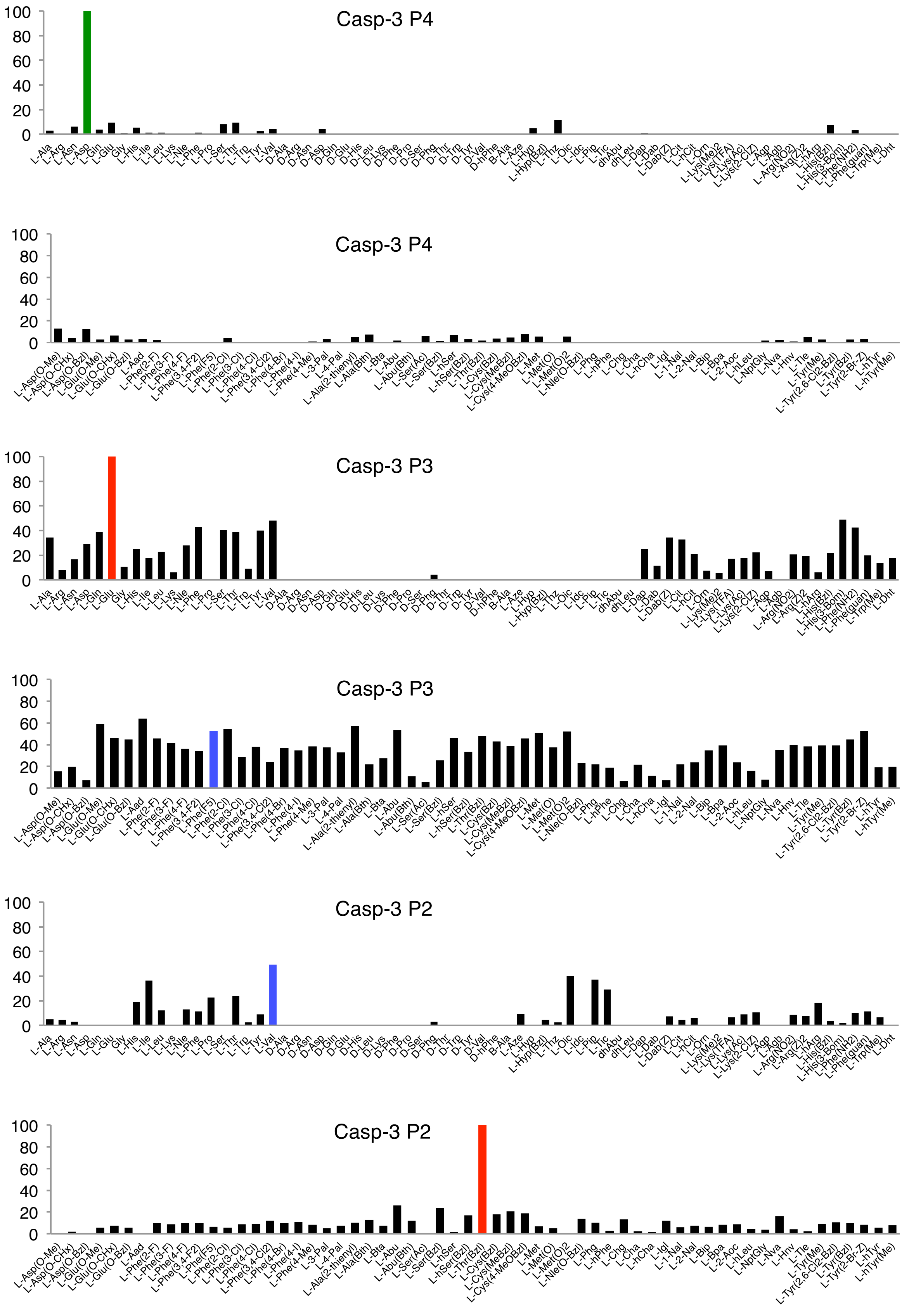
Unnatural amino acids increase sensitivity and provide for the design of highly selective caspase substrates

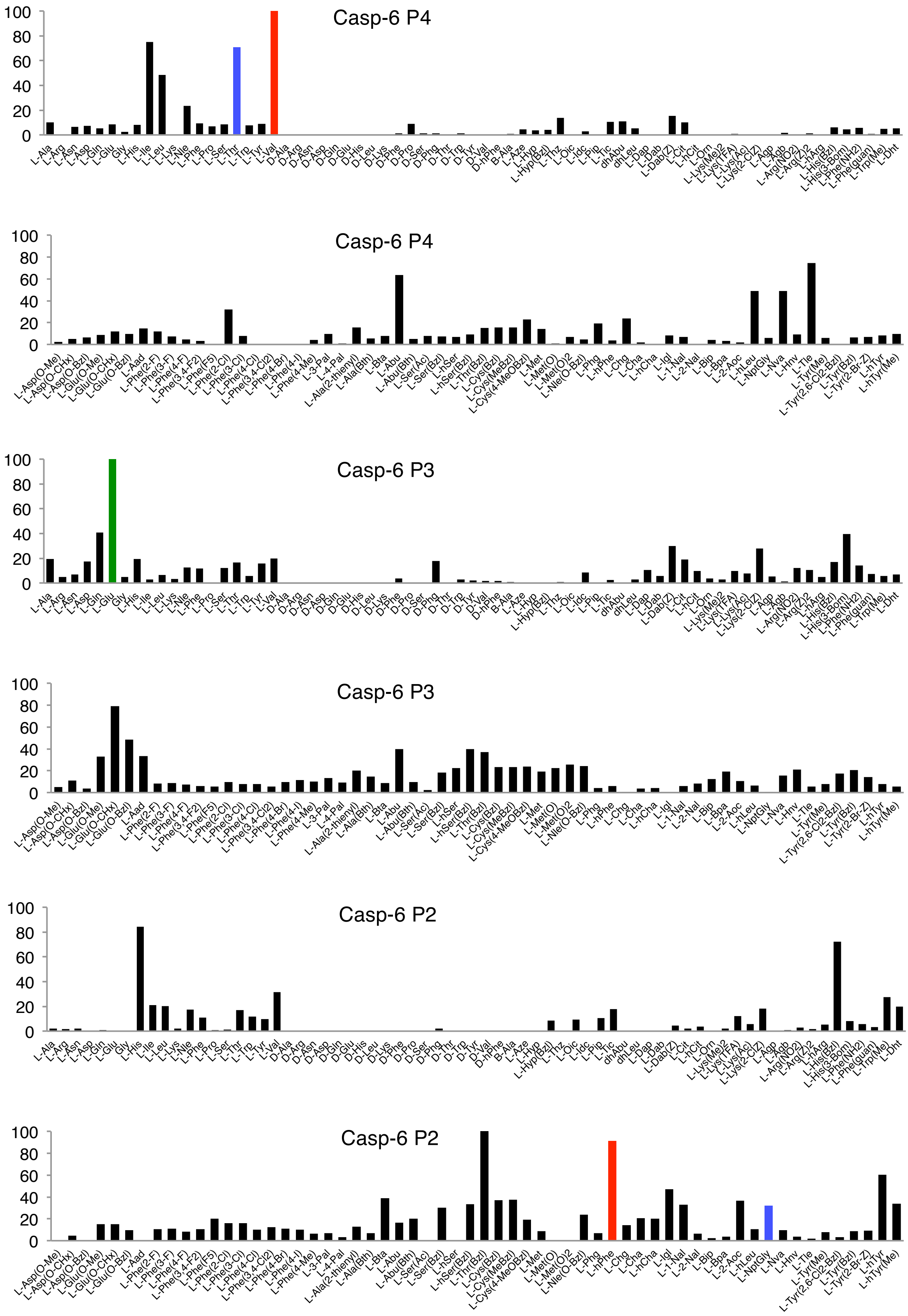
Marcin Poreba[1], Paulina Kasperkiewicz[1], Scott J. Snipas[2], Domenico Fasci[2], Guy S. Salvesen[2,*], Marcin Drag[1,2,*]

[1]Division of Bioorganic Chemistry, Faculty of Chemistry, Wroclaw University of Technology, Wybrzeze Wyspianskiego 27, 50-370 Wroclaw, Poland; [2]Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA

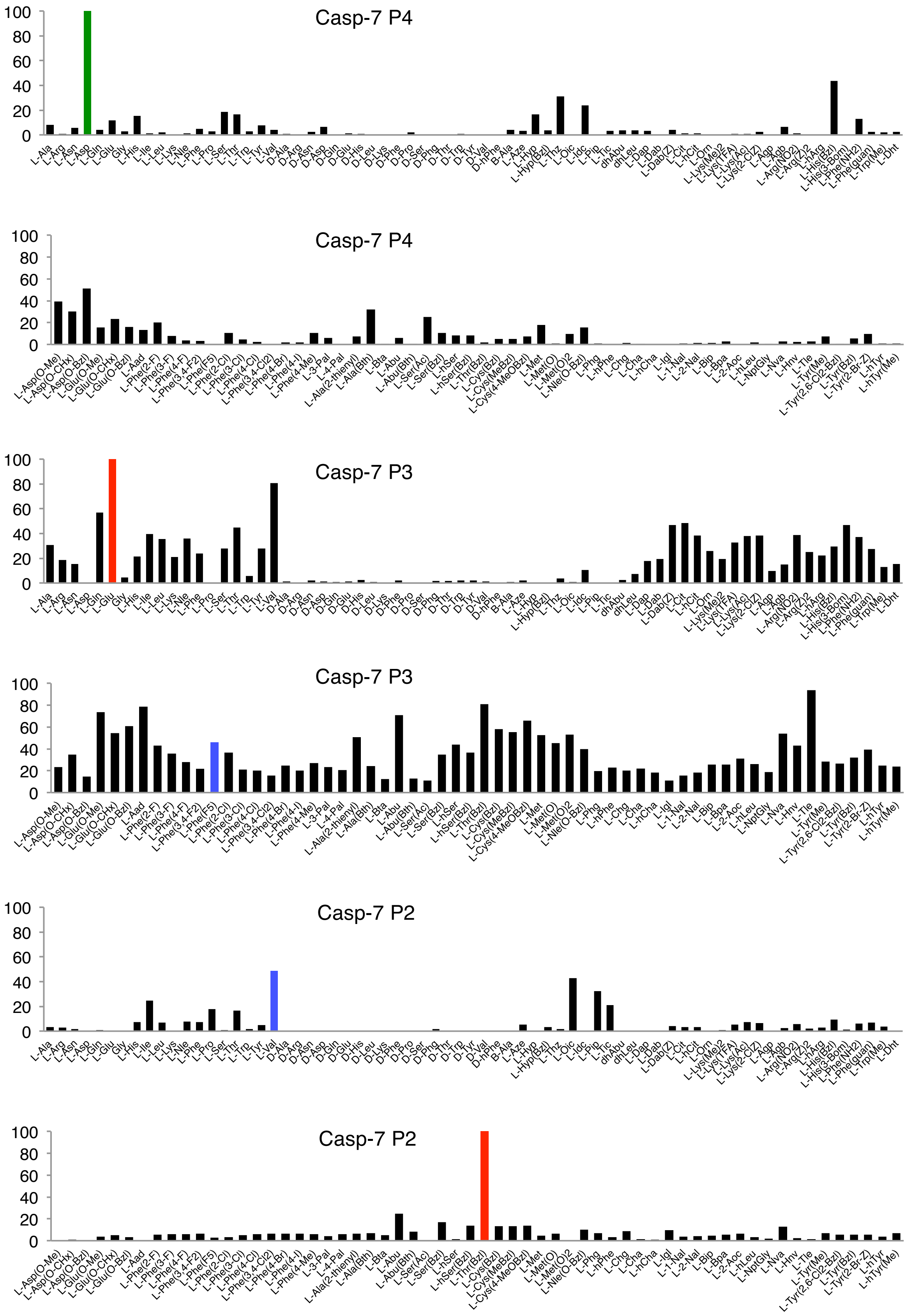
Supplemental section



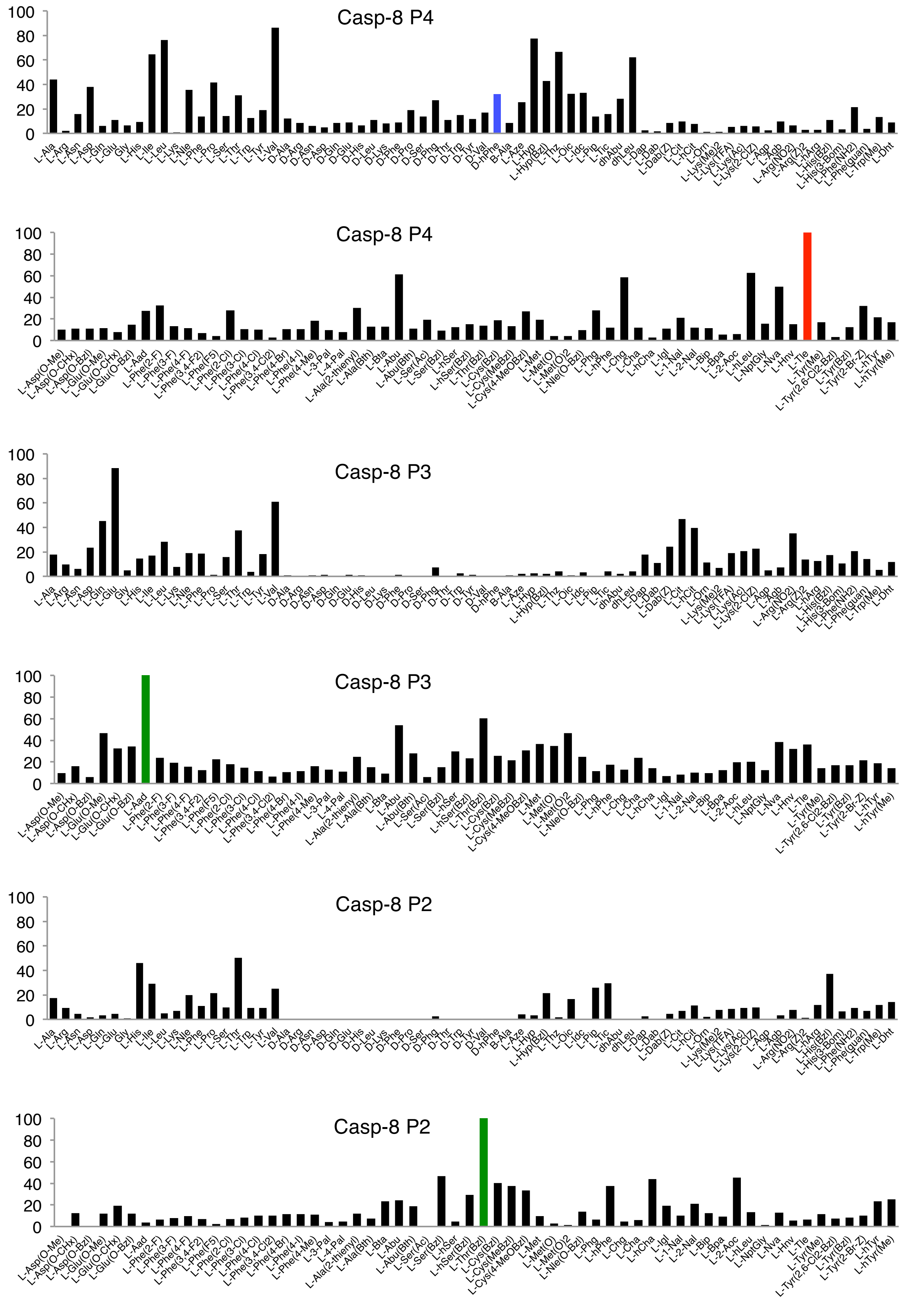
Supplementary Figure 1. Caspase 3 preferences at the P4, P3 and P2. The S4, S3, S2 subsites preferences of recombinant human caspase 3 were determined using three hybrid combinatorial sub-libraries with the general composition: Ac-P4-X-X-Asp-ACC, Ac-X-P3-X-Asp-ACC and Ac-X-X-P2-Asp-ACC, where P4, P3, and P2 are natural and unnatural amino acids, and X is equimolar mixture of 19 natural amino acids (cysteine was omitted and methionine was replaced by norleucine). Abbreviated amino acids names shown on the x axis. The y axis displays the average relative activity expressed as a percentage of the best amino acid. Standard deviations calculated from three screenings were below 10% of values shown in the figure. Red color represents the amino acids selected for most sensitive substrate synthesis, blue for most selective, and green for both sensitive and selective.



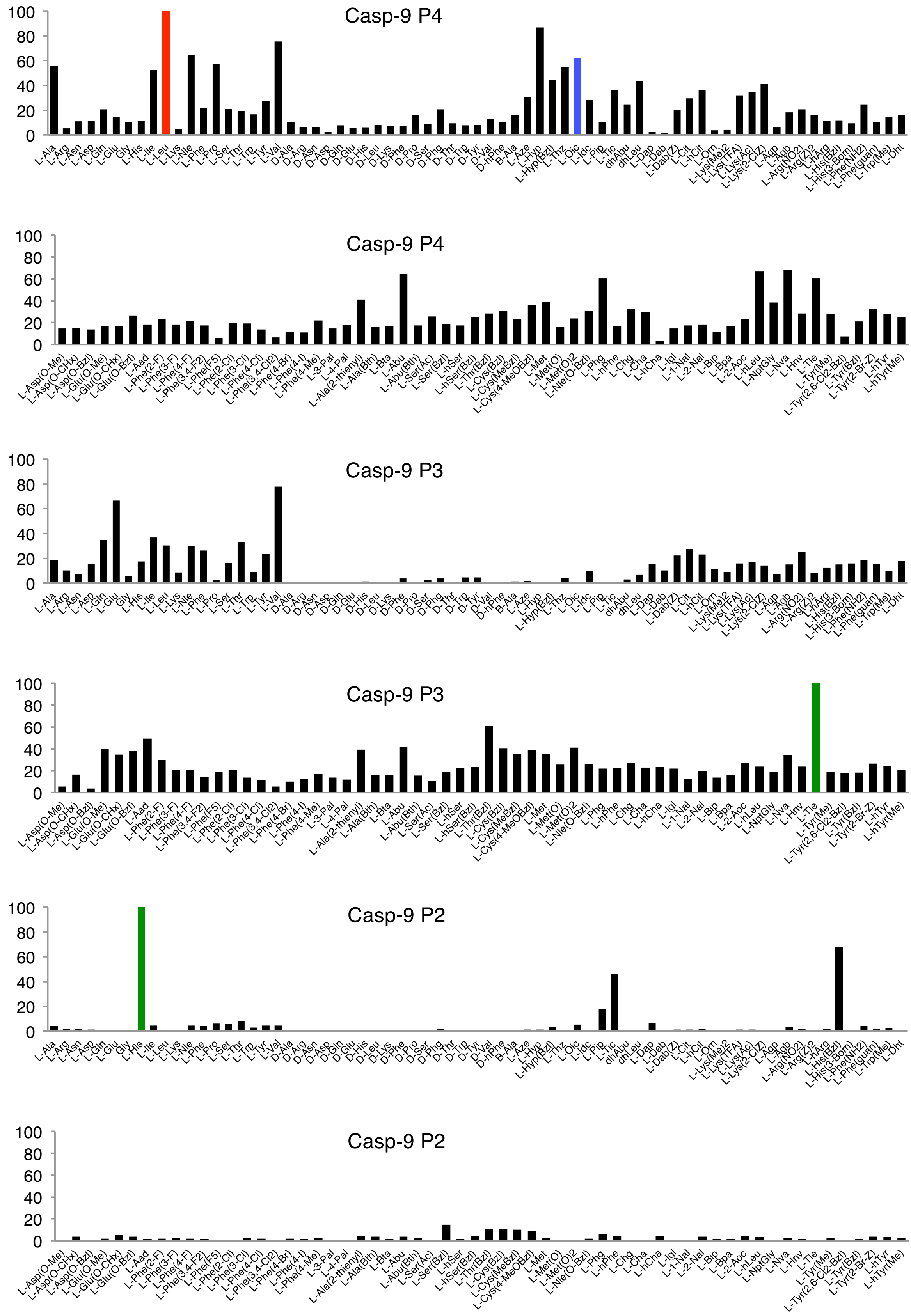
Supplementary Figure 2. Caspase 6 substrate preferences at P4, P3 and P2. The S4, S3, S2 subsites preferences of recombinant human caspase 6 were determined using three hybrid combinatorial sub-libraries with the general composition: Ac-P4-X-X-Asp-ACC, Ac-X-P3-X-Asp-ACC and Ac-X-X-P2-Asp-ACC, where P4, P3, and P2 are natural and unnatural amino acids, and X is equimolar mixture of 19 natural amino acids (cysteine was omitted and methionine was replaced by norleucine). Abbreviated amino acids names shown on the x axis. The y axis displays the average relative activity expressed as a percentage of the best amino acid. Standard deviations calculated from three screenings were below 10% of values shown in the figure. Red color represents the amino acids selected for most sensitive substrate synthesis, blue for most selective, and green for both sensitive and selective.



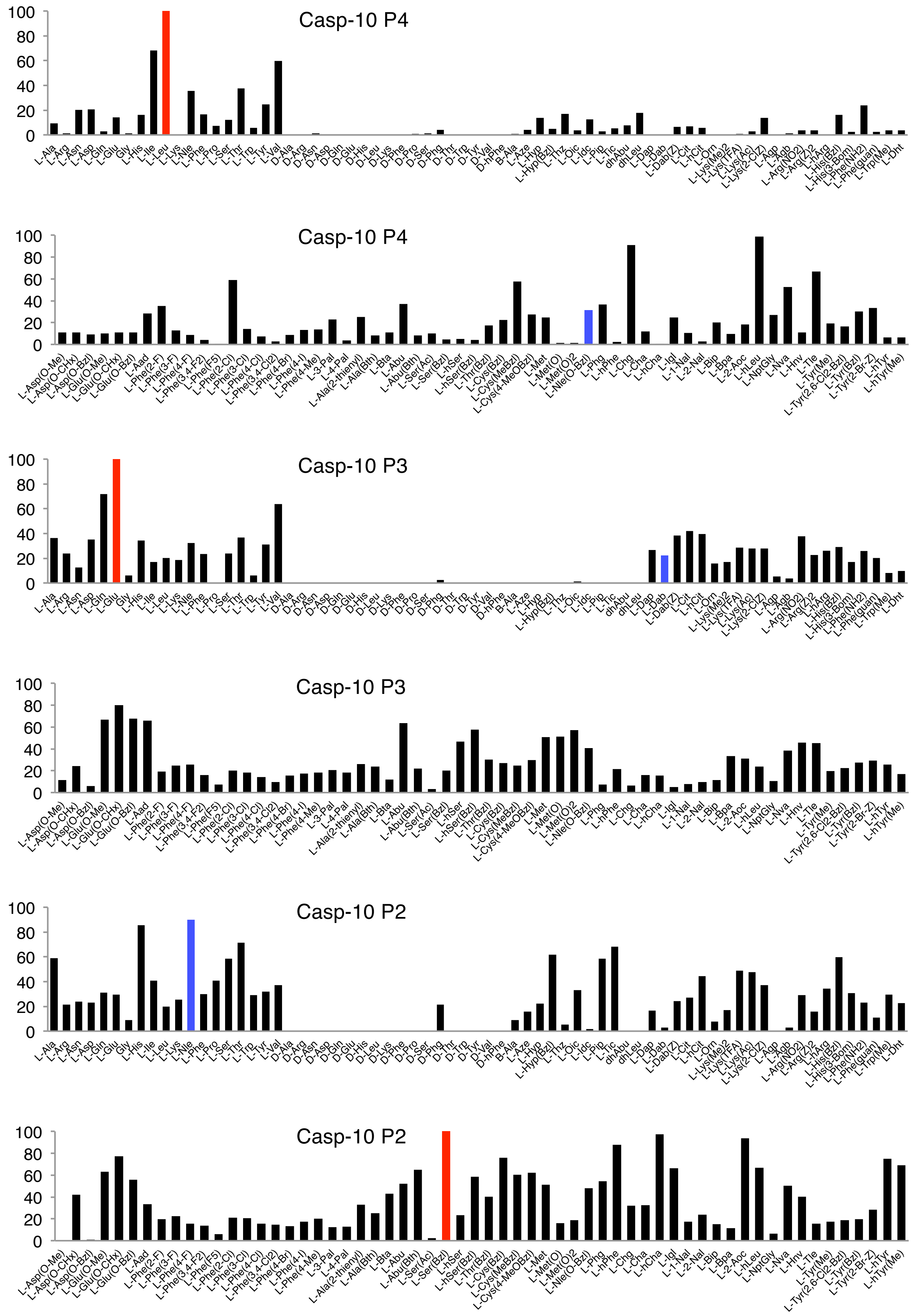
Supplementary Figure 3. Caspase 7 substrate preferences at P4, P3 and P2. The S4, S3, S2 subsites preferences of recombinant human caspase 7 were determined using three hybrid combinatorial sub-libraries with the general composition: Ac-P4-X-X-Asp-ACC, Ac-X-P3-X-Asp-ACC and Ac-X-X-P2-Asp-ACC, where P4, P3, and P2 are natural and unnatural amino acids, and X is equimolar mixture of 19 natural amino acids (cysteine was omitted and methionine was replaced by norleucine). Abbreviated amino acids names are shown on the x axis. The y axis displays the average relative activity expressed as a percentage of the best amino acid. Standard deviations calculated from three screenings were below 10% of values shown in the figure. Red color represents the amino acids selected for most sensitive substrate synthesis, blue for most selective, and green for both sensitive and selective.



Supplementary Figure 4. Caspase 8 substrate preferences at P4, P3 and P2. The S4, S3, S2 subsites preferences of recombinant human caspase 8 were determined using three hybrid combinatorial sub-libraries with the general composition: Ac-P4-X-X-Asp-ACC, Ac-X-P3-X-Asp-ACC and Ac-X-X-P2-Asp-ACC, where P4, P3, and P2 are natural and unnatural amino acids, and X is equimolar mixture of 19 natural amino acids (cysteine was omitted and methionine was replaced by norleucine). Abbreviated amino acids names are shown on the x axis. The y axis displays the average relative activity expressed as a percentage of the best amino acid. Standard deviations calculated from three screenings were below 10% of values shown in the figure. Red color represents the amino acids selected for most sensitive substrate synthesis, blue for most selective, and green for both sensitive and selective



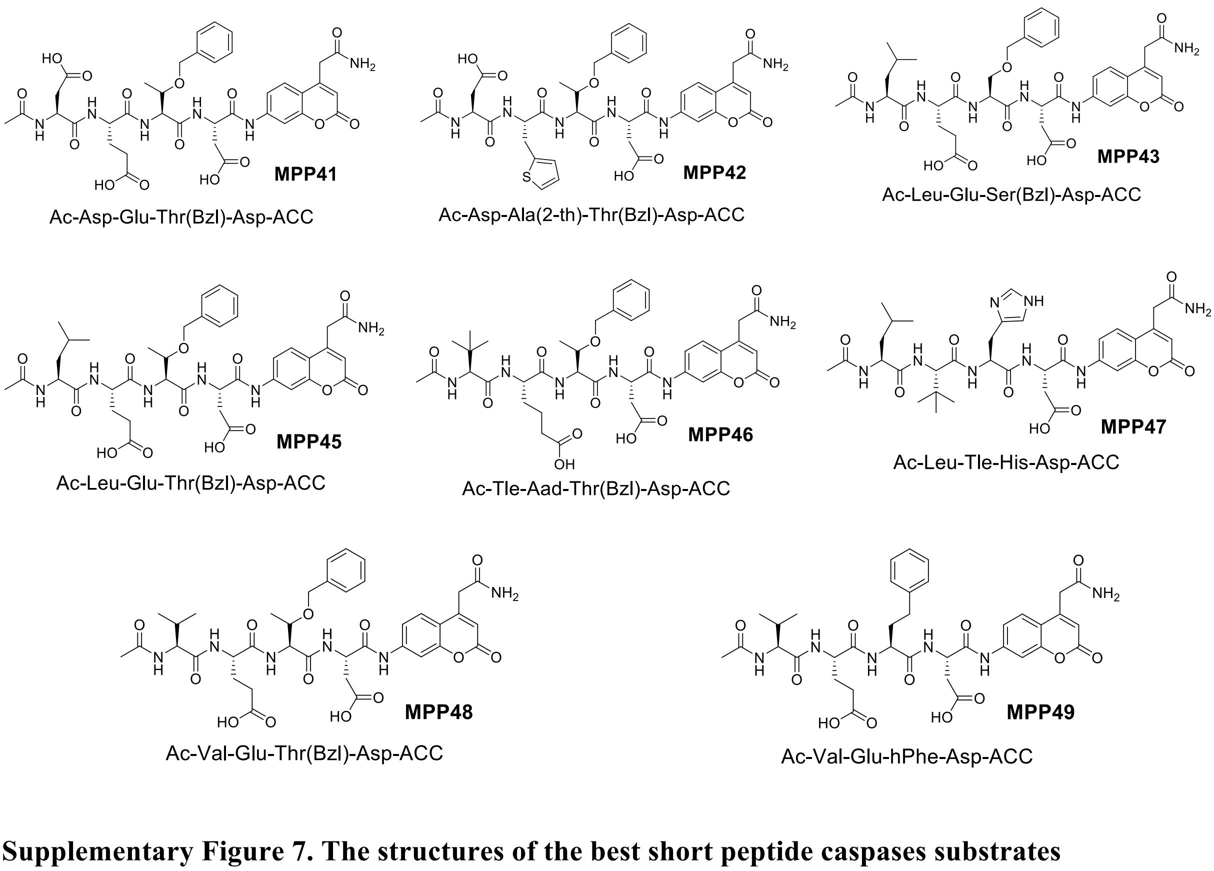
Supplementary Figure 5. Caspase 9 substrate preferences at P4, P3 and P2. The S4, S3, S2 subsites preferences of recombinant human caspase 9 were determined using three hybrid combinatorial sub-libraries with the general composition: Ac-P4-X-X-Asp-ACC, Ac-X-P3-X-Asp-ACC and Ac-X-X-P2-Asp-ACC, where P4, P3, and P2 are natural and unnatural amino acids, and X is equimolar mixture of 19 natural amino acids (cysteine was omitted and methionine was replaced by norleucine). Abbreviated amino acids names are shown on the x axis. The y axis displays the average relative activity expressed as a percentage of the best amino acid. Standard deviations calculated from three screenings were below 10% of values shown in the figure. Red color represents the amino acids selected for most sensitive substrate synthesis, blue for most selective, and green for both sensitive and selective.



Supplementary Figure 6. Caspase 10 substrate preferences at P4, P3 and P2. The S4, S3, S2 subsites preferences of recombinant human caspase 10 were determined using three hybrid combinatorial sub-libraries with the general composition: Ac-P4-X-X-Asp-ACC, Ac-X-P3-X-Asp-ACC and Ac-X-X-P2-Asp-ACC, where P4, P3, and P2 are natural and unnatural amino acids, and X is equimolar mixture of 19 natural amino acids (cysteine was omitted and methionine was replaced by norleucine). Abbreviated amino acids names shown on the x axis. The y axis displays the average relative activity expressed as a percentage of the best amino acid. Standard deviations calculated from three screenings were below 10% of values shown in the figure. Red color represents the amino acids selected for most sensitive substrate synthesis, blue for most selective, and green for both sensitive and selective.

substrate	enzyme	k_{cat}, s^{-1}	K_M, μM	k_{cat}/K_M, $M^{-1}s^{-1}$
MPP41	Casp-3	30.1	28.2	1,050,000
MPP42	Casp-3	27.8	33.5	825,000
MPP48	Casp-6	4.08	23.0	186,300
MPP49	Casp-6	2.94	12.6	229,000
MPP41	Casp-7	6.88	41.6	167,000
MPP42	Casp-7	9.66	61.5	159,000
MPP45	Casp-8	0.361	1.04	349,000
MPP46	Casp-8	0.717	1.31	525,300
MPP47	Casp-9	1.18	32.1	37,000
MPP43	Casp-10	1.59	9.11	176,200

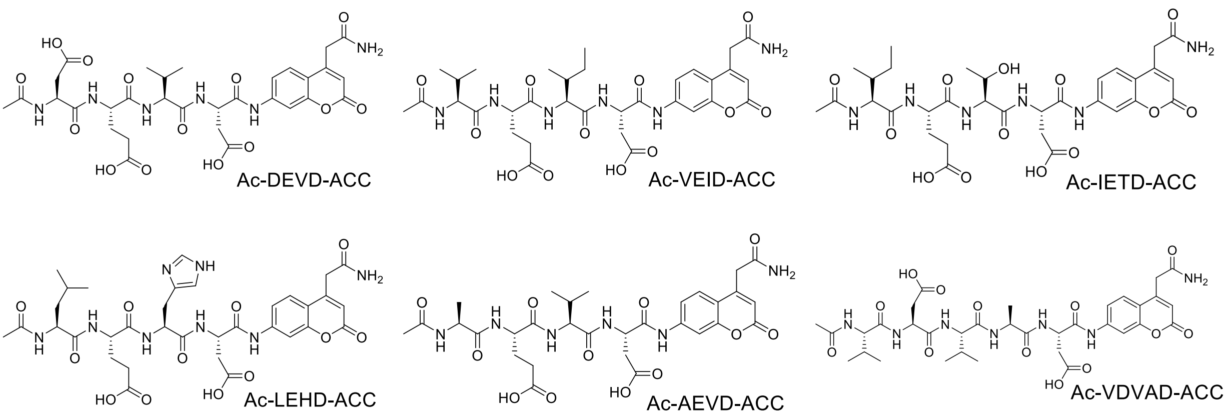
Supplementary Table 1. Kinetic analysis of the best caspases substrates containing unnatural amino acids.



Supplementary Figure 7. The structures of the best short peptide caspases substrates

sequence	enzyme		Casp-3	Casp-6	Cas-7	Casp-8	Casp-9	Casp-10
DEVD	Casp-3/7	K_M, μM	20.7	473	49.6	6.94	104	24.3
		k_{cat}, s^{-1}	10.0	0.642	3.92	0.496	0.033	0.867
		k_{cat}/K_M, $M^{-1}s^{-1}$	474,400	1,360	82,000	71,600	310	34,600
VEID	Casp-6	K_M, μM	218	45.6	285	2.12	25.6	20.9
		k_{cat}, s^{-1}	3.26	2.02	0.553	0.300	0.078	1.25
		k_{cat}/K_M, $M^{-1}s^{-1}$	14,700	44,800	1,870	14,100	3,300	58,400
IETD	Casp-8	K_M, μM	156	141	401	6.43	59.0	20.2
		k_{cat}, s^{-1}	0.962	1.72	0.121	0.607	0.144	1.76
		k_{cat}/K_M, $M^{-1}s^{-1}$	6,120	12,200	300	94,900	2,450	88,200
LEHD	Casp-9	K_M, μM	163	209	419	13.7	102	26.3
		k_{cat}, s^{-1}	1.61	2.83	0.305	2.54	1.35	3.87
		k_{cat}/K_M, $M^{-1}s^{-1}$	9,670	13,600	730	186,000	12,700	143,700
AEVD	Casp-10	K_M, μM	126	n.d.	143	8.59	57.8	33.1
		k_{cat}, s^{-1}	4.04	n.d.	2.64	0.686	0.136	0.510
		k_{cat}/K_M, $M^{-1}s^{-1}$	32,900	1,100	18,500	80,200	2,300	15,700
VDVAD	Casp-2	K_M, μM	30.2	n.d.	98.5	48.5	110	27.9
		k_{cat}, s^{-1}	3.46	n.d.	1.47	0.868	0.00941	0.710
		k_{cat}/K_M, $M^{-1}s^{-1}$	115,000	100	14,900	17,900	80	25,600

Supplementary Table 2. Kinetic analysis of caspases fluorogenic substrates (Ac-P4-P3-P2-Asp-ACC) with reference tetrapeptide sequences and a caspase 2 reference substrate (Ac-VDVAD-ACC). K_M values for Ac-AEVD-ACC and Ac-VDVAD-ACC substrates for caspase 6 were above 500 μM, so we calculated only k_{cat}/K_M parameter (see Equation 2 in Supplementary Figure 12), n.d. not determined.



Supplementary Figure 8. Reference structures of generally used peptidyl caspase-targeting sequences.

substrate	enzyme	Casp-3	Casp-6	Casp-7	Casp-8	Casp-9	Casp-10
MPP38	Casp-3/7	**318,200**	96	**71,600**	2,654	27	2,020
MPP39		**181,000**	36	**40,500**	1,500	16	300
MPP40		**238,000**	39	**38,000**	1,430	7	1,560
MPP36	Casp-6	1,343	**26,600**	560	590	0	5,800
MPP28	Casp-8	520	110	200	**53,400**	600	7,800
MPP30		2,100	140	920	**137,500**	1,880	2,600
MPP8	Casp-9	0	30	0	2,600	**32,200**	960
MPP10		360	350	45	1,400	**27,500**	3,200
MPP12		0	0	0	1,580	**31,500**	1,120
MPP17	Casp-10	3,650	4,300	950	5,400	400	**48,600**
MPP21		40	300	74	380	490	**12,600**
MPP50		580	1,600	34	11,100	1,090	**59,800**
MPP51		120	25	0	10,800	590	**62,400**
MPP52		70	20	36	130	320	**10,300**
MPP53		50	86	51	20	160	**5,800**

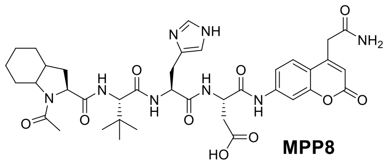
Supplementary Table 3. Kinetic analysis of the most specific substrates tested toward six human recombinant caspases. Values are k_{cat}/K_M with units of $M^{-1}s^{-1}$.

substrate	enzyme	k_{cat}, s^{-1}	K_M, μM	k_{cat}/K_M, $M^{-1}s^{-1}$
MPP38	Caspase-3	8.34	26.2	318,000
MPP39		6.44	35.6	181,000
MPP40		8.03	33.8	238,000
MPP36	Caspase-6	1.63	59.6	26,600
MPP38	Caspase-7	2.11	29.4	71,600
MPP39		2.01	51.8	40,500
MPP40		2.37	64.3	38,000
MPP28	Caspase-8	0.536	10.1	53,400
MPP30		0.222	1.59	137,000
MPP8	Caspase-9	2.07	68.6	32,200
MPP10		0.348	13.1	27,500
MPP12		0.642	21.1	31,500
MPP17	Caspase-10	1.07	22.6	48,600
MPP21		0.102	7.64	12,600
MPP50		0.204	3.55	59,800
MPP51		0.863	13.9	62,400
MPP52		0.121	12.1	10,300
MPP53		0.072	12.7	5,800

Supplementary Table 4. Detailed kinetic analysis of the most selective caspases substrates.

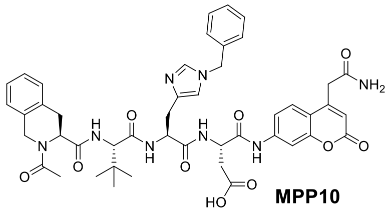
substrate/enzyme	Casp-3	Casp-6	Casp-7	Casp-8	Casp-9	Casp-10
Commercial substrates						
DEVD	**1415**	79	**470**	62	2	120
VEID	172	**371**	16	36	12	188
IETD	60	101	3	**78**	19	326
LEHD	92	307	6	614	**103**	618
AEVD	394	56	134	68	17	**83**
VDVAD	542	0	90	71	1	125
Substrates containing unnatural amino acids						
MPP39	**872**	0	**305**	11	0	3
MPP36	14	**345**	3	5	0	36
MPP28	3	0	0	**94**	5	120
MPP30	34	1	7	**72**	0	3
MPP8	0	0	0	23	**256**	20
MPP10	0	0	0	22	**145**	25
MPP52	0	0	0	0	0	**47**
MPP23	0	0	0	3	1	**26**
MPP50	6	7	0	21	2	**154**

Supplementary Table 5. Selectivity of individual substrates. In this experiment we compared substrates with previously reported sequences with our most selective substrates toward six recombinant caspases. Each substrate concentration was held at 100 μM, and all caspases were used in the final concentration of 50 nM. Results are presented as the initial rate of ACC liberation versus time (RFU/s). Each experiment was performed in standard caspase buffer (buffers for caspases 8, 9, and 10 were supplemented with 1.0 M of sodium citrate).



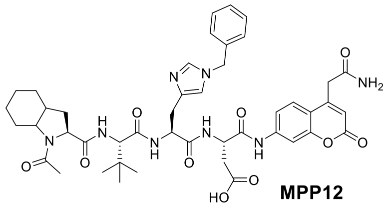
MPP8

Ac-Oic-Tle-His-Asp-ACC



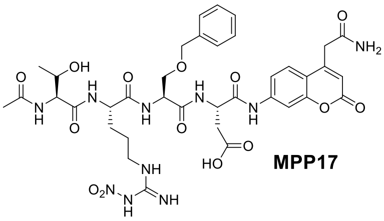
MPP10

Ac-Tic-Tle-His(Bzl)-Asp-ACC



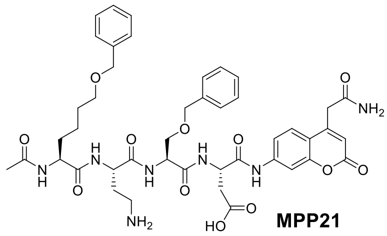
MPP12

Ac-Oic-Tle-His(Bzl)-Asp-ACC



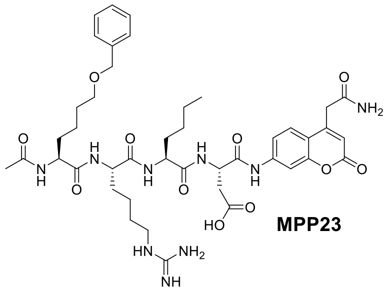
MPP17

Ac-Thr-Arg(NO_2)-Ser(Bzl)-Asp-ACC



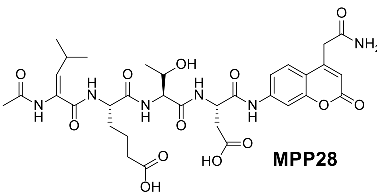
MPP21

Ac-Nle(O-Bzl)-Dab-Ser(Bzl)-Asp-ACC



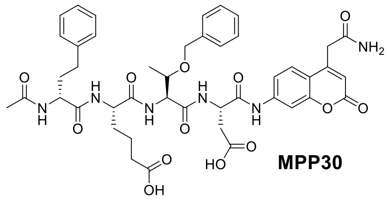
MPP23

Ac-Nle(O-Bzl)-hArg-Nle-Asp-ACC



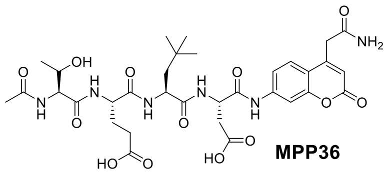
MPP28

Ac-dhLeu-Aad-Thr-Asp-ACC



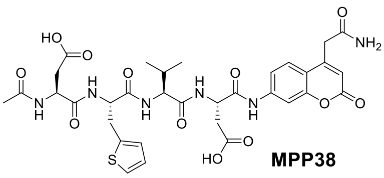
MPP30

Ac-*D*-hPhe-Aad-Thr(Bzl)-Asp-ACC



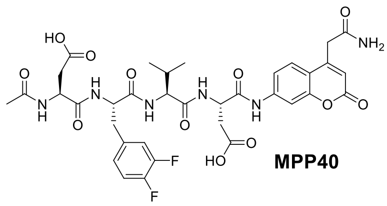
MPP36

Ac-Thr-Glu-NptGly-Asp-ACC



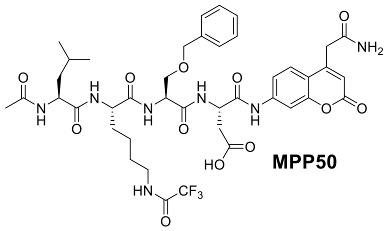
MPP38

Ac-Asp-Ala(2-th)-Val-Asp-ACC



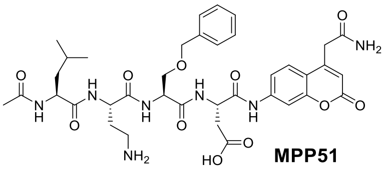
MPP40

Ac-Asp-Phe(3,4-F_2)-Val-Asp-ACC



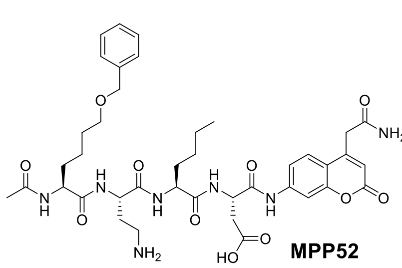
MPP50

Ac-Leu-Lys(TFA)-Ser(Bzl)-Asp-ACC



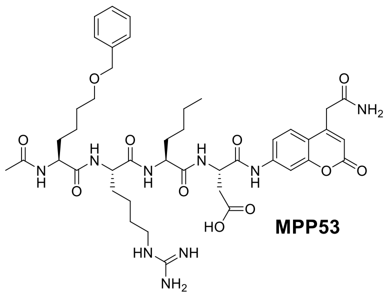
MPP51

Ac-Leu-Dab-Ser(Bzl)-Asp-ACC



MPP52

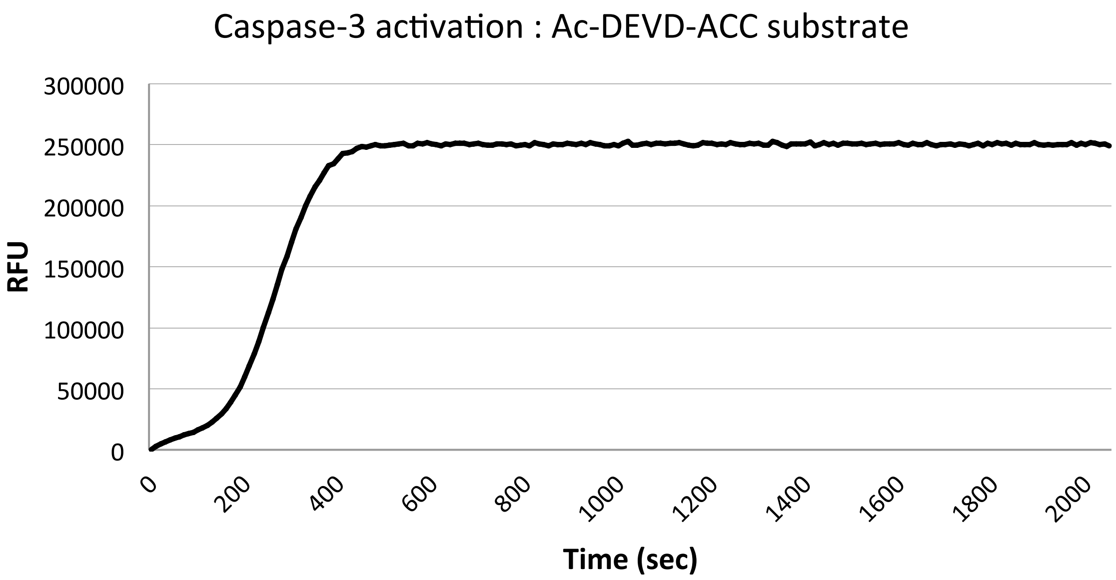
Ac-Nle(O-Bzl)-Dab-Nle-Asp-ACC



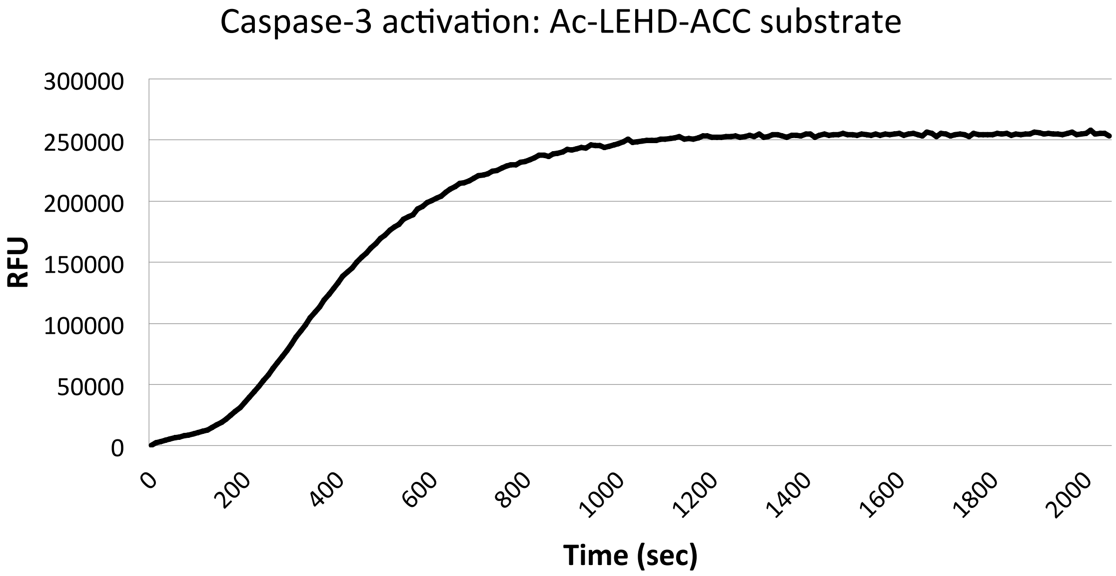
MPP53

Ac-Nle(O-Bzl)-hArg-Nle-Asp-ACC

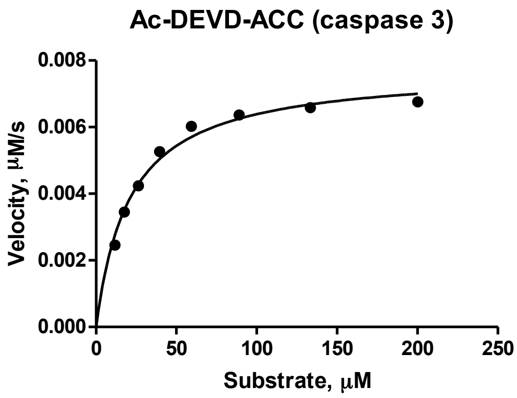
Supplementary Figure 9. Structures of caspases-selective fluorogenic substrates.

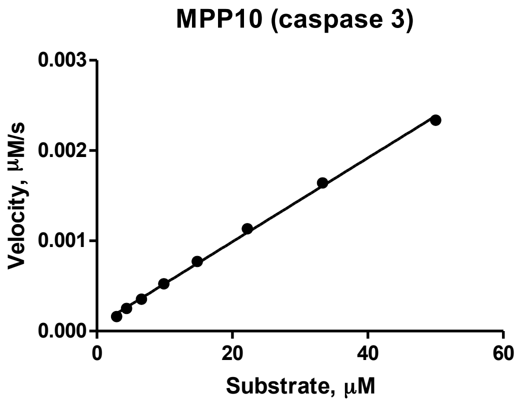


Supplementary Figure 10. Caspase 3 activation measured with Ac-DEVD-ACC. Substrate concentration 250 μM. Cytosolic extract was treated with cytochrome C and dATP (see Materials and Methods) and used without pre-incubation. The assay was performed at 37°C.



Supplementary Figure 11. The Ac-LEHD-ACC hydrolysis time course. Detailed kinetic analysis with the use of XIAP-BIR2 and XIAP-BIR3 clearly demonstrated that Ac-LEHD-ACC is hydrolyzed mainly by caspase 3, making this substrate useless as caspase 9 probe. Substrate concentration 250 μM. Cytosolic extract was stimulated with cytochrome C and dATP (see Materials and Methods) and used without incubation. Assay was performed at 37°C.





Equation 1:

$$Vmax = kcat * [E] \rightarrow \; kcat = \frac{Vmax}{[E]}$$

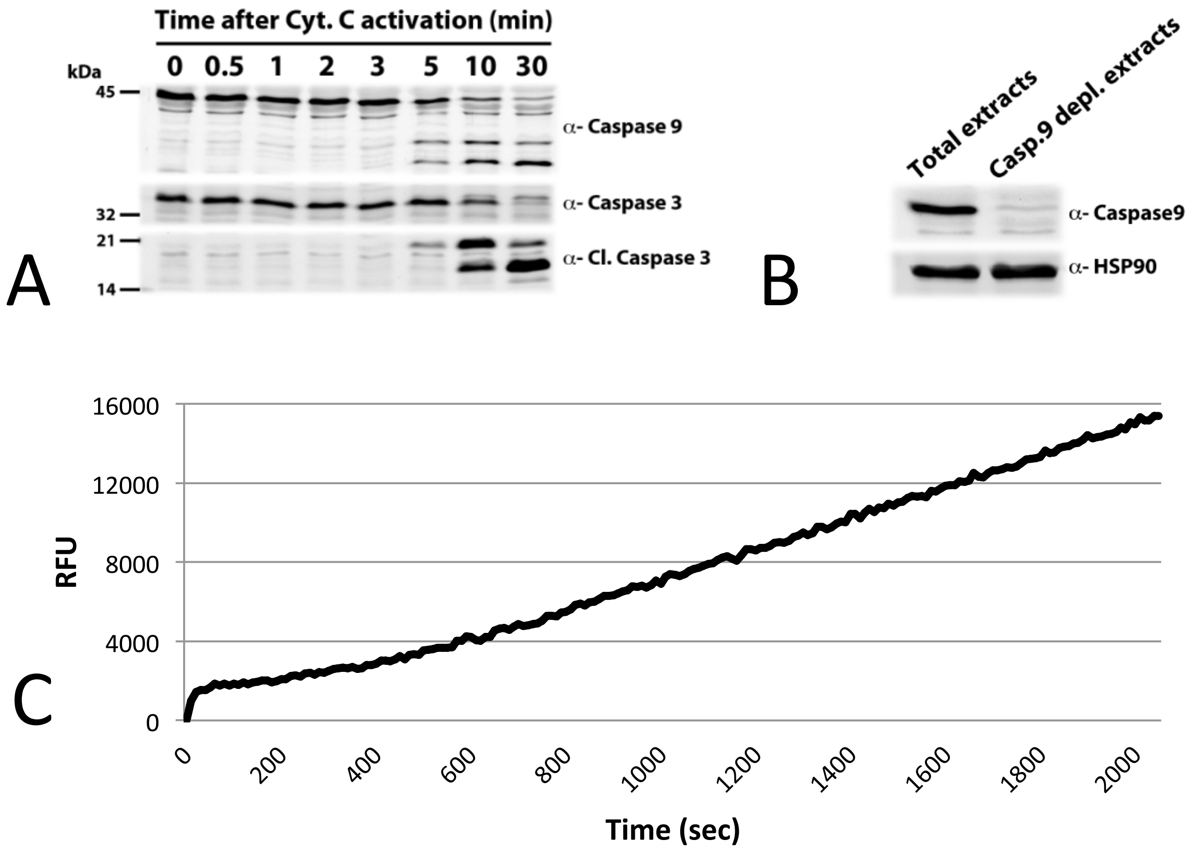
$$\frac{kcat}{Km} = \frac{Vmax}{[E]*Km}$$

Equation 2:

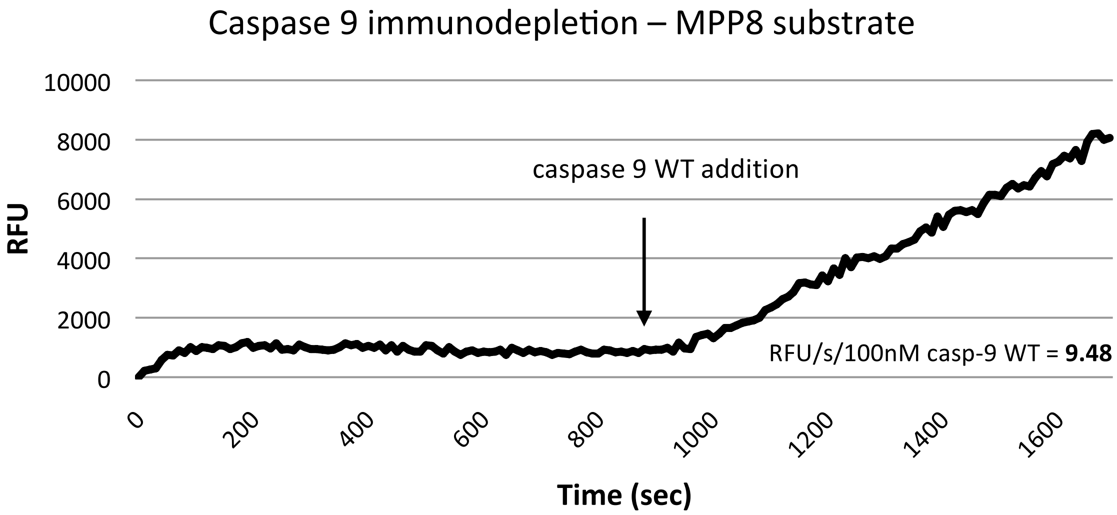
$$slope = \frac{\Delta V}{\Delta [S]}$$

$$\frac{kcat}{Km} = \frac{slope}{[E]} = \frac{\Delta V}{\Delta [S] * [E]}$$

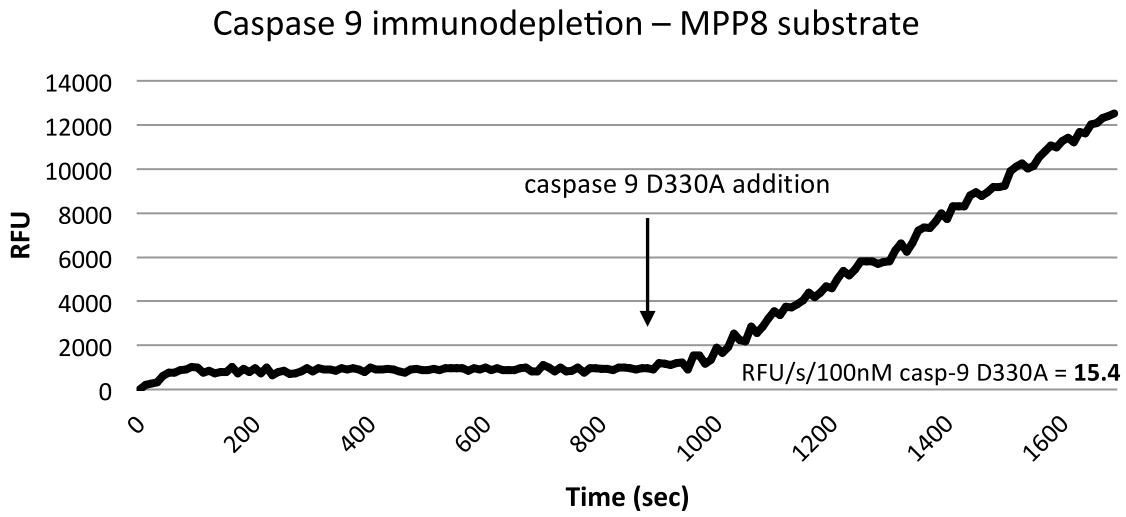
Supplementary Figure 12. Calculation of $k_{cat/}K_M$ parameters – for good substrates (left) and for poor substrates (right).



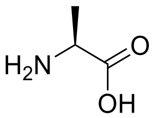
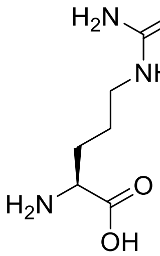
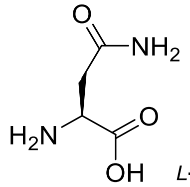
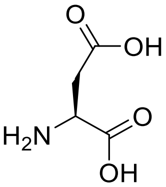
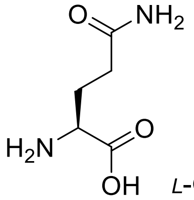
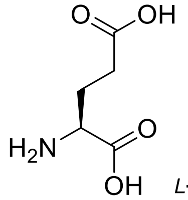
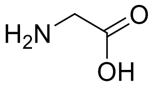
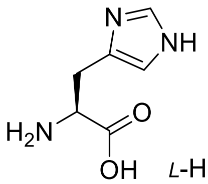
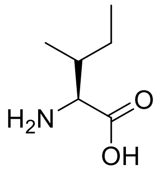
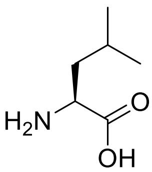
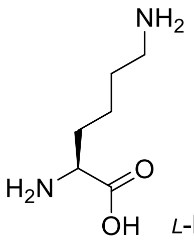
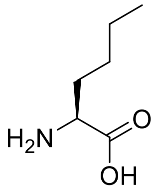
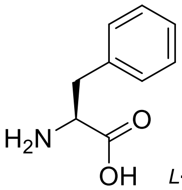
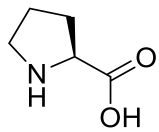
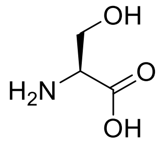
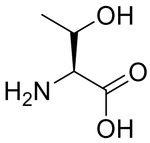
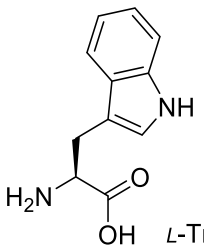
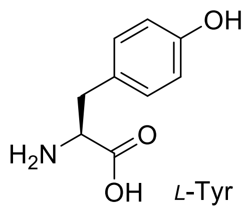
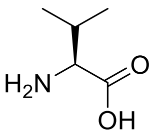
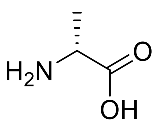
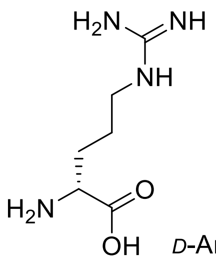
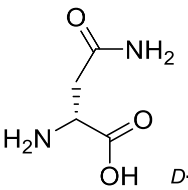
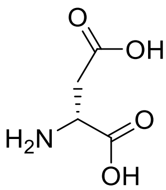
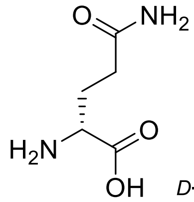
Supplementary Figure 13. Time course of caspase 9 activation. (A) Cytosolic extracts were stimulated with cytochrome C and caspase 9 and caspase 3 cleavage was monitored over the time course. (B) Caspase 9 immunodepletion from cytosolic extracts. Western Blot showing the absence of Caspase 9 in the immunodepleted sample. (C) Caspase 9 activation in cellular extract measured with the use of MPP8, a selective short peptide-based caspase 9 substrate. The "burst" in first 40-60 seconds is caused by cytochrome C and it appears also in the absence of cytosolic extracts (data not shown). The activity of caspase 9 can be measured after 60 seconds after cytochrome stimulation.

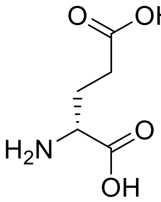


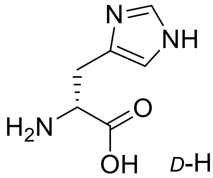
Supplementary Figure 13. Monitoring of the MPP8 hydrolysis by the addition of caspase 9 WT to the caspase 9 immunodepleted cytosolic extract. The proteolytic activity of caspase 9 immunodepleted cytosolic extract has been monitored with the use of MPP8 substrate (200 μM). After 15 minutes a recombinant wild type caspase 9 was added (100 nM) and the measurement was continued.

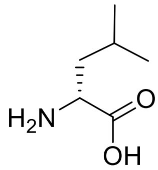


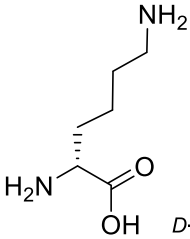
Supplementary Figure 15. Monitoring of the MPP8 hydrolysis by the addition of caspase 9 D330A mutant to the caspase 9 immunodepleted cytosolic extract. The proteolytic activity of caspase 9 immunodepleted cytosolic extract has been monitored with the use of MPP8 substrate (200 μM). After 15 minutes a recombinant caspase 9 D330A mutant was added (100 nM) and the measurement was continued.

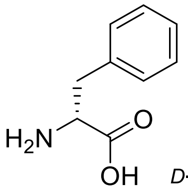
No	Structure	No	Structure	No	Structure
1	 *L*-Ala	2	 *L*-Arg	3	 *L*-Asn
4	 *L*-Asp	5	 *L*-Gln	6	 *L*-Glu
7	 Gly	8	 *L*-His	9	 *L*-Ile
10	 *L*-Leu	11	 *L*-Lys	12	 *L*-Nle
13	 *L*-Phe	14	 *L*-Pro	15	 *L*-Ser
16	 *L*-Thr	17	 *L*-Trp	18	 *L*-Tyr
19	 *L*-Val	20	 *D*-Ala	21	 *D*-Arg
22	 *D*-Asn	23	 *D*-Asp	24	 *D*-Gln

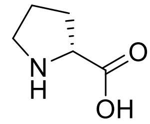
25  D-Glu

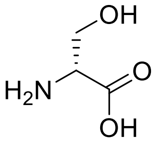
26  D-His

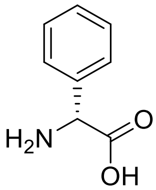
27  D-Leu

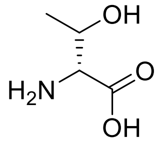
28  D-Lys

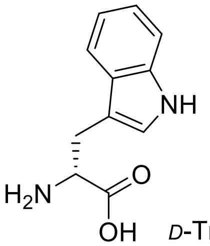
29  D-Phe

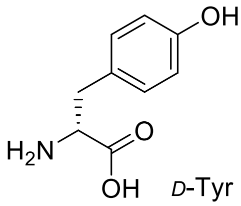
30  D-Pro

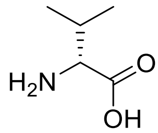
31  D-Ser

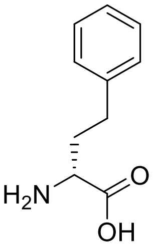
32  D-Phg

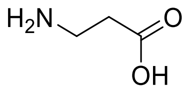
33  D-Thr

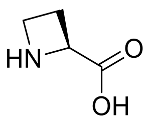
34  D-Trp

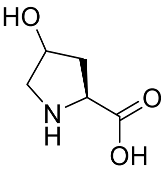
35  D-Tyr

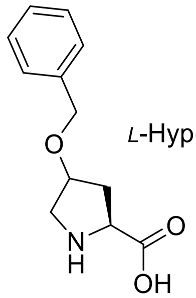
36  D-Val

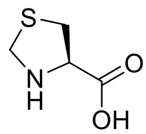
37  D-hPhe

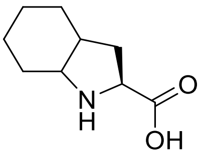
38  β-Ala

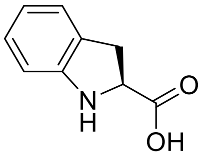
39  L-Aze

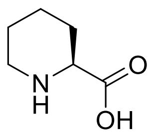
40  L-Hyp

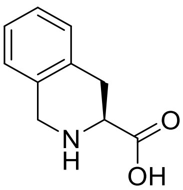
41  L-Hyp(Bzl)

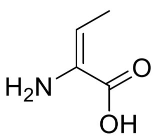
42  L-Thz

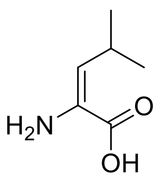
43  L-Oic

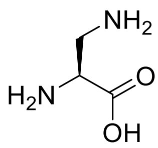
44  L-Idc

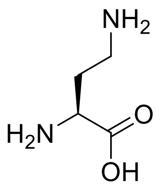
45  L-Pip

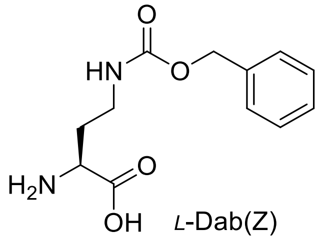
46  L-Tic

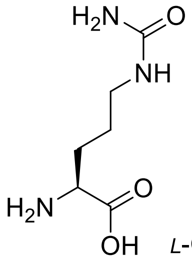
47  dhAbu

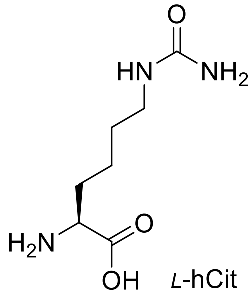
48  dhLeu

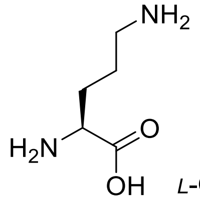
49  L-Dap

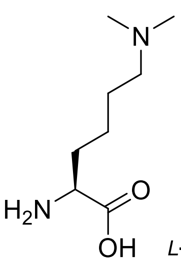
50  L-Dab

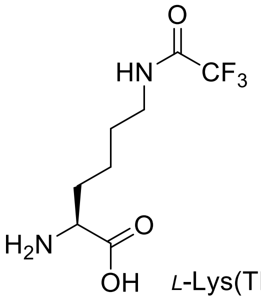
51  L-Dab(Z)

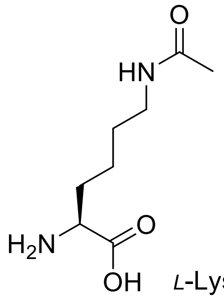
52  L-Cit

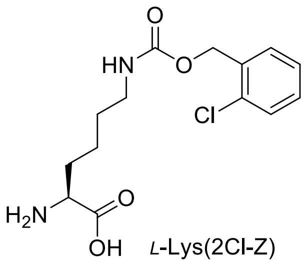
53  L-hCit

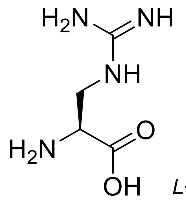
54  L-Orn

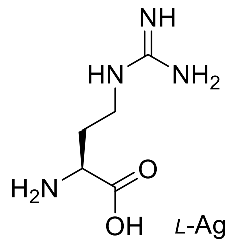
55  L-Lys(Me)$_2$

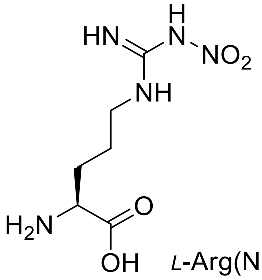
56  L-Lys(TFA)

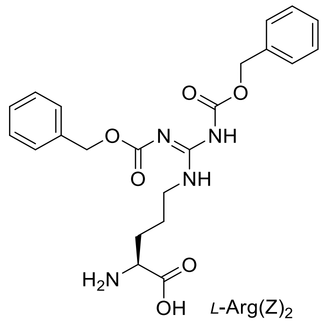
57  L-Lys(Ac)

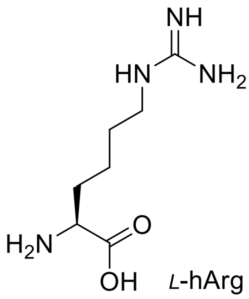
58  L-Lys(2Cl-Z)

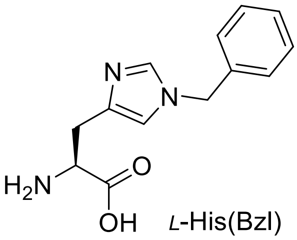
59  L-Agp

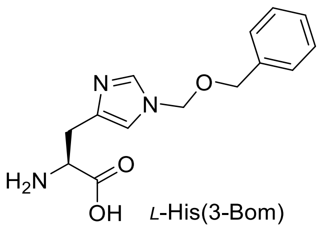
60  L-Agb

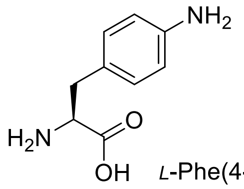
61  L-Arg(NO$_2$)

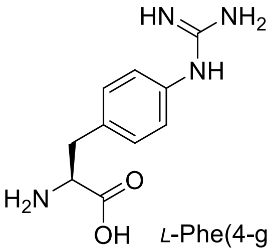
62  L-Arg(Z)$_2$

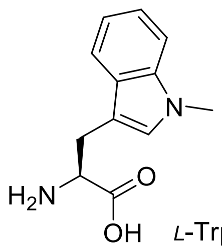
63  L-hArg

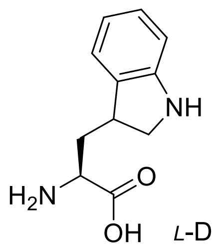
64  L-His(Bzl)

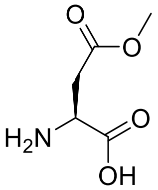
65  L-His(3-Bom)

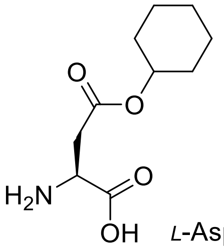
66  L-Phe(4-NH$_2$)

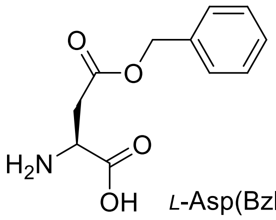
67  L-Phe(4-guan)

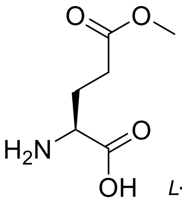
68  L-Trp(Me)

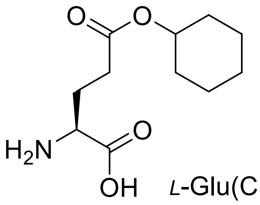
69  L-Dht

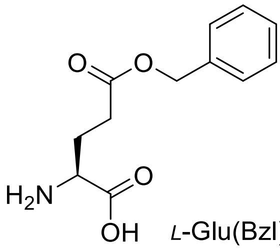
70  L-Asp(Me)

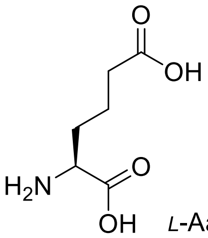
71  L-Asp(Chx)

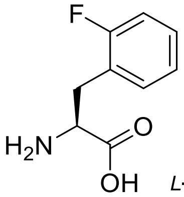
72  L-Asp(Bzl)

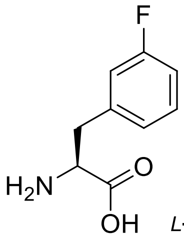
73  L-Glu(Bzl)

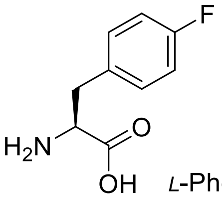
74  L-Glu(Chx)

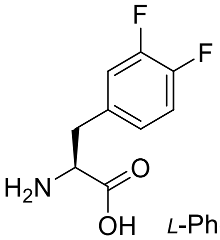
75  L-Glu(Bzl)

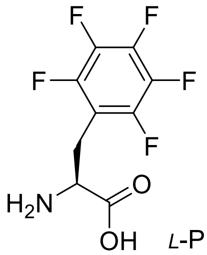
76  L-Aad

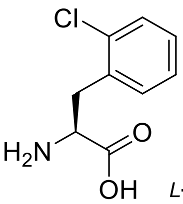
77  L-Phe(2-F)

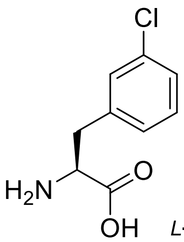
78  L-Phe(3-F)

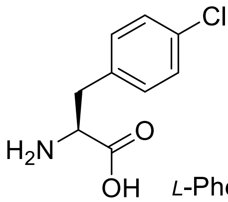
79  L-Phe(4-F)

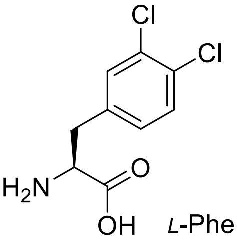
80  L-Phe(4,3-F_2)

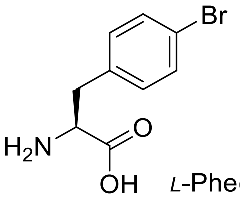
81  L-Phe(F_5)

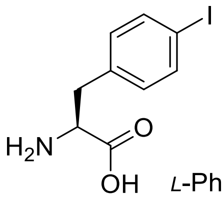
82  L-Phe(2-Cl)

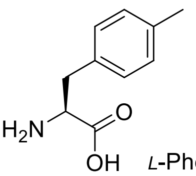
83  L-Phe(3-Cl)

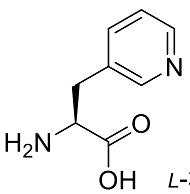
84  L-Phe(4-Cl)

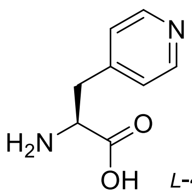
85  L-Phe(3,4-Cl_2)

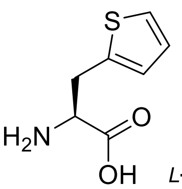
86  L-Phe(4-Br)

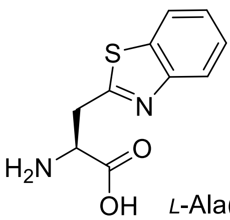
87  L-Phe(4-I)

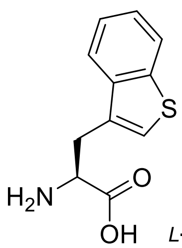
88  L-Phe(4-Me)

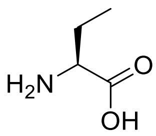
89  L-3-Pal

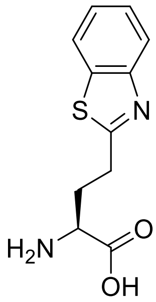
90  L-4-Pal

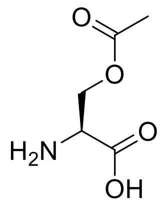
91  L-Ala(2-th)

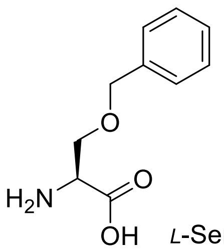
92  L-Ala(Bth)

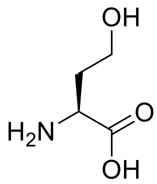
93  L-Bta

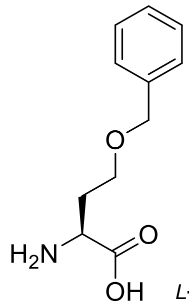
94  *L*-Abu

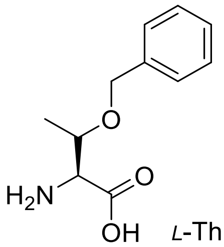
95  *L*-Abu(Bth)

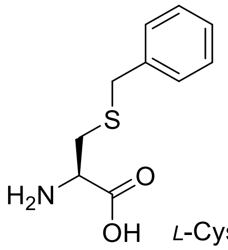
96  *L*-Ser(Ac)

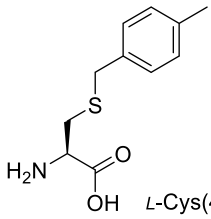
97  *L*-Ser(Bzl)

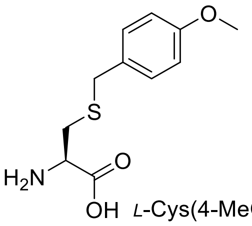
98  *L*-hSer

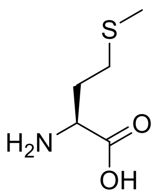
99  *L*-hSer(Bzl)

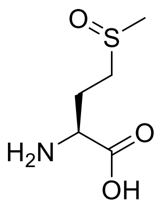
100  *L*-Thr(Bzl)

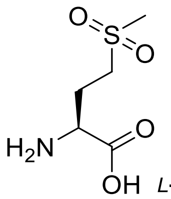
101  *L*-Cys(Bzl)

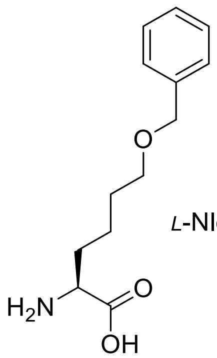
102  *L*-Cys(4-MeBzl)

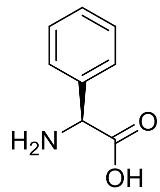
103  *L*-Cys(4-MeOBzl)

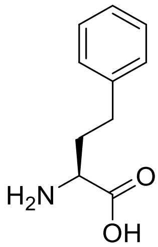
104  *L*-Met

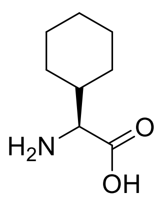
105  *L*-Met(O)

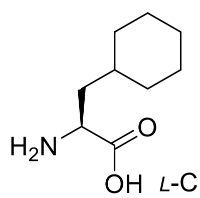
106  *L*-Met(O_2)

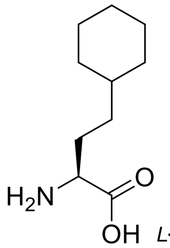
107  *L*-Nle(*O*-Bzl)

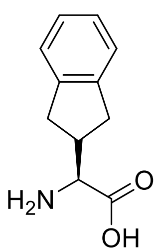
108  *L*-Phg

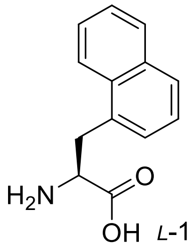
109  *L*-hPhe

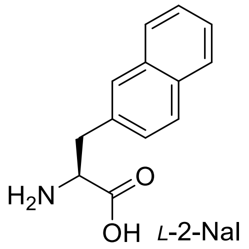
110  *L*-Chg

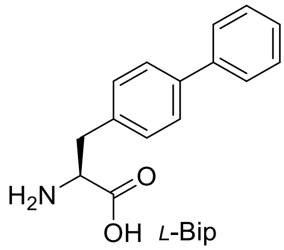
111  *L*-Cha

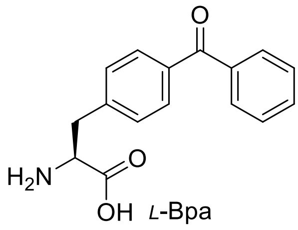
112  *L*-hCha

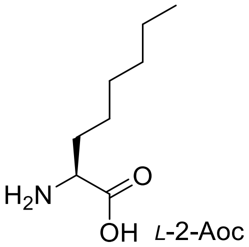
113  *L*-Igl

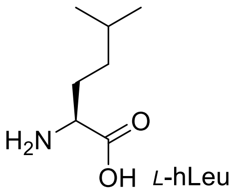
114  *L*-1-Nal

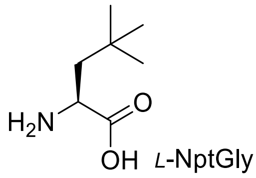
115 

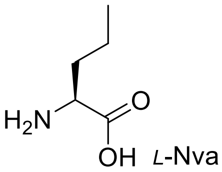
116 

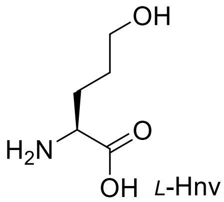
117 

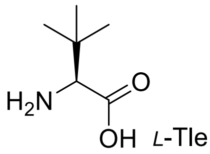
118 

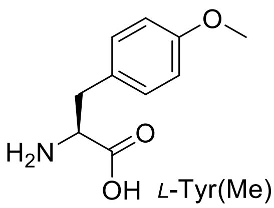
119 

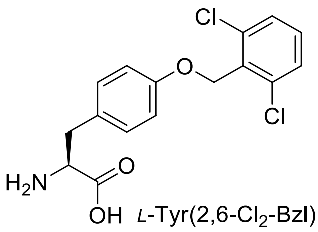
120 

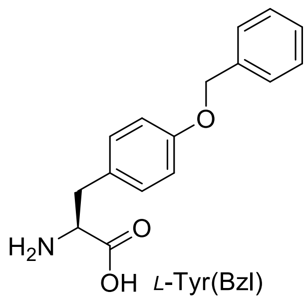
121 

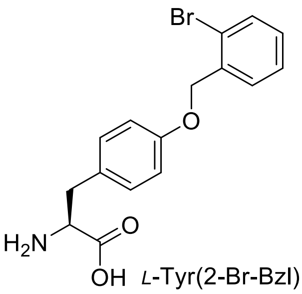
122 

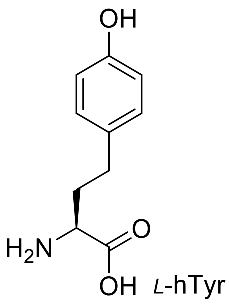
123 

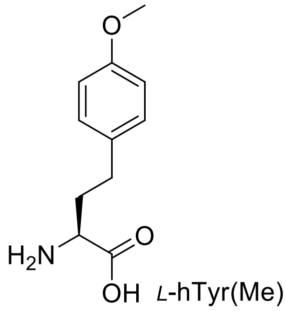
124 

125 

126 

127 

128 

129 

Supplementary Table 5. Structure of amino acids used in HyCoSuL synthesis.

Compounds analysis

All individual compounds used for kinetic and biological studies were purified by HPLC on a Waters M600 solvent delivery module with a Waters M2489 detector system using Waters Spherisorb S5ODS2 column and characterized by high-resolution mass spectrometry (HRMS) using on High Resolution Mass Spectrometer WATERS LCT Premier XE with Electrospray ionization (ESI). Overall yields for the complete synthesis and HRMS data are listed below.

Ac-DEVD-ACC, 32.1% yield. HRMS (m/z): $[M]^+$ calcd for $C_{31}H_{38}N_6O_{14}$, 719.6652 found, 719.2532

Ac-LEHD-ACC, 29.3% yield. HRMS (m/z): $[M]^+$ calcd for $C_{34}H_{42}N_8O_{12}$, 755.2922 found, 755.2966

Ac-IETD-ACC, 25.8% yield. HRMS (m/z): $[M]^+$ calcd for $C_{32}H_{42}N_6O_{13}$, 719.2810 found, 719.2859

Ac-AEVD-ACC, 24.6% yield. HRMS (m/z): $[MNa]^+$ calcd for $C_{30}H_{38}N_6NaO_{12}$, 697.2440 found, 697.2444

Ac-VEID-ACC, 33.3% yield. HRMS (m/z): $[MNa]^+$ calcd for $C_{33}H_{44}N_6NaO_{12}$, 739.2909 found, 739.2908

Ac-VDVAD-ACC, 34.5% yield. HRMS (m/z): $[MNa]^+$ calcd for $C_{34}H_{45}N_7NaO_{13}$, 782.2967 found, 782.2966

MPP8, 28.9% yield. HRMS (m/z): $[M]^+$ calcd for $C_{38}H_{48}N_8O_{10}$, 777.3493 found, 777.3580

MPP10, 39.0% yield. HRMS (m/z): $[M]^+$ calcd for $C_{46}H_{50}N_8O_{10}$, 875.3650 found, 875.3729

MPP12, 35.7% yield. HRMS (m/z): $[M]^+$ calcd for $C_{45}H_{54}N_8O_{10}$, 867.3963 found, 867.3593

MPP17, 35.45% yield. HRMS (m/z): $[M]^+$ calcd for $C_{37}H_{46}N_{10}O_{14}$, 855.3195 found, 855.3266

MPP21, 32.2% yield. HRMS (m/z): $[M]^+$ calcd for $C_{44}H_{53}N_7O_{12}$, 872.3752 found, 872.3831

MPP23, 27.5% yield. HRMS (m/z): $[M]^+$ calcd for $C_{43}H_{59}N_9O_{11}$, 878.4334 found, 878.4421

MPP28, 31.2% yield. HRMS (m/z): $[M]^+$ calcd for $C_{33}H_{42}N_6O_{13}$, 731.2810 found, 731.2879

MPP30, 32.2% yield. HRMS (m/z): $[MNa]^+$ calcd for $C_{44}H_{50}N_6NaO_{13}$, 893.3328 found, 893.3328

MPP36, 17.9% yield. HRMS (m/z): $[M]^+$ calcd for $C_{33}H_{44}N_6O_{13}$, 733.2966 found, 733.3005

MPP38, 27.5% yield. HRMS (m/z): $[MNa]^+$ calcd for $C_{33}H_{38}N_6NaO_{12}S$, 765.2160 found, 765.2180

MPP39, 31.1% yield. HRMS (m/z): $[MNa]^+$ calcd for $C_{35}H_{35}F_5N_6NaO_{12}$, 849.2125 found, 849.2155

MPP40, 32.8% yield. HRMS (m/z): $[MNa]^+$ calcd for $C_{35}H_{38}F_2N_6NaO_{12}$, 795.2408 found, 795.2421

MPP41, 28.6% yield. HRMS (m/z): $[M]^+$ calcd for $C_{37}H_{42}N_6O_{15}$, 811.2708 found, 811.2769

MPP42, 26.5% yield. HRMS (m/z): $[M]^+$ calcd for $C_{39}H_{42}N_6O_{13}S$, 835.2531 found, 835.2628

MPP43, 27.1% yield. HRMS (m/z): $[MNa]^+$ calcd for $C_{38}H_{46}N_6NaO_{13}$, 817.3015 found, 817.2991

MPP45, 30.1% yield. HRMS (m/z): $[MNa]^+$ calcd for $C_{39}H_{48}N_6NaO_{13}$, 831.3162 found, 831.3210

MPP46, 34.2% yield. HRMS (m/z): $[MNa]^+$ calcd for $C_{40}H_{50}N_6NaO_{13}$, 845.3328 found, 845.3335

MPP47, 21.4% yield. HRMS (m/z): $[M]^+$ calcd for $C_{35}H_{46}N_8O_{10}$, 739.3337 found, 739.3400

MPP48, 29.8% yield. HRMS (m/z): $[MNa]^+$ calcd for $C_{38}H_{46}N_6NaO_{13}$, 817.3015 found, 817.3004

MPP49, 24.3% yield. HRMS (m/z): $[MNa]^+$ calcd for $C_{37}H_{44}N_6NaO_{12}$, 787,2909 found, 787,2919

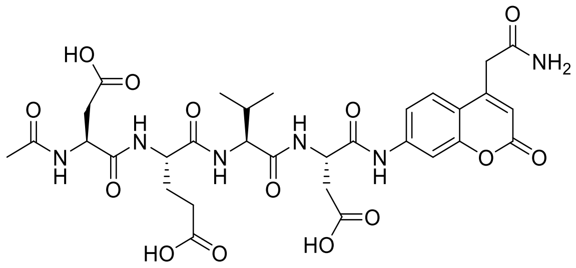
MPP50, 33.3% yield. HRMS (m/z): $[MNa]^+$ calcd for $C_{42}H_{50}F_3N_7NaO_{12}$, 912.3362 found, 912.3362

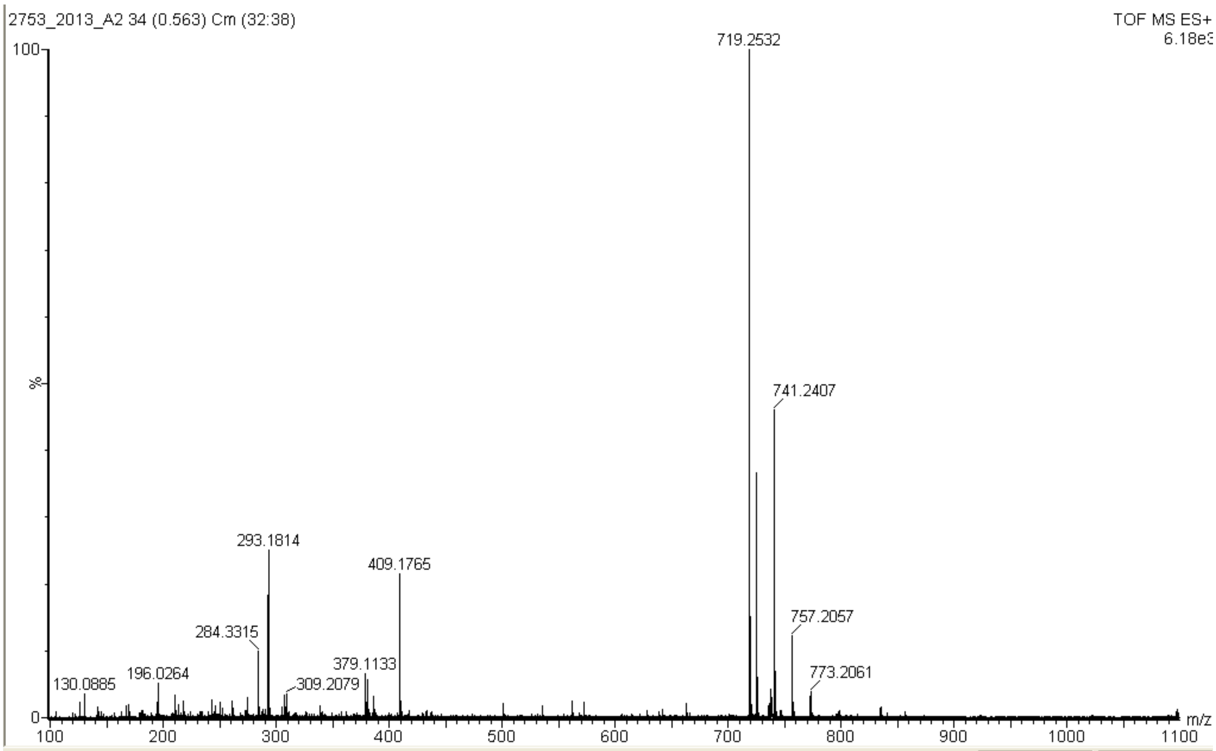
MPP51, 26.4% yield. HRMS (m/z): $[M]^+$ calcd for $C_{37}H_{47}N_7O_{11}$, 766.3334 found, 766.3416

MPP52, 29.6% yield. HRMS (m/z): $[M]^+$ calcd for $C_{40}H_{53}N_7O_{11}$, 808.3803 found, 808.3878

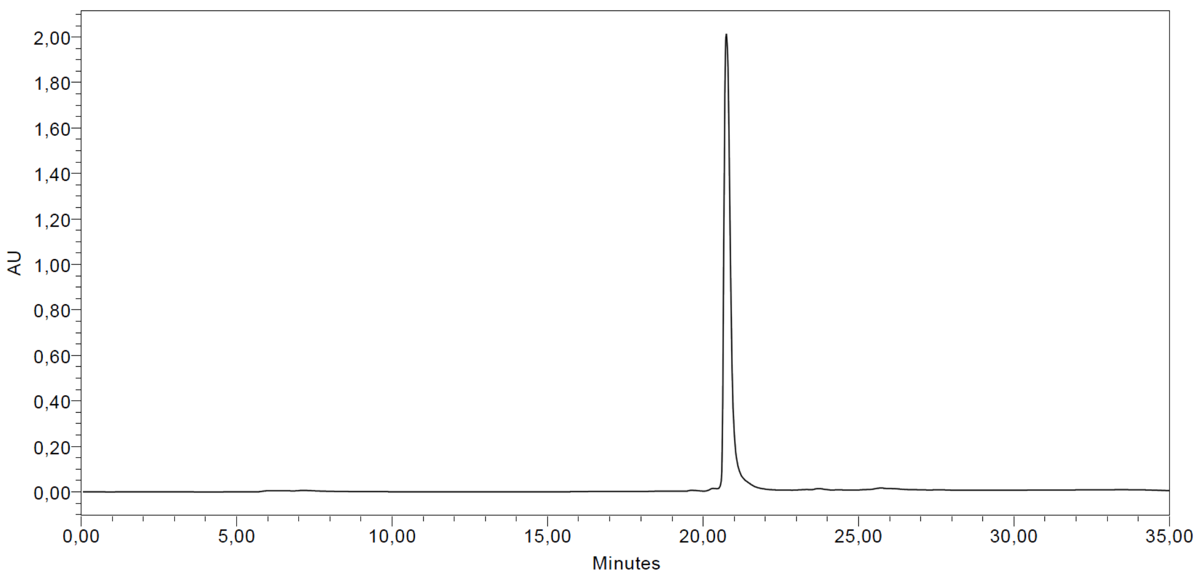
MPP53, 22.5% yield. HRMS (m/z): $[M]^+$ calcd for $C_{43}H_{59}N_9O_{11}$, 878.4334 found, 878.4424

Ac-DEVD-ACC



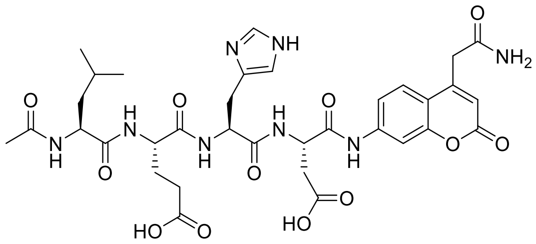


2753_2013_A2 34 (0.563) Cm (32:38)
TOF MS ES+
6.18e3
719.2532
741.2407
293.1814
409.1765
284.3315
757.2057
379.1133
773.2061
130.0885
196.0264
309.2079
%
m/z

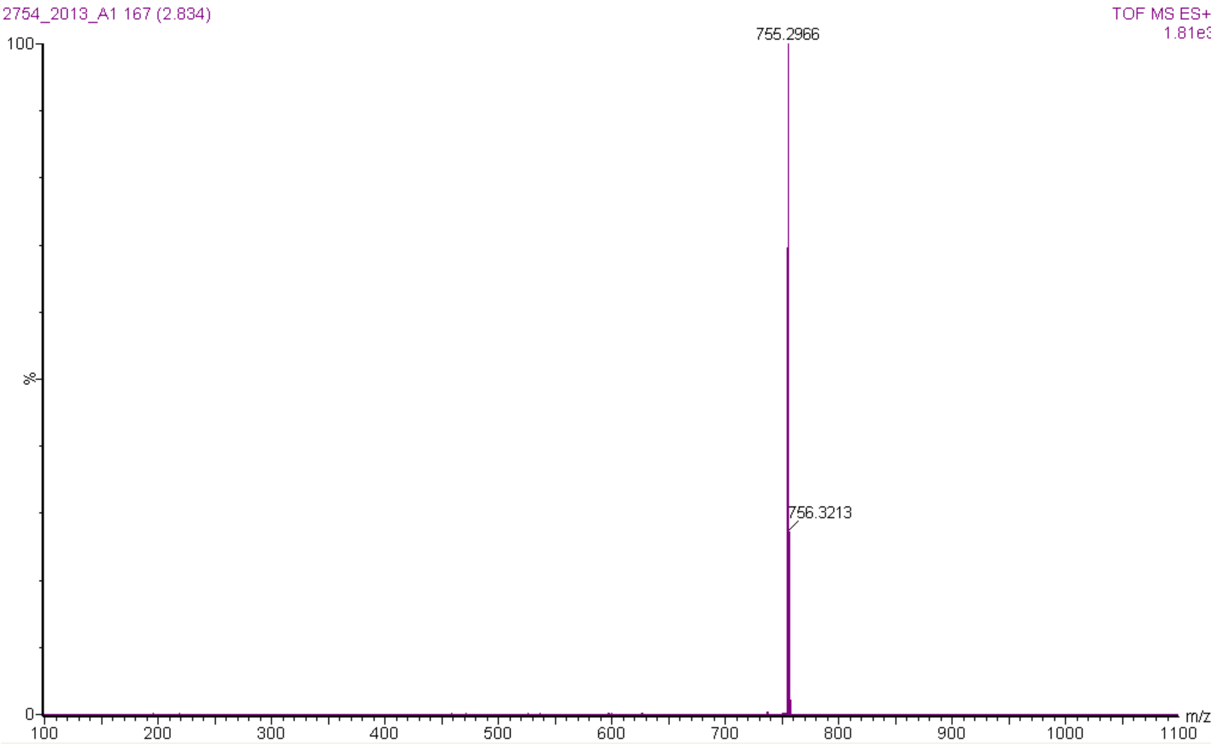


AU
Minutes

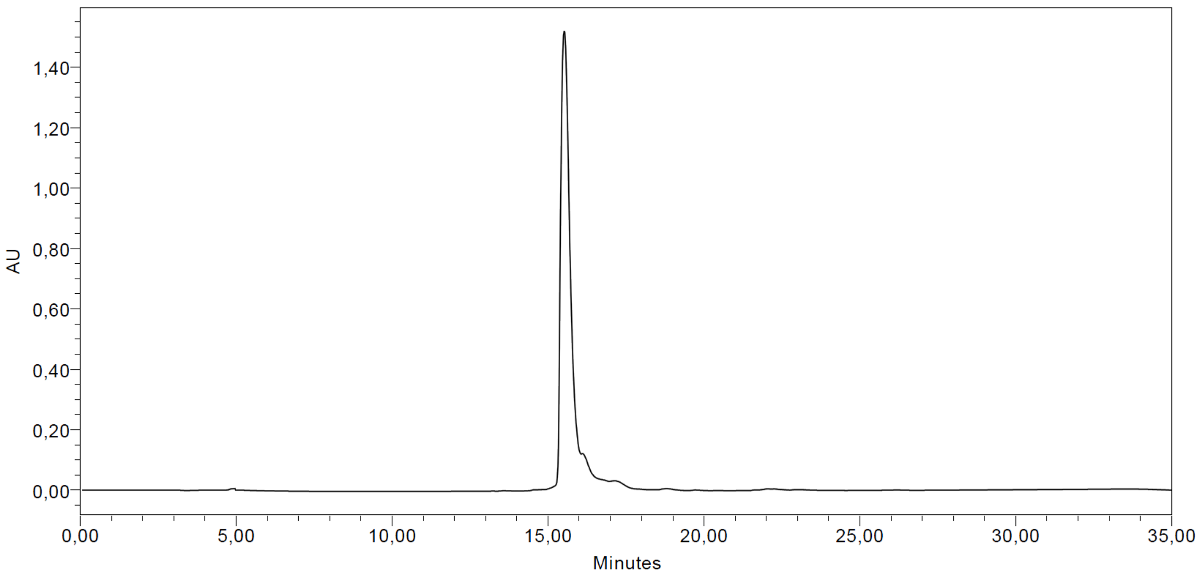
Ac-LEHD-ACC



O
NH2
N
NH
H
N
O
HO

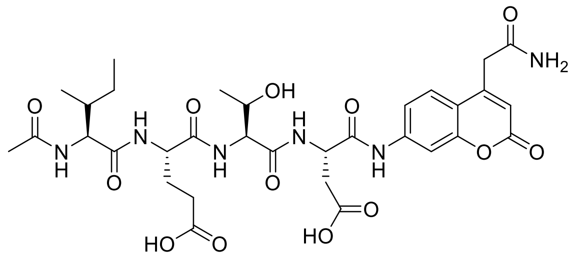


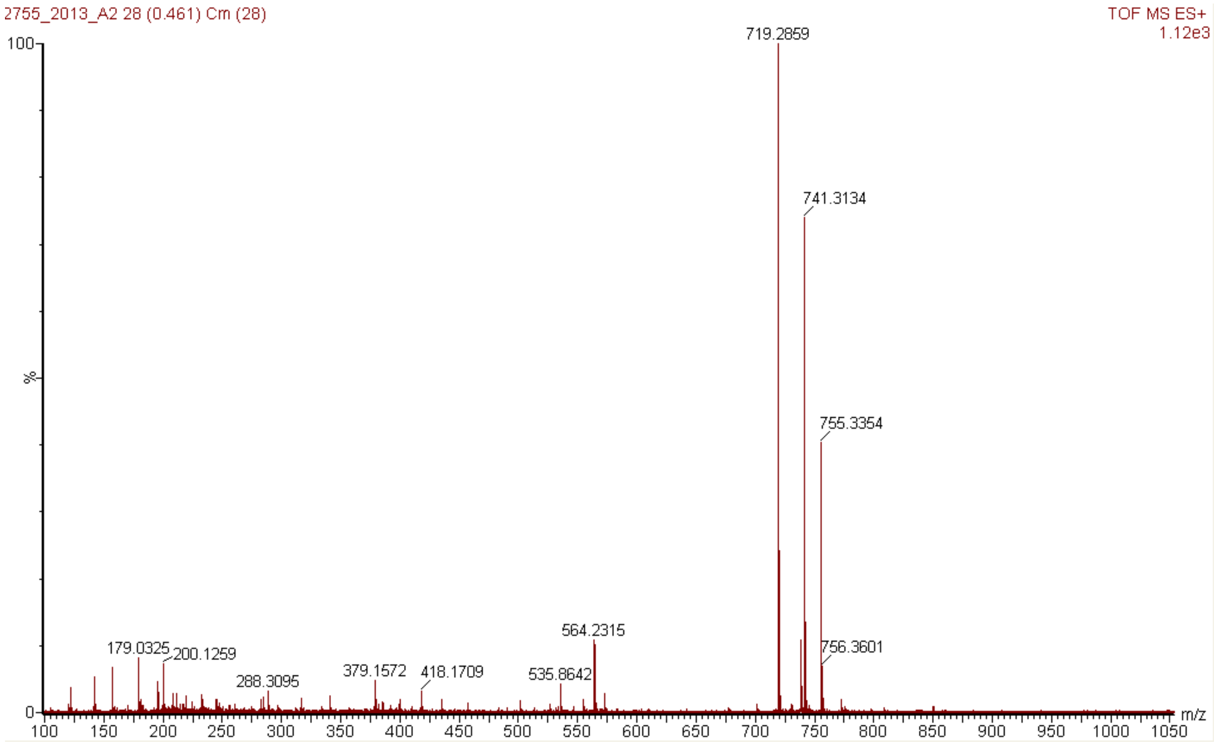
2754_2013_A1 167 (2.834)
TOF MS ES+
1.81e3
755.2966
756.3213
100
%
0
m/z
100
200
300
400
500
600
700
800
900
1000
1100

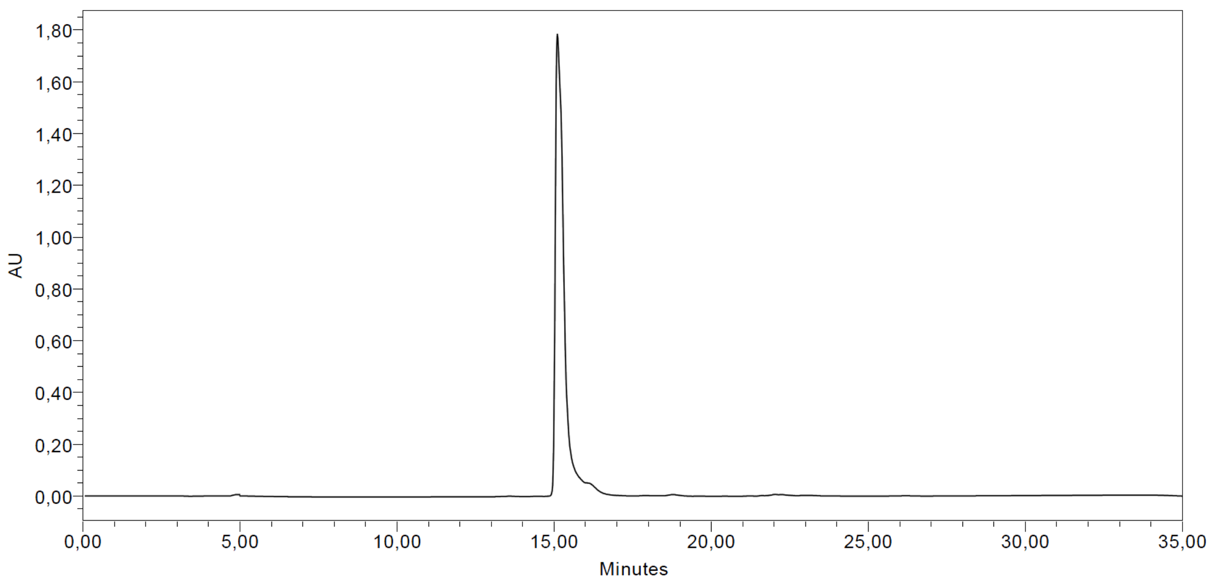


AU
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

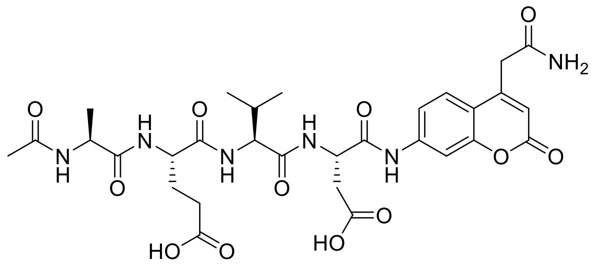
Ac-IETD-ACC

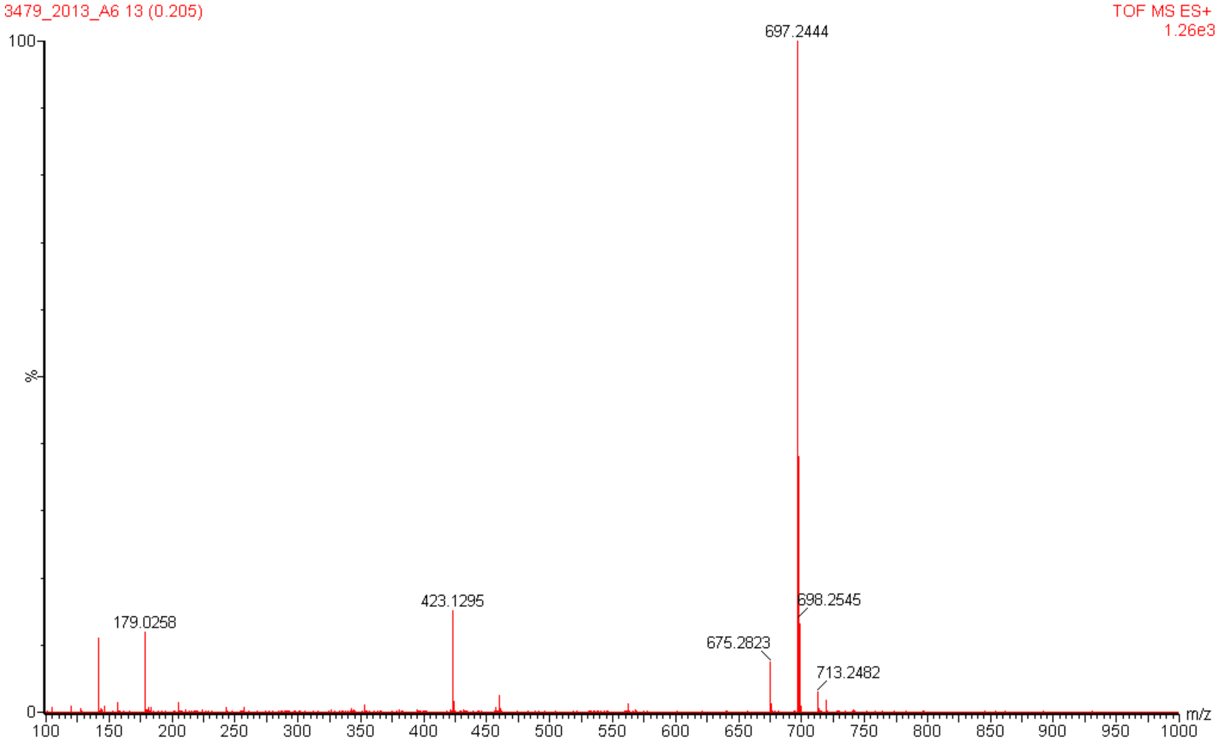

O
NH2
OH
H
N
O
H
N
O
O
N
H
O
N
H
O
N
H
O
O
HO
O
HO
O


2755_2013_A2 28 (0.461) Cm (28)
TOF MS ES+
1.12e3
719.2859
741.3134
755.3354
756.3601
564.2315
535.8642
179.0325
200.1259
288.3095
379.1572
418.1709
100
%
0
m/z
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000 1050

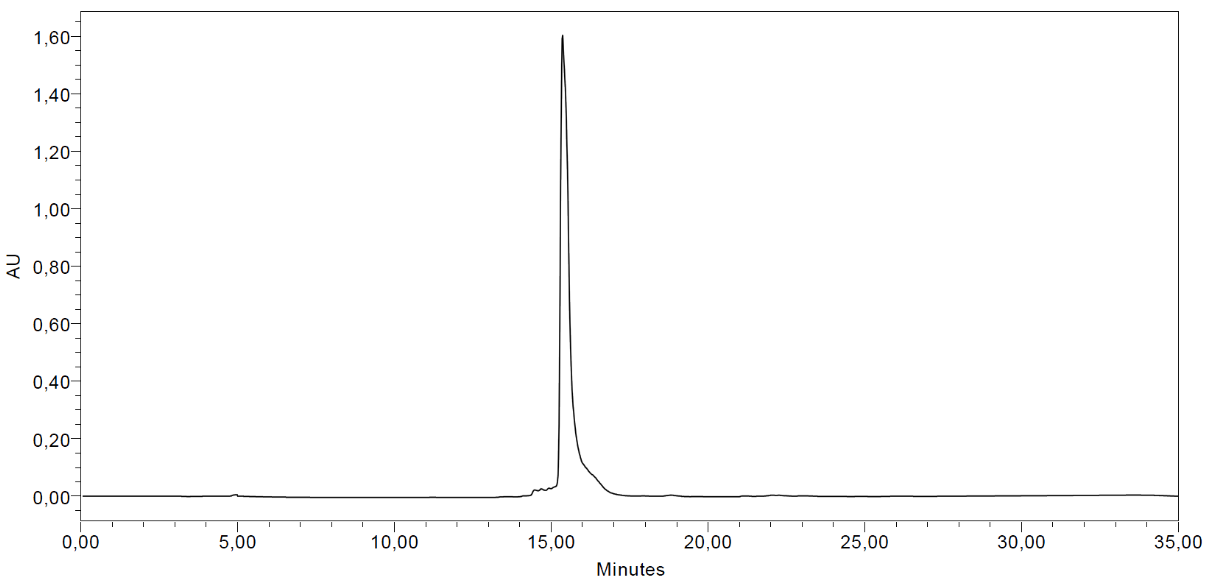

AU
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,00 5,00 10,00 15,00 20,00 25,00 30,00 35,00
Minutes

Ac-AEVD-ACC



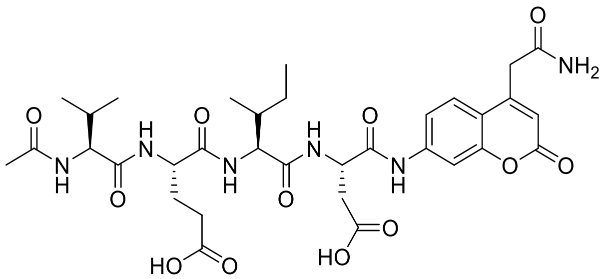


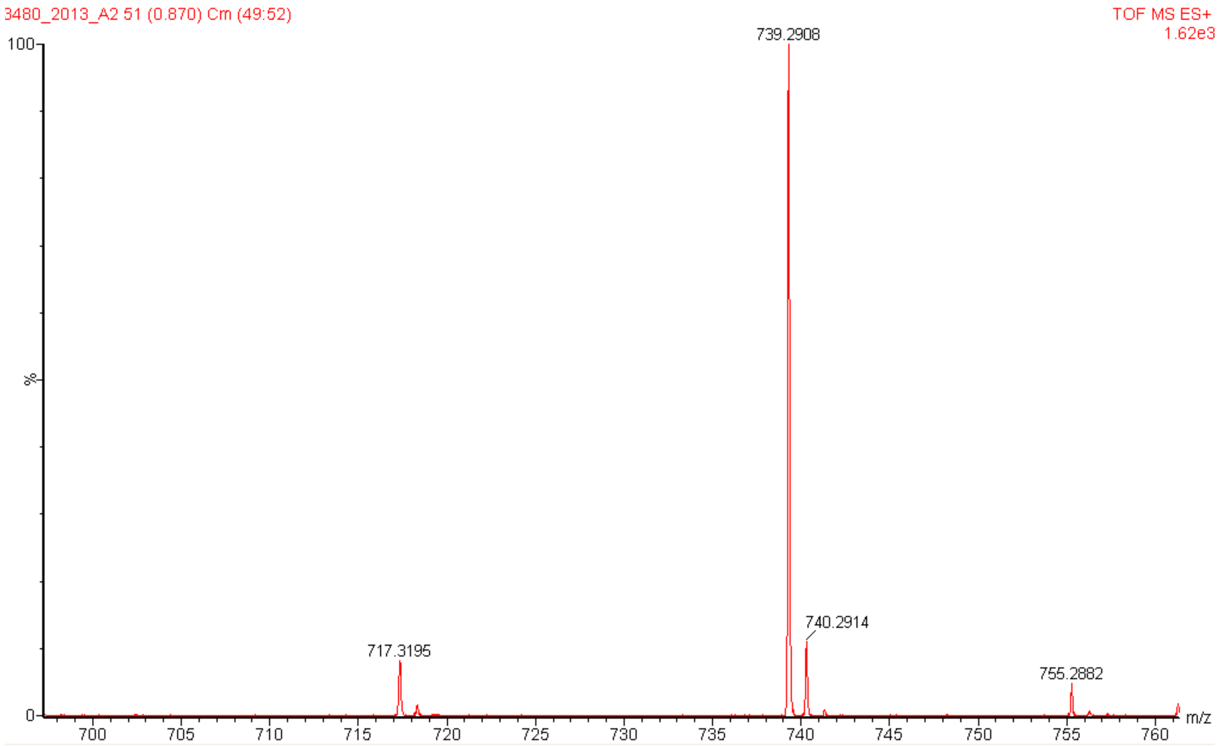
3479_2013_A6 13 (0.205)
TOF MS ES+
1.26e3
697.2444
423.1295
179.0258
675.2823
698.2545
713.2482
%
m/z



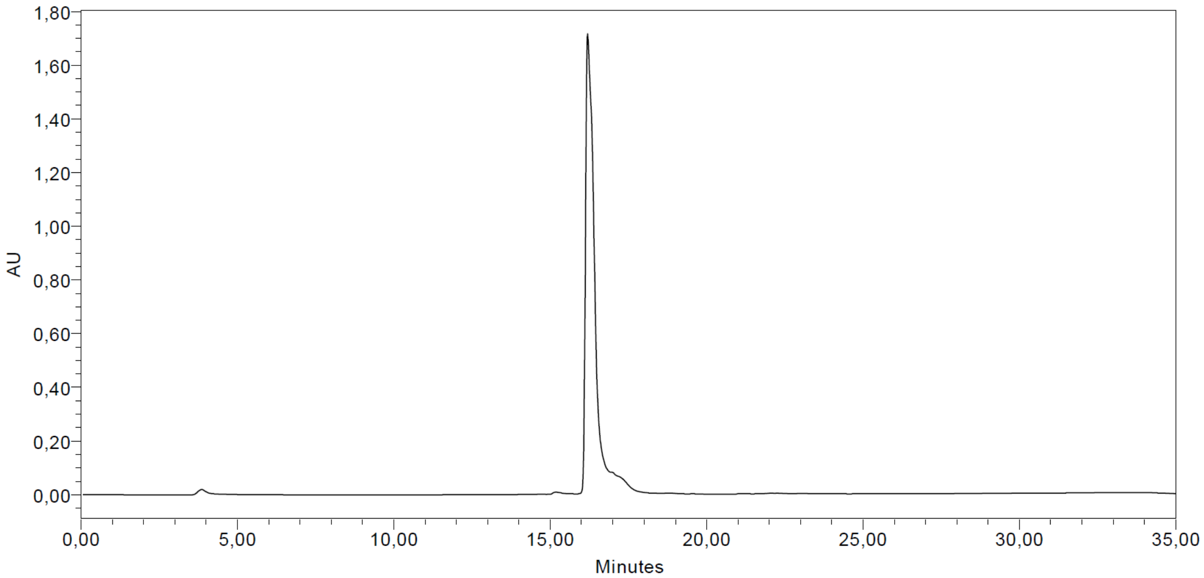
AU
Minutes

Ac-VEID-ACC



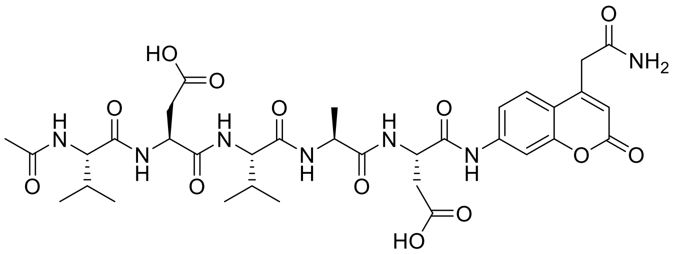


3480_2013_A2 51 (0.870) Cm (49:52)
TOF MS ES+
1.62e3
739.2908
740.2914
717.3195
755.2882
100
%
0
700
705
710
715
720
725
730
735
740
745
750
755
760
m/z

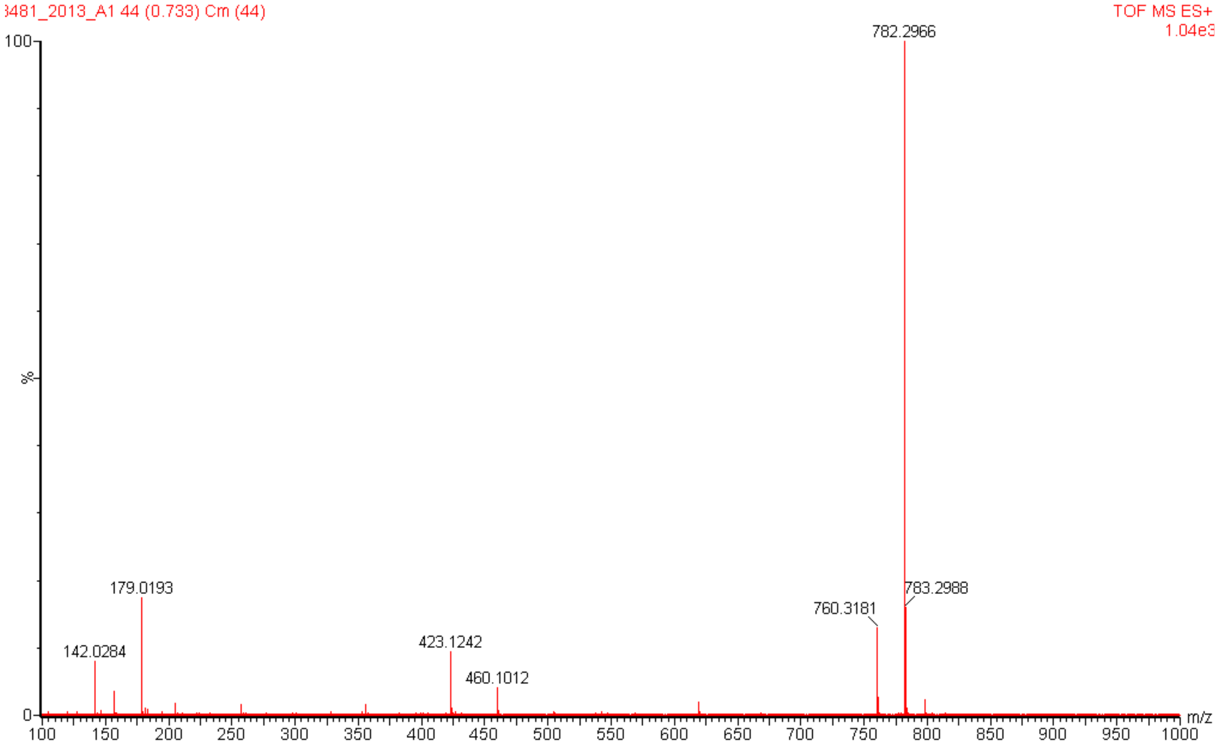


AU
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

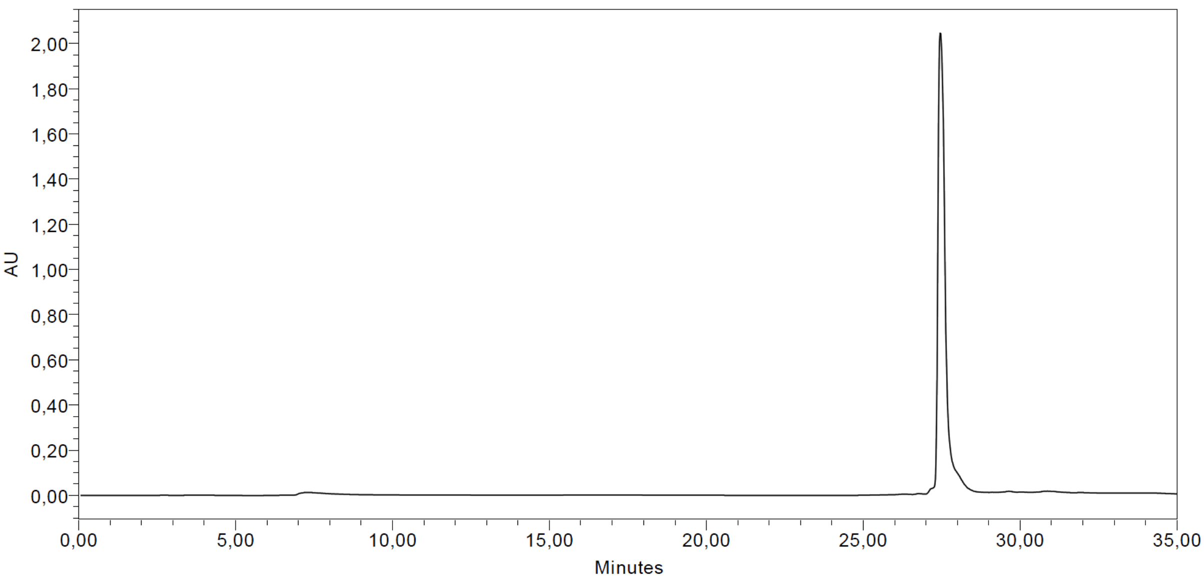
Ac-VDVAD-ACC



HO
O
H
N
O
N
H
O
H
N
O
N
H
O
H
N
O
N
H
O
NH2
O
O
HO
O

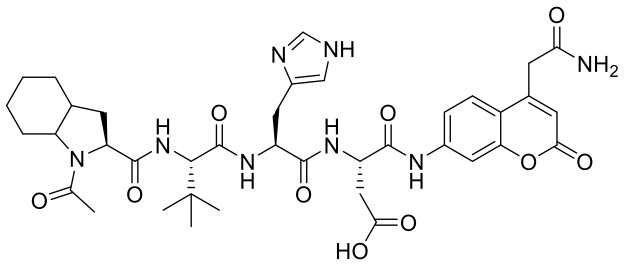


3481_2013_A1 44 (0.733) Cm (44)
TOF MS ES+
1.04e3
782.2966
100
%
0
179.0193
142.0284
423.1242
460.1012
760.3181
783.2988
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
m/z

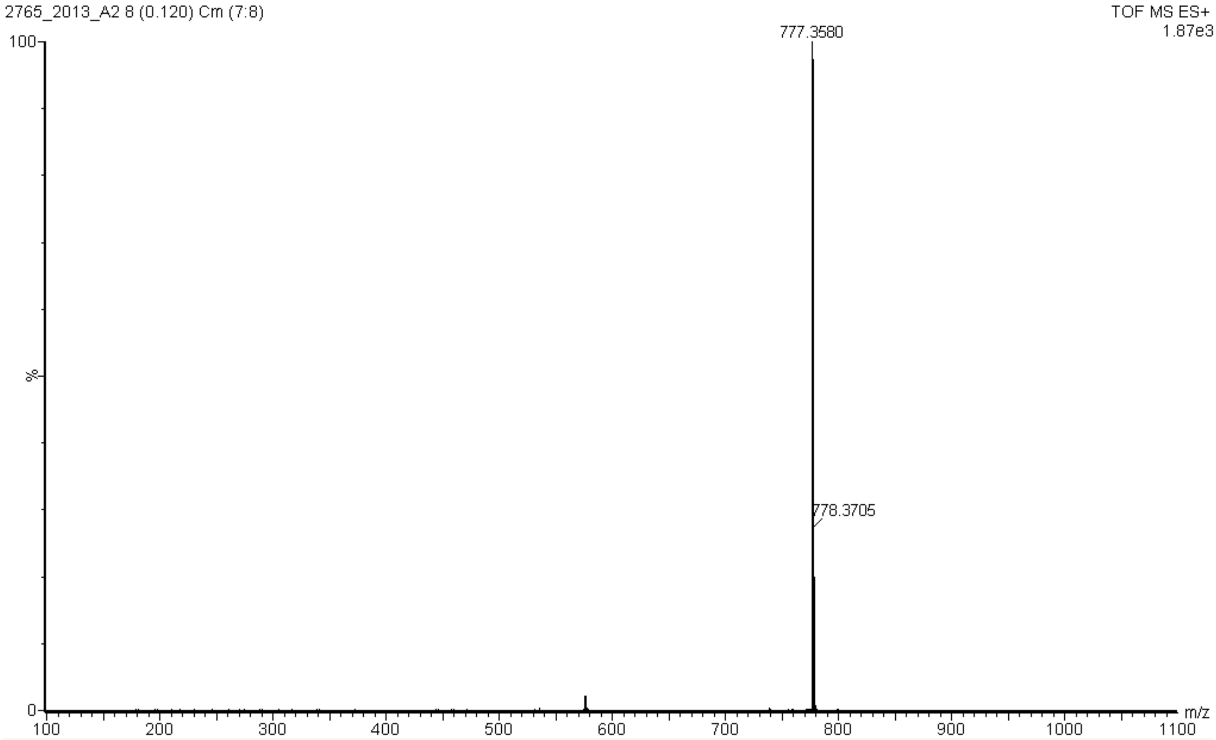


AU
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

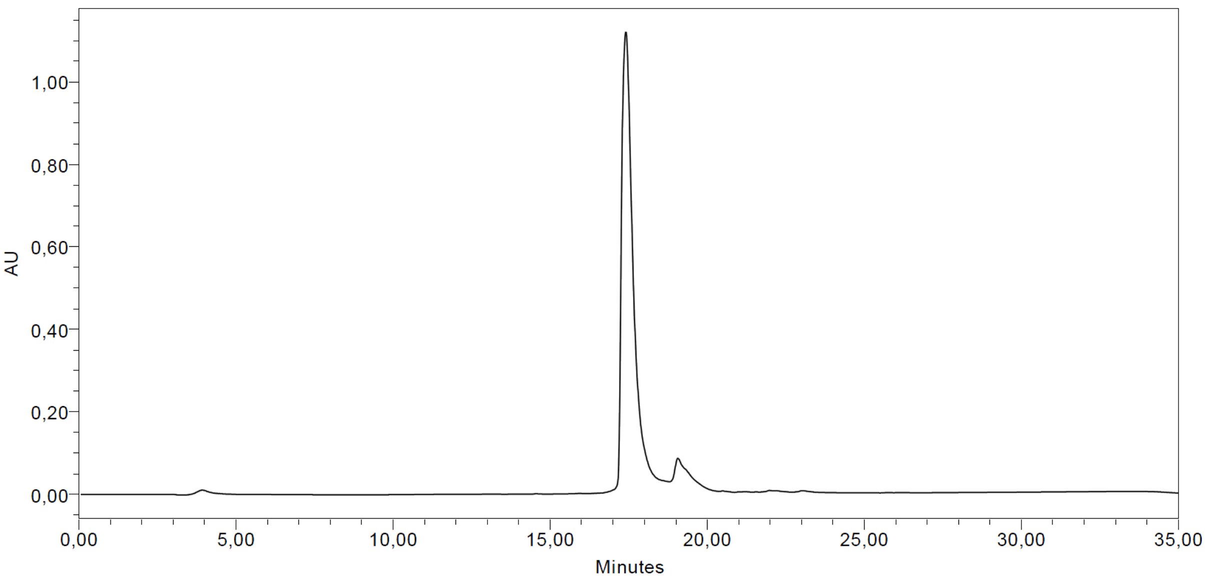
MPP8



N
NH
O
NH2
H
N
O
O
N
H
N
O
O
N
H
N
O
H
N
H
O
O
O
O
HO

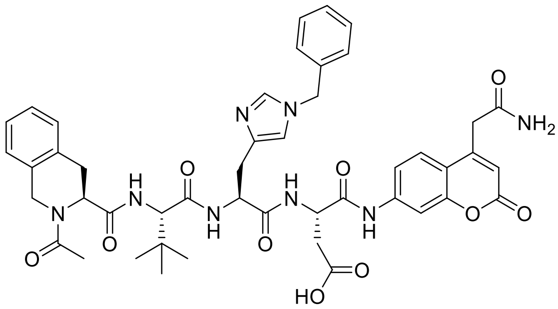


2765_2013_A2 8 (0.120) Cm (7:8)
TOF MS ES+
1.87e3
777.3580
778.3705
100
%
0
100
200
300
400
500
600
700
800
900
1000
1100
m/z

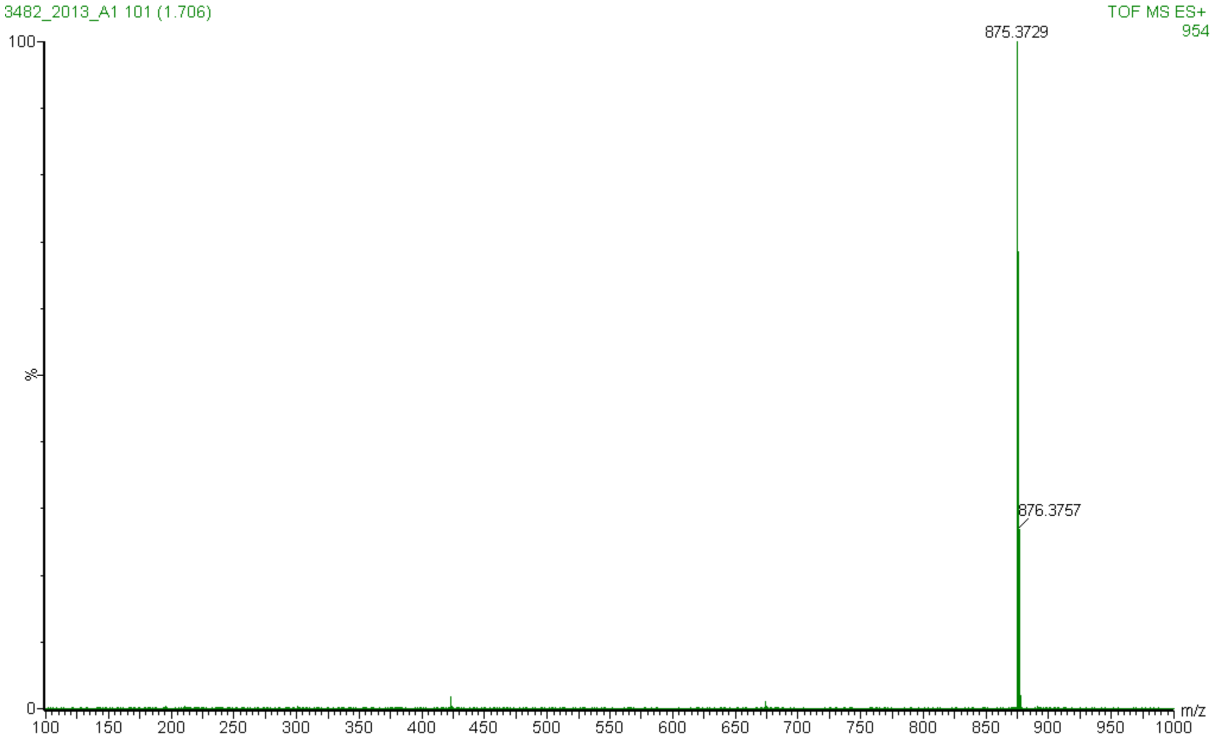


AU
1,00
0,80
0,60
0,40
0,20
0,00
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

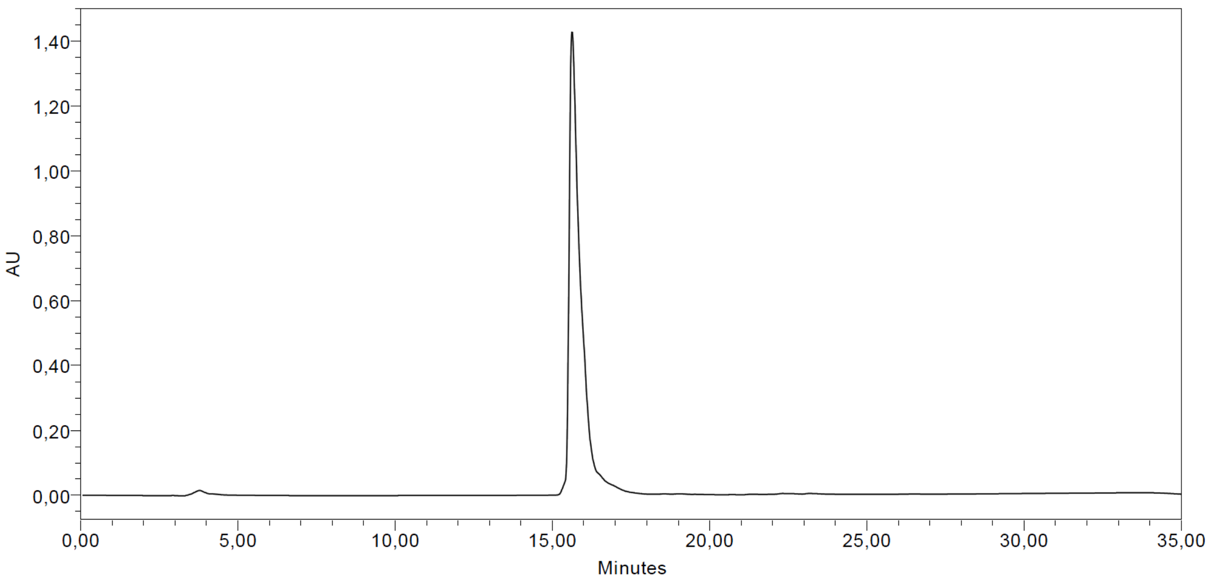
MPP10



N
N
O
NH2
H
N
O
O
N
H
O
H
N
O
N
H
O
O
N
O
O
HO

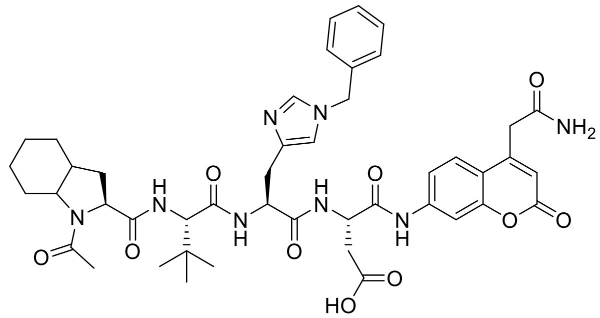


3482_2013_A1 101 (1.706)
TOF MS ES+
954
875.3729
876.3757
100
%
0
m/z
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000

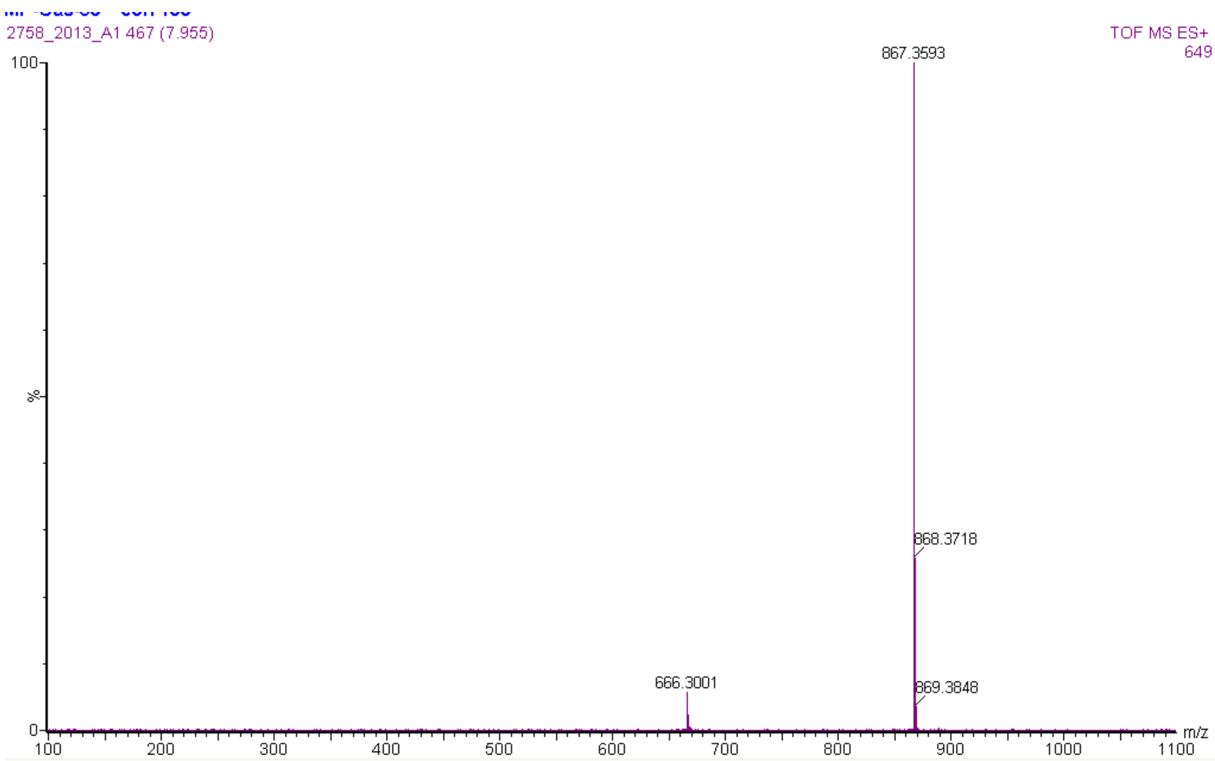


AU
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

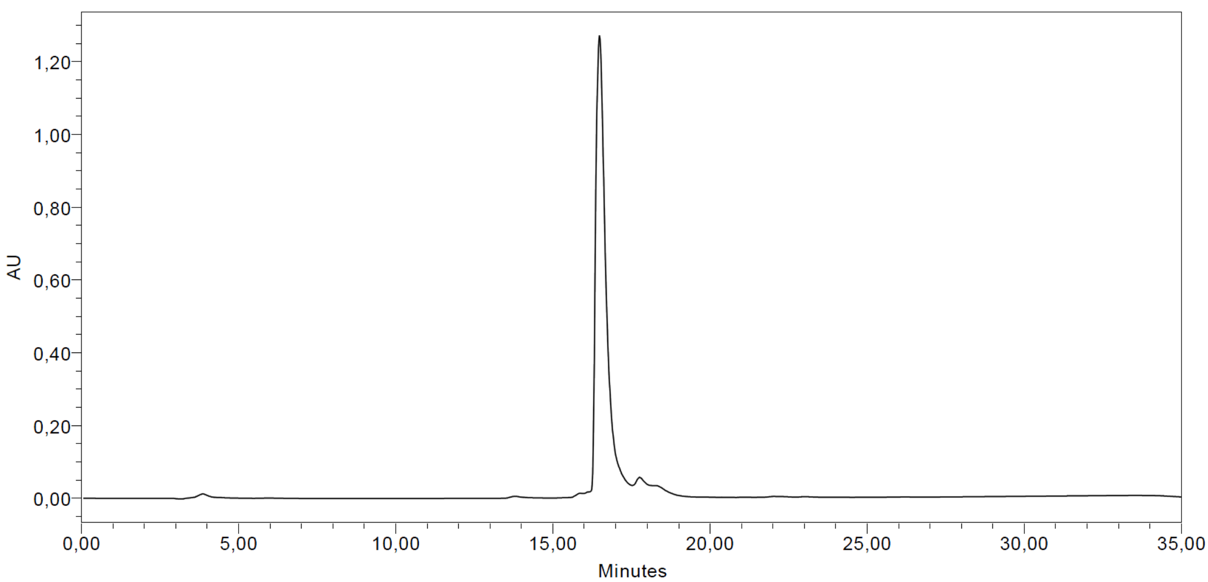
MPP12



NH2
O
N
N
H
HO

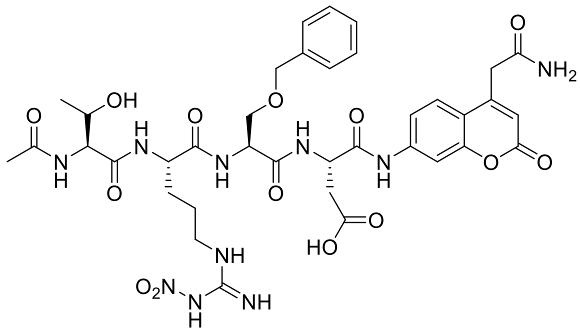


2758_2013_A1 467 (7.955)
TOF MS ES+
649
867.3593
868.3718
869.3848
666.3001
100
%
0
m/z
100
200
300
400
500
600
700
800
900
1000
1100

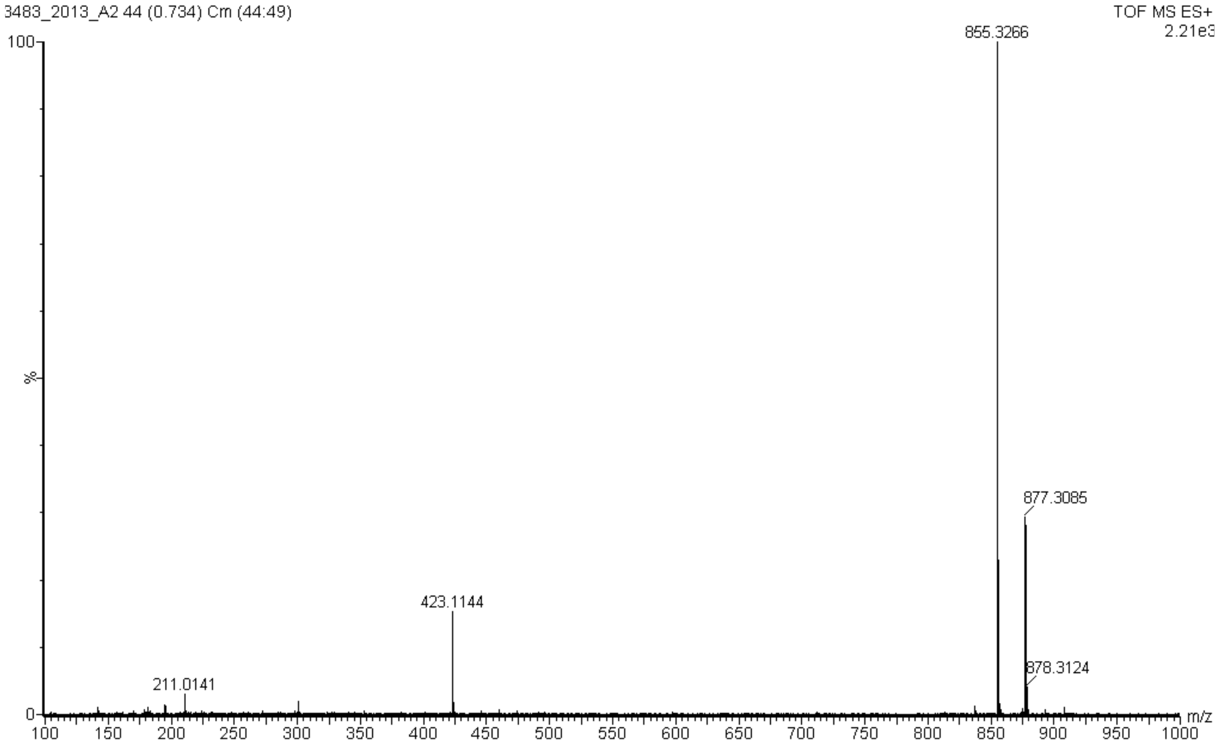


AU
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

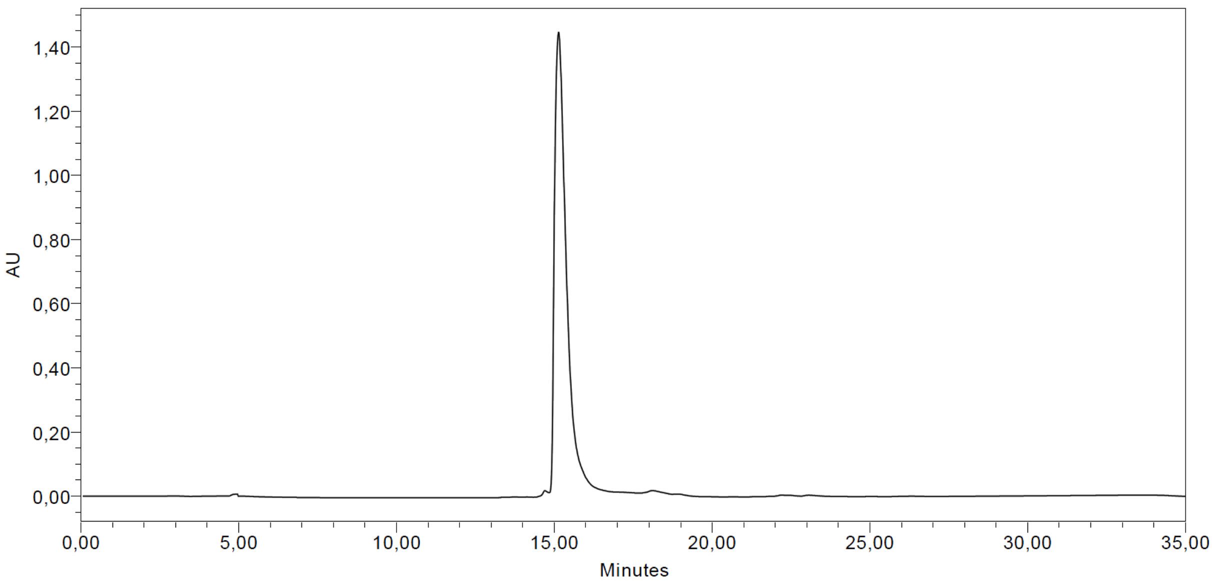
MPP17



OH
O
N
H
O
H
N
O
N
H
O
O
H
N
O
N
H
O
O
O
NH2
NH
O2N
N
H
NH
HO
O

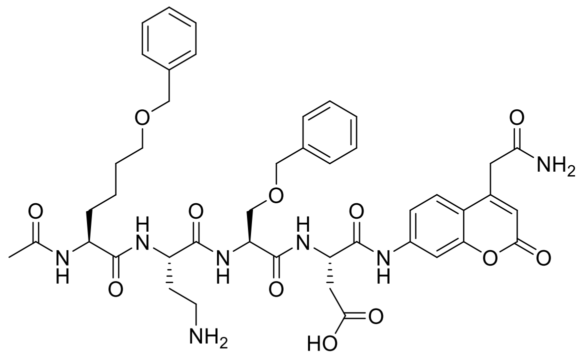


3483_2013_A2 44 (0.734) Cm (44:49)
TOF MS ES+
2.21e3
855.3266
877.3085
878.3124
423.1144
211.0141
%
m/z

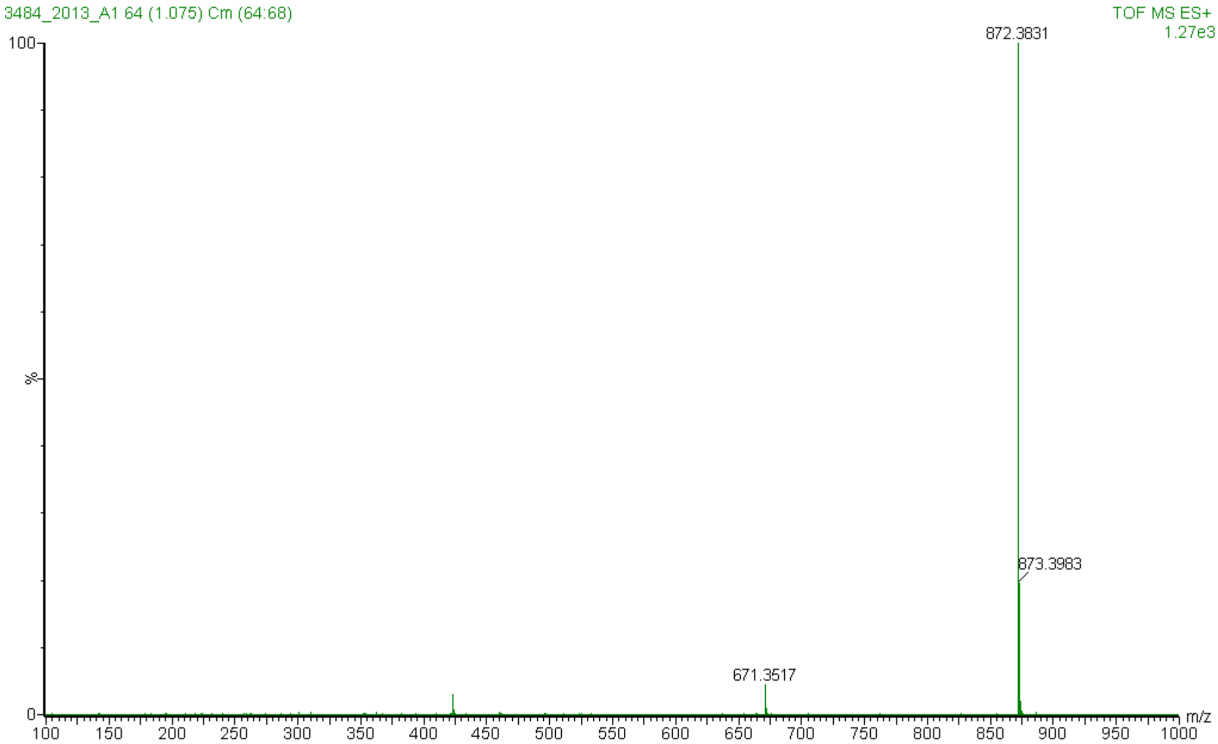


AU
Minutes

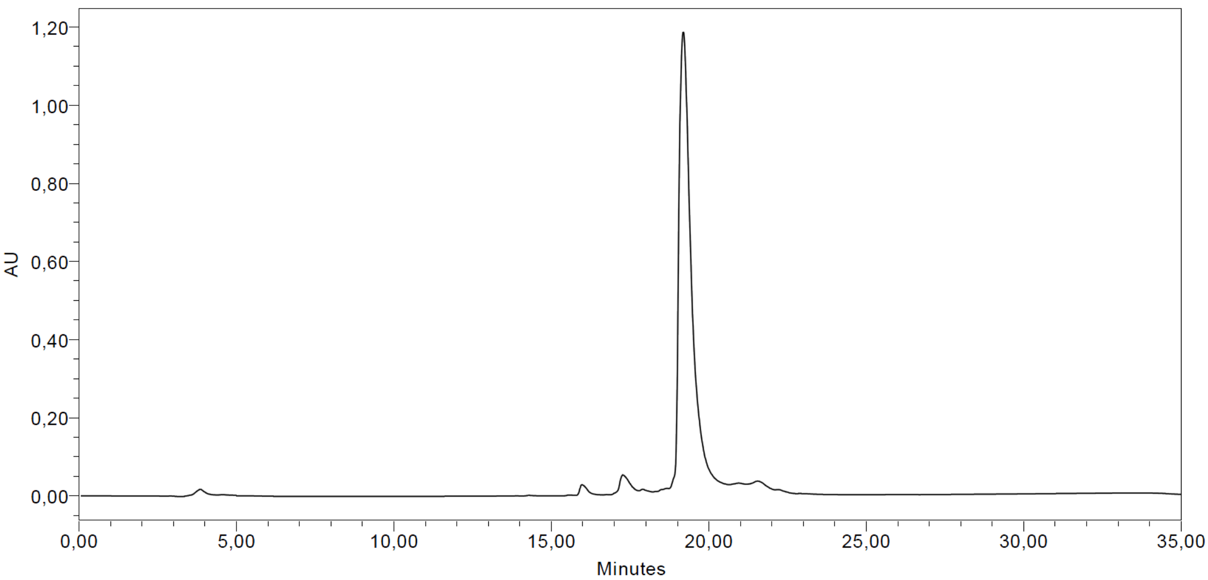
MPP21



O
O
O
O
NH2
O
N
H
H
N
O
O
N
H
H
N
O
N
H
O
O
O
NH2
HO
O

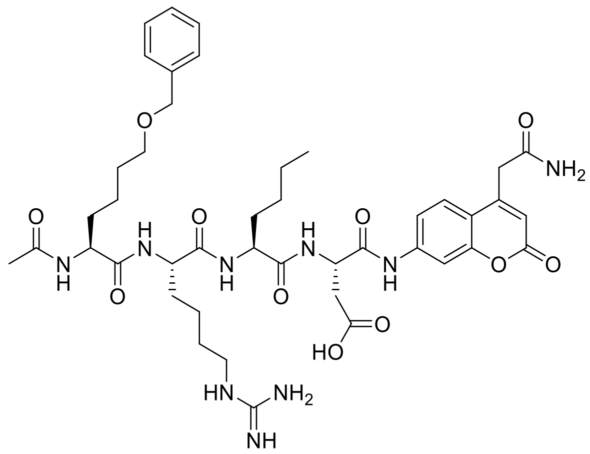


3484_2013_A1 64 (1.075) Cm (64:68)
TOF MS ES+
1.27e3
872.3831
873.3983
671.3517
100
%
0
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
m/z

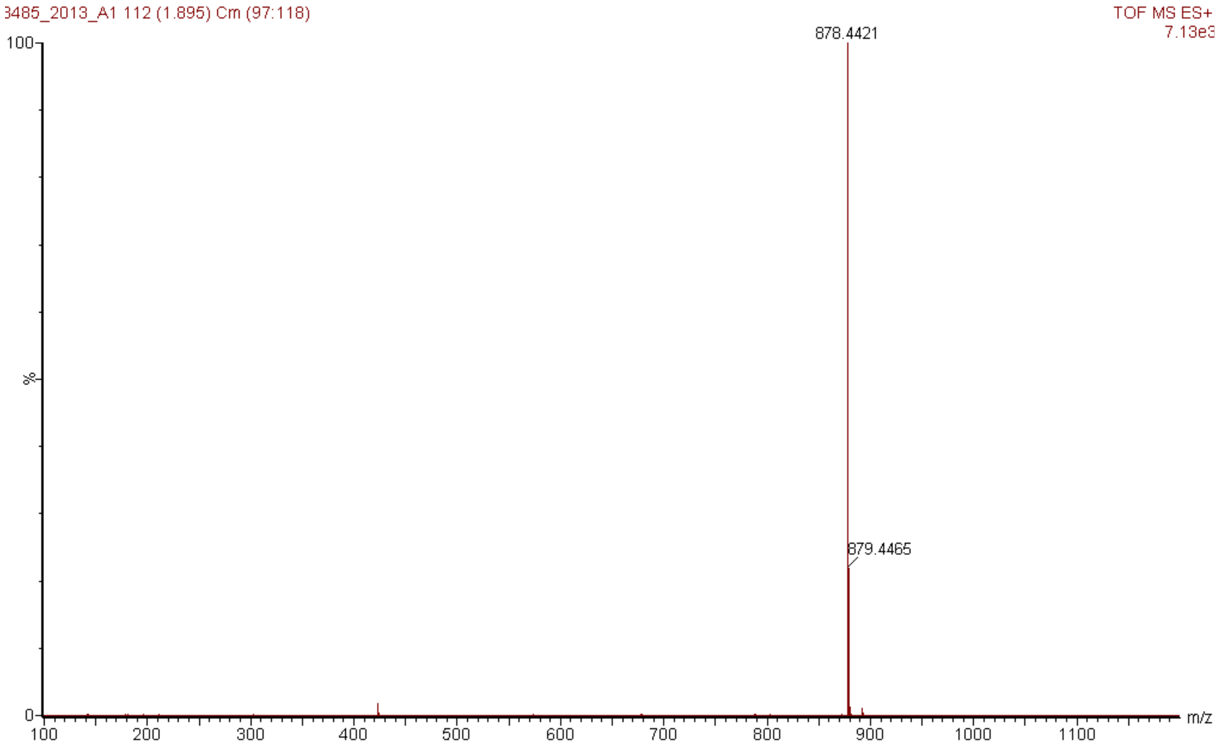


1,20
1,00
0,80
0,60
0,40
0,20
0,00
AU
0,00 5,00 10,00 15,00 20,00 25,00 30,00 35,00
Minutes

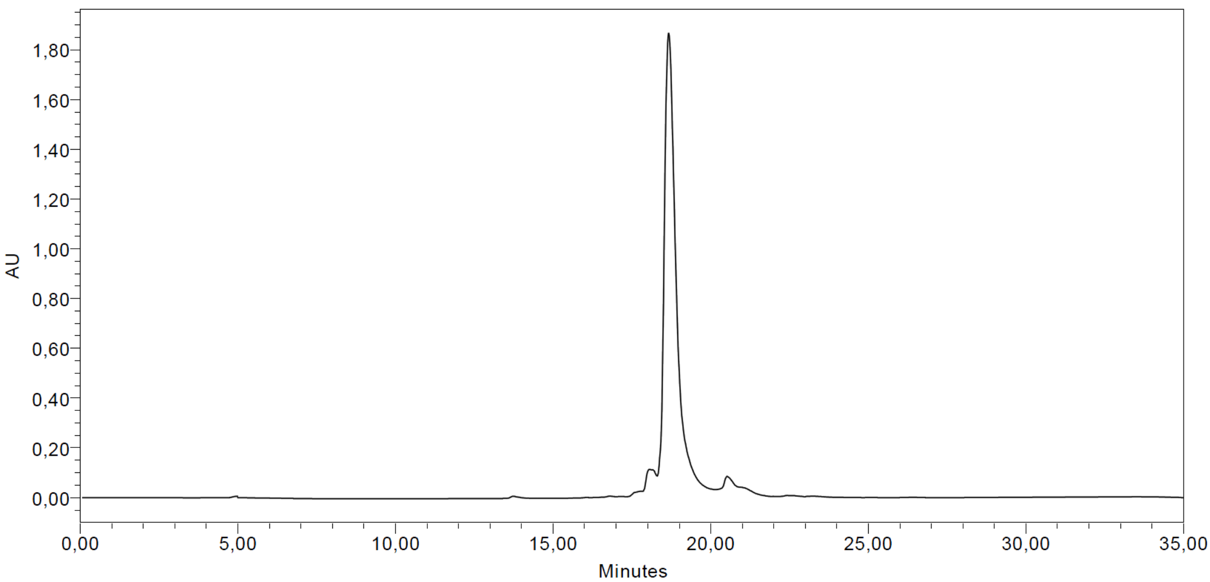
MPP23



O
NH2
O
H
N
H
N
H
N
O
N
H
N
H
O
O
O
O
O
HO
HN
NH2
NH

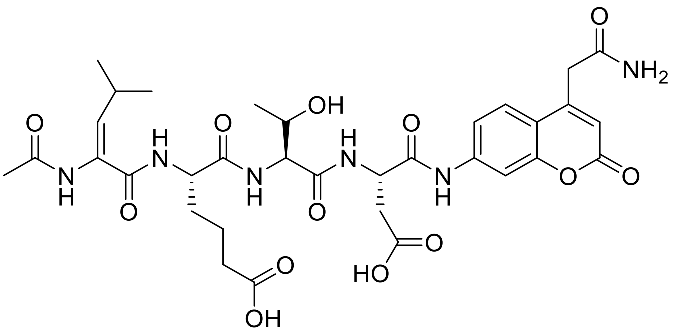


3485_2013_A1 112 (1.895) Cm (97:118)
TOF MS ES+
7.13e3
878.4421
879.4465
100
%
0
100
200
300
400
500
600
700
800
900
1000
1100
m/z

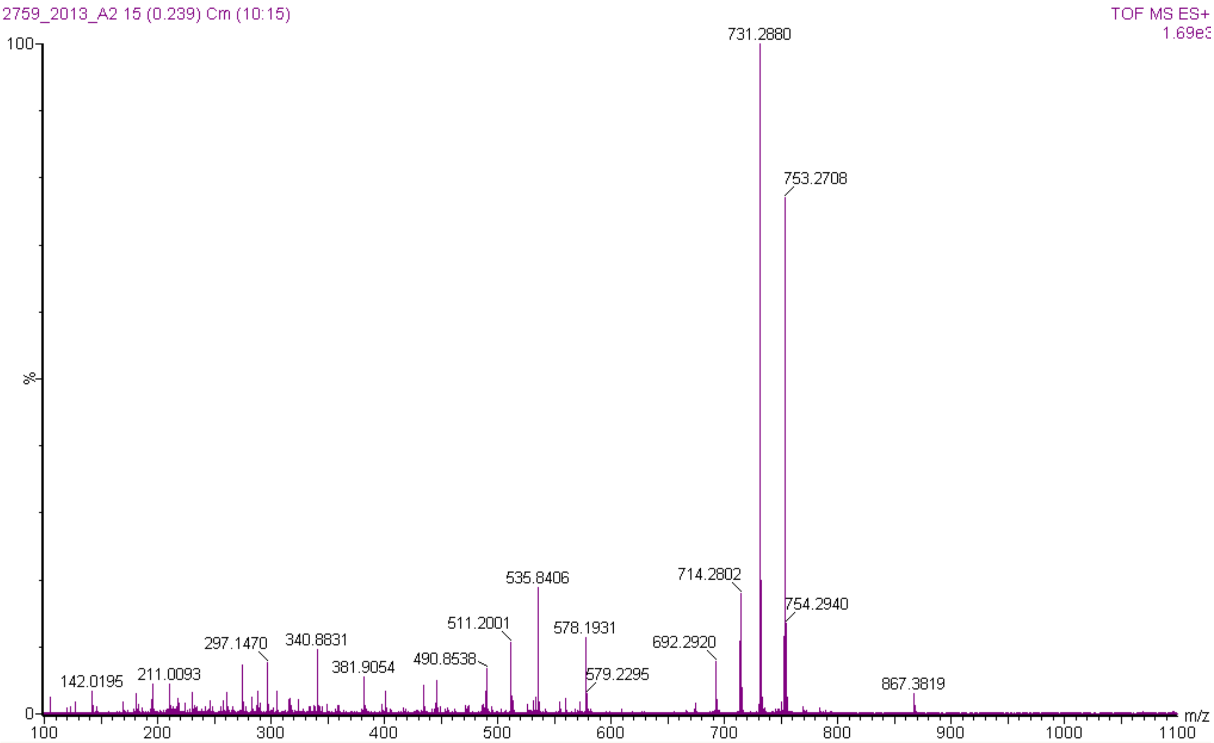


AU
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

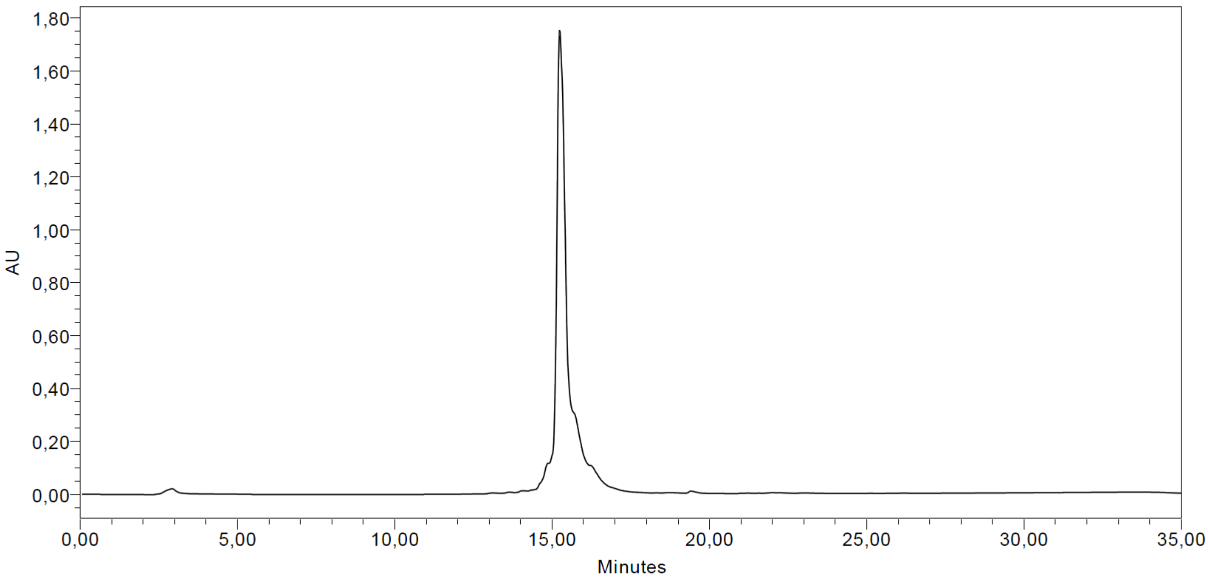
MPP28



O
NH2
OH
O
O
O
H
H
N
N
N
N
N
H
H
H
O
O
O
O
O
HO
O
OH

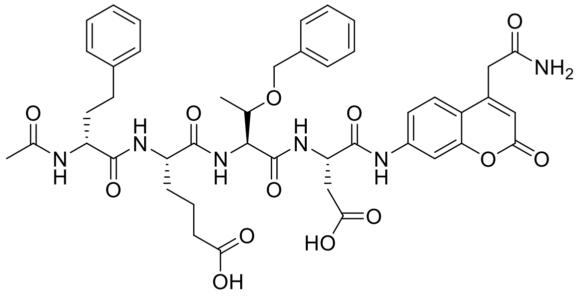


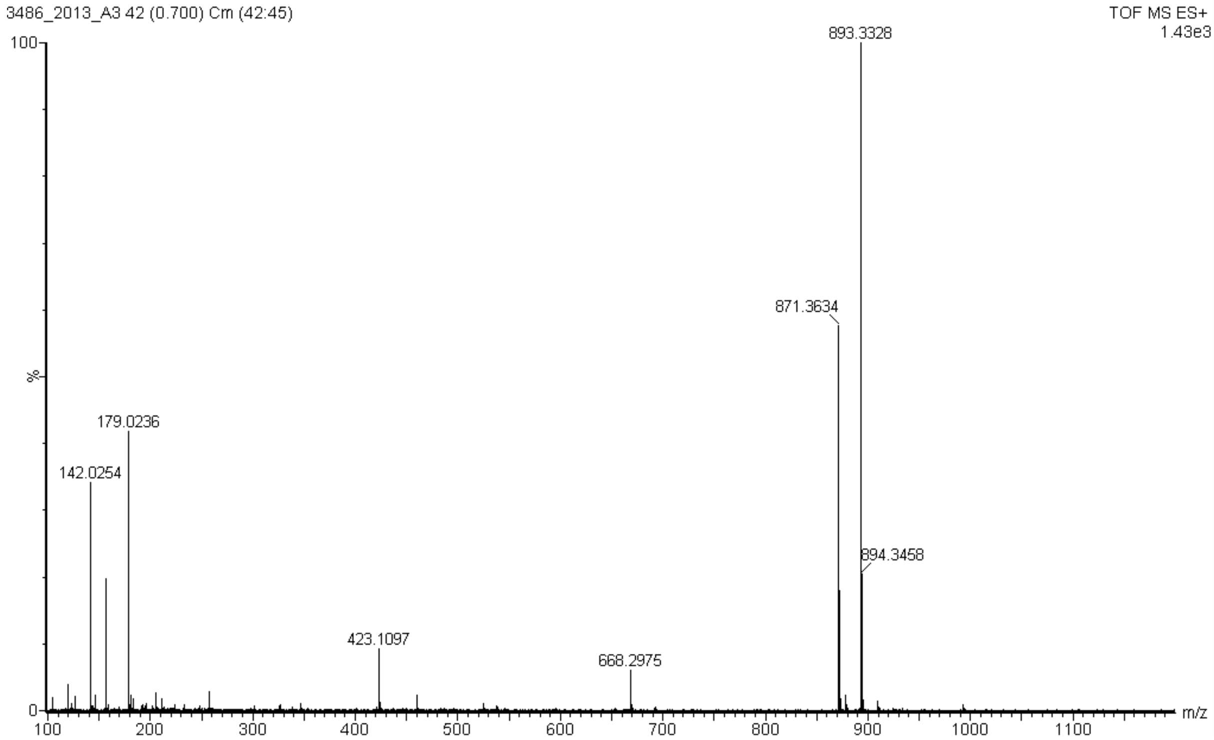
2759_2013_A2 15 (0.239) Cm (10:15)
TOF MS ES+
1.69e3
731.2880
753.2708
535.8406
714.2802
754.2940
511.2001
578.1931
692.2920
297.1470
340.8831
381.9054
490.8538
579.2295
142.0195
211.0093
867.3819
100
%
0
m/z
100
200
300
400
500
600
700
800
900
1000
1100

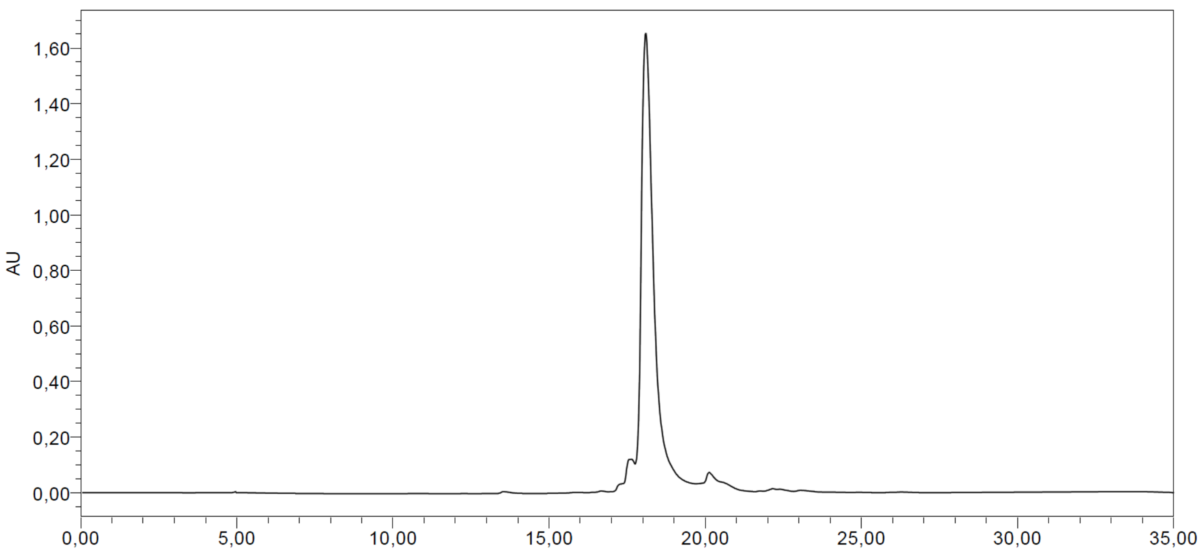


1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
AU
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

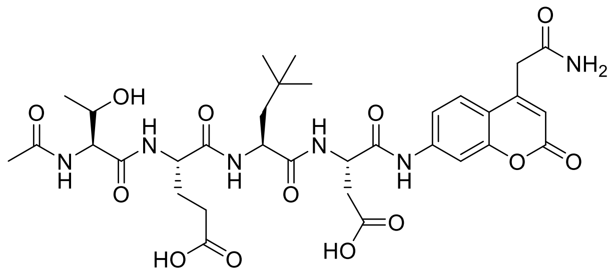
MPP30

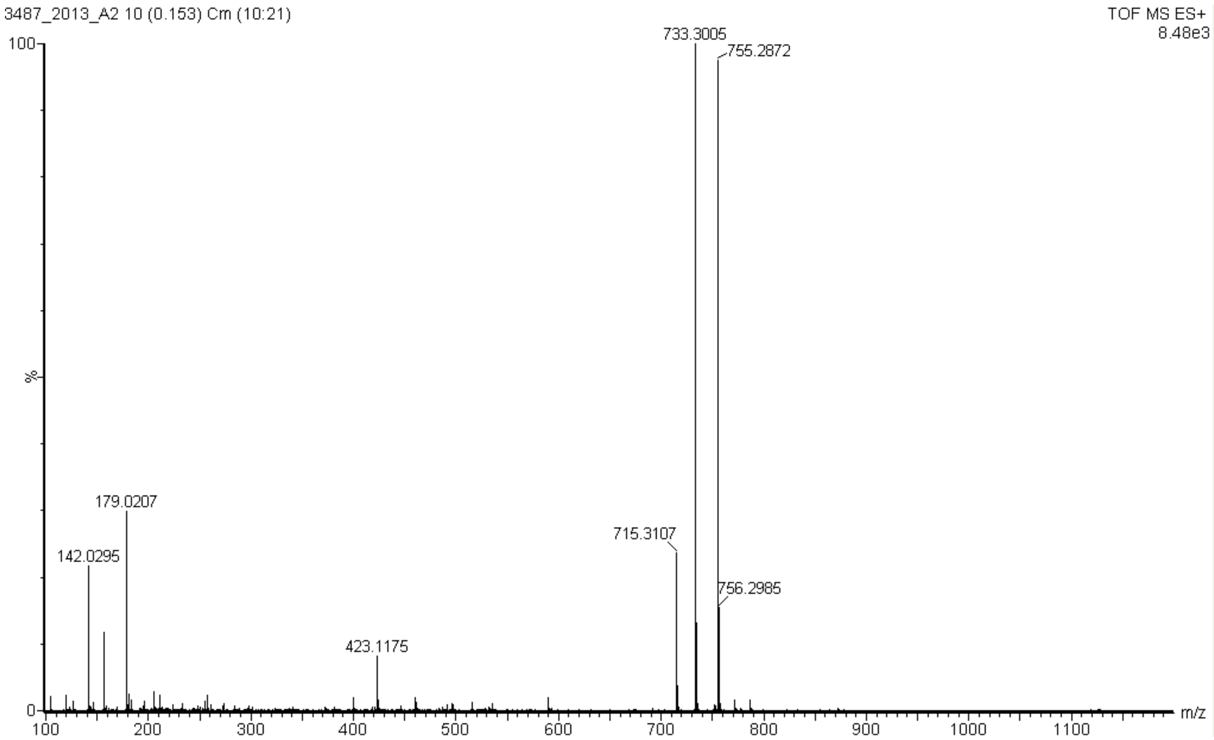

O
NH2
O
O
H
N
O
O
H
N
N
H
N
H
N
H
O
O
O
O
O
HO
O
OH

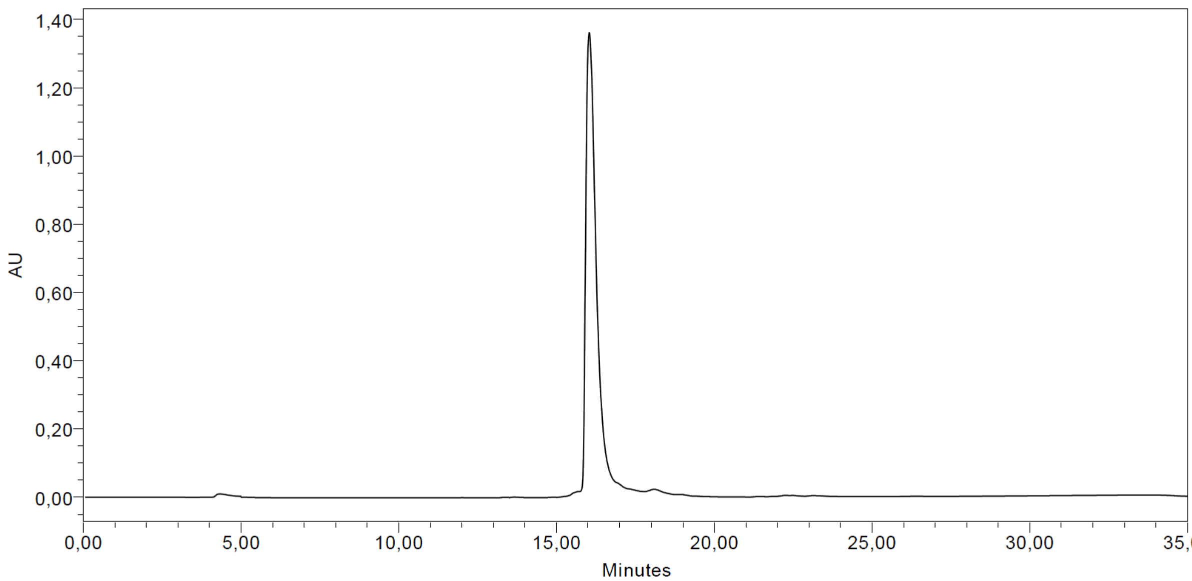

3486_2013_A3 42 (0.700) Cm (42:45)
TOF MS ES+
1.43e3
893.3328
871.3634
179.0236
142.0254
894.3458
423.1097
668.2975
%
m/z


AU

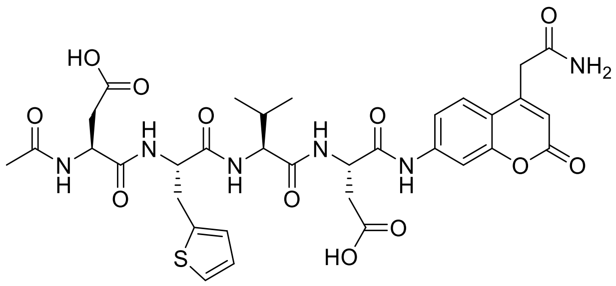
MPP36


OH
O
N
H
O
H
N
O
HO
O
O
N
H
O
H
N
O
HO
O
N
H
O
NH2
O
O

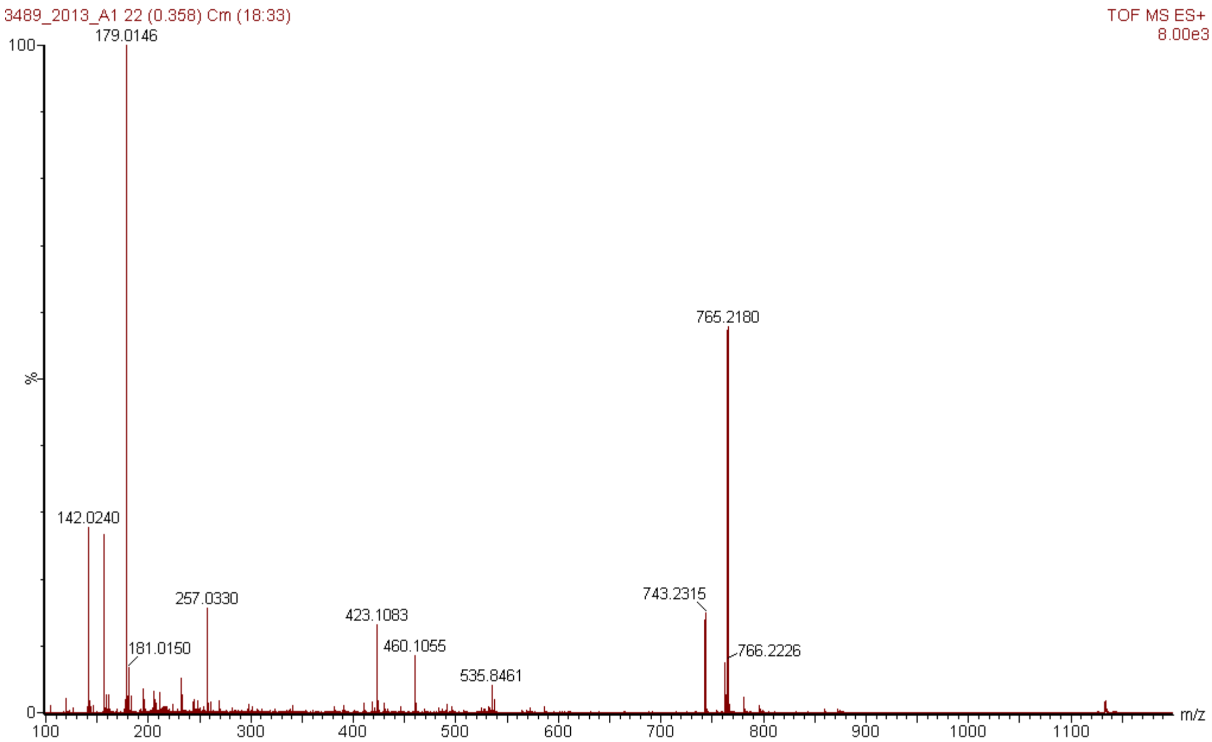

3487_2013_A2 10 (0.153) Cm (10:21)
TOF MS ES+
8.48e3
100
%
0
733.3005
755.2872
179.0207
142.0295
715.3107
756.2985
423.1175
100
200
300
400
500
600
700
800
900
1000
1100
m/z


1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
AU
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

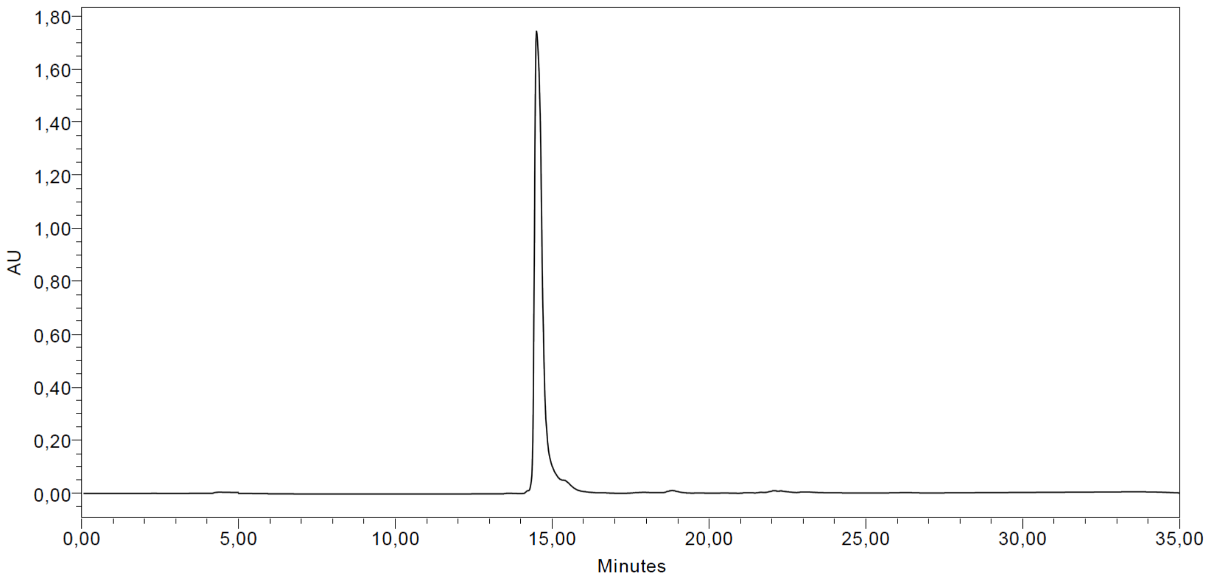
MPP38



HO
O
O
NH2
O
N
H
O
H
N
O
N
H
O
H
N
O
N
H
O
O
S
HO
O

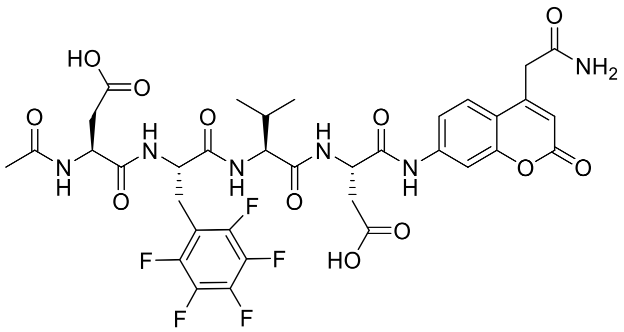


3489_2013_A1 22 (0.358) Cm (18:33)
TOF MS ES+
8.00e3
100
179.0146
765.2180
%
142.0240
257.0330
181.0150
423.1083
460.1055
535.8461
743.2315
766.2226
0
100
200
300
400
500
600
700
800
900
1000
1100
m/z

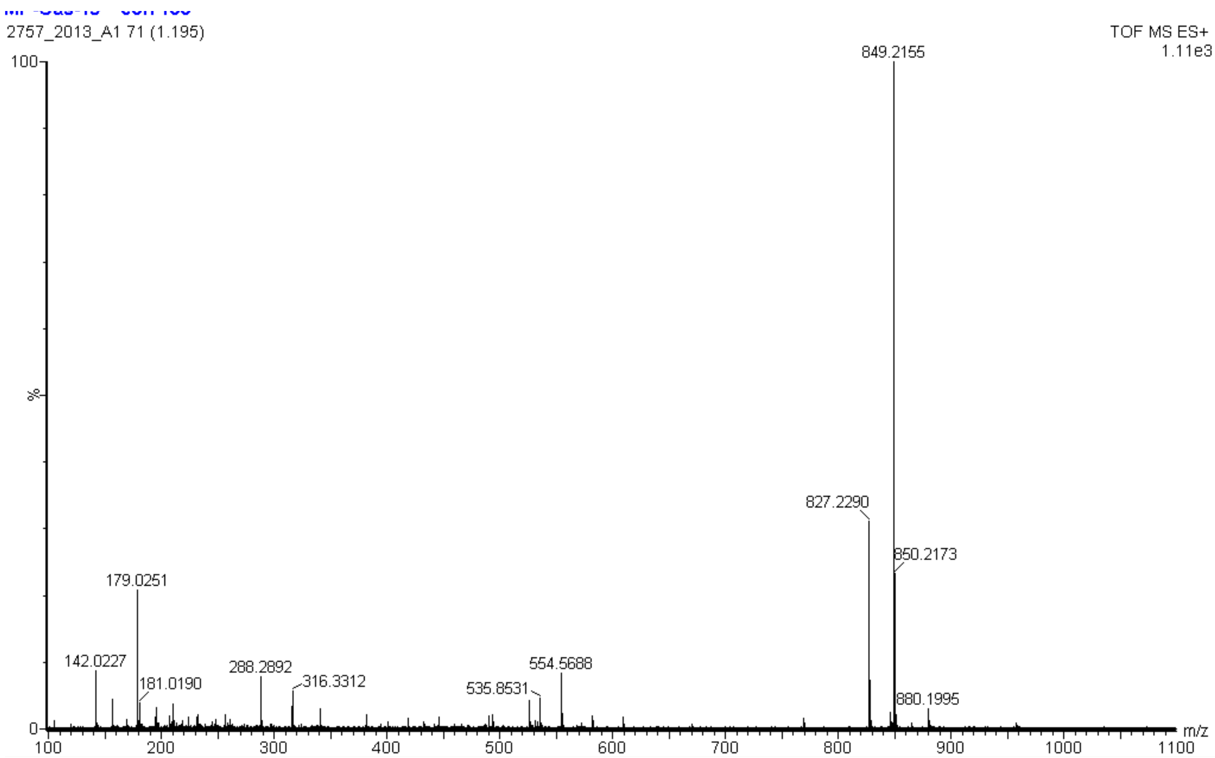


1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
AU
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

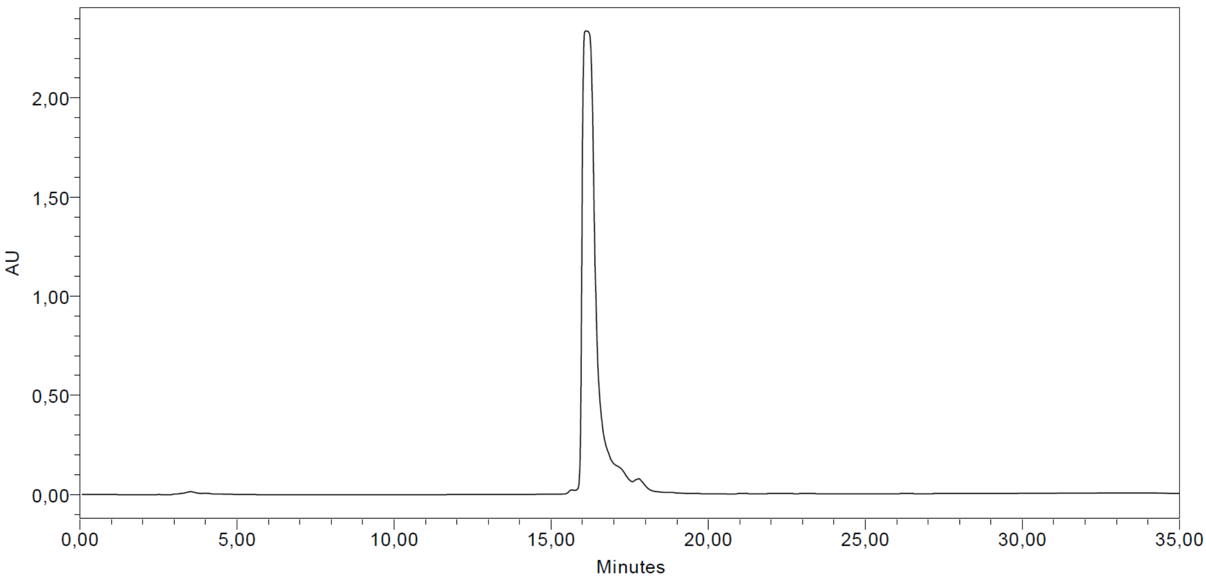
MPP39



HO
O
O
N
H
H
N
O
O
N
H
H
N
O
O
N
H
O
NH2
O
O
F
F
F
F
F
HO
O

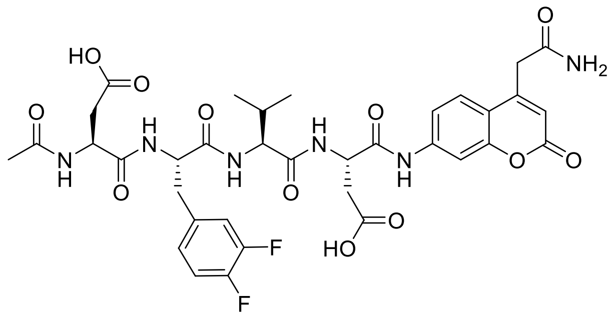


2757_2013_A1 71 (1.195)
TOF MS ES+
1.11e3
849.2155
827.2290
850.2173
179.0251
142.0227
181.0190
288.2892
316.3312
554.5688
535.8531
880.1995
100
%
0
m/z
100
200
300
400
500
600
700
800
900
1000
1100

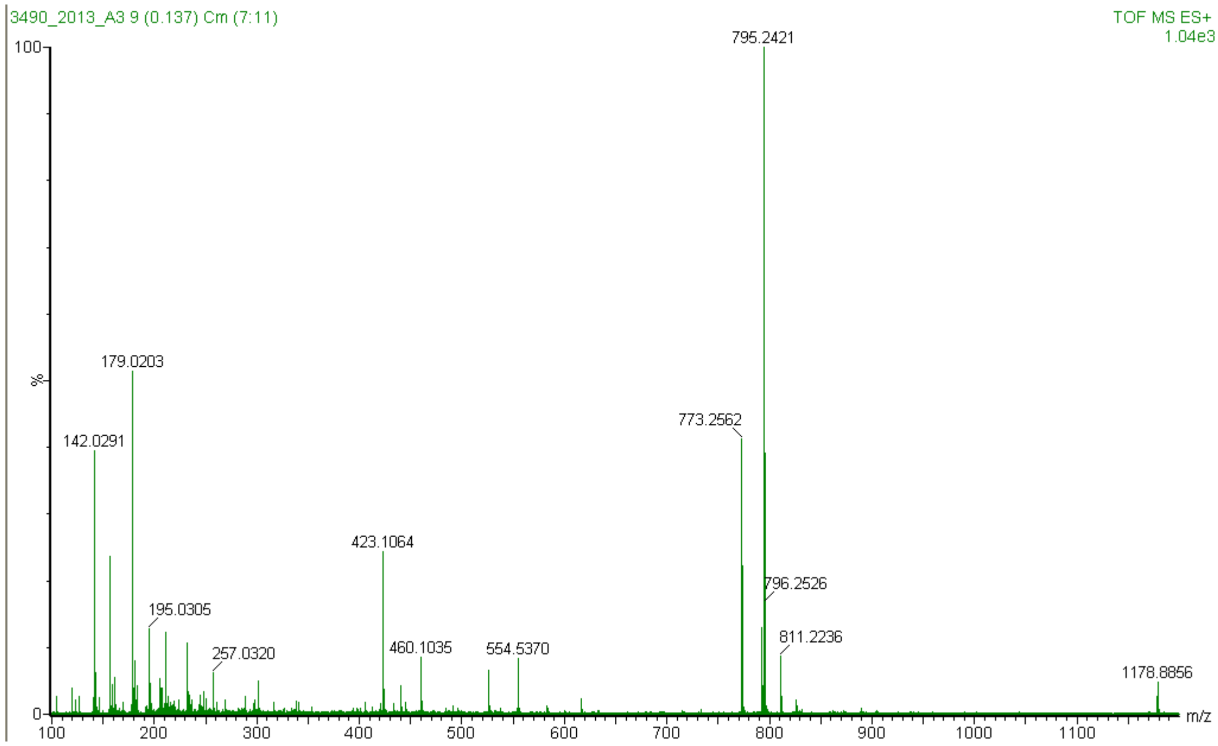


AU
2,00
1,50
1,00
0,50
0,00
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

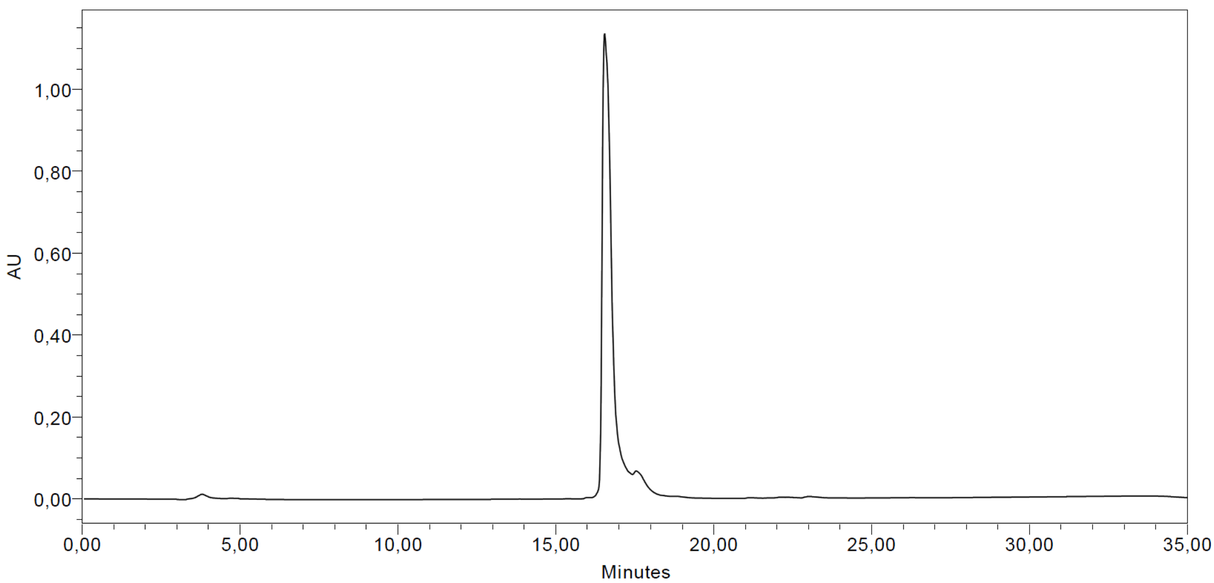
MPP40



HO
O
O
NH2
O
N
H
O
H
N
O
N
H
O
H
N
O
N
H
O
O
F
F
HO
O

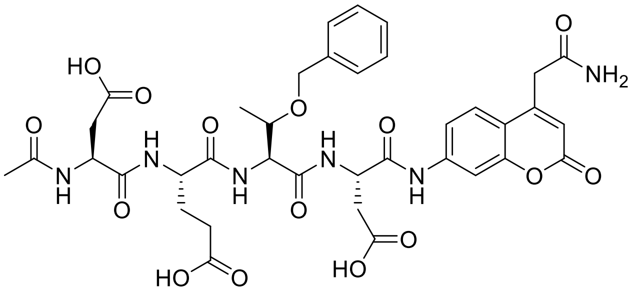


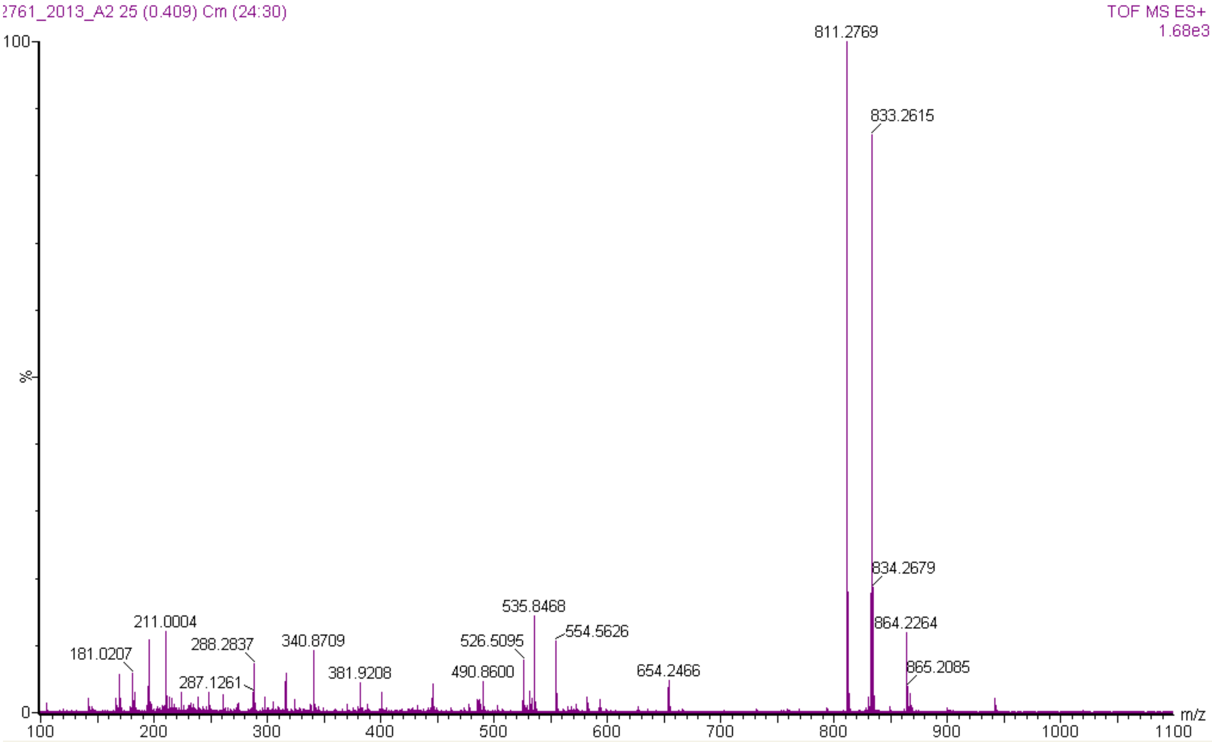
3490_2013_A3 9 (0.137) Cm (7:11)
TOF MS ES+
1.04e3
795.2421
100
%
0
179.0203
142.0291
773.2562
423.1064
796.2526
195.0305
811.2236
257.0320
460.1035
554.5370
1178.8856
100
200
300
400
500
600
700
800
900
1000
1100
m/z

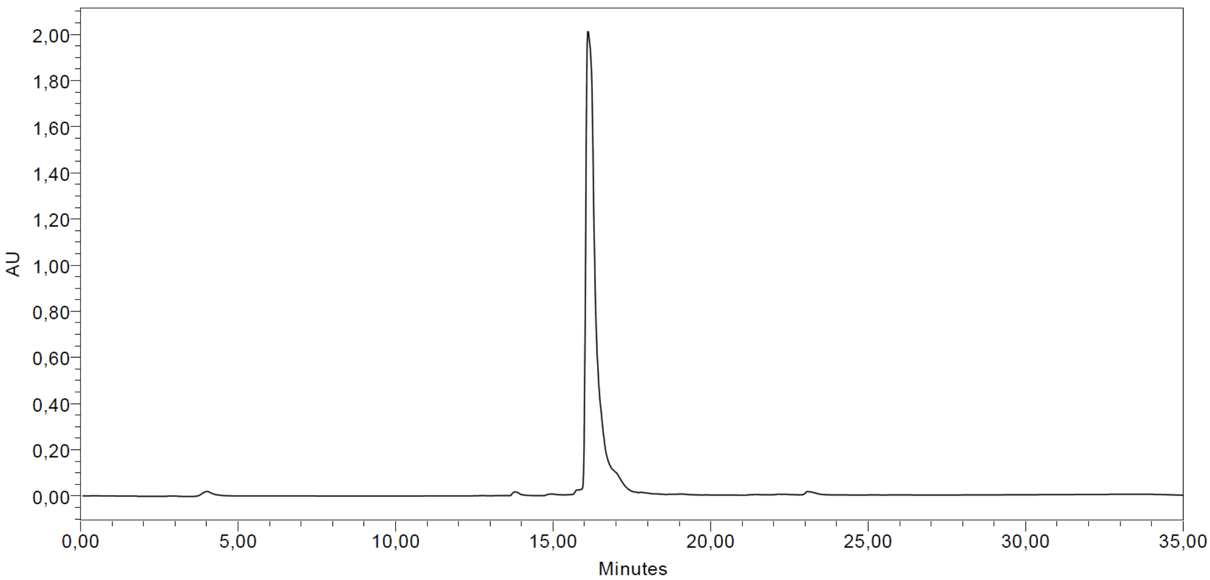


1,00
0,80
0,60
0,40
0,20
0,00
AU
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

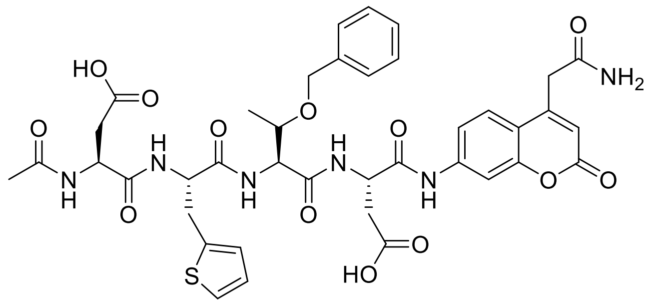
MPP41

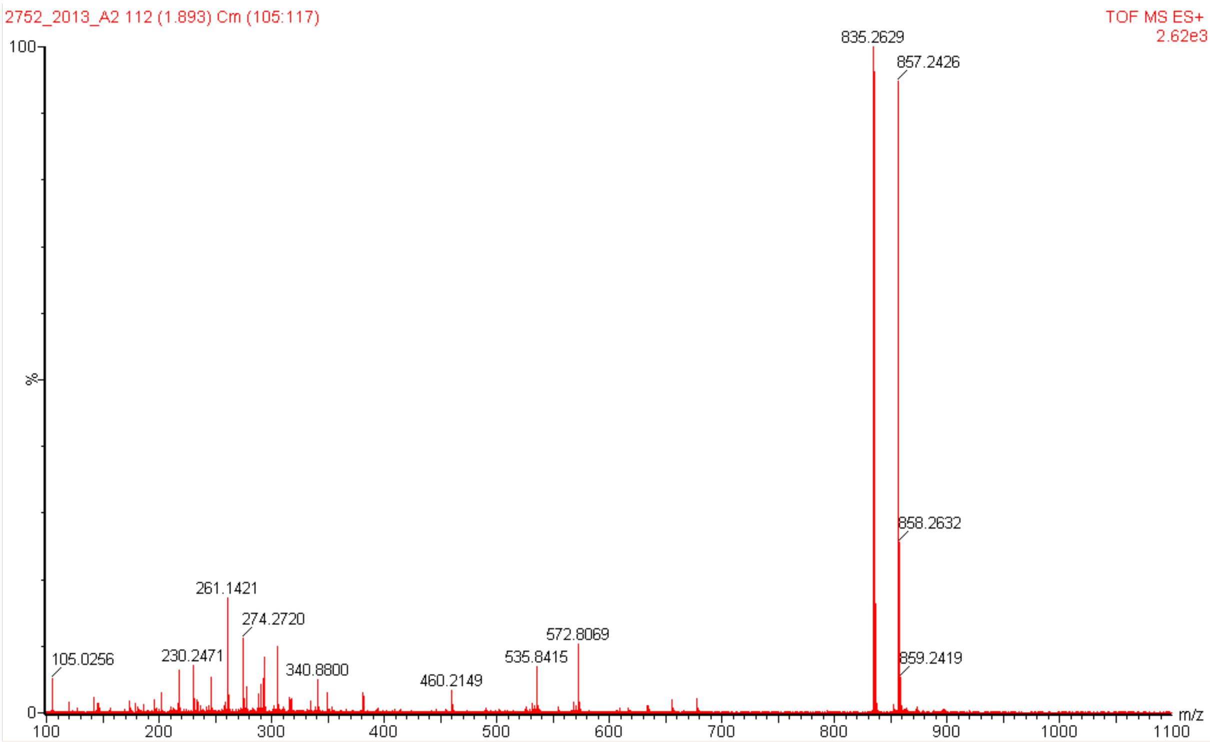


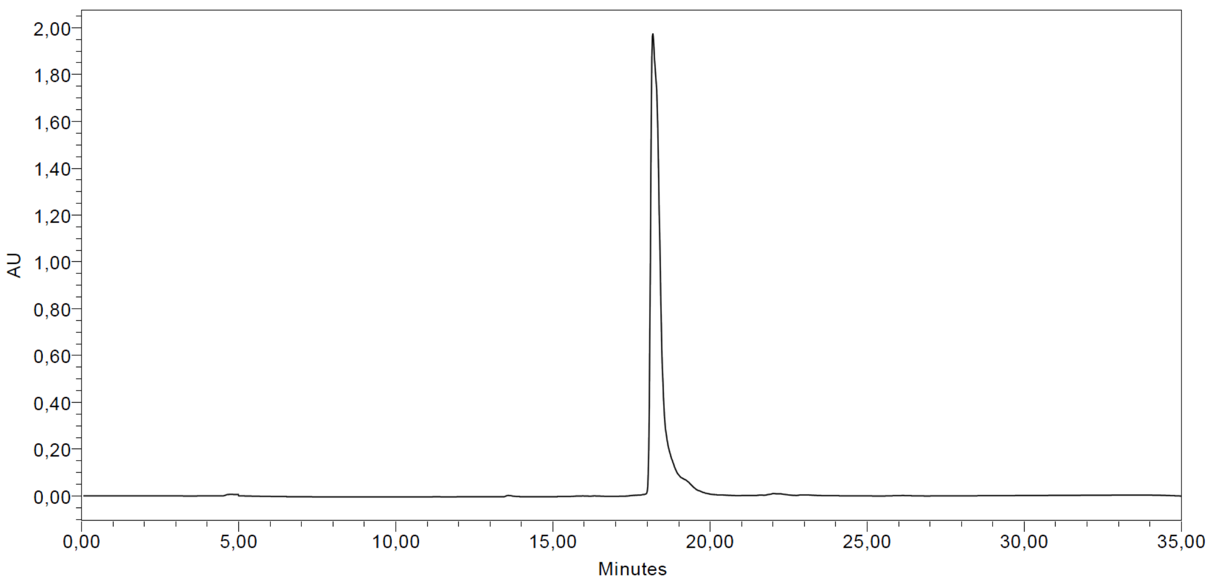

2761_2013_A2 25 (0.409) Cm (24:30)
TOF MS ES+
1.68e3
811.2769
833.2615
834.2679
864.2264
865.2085
654.2466
554.5626
535.8468
526.5095
490.8600
381.9208
340.8709
288.2837
287.1261
211.0004
181.0207
%
m/z


AU
Minutes

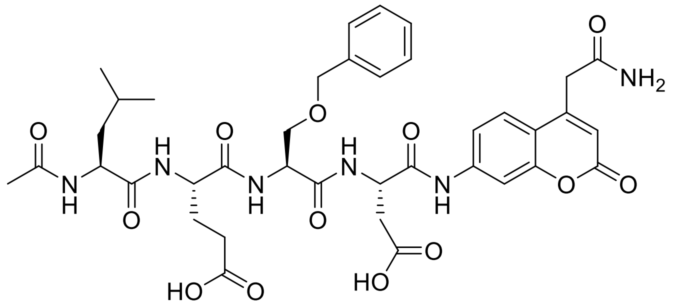
MPP42

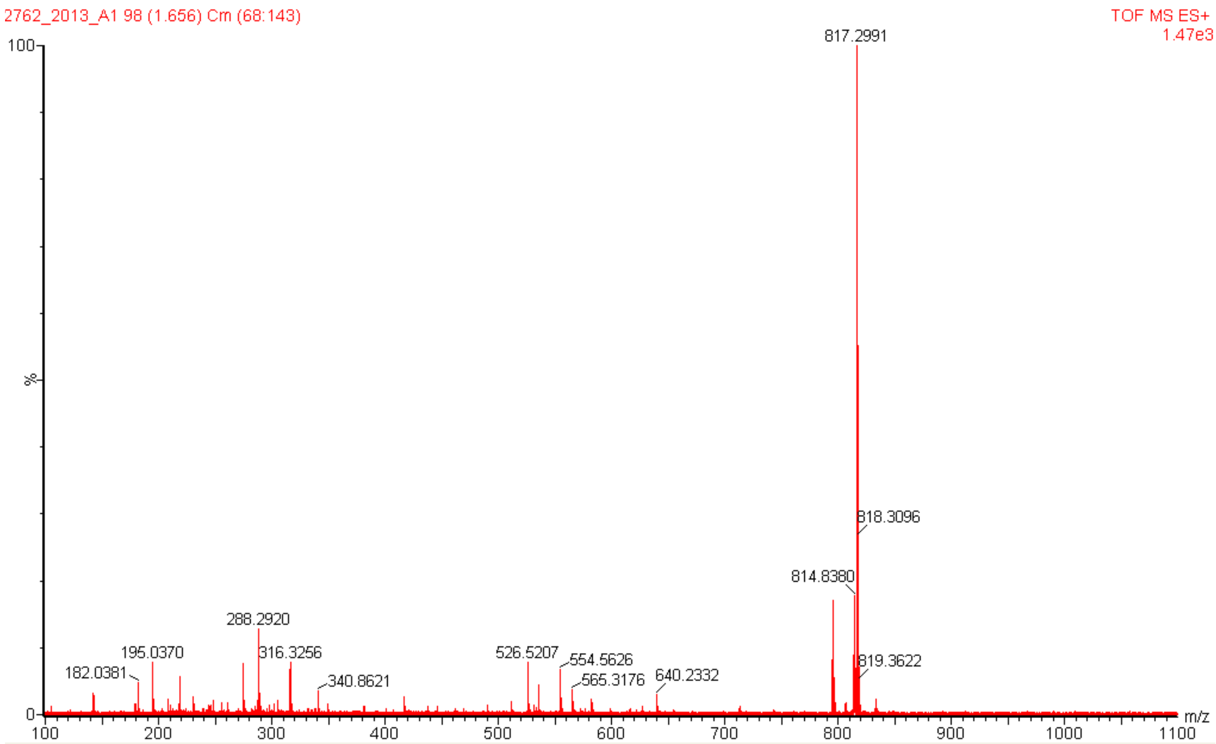

HO
O
O
N
H
H
N
O
O
N
H
O
H
N
O
N
H
O
O
O
NH2
S
HO
O

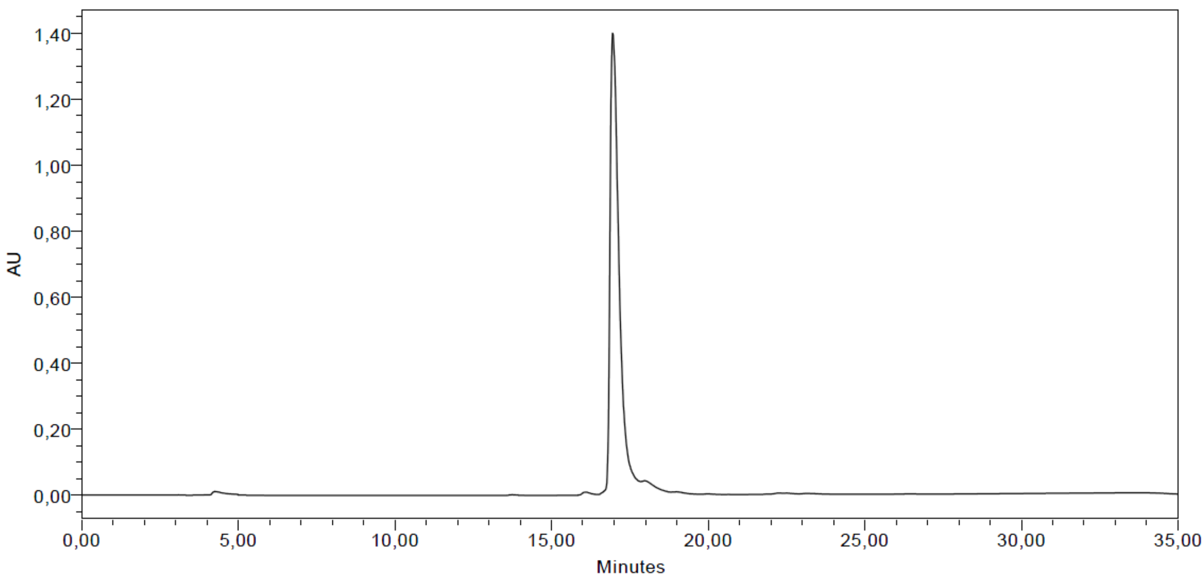

2752_2013_A2 112 (1.893) Cm (105:117)
TOF MS ES+
2.62e3
100
%
0
835.2629
857.2426
858.2632
859.2419
261.1421
274.2720
230.2471
105.0256
340.8800
460.2149
535.8415
572.8069
m/z
100
200
300
400
500
600
700
800
900
1000
1100


AU
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

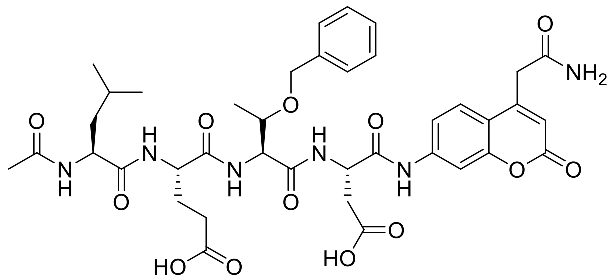
MPP43


O
NH2
O
O
H
N
O
H
N
O
N
H
O
N
H
O
N
H
O
O
O
HO
O
HO
O

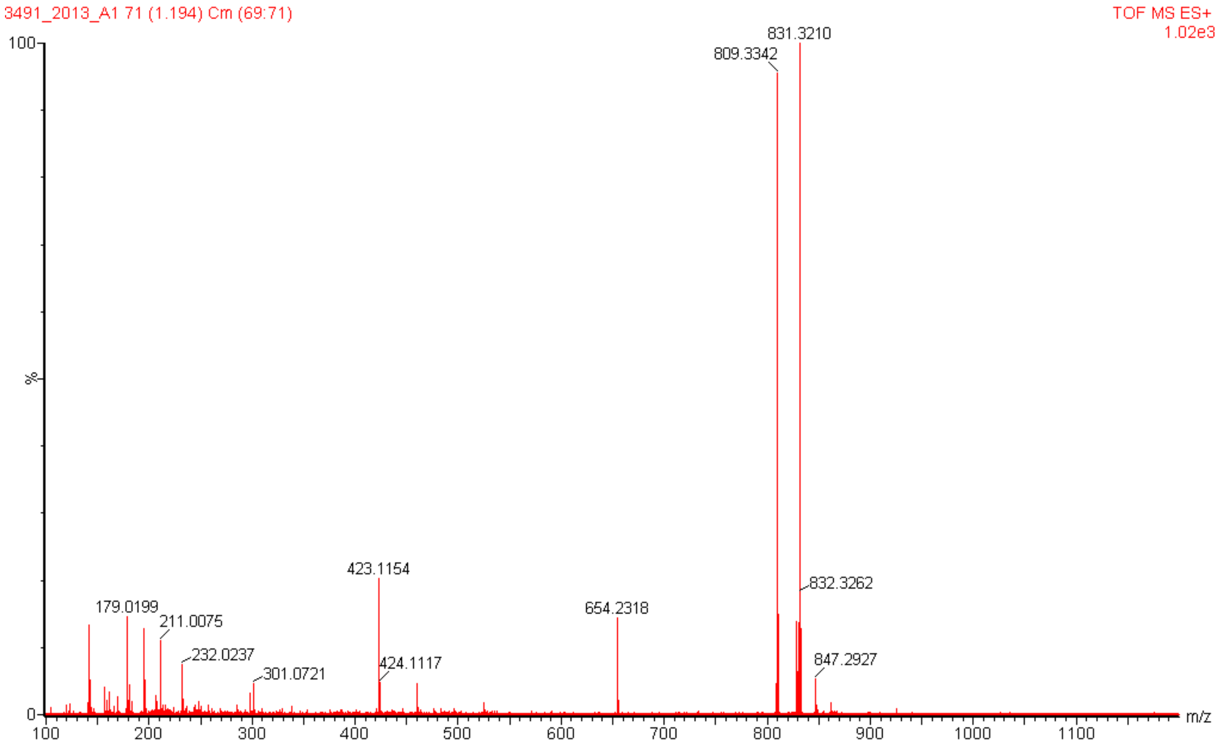

2762_2013_A1 98 (1.656) Cm (68:143)
TOF MS ES+
1.47e3
817.2991
818.3096
814.8380
819.3622
288.2920
195.0370
316.3256
182.0381
340.8621
526.5207
554.5626
565.3176
640.2332
100
%
0
m/z
100
200
300
400
500
600
700
800
900
1000
1100


1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
AU
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

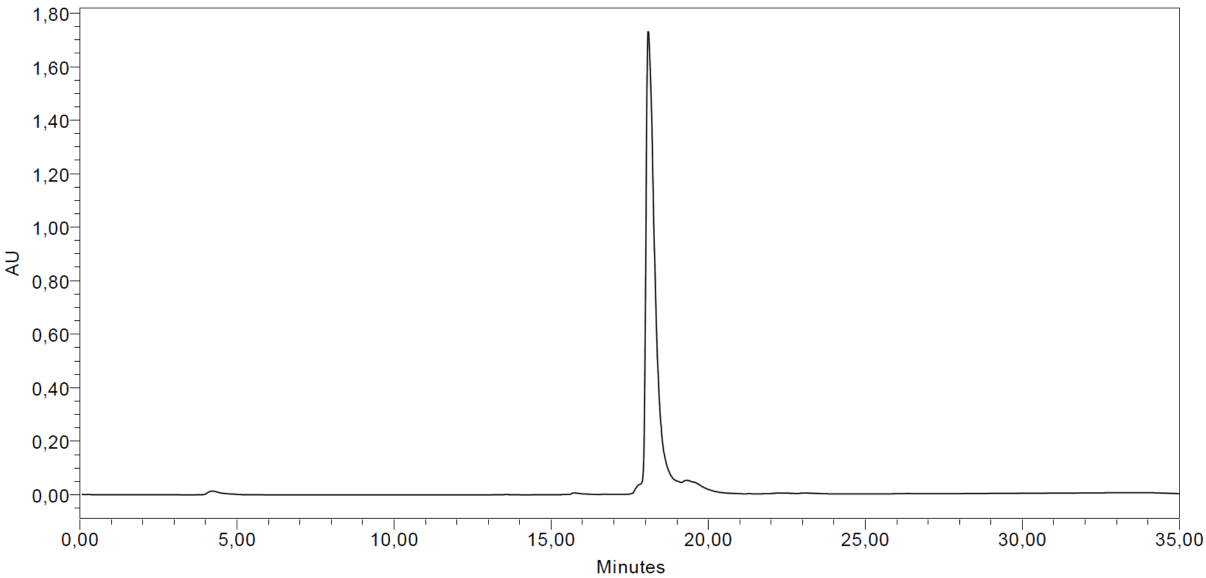
MPP45



O
NH2
O
O
O
N
H
H
N
O
N
H
H
N
O
N
H
O
O
HO
O
HO
O

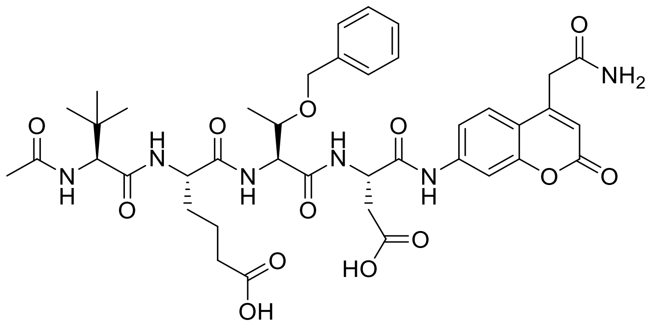


3491_2013_A1 71 (1.194) Cm (69:71)
TOF MS ES+
1.02e3
831.3210
809.3342
423.1154
832.3262
654.2318
179.0199
211.0075
232.0237
301.0721
424.1117
847.2927
100
%
0
m/z
100
200
300
400
500
600
700
800
900
1000
1100

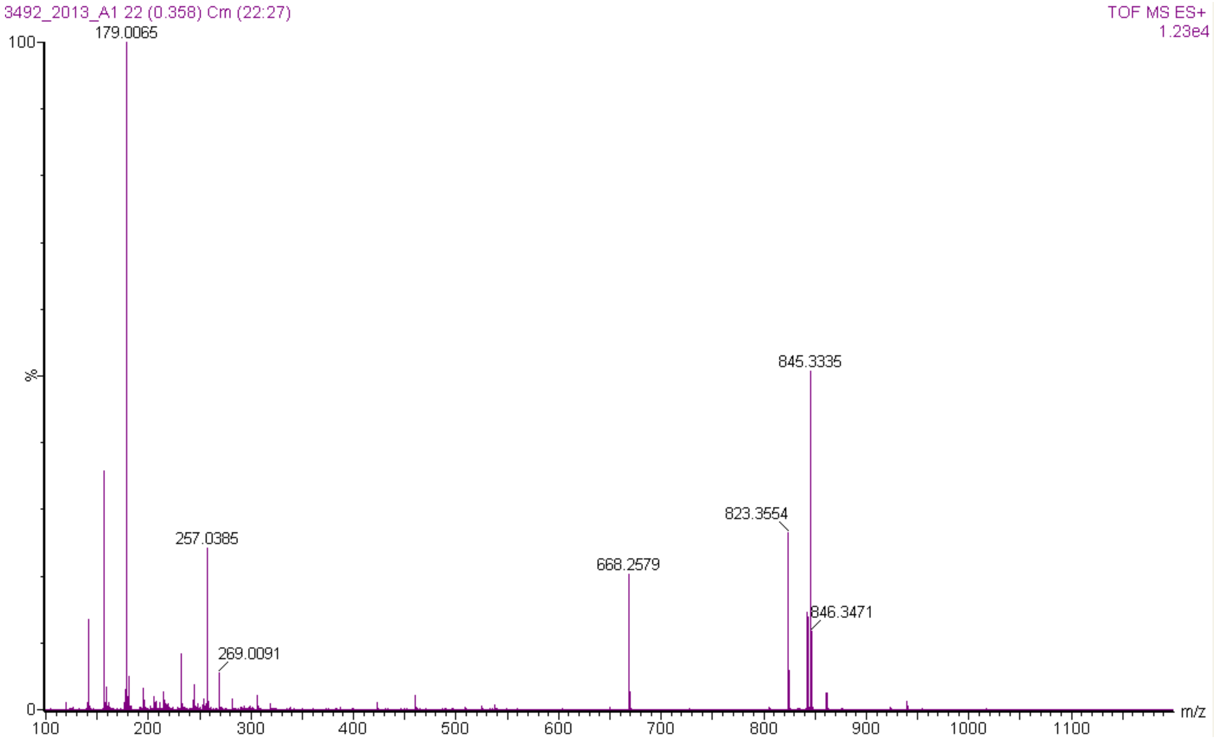


1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
AU
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

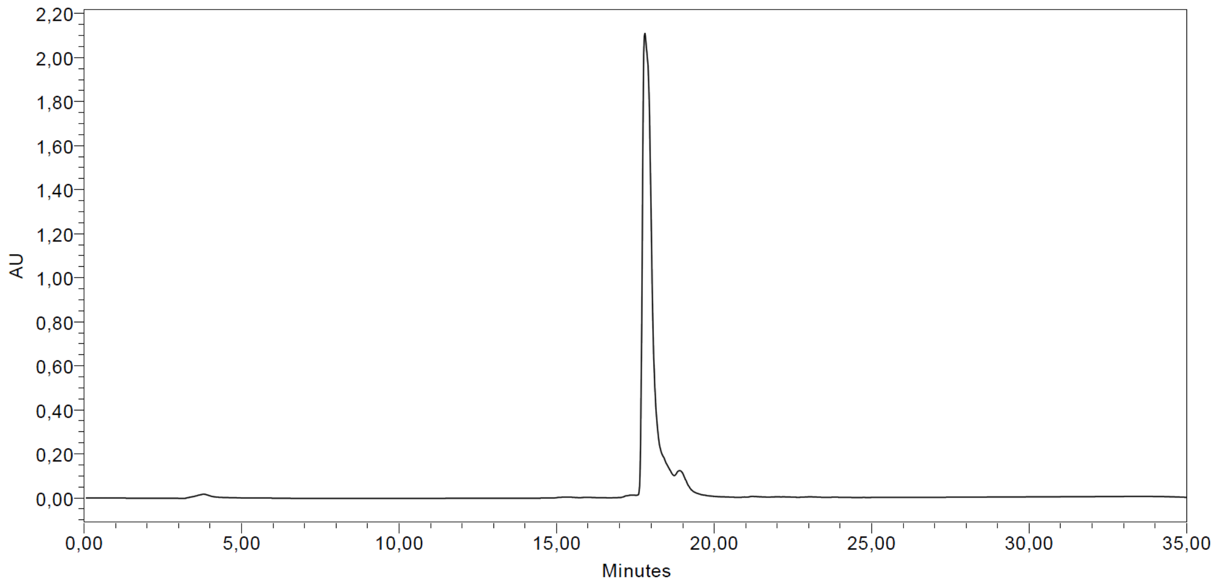
MPP46



O
NH2
O
O
O
H
N
N
H
O
N
H
O
H
N
O
N
H
O
O
O
OH
HO
O

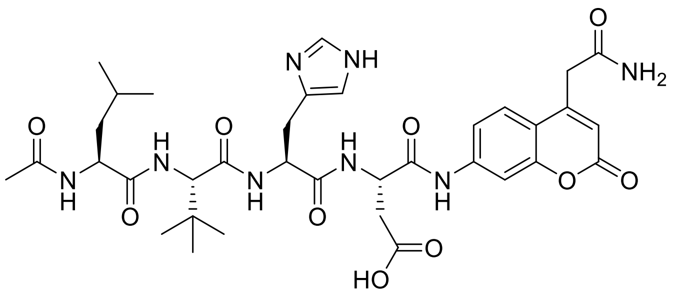


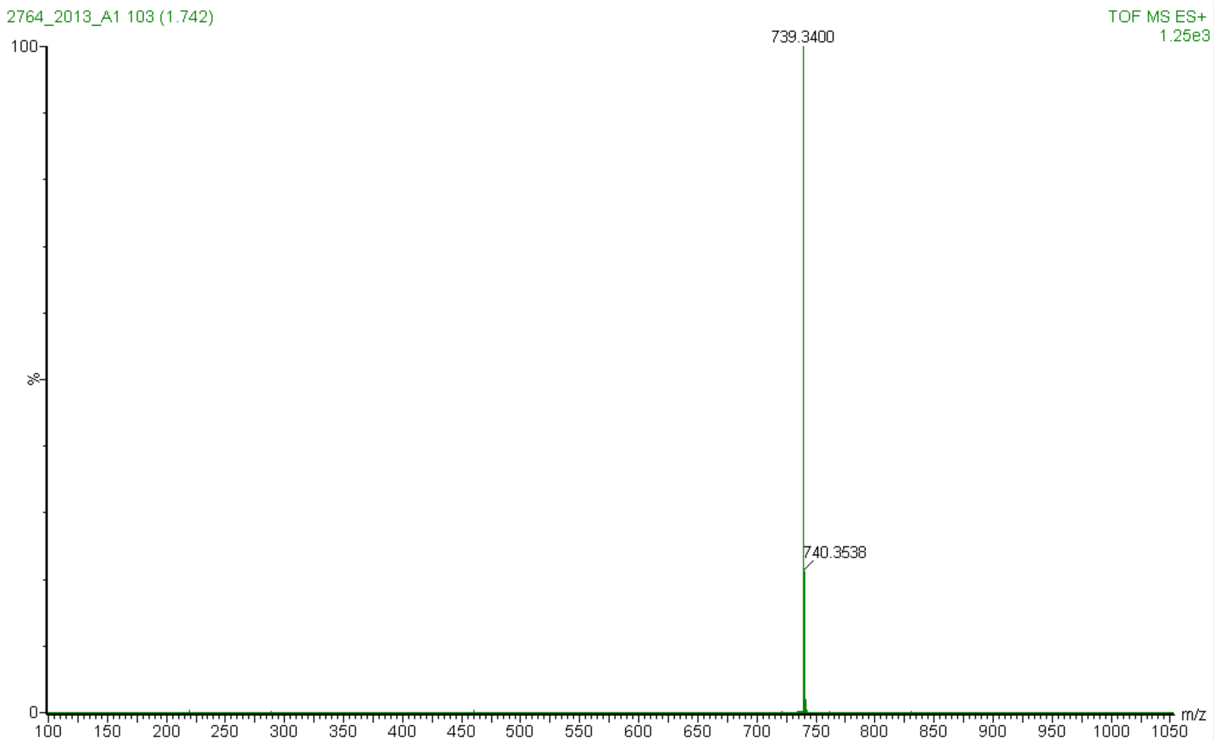
3492_2013_A1 22 (0.358) Cm (22:27)
TOF MS ES+
1.23e4
100
%
0
179.0065
257.0385
269.0091
668.2579
823.3554
845.3335
846.3471
100
200
300
400
500
600
700
800
900
1000
1100
m/z

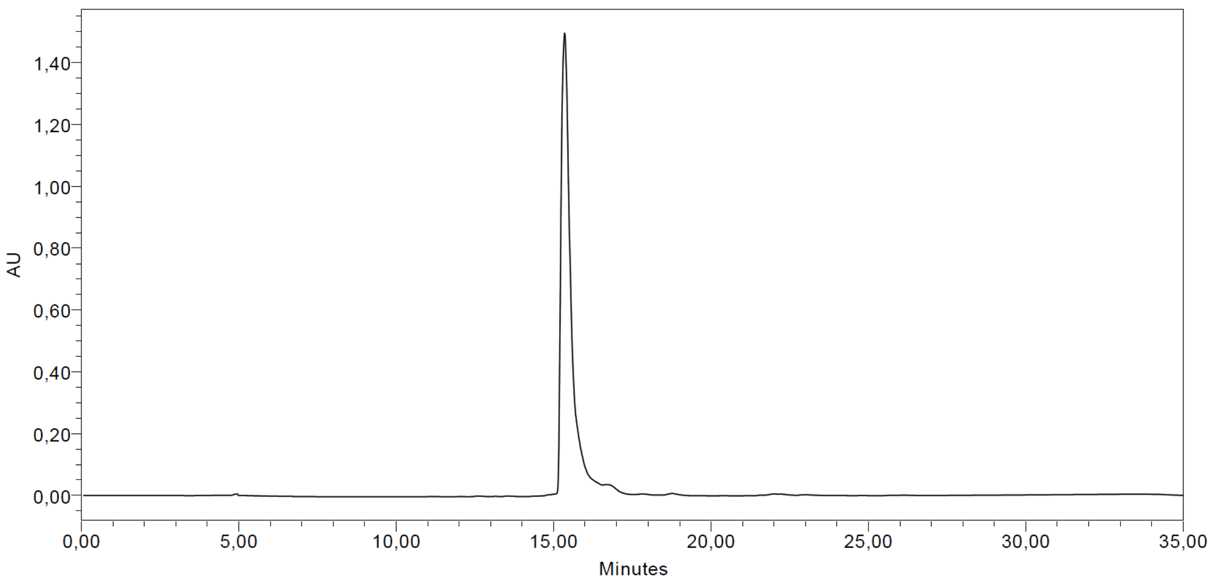


AU
2,20
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

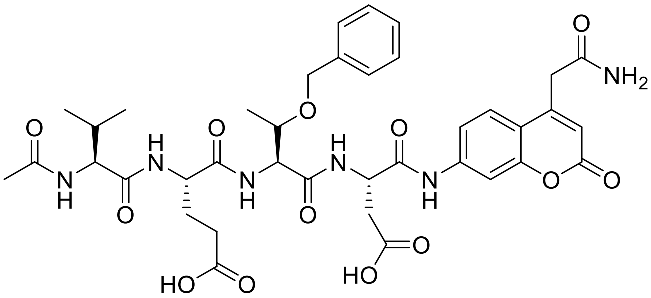
MPP47


N
NH
O
NH2
O
H
N
O
H
N
O
N
H
O
N
H
O
O
O
N
H
O
O
HO

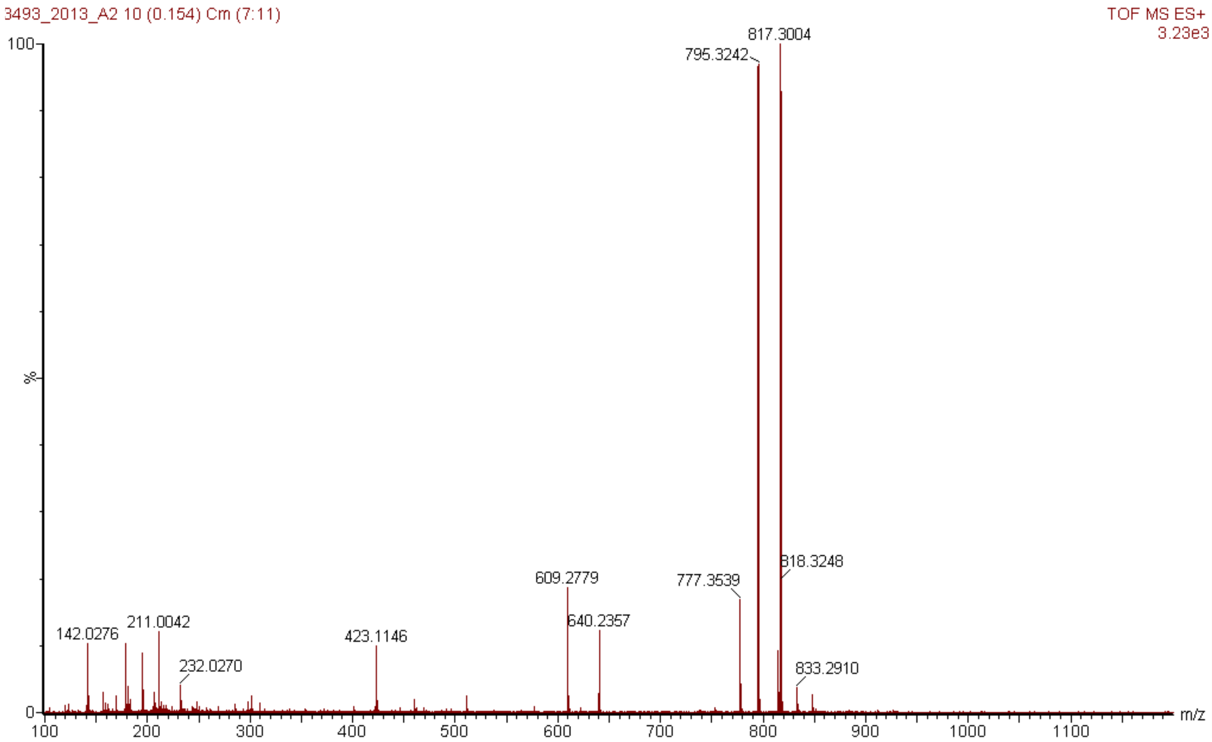

2764_2013_A1 103 (1.742)
TOF MS ES+
1.25e3
739.3400
740.3538
100
%
0
m/z
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
1050


AU
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

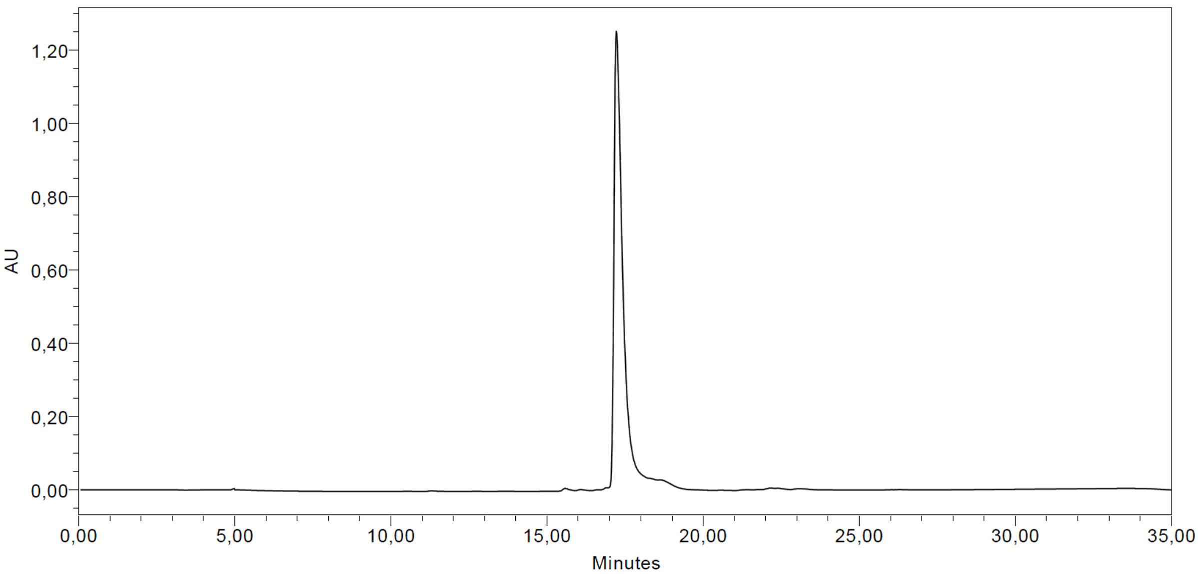
MPP48



O
NH2
O
H
N
O
O
H
N
O
O
N
H
N
H
O
O
N
H
O
O
HO
O
HO
O

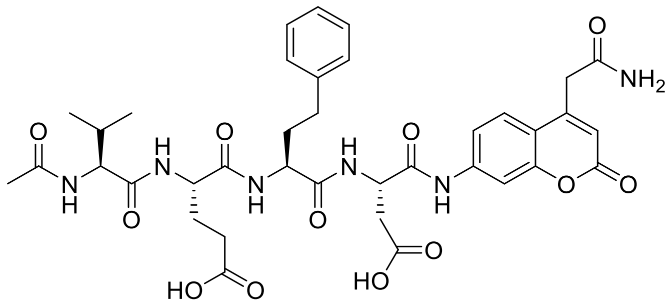


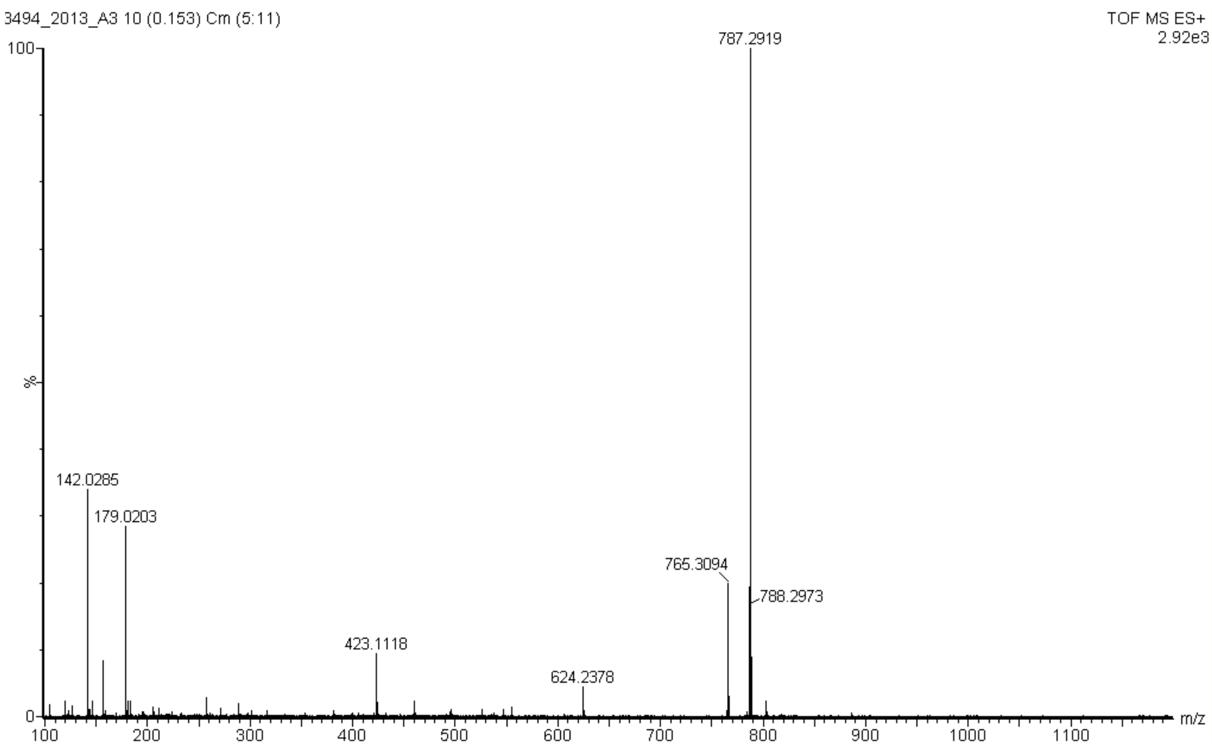
3493_2013_A2 10 (0.154) Cm (7:11)
TOF MS ES+
3.23e3
100
%
0
817.3004
795.3242
818.3248
777.3539
609.2779
640.2357
833.2910
423.1146
211.0042
142.0276
232.0270
100
200
300
400
500
600
700
800
900
1000
1100
m/z

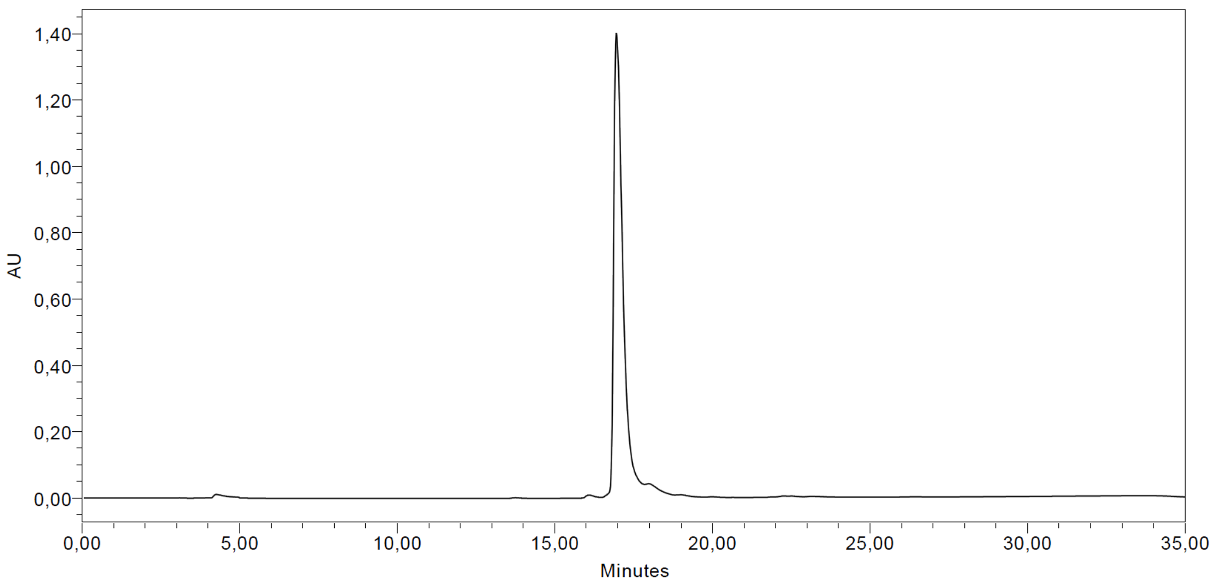


AU
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

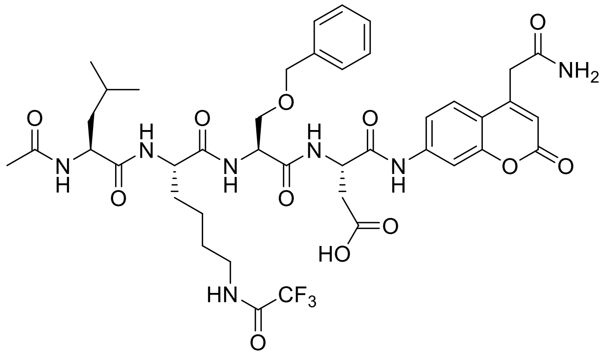
MPP49

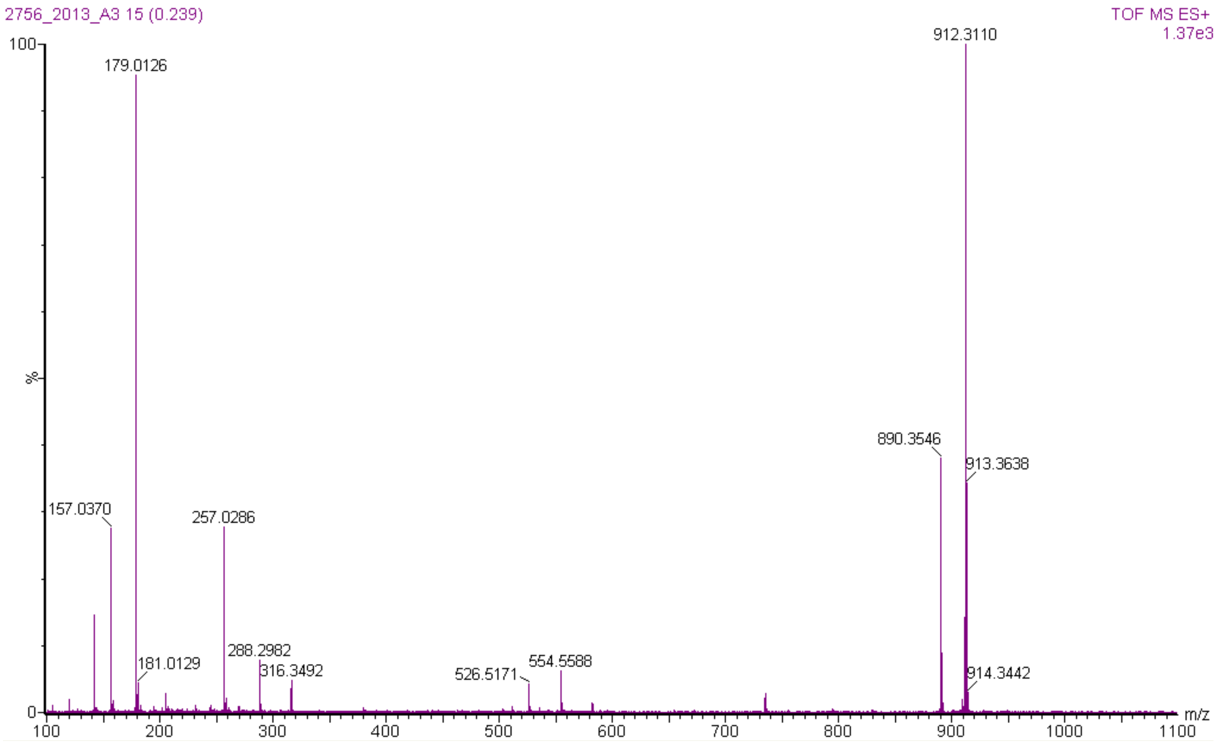

O
NH2
H
N
O
N
H
O
H
N
O
N
H
O
N
H
O
O
HO
O
HO
O

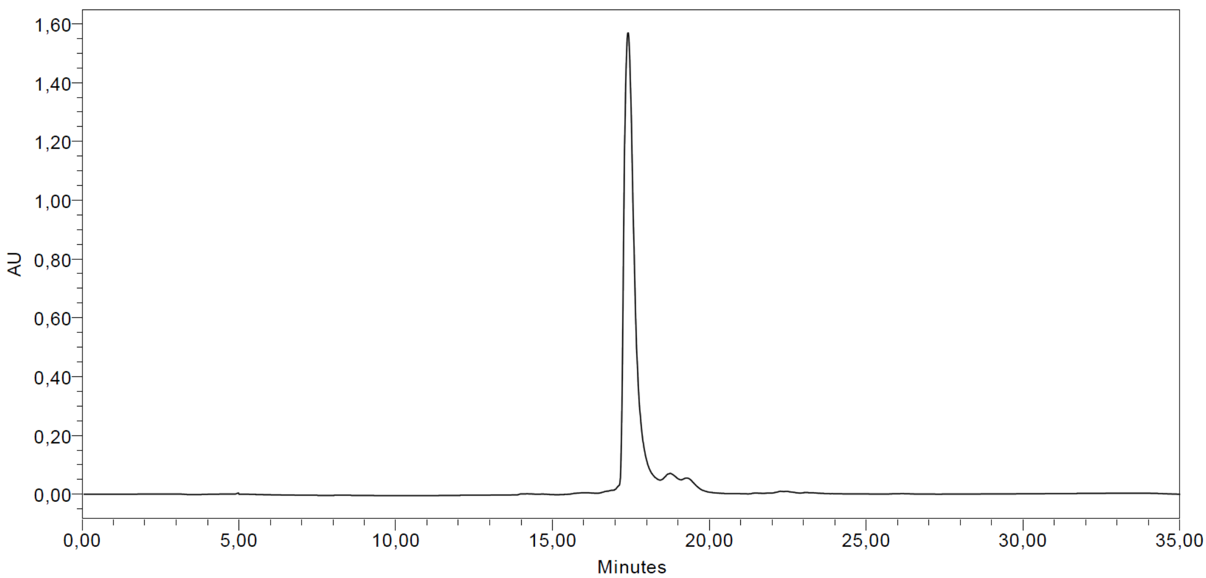

3494_2013_A3 10 (0.153) Cm (5:11)
TOF MS ES+
2.92e3
787.2919
142.0285
179.0203
765.3094
788.2973
423.1118
624.2378
100
%
0
m/z


AU
Minutes

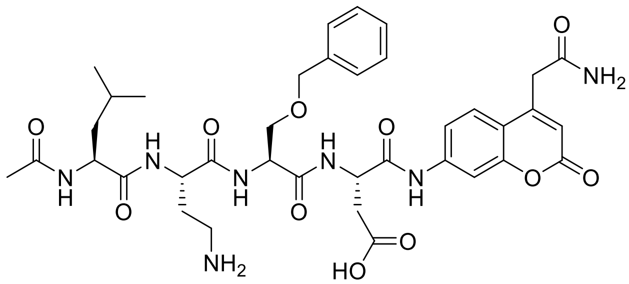
MPP50

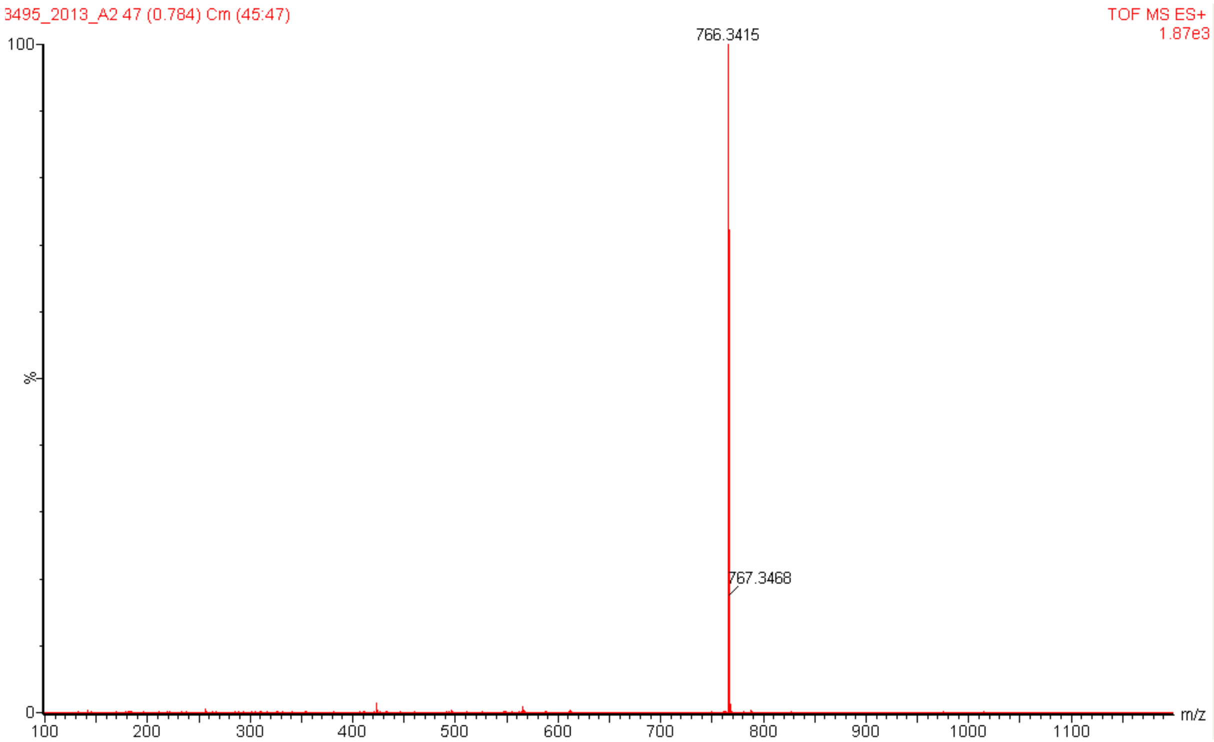


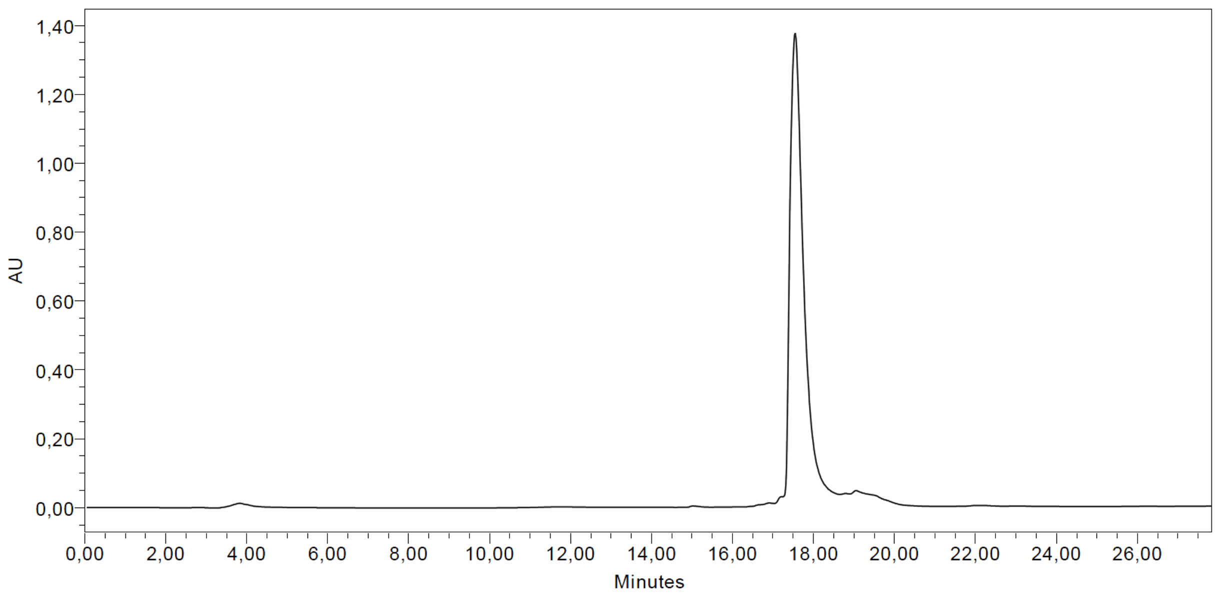

2756_2013_A3 15 (0.239)
TOF MS ES+
1.37e3
912.3110
179.0126
890.3546
913.3638
157.0370
257.0286
288.2982
316.3492
181.0129
526.5171
554.5588
914.3442
%
m/z


AU
Minutes

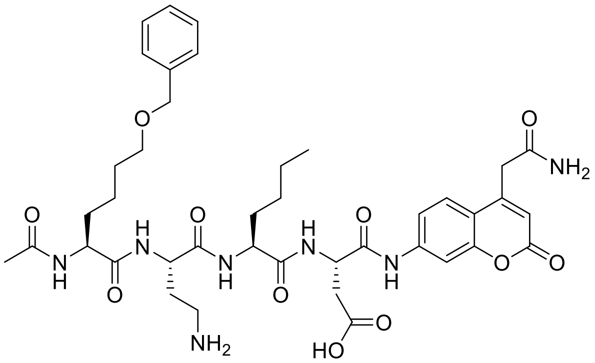
MPP51



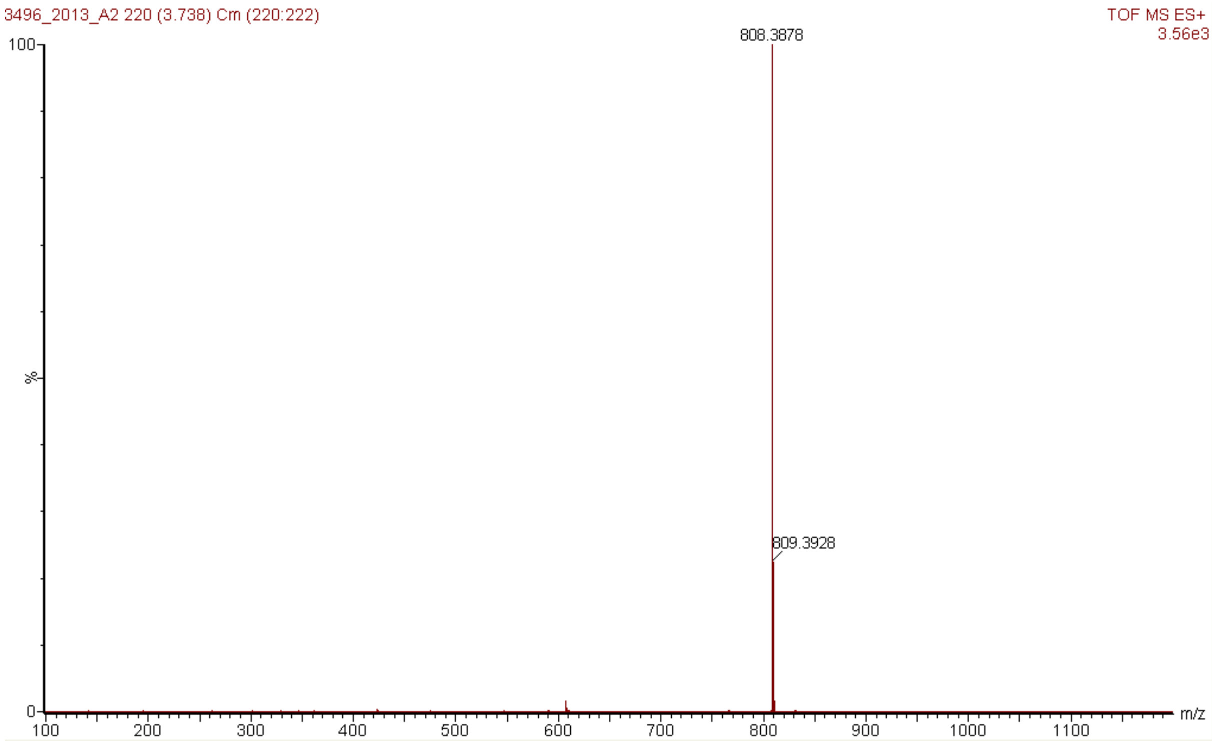

3495_2013_A2 47 (0.784) Cm (45:47)
TOF MS ES+
1.87e3
766.3415
767.3468
100
%
0
m/z
100
200
300
400
500
600
700
800
900
1000
1100


AU
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,00
2,00
4,00
6,00
8,00
10,00
12,00
14,00
16,00
18,00
20,00
22,00
24,00
26,00
Minutes

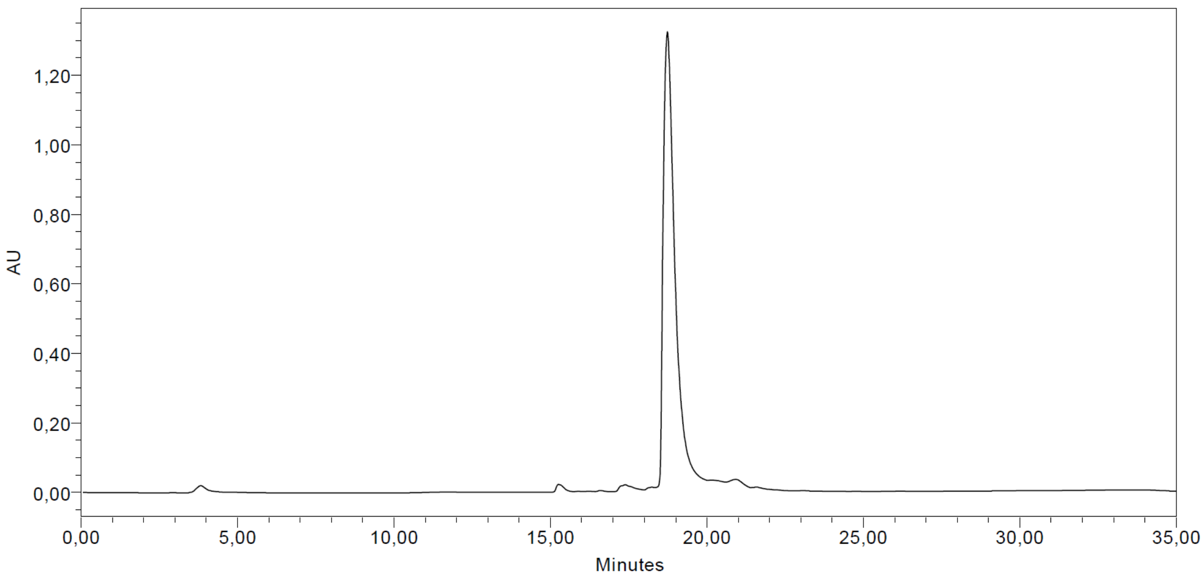
MPP52



O
NH2
O
H
N
O
N
H
O
H
N
O
N
H
O
N
H
O
O
O
NH2
O
HO

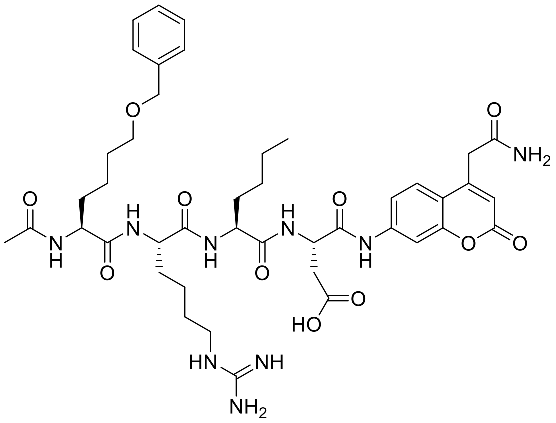


3496_2013_A2 220 (3.738) Cm (220:222)
TOF MS ES+
3.56e3
808.3878
809.3928
100
%
0
m/z
100
200
300
400
500
600
700
800
900
1000
1100

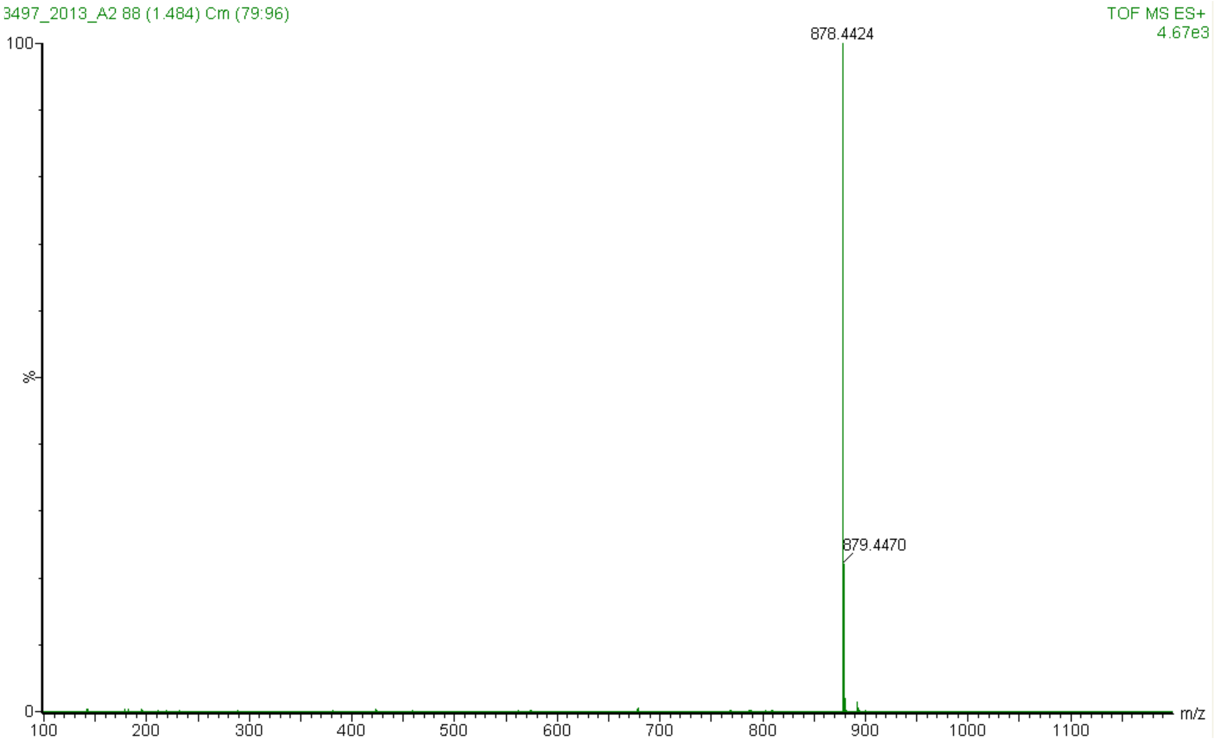


AU
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes

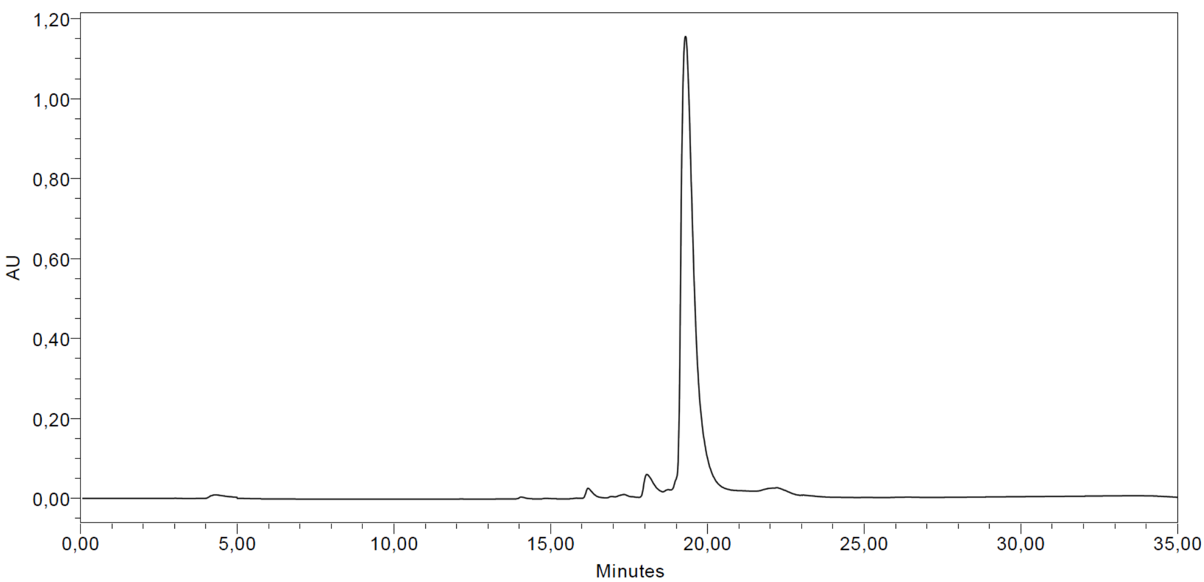
MPP53



O
NH2
O
O
O
H
N
O
H
N
O
O
N
H
O
N
H
O
N
H
O
O
HO
HN
NH
NH2



3497_2013_A2 88 (1.484) Cm (79:96)
TOF MS ES+
4.67e3
878.4424
879.4470
100
%
0
100
200
300
400
500
600
700
800
900
1000
1100
m/z



1,20
1,00
0,80
0,60
0,40
0,20
0,00
AU
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
Minutes