

Selective Inhibition of Initiator Versus Executioner Caspases Using Small Peptides Containing Unnatural Amino Acids

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Methods

Caspase expression and purification.

Caspases-3, -6, -7, -8, and -9 were expressed and purified as previously described.^{1,2}

Caspase-8 versus caspase-3 selectivity screen.

Caspase inhibitors were individually synthesized using standard SPPS from an H-Asp(OtBu)-H pre-loaded aldehyde resin (Anaspec, Inc.). After attachment of N-terminal acetyl groups, side-chain protecting groups were removed from completed peptides with neat trifluoroacetic acid for 2 hrs and cleaved from the resin with acetonitrile/water/trifluoroacetic acid (60/40/1) for 45 min at 60 °C. Dried peptides were dissolved in DMSO to 1 mM stock solutions based on a 15% estimated overall yield. Compounds were screened for caspase-8 versus caspase-3 inhibition without further purification.

IC₅₀ determination against recombinant caspases.

Inhibitor, recombinant caspase, and substrate were all diluted into an assay buffer consisting of 50 mM HEPES pH 7.4, 0.1% CHAPS, 10 mM KCl, 50 mM sucrose, 1 mM MgCl₂, and 10 mM DTT. In addition, 0.70 M sodium citrate was included in the assay buffer for all caspase-9 experiments. 20 µL of 2.5× caspase solution was mixed with an equal volume of inhibitor diluted in assay buffer (2.5×) in NUNC 96-well, black, low-binding, microtiter plates for final concentrations of 10 nM caspase-3, 50 nM caspase-6, 10 nM caspase-7, 25 nM caspase-8, or 500 nM caspase-9. The inhibitor:caspase solution was incubated for 1 min at room temperature followed by addition of 10 µL of 5× fluorescent caspase substrate [Ac-DEVD-AFC: caspases-3/7, Ac-VEID-AFC: caspase-6, Ac-IETD-AFC: caspase-8, and Ac-LEHD-AFC: caspase-9] to a final concentration of 50 µM. The initial rate of substrate turnover (RFU/sec) was then immediately measured on a PerkinElmer EnVision plate reader with excitation at 355 nm and emission detection at 486 nm. At least three replicates were included for each data set with IC₅₀ values calculated using Prism (GraphPad, Inc.).

X-ray crystallography of caspases in complex with CV8/9-AOMK.

CV8/9-AOMK was added to a 2-fold molar excess relative to 8 mg/mL caspase-3 or -8 in a buffer consisting of 2.6 mM HEPES, pH 7.4, 10 mM DTT, and 0.02% sodium azide or 20 mM HEPES, pH 7.4, and 100 mM DTT, respectively. This solution was incubated for 2 hrs at 23 °C and immediately used for co-crystallization experiments. Crystals of CV8/9-AOMK in complex with caspase-3 were grown in 0.15 M sodium citrate, pH 5.5, 10 mM DTT, 0.003% sodium azide, 14% PEG 6000 and CV8/9-AOMK:caspase-8 crystals were grown in 0.1 M sodium citrate, pH 5.5 and 36% PEG 600. All crystals were grown at 22 °C and the His₆-tags were not removed as the proteins crystallized readily. Data for the CV8/9-AOMK co-complex X-ray structures were collected on single, flash-cooled crystals at 100 K in a cryoprotectant consisting of mother liquor only (caspase-8) or 20% glycerol (caspase-3) and were processed with HKL2000 in space groups P2 (caspase-3) and C₂, (caspase-8). X-ray data for the caspase-3 and -8 CV8/9 complex structures were collected to 1.63 Å and 1.73 Å resolution, respectively, on beamline 11.1 at the Stanford Synchrotron Radiation Lightsource (SSRL) (Menlo Park, CA). Data collection and processing statistics are summarized in Supplementary Table S1. Both caspases-3 and -8 structures were determined by molecular replacement (MR) with Phaser³ using PDB identifications 4JJE and 4JJ7 as the initial search models, respectively. Structures were manually built with Coot⁴ and iteratively refined using Phenix⁵ with cycles of conventional positional refinement. For the caspase-3 co-complex structure, the final R_{cryst} and R_{free} are 15.3% and 17.9%, respectively, and for caspase-8 the final R_{cryst} and R_{free} are 15.2% and 18.5%, respectively (Supplementary Table S1).

Coordinates and structure factors have been deposited in the Protein Data Bank⁶ with accession entries 4PS0 (caspase-3) and 4PS1 (caspase-8). Structure refinement statistics are shown in Supplementary Table S1.

Conformational analysis using computational methods.

Torsional and bond angle strain were both calculated based on AMBER 14 molecular mechanics potential parameters. Torsional strain for the N-C δ -C γ -O dihedral angle was calculated for CV8/9-AOMK bound to caspases-3 (142°) and -8 (86°) using eq 1:

$$\text{Torsion energy (kcal/mol)} = [0.156(1 + \cos(3\varepsilon)) + 1.49(1 + \cos(2\varepsilon))] \quad (1)$$

where ε is equal to the observed dihedral angle. The bond angle strain was calculated using eq 2:

$$\text{Angle energy (kcal/mol)} = \zeta[(\theta - \eta)(\pi/180)]^2 \quad (2)$$

where ζ are the bond angle energy constants for each bond angle around the 5-membered hydroxyproline ring, θ is the observed bond angle, and η is the canonical bond angle for hydroxyproline (Supplementary Figure S6).

Caspase inhibitor characterization using irreversible kinetics.

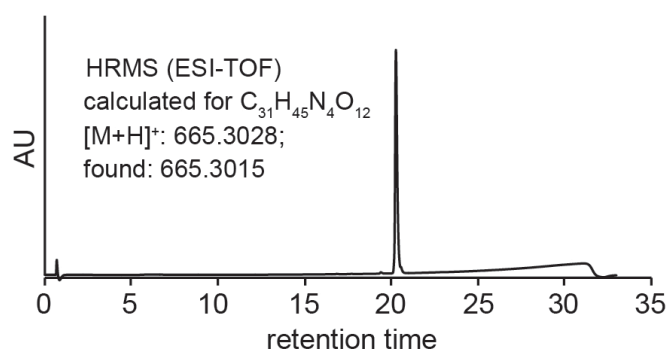
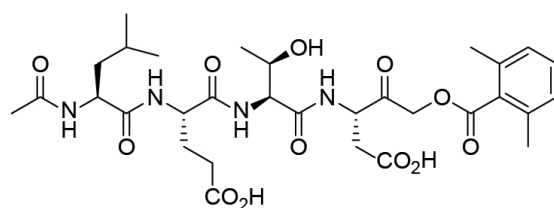
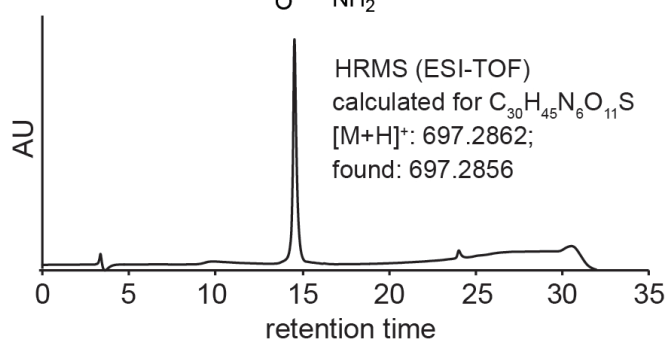
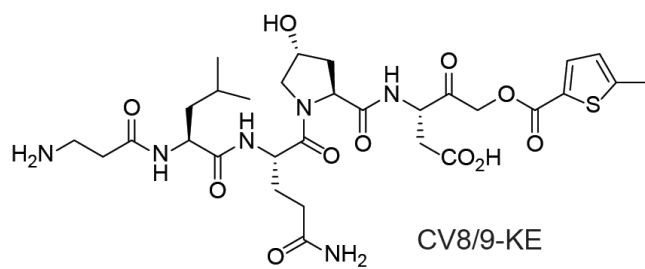
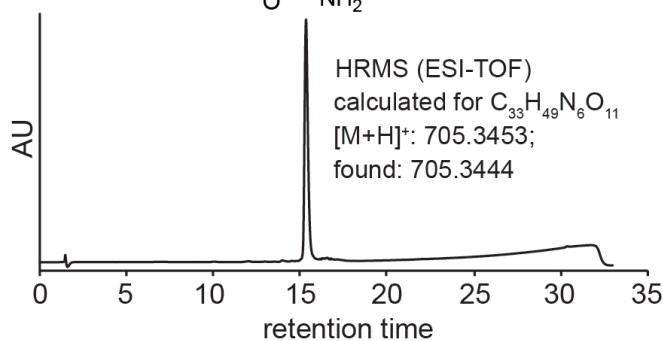
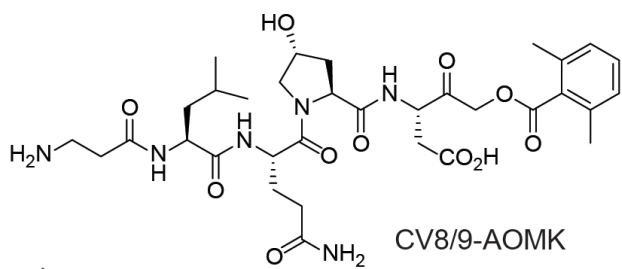
Inhibitor, recombinant caspase, and substrate were all diluted into an assay buffer consisting of 50 mM HEPES pH 7.4, 0.1% CHAPS, 10 mM KCl, 50 mM sucrose, 1 mM MgCl₂, and 10 mM DTT. In addition, 0.70 M sodium citrate was included in the assay buffer for all caspase-9 measurement. 20 μ L of 2.5 \times inhibitor solution and 10 μ L of 5 \times substrate solution [final concentrations: 150 μ M Ac-DEVD-AFC (caspases-3), 50 μ M Ac-IETD-AFC (caspase-8), and 160 μ M Ac-LEHD-AFC (caspase-9)] were added to NUNC 96-well, black, low-binding, microtiter plates. 20 μ L of recombinant caspases-3, -8, or -9 were subsequently added to final concentrations of 5 nM, 25 nM, and 300 nM, respectively. The progress of these reactions were measured by relative fluorescence output overtime (RFU/sec) using a PerkinElmer EnVision plate reader with excitation at 355 nm and emission detection at 486 nm. At least three replicates were included for each data set as well as multiple concentrations of Ac-LETD-AOMK or CV8/9-AOMK. This data was analyzed using Prism (GraphPad, Inc.) to calculate the k_{obs} values for each inhibitor concentration against caspases-3, -8, and -9 and was fit with a non-linear regression curve by eq 3:

$$[F] = V_A(1 - \exp(-k_{obs}t))/k_{obs} \quad (3)$$

where [F] is RFU, V_A is a constant related to the velocity of the reaction (RFU/sec) in the absence of inhibitor for each enzyme, and t is the time in seconds. Plots of k_{obs} versus inhibitor concentration exhibit first-order kinetics that approach saturation at high concentrations of inhibitor. Saturation is important for unambiguous assignment of the dissociation constant (K_i) and the first-order rate of irreversible enzyme inactivation (k_{inact}) by non-linear regression curve fitting using Prism to eq 4:

$$k_{obs} = (k_{inact}[I])/([I] + K_i(1 + [S]/K_M)) \quad (4)$$

where [I] is the concentration of inhibitor, [S] is the substrate concentration, and K_M is the Michaelis constant for caspases-3 (9.6 μ M), -8 (4.0 μ M), and -9 (15 μ M).



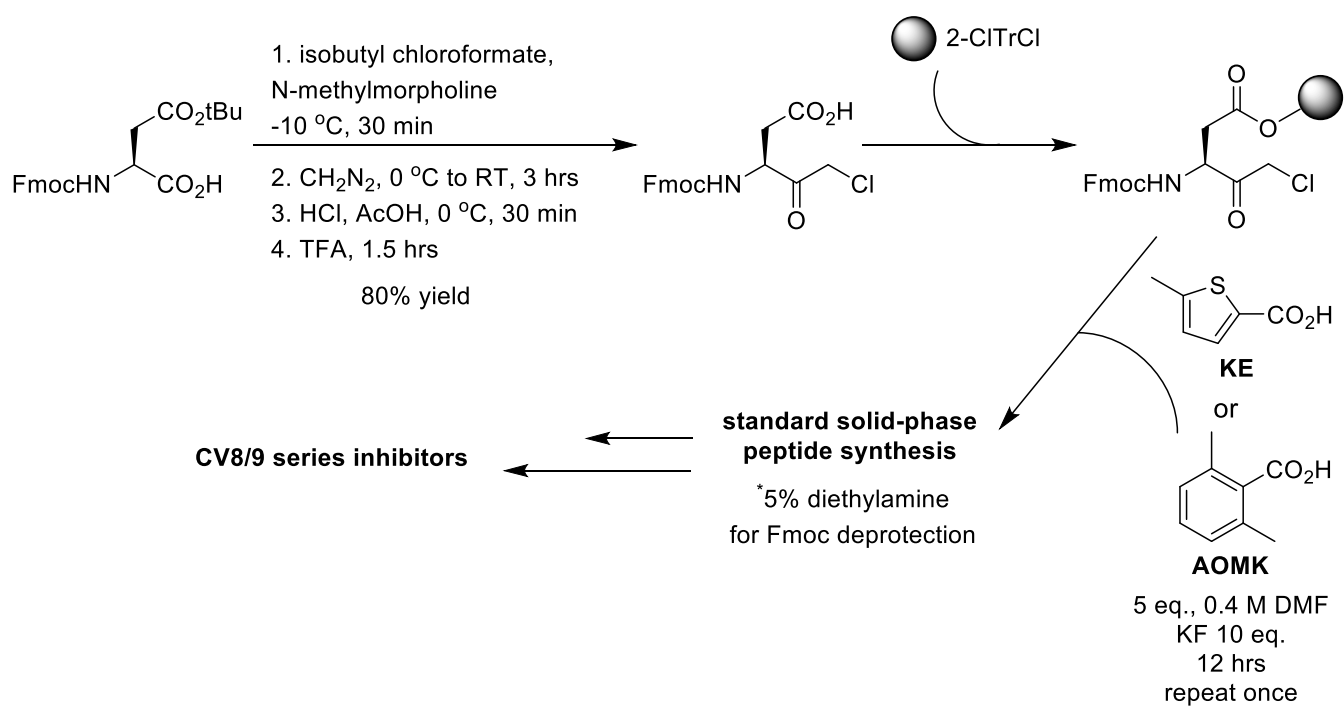


Figure S1. Inhibitor synthesis.

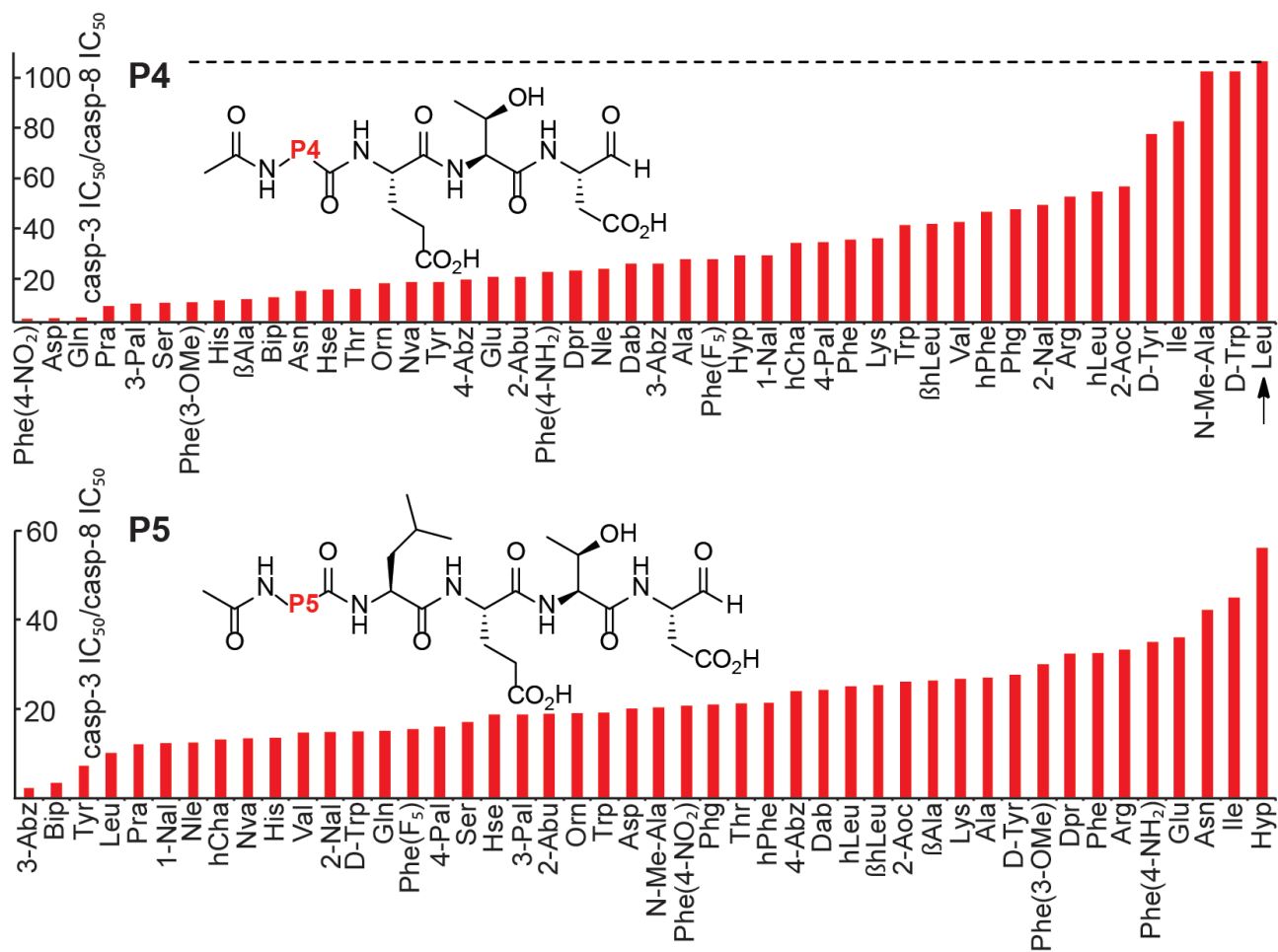


Figure S2. P4-P5 selectivity screen for caspase-8 versus caspase-3 inhibitors. The P5 β-alanine residue was incorporated into CV8/9 to improve solubility and not for caspase-8 selectivity.

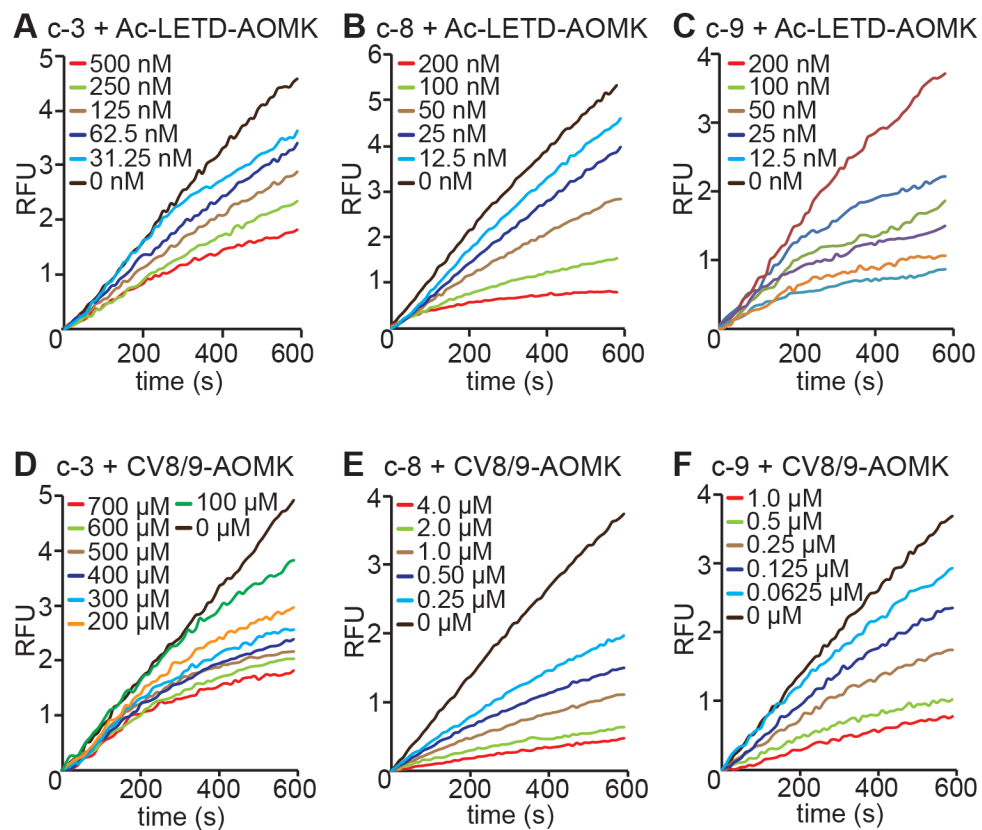


Figure S3. Progress curves for substrate turnover by caspases-3, -8, and -9 in the presence of varying concentrations of Ac-LETD-AOMK or CV8/9-AOMK.

| k_{obs} values (s^{-1}) versus caspase-3 | | | | |
|--|---------|---------|---------|---------|
| Ac-LETD-AOMK (nM) | trial 1 | trial 2 | trial 3 | trial 4 |
| 500 | 0.0045 | 0.0048 | 0.0042 | 0.0033 |
| 250 | 0.0034 | 0.0029 | 0.0036 | 0.0028 |
| 125 | 0.0018 | 0.0018 | 0.0022 | 0.0023 |
| 62.5 | 0.0014 | 0.0012 | 0.0019 | 0.0016 |
| 31.25 | 0.00076 | 0.00066 | 0.00093 | 0.0012 |

| k_{obs} values (s^{-1}) versus caspase-8 | | | | |
|--|---------|---------|---------|---------|
| Ac-LETD-AOMK (nM) | trial 1 | trial 2 | trial 3 | trial 4 |
| 200 | 0.012 | 0.010 | 0.013 | 0.013 |
| 100 | 0.0071 | 0.0069 | 0.0060 | 0.0047 |
| 50 | 0.0032 | 0.0029 | 0.0029 | 0.0038 |
| 25 | 0.0021 | 0.00098 | 0.0013 | 0.0022 |
| 12.5 | 0.00055 | 0.0012 | 0.0011 | 0.00019 |

| k_{obs} values (s^{-1}) versus caspase-9 | | | | |
|--|---------|---------|---------|---------|
| Ac-LETD-AOMK (nM) | trial 1 | trial 2 | trial 3 | trial 4 |
| 200 | 0.0097 | 0.0088 | 0.0081 | 0.0090 |
| 100 | 0.0068 | 0.0043 | 0.0056 | 0.0074 |
| 50 | 0.0046 | 0.0039 | 0.0035 | 0.0039 |
| 25 | 0.0031 | 0.0014 | 0.0025 | 0.0034 |
| 12.5 | 0.0020 | 0.00068 | 0.0019 | 0.0017 |

| k_{obs} values (s^{-1}) versus caspase-3 | | | |
|--|---------|---------|---------|
| CV8/9-AOMK (μ M) | trial 1 | trial 2 | trial 3 |
| 700 | 0.0043 | 0.0045 | 0.0035 |
| 600 | 0.0038 | 0.0031 | 0.0037 |
| 500 | 0.0031 | 0.0032 | 0.0035 |
| 400 | 0.0028 | 0.0030 | 0.0026 |
| 300 | 0.0025 | 0.0021 | 0.0021 |
| 200 | 0.0018 | 0.0013 | 0.0015 |
| 100 | 0.00055 | 0.00031 | 0.00077 |

| k_{obs} values (s^{-1}) versus caspase-8 | | | |
|--|---------|---------|---------|
| CV8/9-AOMK (μ M) | trial 1 | trial 2 | trial 3 |
| 4.0 | 0.022 | 0.015 | 0.018 |
| 2.0 | 0.014 | 0.014 | 0.015 |
| 1.0 | 0.0068 | 0.0070 | 0.0072 |
| 0.50 | 0.0046 | 0.0044 | 0.0046 |
| 0.25 | 0.0030 | 0.0032 | 0.0020 |

| k_{obs} values (s^{-1}) versus caspase-9 | | | |
|--|---------|---------|---------|
| CV8/9AOMK (μ M) | trial 1 | trial 2 | trial 3 |
| 1.0 | 0.013 | 0.016 | 0.012 |
| 0.50 | 0.0070 | 0.0075 | 0.0096 |
| 0.25 | 0.0035 | 0.0045 | 0.0038 |
| 0.125 | 0.0018 | 0.00086 | 0.0018 |
| 0.0625 | 0.00078 | 0.00072 | 0.00017 |

Figure S4. k_{obs} values for caspases-3, -8, and -9 in the presence of varying concentrations of CV8/9-AOMK or Ac-LETD-AOMK.

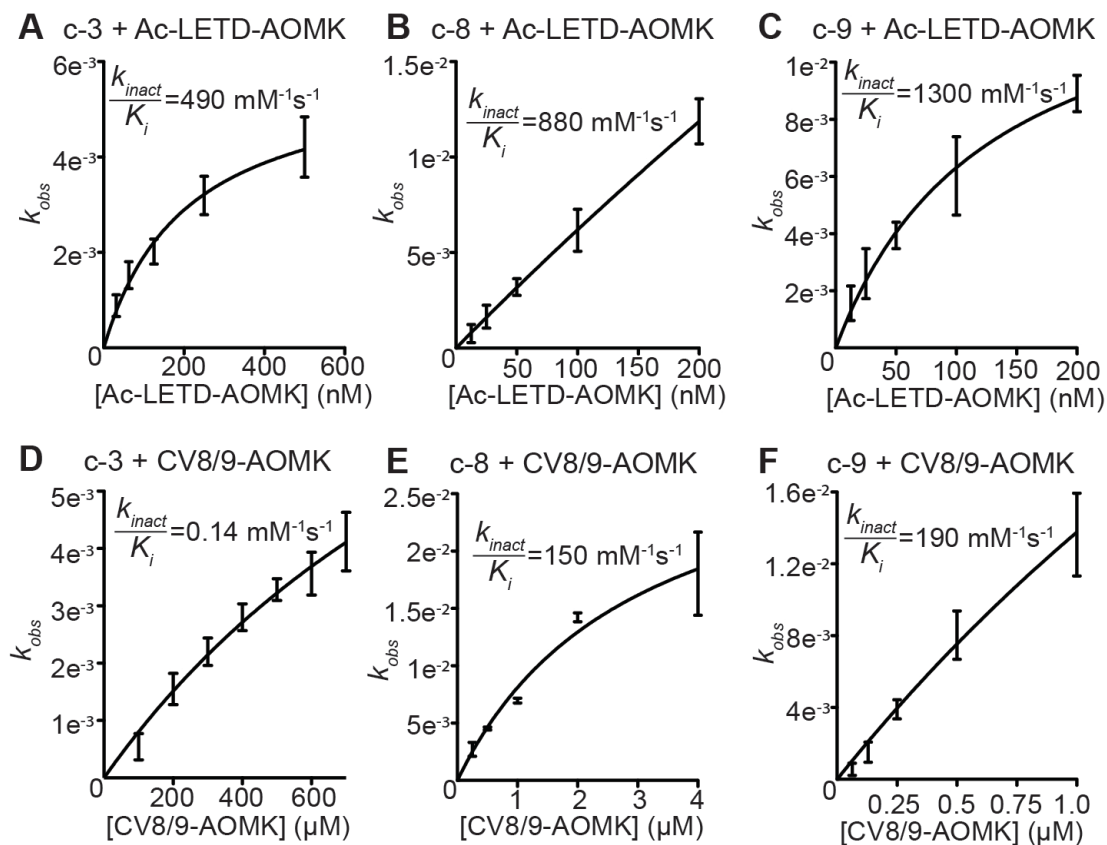


Figure S5. k_{obs} versus inhibitor concentration plots for various concentrations of Ac-LETD-AOMK or CV8/9-AOMK and caspases-3, -8, and -9.

| Hyp atoms | bond angle energy constant (ζ) | canonical | caspase-3:CV8/9 observed | caspase-8:CV8/9 observed |
|-----------------------------------|--|-----------|--------------------------|--------------------------|
| N-C α -C β | 80 | 109.7 | 96.6 | 102 |
| C α -C β -C γ | 40 | 109.5 | 102.9 | 106.8 |
| C β -C γ -C δ | 40 | 109.5 | 107.3 | 109 |
| C γ -C δ -N | 80 | 109.7 | 103.8 | 106.2 |
| C δ -N-C α | 50 | 118 | 110.3 | 108.2 |
| C-N-C α | 50 | 121.9 | 122.2 | 119.7 |
| C-N-C δ | 50 | 121.9 | 127.3 | 132 |
| C-C α -N | 63 | 110.1 | 113.8 | 112.4 |
| C-C α -C β | 63 | 111.1 | 116.4 | 111.9 |
| C β -C γ -O | 50 | 109.5 | 111.9 | 108.3 |
| C δ -C γ -O | 50 | 109.5 | 108.9 | 105.5 |

Figure S6. Angle strain parameters and a comparison of the canonical versus observed bond angles.

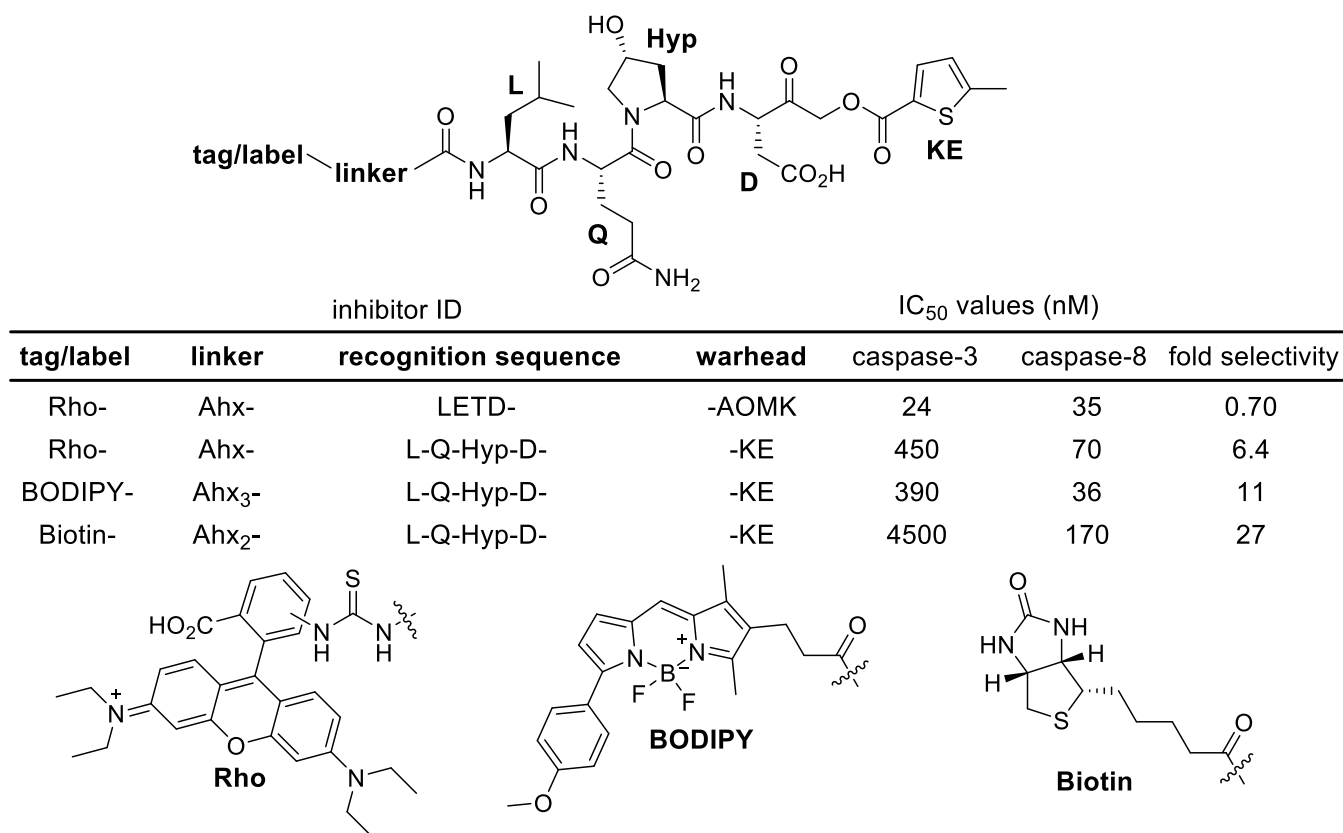


Figure S7. IC₅₀'s for CV8/9-based fluorescent and biotinylated probes.

Table S1. CV8/9-AOMK:caspase co-complex X-ray data processing and structure refinement statistics

| Structure | Caspase-3:CV8/9 | Caspase-8:CV8/9 |
|---|-----------------------|-----------------------|
| PDB ID | 4PS0 | 4PS1 |
| Space group | P21 | C21 |
| Unit Cell Parameters (a,b,c) (Å) | 50.20,69.19,93.14 | 115.87,144.60,86.19 |
| Unit Cell Angles (z,y,z) | 90.0,102.0,90.0 | 90.0,123.9,90.0 |
| Data Processing | | |
| Resolution range (Å) (outer shell) | 50-1.63 (1.66-1.63) | 50-1.73 (1.76-1.73) |
| Unique reflections | 76,222 (3,797) | 119,448 (5,960) |
| Completeness (%) | 98.0 (98.3) | 98.1 (97.9) |
| Redundancy | 3.3 (3.3) | 3.2 (3.1) |
| R _{meas} (%) ¹ | 8.1 (72.3) | 11.6 (52.1) |
| R _{p.i.m.} (%) ² | 4.4 (39.0) | 6.3 (28.5) |
| Average I/σ(I) | 23.6 (2.0) | 18.9 (1.9) |
| Refinement | | |
| Resolution range (Å) | 40.0-1.63 (1.65-1.63) | 45.0-1.73 (1.75-1.73) |
| No. reflections ³ (test set) | 76,158 (2,537) | 119,385 (3,439) |
| R _{cryst} (%) ⁴ | 15.3 (24.9) | 15.2 (20.5) |
| R _{free} (%) ⁴ | 17.9 (26.4) | 18.5 (26.8) |
| Protein atoms / Waters | 3967 / 476 | 7730 / 855 |
| CV ⁵ coordinate error (Å) | 0.15 | 0.16 |
| Rmsd bonds (Å) / angles (°) | 0.016 / 1.55 | 0.015 / 1.50 |
| B-values protein/waters/ligands (Å ²) | 25.5 / 37.4 / 27.4 | 27.8 / 38.1 / 30.5 |
| Ramachandran Statistics (%) | | |
| Most favored | 97.9 | 96.9 |
| Additional allowed | 1.9 | 3.1 |
| Generously allowed | 0.2 | 0 |

1) $R_{meas} = \{\sum_{hkl} [N/(N-1)] 1/2 \sum_i |I_{i(hkl)} - \langle I_{(hkl)} \rangle| \} / \sum_{hkl} \sum_i I_{i(hkl)}$, where $I_{i(hkl)}$ are the observed intensities, $\langle I_{(hkl)} \rangle$ are the average intensities and N is the multiplicity of reflection hkl. 2) $R_{p.i.m.}$ (precision-indicating $R_{merge} = \sum_{hkl} [1/(N_{hkl} - 1)]^{1/2} \sum_i |I_{i(hkl)} - \langle I_{(hkl)} \rangle| / \sum_{hkl} \sum_i I_{i(hkl)}$). 3) Reflections with I > 0 were used for refinement.⁷⁻⁹ 4) $R_{cryst} = \sum_h ||F_{obs}| - |F_{calc}|| / \sum_h |F_{obs}|$, 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.

References

- (1) Denault, J. B., Salvesen, G. S. (2003) Expression, purification, and characterization of caspases. *Curr. Protoc. Protein Sci.* Chapter 21:Unit 21.13.
- (2) Wolan, D. W., Zorn, J. A., Gray, D. C., Wells, J. A. (2009) Small-molecule activators of a proenzyme. *Science* 326, 853–858.
- (3) McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., Read, R. J. (2007) Phaser crystallographic software. *J. Appl. Crystallogr.* 40, 658–674.
- (4) Emsley, P., Lohkamp, B., Scott, W. G., Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* 66, 486–501.
- (5) Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. -W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., Zwart, P. H. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. Biol. Crystallogr.* 66, 213–221.
- (6) Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., Bourne, P. E. The protein data bank. (2000) *Nucleic Acids Res.* 28, 235–242.
- (7) 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.
- (8) Weiss, M. S. (2001) Global indicators of X-ray data quality. *J. Appl. Crystallogr.* 34, 130–135.
- (9) Karplus, P. A., Diederichs, K. (2012) Linking crystallographic model and data quality. *Science* 336, 1030–1033.