Chemistry & Biology *17*

Supplemental Information

Activity-Based Profiling

Reveals Reactivity of the Murine

Thymoproteasome-Specific Subunit β5t

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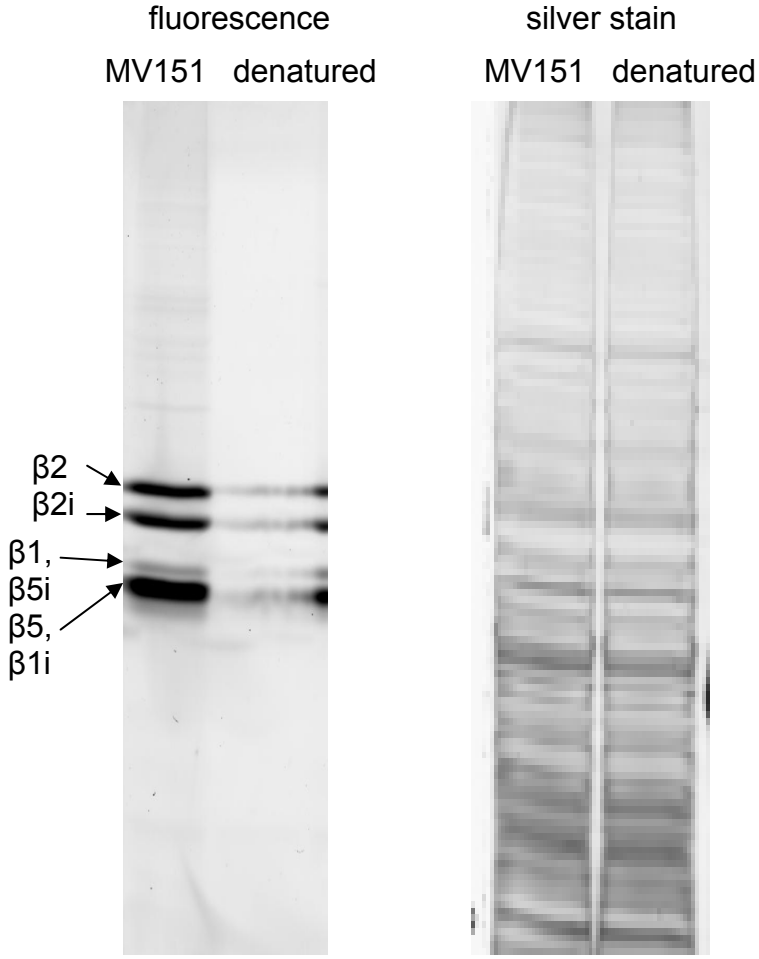


Figure S1, related to Figure 2A.
Fluorescence and silver stain detection of EL4 (murine thymoma cell line) cell lysate incubated with the fluorescent, broad-spectrum proteasome activity-based probe MV151. Some 20 μg protein was incubated with 0.5 μM MV151 for 60' at 37ºC, resolved by 12.5% SDS-PAGE and imaged by fluorescence scanning followed by silver staining of the same gel. In the denatured lane, the lysate was deactivated by boiling with 1% SDS prior to the MV151 incubation.

Table S1, related to Figure 2B. Protein Identification of Silver Stained Bands Captured by Probe 3 in Figure 2B, by In-gel Digestion and LC-MS analysis

prot acc		mass (Da)	cover % AA	z	ppm	pept score	peptide sequence
Psmb11	(β5t)	27834	20	2	-0.29	50	SLEQELEAK
IPI00221461				2	-0.54	39	ESGWEYVSR
				2	0.05	34	LLGTTSGTSADCATWYR
				3	0.00	25	GYHYDMTIQEAYTLAR
Psmb7	(β2)	25235	57	2	-1.20	41	GTTAVLTEK
IPI00136483				2	0.39	64	DGIVLGADTR
				3	0.14	42	FRPDMEEEEAK *
				2	-0.63	45	LDFLRPFSVPNK *
				3	0.58	34	LDFLRPFSVPNKK **
				2	2.14	129	LPYVTMGSGSLAAMAVFEDK
				3	-0.19	64	VTPLEIEVLEETVQTMDTS #
				4	5.34	100	IHFISPNIYCCGAGTAADTDMTTQLISSNLELHSLTTGR
Psmb10	(β2i)	24789	18	2	0.20	25	DGVILGADTR
IPI00316736				2	-0.42	44	ALSTPTEPVQR
				2	1.54	85	EVRPLTLELLEETVQAMEVE #
Psmb6	(β1)	21982	48	2	0.09	56	QVLLGDQIPK
IPI00119239				2	-2.05	76	LAAIQESGVER
				2	-0.46	132	DECLQFTANALALAMER
				2	1.65	100	QSFAIGGSGSSYIYGYVDATYR
				4	4.96	36	SGSAADTQAVADAVTYQLGFHSIELNEPPLVHTAASLFK
Psmb8	(β5i)	22635	42	2	0.71	72	ATAGSYISSLR
IPI00116712				2	0.00	42	FQHGVIVAVDSR
				2	-1.03	73	VESSDVSDLLYK
				2	-0.35	63	GPGLYYVDDNGTR
				2	-0.34	60	QDLSPEEAYDLGR
				2	0.95	72	VIEINPYLLGTMSGCAADCQYWER
Psmb5	(β5)	22514	13	2	0.23	24	ATAGAYIASQTVK
IPI00317902				2	-0.28	48	GPGLYYVDSEGNR
Psmb9	(β1i)	21313	17	2	0.67	79	FTTNAITLAMNR
IPI00309379				2	-0.43	101	DGSSGGVIYLVTITAAGVDHR

Table S1, related to Figure 2B. Protein accession numbers, mass of the active β subunit, % coverage of the protein by amino acids identified by LC-MS, charge of the peptide (z), measurement error (ppm), Mascot peptide scores, one (*) or two (**) miss cleavages, and C-terminal peptides (#). Mascot identifications were manually validated.

Table S2, related to Figure 2B. Protein identification after affinity purification with probe 3, on-bead digestion with trypsin and LC-MS analysis

prot acc		mass (Da)	cover % AA	z	ppm	pept score	peptide sequence
Psmb11	(β5t)	27834	34	2	-0.89	47	HGVIAAADTR
IPI00221461				2	1.30	21	EGQLPSVAGTAK
				2	0.41	76	LLAAMMSCYR
				2	-2.14	93	SSCGSYVACPASR
				2	0.76	71	ACGIYPEPATPQGAR
				2	1.67	130	LLGTTSGTSADCATWYR

				2	1.89	99	ELFVEQEEVTPEDCAIIMK
Psmb7	(β2)	25235	50	2	0.43	27	QMLFR
IPI00136483				2	1.09	70	FRPDMEEEEAK
				2	3.42	55	LDFLRPFSVPNK
				2	-0.80	57	FRPDMEEEEAKK *
				3	0.13	45	LDFLRPFSVPNKK *
				3	2.19	38	SKLDFLRPFSVPNK *
				2	-4.89	62	LPYVTMGSGSLAAMAVFEDK
				2	-1.17	111	VTPLEIEVLEETVQTMDTS #
				2	-0.11	129	LVSEAIAAGIFNDLGSGSNIDLCVISK
				3	3.18	57	KLVSEAIAAGIFNDLGSGSNIDLCVISK *
				3	0.28	173	IHFISPNIYCCGAGTAADTDMTTQLISSNLELHSLTTGR
Psmb10	(β2i)	24789	65	2	-0.29	61	ATNDSVVADK
IPI00316736				2	0.00	40	MELHALSTGR
				2	-0.60	39	FAPGTTPVLTR
				2	-1.12	121	IYCCGAGVAADTEMTTR
				2	0.35	167	LPFTALGSGQGAAVALLEDR
				2	0.73	93	EVRPLTLELLEETVQAMEVE #
				4	0.77	53	YQGHVGASLVVGGVDLNGPQLYEVHPHGSYSR
Psmb6	(β1)	21982	74	2	0.35	58	DGSSGGVIR
IPI00119239				2	-0.21	50	FTIATLPPP #
				2	0.55	78	TTTGSYIANR
				2	1.28	91	LAAIQESGVER
				2	-1.59	55	LTPIHDHIFCCR
				2	1.59	132	DECLQFTANALALAMER
				2	1.27	129	QSFAIGGSGSSYIYGYVDATYR
				3	1.68	32	EGMTKDECLQFTANALALAMER *
				3	1.68	106	YREDLMAGIIIAGWDPQEGGQVYSVPMGGMMVR *
				3	4.81	149	SGSAADTQAVADAVTYQLGFHSIELNEPPLVHTAASLFK
Psmb8	(β5i)	22635	55	2	-0.27	92	ATAGSYISSLR
IPI00116712				2	2.60	66	LLSNMMLQYR
				2	-0.68	72	FQHGVIVAVDSR
				2	-0.74	84	VESSDVSDLLYK
				2	0.56	80	GPGLYYVDDNGTR
				2	1.18	75	DNYSGGVVNMYHMK
				2	1.35	99	GMGLSMGSMICGWDK
				2	-0.75	121	LSGQMFSTGSGNTYAYGVMDSGYR
				3	0.81	60	VIEINPYLLGTMSGCAADCQYWER
Psmb5	(β5)	22514	18	2	0.38	47	VEEAYDLAR
IPI00317902				2	0.91	56	GPGLYYVDSEGNR
Psmb9	(β1i)	21313	58	2	0.21	56	VSAGTAVVNR
IPI00309379				2	-0.71	61	VILGDELPK
				2	-0.07	91	FTTNAITLAMNR
				2	0.05	96	DGSSGGVIYLVTITAAGVDHR
				3	-0.15	99	QPFTIGGSGSSYIYGYVDAAYKPGMTPEECR
				3	-1.11	122	IFCALSGSAADAQAIADMAAYQLELHGLELEEPPLVLAAANVVK

Table S2, related to Figure 2B.
Protein name, mass of the active β subunit, % coverage of the protein by amino acids identified by LC-MS, charge of the peptide (z), measurement error (ppm), Mascot peptide scores, miss cleavage (*), and C-terminal peptides (#). Mascot identifications were manually validated.

Table S3, related to Figure 3C. Calculated exact (*m/z*) masses of the active-site peptides bound to biotin-epoxomicin (probe 3)

		Exact mass		*z*=2		*z*=3	
	y_7 ion sequence	mono-iso	High-peak	mono-iso	High-peak	mono-iso	High-peak
β1	TTIMAVQFNGGVVLGADSR	2659.40773	2660.41061	1330.71114	1331.21258	887.47652	887.81081
β1i	TTIMAVEFDGGVVVGSDSR	2663.35502	2664.35793	1332.68479	1333.18624	888.79228	889.12659
β2	TTIAGVVYK	1674.96302	1674.96302	838.48878	838.48878	559.32828	559.32828
β2i	TTIAGLVFR	1700.98990	1700.98990	851.50223	851.50223	568.00391	568.00391
β5	TTTLAFK	1504.85749	1504.85749	753.43602	753.43602	502.64644	502.64644
β5i	TTTLAFK	1504.85749	1504.85749	753.43602	753.43602	502.64644	502.64644
β5t	TTTLAFR	1532.86364	1532.86364	767.43909	767.43909	511.96182	511.96182

Table S3, related to Figure 3C.
The mono-isotopic mass (mono-iso) and the mass of the most abundant isotope peak (High-peak) are shown at charge (*z*) of 0, 2, and 3. The active site peptide sequence of β5 and β5i is identical.

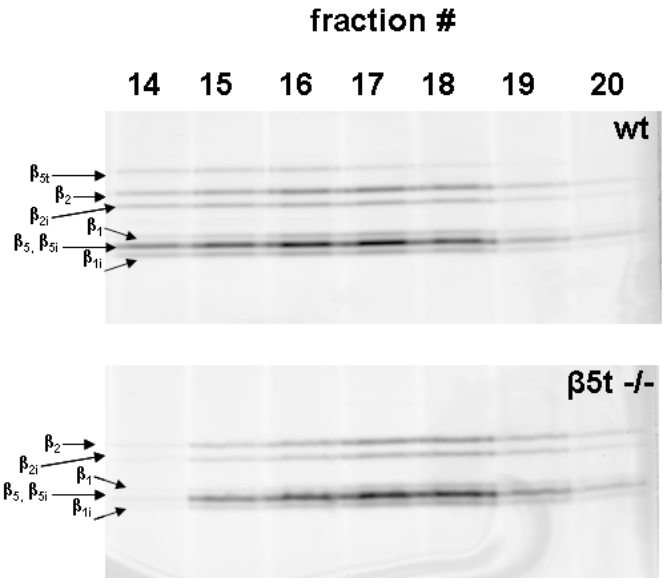


Figure S2.
Lysates from wild type and β5t -/- thymi from 3 weeks old mice were fractionated on 10-40% sucrose gradients by ultra-centrifugation. The (thymo)proteasome activity was assayed by ABP profiling with probe **4**.

Figure S3.

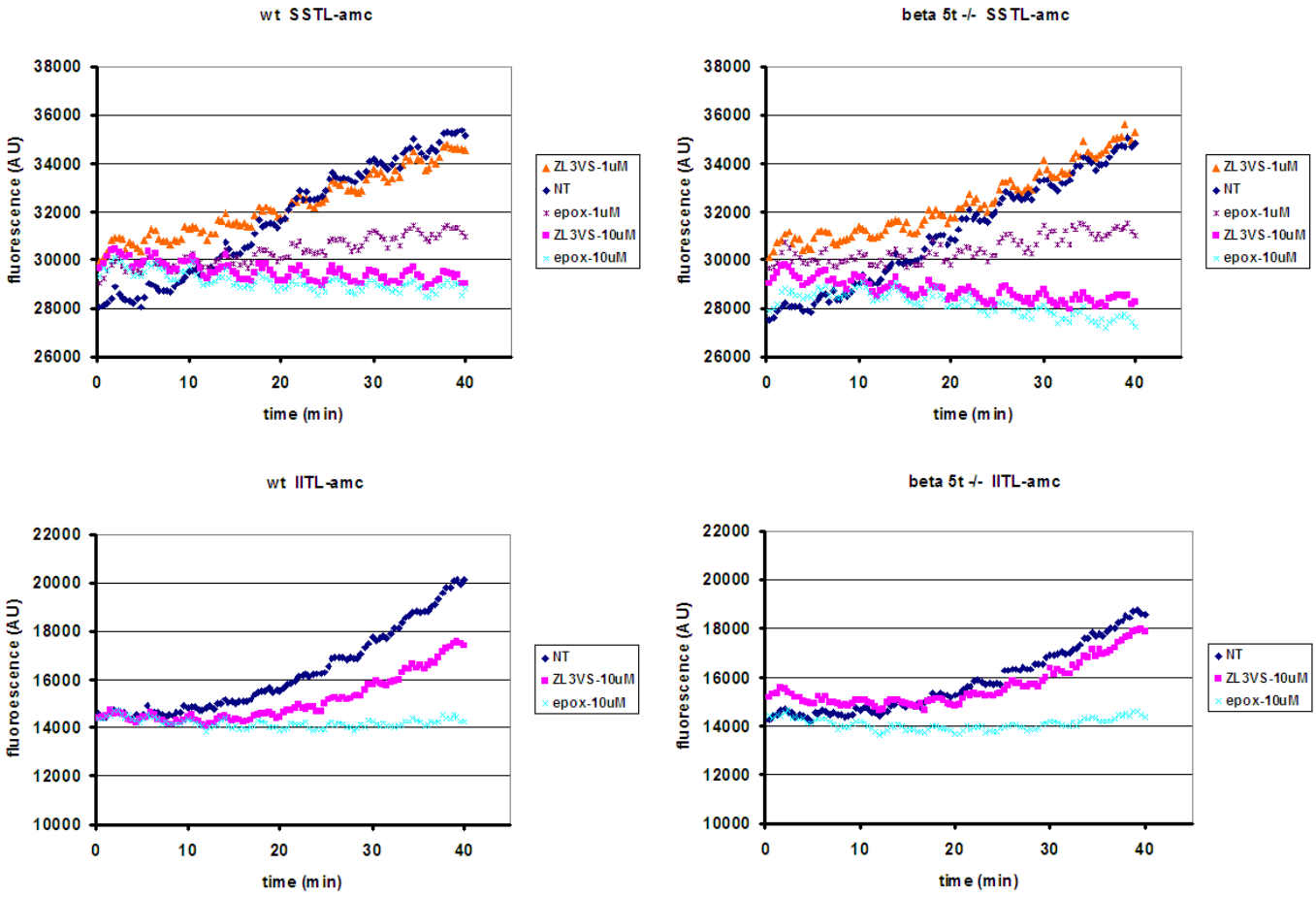


Figure S3.
Fluorogenic assay of enriched wt and β5t -/- thymoproteasomes using the Ac-SSTL-amc and Ac-IITL-amc substrates in combination with inhibition with 1, 10 μM ZL_3VS or epoxomicin.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Synthesis of the Activity-Based Probes 2, 3 and 4

Fmoc-Ile-Thr(*t*Bu)-OMe
L-threonine(*t*Bu) methyl ester HCl salt (2.5 g, 11 mmol) was dissolved in DCM (60 mL). To this solution were added Fmoc-L-isoleucine (4.7 g, 13.3 mmol, 1.2 equiv.), HCTU (5.5 g, 13.3 mmol, 1.2 equiv.) and DiPEA (6.0 mL, 36 mmol, 3.3 equiv.). The mixture was stirred for 2 hours after which TLC analysis indicated a completed reaction. The mixture was concentrated *in vacuo*, dissolved in EtOAc

and extracted with 1 M HCl (2×), saturated $NaHCO_3$ (2×) and brine. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. Purification of the product by column chromatography (10% → 15% EtOAc/petroleum ether) gave the title compound as a colorless solid (yield: 5.16 g, 9.83 mmol, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.76 (d, *J* = 7.48 Hz, 2H), 7.60 (d, *J* = 7.41 Hz, 2H), 7.39 (t, *J* = 7.46, 7.46 Hz, 2H), 7.31 (dt, *J* = 7.43, 7.43, 0.98 Hz, 2H), 6.48 (d, *J* = 8.84 Hz, 1H), 5.58 (d, *J* = 8.70 Hz, 1H), 4.49 (dd, *J* = 9.00, 1.68 Hz, 1H), 4.44-4.33 (m, 2H), 4.28-4.15 (m, 3H), 3.71 (s, 3H), 1.94-1.83 (m, 1H), 1.65-1.53 (m, 1H), 1.33-1.21 (m, 1H), 1.17 (d, *J* = 6.27 Hz, 3H), 1.11 (s, 9H), 1.03-0.93 (m, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ = 171.426, 170.868, 156.074, 143.910, 143.784, 141.249, 127.635, 127.017, 125.077, 119.904, 74.215, 67.193, 66.969, 59.307, 57.832, 52.135, 47.173, 38.179, 28.272, 24.820, 21.046, 15.085, 11.521 ppm.

Boc-Ile-Ile-Thr(*t*Bu)-$NHNH_2$

Fmoc-Ile-Thr(*t*Bu)-OMe (5.16 g, 9.83 mmol) was dissolved in DMF (50 mL) and DBU (1.57 mL, 10.3 mmol, 1.05 equiv.) was added. The reaction was stirred for 5 minutes after which TLC analysis showed complete removal of the Fmoc group. Next, HOBt (1.98 g, 14.7 mmol, 1.5 equiv.) was added and the reaction mixture was stirred for another 30 minutes. To this mixture were added Boc-L-isoleucine (2.73 g, 11.8 mmol, 1.2 equiv.), HCTU (4.88 g, 11.8 mmol, 1.2 equiv.) and DiPEA (4.87 mL, 29.5 mmol, 3 equiv.). The mixture was stirred for 16 hours after which TLC analysis indicated a completed reaction. The mixture was concentrated *in vacuo*, dissolved in DCM and extracted with 1 M HCl (2×), saturated $NaHCO_3$ (2×) and brine. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. Purification of the product by column chromatography (10% → 50% EtOAc/petroleum ether) gave Boc-Ile-Ile-Thr(*t*Bu)-OMe as a colorless solid (yield: 3.69 g, 7.15 mmol, 73%). LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 9.88 (ESI-MS (m/z): 516.13 ($M + H^+$)). The obtained product was dissolved in MeOH (50 mL) and hydrazine hydrate (10.4 mL, 214.5 mmol, 30 equiv.) was added. The reaction mixture was refluxed for 16 hours after which TLC analysis indicated complete conversion. Toluene was added and the mixture was concentrated under reduced pressure. Traces of hydrazine were removed by co-evaporating the mixture with toluene (3 ×) and the title compound was obtained as a colorless solid (yield: 6.67 g, 7.15 mmol, quant.). ^{1}H NMR (400 MHz, MeOD) δ = 4.36 (d, *J* = 3.53 Hz, 1H), 4.32 (d, *J* = 8.12 Hz, 1H), 4.07-4.00 (m, 1H), 3.94 (d, *J* = 7.90 Hz, 1H), 1.93-1.84 (m, 1H), 1.83-1.73 (m, 1H), 1.61-1.50 (m, 2H), 1.44 (s, 9H), 1.19 (s, 9H), 1.19-1.16 (m, 2H), 1.10 (d, *J* = 6.32 Hz, 3H), 0.94-0.87 (m, 12H) ppm. ^{13}C NMR (100 MHz, MeOD) δ = 174.839, 173.393, 171.301, 157.910, 80.568, 75.849, 68.522, 60.624, 59.227, 58.566, 37.949, 37.852, 28.772, 28.668, 25.941, 19.781, 16.231, 15.951, 11.392, 11.325 ppm. LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 6.08 (ESI-MS (m/z): 516.4 ($M + H^+$)).

Boc-Ile-Ile-Thr(*t*Bu)-leucinyl-(*R*)-2-methyloxirane

Boc-Ile-Ile-Thr(*t*Bu)-$NHNH_2$ (2.0 g, 3.87 mmol) was dissolved in DCM (40 mL) and cooled to -30°C under an argon atmosphere. *t*BuONO (566 µL, 4.25 mmol, 1.1 equiv.) and HCl (2.8 equiv., 10.8 mmol, 2.7 mL of a 4 M solution in 1,4-dioxane) were added and the mixture was stirred at -30 °C for 3 hours. (Boc-leucinyl)-(*R*)-2-methyloxirane (1.16 g, 4.25 mmol, 1.1 equiv.) was deprotected

with DCM/TFA (1:1 v/v, 20 mL) for 30 minutes followed by co-evaporation with toluene (3×). The resulting TFA salt was dissolved in DMF (5 mL) and added to the former reaction mixture together with DiPEA (3.31 mL, 20 mmol, 5 equiv.). The reaction mixture was slowly warmed to ambient temperature and stirred for 16 hours. Next, the mixture was extracted with 1 M HCl (2×), H_2O and brine, dried ($MgSO_4$) and concentrated *in vacuo*. The title compound was obtained after column chromatography (20% → 50% EtOAc/petroleum ether) as a colorless solid (yield: 2.25 g, 3.43 mmol, 89%). 1H NMR (400 MHz, $CDCl_3$) δ = 7.64 (d, *J* = 7.47 Hz, 1H), 6.99 (d, *J* = 5.64 Hz, 1H), 6.45 (d, *J* = 8.20 Hz, 1H), 5.22 (d, *J* = 7.85 Hz, 1H), 4.46 (ddd, *J* = 10.45, 7.55, 2.94 Hz, 1H), 4.40-4.32 (m, 2H), 4.14-4.07 (m, 1H), 3.94 (t, *J* = 7.34, 7.34 Hz, 1H), 3.38 (d, *J* = 5.07 Hz, 1H), 2.89 (d, *J* = 5.06 Hz, 1H), 1.93-1.77 (m, 2H), 1.74-1.64 (m, 1H), 1.60-1.55 (m, 1H), 1.52 (s, 3H), 1.51-1.46 (m, 2H), 1.44 (s, 9H), 1.28 (s, 9H), 1.27-1.24 (m, 1H), 1.17-1.08 (m, 2H), 1.06 (d, *J* = 6.44 Hz, 3H), 0.96 (d, *J* = 6.54 Hz, 6H), 0.92-0.86 (m, 12H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ = 208.062, 171.593, 170.738, 169.515, 155.807, 79.761, 75.492, 66.143, 59.249, 57.686, 56.956, 52.395, 50.746, 39.809, 37.300, 36.971, 28.280, 28.082, 25.423, 24.879, 24.695, 23.358, 21.359, 16.754, 15.532, 15.405, 11.285 ppm. LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H2O): R_t (min): 11.31 (ESI-MS (m/z): 655.27 ($M + H^+$)).

Biotin-epoxomicin (3)

Boc-Ile-Ile-Thr(*t*Bu)-leucinyl-(*R*)-2-methyloxirane (13.2 mg, 20.2 µmol) was dissolved in 2 mL DCM. TFA (2 mL) was added and the mixture was stirred for 20 min. The reaction mixture was co-evaporated with toluene (3×). The residue was dissolved in 1 mL DMF. Biotin-OSu (7 mg, 21 µmol, 1.01 equiv.) and DiPEA (8.3 µL, 50 µmol, 2.5 equiv.) were added and the mixture was stirred for 2 hr. The volatiles were removed *in vacuo* and the title compound was obtained after HPLC purification (yield: 5 mg, 6.9 µmol, 34%). ^1H NMR (400 MHz, MeOD) δ = 4.55 (dd, *J* = 10.63, 3.03 Hz, 1H), 4.48 (dd, *J* = 7.72, 4.85 Hz, 1H), 4.32-4.20 (m, 4H), 4.06-3.99 (m, 2H), 3.25 (d, *J* = 5.07 Hz, 1H), 3.23-3.16 (m, 1H), 2.95-2.89 (m, 2H), 2.69 (d, *J* = 12.71 Hz, 1H), 2.33-2.20 (m, 2H), 1.90-1.77 (m, 2H), 1.78-1.30 (m, 13H), 1.24-1.11 (m, 5H), 0.95-0.86 (m, 18H) ppm. LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 6.30 (ESI-MS (m/z): 725.7 ($M + H^+$)).

Azido-BODIPY(Tmr)-epoxomicin

Boc-Ile-Ile-Thr(*t*Bu)-leucinyl-(*R*)-2-methyloxirane (7.9 mg, 12 µmol) was dissolved in TFA (1 mL) and stirred for 30 min., before being coevaporated with toluene (3×). The residue was dissolved in DMF (2 mL) and azido-BODIPY-OSu (6.6 mg, 12 µmol, 1 equiv.) and DiPEA (8 µL, 48 µmol, 4 equiv.) were added and the reaction mixture was stirred for 12 hr. Concentration *in vacuo*, followed by purification by column chromatography (DCM → 2% MeOH/DCM) yielded the title compound as a brown/red solid (yield: 5.4 mg, 5.7 µmol, 47%). ^1H NMR (600 MHz, MeOD) δ = 7.88 (d, *J* = 8.7 Hz, 2H), 7.41 (s, 1H), 7.06 (d, *J* = 3.9 Hz, 1H), 6.99 (d, *J* = 8.7 Hz, 2H), 6.60 (d, *J* = 3.9 Hz, 1H), 4.55 (dd, J_1 = 10.7, J_2 = 2.8 Hz, 1H), 4.30 (d, *J* = 5.0 Hz, 1H), 4.22 (d, *J* = 7.8 Hz, 1H), 4.15-4.12 (m, 3H), 4.02 (p, *J* = 6.1 Hz, 1H), 3.54 (t, *J* = 6.7 Hz, 2H), 3.25 (d, *J* = 5.1 Hz, 1H), 2.92 (d, *J* = 5.1 Hz, 1H), 2.81 (m, 1H), 2.71 (m, 1H), 2.51 (s, 3H), 2.45-2.40 (m, 2H), 2.25 (s, 3H), 2.07 (p, *J* = 6.3 Hz, 2H), 1.89-1.79 (m, 1H), 1.75-1.66 (m, 2H), 1.65-1.52 (m, 2H), 1.53-1.41 (m, 5H), 1.41-1.21 (m, 15H), 1.20-1.06 (m, 5H), 1.05-0.97 (m, 1H), 0.97-0.85 (m, 16H), 0.82 (d, *J* = 6.7 Hz, 3H), 0.76 (t, *J* = 7.4 Hz, 3H) ppm. ^{13}C

NMR (150 MHz, MeOD) δ = 209.51, 174.86, 174.06, 173.59, 172.23, 161.03, 160.67, 156.57, 141.79, 136.67, 135.83, 132.45, 131.92, 131.89, 131.86, 131.67, 131.65, 129.91, 129.28, 127.16, 124.70, 119.10, 115.27, 115.19, 69.14, 68.55, 65.98, 60.13, 59.82, 59.42, 59.41, 53.10, 51.84, 40.38, 38.02, 37.71, 36.45, 30.82, 29.90, 26.26, 26.03, 23.81, 21.52, 21.21, 20.02, 17.05, 15.92, 15.86, 11.47, 11.22, 9.67 ppm.

Biotin-BODIPY(Tmr)-epoxomicin (2)

Azido-BODIPY(Tmr)-epoxomicin (4.1 mg, 4.3 μmol) and Biotin-propargylamide (2.4 mg, 8.6 μmol, 2 equiv.) were dissolved in *t*BuOH (0.25 mL) and toluene (0.25 mL) before $CuSO_4$ (125 μL 3.4 mM, 10 mol%) and sodium ascorbate (125 μL 6.9 mM, 20 mol%) were added. The reaction mixture was stirred at 80 °C for 12 hr., before being cooled to room temperature and concentrated *in vacuo*. Purification by column chromatography (petroleum ether → 50% acetone/petroleum ether) yielded the title compound as a brown/red solid (4.5 mg, 3.7 μmol, 85%). λ_{max} (MeOH): 544.43 nm, ε: 60400 l $mol^{-1}cm^{-1}$. ^{1}H NMR (600 MHz, MeOD) δ = 7.95-7.78 (m, 3H), 7.42 (s, 1H), 7.07 (d, *J* = 4.1 Hz, 1H), 6.95 (d, *J* = 8.9 Hz, 2H), 6.61 (d, *J* = 4.1 Hz, 1H), 4.70-4.52 (m, 5H), 4.46-4.39 (m, 2H), 4.34-4.26 (m, 1H), 4.25-4.19 (m, 1H), 4.17-4.11 (m, 1H), 4.08-3.99 (m, 3H), 3.95 (t, *J* = 2.2 Hz, 1H), 3.25 (d, *J* = 5.0 Hz, 1H), 3.16-3.10 (m, 1H), 2.92 (d, *J* = 5.1 Hz, 1H), 2.71-2.64 (m, 2H), 2.60-2.56 (m, 1H), 2.51 (s, 3H), 2.46-2.37 (m, 4H), 2.26 (s, 3H), 2.24-2.17 (m, 2H), 1.95-1.21 (m, 32H), 1.21-1.10 (m, 5H), 1.06-0.85 (m, 17H), 0.82 (d, *J* = 6.8 Hz, 3H), 0.76 (t, *J* = 7.3 Hz, 3H) ppm. ESI-MS (m/z): 1229.64 ($M + H^+$).

BODIPY(Tmr)-epoxomicin (4)

Boc-Ile-Ile-Thr(*t*Bu)-leucinyl-(*R*)-2-methyloxirane (65 mg, 100 μmol) was dissolved in 2 mL TFA and the solution was stirred for 1 hr. before being coevaporated with toluene (3x). The residue was dissolved in DCM:DMF (1/1, v/v, 10 mL) and DiPEA was added (300 μmol, 50 μL, 3 equiv.) followed by BODIPY(Tmr)-OSu (50 mg, 100 μmol, 1 equiv.) and the reaction mixture was stirred for 12 hr. The solution was concentrated and aplied to column chromatography (0-2% MeOH:DCM, then 0-2% EtOH:DCM, 2x) to yield the title compound as a purple solid (17 mg, 19 μmol, 19%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.71-8.57 (m, 1H), 8.17-7.91 (m, 2H), 7.87 (d, *J* = 8.81 Hz, 2H), 7.53-7.37 (m, 1H), 6.98 (d, *J* = 8.88 Hz, 2H), 6.84 (s, 1H), 6.74 (d, *J* = 3.76 Hz, 1H), 6.40 (d, *J* = 3.94 Hz, 1H), 4.86-4.68 (m, 3H), 4.63-4.56 (m, 1H), 4.09-3.99 (m, 1H), 3.86 (s, 3H), 3.26 (d, *J* = 4.46 Hz, 1H), 2.88 (d, *J* = 4.62 Hz, 1H), 2.73-2.56 (m, 1H), 2.56-2.31 (m, 6H), 2.01 (s, 3H), 1.87-1.73 (m, 99H), 1.51 (s, 3H), 1.09 (d, *J* = 6.12 Hz, 3H), 0.92-0.73 (m, 21H). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 208.16, 172.24, 171.91, 171.74, 170.71, 160.28, 158.28, 155.25, 139.29, 135.04, 134.05, 130.59, 130.15, 128.08, 125.52, 122.75, 118.10, 113.70, 67.32, 59.13, 57.49, 57.24, 56.92, 55.24, 52.35, 50.74, 39.36, 38.24, 37.55, 35.69, 29.68, 25.32, 25.28, 25.05, 23.23, 22.67, 21.25, 19.79, 17.42, 16.83, 15.30, 15.16, 14.11, 12.95, 11.48, 11.44, 9.34. LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 10.21 (ESI-MS (m/z): 879.00 ($M + H^+$)).

Synthesis of the fluorogenic substrates

Boc-Leu-AMC

Boc-Leu-OH · H_2O (0.62 mmol, 155 mg, 1.1 equiv.) was coevaporated with toluene (2x) and dissolved in DMF. HATU (0.67 mmol, 255 mg, 1.2 equiv.) and 2,4,6-trimethylpyridine (0.67 mmol, 90 µL, 1.2 equiv.) were added and the mixture was stirred for 5 min. AMC (0.56 mmol, 100 mg, 1 equiv.) was added and the mixture was stirred for 5 days. The mixture was concentrated, dissolved in DCM and extracted with 1M HCl (2x), sat. aq. $NaHCO_3$ (2x) and brine before drying over Na_2SO_4. Column chromatography (20% EA:PE → 50% EA:PE) yielded the title compound (0.51 mmol, 200 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.70 (s, 1H), 7.80 (s, 1H), 7.35 (d, *J* = 8.55 Hz, 1H), 6.99 (d, *J* = 8.17 Hz, 1H), 6.06 (s, 1H), 5.59 (d, *J* = 7.82 Hz, 1H), 4.53-4.42 (m, 1H), 2.34 (s, 3H), 1.89-1.58 (m, 3H), 1.50 (s, 9H), 1.01-0.92 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 172.28, 160.95, 156.69, 153.58, 152.43, 141.36, 124.53, 115.20, 115.09, 112.74, 106.88, 80.51, 54.04, 40.72, 28.31, 28.18, 24.62, 23.06, 21.13, 18.26.

Fmoc-Ser(tBu)-Thr(tu)OMe

HCl·H-Thr(tBu)OMe (1 mmol, 383 mg, 1 equiv.), FmocSer(tBu)OH (1.05 mmol, 237 mg, 1.05 equiv.) and HBTU (1.2 mmol, 455 mg, 1.2 equiv.) were dissolved in DCM (10 mL) and DiPEA (3.5 mmol, 578 µL, 3.5 equiv.) was added and the mixture was stirred for 1h. The mixture was concentrated, dissolved in EA and washed with 1M HCl (2x), sat. aq. $NaHCO_3$ (4x) and brine, dried over Na_2SO_4 and concentrated to yield the title compound (560 mg, 1 mmol, quant) which was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.74 (d, *J* = 7.50 Hz, 2H), 7.65-7.58 (m, 3H), 7.38 (t, *J* = 7.44, Hz, 2H), 7.30 (dt, *J* = 7.44, 1.00 Hz, 2H), 5.92 (d, *J* = 5.73 Hz, 1H), 4.54 (d, *J* = 9.07 Hz, 1H), 4.40-4.34 (m, 3H), 4.29-4.19 (m, 2H), 3.83 (dd, *J* = 8.21, 3.80 Hz, 1H), 3.70 (s, 3H), 3.47 (t, *J* = 8.56, 1H), 1.29-1.24 (m, 9H), 1.18 (d, *J* = 6.25 Hz, 3H), 1.12-1.11 (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 170.91, 170.66, 155.85, 143.79, 143.62, 141.10, 127.53, 126.90, 125.03, 119.80, 74.14, 73.97, 67.22, 66.94, 61.69, 58.13, 53.91, 51.90, 46.98, 28.27, 27.24, 20.66.

Fmoc-(Ser(tBu))$_2$-Thr(tBu)OMe

Fmoc-Ser(tBu)Thr(tu)OMe (1 mmol) was dissolved in DMF (10 mL). DBU (1 mmol, 152 µmol, 150 µL) was added and the mixture was stirred for 5 min before HOBt (2 mmol, 270 mg, 2 equiv.) was added, After 5 min of stirring, Fmoc-Ser(tBu)OH (1.05 mmol, 403 mg, 1.05 equiv.), HBTU (1.20 mmol, 455 mg, 1.2 equiv.) and DiPEA (4.5 mmol, 743 µL, 4.5 equiv.) were added and the mixture was stirred for 1 hr before being concentrated. The residue was taken up in DCM and washed with 1M HCl (2x), sat. aq. $NaHCO_3$ (4x) and brine, dried over Na_2SO_4 and concentrated. Column chromatography (10% EA/tol → 25% EA/tol) yielded the title compound (548 mg, 785 µmol, 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.77-7.67 (m, 2H), 7.63-7.57 (m, 1H), 7.43 (d, *J* = 9.18 Hz, 1H), 7.37 (t, *J* = 7.45 Hz, 2H), 7.29 (t, *J* = 7.42 Hz, 2H), 7.25-7.19 (m, 2H), 5.90 (d, *J* = 6.24 Hz, 1H), 4.60-4.50 (m, 2H), 4.43-4.26 (m, 3H), 4.24-4.18 (m, 2H), 3.84 (m, 2H), 3.44 (m 2H), 3.67 (s, 3H), 1.27-1.24 (m, 9H), 1.25-1.22 (m, 9H), 1.15 (d, *J* = 5.82 Hz, 3H), 1.10 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 170.60, 170.43, 170.05, 155.78, 143.72, 143.53, 141.03, 127.46, 126.84, 124.96, 119.72, 74.13, 73.97,

73.83, 67.25, 66.91, 61.70, 61.05, 57.95, 54.35, 53.11, 51.76, 46.90, 28.21, 27.19, 20.44.

Ac-(Ser(tBu))$_2$-Thr(tBu)OMe

Fmoc-(Ser(tBu))$_2$Thr(tBu)OMe (548 mg, 785 µmol) was dissolved in DMF (10 mL). DBU (785 µmol, 117 µL, 1 equiv.) was added and the mixture was stirred for 5 min before HOBt (1.18 mmol, 159 mg, 1.5 equiv.) was added. The mixture was stirred for 5 min and Ac_2O (1.18 mmol, 111 µL, 1.5 equiv.) and DiPEA (1.96 mmol, 324 µL, 2.5 equiv.) were added and the mixture was stirred for 30 min before being concentrated. The residue was taken up in DCM and washed with 1M HCl (2x), sat. aq. $NaHCO_3$ (2x) and dried over Na_2SO_4. Column chromatography (20% EA/tol →EA and 50% EA/tol →tol) yielded the title compound (isolated yield 226 mg, 437 µmol, 56%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.64 (d, *J* = 6.31 Hz, 1H), 7.39 (d, *J* = 9.33 Hz, 1H), 6.53 (d, *J* = 6.40 Hz, 1H), 4.57-4.44 (m, 3H), 4.24 (dq, *J* = 6.15, 2.00 Hz, 1H), 3.86-3.78 (m, 2H), 3.70 (s, 3H), 3.44-3.35 (m, 2H), 2.04 (s, 3H), 1.27-1.24 (m, 9H), 1.23 (s, 9H), 1.16 (d, *J* = 6.25 Hz, 3H), 1.12 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 170.79, 170.55, 170.37, 169.93, 74.35, 74.13, 74.02, 67.43, 61.40, 61.18, 58.08, 53.28, 53.07, 51.96, 28.39, 27.34, 23.19, 20.59.

Ac-(Ser(tBu))$_2$-Thr(tBu)NHNH$_2$

Ac-(Ser(tBu))$_2$Thr(tBu)OMe (126 µmol, 65 mg) was dissolved in MeOH (5 mL) and hydrazine monohydrate was added (7.6 mmol, 0.4 mL, 60 equiv.) and the mixture was refluxed for 24h. More hydrazine (1.9 mmol, 0.1 mL, 15 equiv.) was added and the mixture was refluxed for 16 h. The mixture was coevaporated with toluene (3x) and used without further purification. ^{1}H NMR (400 MHz, CD_3OD) δ ppm 4.53-4.42 (m, 2H), 4.32-4.27 (m, 1H), 4.21-4.15 (m, 1H), 3.78 (dd, *J* = 9.18, 4.07 Hz, 1H), 3.68-3.60 (m, 2H), 3.58-3.50 (m, 1H), 2.02 (s, 3H), 1.23 (s, 9H), 1.22 (s, 9H), 1.17 (s, 9H), 1.19 (d, *J* = 1.71 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ ppm 173.40, 172.75, 172.30, 171.36, 75.63, 75.44, 75.11, 68.01, 62.65, 59.43, 55.35, 55.29, 28.72, 27.76, 27.73, 22.50, 20.47.

Fmoc-Ile-Thr(tBu)OMe

HCl·H-Thr(tBu)OMe (2 mmol, 707 mg, 1 equiv.), Fmoc-Ile-OH (2 mmol, 451 mg, 1 equiv.) and HBTU (2.4 mmol, 910 mg, 1.2 equiv.) were dissolved in DCM (40 mL). DiPEA (7 mmol, 1.16 mL, 3.5 equiv.) was added and the mixture was stirred for 90 min. The mixture was concentrated, dissolved in EA and washed with 1M HCl (2x), sat. aq. $NaHCO_3$ (4x) and brine, dried over Na_2SO_4 and concentrated. Column chromatography (10% EA/tol → 25% EA/tol) yielded the title compound (1.05 g, 2 mmol, quant) which was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.76 (d, *J* = 7.46 Hz, 2H), 7.60 (d, *J* = 7.43 Hz, 2H), 7.39 (t, *J* = 7.45 Hz, 2H), 7.30 (dt, *J* = 7.44, 0.95 Hz, 2H), 6.44 (d, *J* = 8.89 Hz, 1H), 5.57 (d, *J* = 8.70 Hz, 1H), 4.49 (dd, *J* = 8.99, 1.67 Hz, 1H), 4.45-4.32 (m, 2H), 4.27-4.16 (m, 3H), 3.70 (s, 3H), 1.95-1.83 (m, 1H), 1.65-1.54 (m, 1H), 1.36-1.20 (m, 1H), 1.17 (d, *J* = 6.27 Hz, 3H), 1.11 (s, 9H), 1.03-0.94 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 171.34, 170.86, 156.02, 143.88, 143.75, 141.21, 127.61, 126.99, 125.10, 125.06, 119.88, 74.14, 67.11, 66.93, 59.25, 57.80, 52.12, 47.13, 38.20, 28.24, 24.80, 21.04, 15.05, 11.52.

H-Ile-Thr(tBu)OMe

Fmoc-IleThr(tBu)OMe (576 µmol, 302 mg) was dissolved in THF (10 mL). EtSH (5.76 mmol, 430 µL, 10 equiv.) was added, followed by DBU (one drop) and the mixture was stirred for 1hr before being concentrated. Column chromatography (50% EA:Tol → EA) yielded the title compound (509 µmol, 154 mg, 88%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.72 (d, *J* = 9.20 Hz, 1H), 4.48 (dd, *J* = 9.20, 2.00 Hz, 1H), 4.26-4.22 (m, 1H), 3.71 (s, 3H), 3.35 (d, *J* = 4 Hz, 1H), 2.00-1.89 (m, 1H), 1.87 (br s, 2H), 1.51-1.49 (m, 1H), 1.25-1.20 (m, 1H), 1.24 (d, *J* = 6.30 Hz, 3H), 1.12 (s, 9H), 0.99 (d, 6.80 Hz, 3H), 0.94 (t, *J* = 2 Hz, 3H).). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 174.81, 171.22, 73.73, 67.12, 59.85, 57.42, 51.86, 37.99, 28.12, 23.76, 20.84, 15.80, 11.66.

Fmoc-Ile$_2$-Thr(tBu)OMe

H-IleThr(tBu)OMe (509 µmol, 154 mg) was dissolved in DCM. Fmoc-Ile-OH (534 µmol, 189 mg, 1.05 equiv.), HBTU (585 µmol, 222 mg, 1.15 equiv.) and DiPEA (1.27 mmol, 210 µL, 2.5 equiv.) were added and the mixture was stirred for 30 min. The mixture was washed with 1M HCl (2x), sat. aq. $NaHCO_3$ (4x) and brine, dried over Na_2SO_4 and concentrated. Column chromatography (10% EA/tol → 50% EA/tol) yielded the title compound (320 mg, 502 µmol, 99%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.72 (d, *J* = 7.50 Hz, 2H), 7.64-7.56 (m, 2H), 7.39-7.31 (m, 3H), 7.29-7.20 (m, 2H), 6.84 (d, *J* = 9.13 Hz, 1H), 6.01 (d, *J* = 9.27 Hz, 1H), 4.61-4.51 (m, 2H), 4.46 (dd, *J* = 10.30, 7.08 Hz, 1H), 4.34-4.22 (m, 2H), 4.22-4.14 (m, 2H), 3.63 (s, 3H), 1.91-1.76 (m, 2H), 1.65-1.51 (m, 2H), 1.29-1.12 (m, 2H), 1.10 (d, *J* = 6.23 Hz, 3H), 1.07 (s, 9H), 0.96 (d, *J* = 6.75 Hz, 3H), 0.92-0.83 (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 171.41, 171.29, 170.71, 156.15, 143.83, 143.77, 141.08, 127.44, 126.84, 125.13, 125.09, 119.71, 73.89, 67.12, 66.85, 59.23, 57.57, 57.38, 51.85, 47.00, 37.94, 37.61, 28.13, 24.90, 24.76, 20.50, 15.17, 14.91, 11.28.

H-Ile$_2$-Thr(tBu)OMe

Fmoc-Ile$_2$Thr(tBu)OMe (320 mg, 502 µmol) was dissolved in THF (10 mL) and EtSH (5.02 µmol, 370 µL, 10 equiv.) was added followed by DBU (1 drop) and the mixture was stirred for 1h before being concentrated. Column chromatography (50% EA/Tol → 5% MeOH:EA) yielded the title compound (168 mg, 404 µmol, 81%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.86 (d, *J* = 8.78 Hz, 1H), 6.51 (d, *J* = 8.89 Hz, 1H), 4.46 (dd, *J* = 8.94, 1.70 Hz, 1H), 4.39 (dd, *J* = 8.77, 6.38 Hz, 1H), 4.24 (dq, *J* = 6.22, 6.21, 6.21, 1.69 Hz, 1H), 3.71 (s, 3H), 3.32 (d, *J* = 4.00 Hz, 1H), 2.11-1.72 (m, 4H), 1.65-1.53 (m, 1H), 1.49-1.32 (m, 1H), 1.11 (s, 9H), 1.16 (d, *J* = 6.27 Hz, 3H), 1.02-0.87 (m, 12H), 1.30-1.21 (m, 2H).

Ac-Ile$_2$-Thr(tBu)OMe

H-Ile$_2$Thr(tBu)OMe (93 mg, 224 µmol) was dissolved in 5 mL DCM. DiPEA (270 µmol, 44 µL, 1.2 equiv.) was added, followed by Ac_2O (246 µmol, 23 µL, 1.1 equiv.). After 1hr, the mixture was washed with 1M HCl (2x), H_2O and dried over Na_2SO_4. Column chromatography (10% EA/tol → 60% EA/tol) yielded the title compound (93 mg, 203 µmol, 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 6.89 (d, *J* = 9.06 Hz, 1H), 6.83 (d, *J* = 8.99 Hz, 1H), 4.57-4.48 (m, 3H), 4.21 (dq, *J* = 6.18, 6.18, 6.16, 1.97 Hz, 1H), 3.71 (s, 3H), 1.91-1.70 (m, 2H), 1.62-1.48 (m, 2H), 1.33-1.20 (m, 2H), 1.13 (d, *J* = 6.30 Hz, 3H), 1.11 (s, 9H), 0.95 (d, *J* = 6.76 Hz, 3H), 0.92-0.83 (m, 9H), 2.02 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 171.51,

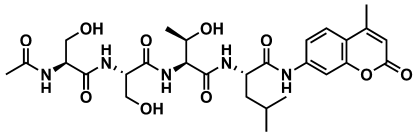
171.32, 170.79, 169.90, 74.02, 67.14, 57.66, 57.46, 51.96, 37.80, 37.55, 28.20, 24.96, 24.85, 23.00, 20.47, 15.15, 15.00, 11.35, 11.27.

Ac-Ile$_2$-Thr(tBu)NHNH$_2$

Ac-Ile$_2$Thr(tBu)OMe (93 mg, 203 µmol) was dissolved in MeOH (7 mL). Hydrazine hydrate (12.2 mmol, 590 µL, 60 equiv.) was added, and the mixture was refluxed for 48 hr after which the mixture was coevaporated with toluene (3x) the residue was used without further purification.

Ac(Ser(tBu))$_2$-Thr(tBu)-Leu-AMC

Boc-Leu-AMC (57 µmol, 25 mg, 1.1 equiv.) was dissolved in 1:1 DCM:TFA and stirred for 30 min before being coevaporated with toluene (3x) to yield TFA·H-Leu-AMC which was used without further purification. Ac(Ser(tBu))$_2$Thr(tBu)NHNH$_2$ (27 mg, 52 µmol, 1 equiv.) was dissolved in 2 mL DMF and 2 mL EA and cooled to -30^{0}C. tBuONO (57 µmol, 6.8 µL, 1.1 equiv.) and HCl (146 µmol, 36 µL 4M/dioxane sln, 2.8 equiv.) were added and the mixture was stirred at -30^{0}C for 3h. The TFA·H-Leu-AMC in DMF was added, followed by DiPEA (260 µmol, 43 µL, 5 equiv.) and the mixture was allowed to warm to RT o/n. The mixture was diluted with EA and washed with H_2O (3x), dried with Na_2SO_4. Column chromatography (DCM → 2% MeOH:DCM) yielded the title compound (11 mg isolated, 14 µmol, 27%). ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD 1/1) δ ppm 7.76 (d, *J* = 2.02 Hz, 1H), 7.66-7.61 (m, 1H), 7.53 (d, *J* = 8.72 Hz, 1H), 6.18-6.15 (m, 1H), 4.53-4.41 (m, 2H), 4.31-4.27 (m, 2H), 3.77 (dd, *J* = 9.31, 3.66 Hz, 1H), 3.67 (dd, *J* = 9.16, 4.49 Hz, 1H), 3.56 (dd, *J* = 9.27, 5.95 Hz, 1H), 3.46 (dd, *J* = 9.13, 7.00 Hz, 1H), 2.42-2.37 (m, 3H), 1.97 (s, 3H), 1.18 (s, 9H), 1.18 (s, 9H), 1.14 (s, 9H), 0.99-0.89 (m, 6H), 1.81-1.63 (m, 3H), 1.26-1.19 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD 1/1) δ ppm 171.17, 171.07, 170.95, 170.20, 161.83, 153.62, 153.06, 141.48, 124.92, 124.85, 115.93, 115.77, 112.55, 107.10, 74.82, 74.08, 65.82, 60.92, 60.56, 59.02, 54.57, 53.22, 52.72, 40.27, 27.83, 26.86, 26.77, 24.51, 22.68, 22.11, 21.01, 18.89, 18.07.



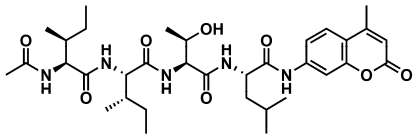
Ac-Ser$_2$-Thr-Leu-AMC

Ac(Ser(tBu))$_2$Thr(tBu)LeuAMC (11 mg, 14 µmol) was dissolved in TFA and the mixture was stirred for 2h. The mixture was coevaporated with tol (3x) and the residue used without further purification. LCMS (gradient 10% → 90% ACN/(0.1% TFA/H_2O)) 13.5 min run: Rt (min): 5.70 (ESI-MS (m/z): 606.00 (M + H+)). ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD 1/1) δ ppm 7.89 (d, *J* = 1.67 Hz, 1H), 7.70-7.64 (m, 2H), 6.25-6.21 (m, 1H), 4.61-4.50 (m, 1H), 4.49-4.40 (m, 2H), 4.36-4.25 (m, 2H), 4.01 (dd, *J* = 11.15, 4.64 Hz, 1H), 3.93-3.75 (m, 3H), 2.47 (s, 3H), 2.06 (s, 3H), 1.80-1.71 (m, 2H), 1.03-0.92 (m, 6H), 1.33-1.23 (m, 4H).

AcIle$_2$-Thr(tBu)-Leu-AMC

BocLeuAMC (48 mg, 124 µmol, 1.1 equiv.) was stirred in 1:1 DCM:TFA for 30 min before being coevaporated with toluene (3x). AcIle$_2$Thr(tBu)NHNH$_2$ (113 µmol, 52 mg) was dissolved in 12 mL DMF:EA 1:1 and cooled to -30^{0}C. tBuONO (124 µmol, 15 µl, 1.1 equiv.) and HCl (316 µmol, 79 µL 4M/dioxane sln, 2.8 equiv.) were added and the mixture was stirred at -30^{0}C for 3 hr. The TFA·H-Leu-AMC in DMF was added, followed by DiPEA (565 µmol, 93 µL, 5 equiv.) and the mixture was allowed to warm to RT o/n. The mixture was diluted with EA and

washed with H_2O (3x), dried with Na_2SO_4. Column chromatography (1% MeOH:DCM → 4% MeOH:DCM, 2x) yielded the title compound (13 mg isolated, 18 µmol, 16%). 1H NMR (400 MHz, CD_3OD, $CDCl_3$) δ ppm 8.06-7.96 (m, 1H), 7.86-7.83 (m, 1H), 7.65-7.59 (m, 2H), 6.24-6.21 (m, 1H), 4.66-4.58 (m, 1H), 4.51-4.46 (m, 1H), 4.35-4.26 (m, 2H), 4.14-4.08 (m, 1H), 2.48-2.45 (m, 3H), 2.03 (s, 3H), 1.92-1.79 (m, 2H), 1.78-1.66 (m, 3H), 1.62-1.49 (m, 2H), 1.22-1.14 (m, 2H), 1.23 (s, 9H), 1.11 (d, *J* = 1.67 Hz, 3H), 1.02-0.86 (m, 18H).



AcIle$_2$-Thr-Leu-AMC

AcIle$_2$Thr(tBu)LeuAMC (13 mg, 18 µmol) was dissolved in TFA and the mixture was stirred for 2h. The mixture was coevaporated with tol (3x) and the residue used without further purification. LCMS (gradient 10% → 90% ACN/(0.1% TFA/H_2O)) 13.5 min run: Rt (min): 8.09 (ESI-MS (m/z): 658.07 (M + H+)). ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD 1/1) δ ppm 8.06-7.99 (m, 1H), 7.88 (d, *J* = 1.32 Hz, 1H), 7.67-7.61 (m, 2H), 6.23 (s, 1H), 4.65-4.57 (m, 1H), 4.42-4.38 (m, 1H), 4.32-4.16 (m, 3H), 2.47 (s, 3H), 2.04 (s, 3H), 1.04-0.82 (m, 18H), 1.98-1.48 (m, 5H), 1.34-1.16 (m, 7H).