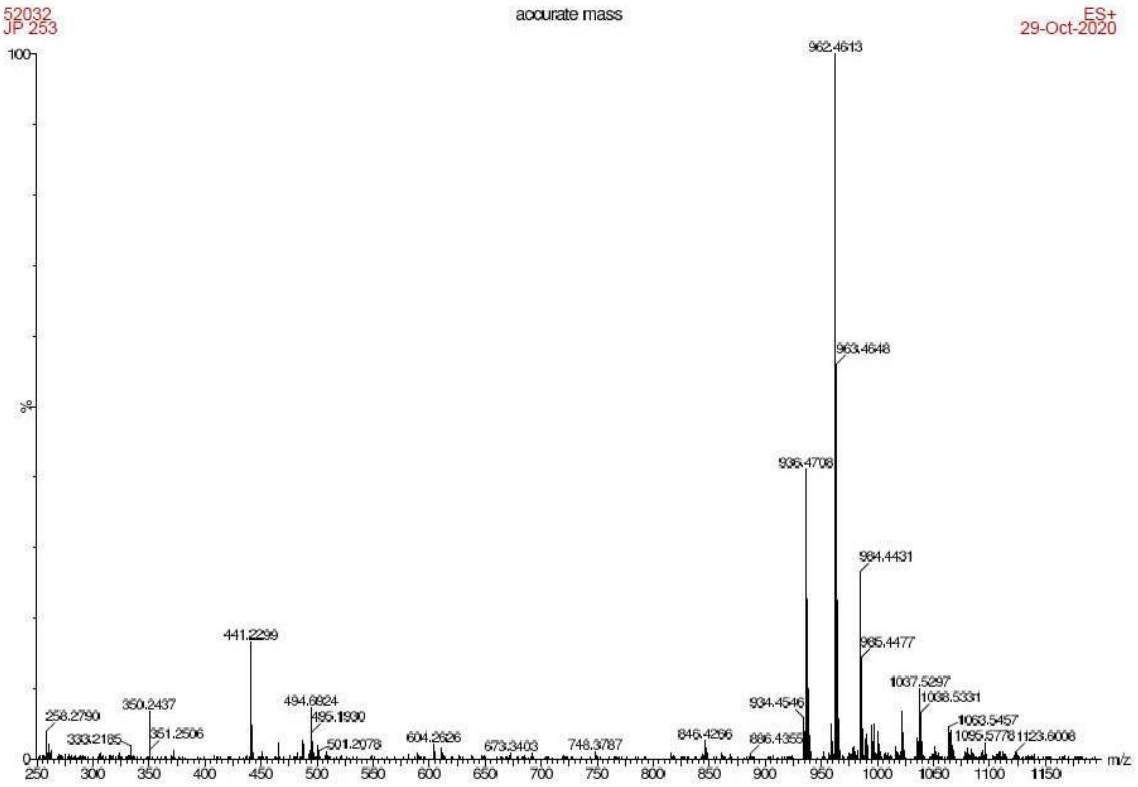
3 Compound 15 – Diastereomer 2

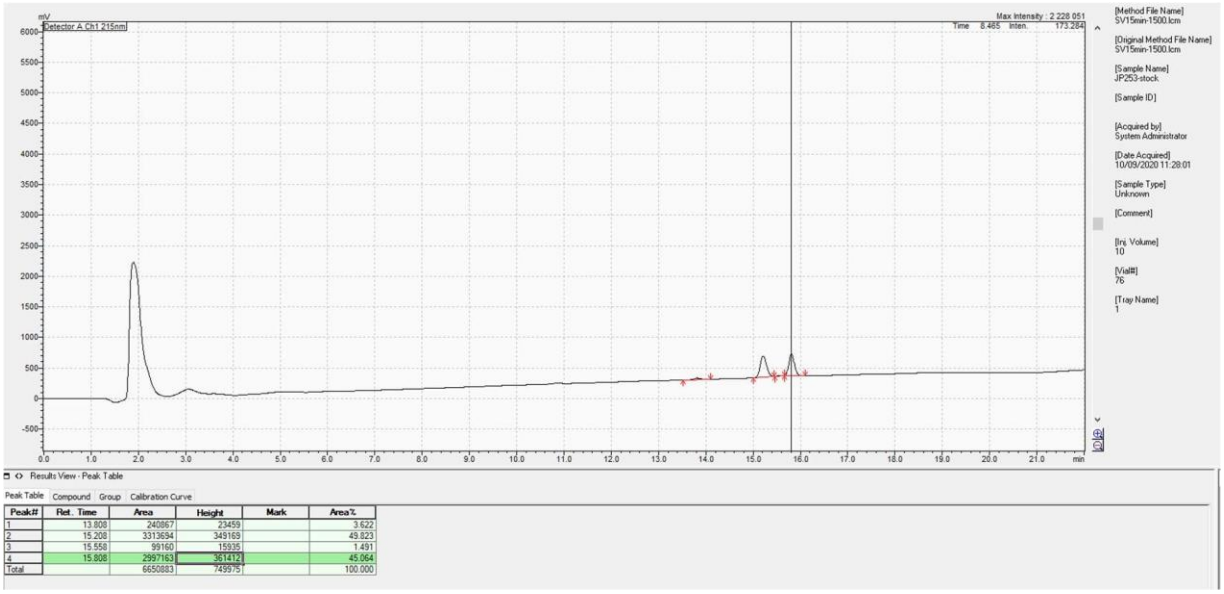




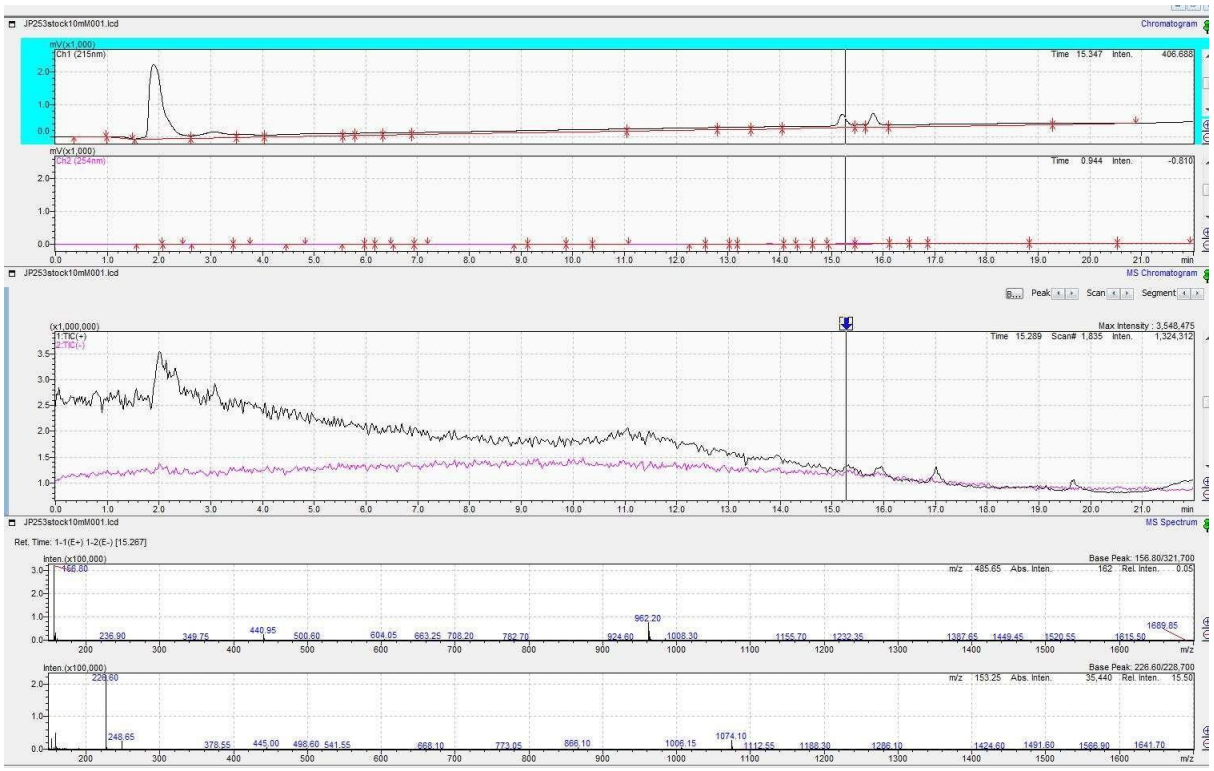

JP252-2-10mM.lcd
Chromatogram
mV(x1,000)
Ch1 (215nm)
Time 14.146 Inten. 453,529
mV(x1,000)
Ch2 (254nm)
Time 15.019 Inten. 9,569
min
JP252-2-10mM.lcd
MS Chromatogram
Peak
Scan
Segment
(x100,000,000)
1:TIC(+)
2:TIC(-)
Max Intensity : 100,205,768
Time 12.676 Scan# 1,521 Inten. 34,498,670
min
JP252-2-10mM.lcd
MS Spectrum
Ret. Time: 1-1(E+) 1-2(E-) [14.083]
Inten.(x10,000,000)
Base Peak: 429.15/10,344,313
m/z 559.00 Abs. Inten. 0 Rel. Inten. 0.00
120.05
157.00
260.00
335.10
401.10
429.15
492.15
570.05
662.55
754.70
879.30
935.75
1009.25
1079.45
1192.35
1242.95
1293.05
1433.95
1474.45
m/z
Inten.(x1,000,000)
Base Peak: 473.15/3,768,187
m/z 342.10 Abs. Inten. 17,794 Rel. Inten. 0.47
90.90
158.90
248.85
311.05
351.05
473.15
541.15
580.90
679.55
739.25
819.45
901.50
956.50
1060.80
1165.30
1239.70
1336.60
1475.95
m/z

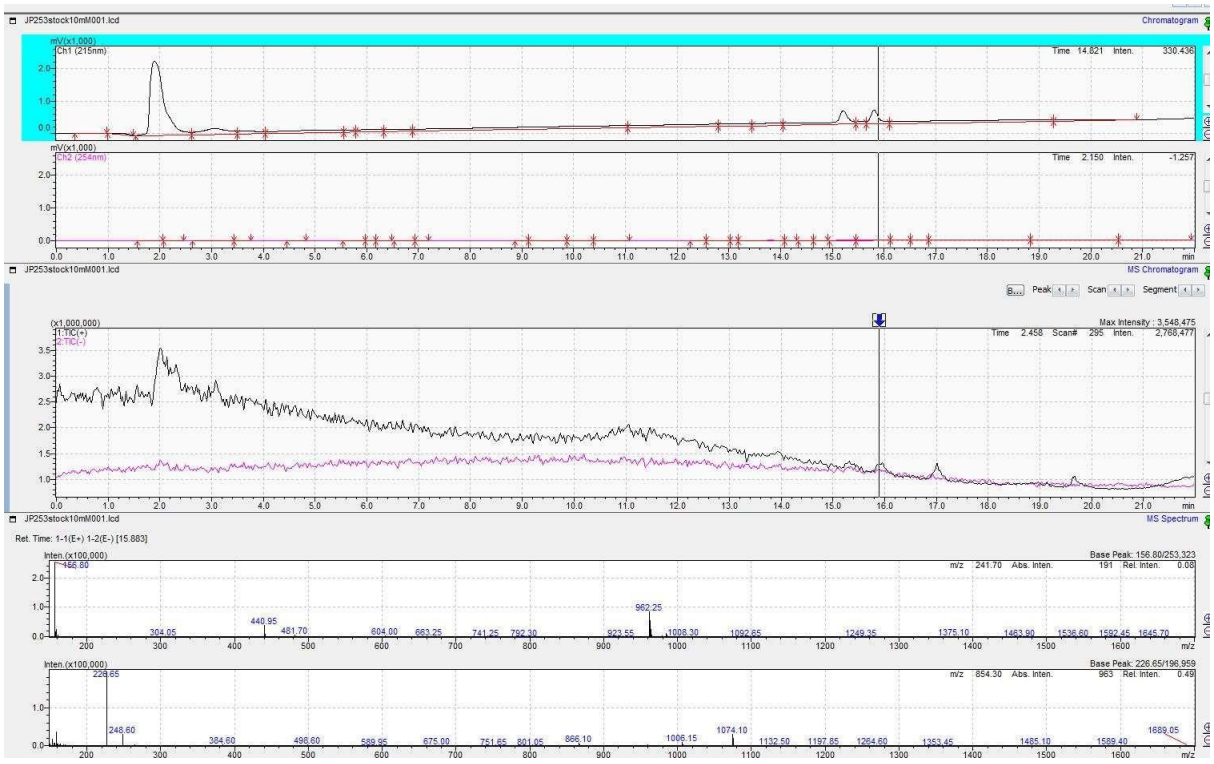
4 Compound 16



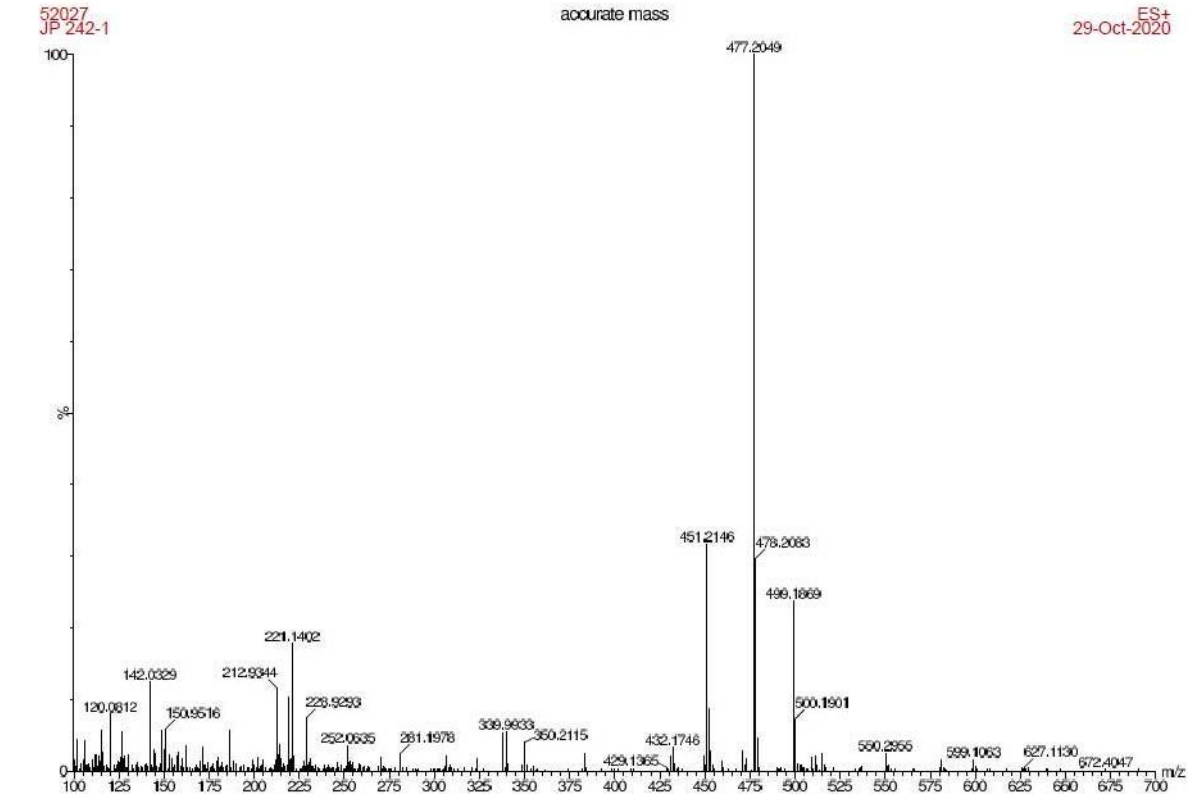


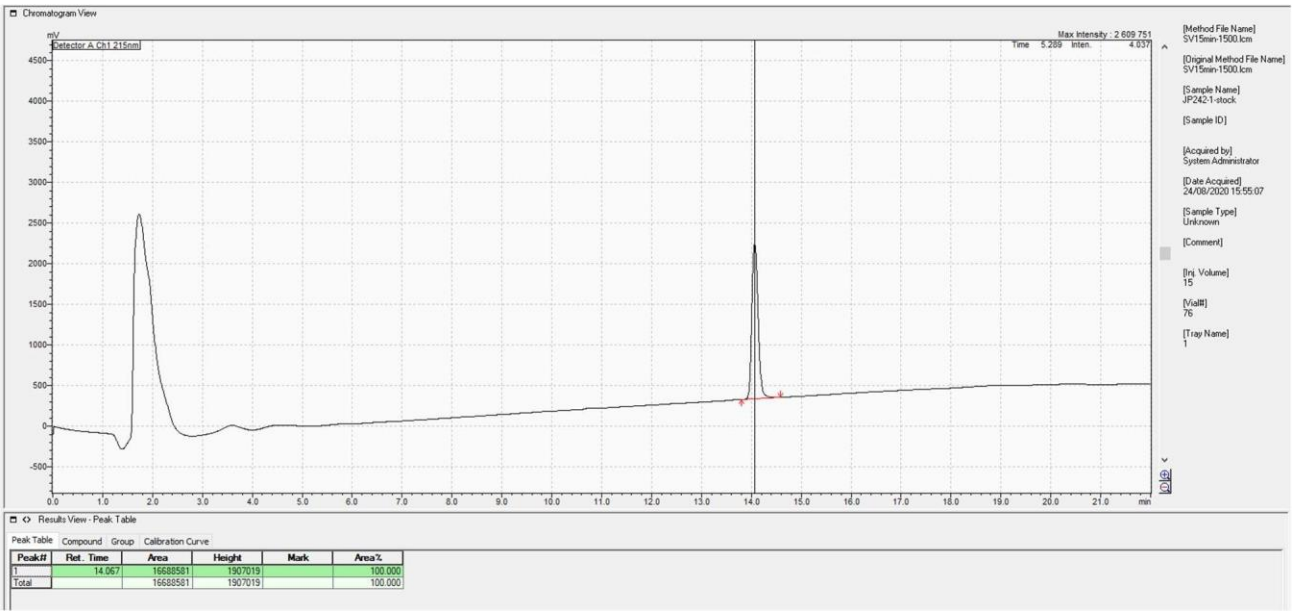
Peak#	Ret. Time	Area	Height	Mark	Area%
1	13.808	240867	23459		3.622
2	15.208	3313694	349169		49.823
3	15.558	99160	15935		1.491
4	15.808	2997163	361412		45.064
Total		6650883	749975		100.000

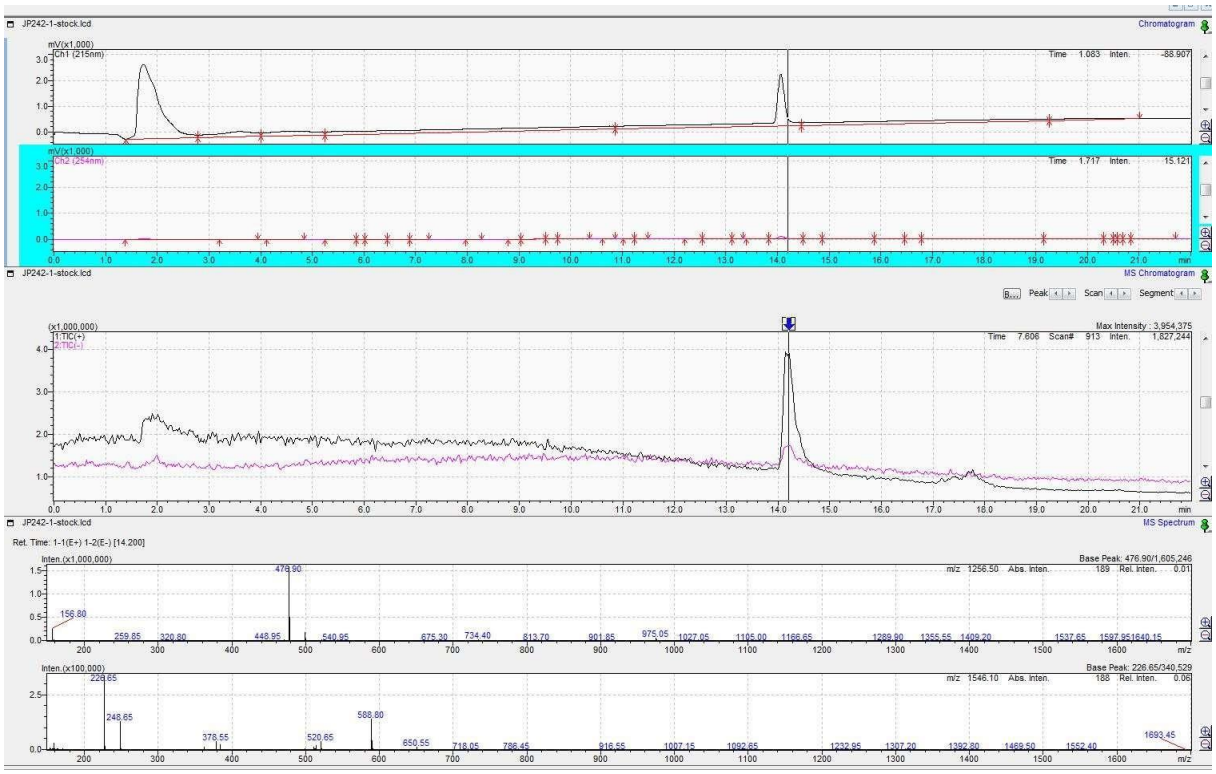

JP253stock10mM001.lcd
Chromatogram
mV(x1,000)
Ch1 (215nm)
Time 15.347 Inten. 406,688
mV(x1,000)
Ch2 (254nm)
Time 0.944 Inten. -0.810
min
JP253stock10mM001.lcd
MS Chromatogram
Peak Scan Segment
(x1,000,000)
TIC(+)
TIC(-)
Max Intensity : 3,548,475
Time 15.289 Scan# 1,835 Inten. 1,324,312
min
JP253stock10mM001.lcd
MS Spectrum
Ret. Time: 1-1(E+) 1-2(E-) [15.267]
Inten.(x100,000)
Base Peak: 156.80/321,700
m/z 485.65 Abs. Inten. 162 Rel. Inten. 0.05
Inten.(x100,000)
Base Peak: 226.60/228,700
m/z 153.25 Abs. Inten. 35,440 Rel. Inten. 15.50
m/z


JP253stock10mM001.lcd
Chromatogram
mV(x1,000)
Ch1 (215nm)
Time 14.821 Inten. 330,436
mV(x1,000)
Ch2 (254nm)
Time 2.150 Inten. -1.257
min
JP253stock10mM001.lcd
MS Chromatogram
Peak Scan Segment
(x1,000,000)
TIC(+)
TIC(-)
Max Intensity : 3,548,475
Time 2.458 Scan# 295 Inten. 2,768,477
min
JP253stock10mM001.lcd
MS Spectrum
Ret. Time: 1-1(E+) 1-2(E-) [15.883]
Inten.(x100,000)
Base Peak: 156.80/253,323
m/z 241.70 Abs. Inten. 191 Rel. Inten. 0.08
Inten.(x100,000)
Base Peak: 226.65/196,959
m/z 854.30 Abs. Inten. 963 Rel. Inten. 0.49
m/z

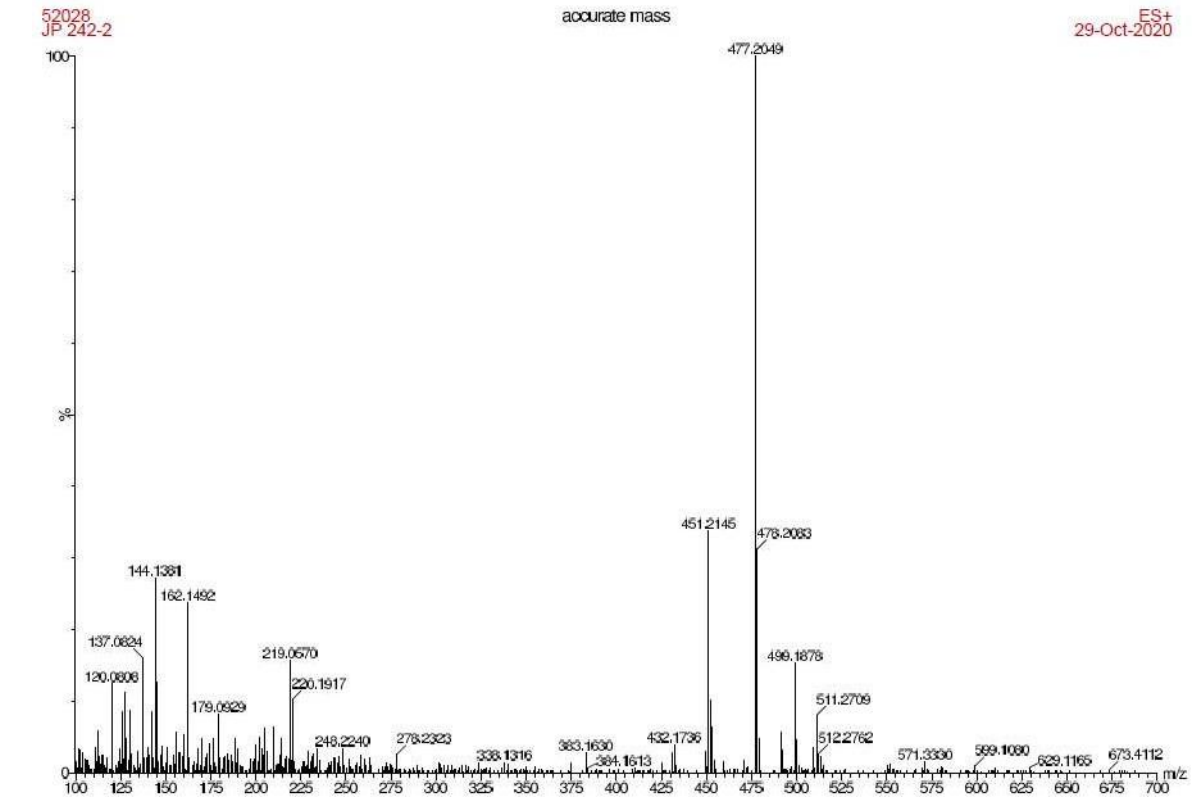
5 Compound 17 – Diastereomer 1

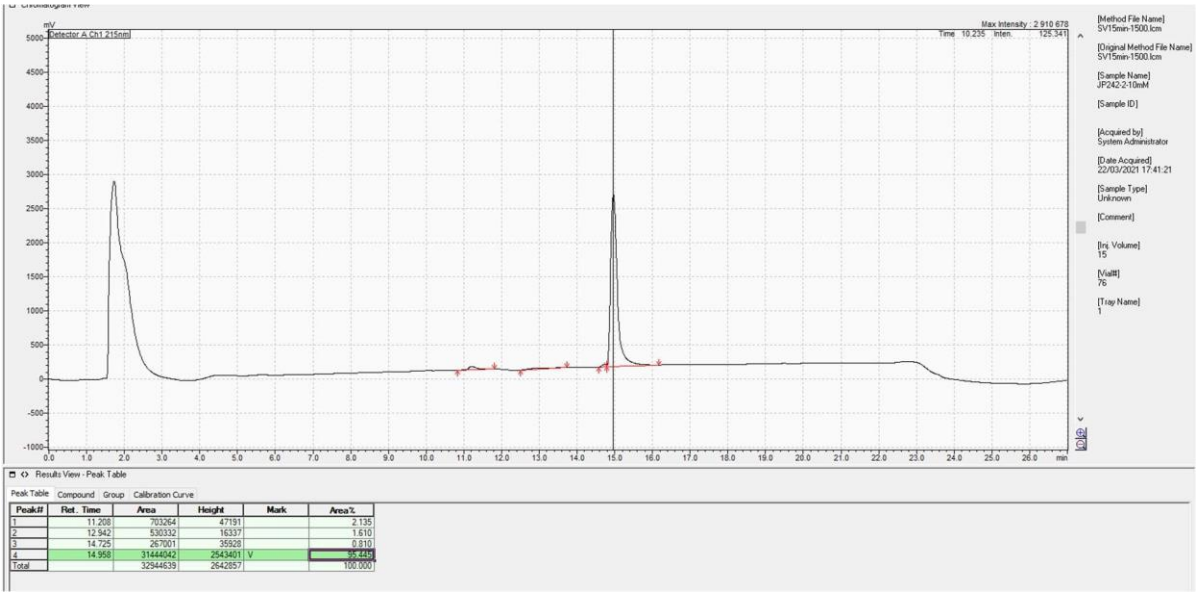


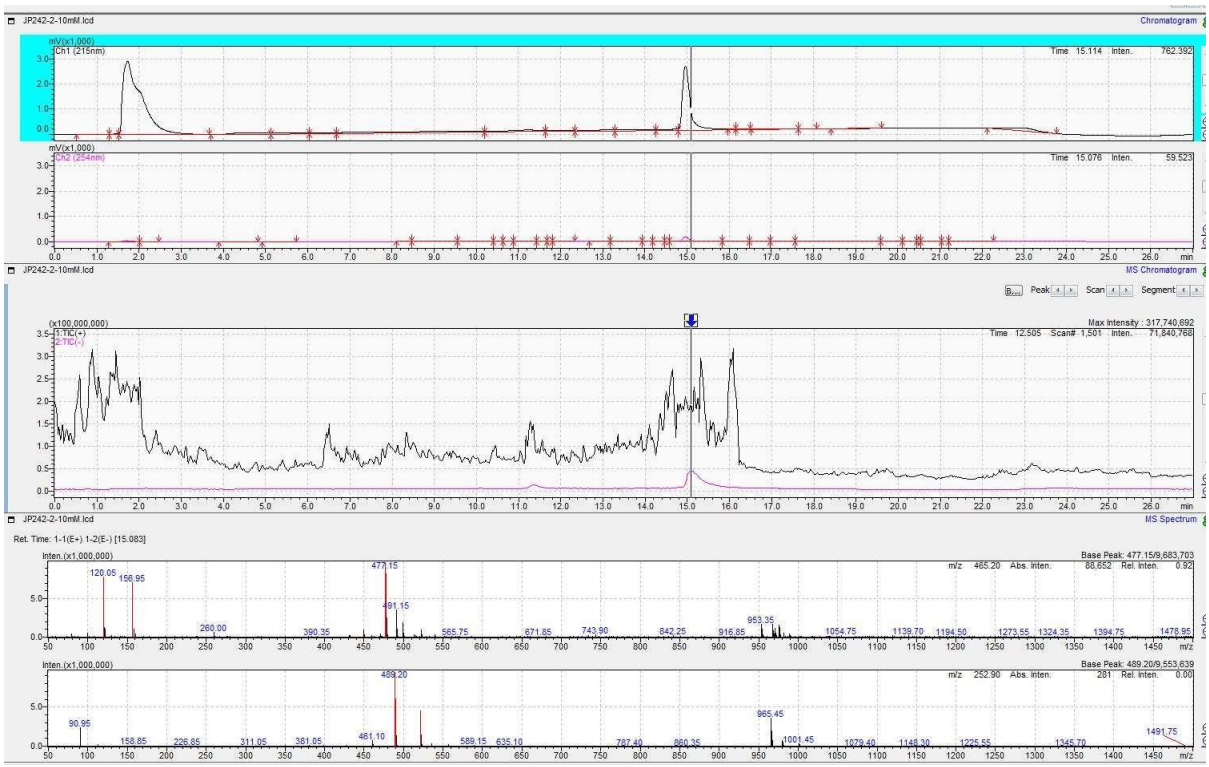



JP242-1-stock.lcd
Chromatogram
mV(x1,000)
Ch1 (215nm)
Time 1.083 Inten. -88.907
mV(x1,000)
Ch2 (254nm)
Time 1.717 Inten. 15.121
min
JP242-1-stock.lcd
MS Chromatogram
Peak: Scan Segment:
(x1,000,000)
TIC(+)
TIC(-)
Max Intensity : 3,954,375
Time 7.606 Scan# 913 Inten. 1,827,244
min
JP242-1-stock.lcd
MS Spectrum
Ret. Time: 1-1(E+) 1-2(E-) [14.200]
Inten.(x1,000,000)
Base Peak: 476.90/1,605,246
m/z 1256.50 Abs. Inten. 189 Rel. Inten. 0.01
476.90
156.80
259.85
320.80
448.95
540.95
675.30
734.40
813.70
901.85
975.05
1027.05
1105.00
1166.65
1289.90
1355.55
1409.20
1537.65
1597.95
1640.15
m/z
Inten.(x100,000)
Base Peak: 226.65/340,529
m/z 1546.10 Abs. Inten. 188 Rel. Inten. 0.06
226.65
248.65
378.55
520.65
588.80
650.55
718.05
786.45
916.55
1007.15
1092.85
1232.95
1307.20
1392.80
1469.50
1552.40
1693.45
m/z

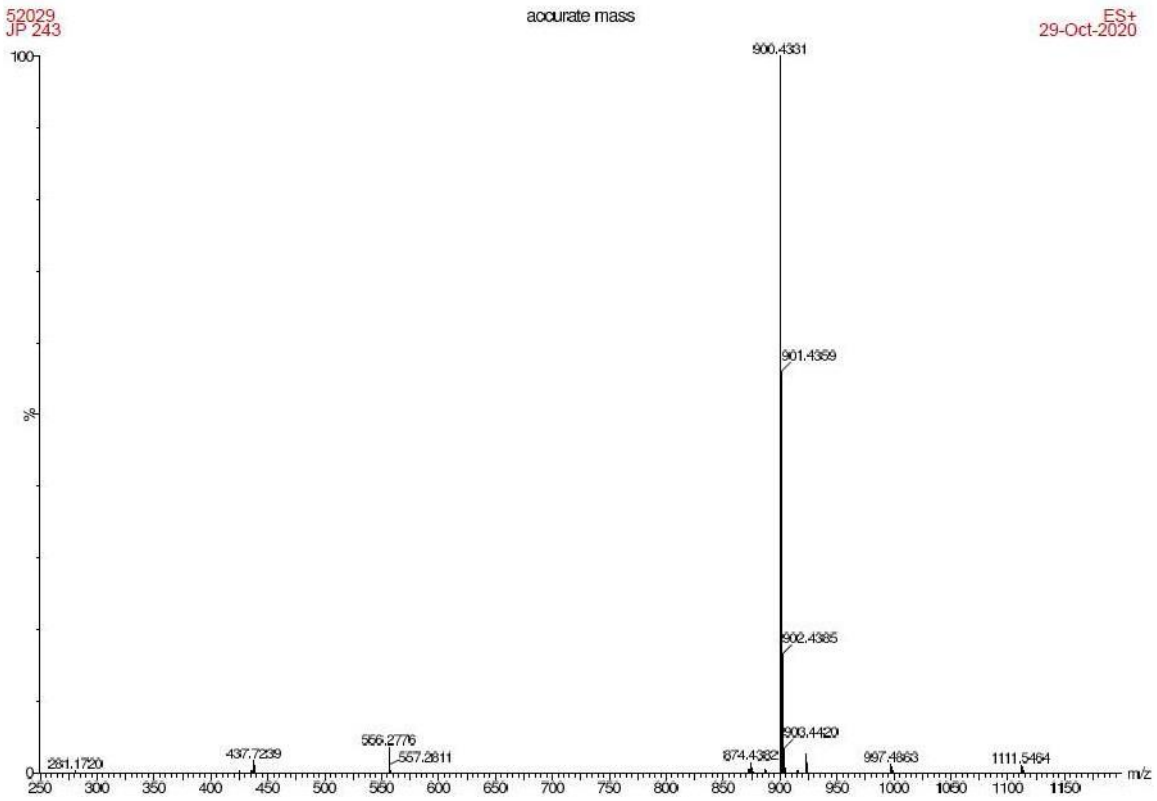
6 Compound 17 – Diastereomer 2

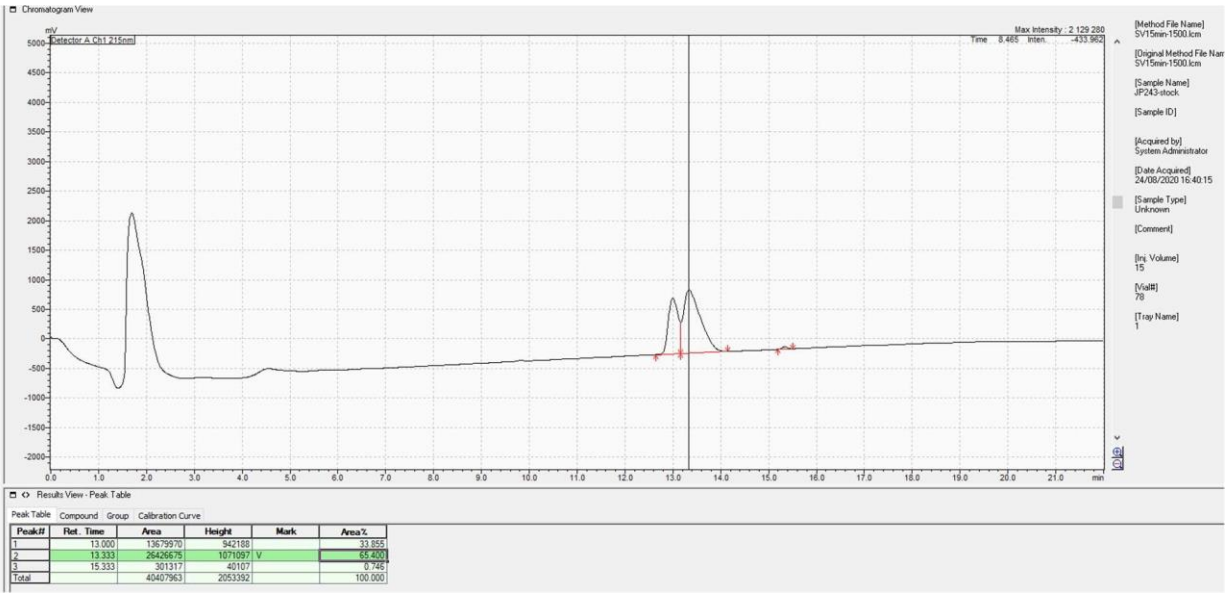




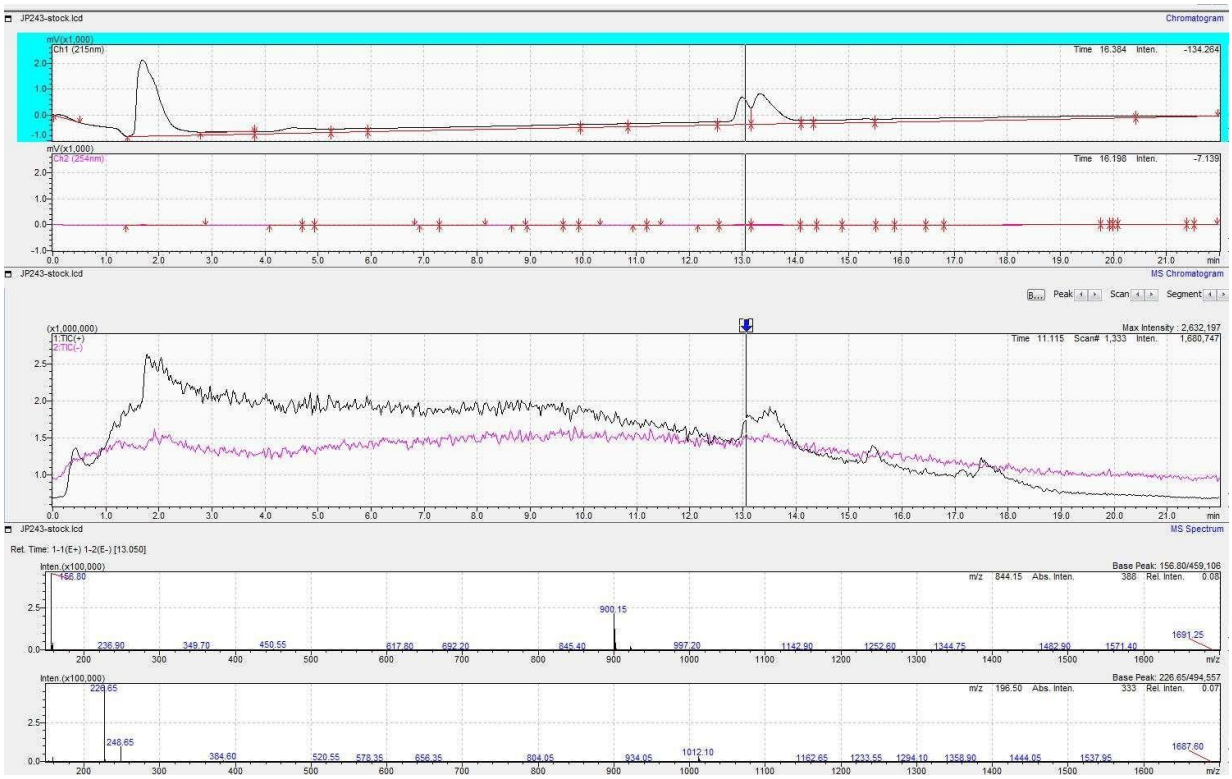

JP242-2-10mM.lcd
Chromatogram
mV(x1,000)
Ch1 (215nm)
Time 15.114 Inten. 762,392
mV(x1,000)
Ch2 (254nm)
Time 15.076 Inten. 59,523
min
JP242-2-10mM.lcd
MS Chromatogram
Peak: Scan: Segment:
(x100,000,000)
1:TIC(+)
2:TIC(-)
Max Intensity : 317,740,692
Time 12.505 Scan# 1,501 Inten. 71,840,768
min
JP242-2-10mM.lcd
MS Spectrum
Ret. Time: 1-1(E+) 1-2(E-) [15.083]
Inten.(x1,000,000)
Base Peak: 477.15/9,683,703
m/z 465.20 Abs. Inten. 88,652 Rel. Inten. 0.92
120.05
156.95
477.15
491.15
260.00
390.35
566.75
671.85
743.90
842.25
916.85
953.35
1054.75
1139.70
1194.50
1273.55
1324.35
1394.75
1478.95
m/z
Inten.(x1,000,000)
Base Peak: 489.20/9,553,639
m/z 252.90 Abs. Inten. 281 Rel. Inten. 0.00
489.20
90.95
158.85
226.85
311.05
381.05
461.10
589.15
635.10
787.40
860.35
965.45
1001.45
1079.40
1148.30
1225.55
1345.70
1491.75
m/z

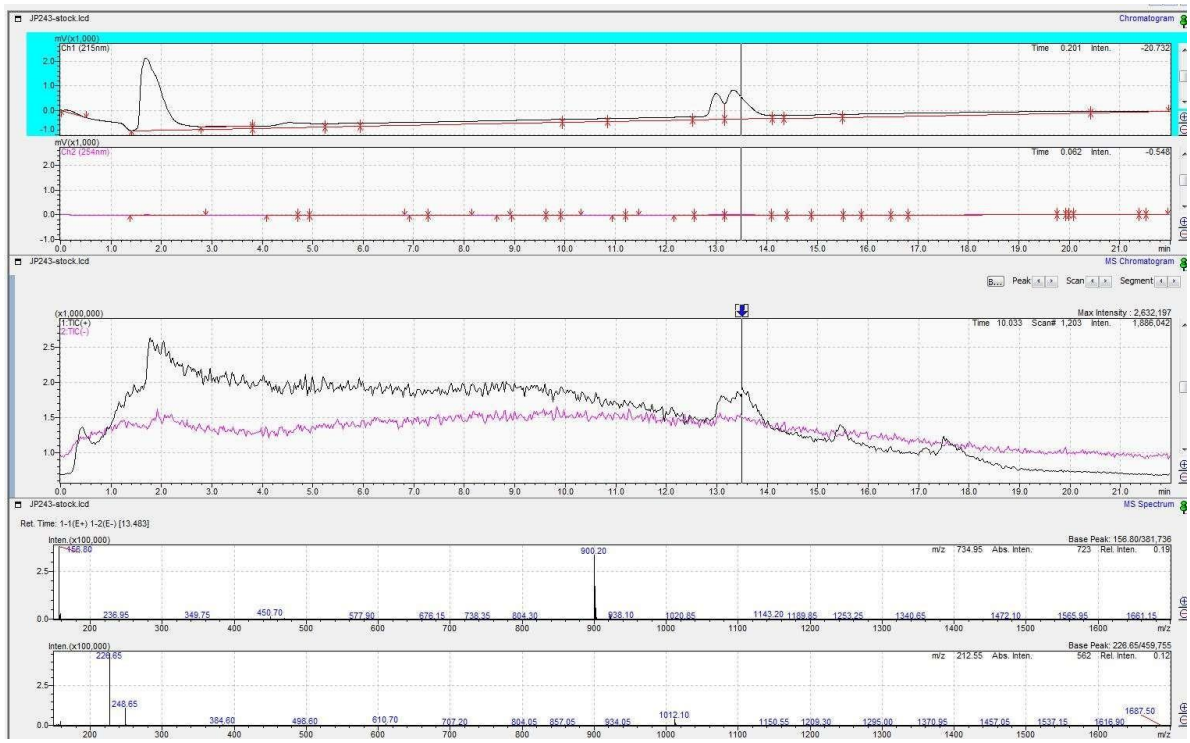
7 Compound 18



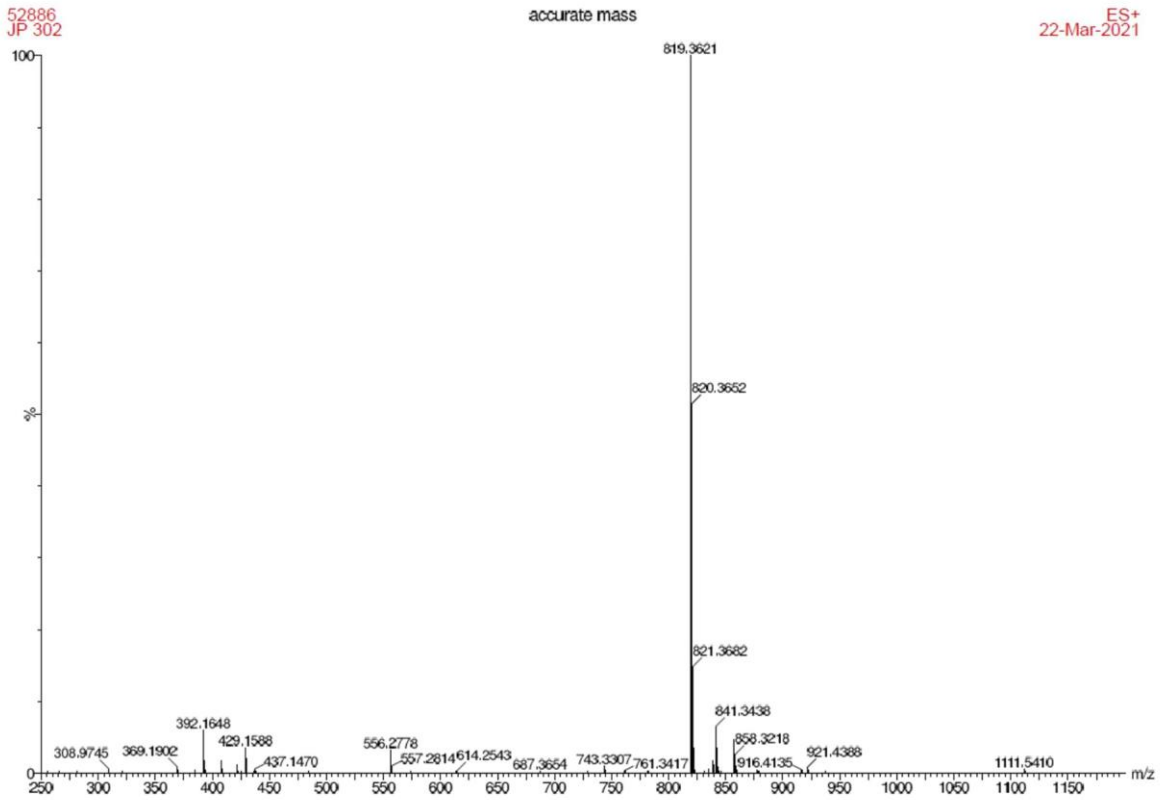


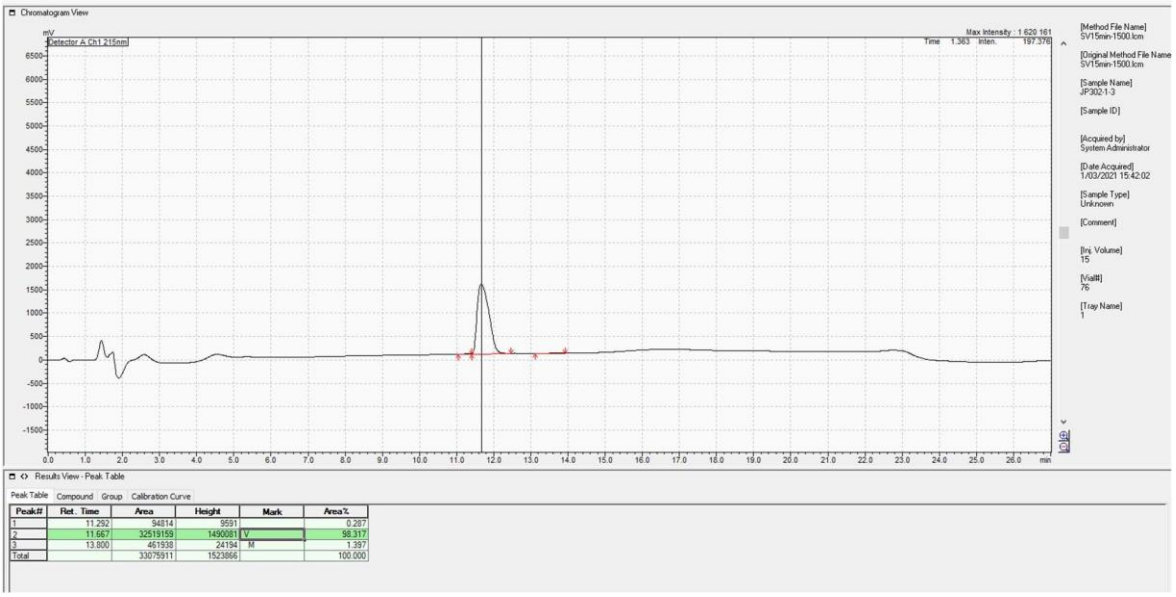
Peak#	Ret. Time	Area	Height	Mark	Area%
1	13.000	13679970	942188		33.855
2	13.333	26426675	1071097	V	65.400
3	15.333	301317	40107		0.746
Total		40407963	2053392		100.000

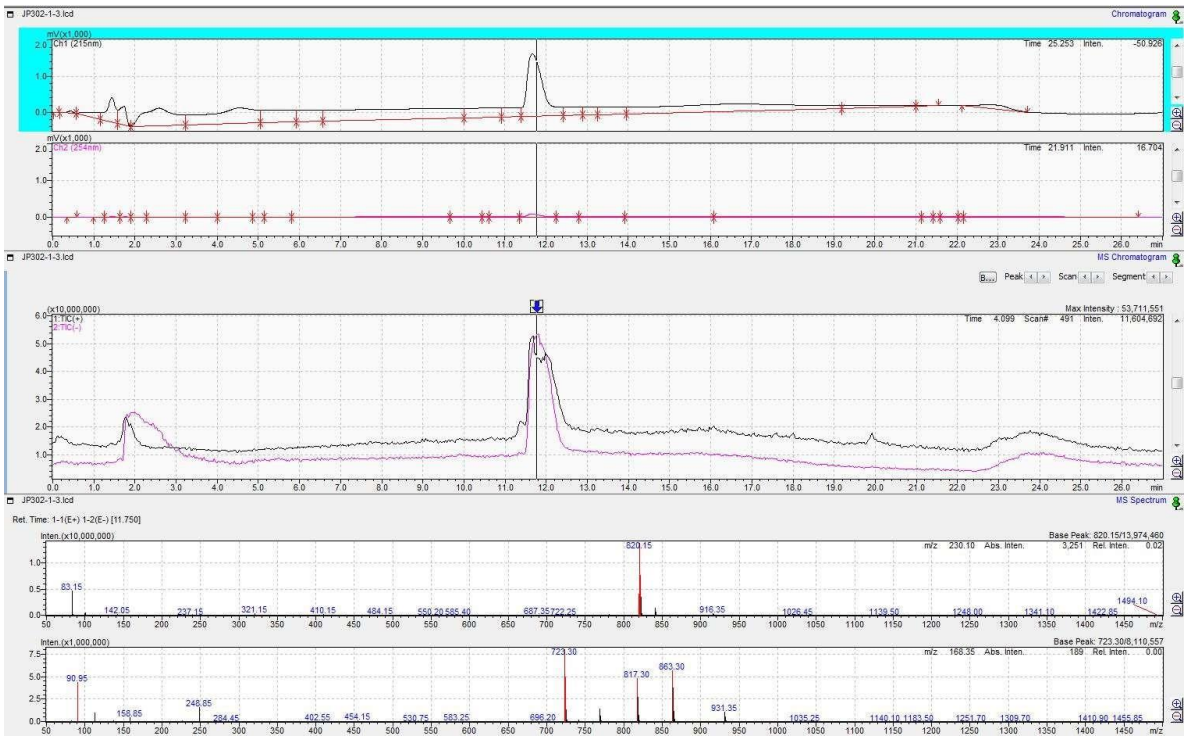

JP243-stock.lcd
Chromatogram
mV(x1,000)
Ch1 (215nm)
Time 16.384 Inten. -134.264
Ch2 (254nm)
Time 16.198 Inten. -7.139
min
MS Chromatogram
Peak Scan Segment
(x1,000,000)
1:TIC(+)
2:TIC(-)
Max Intensity : 2,632,197
Time 11.115 Scan# 1,333 Inten. 1,680,747
MS Spectrum
Ret. Time: 1-1(E+) 1-2(E-) [13.050]
Inten.(x100,000)
Base Peak: 156.80/459,106
m/z 844.15 Abs. Inten. 388 Rel. Inten. 0.08
Base Peak: 226.65/494,557
m/z 196.50 Abs. Inten. 333 Rel. Inten. 0.07


JP243-stock.lcd
Chromatogram
mV(x1,000)
Ch1 (215nm)
Time 0.201 Inten. -20.732
Ch2 (254nm)
Time 0.062 Inten. -0.548
min
MS Chromatogram
Peak Scan Segment
(x1,000,000)
1:TIC(+)
2:TIC(-)
Max Intensity : 2,632,197
Time 10.033 Scan# 1,203 Inten. 1,886,042
MS Spectrum
Ret. Time: 1-1(E+) 1-2(E-) [13.483]
Inten.(x100,000)
Base Peak: 156.80/381,736
m/z 734.95 Abs. Inten. 723 Rel. Inten. 0.19
Base Peak: 226.65/459,755
m/z 212.55 Abs. Inten. 562 Rel. Inten. 0.12

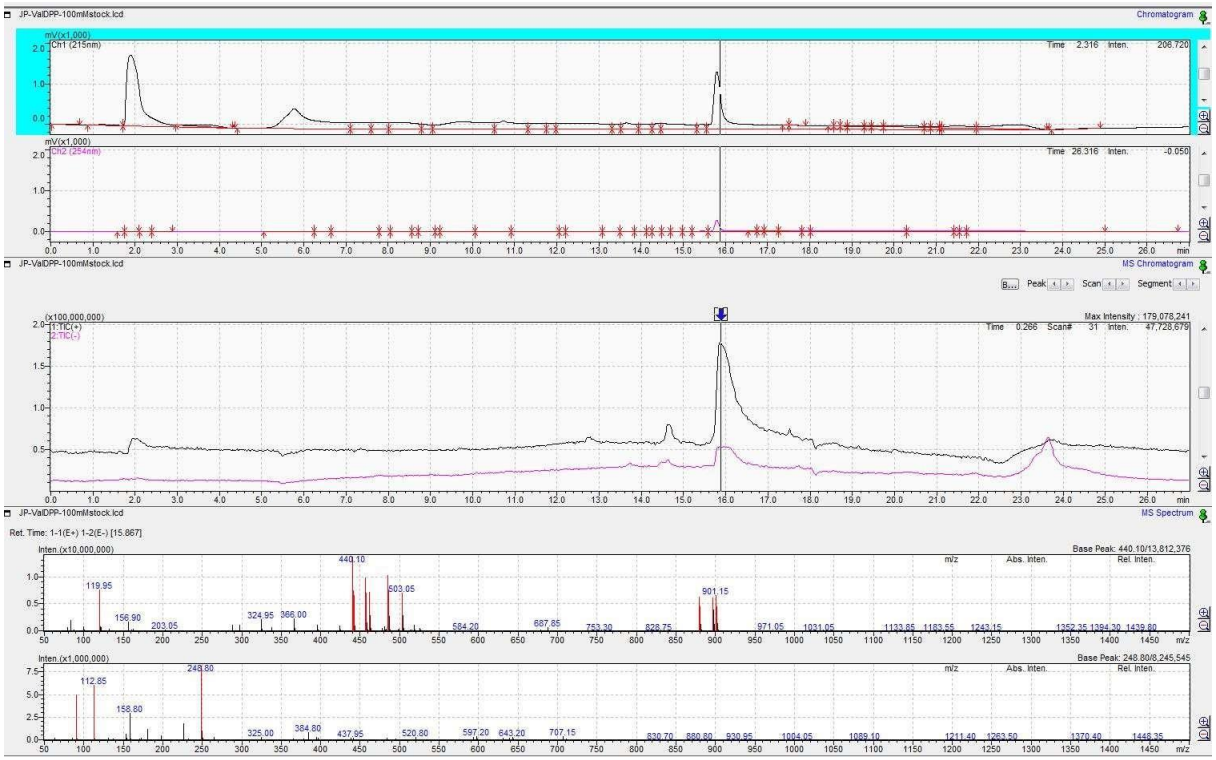
8 Compound S2

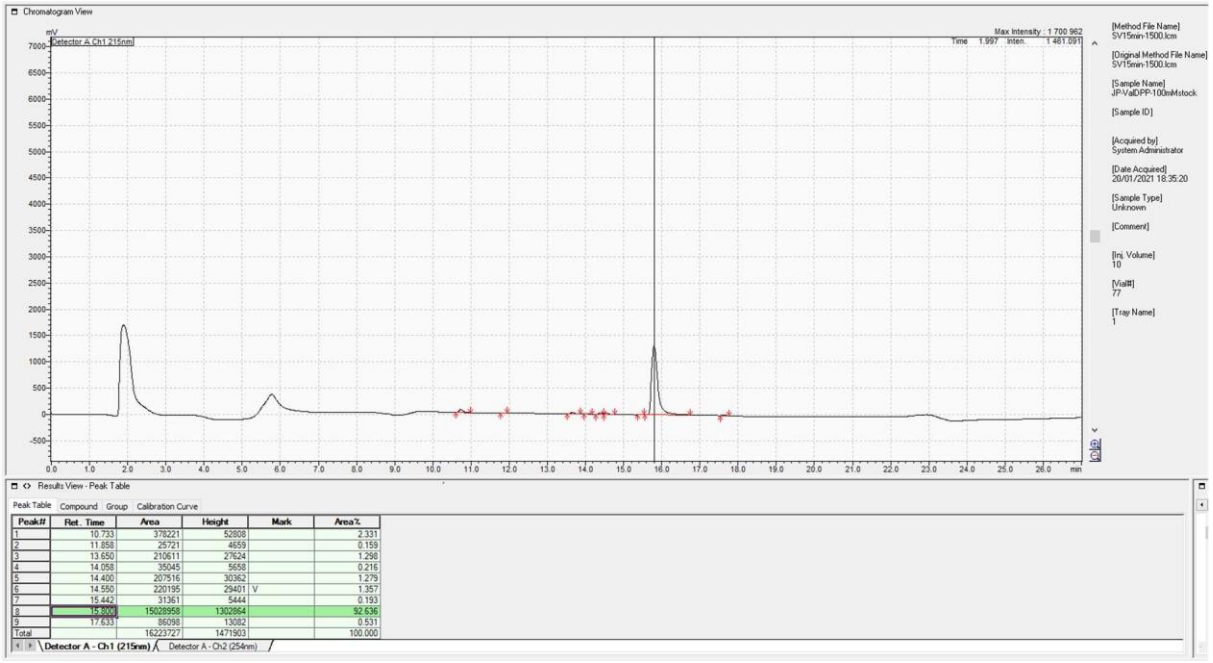





JP302-1-3.lcd
Chromatogram
mV(x1,000)
Ch1 (215nm)
Time 25.253 Inten. -50,926
mV(x1,000)
Ch2 (254nm)
Time 21.911 Inten. 16,704
min
JP302-1-3.lcd
MS Chromatogram
Peak
Scan
Segment
(x10,000,000)
1:TIC(+)
2:TIC(-)
Max Intensity : 53,711,551
Time 4.099 Scan# 491 Inten. 11,604,692
min
JP302-1-3.lcd
MS Spectrum
Ret. Time: 1-1(E+) 1-2(E-) [11.750]
Inten.(x10,000,000)
Base Peak: 820.15/13,974,460
m/z 230.10 Abs. Inten. 3,251 Rel. Inten. 0.02
820.15
83.15
1494.10
Inten.(x1,000,000)
Base Peak: 723.30/8,110,557
m/z 168.35 Abs. Inten. 189 Rel. Inten. 0.00
723.30
817.30
863.30
90.95
931.35
m/z

9 Protease inhibitor CbzNH-Val^{P}

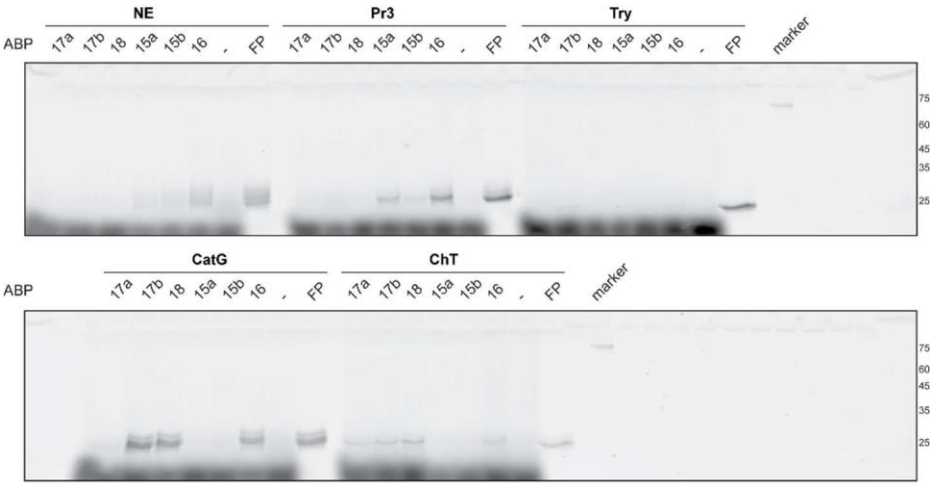




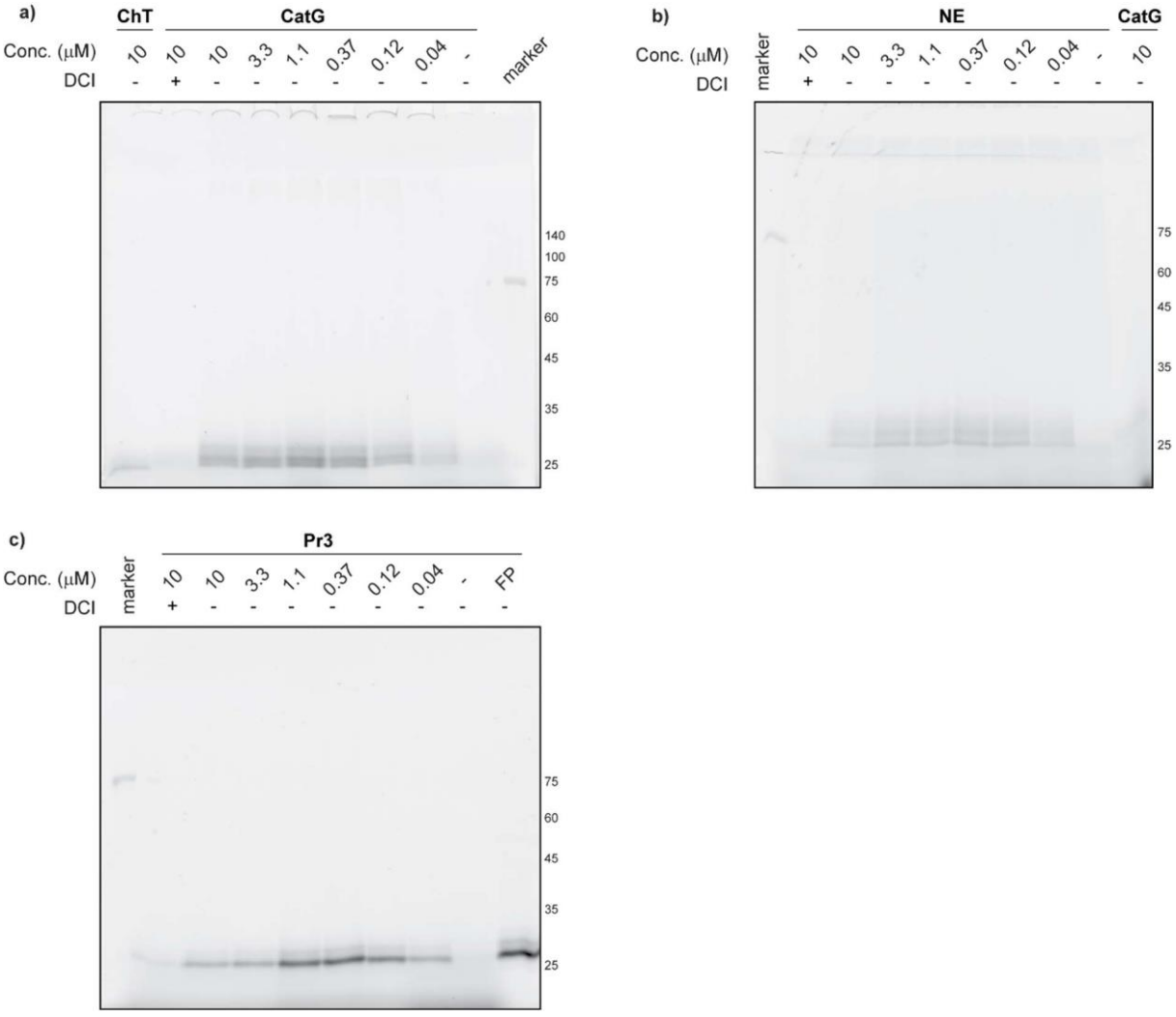
Peak#	Ret. Time	Area	Height	Mark	Area%
1	10.733	378221	52808		2.331
2	11.858	26721	4659		0.159
3	13.650	210611	27624		1.298
4	14.058	35045	5658		0.216
5	14.400	207516	30362		1.279
6	14.550	220195	29401	V	1.357
7	15.442	31361	5444		0.193
8	15.800	15028958	1302864		92.636
9	17.633	86098	13082		0.531
Total		16223727	1471903		100.000

Images of full SDS-PAGE gels

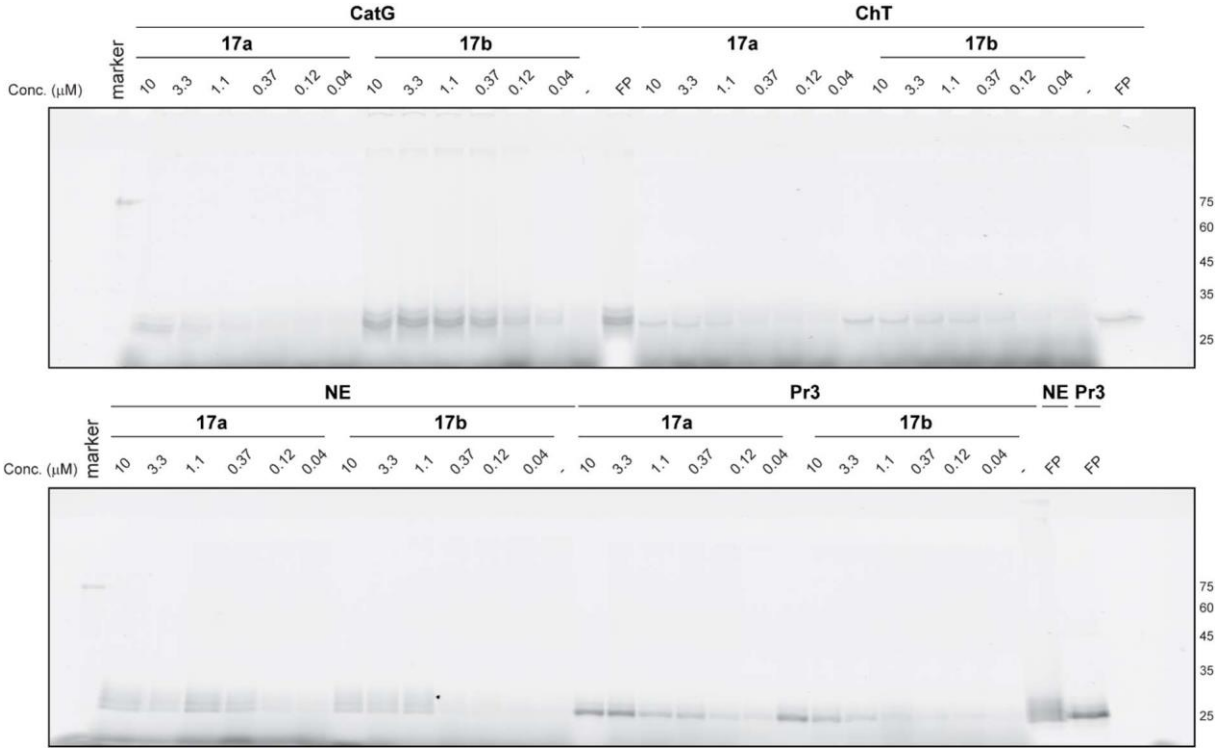
Selectivity determination (shown in **Fig. 2a**)



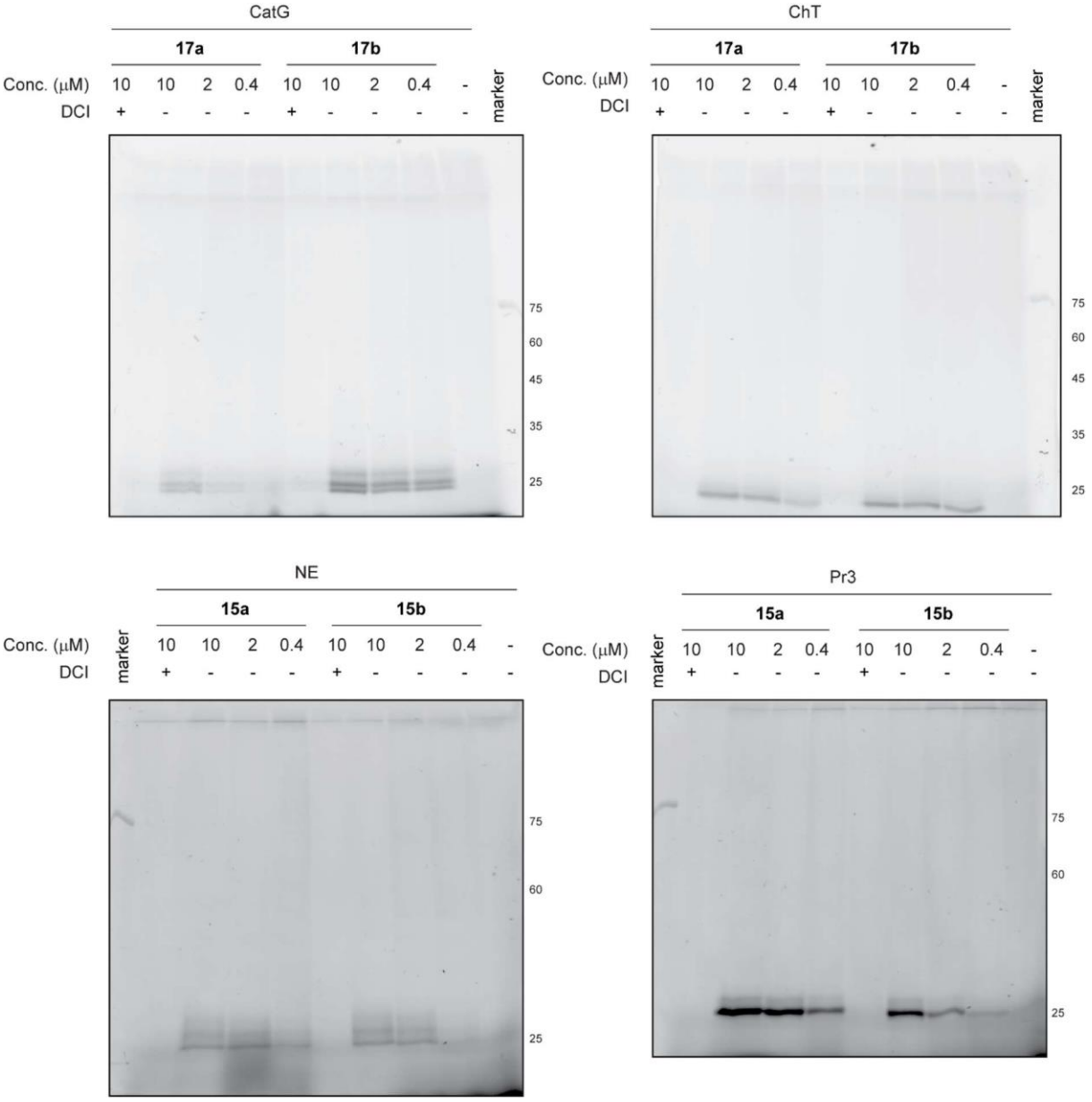
Titration of P4 probes **18** (a) and **16** (b and c) on purified Cat and NE/Pr3, respectively (shown in **Fig. 2b** and **c**)



Titration of P1 probes on purified CatG, ChT, NE and Pr3 (shown in **Fig. 2d** and **e**)



Labelling of purified enzymes by different diastereomers of P1 probes (shown in **Fig. S1**)



Supplementary references

1. Kahler, J. P., Lenders, S., van de Plassche, M. A. T. & Verhelst, S. H. L. Facile Synthesis of Aminomethyl Phosphinate Esters as Serine Protease Inhibitors with Primed Site Interaction. *ACS Med. Chem. Lett.* 0–5 (2020) doi:10.1021/acsmedchemlett.0c00284.

2. Oh, H., Siano, B. & Diamond, S. Neutrophil isolation protocol. *J. Vis. Exp.* 745 (2008) doi:10.3791/745.

3. Schindelin, J. *et al.* Fiji : an open-source platform for biological-image analysis. **9**, (2019).