Selective Detection of Caspase-3 Versus Caspase-7 Using Activity-Based Probes with Key Unnatural Amino Acids

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General Information

Caspase-3/7, -6, and -8 substrates (Ac-DEVD-AFC, Ac-VEID-AFC, Ac-IETD-AFC, and Ac-LEHD-AFC, respectively) were purchased from AnaSpec. Inc. (Fremont, CA), and resuspended as 20 mM stock solutions in DMSO. Antibodies for caspases-3 (9662), -7 (9494), and GAPDH (2118) were purchased from Cell Signaling Technologies and used as instructed. Caspase activity and cell lysate buffers were prepared fresh prior to each experiment. All assay data was collected in triplicate with IC_{50} values determined using GraphPad Prism. All chemicals and solvents were used as received from suppliers. High resolution mass spectra were obtained at the Center for Mass Spectrometry, The Scripps Research Institute.

References

(S1) Weiss, M. S., Hilgenfeld, R. (1997) On the use of the merging R factor as a quality indicator for X-ray data. *J. Appl. Crystallogr. 30*, 203–205.

(S2) Weiss, M. S. (2001) Global indicators of X-ray data quality. *J. Appl. Crystallogr. 34*, 130–135.

(S3) Karplus, P. A., Diederichs, K. (2012) Linking crystallographic model and data quality. *Science 336*, 1030–1033.

Table S1. Caspase-3 and -7 active site identity comparison[a]

ID (+/–)	casp-3	casp-7	ID (+/–)	casp-3	casp-7	ID (+/–)	casp-3	casp-7
+	H121	H144	+	M61	M84	+	Y203	Y229
+	*C163	*C186	–	F128	Y151	–	D167	E190
+	Q161	Q184	+	E123	E146	+	T59	T82
+	R207	R233	+	G60	G83	–	N208	S234
+	R64	R87	+	L119	L142	+	F247	F273
+	S120	S143	–	S209	P235	+	G212	G238
+	Y204	Y230	–	K210	G236	–	F252	D278
+	S205	S231	+	F215	F241	+	H257	H283
+	F215	F241	+	T199	T225	+	S251	S277
–	T62	G85	+	R164	R187	–	A254	P280
+	W206	W232	–	S65	N88	–	D211	R237
+	F256	F282	+	S213	S239	–	F250	Q276
+	W214	W240	+	D80	D93	+	F55	F78
–	T255	H281	+	T166	T189	+	G202	G228
+	D253	D279	+	G165	G188	+	T67	T90
+	Q261	Q287	–	N73	A96	–	N217	Q243
+	I160	I183	–	S63	V86	+	S249	S275
+	S198	S224	+	G66	G89	+	K259	K285
+	L168	L191	+	L118	L141			

[a]Comparison of aligned caspase-3 and -7 crystal structures showing the 56 residues most closely associated with the active site. 43 of the 56 active site residues are identical in caspase-3 and -7 (77% sequence identity). Asterisk labels the catalytic cysteine residues for each enzyme.

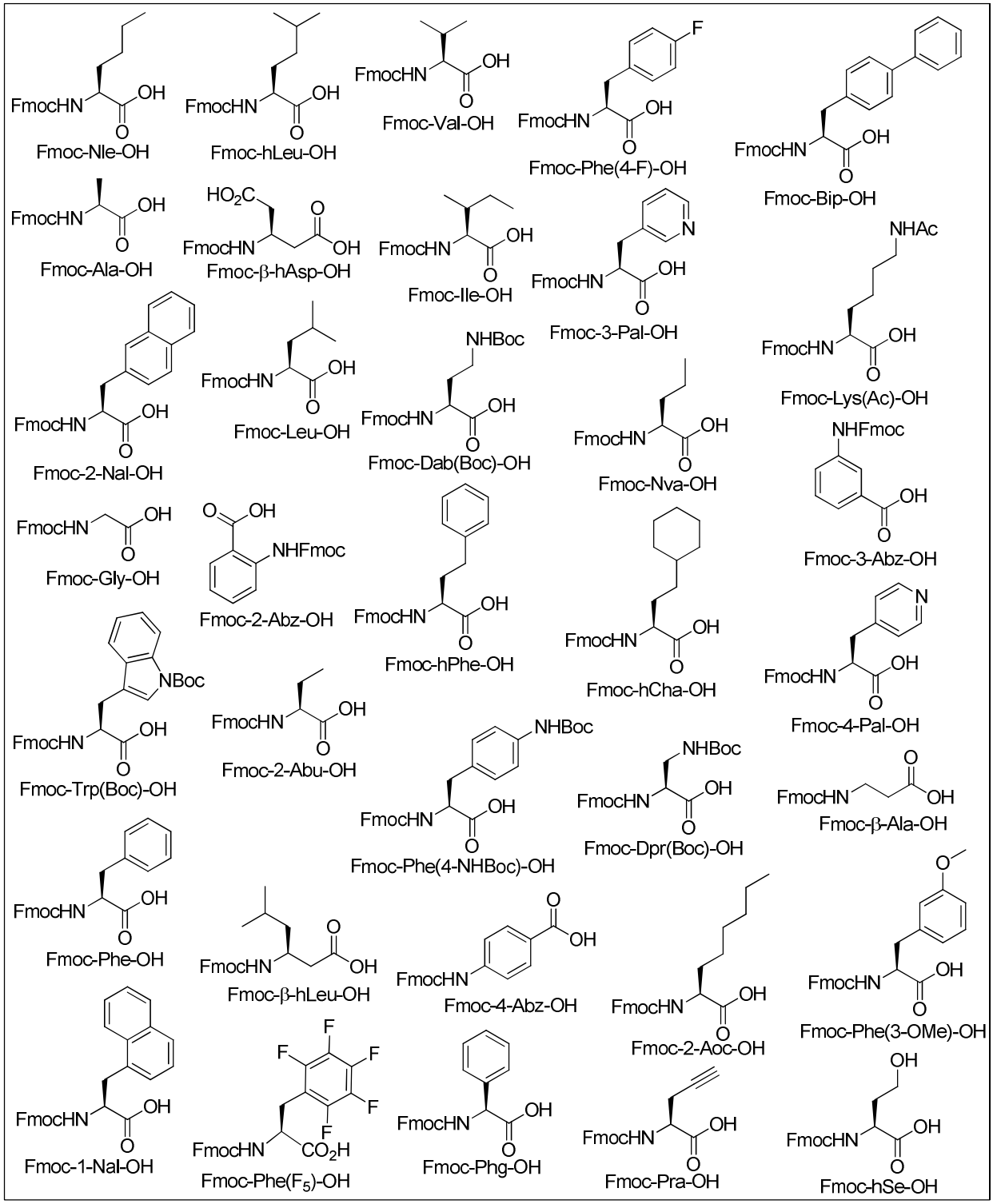


Figure S1. Amino acid structures used in caspase-3 versus caspase-7 selectivity screen.

Table S1. Caspase:**11** co-complex X-ray data processing and structure refinement statistics

Structure	Caspase-3	Caspase-7	Caspase-8
PDB ID	4JJE	4JJ8	4JJ7
Space group	I222	$P3_221$	$P3_121$
Unit Cell Parameters (a,b,c) (Å)	66.11,84.33,96.09	89.09,89.09,185.54	62.84,62.84,129.35
Data Processing			
Resolution range (Å) (outer shell)	50-1.48 (1.51-1.48)	50-2.94 (2.99-2.94)	40-1.18 (1.20-1.18)
Unique reflections	46,048 (2,201)	18,912 (946)	94,065 (4,503)
Completeness (%)	99.6 (96.7)	100.0 (100.0)	95.6 (95.6)
Redundancy	6.3 (5.2)	10.0 (10.0)	9.1 (6.0)
R_{merge} (%)[1]	5.4 (60.1)	14.4 (100.0)[a]	6.2 (34.6)
$R_{p.i.m.}$ (%)[2]	2.3 (28.2)	5.8 (60.4)	2.2 (16.6)
Average $I/\sigma(I)$	36.1 (1.6)	24.4 (2.0)	45.0 (4.3)
Refinement			
Resolution range (Å)	42.2-1.48 (1.51-1.48)	48.3-2.94 (3.1-2.94)	23.4-1.18 (1.19-1.18)
No. reflections[3] (test set)	46,040 (2,330)	18,863 (969)	94,058 (4,671)
R_{cryst} (%)[4]	15.0 (20.7)	19.3 (31.5)	13.5 (14.3)
R_{free} (%)[4]	17.4 (23.9)	21.6 (39.0)	15.7 (16.1)
Protein atoms / Waters	1955 / 214	3745 / 38	1987 / 269
CV[5] coordinate error (Å)	0.14	0.37	0.08
Rmsd bonds (Å) / angles (°)	0.014 / 1.62	0.003 / 0.77	0.017 / 1.73
B-values protein/waters/ligands (Å^2)	22.8 / 35.8 / 38.9	49.9 / 35.2 / 78.3	14.9 / 25.6 / 19.2
Ramachandran Statistics (%)			
Most favored	98.4	98.3	96.7
Additional allowed	1.2	1.3	2.9
Generously allowed	0.4	0.4	0.4

[1] $R_{merge}=\Sigma_{hkl}\Sigma_i|I_{i(hkl)} - \langle I_{(hkl)} \rangle|/\Sigma_{hkl}\Sigma_i I_i(hkl)$. [2] $R_{p.i.m.}$ (precision-indicating R_{merge})$=\Sigma_{hkl}[1/(N_{hkl}-1)]^{1/2}\Sigma_i|I_i(hkl) - \langle I(hkl) \rangle|/\Sigma_{hkl}\Sigma_i I_i(hkl)$. [3] Reflections with $I > 0$ were used for refinement.[1-3] [4] $R_{cryst}=\Sigma_h||F_{obs}|-|F_{calc}||/\Sigma|F_{obs}|$, where F_{obs} and F_{calc} are the calculated and observed structure factor amplitudes, respectively. R_{free} is R_{cryst} with 5.0% test set structure factors. [5] Cross-validated (CV) Luzzati coordinate errors. [a] R_{merge} of the highest resolution shell is high due to high redundancy (10.0). However, the completeness and mean $I/\sigma(I)$ of the highest resolution shell are reasonable, and these data were included in the refinement.

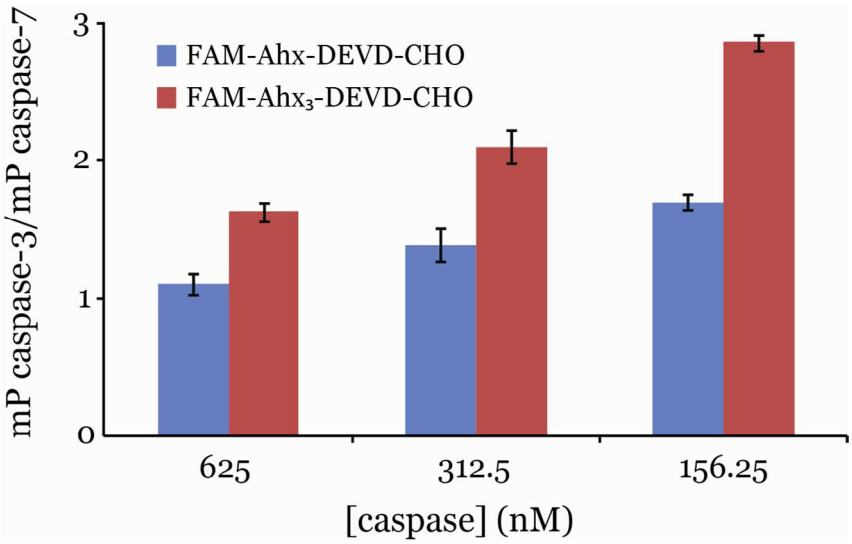


Figure S2. Fluorescence polarization (FP) for caspase-3 over caspase-7 at 625, 312.5, and 156.25 nM caspase. ABPs were used at 600 nM in each experiment and reactions were incubated for 2 minutes before FP measurement. Additional 6-aminohexanoic acid (Ahx) linkers impart increased selectivity for caspase-3 detection over caspase-7.

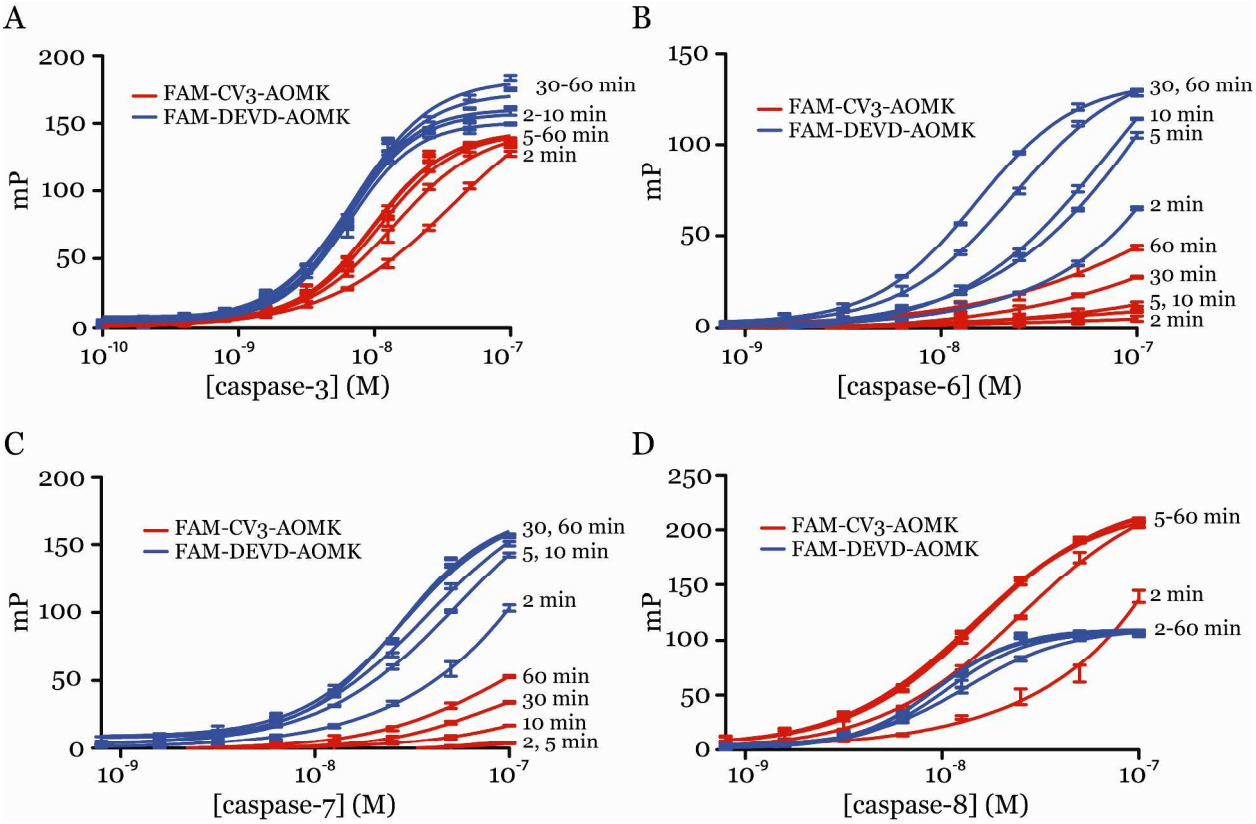


Figure S3. Fluorescence polarization (FP) measured at variable concentrations of recombinant caspase-3 A), -6 B), -7 C), or -8 D) after 2, 5, 10, 30 or 60 minute incubation periods with ABPs. FAM-DEVD-AOMK (**12**) and FAM-CV3-AOMK (**13**) were used at final concentrations of 100 nM and are shown in blue and red, respectively.

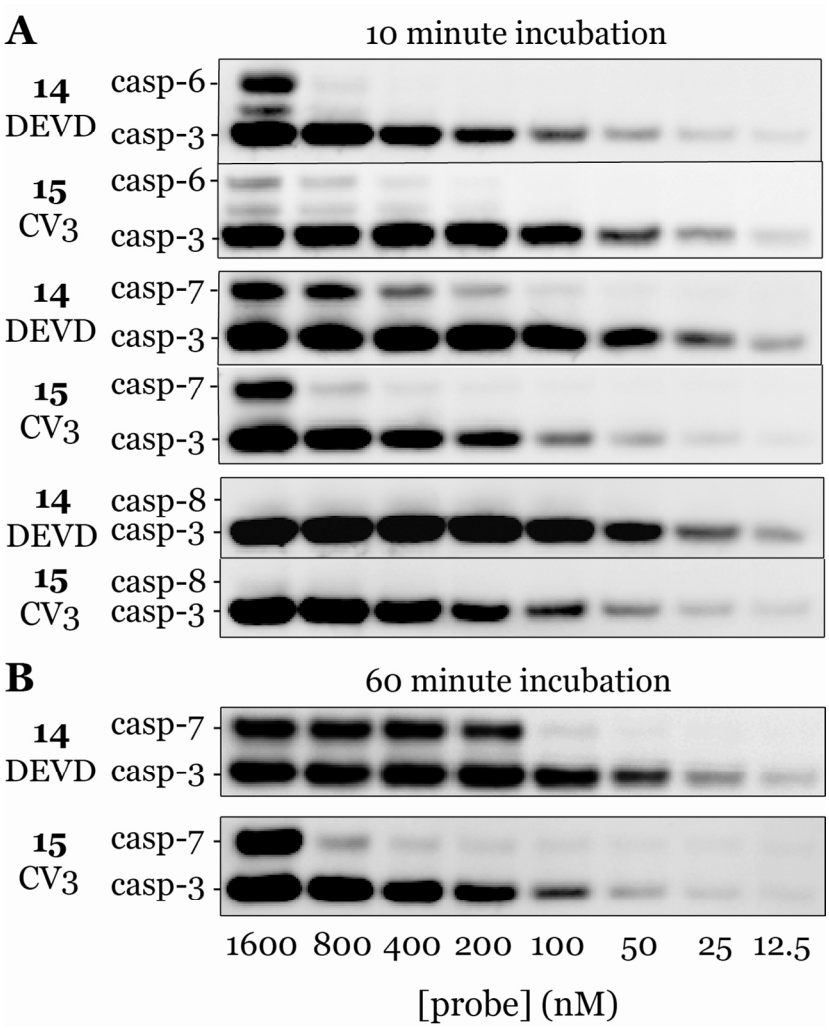


Figure S4. SDS-PAGE of varying concentrations of Biotin-DEVD-AOMK (**14**) or Biotin-CV3-AOMK (**15**) incubated for either A) 10 minutes or B) 60 minutes with a pre-mixed solution of caspase-3 and caspase-6, -7, or -8. Caspases were assayed at 100 nM final concentrations, and gels were visualized with avidin blotting.

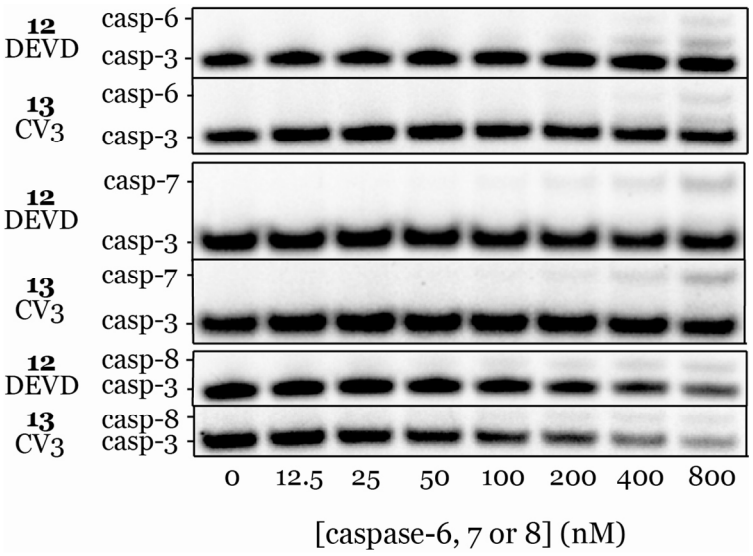
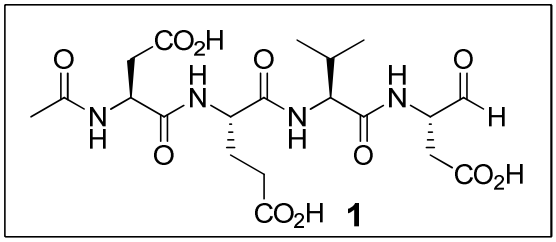


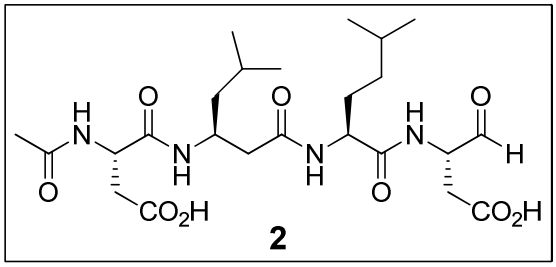
Figure S5. SDS-PAGE competition assay using a pre-mixed solution of recombinant caspases-3 (100 nM final concentration) and -6, -7, or -8 (varying concentrations). FAM-DEVD-AOMK (**12**) or FAM-CV3-AOMK (**13**) was added at a final concentration of 100 nM, and the solution incubated for 10 minutes. No significant covalent binding was visualized for either ABP to caspases-6, -7, or -8 even at 8-fold molar excess when the [ABP] and [caspase-3] are equal. Under these conditions caspase-3 modification by the ABPs is kinetically faster than caspases-6, -7 and -8.

Compound structural information



1

HRMS (ESI-TOF) Calcd. for $C_{20}H_{30}N_4O_{11}$ $[M+H^+]$: 503.1984; found: 503.1981.



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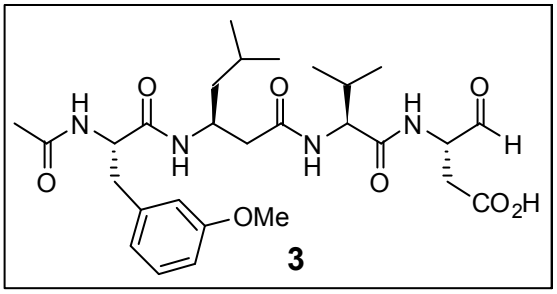
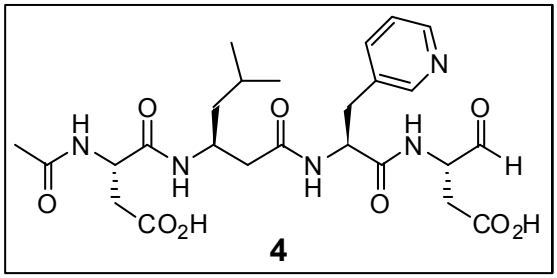
HRMS (ESI-TOF) Calcd. for $C_{24}H_{40}N_4O_9$ $[M+H]^+$: 529.2868; found: 529.2874.



3

HRMS (ESI-TOF) Calcd. for $C_{28}H_{42}N_4O_8$ $[M+H^+]$: 563.3075; found: 563.3091.

HRMS (ESI-TOF) Calcd. for $C_{25}H_{35}N_5O_9$ $[M+H]^+$: 550.2507; found: 550.2527.



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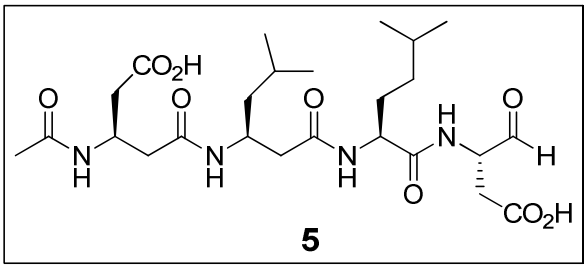

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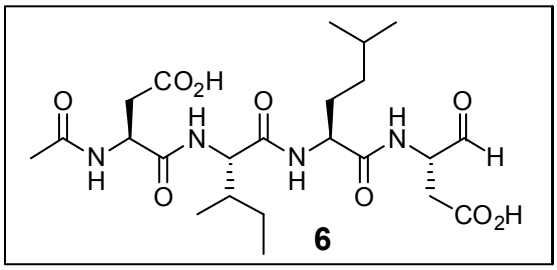

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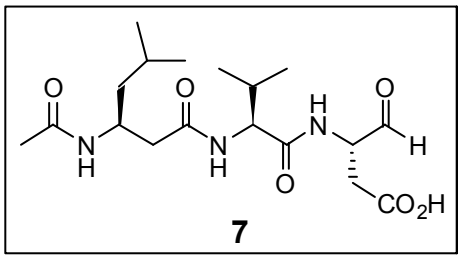
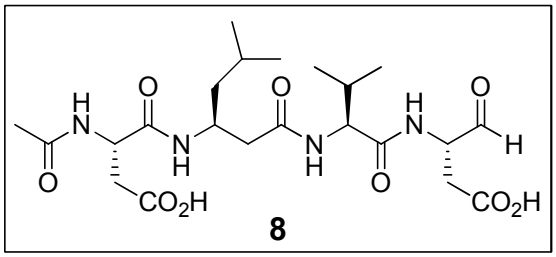
HRMS (ESI-TOF) Calcd. for $C_{23}H_{38}N_4O_9$ $[M+H]^+$: 515.2711; found: 515.2714.



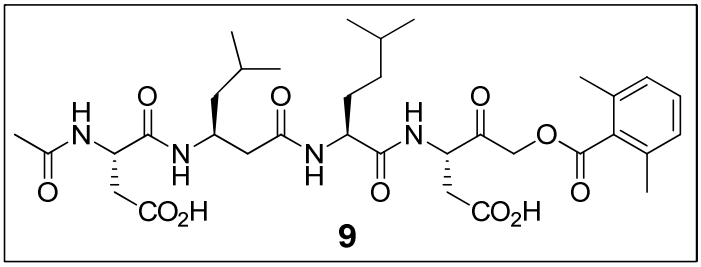
7

HRMS (ESI-TOF) Calcd. for $C_{18}H_{31}N_3O_6$ $[M+H]^+$: 386.2291; found: 386.2293.



8

HRMS (ESI-TOF) Calcd. for $C_{22}H_{36}N_4O_9$ $[M+H]^+$: 501.2555; found: 501.2560.



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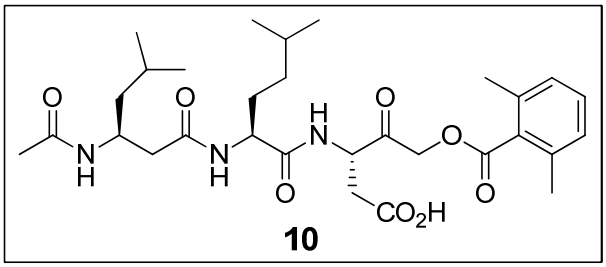
HRMS (ESI-TOF) Calcd.
for $C_{34}H_{50}N_4O_{11}$
$[M+H]^+$: 691.3549; found: 691.3543.



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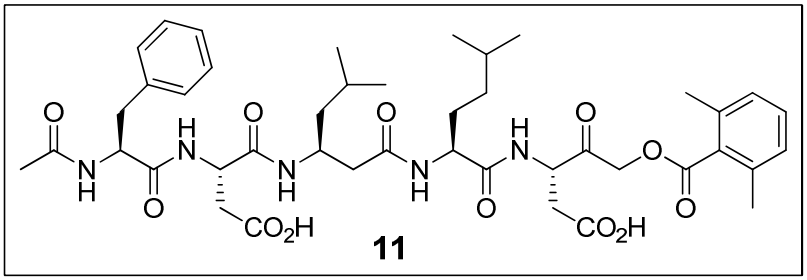
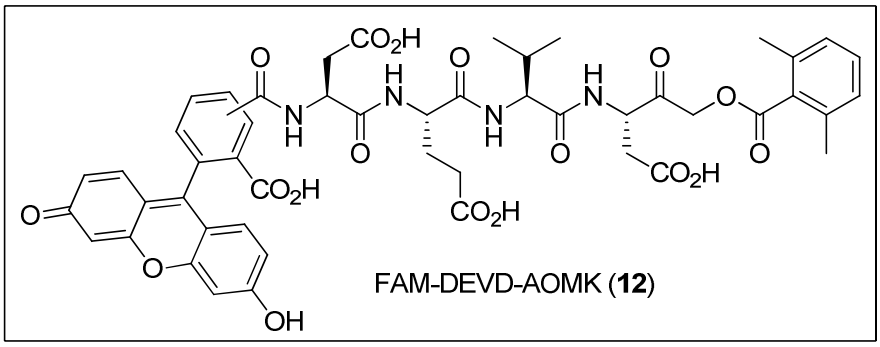
HRMS (ESI-TOF) Calcd.
for $C_{30}H_{45}N_3O_8$
$[M+H]^+$: 576.3279; found: 576.3285.

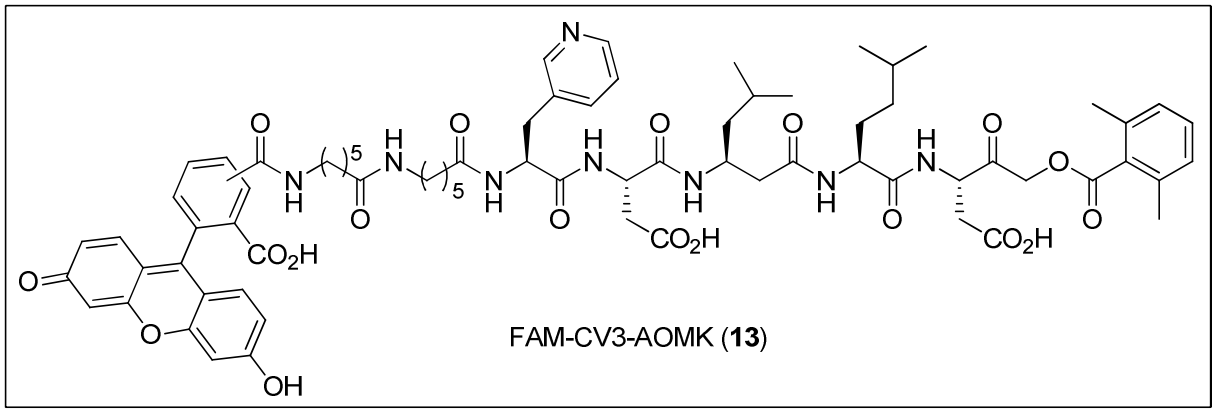


11

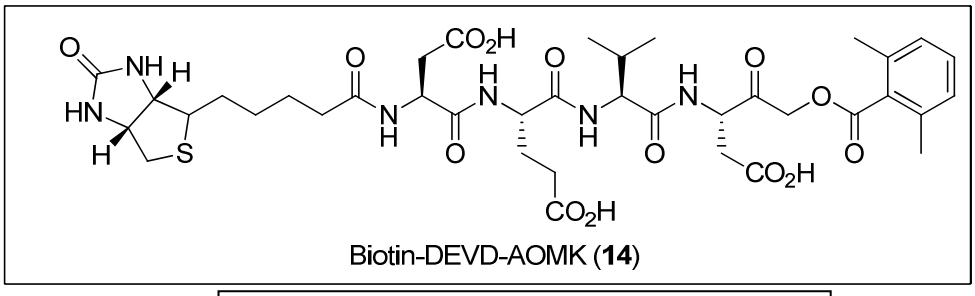
HRMS (ESI-TOF) Calcd.
for $C_{43}H_{59}N_5O_{12}$
$[M+H]^+$: 839.4185; found: 839.4192.



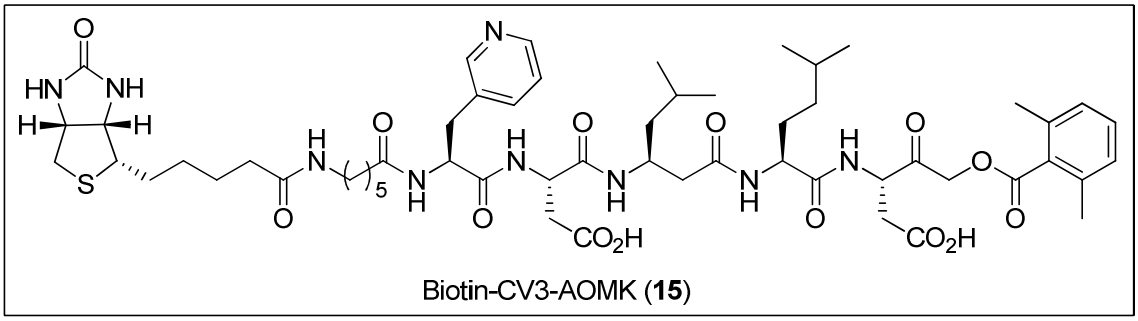
HRMS (ESI-TOF) Calcd. for $C_{49}H_{48}N_4O_{18}$ $[M+H]^+$: 981.3036; found: 981.3017.



HRMS (ESI-TOF) Calcd. for $C_{73}H_{88}N_8O_{19}$ $[M+H]^+$: 1381.6238; found: 1381.6279.

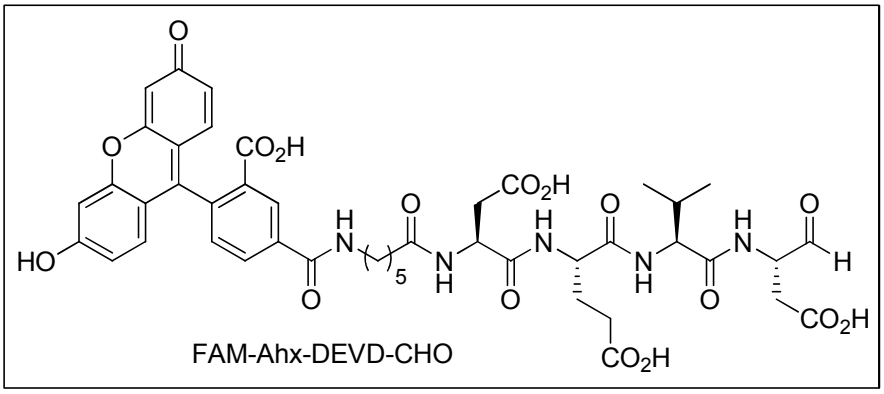


HRMS (ESI-TOF) Calcd. for $C_{38}H_{52}N_6O_{14}S$ $[M+H]^+$: 849.3335; found: 849.3325.



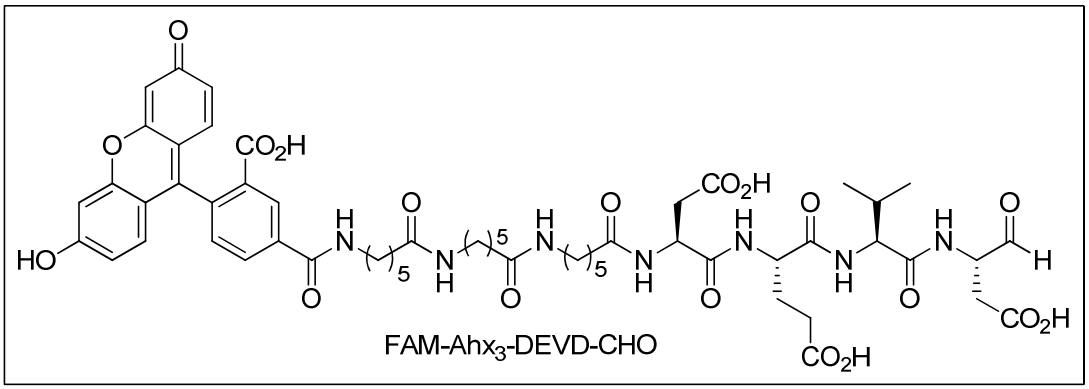
Biotin-CV3-AOMK (**15**)

HRMS (ESI-TOF) Calcd. for $C_{56}H_{81}N_9O_{14}S$ $[M+H]^+$: 1184.5761; found: 1184.5768.



FAM-Ahx-DEVD-CHO

HRMS (ESI-TOF) Calcd. for $C_{45}H_{49}N_5O_{17}[M+H]^+$: 932.3196; found: 932.3174.



FAM-Ahx$_3$-DEVD-CHO

HRMS (ESI-TOF) Calcd. for $C_{57}H_{71}N_7O_{19}[M+H]^+$: 1158.4877; found: 1158.4885.