Chemical tools to monitor and control human proteasome activities

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CHAPTER 3

A set of activity-based probes to visualize human (immuno)proteasome activities*

Introduction

Intracellular proteolysis in eukaryotes is mediated predominantly by 26S proteasomes, which consist of a 20S proteolytic core particles (CP) and one or two 19S regulatory complexes. In vertebrates, constitutive 20S proteasome core particles (cCP) are expressed in all tissues.[1] Proteasomes are therapeutic targets for various cancers and autoimmune diseases. Proteasome core particles are C2-symmetrical complexes of four stacked rings of seven subunits each. The outer rings contain seven α-subunits and the inner rings contain seven β-subunits.[2] Catalytic activity resides in the β-rings, with β1c cleaving preferentially after acidic residues (caspase-like), β2c after basic residues (trypsin-like) and β5c after hydrophobic residues (chymotrypsin-like). Lymphoid tissues express the IFN-γ inducible immunoproteasome core particles (iCP), in which β1c, β2c and β5c are substituted for β1i (LMP2), β2i (MECL-1) and β5i (LMP7) respectively, and substrate preferences of the iCP activities differ from their cCP counterparts.[3] For instance, whereas β1c prefers acidic residues at P1, β1i cleaves preferentially after hydrophobic residues at this position. As a consequence, iCPs produce oligopeptides with more hydrophobic and basic C-termini, which can bind to MHC-I molecules.[4, 5] The assignment of proteasome catalytic activities can be achieved using synthetic, peptide-based fluorogenic substrates (see chapter 1). However, the majority of fluorogenic proteasome substrates do not distinguish between cCP and iCP activities.[6] The ELISA-based ProCISE assay is time-consuming and requires antibodies to all six subunits.[7] Fluorescent, irreversibly binding proteasome probes can be used to assay individual catalytic activities by activity-based protein profiling (ABPP), but β1c, β1i, β5c and β5i subunits cannot be resolved on SDS-PAGE when labelled with the same probe.[8-12] This chapter describes the

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development of a set of activity-based probes that allows simultaneous detection of all catalytic subunits of human cCP and iCP proteasomes in a rapid SDS-PAGE based assay (Figure 1). This cocktail is used to develop selective inhibitors for β1c, β2c, β5c and β2i, to compare active site specificity of clinical proteasome inhibitors and to demonstrate that many hematologic malignancies predominantly express immunoproteasomes. Furthermore, it is shown that selective and complete inhibition of β5i and β1i is cytotoxic to primary cells from acute lymphocytic leukemia (ALL) patients.

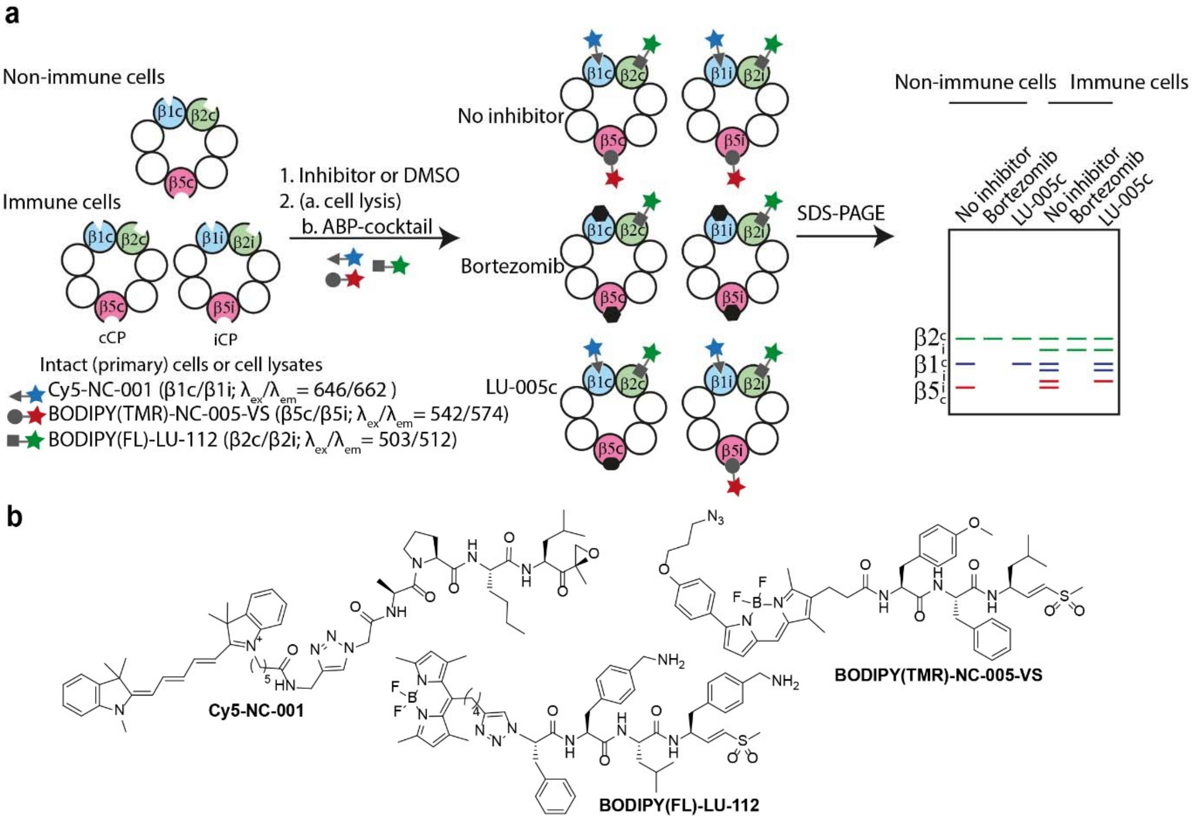
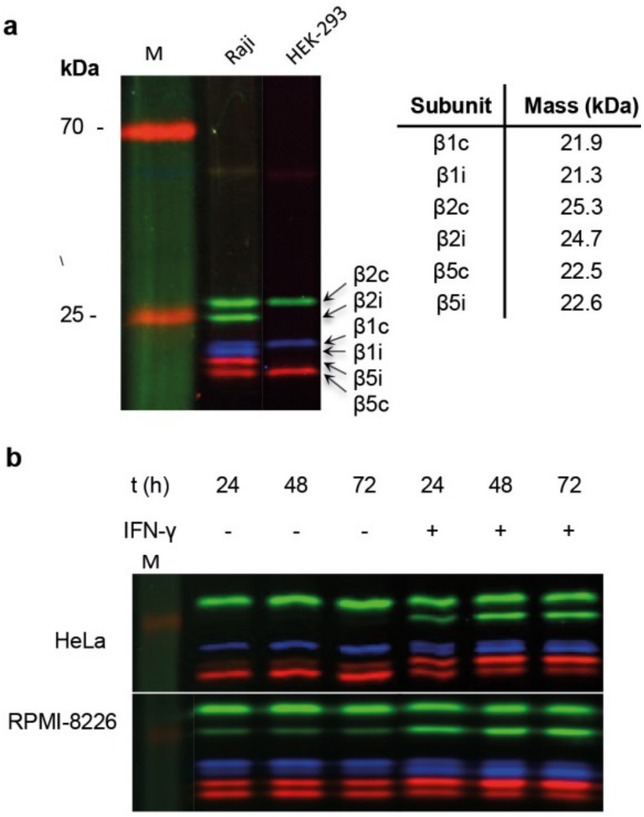


Figure 1. Activity-based probe (ABP) cocktail allows visualisation of the six human active cCP and iCP subunits on SDS-PAGE. A) Schematic representation of competitive activity-based protein profiling (ABPP) using a proteasome ABP cocktail. B) Structures of Cy5-NC-001, BODIPY(FL)-LU-112 and BODIPY(TMR)-NC-005-VS.

Results and discussion

To enable simultaneous resolution of six proteasome subunits, the previously developed site-specific inhibitors NC-001[13], LU-112[14], and NC-005-VS[15] were equipped with three different fluorophores. This approach yielded β1c and β1i-reactive Cy5-NC-001, β2c and β2i-reactive BODIPY(FL)-LU-112[14] and BODIPY(TMR)-NC-005-VS[16] which modifies the β5c and β5i sites (Figure 1B). Complete and optimal modification of the targeted sites is achieved at 100 nM Cy5-NC-001 and BODIPY(TMR)-NC-005-VS and 30 nM BODIPY(FL)-LU-112 (Figure S1, for inhibition constants see Figure S2). Treatment of HEK-293 extracts with a cocktail of the three activity based probes (ABPs) followed by SDS-PAGE and in-gel fluorescent detection yielded three clear bands corresponding to the three cCP activities expressed by HEK-293 cells (Figure 2A). Treatment of lysate from Raji cells, a human B-lymphoblastic cell line expressing both cCP and iCP, with the same ABP cocktail resulted in six well-resolved bands, with the two top bands labelled in green corresponding to β2c and β2i, the two middle bands in blue to β1c and β1i and the two bottom bands in red to β5c and β5i (Figure 2A). The specific fluorescence signal can be quantified and is directly proportional to the amount of cells/protein present per sample. The detection limit for all six subunits corresponds to 1x10^3 cells per sample (Figure S3). The time-dependent, IFN-γ-mediated induction of iCP catalytic subunits can be monitored without the need for proteasome subunit specific antibodies (Figure 2B).[17]



Subunit	Mass (kDa)
β1c	21.9
β1i	21.3
β2c	25.3
β2i	24.7
β5c	22.5
β5i	22.6

Figure 2. ABP cocktail allows visualisation of the human active cCP and iCP subunits on SDS-PAGE. A) Labelling profiles of Raji and HEK-293 lysates with the ABP probe cocktail. The table shows the molecular weight of the active subunits. B) IFN-γ treated HeLa cells (very low endogenous iCP) and RPMI-8226 (MM cell line permanently expressing both cCP and iCP) show induction of iCP upon treatment with IFN-γ.

Selective inhibitors of individual subunits are much sought after agents to study the role of these subunits in antigen presentation and as drug targets in different diseases.[18] An almost complete set of subunit selective inhibitors has been developed in the past years, which will be partially discussed in the chapters following this one. To complement the previously reported subunit specific inhibitors LU-001i (β1i, chapter 5)[19] and LU-015i (β5i, chapter 5)[19] as well as in-class broad-spectrum inhibitors NC-001 (β1c/β1i)[15], LU-102 (β2c/β2i)[14] and NC-005 (β5c/β5i)[13] the ABP cocktail was used to discover β1c, β2c, β2i, and β5c specific inhibitors (Figure 3).

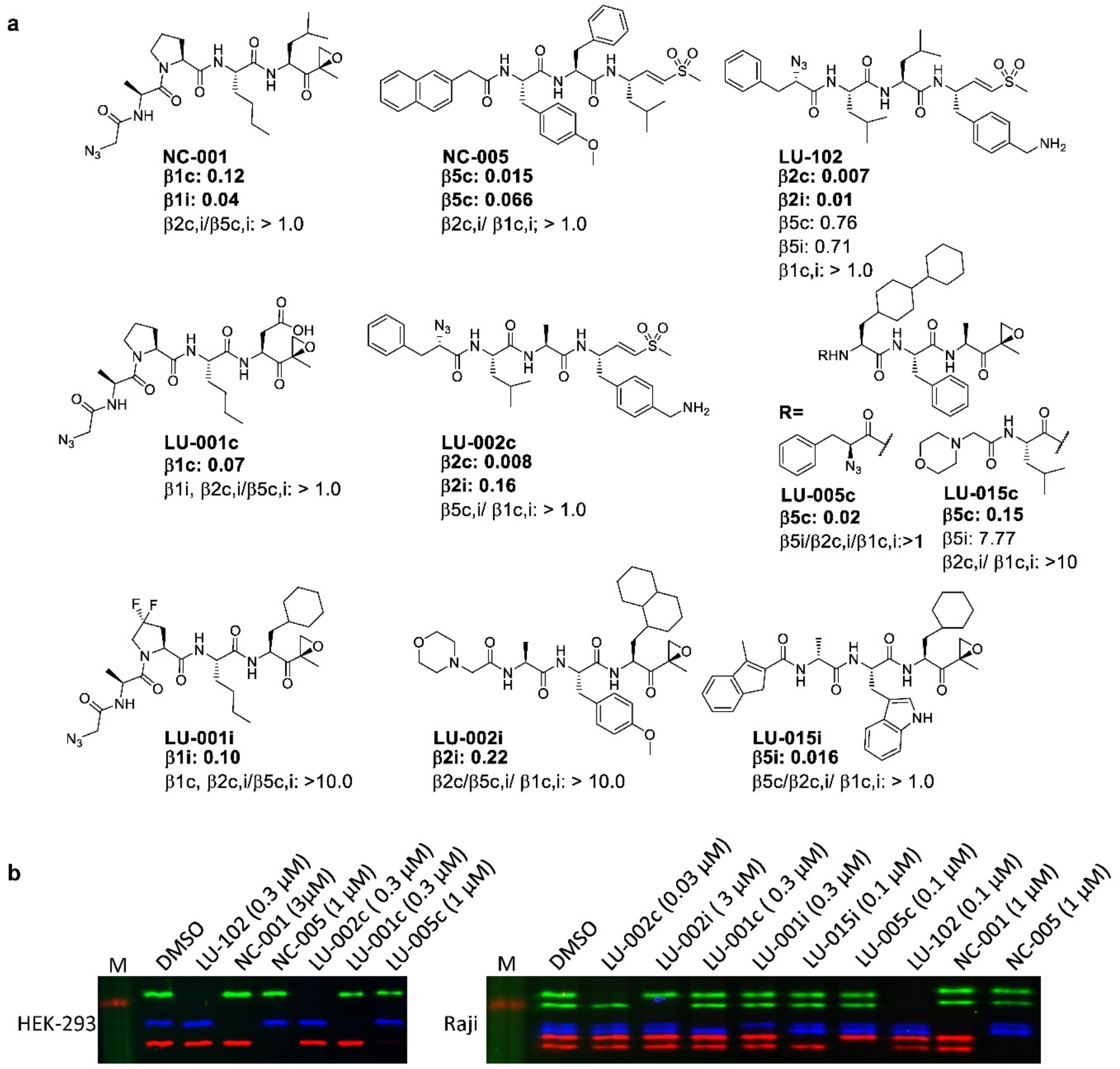


Figure 3. Proteasome subunit specific inhibitors. A) Chemical structures and IC_{50} values (μM) in Raji lysates of (newly developed) proteasome subunit specific inhibitors. B) Selective inhibition of β-subunits in HEK-293 and Raji lysates by subunit specific proteasome inhibitors assessed by competitive ABPP.

LU-001c (chapter 8) is a β1c specific inhibitor and LU-005c/LU-015c are β5c-specific (chapter 7). LU-002c is β2c-specific, and LU-002i is a specific inhibitor of β2i.[†] Subunit specificity of all inhibitors was confirmed in Raji and HEK-293 extracts, and in intact RPMI-8226 cells (Figure S4 and Table S1-2 for apparent IC_{50} values).

Clinical trials of proteasome inhibitors involve pharmacodynamic assessment of proteasome inhibition in blood, where often only combined β5c and β5i activity is measured. At the time of bortezomib development, these β5c and β5i subunits were considered the exclusive targets of antineoplastic agents.[20] Later studies however showed that co-inhibition of other catalytic sites contribute to the antineoplastic activity of bortezomib and carfilzomib.[9, 21] Therefore, it is important to measure inhibition of all six sites. To demonstrate applicability of the here described ABPP assay for this purpose, a side-by-side comparison of proteasome inhibition in RPMI-8226 cells by the clinical drugs carfilzomib and bortezomib, and the clinical candidates oprozomib[22], delanzomib[23] and ixazomib[24] was made (Figure 4A-C).[25, 26] All five compounds proved to be potent β5c and β5i inhibitors. As described in the literature bortezomib co-inhibits β1c/β1i[27], but β2c and β2i are only partially inhibited at micromolar concentrations. Carfilzomib is rather β5c/β5i-specific at lower concentrations, but blocks all 6 sites at higher concentrations. Oprozomib, an orally bioavailable analogue of carfilzomib, is more β5c/β5i-selective. Another boronic acid, delanzomib, matches the activity profile of bortezomib except that delanzomib does not inhibit β2c/β2i even at high concentrations. Ixazomib inhibits four sites, β5c, β5i, β1c and β1i with about equal potency, but at 10-fold higher concentrations than bortezomib. Comparison of recovery of proteasome activity after 1 h inhibitor treatment, followed by inhibitor washout (Figure 4D,E; Figure S5) confirmed that ixazomib has the fastest off-rate.[28] Remarkably, the residence time of the three boronates in β5c/β5i active sites is much shorter than in β1c/β1i active sites.

Information on relative expression of constitutive and immunoproteasomes in primary cells from different hematologic malignancies is limited. Therefore, expression of active immunoproteasomes subunits with their constitutive counterparts in primary cells from ALL, AML, CLL and MM patients were compared by direct quantification based on gel band intensities (Figure 5A). Expression of immunoproteasomes exceeded expression of constitutive proteasomes in all patient samples. In contrast, MM derived cell lines (RPMI-8226 and AMO) show higher expression levels of constitutive subunits (>50% β5c). Even more striking, in all primary patient samples from lymphoid malignancies (2 x B-ALL, 1 x T-ALL, 2 x CLL) over 90% of β5 and 75% of β1 proteasome activity is provided by iCPs. The β2c/β2i ratio was less pronounced, probably reflecting the presence of proteasomes consisting of both

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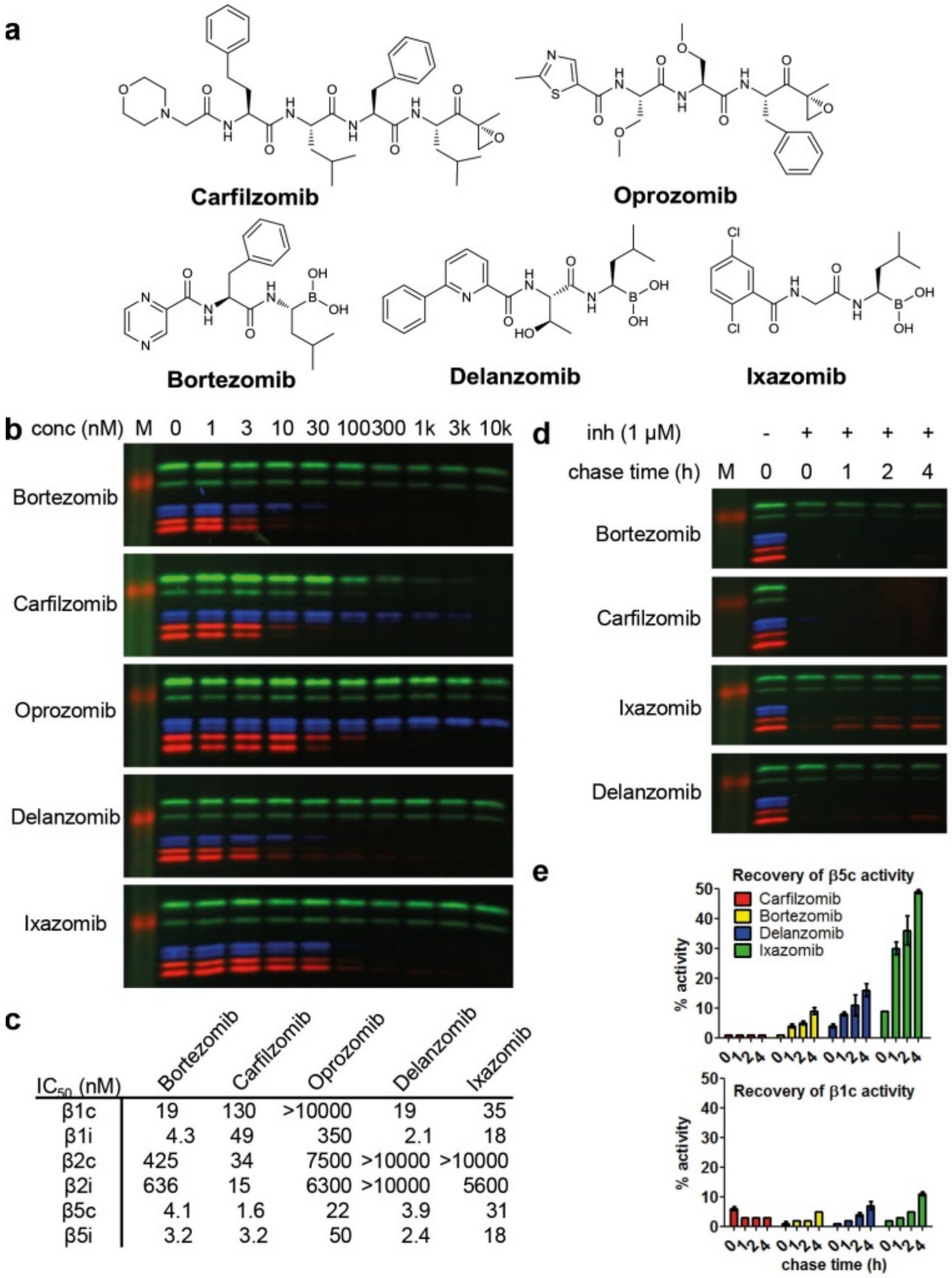
[†] Manuscript regarding β2c and β2i inhibitors in preparation by B.T. Xin *et al.*

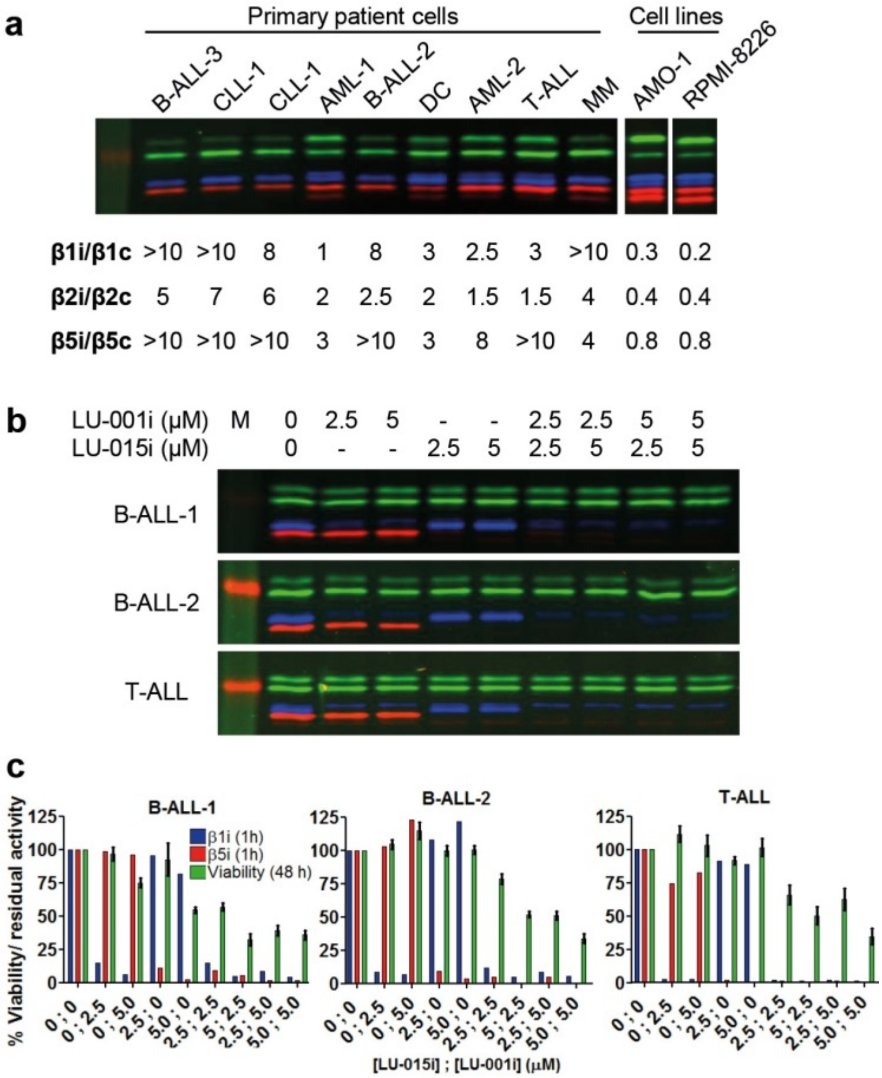
constitutive and immuno-proteasome subunits, termed hybrid or intermediate proteasomes.[29, 30] From a drug development perspective, the here presented data strongly indicate that immunoproteasomes should be regarded as a major drug target in MM and lymphoid neoplasmas such as ALL and chronic lymphocytic leukemia (CLL).



IC_{50} (nM)	Bortezomib	Carfilzomib	Oprozomib	Delanzomib	Ixazomib
β1c	19	130	>10000	19	35
β1i	4.3	49	350	2.1	18
β2c	425	34	7500	>10000	>10000
β2i	636	15	6300	>10000	5600
β5c	4.1	1.6	22	3.9	31
β5i	3.2	3.2	50	2.4	18

Figure 4. Characterization of proteasome inhibitors used clinically (bortezomib and carfilzomib) and undergoing trials (oprozomib, delanzomib, ixazomib). A) Chemical structures of inhibitors. B,C) Inhibition profiles (B) and apparent IC_{50} values (C) in living RPMI-8226 cells after 1 hour treatment. D,E) 1 h treatment with inhibitors, followed by washout and chase for 1, 2 or 4 h showed highest recovery of proteasome activity for ixazomib. Recovery of β5c/β5i is much faster than β1c/β1i (E).

In support of this the effect of selective inhibition of β5i activity in primary B- and T-ALL samples was tested. Continuous treatment with LU-015i for 48 h resulted in dose dependent cytotoxicity, which however did not fully correlate with β5i inhibition (Figure S6). Since β1i co-inhibition was observed at higher concentrations, it stands to reason that combined inhibition of β5i and β1i would lead to more efficient cell death.



	B-ALL-3	CLL-1	CLL-1	AML-1	B-ALL-2	DC	AML-2	T-ALL	MM	AMO-1	RPMI-8226
β1i/β1c	>10	>10	8	1	8	3	2.5	3	>10	0.3	0.2
β2i/β2c	5	7	6	2	2.5	2	1.5	1.5	4	0.4	0.4
β5i/β5c	>10	>10	>10	3	>10	3	8	>10	4	0.8	0.8

LU-001i (µM)	M	0	2.5	5	-	-	2.5	2.5	5	5
LU-015i (µM)		0	-	-	2.5	5	2.5	5	2.5	5

Figure 5. A) Labelling profiles of primary patient cells derived from haematological malignancies, compared to cell lines. Table: ratio between immuno- and constitutive proteasome subunits. B-ALL: B-cell acute lymphoblastic leukemia; T-ALL: T-cell acute lymphoblastic leukemia; CLL: chronic lymphocytic leukemia AML: acute myeloid leukemia; DC: acute leukemia of plasmacytoid dendritic cellsMM: multiple myeloma. B) Inhibition profiles of B- and T-ALL cells treated with indicated concentrations of LU-015i or LU-001i. C) Viability of LU-015i and/or LU-001i treated ALL cells after 1 h pulse treatment followed by 48 h chase compared to β5i and β1i inhibition after 1 h.

To test this hypothesis, patient cells were treated for 1 h with a β5i (LU-015i) and/or β1i (LU-001i) inhibitor, washed out unbound inhibitor to ensure specific inhibition (Figure 5B) and assessed cell viability after 48h. It was found that in B-ALL-2 and T-ALL cells, specific β5i or β1i inhibition did not lead to any cytotoxicity, however, combined β5i and β1i inhibition led to up to 70% cell death (Figure 5C). Also in B-ALL-1 cell combined β5i and β1i inhibition resulted in significant higher cytotoxicity than inhibition of only β5i or β1i. If these findings are confirmed in additional primary cell samples and animal models, the use of selective inhibitors of the iCP for the treatment of lymphoblastic leukemia and perhaps MM may extend the therapeutic window of proteasome inhibitors, because low iCP expression in the majority of tissues should allow administration of higher doses.

Conclusion

The new ABP probe cocktail presented here provides the first means for rapid, antibody-independent measurement of the six catalytic active cCP and iCP subunits, which can be easily adopted to patients. Furthermore the first comprehensive set of specific inhibitors of all six sites should allow dissection of the contribution of individual proteasome subunits to the generation of antigenic peptides. Finally, the panel of ten subunit specific inhibitors in concert with the activity-based probes provides a good entry point to optimize the therapeutic efficacy of β5-targeted proteasome inhibitors by selective, controlled degrees of co-inhibition of additional active sites of the iCP and/or CP that may be tailored to a given clinical application or disease state.

Supporting figures and tables

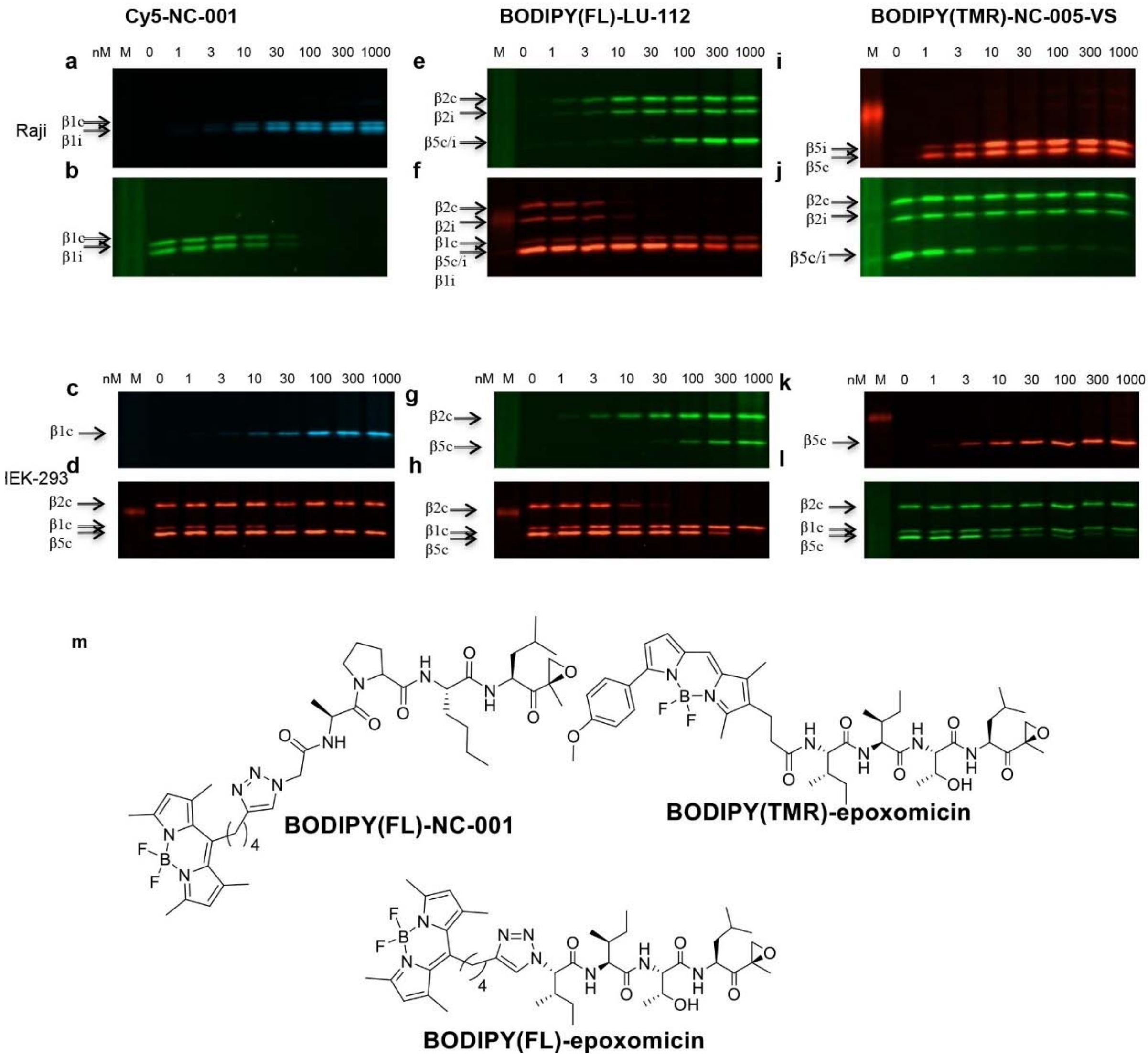
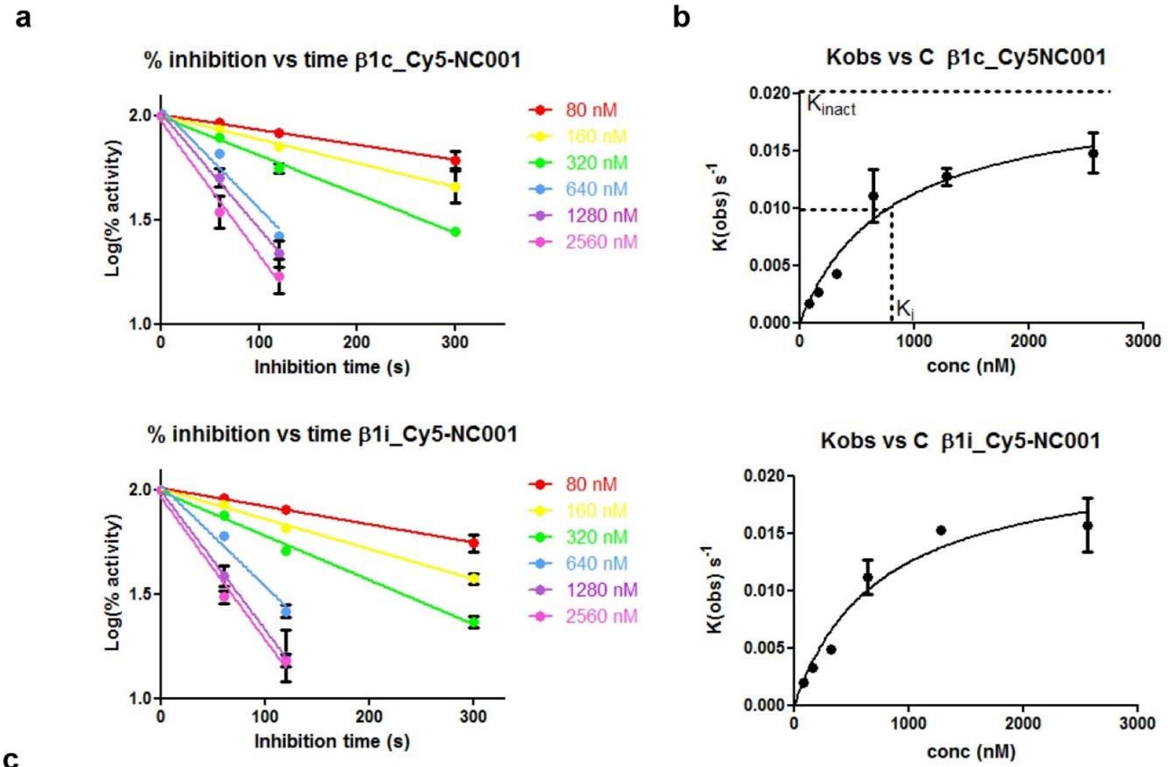


Figure S1. Determination of concentrations of Cy5-NC001 (a-d), BODIPY(FL)-LU-112 (e-h), and BODIPY(TMR)-NC-005-VS (i-l) required for full labelling. Raji/HEK cell lysates were treated with: A-D) Cy5-NC001 for 1 h (A,C), followed by inhibition of β5 by NC005 (5 µM) for 15 min. Residual β1 activity was labelled by BODIPY(FL)-NC-001 (0.5 µM) (B) or BODIPY(TMR)-epoxomicin (0.5 µM) (D); E-H) Extracts were treated with BODIPY(FL)-LU-112 for 1 h (E,H). Residual activity was labelled with BODIPY(TMR)-epoxomicin (0.5 µM) (G,H); I-L) Extracts were treated with BODIPY(TMR)-NC-005-VS for 1 h (I,K), followed by inhibition of β1 by NC-001 (5 µM) for 15 min. Residual activity was labelled by BODIPY(FL)-epoxomicin (J,L). M) Chemical structures of BODIPY(FL)-NC-001, BODIPY-FL-epoxomicin, BODIPY-TMR-epoxomicin.



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	Cy5-NC-001		BODIPY(FL)-LU-112		BODIPY(TMR)-NC-005-VS	
	β1c	β1i	β2c	β2i	β5c	β5i
$K_{(inact)}$ (s^{-1})	0.0201 ± 0.0030	0.0218 ± 0.0029	0.0271 ± 0.0026	0.0269 ± 0.0034	0.00383 ± 0.00027	0.00108 ± 0.00005
K_i (nM)	780 ± 282	745 ± 249	150 ± 29	119 ± 32	14.6 ± 4.0	20.4 ± 3.8
$K_{(inact)}/K_i$ ($M^{-1}s^{-1}$)	25.8×10^3 $\pm 9.3 \times 10^3$	29.3×10^3 $\pm 9.8 \times 10^3$	180.7×10^3 $\pm 34.9 \times 10^3$	226.1×10^3 $\pm 60.8 \times 10^3$	262.3×10^3 $\pm 71.9 \times 10^3$	52.9×10^3 $\pm 9.9 \times 10^3$
$T_{1/2}$(s)	34.5	31.8	25.5	25.8	181	641

Figure S2. Kinetic constants of Cy5-NC-001, BODIPY(FL)-LU-112, and BODIPY(TMR)-NC-005-VS in Raji lysates. A) Normalized inhibition curves for Cy5-NC001 at various concentrations, providing k_{obs} values (=slope) for each concentration, which are plotted against [probe] in (B). K_{inact}= max k_{obs}, Ki= [probe] at 0.5 K_{inact}. Similar curves were obtained for BODIPY(FL)-LU-112 and BODIPY(TMR)-NC-005-VS. C) Inhibition constants of the ABP probes.

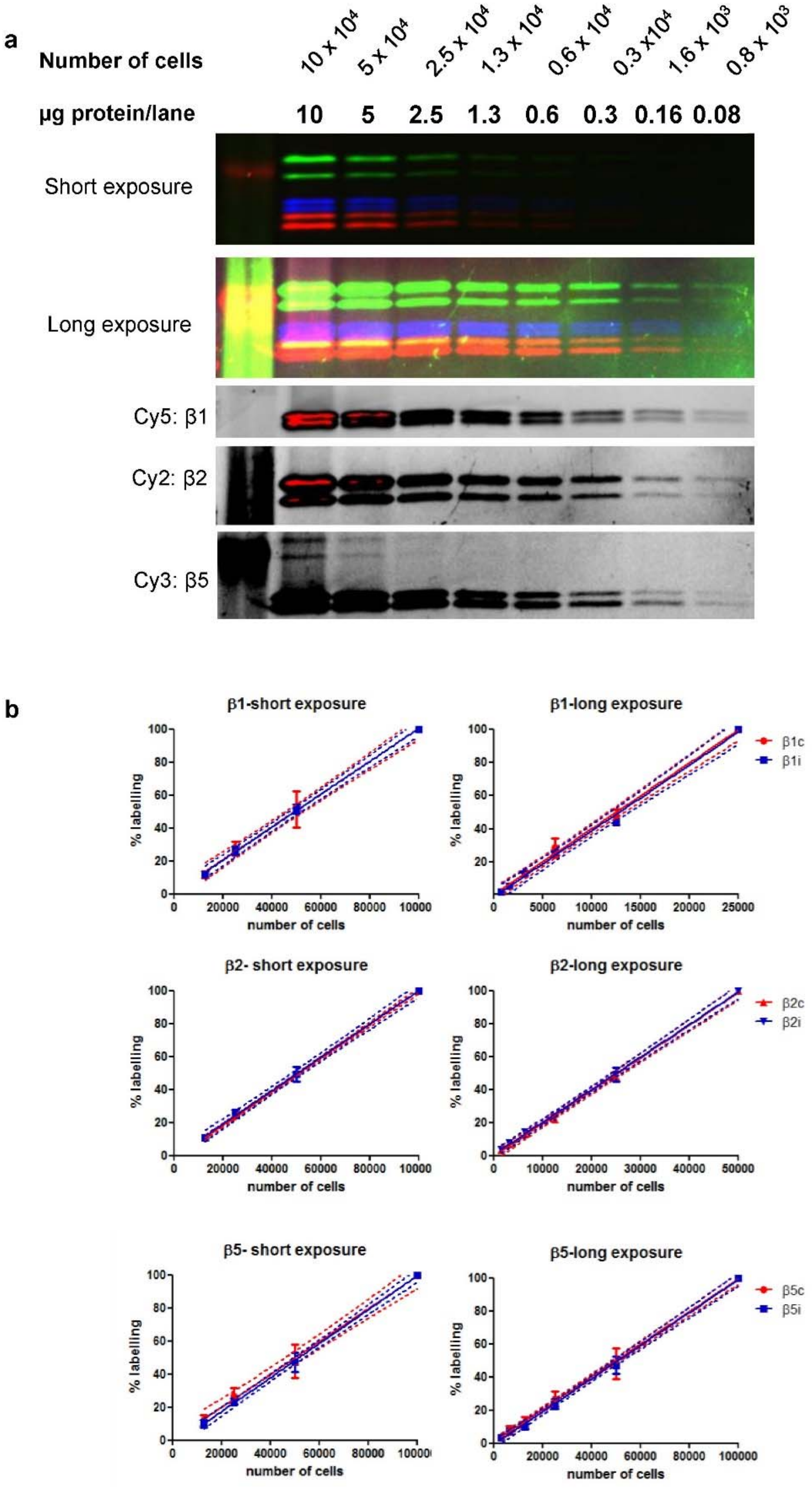


Figure S3. Determination of the linear dynamic range and limit of detection of the ABP cocktail in Raji lysates. A) Labelling profiles of decreasing protein/cell concentrations with ABP cocktail. Gels were exposed for 15/15/5 sec (short) and for 120/120/60 sec (long), respectively for Cy2, Cy3 and Cy5 channels. B) Quantification of gel band intensities shows a linear relation between signal and amount of protein present per lane for each subunit.

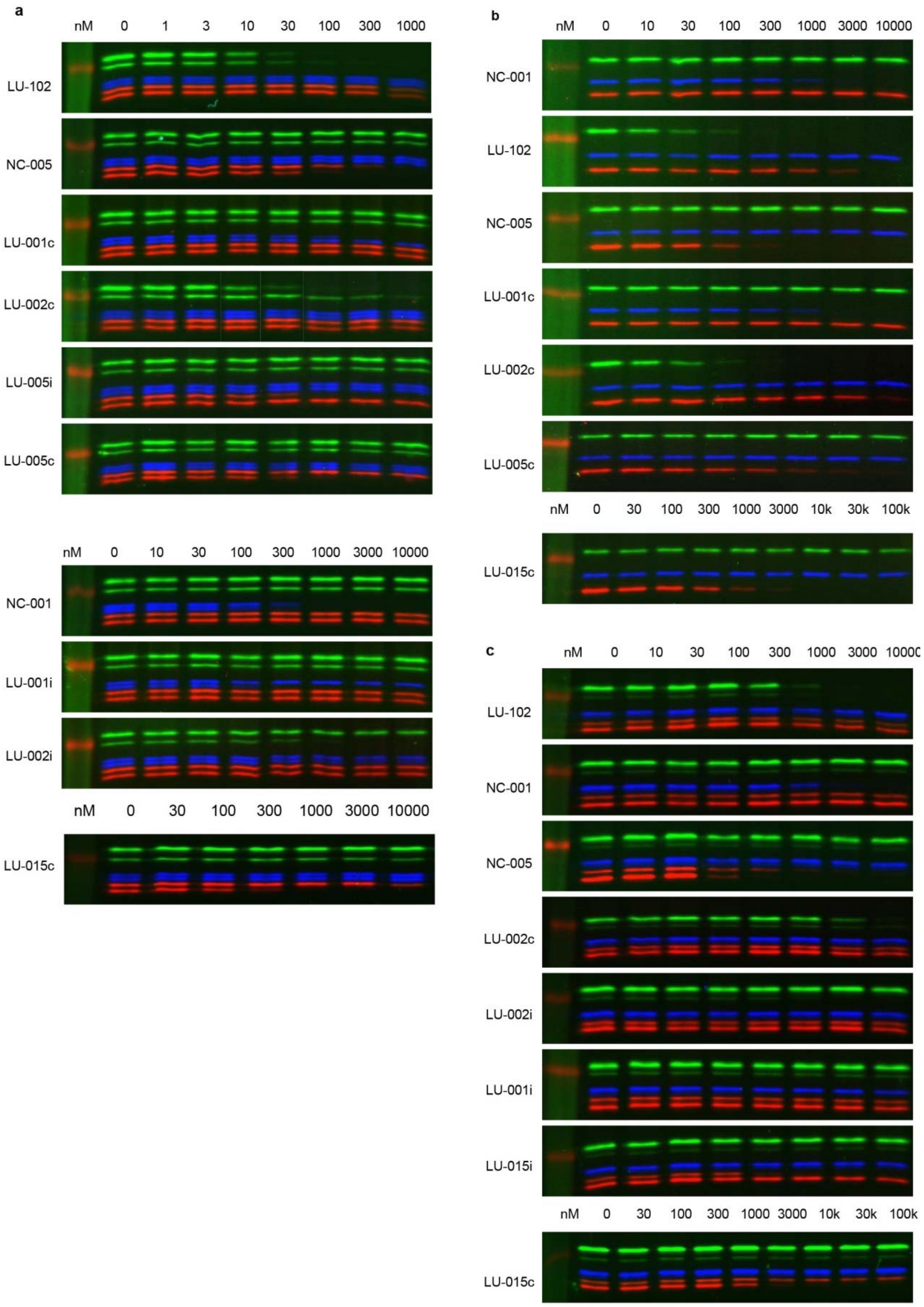


Figure S4. Inhibitory profiles of proteasome subunit selective inhibitors in cell lysates and living cells. A) Raji cell lysates. B) HEK-293 cell lysates. C) Living RPMI-8226 cells.

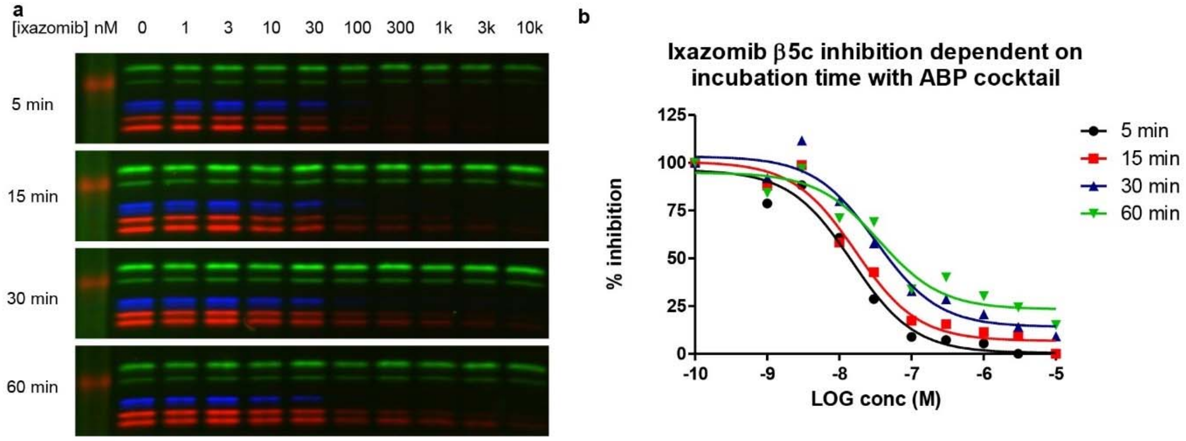


Figure S5. β5c/β5i probe BODIPY(TMR)-NC-005-VS outcompetes ixazomib in time. A) Inhibitory profiles of Raji cell lysates incubated with increasing concentrations of Ixazomib for 1h, followed by 5, 15, 30 or 60 min incubation with probe cocktail. B) Quantification of β5c inhibition.

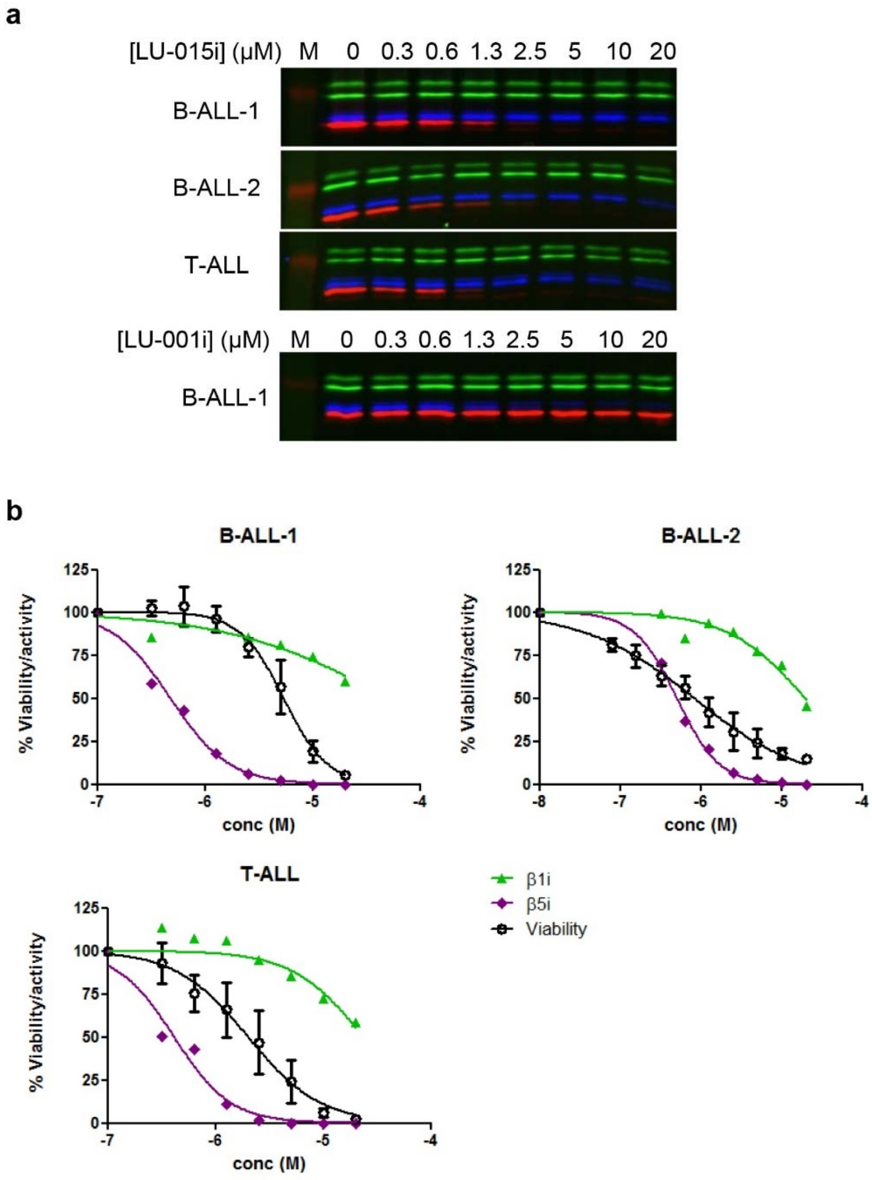


Figure S6. Treatment of ALL cells with LU-015i or LU-001i. A) Inhibition profiles of various primary ALL cells with LU-015i or LU-001i. Cells were treated LU-015i or LU-001i for 1 hour, followed by lysis and labelling with ABP cocktail. B) Viability of LU-015i treated cells after 48 h of continuous exposure compared to β5i and β1i inhibition after 1 h.

Table S1. Apparent IC_{50} (nM) of subunit specific inhibitors as determined in Raji and HEK-293 cell lysates.

	Raji						HEK-293		
Compound	**β1c**	**β1i**	**β2c**	**β2i**	**β5c**	**β5i**	**β1c**	**β2c**	**β5c**
NC-001	121	44.9	>1000	>1000	>1000	>1000	343	>10000	>10000
LU-102	>1000	>1000	6.98	10.8	766	711	>10000	19.4	837
NC-005	>1000	>1000	>1000	>1000	14.8	65.6	>10000	>10000	59.8
LU-001c	65.5	>1000	>1000	>1000	>1000	>1000	425	>10000	>10000
LU-001i	>10000	98.8	>10000	>10000	>10000	>10000	n.d.	n.d.	n.d.
LU-002c	>1000	>1000	7.90	156	>1000	>1000	>10000	13.9	2838
LU-002i	>10000	>10000	>10000	220	>10000	>10000	n.d.	n.d.	n.d.
LU-005c	>1000	>1000	>1000	>1000	22.9	>1000	>10000	>10000	204
LU-015c	>10000	>10000	>10000	>10000	148	7766	>100000	>100000	255
LU-015i	>1000	>1000	>1000	>10000	>1000	16.0	n.d.	n.d.	n.d.

Table S2. Apparent IC_{50} (nM) of subunit specific inhibitors as determined in living RPMI-8226 cells

Compound	**β1c**	**β1i**	**β2c**	**β2i**	**β5c**	**β5i**
NC-001	714	461	>10000	>10000	>10000	>10000
LU-102	>10000	>10000	294	407	>10000	>10000
NC-005	>10000	>10000	>10000	>10000	61.4	224
LU-001c	>10000	>10000	>10000	>10000	>10000	>10000
LU-001i	>10000	122	>10000	>10000	>10000	>10000
LU-002c	>10000	>10000	1801	>10000	>10000	>10000
LU-002i	>10000	>10000	>10000	>541	>10000	>10000
LU-015c	>100000	>100000	>100000	>100000	1017	>100000
LU-015i	>10000	>10000	>10000	>10000	>10000	211

Experimental

Biochemical methods

General

Lysates of cells were prepared by treating cell pellets with 4 volumes of lysis buffer containing 50 mM Tris pH 7.5, 2 mM DTT, 5 mM $MgCl_2$, 10% glycerol, 2 mM ATP, and 0.05% digitonin for 15-60 min. Protein concentration was determined using Qubit® protein assay kit (Thermo Fisher). All cell lysate labelling experiments were performed in assay buffer containing 50 mM Tris pH 7.5, 2 mM DTT, 5 mM $MgCl_2$, 10% glycerol, 2 mM ATP. Cell lysate labelling and competition experiments were performed at 37^{o}C. Prior to fractionation on 12.5% SDS-PAGE (TRIS/glycine), samples were boiled for 3 min in a reducing gel loading buffer. The 7.5x10 cm (L x W) gels were run for 15 min at 80V followed by 120 min at 130V. In-gel detection of (residual) proteasome activity was performed in the wet gel slabs directly on a ChemiDoc™ MP System using Cy2 setting to detect BODIPY(FL)-LU-112, BODIPY(FL)-epoxomicin and BODIPY(FL)-NC-001, Cy3 settings to detect BODIPY(TMR)-NC-005-VS and BODIPY(TMR)-epoxomicin and Cy5 settings to detect Cy5-NC-001. When the probes were used as a mixture the following concentrations were used: 100 nM Cy5-NC-001, 30 nM BODIPY(FL)-LU-112, 100 nM BODIPY(TMR)-NC-005-VS, as premixed 10x concentrated cocktail in DMSO which was incubated with cell lysate for 60 min, unless stated otherwise.

Determination of probe concentration required for full subunit labelling

Cy5-NC001: Raji or HEK-293 cell lysates (10 µg/ 9µL) were treated with increased concentration of Cy5-NC-001 (1 µL from 10x stock in DMSO) for 1 h. Next, for Raji lysates residual β5 activity was blocked (BODIPY(FL)-NC-001 may partially label β5) by NC-005 (5 µM) for 15 min at 37^{o}C followed by labelling of residual β1 activity with BODIPY(FL)-NC001 (0.5 µM) for 1 h. For HEK-293 lysates: residual activity was labelled with BODIPY(FL)-epoxomicin (0.5 µM) for 1 h.

BODIPY(FL)-LU-112: Raji or HEK-293 cell lysates (10 µg/ 9µL) were treated with increased concentration of BODIPY(FL)-LU-112 (1 µL from 10x stock in DMSO) for 1 h. Next, residual proteasome activity was labelled by BODIPY(TMR)-epoxomicin (0.5 µM) for 1 h.

BODIPY(TMR)-NC-005-VS: Raji or HEK-293 cell lysates (10 µg/ 9µL) were treated with increased concentration of BODIPY(TMR)-NC-005-VS (1 µL from 10x stock in DMSO) for 1 h, followed by inhibition of β1 by NC-001 (5 µM) for 15 min. Next, residual proteasome activity was labelled by BODIPY(FL)-epoxomicin (0.5 µM) for 1 h.

Labelling of all six subunits was performed by a mixture of 100 nM Cy5-NC001, 30 nM BODIPY(FL)-LU-112, 100 nM BODIPY(TMR)-NC-005-VS, which were used premixed as 10x concentrated cocktail in DMSO. Chemical structures of BODIPY(FL)-NC-001, BODIPY(FL)-epoxomicin, BODIPY(TMR)-epoxomicin are shown in Figure S1.

Determination of the linear dynamic range and limit of detection of ABP cocktail in Raji lysates.

1 million Raji cells were lysed according to the general method for cell lysis and diluted to the appropriate concentrations followed by labelling with probes according to the general methods, followed by SDS-PAGE. Gels were exposed for 15/15/5 sec (short), and for 120/120/60 sec (long), respectively for the Cy2, Cy3 and Cy5 channels.

Competition experiments in cell lysate

Cell lysates (diluted to 10-15 µg total protein in 9 µL buffer) were exposed to the inhibitors (10x stock in DMSO) at indicated concentrations for 1 h at 37 °C, followed by addition of probe cocktail (10x stock, 1.1 µL) and SDS-PAGE as described in general methods.

Competition experiments in living RPMI-8226 cells

RPMI-8226 were cultured in RPMI-1640 media supplemented with 10% fetal calf serum, GlutaMAX™, penicillin, streptomycin in a 5% CO_2 humidified incubator. 5-8 × 10^5 cells/mL were exposed to inhibitors for 1 h at 37 °C. Cells were harvested and washed twice with PBS. Cell pellets were treated with lysis buffer on ice for 15 min, followed by centrifugation at 14000 rpm for 5 min. Proteasome inhibition in the obtained cell lysates was determined using the method described above, with 15 min (in case of bortezomib, MLN2238 and delanzomib, because longer incubation times cause out-competition of boronates, see Figure S4.) or 60 min (all other inhibitors) incubation with ABP cocktail. Intensities of bands were measured by fluorescent densitometry and divided by the intensity of bands in mock-treated extracts. Average values of three independent experiments were plotted against inhibitor concentrations. IC_{50} (inhibitor concentrations giving 50% inhibition) values were calculated using GraphPad Prism software.

Determination of kinetic constants

Raji cell lysates (10 µg/ 9 µL) were incubated with increasing concentrations of probe for 0, 1, 2 or 5 min. Concentrations used for Cy5-NC001: 80, 160, 320, 640, 1280, 2560 nM; BODIPY(FL)-LU-112 and BODIPY(TMR)-NC-005-VS: 8, 16, 32, 64, 128, 256 nM. The reaction was stopped by snap-freezing in liquid nitrogen and while still frozen, the denaturing sample buffer is added. Next, SDS-PAGE analysis is performed as described in the general methods. Intensities of bands were measured by fluorescent densitometry and normalized to full labelling by either 100 nM Cy5-NC-001, 30 nM BODIPY(FL)-LU-112 or 100 nM BODIPY(TMR)-NC-005-VS for 1 h. When the Log (% activity) is plotted versus time, a straight line is observed, from which the first order rate constants (K_{obs}) can be derived for each concentration. K_{obs} were plotted versus probe concentration, from which inhibition constants K_I and K_{inact} were calculated using Graphpad Prism software.

Inhibitor washout experiments

5x 10^5 RPMI-8226 cells were treated with 1 µM of inhibitor (1% DMSO end concentration) at 37°C. After 1 h, the cells were washed with medium (2x) and incubated at 37°C for 0, 1, 2 or 4 hours. The cells were harvested and washed with PBS, lysed in standard lysis buffer for 15 min, followed by centrifugation at 14000 rpm for 5 min. Proteasome inhibition in the obtained cell lysates was determined using the method described above (5 min incubation with probe mixture).

Proteasome labelling in primary patient cells

Primary MM, T-ALL, AML, CLL and DC patient cells were thawed and put in high glucose DMEM medium for 4 hours. Cells were harvested and washed twice with PBS. Cell pellets were treated with lysis buffer on ice for 15 min, followed by centrifugation at 14000 rpm for 10 min. Proteasome inhibition in the obtained cell lysates was determined using the method described above.

Primary ALL cell viability assay

B- and T-ALL primary patient cells (either continuously treated for 48 h with LU-015i at indicated concentrations or pulse treated for 1 h with LU-015i and/or LU-001i at indicated concentrations and washed with medium (2x), followed by a 48 h chase), were assayed for viability by CellTiter Glo-assay (Promega) according to manufacturer's instructions. Luminescence was determined in 96 well microplates in GloMax luminometer (Promega). In parallel, B- and T-ALL cells were treated with indicated concentrations of LU-015i or LU-001i for 1 h and washed with PBS (2x). Cell pellets were treated with lysis buffer on ice for 60 min, followed by centrifugation at 14000 rpm for 10 min. Proteasome inhibition in the obtained cell lysates was determined using the method described above.

Synthetic procedures

General

Acetonitrile (ACN), dichloromethane (DCM), N,N-dimethylformamide (DMF), methanol (MeOH), diisopropylethylamine (DiPEA) and trifluoroacetic acid (TFA) were of peptide synthesis grade, purchased at Biosolve, and used as received. All general chemicals (Fluka, Acros, Merck, Aldrich, Sigma, Iris Biotech) were used as received. Traces of water were removed from reagents used in reactions that require anhydrous conditions by co-evaporation with toluene. Diethylether was stored over 4 Å molecular sieves. Column chromatography was performed on Screening Devices b.v. Silica Gel, with a particle size of 40-63 µm and pore diameter of 60 Å. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F254). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of $(NH_4)_6Mo_7O_{24}\cdot4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4\cdot2H_2O$ (10 g/L) in 10% sulphuric acid, a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 mL/L) in ethanol, where appropriate, followed by charring at ca. 150°C. ^1H and ^{13}C-NMR spectra were recorded on a Bruker AV-400 (400 MHz) or AV-600 (600 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane, CD_3OD or $CDCl_3$ as internal standard. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile 50/50 (v/v) and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60,000 at m/z 400 (mass range m/z = 150-2,000) and dioctylphthalate (m/z = 391.28428) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). LC-MS analysis was performed on a Finnigan Surveyor HPLC system with a Gemini C18 50 × 4.60 mm column (detection at 200−600 nm) coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI. The applied buffers were H_2O, MeCN and 1.0% TFA in H_2O (0.1% TFA end concentration). Methods used are: 10→90% MeCN, 13.5 min (0→0.5 min: 10% MeCN; 0.5→8.5 min: gradient time; 8.5→10.5 min: 90% MeCN; 10.5→13.5 min: 90% → 10% MeCN), 0→50% MeCN, 13.5 min (0→0.5 min: 10% MeCN; 0.5→8.5 min: gradient time; 8.5→10.5 min: 90% MeCN; 10.5→13.5 min: 90% → 10% MeCN). HPLC purification was performed on a Gilson HPLC system coupled to a Phenomenex Gemini 5µm 250×10 mm column. All tested compounds are >95% pure on the basis of LC-MS and NMR.

General procedure for azide couplings.

Compounds **5**, **10**, **18**, **25** and **28** were prepared via azide coupling of properly protected peptide hydrazide and properly deprotected epoxyketone amines (Boc-AA-EK were treated with TFA for 30 min, followed by co-evaporation with toluene (2x)). The appropriate hydrazide was dissolved in 1:1 DMF (v/v) and cooled to -30 °C. tBuONO (1.1 equiv.) and HCl (4M solution in 1,4-dioxane, 2.8 equiv.) were added, and the mixture was stirred for 3 h at -30 ˚C after which TLC analysis (10% MeOH/DCM, v/v) showed complete consumption of the starting material. The epoxyketone as free amine was added to the reaction mixture as a solution in DMF. DiPEA (5 equiv.) was added to the reaction mixture, and this mixture was allowed to warm to RT slowly overnight. The mixture was diluted with EtOAc or DCM and extracted with H_2O (3×). The organic layer was dried over $MgSO_4$ concentrated and purified by flash column chromatography (1-5% MeOH in DCM) and HPLC-purification (if necessary).

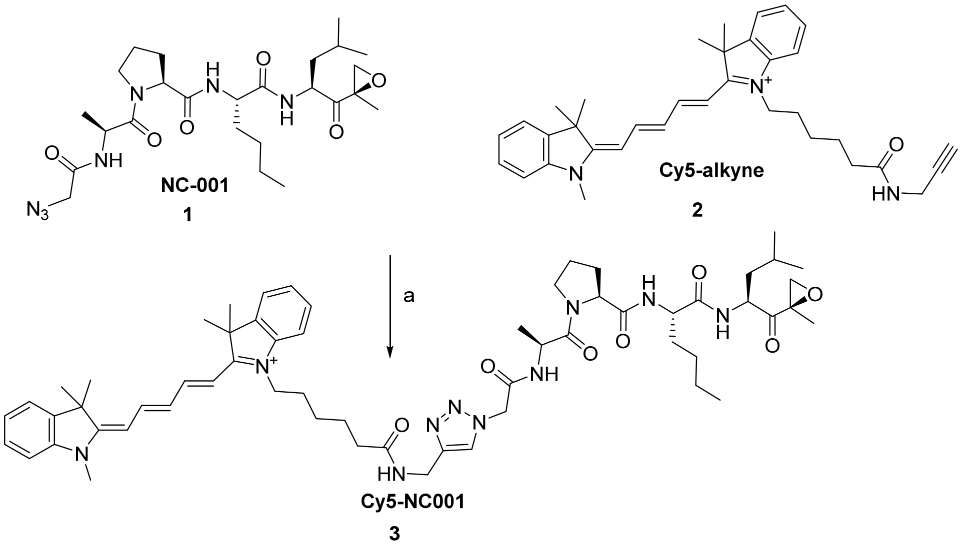
General procedure for Boc removal

Boc protected compounds were treated with TFA (0.1 M) for 30 minutes, followed by co-evaporation with toluene (2x).

General procedure for peptide couplings

Free acid (1.2 equiv.), HCTU (1.2 equiv.) and free amine (1 equiv.) are dissolved in DCM (0.1 M), followed by the addition of DiPEA (3.5 equiv or 4.5 equiv in case of 2-morpholinoacetic acid HCl). After stirring overnight (or alternatively 1-3 hours, until completion), the reaction mixture is concentrated and re-dissolved in EtOAc, washed with 1 N HCl (2x), sat. $NaHCO_3$ (2x) and brine (in case of morpholino acetic acid coupling, no 1N HCl washings). The organic layer is dried over Na_2SO_4, filtered and concentrated, followed by purification by column chromatography.

Synthesis of Cy5-NC-001

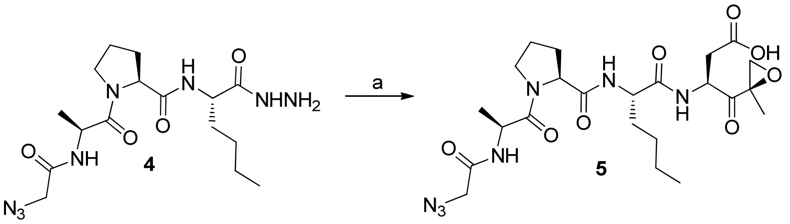


Scheme 1. Synthesis of Cy5-NC001. Reagents and conditions: a. $CuSO_4$, NaAsc, DMF, H_2O, 54%

Cy5-NC001 (3)

To a degassed solution of NC-001[13] **1** (7.0 mg, 13 µmol) and Cy5-alkyne **2** (6.0 mg, 12 µmol) in DMF (0.8 mL) under an argon atmosphere was added $CuSO_4 \cdot 5H_2O$ (0.5 equiv, 6.5 µmol (100 µL from degassed stock solution of 65 µmol/mL)) and NaAsc (0.75 equiv. 9.8 µmol (100 µL from degassed stock solution of 98 µmol/mL)). After stirring overnight, water and EtOAc were added and the layers were separated. The aqueous layer was extracted with DCM (5x). The combined organic layers were evaporated and purified by column chromatography (0-3-5-10-20% MeOH in DCM) providing the product after lyophilisation as a blue powder (7.5 mg, 7 µmol, 54%). ^1H NMR (850 MHz, $CDCl_3$) δ 7.94 (s, 2H), 7.91 – 7.82 (m, 2H), 7.56 (d, *J* = 39.1 Hz, 2H), 7.40 – 7.36 (m, 2H), 7.37 – 7.33 (m, 2H), 7.23 (dd, *J* = 7.5, 3.7 Hz, 2H), 7.15 – 7.12 (m, 1H), 7.11 (d, *J* = 7.9 Hz, 1H), 6.82 (d, *J* = 12.8 Hz, 2H), 6.43 (d, *J* = 13.5 Hz, 1H), 6.31 (d, *J* = 13.3 Hz, 1H), 5.23 (d, *J* = 14.9 Hz, 1H), 5.14 (d, *J* = 15.8 Hz, 1H), 4.66 – 4.45 (m, 4H), 4.24 (d, *J* = 46.0 Hz, 1H), 4.07 (d, *J* = 7.4 Hz, 2H), 3.65 (d, *J* = 23.0 Hz, 3H), 3.32 (d, *J* = 4.6 Hz, 1H), 2.84 (d, *J* = 5.1 Hz, 1H), 2.42 – 2.29 (m, 2H), 2.05 (d, *J* = 19.8 Hz, 3H), 1.94 (s, 3H), 1.87 – 1.74 (m, 4H), 1.71 (s, 7H), 1.69 – 1.50 (m, 5H), 1.47 (d, *J* = 16.3 Hz, 3H), 1.45 – 1.39 (m, 3H), 1.39 – 1.16 (m, 12H), 0.98 – 0.79 (m, 9H). ^{13}C NMR (214 MHz, $CDCl_3$) δ 208.49, 173.50, 173.11, 172.95, 172.22, 172.08, 171.57, 165.52, 153.30, 152.79, 145.65, 142.90, 142.04, 141.17, 140.86, 129.00, 128.87, 126.42, 125.52, 125.21, 124.44, 122.30, 122.21, 111.09, 110.56, 104.44, 103.99, 60.59, 59.24, 53.83, 52.72, 52.63, 50.38, 49.48, 49.20, 48.61, 47.72, 44.59, 39.90, 36.06, 35.33, 32.07, 31.87, 29.85, 29.52, 28.28, 28.24, 27.96, 27.16, 26.46, 25.44, 25.21, 25.15, 23.55, 22.85, 22.39, 21.41, 17.34, 16.95, 14.29, 14.13. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 7.45 (ESI-MS (m/z): 1055.53 (M^+)). HRMS: calculated for $C_{60}H_{83}N_{10}O_7$ 1055.64407 $[M]^+$; found 1055.64425

Synthesis of LU-001c

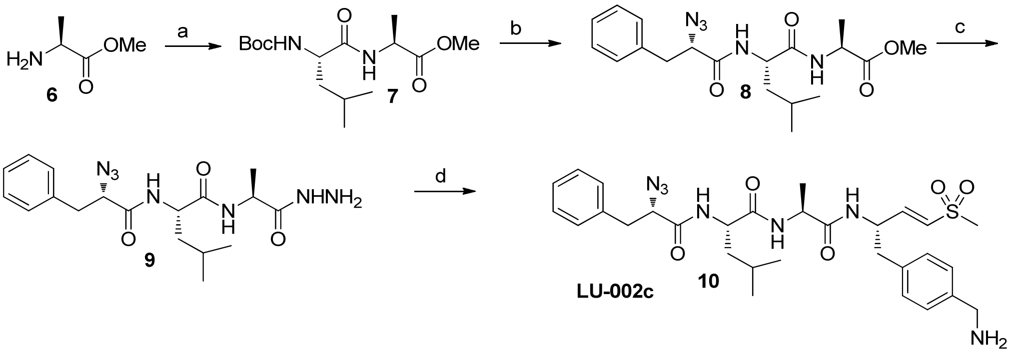


Scheme 2. Synthesis of LU-001c. Reagents and conditions: a. 1. i. tBuONO, HCl, DMF, -35°C. ii DiPEA, H_2N-Asp(OtBu)-EK, -35°C-RT. 2. TFA, 1.9 %.

N_3Ac-Ala-Pro-Nle-Asp-EK (LU-001c ,5)

Compound **4** was synthesized as reported before.[13] This compound was obtained by the general protocol for azide coupling on a 144 µmol scale. Purification by column chromatography (0→3% MeOH in DCM), provided the tert-butyl protected product, which was deprotected by treatment with dry TFA (1 mL) for 10 min, followed by the addition of toluene and concentration. Purification by HPLC (C_{18}, 10-30% MeCN, 0.1% TFA, 15 min gradient), followed by lyophilization provided the product (1.46 mg, 1.9%). Complex NMR due to presence of rotamers, peaks of major rotamer are reported. ^{1}H NMR (600 MHz, MeOD) δ 4.80 – 4.74 (m, 1H), 4.63 (q, *J* = 7.0 Hz, 1H), 4.46 (dd, *J* = 8.4, 4.5 Hz, 1H), 4.29 – 4.23 (m, 1H), 3.92 – 3.85 (m, 2H), 3.80 (dt, *J* = 9.8, 6.8 Hz, 1H), 3.65 (dt, *J* = 10.1, 6.7 Hz, 1H), 3.23 (d, *J* = 4.7 Hz, 1H), 2.92 (dd, *J* = 12.3, 4.9 Hz, 1H), 2.79 (dd, *J* = 16.5, 4.2 Hz, 1H), 2.70 (dd, *J* = 16.3, 7.6 Hz, 1H), 2.21 (tdd, *J* = 15.0, 9.7, 5.6 Hz, 1H), 2.15 – 2.07 (m, 1H), 2.07 – 1.97 (m, 2H), 1.80 (ddt, *J* = 15.2, 10.8, 5.2 Hz, 1H), 1.71 – 1.57 (m, 1H), 1.40 – 1.30 (m, 7H), 1.22 (s, 3H), 0.97 – 0.88 (m, 3H). ^{13}C NMR (151 MHz, MeOD) δ 207.14, 174.28, 174.11, 173.74, 173.24, 169.87, 61.51, 54.67, 53.25, 52.47, 50.51, 49.57, 48.63, 36.13, 32.77, 30.48, 28.93, 26.04, 23.45, 16.82, 14.27. LC-MS (linear gradient 0 → 50% MeCN, 0.1% TFA, 13.5 min): R_t (min): 7.70 (ESI-MS (m/z): 538.07 $(M+H)^+$). HRMS: calculated for $C_{23}H_{35}N_7O_8$ 538.26199 $[M+H]^+$; found 538.26202

Synthesis of LU-002c



Scheme 3. Synthesis of LU-002c. Reagents and conditions: a. HCTU, Boc-Leu-OH, DiPEA, DCM, 78%. b. 1. TFA. 2. HCTU, N_3Phe-OH, DiPEA, DCM, 44%. c. Hydrazine hydrate, MeOH, quant. d. 1. i. tBuONO, HCl, DMF, -35°C. ii DiPEA, H_2N- Phe(4-CH_2NHBoc)-VS, -35°C-RT. 2. TFA, 25%

Boc-Leu-Ala-OMe (7)

The title compound was prepared by the general procedure for peptide coupling on a 0.5 mmol scale. Column chromatography (10-30% EtOAc/pentane) provided the product (124 mg, 78%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.07 (d, *J* = 7.3 Hz, 1H), 5.22 (d, *J* = 8.4 Hz, 1H), 4.58-4.51 (m, 1H), 4.24-4.18 (m, 1H), 3.74 (s, 3H), 1.82-1.31 (m, 15H), 0.95-0.88 (m, 6H).^{13}C NMR (101 MHz, $CDCl_3$) δ 173.25, 172.55, 155.82, 79.93, 52.91, 52.42, 47.96, 41.43, 28.36, 24.68, 23.01, 21.99, 18.03.

N_3Phe-Leu-Ala-OMe (8)

Boc-Leu-Ala-OMe (124 mg, 0.39 mmol) was deprotected using the standard procedure for Boc removal, followed by peptide coupling with N_3Phe-OH using the standard procedure for peptide couplings. Column chromatography (10-30% EtOAc/pentane), provided the product (67 mg, 44%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38-7.19 (m, 5H), 6.78 (d, *J*= 7.6 Hz, 2H), 4.59-4.42 (m, 2H), 4.30-4.23 (m, 1H), 3.75 (s, 3H), 3.34-3.29 (m, 1H), 3.09-3.03 (m, 1H), 1.63-1.33 (m, 6H), 0.91-0.86 (m, 6H).^{13}C NMR (101 MHz, $CDCl_3$) δ 173.20, 171.19, 168.59, 135.94, 129.62, 128.73, 127.35, 65.30, 52.62, 51.56, 48.16, 41.28, 38.38, 24.53, 22.95, 22.15, 18.26.

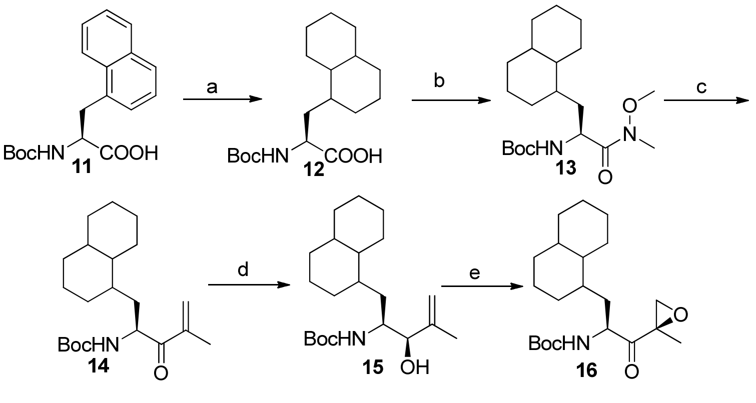
N_3Phe-Leu-Ala-$NHNH_2$ (9)

N_3Phe-Leu-Ala-OMe (67 mg, 0.17 mmol) was dissolved in MeOH (5 mL), followed by the addition of $NH_2NH_2 \cdot H_2O$ (0.25 mL, 5.1 mmol, 30 equiv.). The reaction mixture was stirred overnight and refluxed for 2 h, concentrated and co-evaporated with toluene (2x) thereby providing the product in a quantitative yield. ^{1}H NMR (400 MHz, MeOD/$CDCl_3$) δ 7.33-7.14 (m, 5H), 4.40-4.24 (m, 2H), 4.18-4.13 (m, 1H), 3.23-3.18 (m, 1H), 3.02-2.96 (m, 1H), 1.54-1.41 (m, 3H), 1.35-1.33 (m, 3H), 0.88-0.83 (m, 6H).^{13}C NMR (101 MHz, MeOD/$CDCl_3$) δ 194.90, 173.14, 170.69, 136.81, 131.80, 129.93, 129.11, 127.64, 65.09, 52.40, 41.29, 38.37, 25.11, 23.26, 21.79, 17.99.

N_3Phe-Leu-Ala-Phe(4-CH_2NH_2)-VS TFA salt (LU-002c, 10)

Compound **10** was prepared by the general procedure for azide peptide couplings on a 50 µmol scale (Cbz-Phe(4-CH_2NHBoc)-VS synthesized and deprotected as described before[14]). After workup, the crude product was treated with TFA for 30 min, co-evaporation with toluene, subsequent purification by preparative HPLC, followed by lyophilisation, provided the title compound (7.8 mg, 25%). ^{1}H NMR (600 MHz, MeOD) δ 7.42 – 7.33 (m, 4H), 7.33 – 7.21 (m, 5H), 6.80 (dd, *J* = 15.2, 5.1 Hz, 1H), 6.59 (dd, *J* = 15.2, 1.6 Hz, 1H), 4.85 – 4.79 (m, 1H), 4.34 (dd, *J* = 9.9, 4.7 Hz, 1H), 4.27 – 4.13 (m, 2H), 4.09 (s, 2H), 3.21 (dd, *J* = 14.1, 5.0 Hz, 1H), 3.08 – 2.96 (m, 3H), 2.94 (s, 3H), 1.57 (td, *J* = 6.5, 5.5, 2.5 Hz, 3H), 1.32 (d, *J* = 7.2 Hz, 3H), 0.93 (dd, *J* = 19.8, 6.0 Hz, 6H). ^{13}C NMR (150 MHz, MeOD) δ 174.43, 174.21, 171.97, 146.60, 139.64, 137.81, 133.00, 131.81, 131.29, 130.43, 130.21, 129.63, 128.10, 65.43, 53.54, 52.40, 50.78, 44.07, 42.75, 41.48, 40.30, 38.66, 25.80, 23.47, 21.88, 18.04. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 15.0 min):R_t (min): 6.24 (ESI-MS (m/z): 612.13 ($M+H^+$)). HRMS calculated for $C_{30}H_{41}N_7O_5S$ 612.29626 $[M+H]^+$; found 612.29624.

Synthesis of LU-002i



Scheme S4. Synthesis of Boc-DecAla-EK. Reagents and conditions: a. Rh/Al, H_2, MeOH, quant. b. N,O-Dimethylhydroxylamine, HCTU, DiPEA, DCM, 60%. c. 2-bromopropene, tBuLi, Et_2O, -78°C, 67%. d. $CeCl_3 \cdot 7H_2O$, $NaBH_4$, MeOH, 0°C, quant. e. 1. Vo(acac)$_2$, tBuOOH, DCM, 0°C. 2. Dess-Martin periodane, DCM, 0°C, 19%.

(2S)-2-((tert-butoxycarbonyl)amino)-3-(decahydronaphthalen-1-yl)propanoic acid (12)

Boc-1-Nal-OH **11** (1.0 g, 3.2 mmol) was dissolved in MeOH (30 mL), followed by the addition of Rh on alumina (5 wt. %, 300 mg). The mixture was placed under H_2 (4 bar, Parr apparatus) for 48h. Subsequent filtration and concentration provided the product in a quantitative yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.12 (s, 1H), 6.67-6.20 (m, 0.5H), 5.11-4.86 (m, 0.5H), 4.36-4.13 (m, 1H), 2.11-0.59 (m, 28H).^{13}C NMR (101 MHz, $CDCl_3$) δ 179.04, 178.68, 178.14, 156.94, 155.91, 155.78, 81.67, 80.13, 52.75, 48.12, 47.61, 43.09, 38.61, 37.86, 37.50, 37.40, 36.95, 36.46, 36.33, 36.22, 35.94, 34.93, 34.70, 34.57, 34.37, 33.35, 32.65, 31.93, 30.80, 30.25, 29.26, 28.40, 27.99, 27.69, 27.01, 26.90, 26.85, 26.76, 26.64, 26.52, 26.48, 26.20, 26.07, 25.95, 25.50, 21.56, 21.40, 21.37, 20.72, 20.39, 20.16, 19.67. HRMS calculated for $C_{18}H_{31}NO_4$326.23258[M+H]$^+$; found 326.23265.

tert-butyl ((2S)-3-(decahydronaphthalen-1-yl)-1-(methoxy(methyl)amino)-1-oxopropan-2-yl)carbamate (13)

The title compound was prepared by the general procedure for peptide coupling on a 2 mmol scale. Column chromatography (10-40% EtOAc/pentane provided the title compound (427 mg, 60%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.26-5.06 (m, 1H), 4.70 (s, 1H), 3.78 (d, *J* = 4.9 Hz, 3H), 3.31-3.13 (m, 3H), 2.04-0.56 (m, 28H).^{13}C NMR (101 MHz, $CDCl_3$) δ 173.95, 155.69, 155.20, 79.20, 77.36, 61.46, 48.63, 48.50, 48.32, 48.28, 48.14, 47.52, 43.01, 41.73, 39.47, 38.47, 37.70, 37.52, 37.22, 36.79, 36.18, 35.99, 35.89, 34.84, 34.72, 34.58, 34.44, 34.39, 34.27, 34.15, 32.48, 32.25, 31.85, 30.63, 30.44, 30.05, 29.99, 29.57, 29.09, 28.55, 28.24, 27.79, 26.87, 26.84, 26.76, 26.73, 26.69, 26.67, 26.53, 26.41, 26.38, 26.32, 26.09, 25.83, 25.71, 25.35, 25.32, 21.58, 21.30, 21.20, 21.14, 20.70, 20.15, 19.31. HRMS calculated for $C_{20}H_{36}N_2O_4$369.27478[M+H]$^+$; found 369.27533.

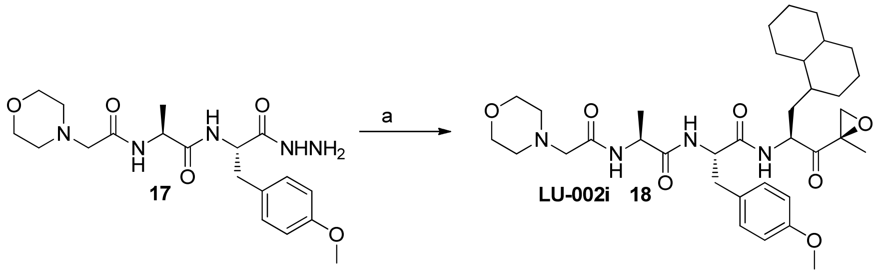
tert-butyl ((2S)-1-(decahydronaphthalen-1-yl)-4-methyl-3-oxopent-4-en-2-yl)carbamate (14)

To a solution of 2-bromopropene (435 mg, 320 µL, 3.6 mmol, 3 equiv.) in Et_2O at -78°C was added tBuLi (3.20 mL. 4.5 equiv, 1.7 M in pent) in 10 min. After stirring for 15 min. at -78°C, the Weinreb amide **13** (427 mg. 1.2 mmol, 1 equiv.) in Et_2O was added slowly in 10 min. The reaction mixtures is stirred for 2 h at -40°C. The reaction was quenched by the addition of sat. NH_4Cl and warmed to RT. The mixture was transferred to a separatory funnel and the water layer was extracted with EtOAc (3X). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated. The crude product was purified by column chromatography (1-10% EtOAc/pentane) providing the product (278 mg, 67%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.21-6.00 (m, 1H), 5.94-5.80 (m, 1H), 5.33-4.89 (m, 2H), 2.18-0.51 (m, 31H).^{13}C NMR (101 MHz, $CDCl_3$) δ 201.83, 201.61, 155.67, 142.73, 142.20, 126.12, 126.04, 125.87, 125.69, 114.89, 109.66, 79.45, 52.89, 52.29, 47.65, 43.13, 41.81, 38.97, 38.11, 37.64, 37.40, 37.31, 37.21, 36.24, 34.89, 34.79, 34.66, 34.61, 34.53, 34.48, 34.34, 34.18, 32.56, 32.25, 31.65, 31.15, 30.74, 30.37, 30.12, 29.31, 28.33, 27.86, 26.92, 26.83, 26.78, 26.71, 26.62, 26.48, 26.45, 26.40, 26.11, 26.00, 25.91, 25.40, 25.32, 21.61, 21.35, 21.28, 20.66, 20.63, 20.23, 19.97, 17.84.

tert-butyl ((2S,3R)-1-(decahydronaphthalen-1-yl)-3-hydroxy-4-methylpent-4-en-2-yl)carbamate (15)

To a solution of alkene **14** (278 mg, 0.8 mg, 1 equiv.) in MeOH was added $CeCl_3{\cdot}7H_2O$ (477 mg, 1.28 mmol,1.6 equiv.) and the mixture was stirred until the solution became clear. The mixture is cooled to 0°C and $NaBH_4$ (39 mg, 1.04 mmol, 1.3 equiv.) is added in portions in 10 min. After 30 min, TLC analysis showed completion of the reaction. The reaction was quenched by the addition of AcOH. The mixture was stirred for 15 min. followed by the addition of toluene and removal of the solvent. The residue was redissolved in a H_2O/ EtOAc mixture, which is then transferred to a separatory funnel. The layers were separated and the aqueous layer was extracted with EtOAc (2X). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated. The crude product was purified by column chromatography (10-30% EtOAc/pentane) providing the product in a quantitative yield (283 mg, 0.8 mmol). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.06-5.03 (m, 1H), 4.97-4.71 (m, 2H), 4.16-4.04 (m, 1H), 4.00-3.56 (m, 1H), 3.00 (s, 1H), 2.00-0.53 (m, 31H).^{13}C NMR (101 MHz, $CDCl_3$) δ 156.24, 156.13, 144.99, 144.55, 111.71, 111.32, 111.21, 111.11, 79.95, 79.34, 79.23, 77.93, 77.80, 77.48, 77.16, 76.84, 50.72, 50.09, 47.69, 43.26, 43.24, 42.32, 40.62, 38.55, 38.44, 38.00, 37.81, 36.18, 35.02, 34.88, 34.81, 34.65, 34.62, 34.55, 34.52, 34.46, 34.35, 34.06, 32.80, 32.66, 32.45, 32.26, 31.36, 30.88, 30.43, 30.30, 29.71, 29.35, 28.43, 28.38, 28.05, 27.03, 26.96, 26.90, 26.88, 26.74, 26.57, 26.52, 26.40, 26.35, 26.05, 25.91, 25.56, 23.37, 21.84, 21.40, 21.33, 20.89, 20.38, 19.51.

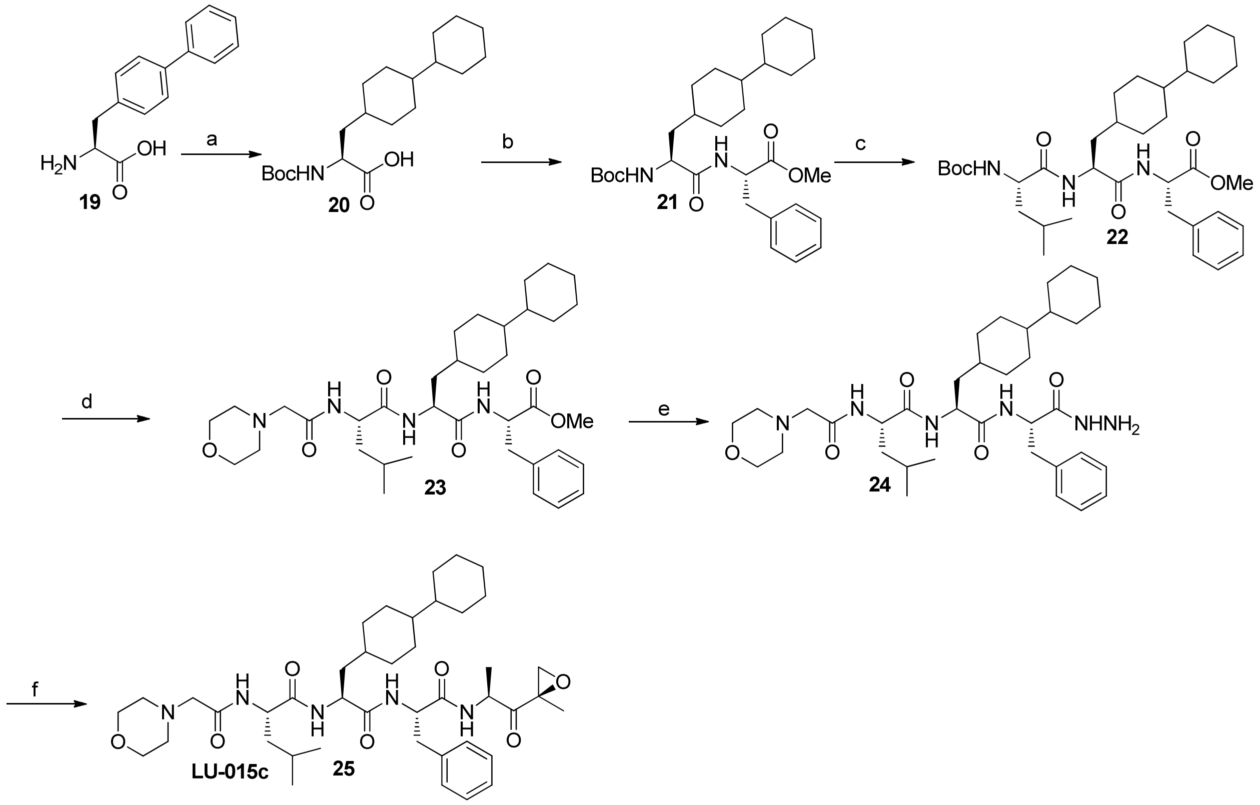
tert-butyl ((2S)-3-(decahydronaphthalen-1-yl)-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)carbamate (16)
To a solution of alcohol **15** (283 mg, 0.8 mmol) in DCM at 0°C is added VO(acac)$_2$ (21 mg, 0.08 mmol, 0.1 equiv.) followed by the addition of tBuOOH (5.5 M in decane, 0.44 mL, 2.4 mmol, 3 equiv.). The reaction mixture is stirred at 0°C for 2-3 h after which TLC analysis showed completion of the reaction. The reaction mixture is concentrated, redissolved in EtOAc and washed with 0.5 sat. aq. $NaHCO_3$ (2x), H_2O and brine. The organic layer is dried over Na_2SO_4, filtered and concentrated. The crude product is added as a solution in DCM to a solution of Dess-Martin-Periodane (679 mg, 1.6 mmol, 2.0 equiv.) in DCM at 0°C. After stirring overnight, the reaction was quenched by the addition of sat. $NaHCO_3$. The mixture was transferred to a separatory funnel and the layers were separated. The aqueous layer was extracted with DCM (1x) and the combined organics were washed with sat. $NaHCO_3$ (1x) and brine and dried over Na_2SO_4, filtered and concentrated. The crude product was purified by column chromatography (1-10% EtOAc/pentane) providing the title compound (56.2 mg, 0.15 mmol, 19%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.97-4.75 (m, 1H), 4.35-4.25 (m, 1H), 3.33-3.27 (m, 1H), 2.90-2.87 (m, 1H), 2.01-0.57 (m, 31H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 210.04, 209.78, 155.86, 79.85, 59.14, 59.07, 52.58, 52.47, 52.39, 52.25, 51.31, 50.82, 47.68, 43.23, 42.15, 37.76, 37.44, 36.50, 36.31, 36.01, 35.41, 35.00, 34.92, 34.73, 34.65, 34.58, 34.55, 34.37, 34.29, 32.66, 32.61, 31.80, 30.90, 30.66, 30.32, 30.22, 29.80, 29.00, 28.42, 28.42, 28.13, 27.52, 27.05, 26.92, 26.89, 26.85, 26.80, 26.62, 26.58, 26.54, 26.50, 26.23, 25.91, 25.80, 25.49, 21.75, 21.45, 21.37, 21.20, 20.85, 20.36, 20.24, 19.34, 16.91, 16.88. HRMS calculated for $C_{21}H_{35}NO_4$366.26389[M+H]$^+$; found 366.26401.



Scheme S5 Synthesis of LU-002i. Reagents and conditions: a. 1. i. tBuONO, HCl, DMF, -35°C. ii DiPEA, H_2N- DecAla-EK, -35°C-RT, 41%.

MorphAc-Ala-Tyr(OMe)-DecAla-EK (LU-002i, 18)
Compound **17** was synthesized as reported before.[31] Compound 18 was prepared by the general procedure for azide peptide couplings on a 50 µmol scale. Purification by column chromatography (10-30% EtOAc/pent), followed by lyophilisation, provided the title compound (13.2 mg, 20 µmol, 41%). ^{1}H NMR (600 MHz, MeOD) δ 7.14 (d, *J* = 8.6 Hz, 2H), 6.81 (d, *J* = 8.4 Hz, 2H), 4.67 – 4.49 (m, 2H), 4.42 (p, *J* = 6.9 Hz, 1H), 3.78 (s, 3H), 3.75 – 3.67 (m, 4H), 3.23 (dd, *J* = 23.4, 5.1 Hz, 1H), 3.08 – 3.01 (m, 2H), 2.99 (d, *J* = 4.7 Hz, 1H), 2.94 (tt, *J* = 9.0, 4.5 Hz, 1H), 2.86 – 2.79 (m, 1H), 2.49 (s, 4H), 2.11 – 1.50 (m, 10H), 1.48 (d, *J* = 1.6 Hz, 3H), 1.47 – 1.36 (m, 2H), 1.33 (dd, *J* = 7.1, 1.8 Hz, 3H), 1.31 – 0.60 (m, 7H). ^{13}C NMR (151 MHz, MeOD) δ 209.38, 209.07, 173.75, 173.70, 173.02, 172.94, 171.58, 171.53, 159.56, 131.02, 129.64, 129.63, 114.37, 67.48, 62.02, 59.70, 59.53, 59.50, 55.42, 55.38, 55.37, 55.22, 54.32, 52.61, 52.59, 50.62, 50.04, 49.25, 49.17, 44.17, 44.06, 42.69, 41.19, 39.47, 39.03, 38.73, 38.71, 38.68, 38.31, 37.83, 37.78, 37.75, 37.72, 37.15, 37.12, 36.67, 36.17, 36.05, 35.79, 35.67, 35.51, 35.40, 35.30, 35.26, 35.23, 35.11, 35.03, 34.64, 34.58, 34.40, 33.46, 33.36, 32.80, 32.23, 31.51, 30.98, 30.74, 30.24, 29.54, 28.70, 27.76, 27.62, 27.56, 27.52, 27.30, 27.17, 26.88, 26.61, 26.34, 26.25, 26.17, 22.02, 21.95, 21.40, 20.84, 19.84, 18.43, 18.37, 18.32, 16.54, 16.51, 16.43, 16.39. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 13.5 min): R_t (min): 6.71 (ESI-MS (m/z): 641.40 (M+H$^+$)). HRMS calculated for $C_{35}H_{52}N_4O_7$ 641.39088 [M+H]$^+$; found 641.39093.

Synthesis of LU-015c



Scheme S6. Synthesis of LU-015c. Reagents and conditions. a. 1. Rh/Al, H_2, MeOH, quant. 2. Boc_2O, THF/H_2O, 85%. b. HCTU, H-Phe-OMe, DiPEA, 80%. c. 1. TFA. 2. HCTU, Boc-Leu-OH, DiPEA, 86%. d. 1. TFA. 2. HCTU, Morpholinoacetic acid HCl, DiPEA, 78%. e. hydrazine hydrate, MeOH, quant. f. 1. i. tBuONO, HCl, DMF, -35°C. ii DiPEA, H_2N- Ala-EK, -35°C-RT, 19.8%.

Boc-BiCha-OH (20)

H-BiPhe-OH **19** (0.5 g, 2.1 mmol) was dissolved in MeOH (20 mL), followed by the addition of Rh on alumina (5 wt. %, 200 mg). The mixture was placed under H_2 (4 bar, Parr apparatus) for 48h. Subsequent filtration and concentration provided the product in a quantitative yield. The crude product was dissolved in 1:1 THF/H_2O (16 mL), followed by the addition of Boc_2O (917 mg, 4.2mmol, 2 eq.) and Et_3N (1.2 mL, 8.4 mmol, 4 equiv.). After stirring overnight, the reaction mixture was concentrated and co-evaporated with toluene. Purification by column chromatography (0-10% MeOH/EtOAc) provided the product (632 mg, 1.79 mmol, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.79 (s, 1H), 6.58 (s, 0.5H), 5.11 (s, 0.5H), 4.49-3.83 (m, 1H), 2.07-0.54 (m, 32H).^{13}C NMR (101 MHz, $CDCl_3$) δ 178.23, 177.80, 157.07, 155.72, 114.12, 81.58, 79.94, 77.48, 77.16, 76.84, 53.01, 51.92, 43.28, 41.64, 40.30, 40.17, 36.52, 36.31, 34.28, 33.83, 33.80, 32.71, 31.94, 30.97, 30.55, 30.27, 30.25, 30.15, 29.77, 29.70, 29.67, 29.63, 29.52, 29.37, 29.16, 28.95, 28.63, 28.46, 28.26, 28.22, 28.17, 28.02, 26.86, 26.75, 25.92, 25.59, 25.32, 22.70. HRMS calculated for $C_{20}H_{35}NO_4$ 354.26389[M+H]$^+$; found 354.26399.

Boc-BiCha-Phe-OMe (21)

The title compound was prepared by the general procedure for peptide coupling on a 0.5 mmol scale. Column chromatography (10-30% EtOAc/pentane) provided the product (207 mg, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41-7.03 (m, 5H), 6.69 (d, *J* = 7.8 Hz, 1H), 5.03-4.99 (m, 1H), 4.88-4.68 (m, 1H), 4.26-3.99 (m, 1H), 3.69 (s, 3H), 3.20-2.94 (m, 2H), 1.88-0.72 (m, 32H).^{13}C NMR (101 MHz, $CDCl_3$) δ 172.38, 171.72, 171.71, 155.58, 135.87, 129.33, 128.53, 127.07, 79.94, 77.48, 77.16, 76.84, 53.22, 52.26, 43.28, 41.58, 40.17, 39.92, 37.95, 36.07, 34.33, 33.85, 32.87, 31.02, 30.56, 30.54, 30.25, 29.99, 29.77, 29.62, 28.70, 28.33, 26.86, 26.77, 26.75, 25.61, 25.38.

Boc-Leu-BiCha-Phe-OMe (22)

Boc-BiCha-Phe-OMe **21** (385 mg, 0.75 mmol) was deprotected using the standard procedure for Boc removal, followed by peptide coupling with Boc-Leu-OH using the standard procedure for peptide couplings. Column chromatography (20-40% EtOAc/pentane), provided the product (408 mg, 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27 (dt, *J* = 14.0, 6.7 Hz, 3H), 7.11 (d, *J* = 6.9 Hz, 2H), 6.75 (d, *J* = 7.5 Hz, 1H), 6.64 (dd, *J* = 14.0, 8.1 Hz, 1H), 5.10 – 5.01 (m, 1H), 4.89 – 4.76 (m, 1H), 4.45 (dq, *J* = 13.7, 7.5, 6.9 Hz, 1H), 4.18 – 4.08 (m, 1H), 3.70 (s, 3H), 3.10 (dd, *J* = 5.8, 3.3 Hz, 2H), 1.92 – 1.48 (m, 12H), 1.45 (s, 9H), 1.41 – 0.98 (m, 11H), 1.03 – 0.77 (m, 9H).

Morph-Leu-BiCha-Phe-OMe (23)

Boc-Leu-BiCha-Phe-OMe **22** (408 mg, 0.65 mmol) was deprotected using the standard procedure for Boc removal, followed by peptide coupling with 2-morpholinoacetic acid HCl using the standard procedure for peptide couplings. Column chromatography (20-40% EtOAc/pentane), provided the product (333 mg, 78%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 (d, *J* = 8.6 Hz, 1H), 7.27 – 7.14 (m, 3H), 7.09 – 6.96 (m, 3H), 6.73 (d, *J* = 7.8 Hz, 1H), 4.84 – 4.69 (m, 1H), 4.55 – 4.41 (m, 1H), 4.41 – 4.29 (m, 1H), 3.73 – 3.65 (m, 4H), 3.63 (s, 3H), 3.04 (dd, *J* = 5.7, 2.4 Hz, 2H), 3.00 – 2.91 (m, 2H), 2.47 (s, 4H), 1.86 – 0.70 (m, 32H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.97, 171.86, 171.54, 171.46, 169.93, 135.79, 129.22, 128.48, 127.04, 66.79, 61.72, 53.72, 53.47, 53.24, 52.20, 51.65, 51.27, 51.03, 43.16, 43.11, 41.45, 40.90, 40.05, 39.43, 37.80, 35.55, 34.27, 33.58, 32.89, 30.88, 30.45, 30.16, 29.89, 29.58, 28.59, 26.75, 26.64, 25.50, 25.32, 24.86, 22.93, 22.07.

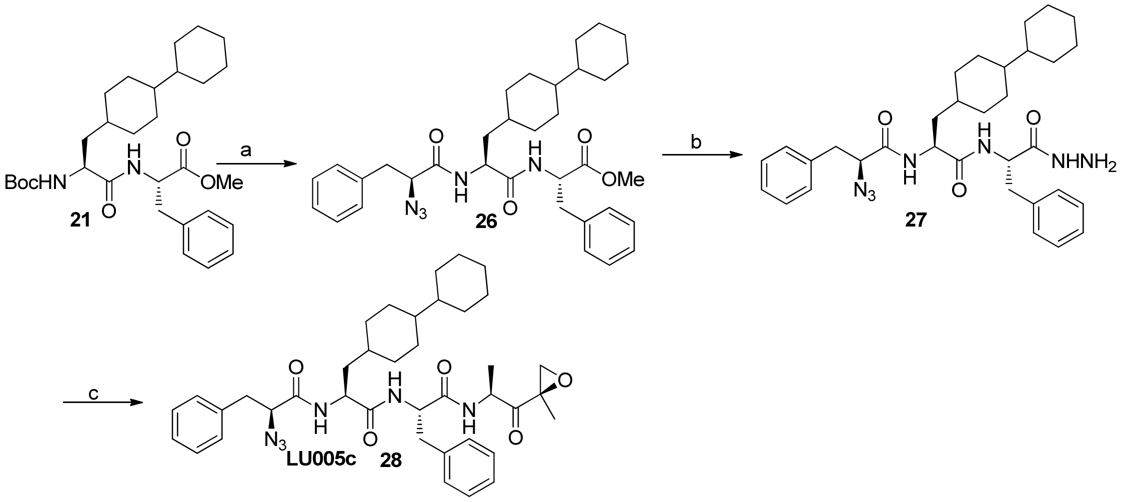
Morph-Leu-BiCha-Phe-$NHNH_2$ (24)

Morph-Leu-BiCha-Phe-OMe **23** (333 mg, 0.51 mmol) was dissolved in MeOH (7.5 mL), followed by the addition of $NH_2NH_2 \cdot H_2O$ (1 mL, 40 equiv.). After stirring for 3 hours, the reaction mixture was concentrated and co-evaporated with toluene (2x) providing the product in a quantitative yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.60 (s, 1H), 7.82 (d, *J* = 7.5 Hz, 1H), 7.63 (d, *J* = 7.9 Hz, 1H), 7.22 (ddd, *J* = 28.6, 14.4, 6.7 Hz, 6H), 4.91 (q, *J* = 7.4 Hz, 1H), 4.77 – 4.63 (m, 1H), 4.53 (dt, *J* = 15.4, 8.1 Hz, 1H), 3.80 – 3.67 (m, 4H), 3.17 – 2.94 (m, 4H), 2.54 (s, 4H), 1.80 – 1.44 (m, 12H), 1.45 – 1.23 (m, 7H), 1.11 (dt, *J* = 22.2, 12.4 Hz, 6H), 0.90 (t, *J* = 6.1 Hz, 6H), 0.86 – 0.74 (m, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.86, 171.80, 171.69, 170.95, 169.76, 136.14, 128.95, 128.24, 126.68, 66.54, 61.49, 53.52, 52.52, 51.63, 51.16, 50.38, 42.97, 42.91, 41.20, 40.10, 39.78, 38.41, 36.37, 34.21, 33.60, 32.66, 30.92, 30.31, 30.00, 29.73, 29.56, 29.43, 28.61, 26.54, 26.44, 25.44, 25.26, 24.72, 22.68, 22.19.

Morph-Leu-BiCha-Phe-Ala-EK (LU015c, 25)

This compound was obtained by the general protocol for azide coupling on a 100 µmol scale. Purification by column chromatography (0-1-2% MeOH/DCM), followed by lyophilisation provided the product (52.7 mg, 69.2 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55 (s, 1H), 7.37 – 7.14 (m, 7H), 7.03 (s, 1H), 6.81 (s, 1H), 4.74 (q, *J* = 7.1 Hz, 1H), 4.55 – 4.42 (m, 2H), 4.35 (s, 1H), 3.80 (s, 4H), 3.22 (d, *J* = 5.0 Hz, 1H), 3.19 – 2.96 (m, 4H), 2.91 (d, *J* = 5.0 Hz, 1H), 2.60 (d, *J* = 28.7 Hz, 4H), 1.84 – 1.56 (m, 10H), 1.53 (s, 3H), 1.50 – 1.30 (m, 7H), 1.27 (d, *J* = 7.1 Hz, 3H), 1.16 (dt, *J* = 21.8, 12.3 Hz, 5H), 0.94 (dd, *J* = 12.3, 5.9 Hz, 6H), 0.90 – 0.78 (m, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 207.77, 172.15, 172.06, 171.81, 170.40, 136.68, 59.05, 54.09, 52.50, 52.25, 47.94, 41.61, 40.61, 38.09, 34.53, 33.84, 31.14, 30.64, 30.13, 28.62, 26.94, 26.83, 25.67, 25.48, 25.07, 23.09, 22.18, 17.08, 16.93. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 13 min): R_t (min): 7.44 (ESI-MS (m/z): 752.20 $(M+H)^+$). HRMS: calculated for $C_{42}H_{65}N_5O_7$ 752.49568 $[M+H]^+$; found 752.49561

Synthesis of LU-005c



Scheme S7. Synthesis of LU-005c. Reagents and conditions. a. 1. TFA. 2. HCTU, N_3Phe-OH, DiPEA, 86%. b. Hydrazine hydrate, MeOH, quant. d. 1. i. tBuONO, HCl, DMF, -35°C. ii DiPEA, H_2N- Ala-EK, -35°C-RT, 19.8%.

N_3Phe-BiCha-Phe-OMe (26)

Boc-BiCha-Phe-OMe **21** (207 mg, 0.40 mmol) was deprotected using the standard procedure for Boc removal, followed by peptide coupling with 2-morpholinoacetic acid HCl using the standard procedure for peptide couplings. Column chromatography (10-30% EtOAc/pentane), provided the product (108 mg, 45%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37-7.19 (m, 8H), 7.13 -6.99 (m, 2H), 6.94-6.88 (m, 1H), 6.72-6.69 (m, 1H), 4.90-4.84 (m, 1H), 4.56-4.46 (m, 1H), 4.05-4.01 (m, 1H), 3.71 (s, 3H), 3.41-2.84 (m, 4H), 1.83-0.71 (m, 23H).^{13}C NMR (101 MHz, $CDCl_3$) δ 171.66, 171.65, 171.40, 168.55, 136.03, 135.96, 135.83, 129.58, 128.64, 128.60, 127.19, 77.48, 77.16, 76.84, 65.12, 53.25, 52.43, 51.38, 51.01, 43.23, 41.73, 40.50, 39.65, 38.41, 38.34, 37.92, 37.67, 35.55, 34.20, 33.74, 33.03, 30.67, 30.55, 30.29, 29.94, 29.69, 29.62, 28.97, 26.90, 26.80, 25.46, 25.30.

N_3Phe-BiCha-Phe-$NHNH_2$ (27)

N_3Phe-BiCha-Phe-OMe **26** (108 mg, 0.18 mmol) was dissolved in MeOH (5 mL), followed by the addition of NH_2-$NH_2 \cdot H_2O$ (0.26 mL, 30 equiv.). After stirring for overnight followed by 2 hours at reflux temperature, the reaction mixture was concentrated and co-evaporated with toluene (2x) providing the product in a quantitative yield. ^{1}H NMR (400 MHz, , $CDCl_3$) δ 8.56 (s, 1H), 7.74 (d, J = 8.7 Hz, 1H), 7.37-7.07 (m, 10H), 4.82 (q, J = 7.7 Hz, 1H), 4.70-4.43 (m, 1H), 4.13-4.10 (m, 2H), 3.32-2.86 (m, 4H), 1.77-0.67 (m, 23H).^{13}C NMR (101 MHz, $CDCl_3$) δ 172.09, 171.97, 171.41, 169.65, 169.19, 169.16, 136.62, 136.36, 136.16, 136.06, 129.80, 129.37, 129.27, 128.97, 128.65, 128.58, 127.29, 127.01, 64.86, 53.14, 51.80, 51.32, 43.22, 39.72, 38.51, 38.18, 37.66, 35.72, 34.29, 33.90, 32.85, 30.93, 30.55, 30.26, 29.96, 29.75, 29.71, 29.57, 29.41, 28.96, 26.86, 26.74, 25.57, 25.36, 22.76.

N_3Phe-BiCha-Phe-Ala-EK (LU-005c, 28)

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by HPLC (C_{18}, 70-90% MeCN, 0.1% TFA, 10 min gradient), followed by lyophilization provided the product (6.77 mg, 19.8 %). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.34 – 7.30 (m, 2H), 7.28 (dt, J = 7.0, 2.2 Hz, 3H), 7.22 (ddd, J = 9.3, 4.6, 2.0 Hz, 3H), 7.19 (d, J = 7.2 Hz, 2H), 6.63 (t, J = 6.9 Hz, 1H), 6.49 (t, J = 7.5 Hz, 1H), 6.37 (d, J = 7.0 Hz, 1H), 4.60 (q, J = 7.2 Hz, 1H), 4.47 (td, J = 7.1, 2.7 Hz, 1H), 4.36 – 4.29 (m, 0.3H), 4.26 (m, 0.7H), 4.07 (ddd, J = 12.2, 7.8, 4.1 Hz, 1H), 3.27 (dt, J = 14.1, 3.6 Hz, 1H), 3.18 – 3.15 (m, 1H), 3.07 – 3.03 (m, 2H), 3.03 – 2.97 (m, 1H), 2.89 (d, J = 4.9 Hz, 1H), 1.78 – 1.54 (m, 9H), 1.51 (s, 3H), 1.41 – 1.25 (m, 6H), 1.24 (d, J = 7.1 Hz, 3H), 1.21 – 0.73 (m, 8H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 207.80, 171.42, 170.11, 168.79, 136.49, 135.84, 129.63, 129.61, 129.40, 128.85, 128.81, 128.73, 127.48, 127.45, 127.16, 65.20, 65.16, 59.02, 54.21, 52.53, 51.67, 51.23, 48.12, 43.37, 43.26, 39.01, 38.42, 38.36, 37.95, 37.91, 34.30, 33.79, 32.96, 30.61, 30.34, 30.01, 29.69, 29.62, 28.88, 26.97, 26.87, 25.48, 25.32, 17.31, 16.92. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 10.82 (ESI-MS (m/z): 685.33 $(M+H)^+$). HRMS: calculated for $C_{39}H_{52}N_6O_5$ 685.40720 $[M+H]^+$; found 685.40717

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