Supplementary Information Document

Table of Contents

Supplementary Tables and Figures

Entry	Sequence	Enzyme	k_m (μM)	k_{cat} (s^{-1})	k_{cat}/k_m ($s^{-1}M^{-1}$)
1	NAC_{20}KKNT	PPT1	7.9 ± 0.9	0.28 ± 0.01	35,000
		APT1	6.2 ± 0.8	0.38 ± 0.02	61,000
		APT2	9.4 ± 0.9	0.199 ± 0.006	21,000
2	GAC_{20}KKNT	PPT1	2.0 ± 0.1	0.28 ± 0.04	140,000
		APT1	2.7 ± 0.3	0.46 ± 0.01	170,000
		APT2	5.8 ± 0.6	0.106 ± 0.004	18,000
3	WAC_{20}KKNT	PPT1	12.0 ± 2.0	0.0063 ± 0.0004	530
		APT1	8.9 ± 2.0	0.027 ± 0.003	3,300
		APT2	7.9 ± 0.7	0.012 ± 0.004	1,500
4	ASC_{20}KKNT	PPT1	4.4 ± 0.5	0.43 ± 0.02	98,000
		APT1	1.2 ± 0.2	0.56 ± 0.02	470,000
		APT2	4.8 ± 0.4	0.29 ± 0.009	60,000
5	GSC_{20}KKNT	PPT1	8.2 ± 0.6	0.41 ± 0.009	50,000
		APT1	1.7 ± 0.3	0.66 ± 0.02	390,000
		APT2	5.6 ± 0.4	0.266 ± 0.006	48,000
6	WYC_{20}KKNT	PPT1	4.3 ± 0.6	0.0078 ± 0.0003	1,500
		APT1	8.4 ± 1.0	0.024 ± 0.002	2,900
		APT2	10.0 ± 2.0	0.0045 ± 0.0003	450
7	ASC_{20}KRNT	PPT1	5.7 ± 0.6	0.90 ± 0.03	160,000
		APT1	4.9 ± 0.7	0.95 ± 0.04	190,000
		APT2	5.6 ± 0.7	0.227 ± 0.009	41,000
8	GAC_{20}KRNT	PPT1	7.5 ± 0.7	0.77 ± 0.02	100,000
		APT1	5.9 ± 0.4	0.64 ± 0.01	110,000
		APT2	16.0 ± 2.0	0.056 ± 0.003	3,500
9	WYC_{20}KRNT	APT1	4.4 ± 0.6	0.044 ± 0.002	10,000
10	GVC_{20}DHNT	APT1	14.0 ± 1.0	0.03 ± 0.001	2,100
11	DEC_{20}WRNT	APT1	155 ± 84	0.003 ± 0.001	19
		APT2	91 ± 73	0.003 ± 0.001	33
12	GAC_{20}AANT	APT1	9.0 ± 3.0	0.016 ± 0.002	1,800
13	AHC_{20}DRNT	PPT1	6.7 ± 0.6	0.054 ± 0.001	8,060
		APT1	4.8 ± 0.7	0.163 ± 0.007	34,000
		APT2	5.3 ± 0.5	0.00259 ± 0.00008	490
14	PAC_{20}EANT	APT1	23 ± 8	0.0022 ± 0.0003	97
15	SYC_{20}IANT	APT1	23 ± 4	0.040 ± 0.003	1,700
16	ADC_{20}SRNT	PPT1	11 ± 6	0.012 ± 0.002	1,090
		APT1	7 ± 1	0.031 ± 0.002	4,400
17	FSC_{20}RANT	PPT1	42 ± 25	0.007 ± 0.002	170
		APT1	59 ± 19	0.036 ± 0.006	610
18	FRC_{20}KANT	PPT1	2.3 ± 0.4	0.032 ± 0.002	14,000
		APT1	1.7 ± 0.1	0.226 ± 0.006	130,000
		APT2	2.7 ± 0.3	0.062 ± 0.003	23,000

Table S1. Catalytic properties of APTs, for the hydrolysis of fluorogenic peptides, Related to figures 3 and 5. Amino acid substitutions at positions P2, P1, P1' and P2' are in red letters.

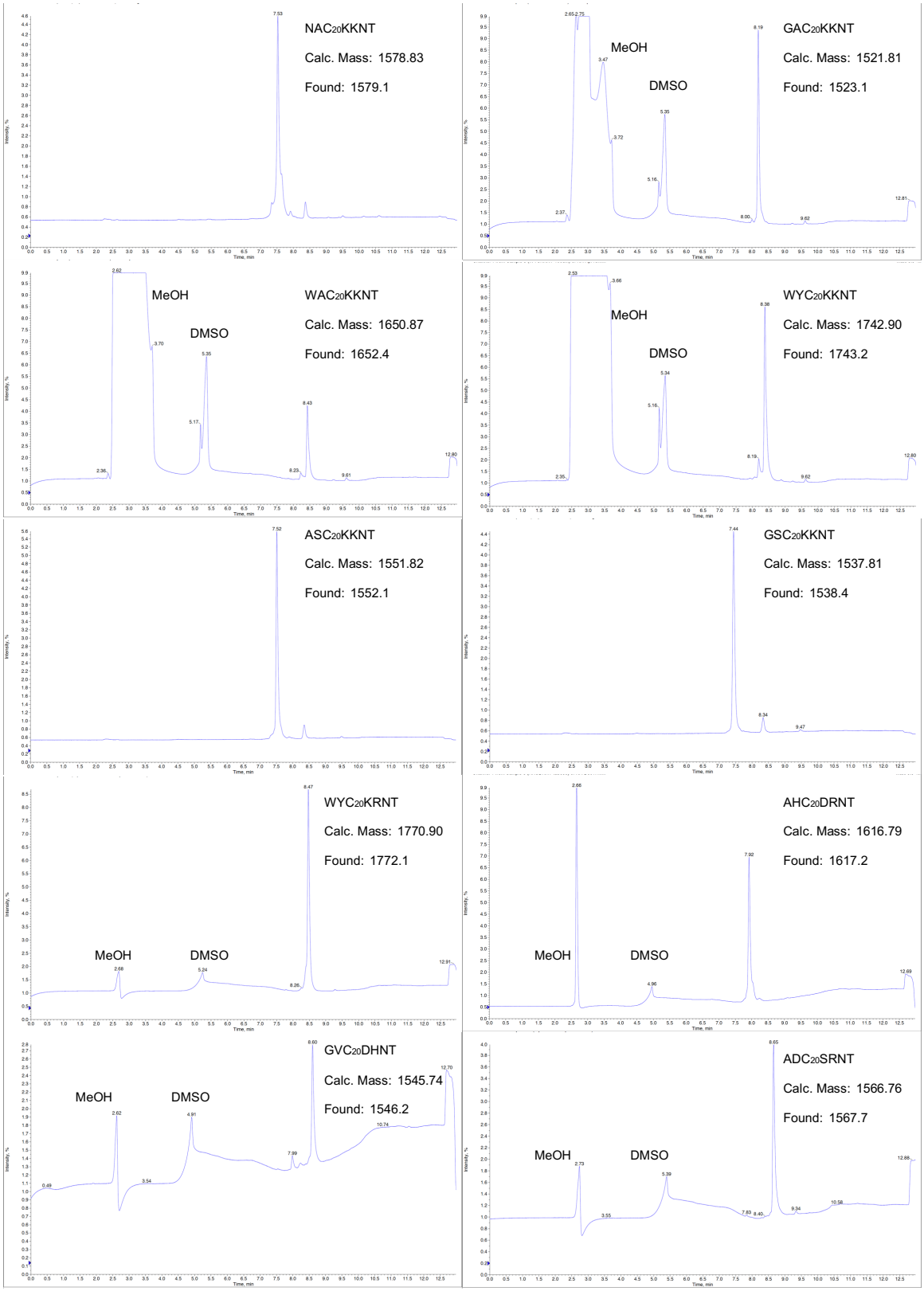


Figure S1. Characterization of fluorogenic peptides, Related to figures 3 and 5. HPLC purified peptides were characterized by LCMS, UV traces are measured at a wavelength of 220 nm. $[M+H]^{+}$ parent ions as well as $[M+2H]^{2+}$ were compared to calculated masses.

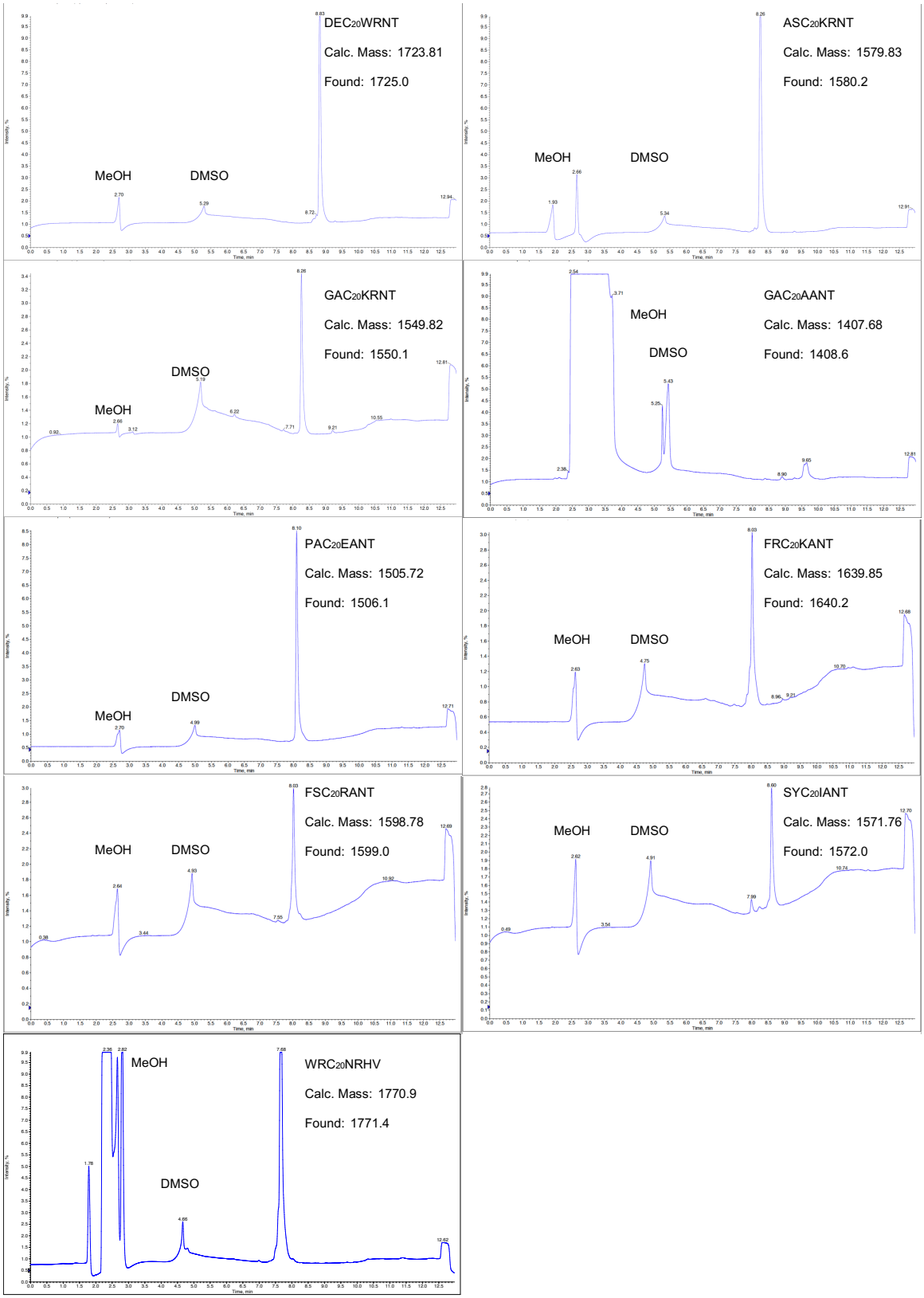


Figure S2. Characterization of fluorogenic peptides, Related to figures 3 and 5. HPLC purified peptides were characterized by LCMS, UV traces are measured at a wavelength of 220 nm. $[M+H]^{+}$ parent ions as well as $[M+2H]^{2+}$ were compared to calculated masses.

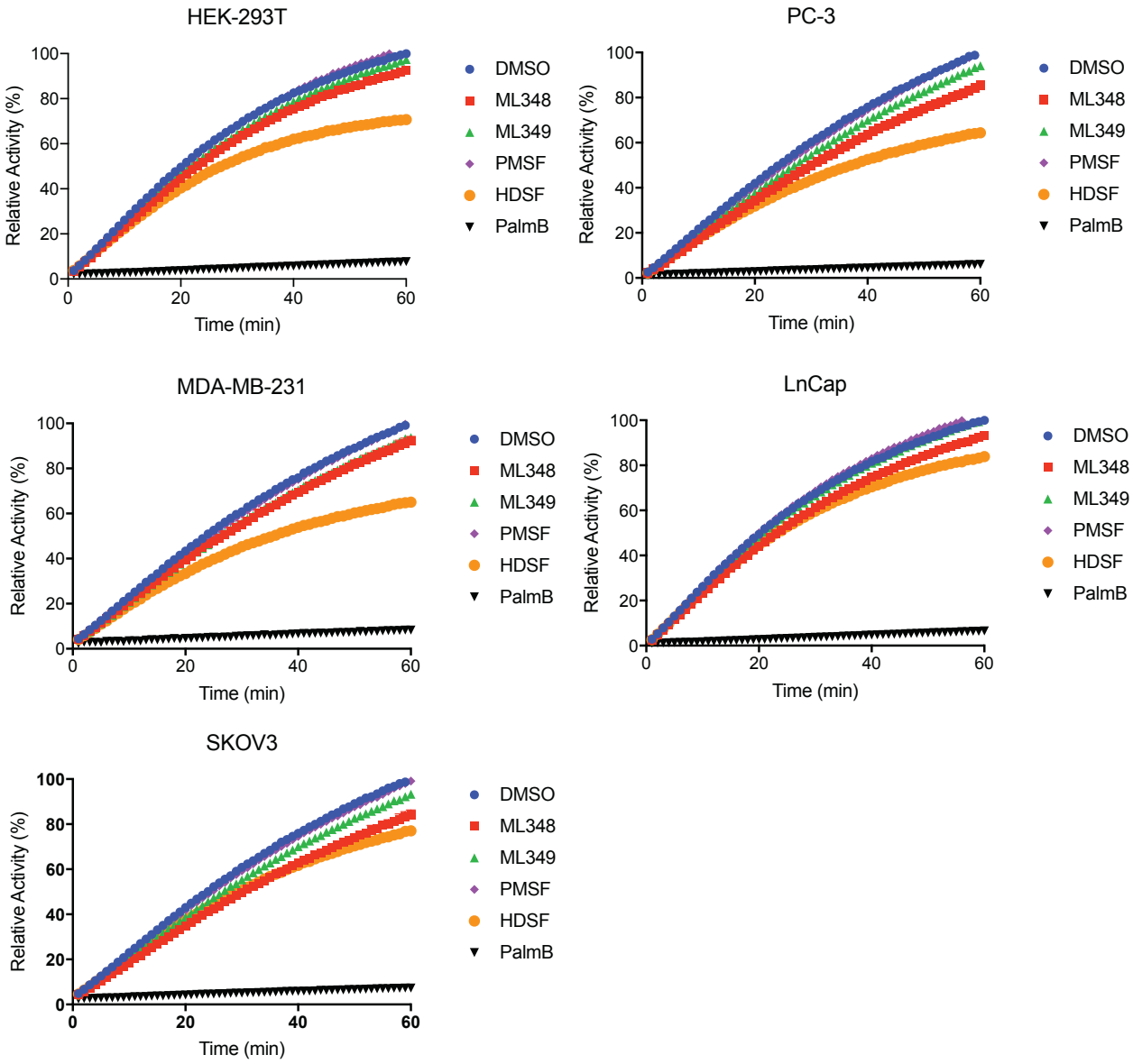


Figure S3. Depalmitoylase activity in cell lysates can be interrogated with selective inhibitors, Related to figure 1. Cell lysates (20 μg per reaction) were pre-incubated with inhibitors (10 μM) or DMSO as control, and then QStE (10 μM) was added to measure residual activity (Ex = 410 nm, Em = 450 nm) over the course of 60 minutes.

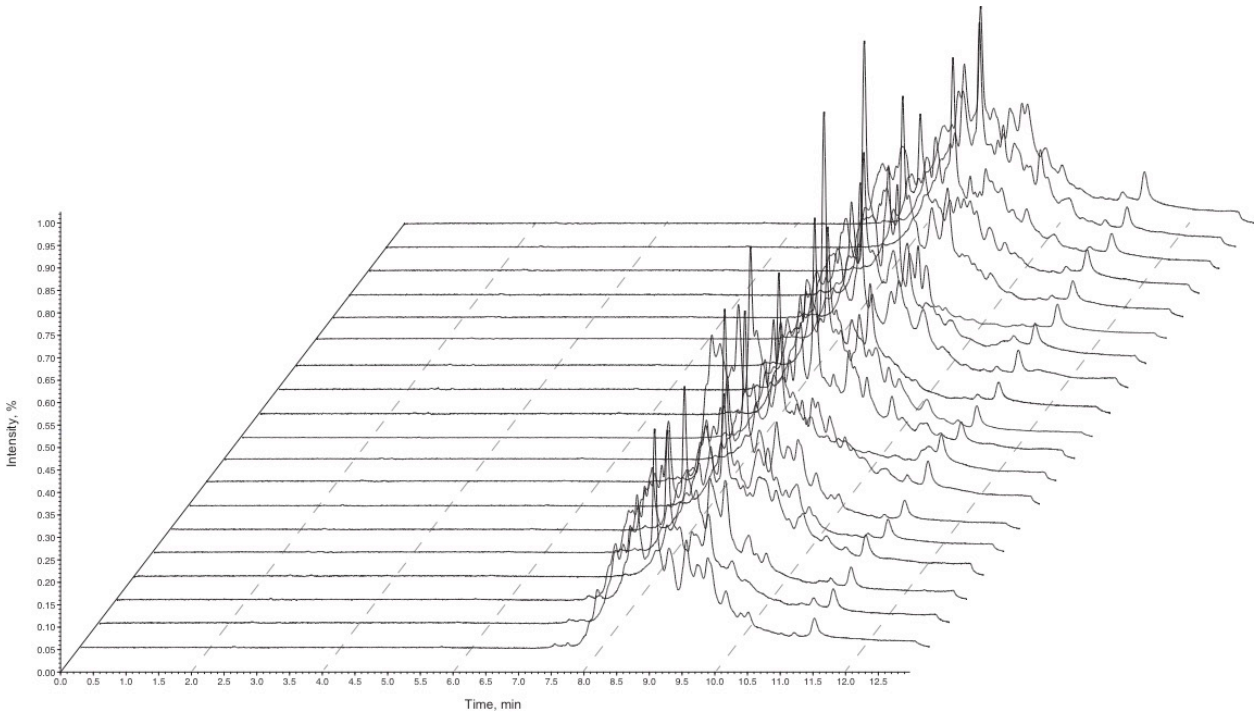


Figure S4. Determination of library integrity, Related to figures 2 and 4. Each combinatorial library is composed of 19 samples that correspond to each natural amino acid (excluding cysteine) at the varied position (X). Each sample contains a mixture of 19 peptides that are produced by the isokinetic mixture (IK) used at the position adjacent to X. UV traces of the LCMS analysis of library P2' are depicted, peak distribution and intensities are similar across the samples suggesting equimolar representation of peptide mixtures. UV traces are measured at a wavelength of 500 nm.

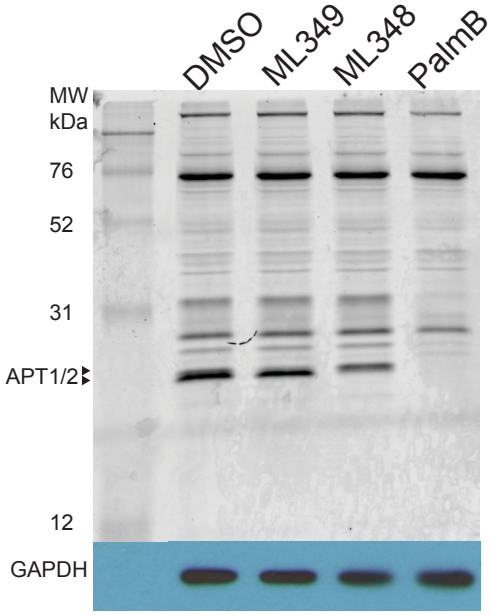


Figure S5. FP-Rho competition labeling confirms selective inhibition of APT1 and APT2, Related to figure 6. Tumor tissue lysate (20 µg) was incubated with DMSO as control or inhibitors at 10 µM for 30 minutes on ice, then 1 µM of FP-Rho was added and samples were incubated for additional 20 min on ice. Lysates were resolved by SDS-PAGE, and the gel was scanned for fluorescence (532-nm laser, 610-nm filter, PMT800). Western blot for GAPDH is shown for loading control.

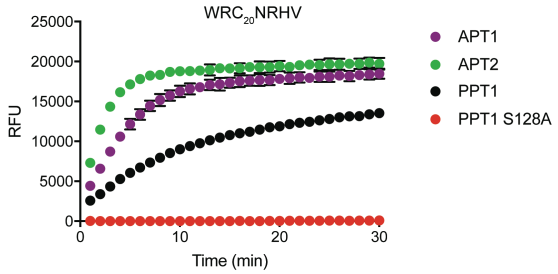
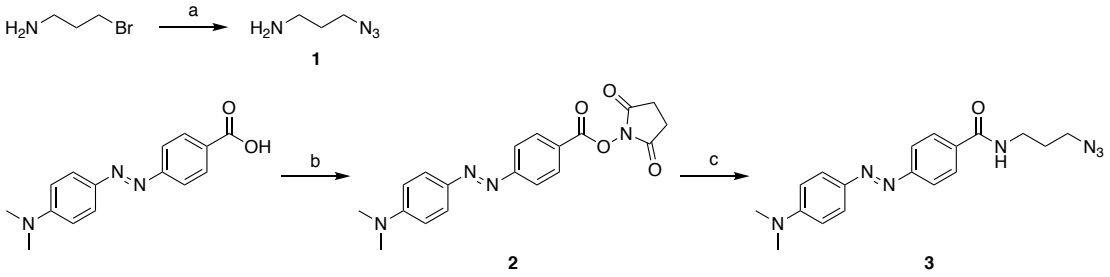
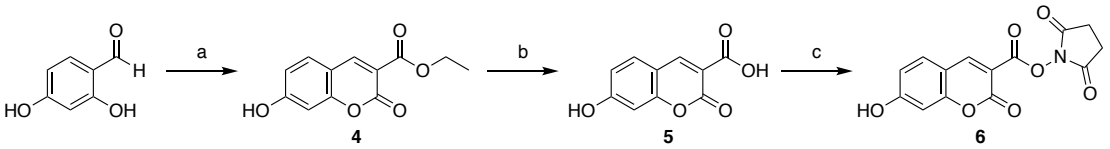


Figure S6. APT2 exhibits selectivity towards a Scribble-derived peptide sequence, Related to figure 5. The peptide WRCNRVH was synthesized based on the sequence of human Scribble. Activity of the recombinant depalmitoylases (50 nM) for the hydrolysis of the peptide (1 μM) was measured over 30 minutes. Error bars represent the S.D. of three replicates.

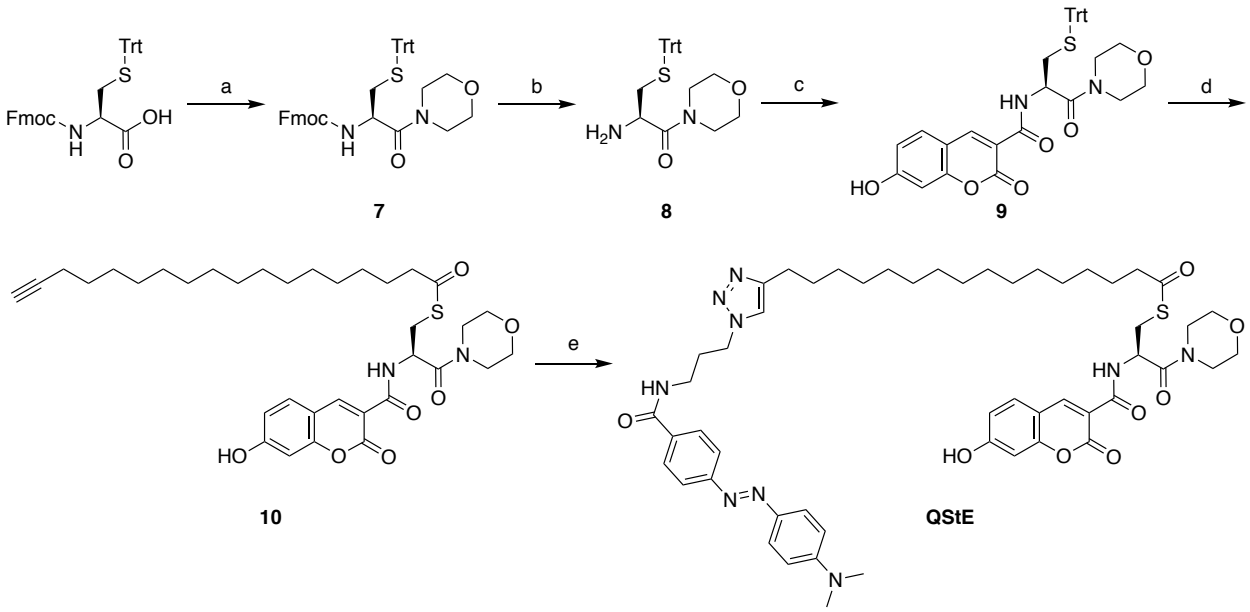
1) Supplementary Synthesis schemes



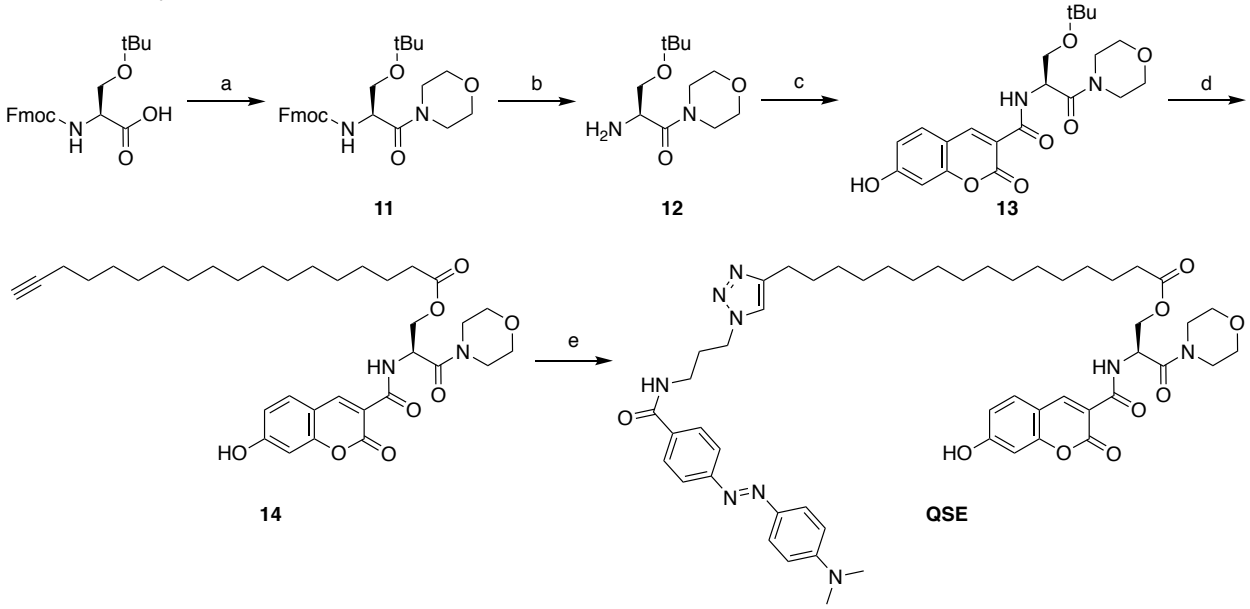
Scheme S1. Synthesis of DABCYL-azide, Related to Compound Synthesis methods in STAR Method Section. Reaction conditions; a) LiN_3, H_2O, 12 hr. b) NHS, EDC, DMF, 16 hr. c) 1, DIPEA, DMF, 4 hr.



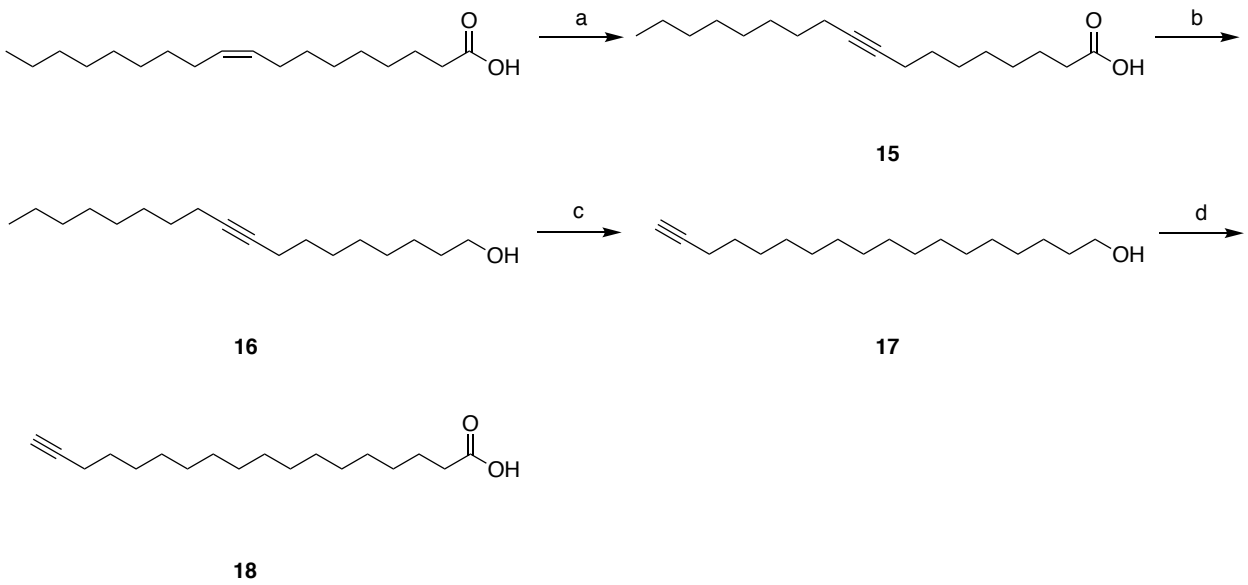
Scheme S2. Synthesis of 7-Hydroxycoumarin-3-carboxylic acid N-succinimidyl ester, Related to Compound Synthesis methods in STAR Method Section. Reaction conditions; a) Diethyl malonate, EtOH, piperidine, 12 hr. b) NaOH, H_2O, 1 hr. c) NHS, EDC, DMF, 3 hr.



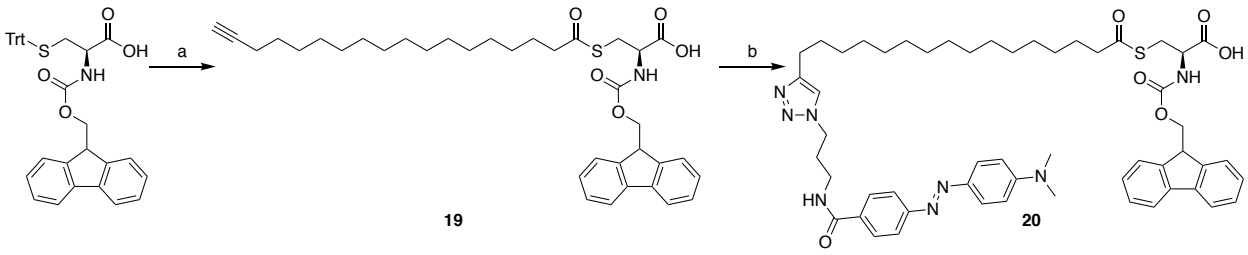
Scheme S3. Synthesis of QStE. Related to Compound Synthesis methods in STAR Method Section. Reaction conditions; a) HBTU, Morpholine, DMF, 30 min. b) 20% piperidine, DMF, 30 min. c) **6**, DIPEA, DMF, 4 hr. d) 1. 5% TFA, DCM, 2 hr. 2. 17-ODYA, $(COCl)_2$, DMF, DCM 3. TFA, 3 hr. e) **3**, $CuSO_4$, L-ascorbic acid, DMF, 40 ^{0}C, 2 hr.



Scheme S4. Synthesis of QSE. Related to Compound Synthesis methods in STAR Method Section. Reaction conditions; a) HBTU, Morpholine, DMF, 30 min. b) 20% piperidine, DMF, 30 min. c) **6**, DIPEA, DMF, 4 hr. d) 1. 50% TFA, DCM, 2 hr. 2. 17-ODYA, $(COCl)_2$, DMF, DCM 3. TFA, reflux, 4 hr. e) **3**, $CuSO_4$, L-ascorbic acid, DMF, 40 ^{0}C, 2 hr.



Scheme S5. Synthesis of 17-ODYA, Related to Compound Synthesis methods in STAR Method Section. Reaction conditions; a) Br_2, KOH, DMSO, 100 ^{0}C, 1hr. b) $LiAlH_4$, diethyl ether, 4 hr. c) KH, APA, 16 hr. d) PDC, DMF, 12 hr.



Scheme S6. Synthesis of cysteine building block (C_{20}), Related to Compound Synthesis methods in STAR Method Section. Reaction conditions; a) 1. TFA, TIS, DCM 2. $CHCl_3$, 17-ODYA, $SOCl_2$, reflux, 1hr. 3. 40 ^{0}C, 4 hr. b) Cu(II)SO_4, Ascorbic acid, **3**, DMF, 40 ^{0}C, 2 hr.