



A strategy for dual inhibition of the proteasome and fatty acid synthase with belactosin C-orlistat hybrids



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ABSTRACT

The proteasome, a validated cellular target for cancer, is central for maintaining cellular homeostasis, while fatty acid synthase (FAS), a novel target for numerous cancers, is responsible for palmitic acid biosynthesis. Perturbation of either enzymatic machine results in decreased proliferation and ultimately cellular apoptosis. Based on structural similarities, we hypothesized that hybrid molecules of belactosin C, a known proteasome inhibitor, and orlistat, a known inhibitor of the thioesterase domain of FAS, could inhibit both enzymes. Herein, we describe proof-of-principle studies leading to the design, synthesis and enzymatic activity of several novel, β -lactone-based, dual inhibitors of these two enzymes. Validation of dual enzyme targeting through activity-based proteome profiling with an alkyne probe modeled after the most potent inhibitor, and preliminary serum stability studies of selected derivatives are also described. These results provide proof of concept for dual targeting of the proteasome and fatty acid synthase-thioesterase (FAS-TE) enabling a new approach for the development of drug-candidates with potential to overcome resistance.

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1. Introduction

Intentional and selective polypharmacology has emerged as an exciting concept for rational drug design.¹ In cancer therapy, prototypical chemotherapeutic agents are generally based on the concept of “one-drug, one-target,” whereas selective polypharmacology targets two or more enzyme targets with a single drug. Selective polypharmacological drugs can be efficacious, a notable example being the FDA approved multi-kinase inhibitor lapatinib, which targets receptor tyrosine kinases (EGFR) and ErbB-2 (HER2), and is currently used for the treatment of metastatic breast cancer.² Drugs with single targets may fall victim to drug resistance due to mutations or up-regulation of the target protein, whereas polypharmacological anticancer drugs are thought to evade drug resistance due to the need for cells to become resistant

across multiple pathways through simultaneous protein/enzyme mutations.³

The human proteasome is responsible for the vast majority of intracellular protein degradation, and thus plays a central role in cellular homeostasis.⁴ The 20S proteasome is the core particle (CP) of the 26S proteasome, and is composed of 28 subunits arranged as four stacked rings, in which each of the rings contains either seven unique α -chains or seven unique β -chains. The organization of the CP is $\alpha_1\text{--}\beta_1\text{--}\beta_1\text{--}\alpha_1$, and possesses C_2 symmetry.⁵ Three of the 7 β -subunits are proteolytically active: β_1 , β_2 , and β_5 , and have been characterized to have peptide cleavage preferences corresponding to caspase-like (C-L, β_1), trypsin-like (T-L, β_2) and chymotrypsin-like (ChT-L, β_5) activity.⁶ The proteasome is a member of the *N*-terminal hydrolase super family, and all three catalytic subunits act to degrade substrates utilizing an *N*-terminal Thr1 residue. Due to accelerated cell cycles and metabolism in tumor cells, the CP is often upregulated to increase turnover rates of proteins.⁷ This dependence on CP activity renders neoplastic

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cells highly susceptible to proteasome inhibition. In addition, proteasome inhibitors increase the level of tumor suppressors (e.g. cyclin kinase inhibitor p27) which induce tumor apoptosis without significantly affecting quiescent cells.⁸ Thus, the proteasome CP has been extensively explored as a drug target against various types of cancer, and many classes of compounds have been found to inhibit proteasome function, both by covalently binding to Thr1 and also non-covalent inhibition.⁹ The ChT-L site has been the main focus for the design of antineoplastic therapeutics, however, the T-L and C-L sites have also recently gained attention.¹⁰ Two covalent inhibitors with preference for the ChT-L site have received FDA approval: the boronic acid, bortezomib (**1**, Velcade®), for the treatment of multiple myeloma and relapsed or refractory mantle cell lymphoma; and the epoxyketone carfilzomib (**2**, Kyprolis®), for the treatment of refractory multiple myeloma. A third promising class of proteasome inhibitors under development possess a β -lactone which covalently modifies Thr1 to generate an acyl-enzyme adduct.¹¹ An example is salinosporamide A (Marizomib, **3**), which is a β -lactone containing proteasome inhibitor designated as an orphan drug for multiple myeloma¹² and is in Phase I/II clinical studies for the treatment of multiple myeloma, leukemia, and solid tumors (Fig. 1).¹³ Despite these advances, drug resistance to proteasome inhibitors through active site mutations and upregulation of ChT-L subunits, continues to necessitate the development of novel inhibitors for cancer therapy.¹⁴

Fatty acid synthase (FAS) is a second vital enzyme which is upregulated in rapidly proliferating cancers, including breast, prostate, ovarian, and multiple myeloma.¹⁵ FAS catalyzes the *de novo* synthesis of fatty acids to provide lipids for membrane formation and energy production via β -oxidation and lipid modification of proteins. FAS inhibition suppresses cell proliferation, adhesion, migration, and invasion. It also leads to suppression of genes involved in production of arachidonic acid and androgen hormones, both of which promote tumor progression.¹⁶ Studies have shown that FAS inhibition induces apoptosis in various cancer cell lines,¹⁷ synergizes with common anti-cancer therapies (Herceptin® and Taxol®) and in some cases reverses auto-resistance to those therapies, inhibits tumor angiogenesis, and FAS inhibitors may even act as chemopreventative agents for cancer making FAS an

attractive target for further development.^{15a,18} Tetrahydrolipstatin (**4**, Orlistat®, Alli®, Xenical®) is a reduced form of the β -lactone-containing natural product lipstatin, and was approved by the FDA in 1999 for the treatment of obesity due to its ability to inhibit pancreatic lipase in the gut. Recently, however, it was determined that orlistat also has antitumor activity, through inhibition of the thioesterase domain of FAS, FAS-TE (IC_{50} = 1.35 μ M).¹⁹ X-ray analysis of the human FAS-TE bound with orlistat by Kridel and Lowther revealed the mechanism of inhibition to be covalent bond formation between Ser2308 of FAS-TE and the β -lactone of orlistat through acylation.²⁰ Poor solubility and poor oral bioavailability limit the practicality of orlistat as an antitumor drug, therefore it was crucial to overcome these problems by modifying the structure of orlistat to develop the next generation of FAS-TE inhibitors.^{18e}

Our group previously reported the synthesis of orlistat and a series of analogs with FAS-TE inhibitory activity.²¹ We also described the synthesis of (–)-belactosin C (**6**) and derivatives,²² which along with (–)-belactosin A (**5**) and congeners possess inhibitory activity toward the 20S proteasome. X-ray studies of the belactosin C analog, *N*-Cbz-O-Bn homobelactosin C (**7**), bound to the yeast 20S proteasome by Groll and coworkers²³ revealed that the Thr1 is acylated by the β -lactone moiety, and that the orientation of the inhibitor differs from other β -lactone-containing proteasome inhibitors such as omuralide, which may contribute to its selectivity for ChT-L subunits. A recent study demonstrated the potential of belactosin derivatives bearing minimal β -lactone fragments to have selectivity for the immunoproteasome.²⁴ Our concurrent studies of the belactosins and orlistat derivatives led to a hypothesis that hybrid structures of these β -lactones might have the potential to inhibit both the proteasome and FAS-TE. The common reactive acylating moiety in these inhibitors is a (3*S*, 4*R*)-*trans*-disubstituted β -lactone providing a starting point for the design of analogs with dual inhibitory activity. Herein, we report our successful design and synthesis of dual inhibitors of the proteasome and FAS-TE, structure-activity studies (SAR) using fluorogenic assays for each enzyme, and verification of the ability of these dual inhibitors to target both FAS and the proteasome in HeLa cells using activity-based protein profiling (ABPP).

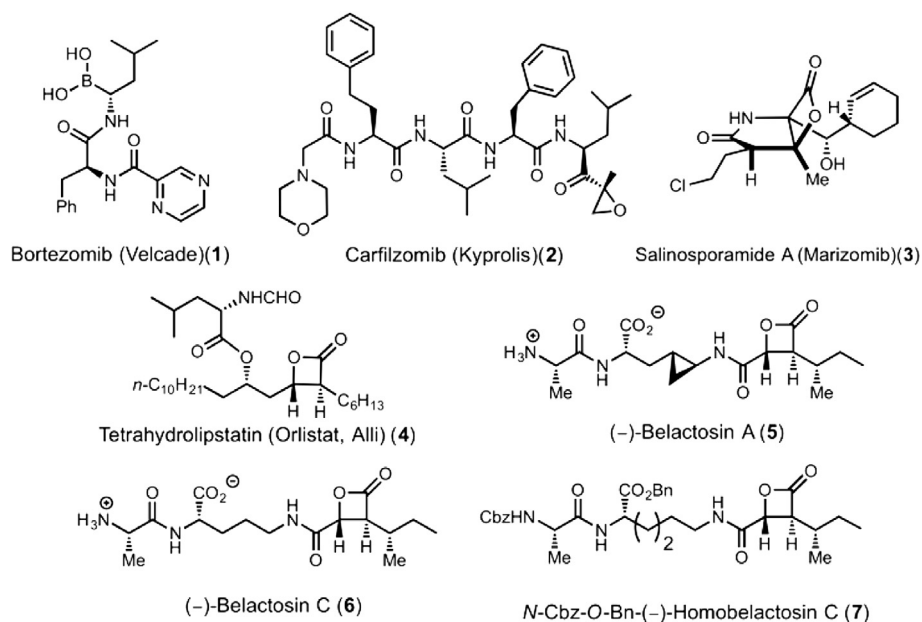


Fig. 1. Representative structures of human or yeast 20S proteasome and fatty acid synthase inhibitors.

2. Results and discussion

2.1. Initial dual inhibitors

Given the presence of the common (3*S*, 4*R*)-*trans*-disubstituted- β -lactone (red) and similar β -side chains of orlistat and protected belactosins (e.g. *N*-Cbz-*O*-Bn-(–)-homobelactosin C (**7**)), we reasoned that β -lactones **8a–c** that were originally synthesized as belactosin derivatives to provide SAR information regarding inhibition of the proteasome,^{22a} might also have inhibitory activity toward FAS (Fig. 2).

These initial analogs **8a–c** were synthesized as either a 1:1 (**8a**, **8b**) or 1.3:1 (**8c**) mixture of C3, C4-diastereomers as a result of a non-diastereoselective, tandem Mukaiyama aldol-lactonization with a glyoxamide dipeptide β -side chain, as previously described.^{22a} Since these diastereomers were not readily separable, they were assayed as diastereomeric mixtures using fluorogenic substrates for both FAS-TE and the proteasome as previously described.^{18e,25} All three compounds exhibited inhibition of the proteasome with **8c** (1.3:1 mixture of *N*-Cbz, *O*-Bn belactosin C^{23b} and its C3, C4 diastereomer) showing the greatest activity as expected. Importantly, all three derivatives were more potent than orlistat toward FAS-TE with IC₅₀ values ranging from ~0.16 to 4.0 μ M.²⁶

The inhibitory activity of these initial β -lactones verified our hypothesis that simultaneous inhibition of the proteasome and FAS was possible with a single compound by maintaining a (3*S*, 4*R*)-*trans*-disubstituted β -lactone pharmacophore with a homobelactosin C dipeptide β -sidechain. Encouraged by these preliminary results, we set out to design and synthesize a series of belactosin C-orlistat hybrids with the great challenge of increasing potency toward *both* enzymes.

2.2. Design and synthesis of dual inhibitors of FAS and the proteasome

A crystal structure of bis-benzylhomobelactosin C bound to the yeast 20S proteasome suggests that the β -dipeptide side-chain plays a role in dictating which proteasomal active site will be targeted via interaction with both the S1 specificity pocket and the primed substrate binding site.^{23a} Belactosins are the only class of proteasome inhibitors which bind to the primed region, whereas all other proteasome inhibitors bind to the non-primed sites.^{23a}

The cyclopropane ring of belactosin A was proposed to adopt a position which blocks the nucleophilic addition of water for enzyme turnover, and in conjunction with cyclopropane stereochemistry, forces the remainder of the compound into the primed site of the CP.

In the case of FAS, the co-crystal structure of FAS-TE with bound orlistat shows the 11-carbon β -chain of orlistat binding predominantly in a hydrophobic channel, termed the specificity channel.²⁰ Given that orlistat possesses a relatively hydrophobic β -side chain also bearing an *N*-formyl amino ester while *N*-Cbz, *O*-Bn-(–)-belactosin C has a protected dipeptide β -chain, we reasoned that this dipeptide might display a similar binding mode and interact with both the specificity channel and interface cavity of FAS-TE. As described previously, the β -chain dipeptide binds to the *primed site* of the proteasome and optimization of binding interactions with primed sites has led to success in improving potency and selectivity of proteasome inhibitors.²⁷ Thus, it was reasonable to consider modifications of the β -dipeptide that might alter selectivity to the three proteolytically active sites while at the same time improving interactions with the specificity channel and interface cavity of FAS-TE. We utilized *L*-(*S*)-lysine rather than *L*-(*S*)-ornithine as the first amino acid of the β -dipeptide side-chain resulting in a four methylene unit spacer rather than three and protection of the terminal nitrogen with a carbobenzyloxy group and the lysine carboxylic acid as the benzyl ester was also maintained in all derivatives given the high potency of *N*-Cbz, *O*-Bn homobelactosin C toward the proteasome reported previously.^{23a} On the other hand, less is known about the role the small, hydrophobic *sec*-butyl α -chain might play in binding to the proteasome but the C3-*n*-hexyl group of orlistat binds to the promiscuous C3-short-chain binding site in FAS-TE.^{18e,21} As described above, replacement of the α -*sec*-butyl chain with an α -*n*-hexyl group was tolerated by the proteasome and given the structure of orlistat, we elected to maintain this hydrophobic α -chain in planned derivatives for this study. Both the belactosins and orlistat bear the same relative (*trans*) and absolute configuration (3*S*, 4*R*) at C2 and C3 of the β -lactone so this was maintained in most derivatives, however the enantiomeric (3*R*, 4*S*)-*trans*- β -lactone was also investigated.

To synthesize the targeted belactosin-orlistat hybrids, we utilized our previously reported strategy toward (–)-belactosin C.^{22a} Synthesis of the disubstituted- β -lactones was enabled through a

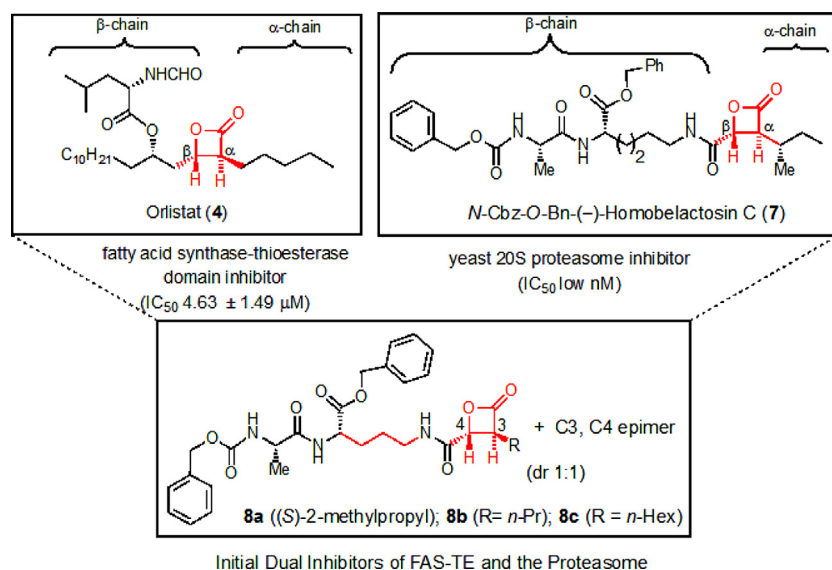
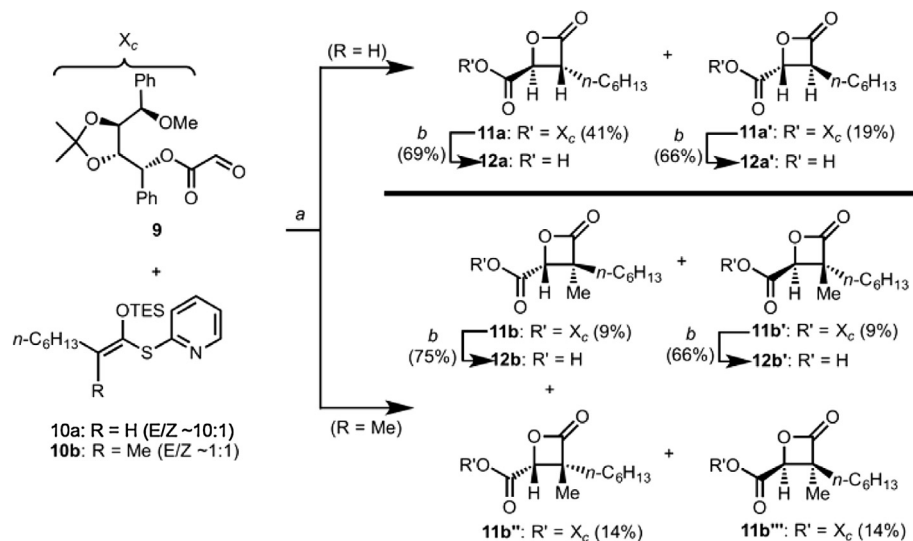
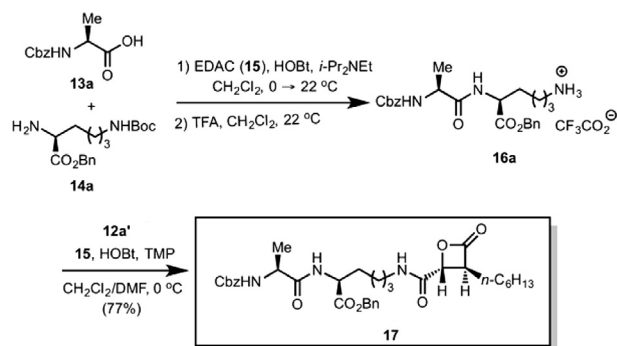


Fig. 2. Representative initial dual inhibitors **8a–c** displaying inhibitory activity toward both FAS-TE and the proteasome and design process based on structures of orlistat (**4**) and the belactosin C derivative **7**.



Scheme 1. Synthesis of disubstituted α -lactone acids **12a** and **12a'** and α -methyl- β -lactone acids **12b** and **12b'** employing a chiral auxiliary-based, tandem Mukaiyama aldol- β -lactonization. (a) ZnCl_2 , CH_2Cl_2 , 23 °C, 30 min (R = H) or 15 h (R = Me); (b) H_2 , Pd/C (10% w/w), THF.



Scheme 2. Representative coupling strategy for synthesis of dual inhibitors as shown for dipeptide β -lactone **17**.

chiral auxiliary-based, tandem Mukaiyama aldol-lactonization (TMAL) process between glyoxylate ester **9** and the silyl ketene acetal **10a** to deliver the *trans*- β -lactone esters **11a** and **11b'** (Scheme 1). The tartrate-derived chiral auxiliary, while not significantly impacting the absolute configuration of the newly created C2, C3 stereocenters (dr ~2:1) during the TMAL process, served to facilitate separation of the diastereomers **11a**/**11a'** produced. The two were separated and underwent hydrogenolysis to cleave the chiral and auxiliary and enabled exploration of the enantiomeric *trans*- β -lactone for SAR purposes. In a similar manner, the α -methyl β -lactone esters **11b**, **11b'**, **11b''**, and **11b'''** were synthesized and despite the absence of diastereoselectivity, separation of the diastereomers was again possible and subsequent deprotection of the two *trans*-diastereomers allowed access to the α -methyl β -lactone acids **12b** and **12b'**. A series of dipeptides, represented by dipeptide amine **16a**, were coupled to the β -lactone carboxylic acids, e.g. **11b**, to deliver the dipeptide β -lactone **17** (Scheme 2). In this manner, a series of potential dual inhibitors **17–27** were synthesized through variations of the dipeptide and β -lactone fragments.

2.3. Enzymatic assays: inhibition of the 20S proteasome and FAS-TE

With the new analogs in hand, we performed an *in vitro* enzyme assay to determine their potency as inhibitors of the proteasome

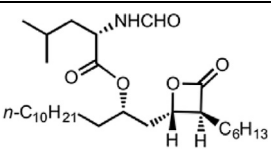
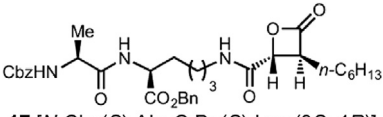
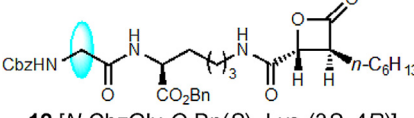
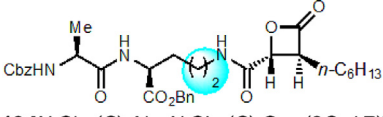
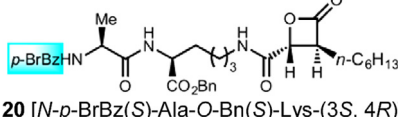
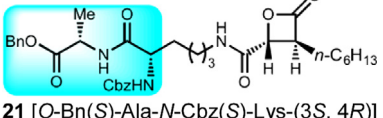
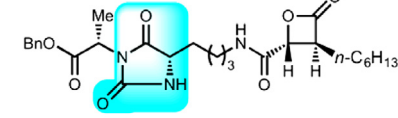
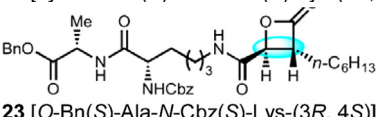
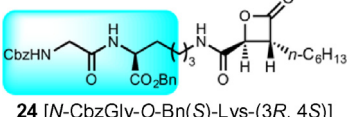
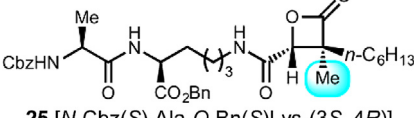
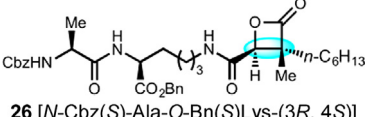
and FAS-TE. Both assays measure the rate of hydrolysis of fluorogenic substrates in the presence and absence of inhibitors (see SI for details). In the case of the proteasome, the assay was run three times with a different substrate specific for each of its catalytic sites; chymotrypsin-like (ChT-L(b5)), caspase-like (C-L(b1)), or trypsin-like (T-L(b2)). As summarized in Table 1, the 20S proteasome inhibition assay reveals that all analogs have selectivity for the ChT-L proteasomal subunit, with IC_{50} values ranging from 0.26 to 2.71 μM . Inhibition is also seen against the C-L subunits with generally higher IC_{50} values of 2.16 to > 100 μM , however, no inhibition was seen against the TL subunit. Orlistat, used as a control for the FAS-TE assay, showed no inhibition against any of the proteasome catalytic sites at concentrations up to 100 μM (Table 1, entry 1). All of the analogs tested inhibited FAS-TE in the range of ~1.2 to 9.6 μM . The most potent dual inhibitor when considering inhibitory activity toward both enzymes was dipeptide β -lactone **21**, which exhibited an IC_{50} value of $1.50 \pm 0.28 \mu\text{M}$ against FAS-TE, which was 3X more potent compared to orlistat (IC_{50} $4.63 \pm 1.49 \mu\text{M}$), while also having an IC_{50} of $0.37 \pm 0.01 \mu\text{M}$ against the proteasome with high selectivity for ChT-L sites. A more detailed discussion of SAR established from these derivatives is provided below with systematic and sequential changes made in derivatives **18–33** highlighted (aqua) in Table 1.

2.3.1. Variations of the dipeptide fragment

We first targeted variations of the dipeptide side-chain to probe the effects these changes would have on presumed binding to the S1 specificity pocket of the proteasome and the specificity (hydrophobic) channel of FAS-TE. Building on our preliminary data (*vide infra*), our initial derivative **17** maintained the α -*n*-hexyl side chain and the *N*-Cbz-(*S*)-Ala-*O*-Bn(*S*)-Lys dipeptide and served as an embarkation point for further structural modifications. Derivative **17** exhibited IC_{50} values of 0.50 ± 0.06 and $3.17 \pm 0.17 \mu\text{M}$ toward the proteasome and FAS-TE, respectively (Table 1, entry 2). Removal of a methyl group in the dipeptide by replacement of alanine with glycine in derivative **18** did not impact proteasome inhibition, but led to an ~3-fold drop in potency against FAS-TE (Table 1, entry 3). Removing one methylene unit by replacing *L*-(*S*)-lysine with *L*-(*S*)-ornithine, as found in the belactosins, provided derivative **19** with similar activity to the ChT-L site of the proteasome and reduced activity toward FAS-TE, thus in all further derivatives (*S*)-lysine was retained in the dipeptide (Table 1, entry 2 vs. 4). Replacing the terminal benzyl carbamate of derivative **17**

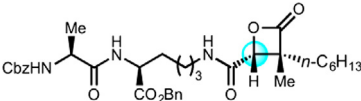
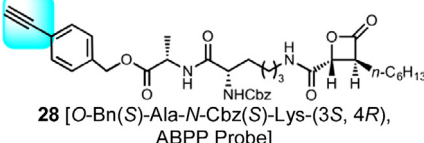
Table 1

Structure-activity relationships of novel orlistat-belactosin hybrids against the three proteolytic sites of the human 20S proteasome, human FAS-TE, and HeLa cells.

Entry	Compound	Chymotrysin-like (ChT-L) IC ₅₀ (μM)	Caspase-like (C-L) IC ₅₀ (μM)	Trypsin-like (T-L) IC ₅₀ (μM)	FAS-TE IC ₅₀ (μM)	HeLa cell growth IC ₅₀ (μM)
1	 Orlistat (4)	>100	>100	>100	4.63 ± 1.49	ND ^a
2	 17 [N-Cbz(S)-Ala-O-Bn(S)-Lys-(3S, 4R)]	0.50 ± 0.06	3.03 ± 0.05	>100	3.17 ± 0.17	62.5
3	 18 [N-CbzGly-O-Bn(S)-Lys-(3S, 4R)]	0.57 ± 0.03	3.51 ± 0.04	>100	9.56 ± 0.58	51.1
4	 19 [N-Cbz(S)-Ala-N-Cbz(S)-Orn-(3S, 4R)]	0.70 ± 0.05	2.16 ± 0.05	>100	4.35 ± 1.11	ND ^a
5	 20 [N-p-BrBz(S)-Ala-O-Bn(S)-Lys-(3S, 4R)]	0.51 ± 0.01	2.81 ± 0.24	>100	4.81 ± 1.84	ND ^a
6	 21 [O-Bn(S)-Ala-N-Cbz(S)-Lys-(3S, 4R)]	0.37 ± 0.01	>100	>100	1.50 ± 0.28	45.8
7	 22 [cyclo-O-Bn(S)-Ala-O-Bn(S)-Lys-(3S, 4R)]	0.51 ± 0.02	7.93 ± 0.21	>100	5.55 ± 1.33	ND ^a
8	 23 [O-Bn(S)-Ala-N-Cbz(S)-Lys-(3R, 4S)]	2.71 ± 0.17	>100	>100	12.6 ± 0.6	ND ^a
9	 24 [N-CbzGly-O-Bn(S)-Lys-(3R, 4S)]	1.38 ± 0.03	8.72 ± 0.05	>100	8.37 ± 0.17	ND ^a
10	 25 [N-Cbz(S)-Ala-O-Bn(S)-Lys-(3S, 4R)]	2.43 ± 0.25	3.74 ± 0.04	>100	2.61 ± 0.94	10.0
11	 26 [N-Cbz(S)-Ala-O-Bn(S)-Lys-(3R, 4S)]	2.18 ± 0.08	3.98 ± 0.63	>100	3.11 ± 0.75	37.5

(continued on next page)

Table 1 (continued)

Entry	Compound	Chymotrysin-like (ChT-L) IC ₅₀ (μM)	Caspase-like (C-L) IC ₅₀ (μM)	Trypsin-like (T-L) IC ₅₀ (μM)	FAS-TE IC ₅₀ (μM)	HeLa cell growth IC ₅₀ (μM)
12		3.39 ± 0.08	3.62 ± 0.08	>100	1.19 ± 0.42	ND ^a
13	27 [N-Cbz(S)-Ala-O-Bn(S)-Lys-(3R, 4R)] 	0.26 ± 0.01	>100	>100	3.98 ± 0.74	ND ^a
	28 [O-Bn(S)-Ala-N-Cbz(S)-Lys-(3S, 4R), ABPP Probe]					

^a ND = not determined.

with a bromobenzoyl group provided derivative **20**, which maintains the hydrophobicity and hydrogen-bonding capabilities, did not significantly alter inhibitory activity (Table 1, entry 5). We also investigated reversed-amide variants in the dipeptide wherein linkage is made through the carboxylic acid rather than the amine of lysine leading to derivative **21** bearing a *N*-Cbz substituent and terminal benzyl ester. This derivative exhibited the most potent activity toward FAS-TE (1.50 ± 0.28 M) to this point, while also dramatically increasing specificity toward the ChT-L sites of the proteasome (Table 1, entry 6). A more rigid dipeptide was also explored through synthesis of the cyclic imide urea **22** which also lacked a hydrophobic benzyl substituent relative to other derivatives. The restricted flexibility did not greatly alter ChT-L activity, but reduced active site specificity with activity observed toward the C-L site of the proteasome (IC₅₀ 7.93 ± 0.21 μM) and reduced FAS-TE activity (Table 1, entry 7).

2.3.2. Effect of β-lactone absolute configuration

To probe the effect of the β-lactone relative and absolute configuration, analogs **23** and **24** were prepared from diastereomeric β-lactones (cf. **12**, Scheme 1). Derivative **23** maintained the reverse amide variation in the dipeptide in analog **21**, however the absolute configuration was inverted at both the α- and β-positions of the β-lactone to the 3*R*, 4*S* configuration. This alteration reduced the proteasome ChT-L and FAS-TE activity ~8-fold of analog **23** compared to the diastereomeric β-lactone **21** (Table 1, entry 6 vs entry 8). Maintaining the same β-lactone configuration but returning to the previous amide connectivity with a Gly-Lys dipeptide provided analog **24** (Table 1, entry 9) which regained some activity toward both enzymes but did not rival activity previously observed with the 3*S*, 4*R* configuration (cf. **18**, Table 1, entry 3). The results validated the importance of the β-lactone configuration and is consistent with previous reports indicating the importance of both relative and absolute stereochemistry for β-lactone inhibitors of both FAS-TE and the proteasome. Overall, it is interesting to note that both the reversed amide variation and alternate β-lactone absolute configuration (Table 1, entries 6, 8) led to inhibitors that had complete selectivity for the ChT-L sites of the proteasome over the C-L site.

2.3.3. Effects of the C2-methyl group

Given the potential for hydrolysis of these enzyme inhibitors prior to reaching their targets, we explored introduction of an α-methyl substituent on the β-lactone to slow the rate of hydrolysis. Maintaining the best dipeptide, namely *N*-Cbz-(*S*)-Ala-O-Bn-(*S*)-Lys, we studied three diastereomeric β-lactones **25–27** prepared through a TMAL process with an α-methyl silylketene acetal (cf. **10b**, Scheme 1; see SI for further details). In general, and not

unexpectedly, the α-quaternary carbon reduced the potency toward both enzymatic targets (IC₅₀ ~ 1–3 μM, Table 1, entries 10–12) given the expected slower rate of acylation of the active site serine and threonine of the FAS-TE and the proteasome, respectively. However, the stability of these α-quaternary carbon-containing β-lactones was improved in culture media leading to greater cytotoxicity in cellular assays (*vide infra*). Interestingly, β-lactone **27** with an α-quaternary carbon to the β-lactone but inverted C3 stereochemistry relative to the most potent dual inhibitor **21**, rivaled the inhibitory activity against FAS-TE.

2.4. Cytotoxicity toward HeLa cells

Several of the more potent dual inhibitors, derivatives **17**, **18**, **21**, **25**, and **26**, identified through enzymatic assays, were selected to assess their cytotoxicity toward HeLa cells using a colorimetric cell viability assay (MTT). All analogs showed IC₅₀ values in the 10–60 μM range with the (3*S*, 4*R*)-α-disubstituted β-lactone **25** demonstrating the highest cytotoxicity with an IC₅₀ of 10.0 μM, which was approximately 6X more potent than our initial lead inhibitor **17** (IC₅₀ 62.5 μM). While cell permeability or efflux pump effects could be responsible for the disparity observed between cell cytotoxicity and enzymatic inhibition, we reasoned that the differential instability of these β-lactones was a more likely explanation. For example, the greatest cytotoxicity, which was observed with derivative **25**, did not correspond to the best IC₅₀ values in enzymatic assays, but this could be a reflection of the greater stability to hydrolysis of the α-disubstituted β-lactone. This led us to study the relative rates of hydrolysis of selected β-lactone inhibitors in the buffer and serum used in both enzymatic and cell-based activity studies.

2.5. Stability of selected inhibitors in buffer with and without FBS

Given the disparity observed between enzymatic activity and whole cell cytotoxicity and the potential for hydrolysis of these β-lactone containing dual inhibitors, we studied the relative stability of two representative dual inhibitors in media employed in cytotoxicity assays. We chose β-lactone **21** which displayed the greatest potency in the enzymatic assays (IC₅₀ 0.37 ± 0.01 and 1.50 ± 0.28 μM vs ChT-L and FAS-TE, respectively) and β-lactone **25** which displayed the greatest cellular cytotoxicity ((IC₅₀ 10.0 μM) for these studies. The cell viability assay was run in DMEM buffer (pH 7.5) containing 5% fetal bovine serum (FBS) and for comparison, stability studies were also run in DMEM only. The half-lives of β-lactone inhibitors in buffer with and without added serum was studied through HPLC monitoring over time. In the case of α-monosubstituted β-lactone **21**, the impact of added

Table 2

Serum stability studies ($t_{1/2}$) with an α -monosubstituted **21** versus an α -disubstituted **25** β -lactone in DMEM with and without serum (FBS).^a

β -lactone inhibitor ^a	DMEM (pH = 7.5)	DMEM + 5% FBS ^a
21	20	<3
25	70	120

^a Stability studies were run in triplicate. DMEM = Dulbecco's modified eagle medium; FBS = fetal bovine serum.

serum is dramatic leading to a change in half-life from an already brief 20 min in buffer to <3 min with added serum (Table 2). However, the stability of the quaternary α,α -disubstituted- β -lactone **25** did not follow the same trend. The half-life of β -lactone **25** in DMEM buffer alone was nearly 3X that of the α -monosubstituted ($t_{1/2} \sim 70$ min). Furthermore, the presence of serum led to a dramatically extended half-life ($t_{1/2} \sim 120$ min) for this β -lactone and is consistent with improved stability of labile molecules often observed due to interaction of hydrophobic molecules with serum proteins protecting them from the somewhat basic medium.²⁸ Furthermore, the hydrolysis products of several α -monosubstituted- β -lactones were observed by LC-MS analysis despite the purity indicated by NMR analysis and this was observed most often with α -monosubstituted β -lactones as expected. Thus, it is reasonable to deduce that α,α -disubstituted β -lactones are generally better drug leads since their increased cytotoxicity may be a reflection of their greater stability to hydrolysis and would be expected to have a longer half-life in the blood stream. These findings are consistent with observations made in recent studies with belactosin A derivatives bearing α,α -disubstituted β -lactones with the exception that increased stability was not observed in the presence of human AB serum.^{27a}

2.6. Dual target validation via activity-based protein profiling

To determine if cytotoxicity observed in cancer cells might be due to dual inhibition of both FAS-TE and the proteasome, we

conducted activity-based protein profiling (ABPP) experiments with cell lysates.²⁹ Previous whole cell ABPP studies led to orlistat as an inhibitor of FAS.^{18h} We sought to verify that both FAS-TE and the proteasome were indeed labeled in a sensitive cancer cell line with the designed activity-based probe **28**, which is based on the most potent dual inhibitor **21** in terms of enzymatic inhibition. Activity-based probe **28** includes an alkyne group in the *para*-position of the benzyl ester moiety for subsequent Sharpless-Huisgen cycloaddition following incubation with a proteome. Given their potency in enzymatic and cell-based assays, dual inhibitors **21** and **25** were selected for competition experiments to validate selectivity if any.³⁰ Satisfyingly, probe **28** retained good activity compared to the parent inhibitor **21** with slightly improved activity to the ChT-L site of the proteasome and ~ 2.5 X drop in activity toward FAS-TE suggesting no major adverse effects of the alkyne tag (Table 1). Both MCF-7 (breast adenocarcinoma) and HeLa (cervix adenocarcinoma) cells were incubated *in situ* with varying concentrations (0 $\rightarrow$ 50 μ M) of probe **28** for 1 h at 37 °C. The labeling was followed by cell lysis and attachment of the TAMRA fluorophore under copper (I) catalyzed Sharpless-Huisgen cycloaddition conditions.³¹ SDS-PAGE and in-gel fluorescence scanning were employed to separate and detect labeled proteins, respectively (Fig. 3A). Importantly, labeling of several protein targets could be observed with dominant bands at molecular weights of ca. 250, 50, and 20 kDa. To validate the identity of these proteins, we applied western blot analysis with anti-FAS and anti-CT-L (PSB-5) antibodies. The fluorescent bands at 250 kDa and 20 kDa overlapped with the corresponding antibody signals suggesting that these two protein bands represent the expected FAS and CT-L targets (Fig. 3B). Finally, we first added the dual inhibitors **21** and **25** in various concentrations (0 $\rightarrow$ 100 μ M) to MCF-7 cells prior to probe addition (Fig. 3C). Subsequent labeling with probe **28** revealed a clear concentration dependent reduction in signal intensity only for **21** emphasizing that this inhibitor competes with the ABPP probe **28** for the same active sites. FAS was less sensitive to this competition (10-fold excess resulted in signal reduction) compared to ChT-L (2.5-fold excess resulted in signal reduction) which is in line with the lower

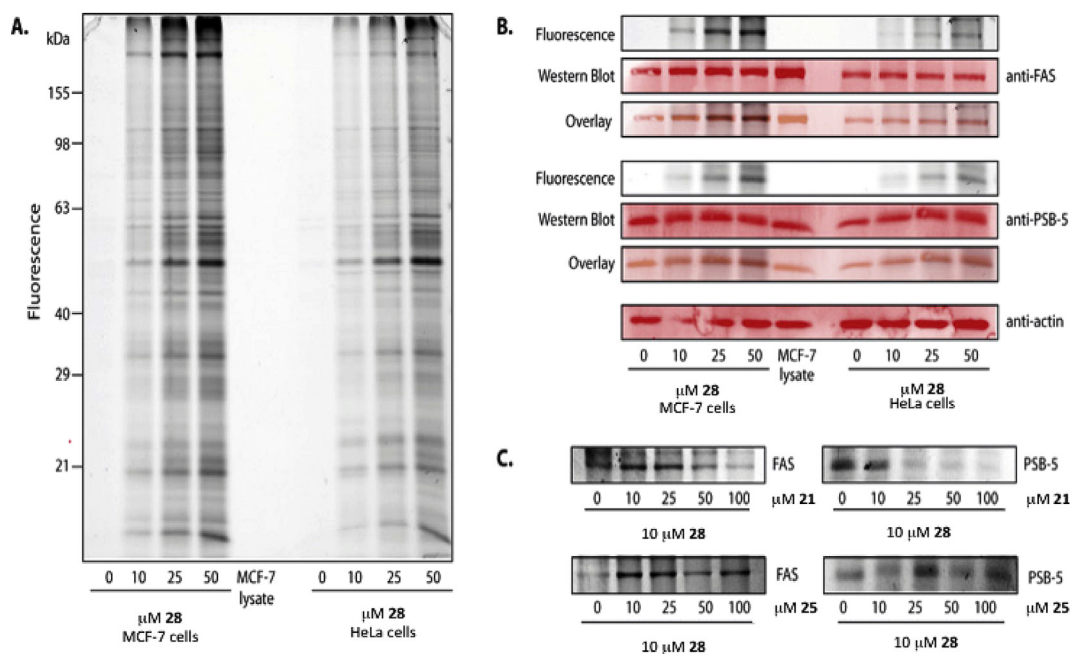


Fig. 3. (A) *In situ* ABPP labeling of MCF-7 and HeLa cells with increasing concentrations of alkyne ABPP probe **28**. (B) Identification of FAS and proteasome beta-5 by Western Blotting using specific anti-FAS and anti-PSB-5 antibodies. (C) Competitive labeling with inhibitors **21**, **25** and ABPP probe **28**. (PSB-5: proteasome subunit beta type, FAS: Fatty acid synthase).

IC₅₀ of **21** for FAS. On the other hand, inhibitor **25** was not able to compete effectively with labeling by probe **28** even at 10-fold excess of the compound. This is likely due to slower reaction rates of this inhibitor with active site nucleophiles (i.e. serine and threonine hydroxyl groups) due to the presence of the more hindered, α -disubstituted β -lactone and the short (30 min) pre-incubation time utilized prior to adding the ABPP probe **28**. This observation is consistent with findings made during enzymatic assays with inhibitor **25** which required longer incubation times to achieve comparable IC₅₀ values to other α -monosubstituted β -lactones and also the greater stability to buffer/serum (120 vs <3 min) leading to greater whole cell cytotoxicity for inhibitor **25** compared to **21** (10.0 vs 45.8 μ M, respectively).

3. Conclusions

Several dual inhibitors of the proteasome and fatty acid synthase were designed and synthesized based on the original structures of orlistat and belactosin C. Exploiting the *trans*-disubstituted β -lactone pharmacophore which covalently binds to these two enzyme active sites and modifying the dipeptide, which was optimized based on analysis of the X-ray structures of proteasome-homobelactosin C and FAS-TE-orlistat complexes and structure-activity relationships, we successfully identified a dual inhibitor **21** with greater enzymatic inhibition to both targets (ChT-L: IC₅₀ = 0.37 μ M; FAS IC₅₀ = 1.5 μ M; HeLa cells IC₅₀ = 45.8 μ M) compared to our original lead compound **17**. In addition, an α -disubstituted β -lactone dual inhibitor **25** displayed the highest potency in a cell-based cytotoxicity assay (HeLa cells IC₅₀ = 10.0 μ M) which along with serum stability studies suggest greater hydrolytic stability of this β -lactone with attendant improved cell-based activity. We confirmed targeting of the proteasome and FAS-TE in HeLa cells through activity based-protein profiling with an alkyne probe modeled after our best enzymatic inhibitor **21**. These studies provide proof of concept for the potential of dual inhibition of FAS-TE and the proteasome with orlistat-belactosin hybrid molecules. In addition, it raises the possibility that previously described belactosin derivatives should be analyzed for cross-reactivity with FAS-TE. The ability to target an enzyme that is a validated target for chemotherapy, namely the proteasome, and an enzyme that has attracted great interest for cancer chemotherapy, namely FAS-TE, by a single molecule provides proof-of-principle for targeting both these enzymes with β -lactone-based dual inhibitors.

4. Experimental section

4.1. General information

All reactions were carried out under a nitrogen atmosphere in oven-dried glassware. Acetonitrile, dichloromethane, tetrahydrofuran, and methanol were purified by passage through activated molecular sieves or alumina (solvent drying system). DMF was dried over molecular sieves before use. All commercial reagents were used as received. ¹H and ¹³C NMR spectra were recorded on INOVA-500. ¹H NMR chemical shifts are reported as δ values in ppm relative to CDCl₃ (7.26 ppm), coupling constants (*J*) are reported in Hertz (Hz), and multiplicity follows convention. Unless indicated otherwise, deuteriochloroform (CDCl₃) served as an internal standard (77.2 ppm) for all ¹³C spectra. Flash column chromatography was performed using 60 Å silica gel as a stationary phase using a gradient solvent system (EtOAc/*n*-hexane as eluent unless specified otherwise). Mass spectra were obtained at the Center for Chemical Characterization and Analysis (Texas A&M University). Thin layer chromatography (TLC) was performed using

glass-backed silica gel 60F₂₅₄. LC/MS was carried out using an ion trap HPLC/MS instrument with an Agilent Poroshell EC-C-18 2.7 μ m column (50 $\times$ 3.0 mm), eluting with a gradient of 5% acetonitrile/95% water $\rightarrow$ 95% acetonitrile/5% water over 12 min or 20 min. Signals were detected with UV 254 nm and MS ion trap (ionization modes: negative or positive APCI or positive or negative ESI, scan range 100–1000 a.m.u.). Synthetic details and characterization data for thioester (**S3**) required for the preparation of ketene acetal **10b** are provided in the SI. Aldehyde **9** was prepared according to our previously described procedure.^{22a} Dipeptide **15c** was prepared according to the previously published procedure and data matched that previously reported.^{22a} Commercially available protected amino acids, **13a**, **13b**, **13c**, **13d**, **13e**, **14b** were purchased and used as received. Protected amino acids, **14a**^{23b} and 4-ethynylbenzyl (*tert*-butoxycarbonyl)-L-alanine³² were prepared based on known procedures.

4.1.1. (*E/Z*)-2-((1-((Triethylsilyl)oxy)oct-1-en-1-yl)thio)pyridine (**10a**)

The synthesis was performed as previously described²⁶ and related to a published procedure.^{22a} To a solution of *S*-(pyridin-2-yl) octanethioate³³ (5.30 g, 22.7 mmol, 1 equiv), DMF (2.11 mL, 27.2 mmol, 1.2 equiv) and Et₃N (3.78 mL, 27.2, 1.2 equiv) in 100 mL of DCM was added LiHMDS (1 M in THF, 52.3 mL, 52.3 mmol, 2.3 equiv) at -78°C under N₂. After stirring for 30 min, TESCl (7.69 mL, 45.4 mmol, 2 equiv) was added. The mixture was continued to stir at -78°C for another 2 h. The reaction was quenched with pH 7 buffer (40 mL) and the organic layer was separated, dried over MgSO₄ and concentrated. The residue was purified by flash chromatography (SiO₂, hexanes:MTBE = 30:1) to give ketene acetal **10a** as a yellow oil (5.1 g, 64%, $\sim$ 13:1 ratio of *E/Z* geometrical isomers). Data provided for major *E*-olefin isomer: ¹H NMR (500 MHz, CDCl₃) δ 8.42–8.41 (m, 1H), 7.56–7.52 (m, 1H), 7.34 (d, *J* = 8.0 Hz, 1H), 7.01–6.98 (m, 1H), 5.38 (t, *J* = 7.3 Hz, 1H), 2.18 (q, *J* = 7.3 Hz, 2H), 1.44–1.23 (m, 11H), 0.90–0.84 (m, 9H), 0.67–0.62 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 160.6, 149.6, 139.4, 136.7, 124.5, 121.6, 119.8, 31.8, 29.2, 27.0, 22.8, 14.3, 6.7 (2C), 6.0, 5.3 (2C).

4.1.2. Ketene acetal **10b**

To a solution of LiHMDS in THF (1 M, 8.76 mL, 2.0 equiv) was added DMF (406 μ L, 5.26 mmol, 1.2 equiv) at -78°C followed by TESCl (742 μ L, 4.38 mmol, 1 equiv). After stirring for 10 min, a solution of thioester **S3** (1.10 g, 4.38 mmol, 1.0 equiv) in 40 mL of THF was added and the solution was stirred for another 5 h at the same temperature. The reaction was quenched by aq. satd. NH₄Cl solution, and was extracted with MTBE (3 $\times$ 50 mL). The combined organic layers were washed with brine, dried over anhydrous MgSO₄ and concentrated in vacuo. The residue was purified by flash chromatography (SiO₂, hexanes:MTBE = 100:1 $\rightarrow$ 50:1) to give **10b** as an $\sim$ 1:1 mixture of *E*- and *Z*-isomers (1.01 g, 63%). ¹H NMR (300 MHz, CDCl₃) δ 8.43 (m, 1H), 7.54 (td, *J* = 7.9, 2.0 Hz, 1H), 7.26 (t, *J* = 7.9 Hz, 1H), 7.00 (m, 1H), 2.29 (m, 2H), 1.89/1.86 (s, 3H), 1.36–1.24 (m, 8H), 1.03–0.86 (m, 12H), 0.69–0.60 (m, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 200.9 181.7, 151.8, 150.3, 137.4, 130.4, 123.6, 105.2, 34.2, 31.8, 29.4, 27.3, 22.8, 17.7, 14.3, 7.0 (3C), 6.6 (3C).

4.1.3. Tandem Mukaiyama Aldol-Lactonization: synthesis of β -lactone esters **11** and **11a'**

ZnCl₂ (1.47 g, 10.82 mmol, 2 equiv) was heated to fuse under vacuum and was cooled to ambient temperature, to which a solution of aldehyde **9** (2.08 g, 5.41 mmol, 1.0 equiv) in 50 mL of CH₂Cl₂ was added. After stirring at ambient temperature (23 $^\circ\text{C}$) for 10 min, a solution of ketene acetal **10a** (2.09 g, 5.95 mmol, 1.1 equiv) in 10 mL of CH₂Cl₂ was added. After 30 min the dark suspension was diluted with MTBE (100 mL), washed with H₂O

(5 mL) and brine (5 mL), and dried over MgSO_4 . After concentration the residue was purified by flash chromatography (SiO_2 , hexanes:MTBE = 5:1) to obtain a diastereomeric mixture of **11a** and **11a'**. The diastereomeric mixture was further separated by a second flash chromatography (SiO_2 , hexanes:DCM:MTBE = 30:10:1) to give **11a** (1.13 g, 41%) and **11a'** (530 mg, 19%) as colorless oils. Data for **11a**: R_f = 0.33 (hexanes:MTBE = 5:1), $[\alpha]_D^{23}$ = 22 (c = 1.0, CHCl_3). ^1H NMR (500 MHz, CDCl_3) δ 7.34–7.32 (m, 3H), 7.30–7.27 (m, 3H), 7.14–7.12 (m, 2H), 7.09–7.07 (m, 2H), 5.16 (d, J = 5.3 Hz, 1H), 4.60 (d, J = 4.4 Hz, 1H), 4.17 (dd, J = 6.8, 5.4 Hz, 1H), 4.04 (t, J = 6.7 Hz, 1H), 3.73 (d, J = 6.2 Hz, 1H), 3.63–3.59 (m, 1H), 3.16 (s, 3H), 1.93–1.78 (m, 2H), 1.46 (s, 3H), 1.45 (s, 3H), 1.45–1.41 (m, 2H), 1.35–1.26 (m, 6H), 0.90 (t, J = 6.9 Hz, 3H); ^{13}C NMR (75 MHz, CDCl_3) δ 169.1, 167.2, 137.3, 136.0, 129.1, 128.89 (2C), 128.83 (2C), 128.79, 127.8 (2C), 127.5 (2C), 111.1, 84.4, 80.8, 79.4, 77.1, 71.5, 57.7, 57.0, 31.6, 28.8, 27.84, 27.78, 27.5, 26.8, 22.7, 14.2. LRMS (ESI^+): Calcd. For $\text{C}_{30}\text{H}_{38}\text{O}_7\text{Na}$ ($[\text{M}+\text{Na}]^+$), 533.3. Found: 533.9. Data for **11a'**: R_f = 0.33 (hexanes:MTBE = 5:1), $[\alpha]_D^{23}$ = −0.50 (c = 1.0, CHCl_3). ^1H NMR (500 MHz, CDCl_3) δ 7.34–7.32 (m, 3H), 7.31–7.27 (m, 3H), 7.15–7.11 (m, 4H), 5.19 (d, J = 5.4 Hz, 1H), 4.59 (d, J = 4.3 Hz, 1H), 4.18 (dd, J = 7.0, 5.4 Hz, 1H), 4.03 (dd, J = 7.0, 6.2 Hz, 1H), 3.71 (d, J = 6.2 Hz, 1H), 3.68 (ddd, J = 8.7, 6.6, 4.3 Hz, 1H), 3.15 (s, 3H), 1.94–1.79 (m, 2H), 1.47 (s, 3H), 1.45 (s, 3H), 1.34–1.26 (m, 8H), 0.89 (t, J = 6.9 Hz, 3H); ^{13}C NMR (75 MHz, CDCl_3) δ 169.2, 167.2, 137.3, 136.0, 129.1, 128.83 (2C), 128.80 (2C), 128.75, 127.7 (2C), 127.5 (2C), 111.0, 84.2, 80.7, 79.4, 77.1, 71.5, 57.6, 57.0, 31.5, 28.8, 27.8, 27.7, 27.4, 26.7, 22.6, 14.2. LRMS (ESI^+): Calcd. For $\text{C}_{30}\text{H}_{38}\text{O}_7\text{Na}$ ($[\text{M}+\text{Na}]^+$), 533.3. Found: 533.9.

4.1.4. Tandem Mukaiyama Aldol-Lactonization: synthesis of β -lactone esters **11b**, **11b'**, **11b''** and **11b'''**

ZnCl_2 (559 mg, 4.10 mmol, 2.5 equiv) was fused by heating under vacuum and then cooled to ambient temperature and then 0 °C. A solution of aldehyde **9** (630 mg, 1.64 mmol, 1.0 equiv) in 5 mL of CH_2Cl_2 was added. After stirring for 10 min, a solution of ketene acetal **10b** (599 mg, 1.64 mmol, 1 equiv) in 5 mL of CH_2Cl_2 was added. The mixture was stirred at ambient temperature (23 °C) for 15 h and then the reaction was quenched by adding water (5 mL). The mixture was poured into 125 mL of MTBE and the organic layer was washed with brine. After drying over MgSO_4 , the organic solution was concentrated. The residue was purified by a flash chromatography (SiO_2 , hexanes:MTBE = 5:1) to isolate the *trans*-diastereomers (containing **11b** and **11b'**) and the *cis*-diastereomers (containing **11b''** and **11b'''**). The *trans*-diastereomers were submitted to a 2nd flash chromatographic separation (SiO_2 , hexanes: CH_2Cl_2 :MTBE = 5:4:0.08) to isolate **11b** and **11b'**. A 2nd flash chromatographic separation enabled separation of the *syn*-diastereomers **11b''** and **11b'''** (SiO_2 , hexanes: CH_2Cl_2 :MTBE = 20:20:1).

4.1.5. (R)-((4S,5S)-5-((R)-methoxy(phenyl)methyl)-2,2-dimethyl-1,3-dioxolan-4-yl)(phenyl)methyl (3S, 4R)-3-hexanoyl-3-methyl-4-oxooxetane-2-carboxylate (**11b**)

Obtained as a colorless oil (72 mg, 9%). $[\alpha]_D^{25}$ + 59 (c = 1.0, CHCl_3). ^1H NMR (500 MHz, CDCl_3) δ 7.35–7.27 (m, 6H), 7.15–7.11 (m, 4H), 5.21 (d, J = 5.8 Hz, 1H), 4.69 (s, 1H), 4.22 (dd, J = 6.8, 5.8 Hz, 1H), 4.00 (t, J = 6.3 Hz, 1H), 3.68 (d, J = 6.0 Hz, 1H), 3.16 (s, 3H), 1.80–1.77 (m, 2H), 1.48 (s, 3H), 1.47 (s, 3H), 1.39–1.30 (m, 8H), 1.22 (s, 3H), 0.91 (t, J = 6.6 Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.7, 166.64, 137.34, 136.06, 129.11, 128.81 (2C), 128.74 (2C), 128.72, 127.79 (2C), 127.70 (2C), 110.99, 84.13, 80.91, 79.15, 76.94, 75.48, 62.26, 56.95, 35.31, 31.63, 29.30, 27.76, 27.55, 24.43, 22.63, 15.73, 14.17. LRMS (ESI^+): Calcd. For $\text{C}_{31}\text{H}_{40}\text{O}_7\text{Na}$ ($[\text{M}+\text{Na}]^+$), 547.3. Found: 547.4.

4.1.6. (R)-((4S,5S)-5-((R)-methoxy(phenyl)methyl)-2,2-dimethyl-1,3-dioxolan-4-yl)(phenyl)methyl (3R, 4S)-3-hexyl-3-methyl-4-oxooxetane-2-carboxylate (**11b'**)

Obtained as a colorless oil (72 mg, 9%). $[\alpha]_D^{25}$ + 43 (c = 1.0, CHCl_3). ^1H NMR (500 MHz, CDCl_3) δ 7.34–7.25 (m, 6H), 7.12–7.10 (m, 4H), 5.25 (d, J = 5.8 Hz, 1H), 4.70 (s, 1H), 4.22 (dd, J = 6.6, 5.8 Hz, 1H), 4.02 (t, J = 6.2 Hz, 1H), 3.71 (d, J = 6.2 Hz, 1H), 3.15 (s, 3H), 1.76–1.72 (m, 2H), 1.47 (s, 6H), 1.35–1.25 (m, 8H), 1.01 (s, 3H), 0.90 (t, J = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.61, 166.68, 137.28, 136.03, 129.15, 128.85 (2C), 128.73, 128.68 (2C), 127.93 (2C), 127.76 (2C), 111.14, 84.27, 80.98, 79.20, 77.08, 75.31, 62.26, 56.97, 35.22, 31.66, 29.30, 27.87, 27.59, 24.45, 22.63, 15.46, 14.18. LRMS (ESI^+): Calcd. For $\text{C}_{31}\text{H}_{40}\text{O}_7\text{Na}$ ($[\text{M}+\text{Na}]^+$), 547.3. Found: 547.4.

4.1.7. (R)-((4S,5S)-5-((R)-methoxy(phenyl)methyl)-2,2-dimethyl-1,3-dioxolan-4-yl)(phenyl)methyl (3S, 4S)-3-hexyl-3-methyl-4-oxooxetane-2-carboxylate (**11b''**)

Obtained as a colorless oil (125 mg, 14%). $[\alpha]_D^{25}$ + 66 (c = 1.0, CHCl_3). ^1H NMR (500 MHz, CDCl_3) δ 7.35–7.26 (m, 6H), 7.15–7.11 (m, 4H), 5.19 (d, J = 5.5 Hz, 1H), 4.60 (s, 1H), 4.22 (dd, J = 6.9, 5.5 Hz, 1H), 4.01 (t, J = 6.4 Hz, 1H), 3.70 (d, J = 6.1 Hz, 1H), 3.16 (s, 3H), 1.52 (s, 3H), 1.49 (s, 3H), 1.48 (s, 3H), 1.40–1.30 (m, 2H), 1.27–1.20 (m, 4H), 1.16–1.03 (m, 4H), 0.85 (t, J = 7.2 Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.51, 166.43, 137.36, 136.04, 129.10, 128.82 (2C), 128.72 (2C), 128.66, 127.90 (2C), 127.71 (2C), 111.12, 84.17, 80.90, 79.19, 77.63, 77.17, 61.83, 56.93, 31.61, 31.44, 29.42, 27.87, 27.51, 23.88, 22.63, 19.59, 14.10. LRMS (ESI^+): Calcd. For $\text{C}_{31}\text{H}_{40}\text{O}_7\text{Na}$ ($[\text{M}+\text{Na}]^+$), 547.3. Found: 547.4.

4.1.8. (R)-((4S,5S)-5-((R)-methoxy(phenyl)methyl)-2,2-dimethyl-1,3-dioxolan-4-yl)(phenyl)methyl (3R, 4R)-3-hexyl-3-methyl-4-oxooxetane-2-carboxylate (**11b'''**)

Obtained as a colorless oil (125 mg, 14%). $[\alpha]_D^{25}$ + 48 (c = 1.0, CHCl_3). ^1H NMR (500 MHz, CDCl_3) δ 7.34–7.24 (m, 6H), 7.15–7.09 (m, 4H), 5.31 (d, J = 5.9 Hz, 1H), 4.62 (s, 1H), 4.26 (t, J = 6.3 Hz, 1H), 4.001 (t, J = 6.3 Hz, 1H), 3.67 (d, J = 6.0 Hz, 1H), 3.14 (s, 3H), 1.494 (s, 3H), 1.486 (s, 3H), 1.47 (s, 3H), 1.30–1.27 (m, 2H), 1.20–1.12 (m, 4H), 1.04–0.97 (m, 4H), 0.82 (t, J = 7.3 Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.40, 166.46, 137.35, 135.83, 129.16, 128.80 (2C), 128.71 (2C), 128.64, 128.08 (2C), 127.74 (2C), 111.16, 84.07, 81.06, 79.10, 77.45, 77.32, 61.79, 56.97, 31.60, 31.34, 29.37, 27.90, 27.61, 23.88, 22.62, 19.67, 14.10. LRMS (ESI^+): Calcd. For $\text{C}_{31}\text{H}_{40}\text{O}_7\text{Na}$ ($[\text{M}+\text{Na}]^+$), 547.3. Found: 547.4.

Representative procedure for hydrogenolytic cleavage of the chiral auxiliary: Synthesis of (3S, 4R)-3-hexyl-4-oxooxetane-2-carboxylic acid (**12a**).

In a round-bottomed flask was added Pd/C (80 mg, 10% w/w, 0.076 mmol, 0.09 equiv) and the flask was evacuated by vacuum for ~30 min, and then refilled with N_2 . A THF solution of **11a** (429 mg, 0.840 mmol, 1 equiv, 0.05 M) was added. The flask was charged with a hydrogen balloon and the reaction was kept at ambient temperature (23 °C) for 15 h with stirring. The mixture was filtered through a short Celite pad and washed with EtOAc (5 mL). After concentration the crude product was purified by silica gel flash chromatography (hexanes:MTBE = 2:1) to give **12a** as a colorless oil (116 mg, 69%). $[\alpha]_D^{23}$ − 20 (c = 5.8, CHCl_3). ^1H NMR (300 MHz, CDCl_3) δ 10.19 (brs, 1H), 4.65 (d, J = 4.4 Hz, 1H), 3.82 (ddd, J = 8.7, 6.7, 4.3 Hz, 1H), 2.01–1.80 (m, 2H), 1.54–1.23 (m, 8H), 0.88 (t, J = 6.8 Hz, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ 173.8, 168.9, 71.0, 58.2, 31.6, 28.8, 27.9, 26.7, 22.6, 14.2. HRMS (ESI^-): Calcd. for $\text{C}_{10}\text{H}_{15}\text{O}_4$ ($[\text{M}-\text{H}]^-$), 199.0976. Found: 199.0982.

4.1.9. (3S, 4R)-3-Hexyl-4-oxooxetane-2-carboxylic acid (**12a'**)

Based on the representative hydrogenolysis procedure, the enantiomeric β -lactone carboxylic acid **12a'** was obtained as a

colorless oil (135 mg, 66%). $[\alpha]_D^{25} + 18$ ($c = 6.8$, CHCl_3). ^1H NMR and ^{13}C NMR spectra were identical to that of **12a**. HRMS (ESI⁺): Calcd. For $\text{C}_{10}\text{H}_{15}\text{O}_4$ ($[\text{M}-\text{H}]^-$), 199.0976. Found: 199.0963.

4.1.10. (3S, 4R)-3-hexyl-3-methyl-4-oxooxetane-2-carboxylic acid (**12b**)

According to the representative hydrogenolysis procedure, anti- β -lactone acid **12b** was prepared from ester **11b** as a colorless oil (20.5 mg, 75%). $[\alpha]_D^{25} + 11.6$ ($c = 2.1$, CHCl_3). ^1H NMR (500 MHz, CDCl_3) δ 8.49 (brs, 1H), 4.75 (s, 1H), 1.83–1.80 (m, 2H), 1.53–1.46 (m, 1H), 1.36 (s, 3H), 1.33–1.24 (m, 7H), 0.89 (t, $J = 7.0$ Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.52, 172.36, 75.2, 62.45, 35.24, 31.66, 29.32, 24.47, 22.67, 15.81, 14.19. HRMS (ESI⁺): Calcd. For $\text{C}_{11}\text{H}_{17}\text{O}_4$ ($[\text{M}-\text{H}]^-$), 213.1132. Found: 213.1131.

4.1.11. (3R, 4S)-3-hexyl-3-methyl-4-oxooxetane-2-carboxylic acid (**12b'**)

According to the general hydrogenolysis procedure, the enantiomeric anti- β -lactone **12b'** was prepared from ester **11b'** as a colorless oil (12.8 mg, 68%). $[\alpha]_D^{25} - 10.5$ ($c = 1.3$, CHCl_3). The ^1H NMR and ^{13}C NMR were identical to the enantiomeric anti- β -lactone acid **12b**. HRMS (ESI⁺): Calcd.

4.1.12. (3R, 4R)-3-hexyl-3-methyl-4-oxooxetane-2-carboxylic acid (**12b''**)

According to the general hydrogenolysis procedure, the diastereomeric syn- β -lactone **12b''** was prepared as a colorless oil (16.7 mg, 64%). $[\alpha]_D^{25} - 3.7$ ($c = 1.7$, CHCl_3). ^1H NMR (500 MHz, CDCl_3) δ 8.49 (brs, 1H), 4.75 (s, 1H), 1.83–1.80 (m, 2H), 1.53–1.46 (m, 1H), 1.36 (s, 3H), 1.33–1.24 (m, 7H), 0.89 (t, $J = 7.0$ Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.52, 172.36, 75.2, 62.45, 35.24, 31.66, 29.32, 24.47, 22.67, 15.81, 14.19. HRMS (ESI⁺): Calcd. For $\text{C}_{11}\text{H}_{17}\text{O}_4$ ($[\text{M}-\text{H}]^-$), 213.1132. Found: 213.1131.

The relative configuration of β -lactone acids **12a**, **12a'**, **11b**, **11b'**, **11b''**, **11b'''** was assigned based on coupling constant analysis or NOE correlations (via ROESY experiments). The absolute configuration was assigned by comparison of optical rotations to known, related β -lactones.^{27e,34} See [Supporting Information](#) for details.

4.1.13. 4-Ethynylbenzyl-L-ala (**14c**)

To the solution of 4-ethynylbenzyl (*tert*-butoxycarbonyl)-L-alaninate³² (1.00 g, 3.30 mmol) in 12 mL of DCM was slowly added TFA (1.5 mL) at 0 °C. The solution was kept in a 4 °C refrigerator for 40 h. After concentration the crude product was purified on silica gel chromatography (DCM:MeOH = 20:1) to give the product as a yellow oil (880 mg, 89%) in the form of a TFA salt. ^1H NMR (500 MHz, CDCl_3) δ 8.43 (brs, 3H), 7.44 (d, $J = 8.1$ Hz, 2H), 7.23 (d, $J = 8.1$ Hz, 2H), 5.14 (d, $J = 12.5$ Hz, 1H), 5.08 (d, $J = 12.5$ Hz, 1H), 4.08 (q, $J = 7.2$ Hz, 1H), 3.09 (s, 1H), 1.55 (d, $J = 7.2$ Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 170.2, 135.1, 132.6, 128.2, 122.8, 83.1, 78.2, 67.9, 49.3, 15.9. HRMS (ESI⁺): Calcd. for $\text{C}_{12}\text{H}_{14}\text{N}_2\text{O}^+$ ($[\text{M}]^+$), 204.1019. Found: 204.1023.

4.1.14. Cbz-Ala-BnO-Lys(Boc) dipeptide (**15a**)

Based on the literature procedure,^{23b} N-Cbz-Ala (1.1 g, 4.92 mmol) (**13a**) coupled with amine **14a** (1.51 g, 4.47 mmol) to give the known dipeptide **15a** (2.12 g, 87% yield) as a white solid. The characterization data are identical to the literature.^{23b}

4.1.15. Cbz-Gly-BnO-Lys(Boc) dipeptide (**15b**)

N-Cbz-Gly (**13b**) (1.03 g, 4.92 mmol) was dissolved in dry CH_2Cl_2 (20 mL) in a dry, round-bottomed flask under a positive atmosphere of nitrogen at 22 °C and then the solution was cooled to 0 °C. The EDAC (1.03 g, 5.37 mmol), HOBT (0.726 g, 5.37 mmol) and *i*-Pr₂NEt (2.34 mL, 13.43 mmol) were then added sequentially and the resulting solution was stirred at 0 °C for 30 min. BnO-Lys

(Boc) (**14a**) (1.51 g, 4.47 mmol) was then added via cannula dissolved in dry CH_2Cl_2 (10 mL). After the addition was complete, the reaction mixture was allowed to warm to 22 °C and stirred at this temperature for 4 h. The reaction was quenched by addition of water (30 mL), the layers were separated, and the organic fraction was dried over Na_2SO_4 , filtered and concentrated under reduced pressure. The crude mixture was purified by MPLC (SiO_2 , gradient: 100% hexane $\rightarrow$ 100% EtOAc) to afford the desired dipeptide (2.01 g, 85% yield) as a viscous colorless oil. ^1H NMR (500 MHz, CDCl_3) δ 7.36–7.30 (m, 10H), 6.89 (bs, 1H), 5.75 (bs, 1H), 5.18 (d, $J = 12.0$ Hz, 1H), 5.12 (d, $J = 12.0$ Hz, 1H), 5.11 (s, 2H), 4.72 (bs, 1H), 4.64–4.60 (m, 1H), 3.91 (d, $J = 5.0$ Hz, 2H), 3.05–2.96 (m, 2H), 1.87–1.78 (m, 1H), 1.70–1.62 (m, 1H), 1.46–1.38 (m, 2H), 1.42 (s, 9H), 1.29–1.22 (m, 2H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.16, 169.19, 156.79, 156.26, 136.33, 135.37, 128.78, 128.66 (2C), 128.45 (2C), 128.31, 128.19, 79.27, 67.34, 67.26, 52.23, 44.54, 40.11, 31.81, 29.55, 28.57 (3C), 22.39. FT-IR (neat, cm^{-1}): 3326, 2934, 1682, 1520. FT-IR (neat, cm^{-1}): 3326, 2934, 1682, 1520. HRMS (ESI⁺): Calcd. for $\text{C}_{28}\text{H}_{38}\text{N}_3\text{O}_7$ ($[\text{M}+\text{H}]^+$), 528.2710. Found: 528.2681.

4.1.16. 4-BrBzAla-BnO-Lys(Boc) dipeptide (**15d**)

4-BrBz-Ala-OH (**13c**) (0.536 g, 1.97 mmol) was dissolved in dry CH_2Cl_2 (10 mL) in a round-bottomed flask under positive atmosphere of nitrogen at 22 °C then the solution was cooled to 0 °C. EDAC (0.412 g, 2.15 mmol), HOBT (0.290 g, 2.15 mmol), and *i*-Pr₂NEt (0.935 mL, 5.37 mmol) were then added sequentially and the resulting solution was stirred at 0 °C for 30 min. BnO-Lys(Boc) (**14a**) (0.602 g, 1.79 mmol) was then added via cannula dissolved in dry CH_2Cl_2 (5 mL). After the addition was completed, the reaction mixture was allowed to warm to 22 °C and stirred at this temperature for 12 h. To quench the reaction, the solution was diluted with deionized water (15 mL), the organic fraction was dried over MgSO_4 , filtered and concentrated under reduced pressure. The crude was purified by MPLC (SiO_2 , gradient: 100% hexane $\rightarrow$ 100% EtOAc) to afford **15d** (0.326 g, 31% yield) as a white solid and as a 2:1 mixture of rotamers. Data for the major rotamer: ^1H NMR (500 MHz, CDCl_3) δ 7.65 (d, $J = 8.5$ Hz, 2H), 7.54 (d, $J = 8.5$ Hz, 2H), 7.35–7.29 (m, 5H), 7.18 (d, $J = 8.0$ Hz, 1H), 7.10 (d, $J = 4.5$ Hz, 1H), 5.16 (d, $J = 12.0$ Hz, 1H), 5.08 (d, $J = 12.0$ Hz, 1H), 4.81–4.74 (m, 1H), 4.66–4.59 (m, 2H), 3.09–2.97 (m, 2H), 1.90–1.84 (m, 1H), 1.76–1.69 (m, 1H), 1.49 (d, $J = 7.0$, 3H) 1.47–1.40 (m, 2H), 1.44 (s, 9H), 1.36–1.26 (m, 2H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.49, 172.04, 166.40, 156.29, 135.37, 132.77, 131.96 (2C), 128.94 (2C), 128.79 (2C), 128.69 (2C), 128.46, 126.67, 79.38, 67.37, 52.38, 49.37, 40.10, 31.79, 29.60, 28.62 (3C), 22.52, 18.89. FT-IR (neat, cm^{-1}): 3417, 1642, 1535, 1171. HRMS (ESI⁺): Calcd. For $\text{C}_{28}\text{H}_{37}\text{BrN}_3\text{O}_6$ ($[\text{M}+\text{H}]^+$), 590.1866. Found: 590.1895.

4.1.17. BnO-Ala-Cbz-Lys(Boc) dipeptide (**15e**)

Cbz-Lys(Boc)-OH (**13d**) (2.0 g, 5.26 mmol) was dissolved in dry CH_2Cl_2 (20 mL) in a dry round-bottomed flask under a positive atmosphere of nitrogen at 22 °C and then the solution was cooled to 0 °C. The EDAC (1.21 g, 6.31 mmol), HOBT (0.852 g, 6.31 mmol) and *i*-Pr₂NEt (2.75 mL, 15.77 mmol) were then added sequentially and the resulting solution was stirred at 0 °C for 30 min. The H-Ala-OBn *p*-tosylate (**14b**) (2.03 g, 5.78 mmol) was then added via cannula as a solution in dry CH_2Cl_2 (10 mL). After the addition was completed, the reaction mixture was allowed to warm to 22 °C and stirred at this temperature for 4 h. To quench the reaction, the solutions was diluted with water (30 mL), the organic fraction was dried over Na_2SO_4 , filtered and concentrated under reduced pressure. The crude was purified by MPLC (SiO_2 , gradient: 100% hexane $\rightarrow$ 100% EtOAc) to afford the desired dipeptide **15e** (2.52 g, 89% yield) as a white solid. ^1H NMR (500 MHz, CDCl_3) δ 7.37–7.32 (m, 10H), 6.93 (bs, 1H), 5.68 (bs, 1H), 5.20 (d,

$J = 12.0$ Hz, 1H), 5.12 (d, $J = 12.0$ Hz, 1H), 5.08 (s, 2H), 4.80 (bs, 1H), 4.60 (p, $J = 7.0$ Hz, 1H), 4.24 (t, $J = 7.0$ Hz, 1H), 3.09–3.03 (m, 2H), 1.84–1.78 (m, 1H), 1.67–1.60 (m, 1H), 1.48–1.35 (m, 4H), 1.41 (s, 9H), 1.37 (d, $J = 7.0$ Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.67, 171.72, 156.39, 156.29, 136.33, 135.42, 128.72 (2C), 128.60 (2C), 128.54, 128.27 (2C), 128.24, 128.15 (2C), 79.17, 67.26, 67.08, 54.64, 48.25, 39.88, 32.37, 29.50, 28.55 (3C), 22.36, 18.01. FT-IR (neat, cm^{-1}): 3306, 2937, 1694, 1538. FT-IR (neat, cm^{-1}): 3306, 2937, 1694, 1538. HRMS (ESI+) Calcd. for $\text{C}_{29}\text{H}_{40}\text{N}_3\text{O}_7$ ($[\text{M}+\text{H}]^+$): 542.2866. Found: 542.2850.

4.1.18. BnO-Ala-Fmoc-Lys(Boc) dipeptide (**15f**)

Fmoc-Lys(Boc)-OH (**13e**) (2.0 g, 4.27 mmol) was dissolved in dry CH_2Cl_2 (20 mL) in a dry round-bottomed flask under a positive atmosphere of nitrogen at 22 °C and then the solution was cooled to 0 °C. The EDAC (0.982 g, 5.12 mmol), HOBT (0.692 g, 5.12 mmol) and *i*-Pr₂NEt (2.23 mL, 12.81 mmol) were then added sequentially and the resulting solution was stirred at 0 °C for 30 min. The H-Ala-OBn *p*-tosylate (**14b**) (1.65 g, 4.70 mmol) was then added via cannula as a solution in dry CH_2Cl_2 (10 mL). After the addition was complete, the reaction was allowed to warm to 22 °C and stirred at this temperature for 1 h. To quench the reaction, the solution was diluted with water (30 mL), the organic fraction was dried over Na_2SO_4 , filtered and concentrated under reduced pressure. The crude was purified by MPLC (SiO_2 , gradient: 100% hexane → 100% EtOAc) to afford the desired dipeptide **15f** (2.28 g, 85% yield) as a white solid. ^1H NMR (500 MHz, CDCl_3) δ 7.77 (d, $J = 7.5$ Hz, 2H), 7.60 (d, $J = 7.0$ Hz, 2H), 7.40 (t, $J = 7.5$ Hz, 2H), 7.36–7.29 (m, 7H), 6.73 (bs, 1H), 5.60 (bs, 1H), 5.20 (d, $J = 12.0$ Hz, 1H), 5.13 (d, $J = 12.0$ Hz, 1H), 4.73 (bs, 1H), 4.62 (p, $J = 7.0$ Hz, 1H), 4.38 (d, $J = 7.0$ Hz, 2H), 4.21 (t, $J = 7.0$ Hz, 2H), 3.15–3.03 (m, 2H), 1.89–1.82 (m, 1H), 1.70–1.63 (m, 1H), 1.48–1.39 (m, 4H), 1.44 (s, 9H), 1.42 (d, $J = 7.0$ Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.69, 171.57, 156.36, 144.02, 141.4, 135.43, 128.80 (2C), 128.64 (2C), 128.38 (2C), 128.33 (2C), 127.90 (2C), 127.26 (2C), 125.29 (2C), 120.16 (2C), 79.34, 77.43, 67.42, 54.72, 48.39, 47.30, 39.93, 32.45, 29.62, 28.63 (3C), 22.45, 18.24. FT-IR (neat, cm^{-1}): 3311, 2934, 1684, 1532. HRMS (ESI+) Calcd. for $\text{C}_{36}\text{H}_{44}\text{N}_3\text{O}_7$ ($[\text{M}+\text{H}]^+$): 630.3179. Found: 630.3203.

4.1.19. Boc-hydantoin (**15ff**)

BnO-Ala-Fmoc-Lys(Boc) dipeptide (**15f**) (0.5 g, 0.793 mmol) was dissolved in dry CH_2Cl_2 (5 mL) in a dry round-bottomed flask under a positive atmosphere of nitrogen at 22 °C and then the triphosgene was added (94.2 mg, 0.318 mmol). The solution was then cooled to 0 °C and then the DBU (0.475 mL, 3.18 mmol) was added dropwise. The reaction mixture was stirred at 0 °C for 3 h and then at 50 °C for 8 h. The mixture was then concentrated under reduced pressure and the crude residue was purified by MPLC (SiO_2 , gradient: 100% hexanes → 100% EtOAc) to afford the desired hydantoin **15ff** (0.141 g, 41% yield) as a colorless viscous oil. ^1H NMR (500 MHz, CDCl_3) δ 7.37–7.31 (m, 5H), 6.43 (bs, 1H), 5.20 (d, $J = 12.5$ Hz, 1H), 5.13 (d, $J = 12.5$ Hz, 1H), 4.79 (q, $J = 7.5$ Hz, 1H), 4.62 (bs, 1H), 4.01 (t, $J = 6.0$ Hz, 1H), 3.12–3.03 (m, 2H), 1.89–1.82 (m, 1H), 1.70–1.61 (m, 1H), 1.66 (d, $J = 7.5$ Hz, 3H), 1.48–1.32 (m, 4H), 1.45 (s, 9H). ^{13}C NMR (125 MHz, CDCl_3) δ 173.55, 169.56, 156.78, 156.42, 135.40, 128.78 (2C), 128.61, 128.48 (2C), 79.58, 67.77, 57.05, 48.38, 40.04, 31.15, 29.72, 28.67 (3C), 21.63, 15.00. FT-IR (neat, cm^{-1}): 3333, 2931, 1717, 1510. HRMS (ESI+) Calcd. for $\text{C}_{22}\text{H}_{32}\text{N}_3\text{O}_6$ ($[\text{M}+\text{H}]^+$): 434.2291. Found: 434.2276.

4.1.20. 4-Ethynylbenzyl N-Cbz-N-Boc-L-lysyl-L-alaninate (**15g**)

A solution of N-Cbz-N-Boc-L-lysine (**13d**) (253 mg, 0.666 mmol, 1.0 equiv), TFA salt of 4-ethynylbenzyl-L-ala (**14c**) (200 mg, 0.666 mmol, 1 equiv), EDCI (255 mg, 1.33 mmol, 2 equiv), HOBT (90 mg, 0.666 mmol, 1 equiv) in 3 mL of DMF and 12 mL of DCM

was cooled to 0 °C and TMP (87 mL, 0.666 mmol, 1 equiv) was added. The solution was kept in a 4 °C refrigerator for 15 h, diluted with 50 mL of EtOAc and washed with H_2O (5 mL) and brine (5 mL). The organic layer was dried over MgSO_4 and concentrated. The residue was purified on a silica gel chromatography (hexanes: EtOAc = 4:1) to give the product as a yellow oil (250 mg, 66%). ^1H NMR (500 MHz, CDCl_3) δ 7.41 (d, $J = 8.1$ Hz, 2H), 7.28–7.23 (m, 5H), 7.22 (d, $J = 8.1$ Hz, 2H), 5.76 (d, $J = 8.0$ Hz, 1H), 5.12 (d, $J = 12.6$ Hz, 1H), 5.04 (d, $J = 12.6$ Hz, 1H), 5.02 (s, 2H), 4.82 (brs, 1H), 4.58–4.48 (m, 1H), 4.21–4.14 (m, 1H), 3.06 (s, 1H), 30.3–2.95 (m, 2H), 1.80–1.69 (m, 1H), 1.63–1.51 (m, 1H), 1.42–1.30 (m, 4H), 1.36 (s, 9H), 1.33 (d, $J = 7.3$ Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.6, 171.9, 156.5, 136.1, 132.5 (2C), 128.7 (2C), 128.2 (2C), 128.1 (2C), 127.2, 122.4, 116.5, 111.8, 83.3, 79.5, 78.0, 67.2, 66.7, 54.8, 48.4, 30.9, 32.2, 29.5, 28.5, 22.4, 18.0. HRMS (ESI+): Calcd. for $\text{C}_{31}\text{H}_{40}\text{N}_3\text{O}_7$ ($[\text{M}+\text{H}]^+$), 566.2861. Found: 566.2884.

4.1.21. Cbz-Ala-BnO-Lys dipeptide (**16a**)

Cbz-Ala-BnO-Lys(Boc) dipeptide (**15a**) (1.0 g, 1.85 mmol) was dissolved in dry CH_2Cl_2 (10 mL) in a dry, round-bottomed flask under a positive atmosphere of nitrogen at 22 °C and then TFA was added (1.41 mL, 18.46 mmol). The reaction mixture was stirred for 8 h at 22 °C and then concentrated under reduced pressure and diluted with toluene (2×10 mL) and concentrated to provide the desired Cbz-Ala-BnO-Lys dipeptide (1.03 g, 98% yield) in the form a TFA salt as a viscous colorless oil. ^1H NMR (500 MHz, CDCl_3) δ 7.97 (bs, 3H), 7.44 (d, $J = 7.5$ Hz, 1H), 7.35–7.27 (m, 10H), 6.01 (d, $J = 7.5$ Hz, 1H), 5.16 (d, $J = 12.0$ Hz, 1H), 5.08 (d, $J = 12.0$ Hz, 1H), 5.06 (d, $J = 12.0$ Hz, 1H), 4.99 (d, $J = 12.0$ Hz, 1H), 4.56–4.51 (m, 1H), 4.32–4.29 (m, 1H), 2.82–2.74 (m, 2H), 1.82–1.75 (m, 1H), 1.67–1.56 (m, 2H), 1.55–1.46 (m, 1H), 1.30 (d, $J = 7.0$ Hz, 3H), 1.37–1.25 (m, 2H). ^{13}C NMR (125 MHz, CDCl_3) δ 173.41, 171.84, 162.67 (q, $J = 26.75$ Hz, CF_3CO_2^-), 156.64, 136.28, 135.38, 128.77 (2C), 128.72, 128.67 (2C), 128.46, 128.35 (2C), 127.95 (2C), 116.75 (q, $J = 288.75$ Hz, CF_3CO_2^-), 67.42, 67.18, 52.19, 50.50, 39.28, 30.88, 26.63, 21.93, 18.61. FT-IR (neat, cm^{-1}): 3300, 2939, 1673, 1531. HRMS (ESI+): Calcd. for $\text{C}_{24}\text{H}_{32}\text{N}_3\text{O}_5$ ($[\text{M}]^+$), 442.2336. Found: 442.2365.

4.1.22. Cbz-Gly-BnO-Lys dipeptide (**16b**)

Cbz-Gly-BnO-Lys(Boc) dipeptide (**15b**, 1.0 g, 1.90 mmol) was dissolved in dry CH_2Cl_2 (10 mL) in a dry, round-bottomed flask under a positive atmosphere of nitrogen at 22 °C and then TFA was added (1.41 mL, 18.46 mmol). The reaction mixture was stirred for 8 h at 22 °C and then concentrated under reduced pressure and diluted with toluene (2×10 mL) and concentrated to provide the desired Cbz-Gly-BnO-Lys dipeptide (**16b**, 1.04 g, 99% yield) as a viscous colorless oil. ^1H NMR (500 MHz, CDCl_3) δ 7.67 (bs, 3H), 7.44 (d, $J = 7.5$ Hz, 1H), 7.35–7.26 (m, 10H), 6.12 (bs, 1H), 5.14 (d, $J = 12.5$ Hz, 1H), 5.09 (d, $J = 12.5$ Hz, 1H), 5.04 (s, 2H), 4.55–4.50 (m, 1H), 3.83 (d, $J = 5.0$ Hz, 2H), 2.86–2.77 (m, 2H), 1.83–1.75 (m, 1H), 1.65–1.49 (m, 3H), 1.34–1.23 (m, 2H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.05, 170.66, 162.67 (q, $J = 37.50$ Hz, CF_3CO_2^-), 157.46, 136.16, 135.20, 128.84 (2C), 128.79 (2C), 128.75 (2C), 128.43 (2C), 127.96, 116.05 (q, $J = 290.62$ Hz, CF_3CO_2^-), 67.67, 67.49, 52.24, 44.21, 39.68, 30.96, 26.52, 21.80. FT-IR (neat, cm^{-1}): 3385, 2987, 1781, 1650, 1586. HRMS (ESI+) Calcd. for $\text{C}_{23}\text{H}_{30}\text{N}_3\text{O}_5$ ($[\text{M}]^+$): 428.2180. Found: 428.2205.

4.1.23. N-Cbz-Ala-O-Bn-Orn dipeptide (**16c**)

N-Cbz-Ala-O-Bn-Orn(Boc) (**15c**)³² (300 mg, 569 μmol) was dissolved in dry CH_2Cl_2 (5 mL) in a round bottomed flask under positive atmosphere of nitrogen at 22 °C. TFA was added (0.4 mL, 5.54 mmol) to the solution. The reaction mixture was stirred for 8 h at 22 °C and then concentrated under reduced pressure and

diluted with toluene (2×4 mL) and concentrated to provide **16c** (315 mg, 99% yield) as viscous yellow oil in the form of a TFA salt. ^1H NMR (500 MHz, CD_3OD) δ 7.37–7.30 (m, 10 H), 5.18 (d, $J = 12.2$ Hz, 1H), 5.16 (d, $J = 12.2$ Hz, 1H), 5.10 (d, $J = 12.4$ Hz, 1H), 5.04 (d, $J = 12.4$ Hz, 1H), 4.56–4.52 (m, 1H), 4.11 (q, $J = 7.3$ Hz, 1H), 2.92 (t, $J = 7.5$ Hz, 2H), 2.04–1.97 (m, 1H), 1.78–1.73 (m, 2H), 1.30 (d, $J = 7.2$ Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 176.0, 172.6, 163.0 (q, $J = 34.5$ Hz, CF_3CO_2^-), 158.3, 138.1, 137.0, 129.6 (2C), 129.5 (2C), 129.42, 129.37 (2C), 129.0, 128.7, 118.2 (q, $J = 289.4$ Hz, CF_3CO_2^-), 68.2, 67.6, 52.8, 51.9, 40.1, 29.2, 24.9, 18.1. HRMS (ESI+) Calcd. for $\text{C}_{23}\text{H}_{30}\text{N}_3\text{O}_5^+$ ($[\text{M}]^+$): 428.2180. Found: 428.2289.

4.1.24. 4-BrBzAla-BnO-Lys dipeptide (**16d**)

4-BrBz-Ala-BnO-Lys(Boc) (**15d**) (326 mg, 552.06 μmol) was dissolved in dry CH_2Cl_2 (5 mL) in a round-bottomed flask under a positive atmosphere of nitrogen at 22 °C. TFA was added (0.4 mL, 5.54 mmol) to the solution. The reaction mixture was stirred for 8 h at 22 °C and then concentrated under reduced pressure and diluted with toluene (2×4 mL). Concentration in vacuo provided the desired 4-BrBz-Ala-BnO-Lys dipeptide (330 mg, 99% yield) as viscous yellow oil and as a 2:1 mixture of rotamers. Data for the major rotamer: ^1H NMR (500 MHz, CDCl_3) δ 7.98 (bs, 3H), 7.67 (bs, 1H), 7.59 (d, $J = 8.5$ Hz, 2H), 7.50 (d, $J = 8.5$ Hz, 2H), 7.34–7.24 (m, $J = 7.8$, 5H), 5.43 (bs, 1H), 5.17–5.09 (m, 2H), 4.69–4.46 (m, 2H), 3.05–2.85 (m, 2H), 1.85–1.57 (m, 4H), 1.4–1.35 (m, 2H), 1.41 (d, $J = 6.0$, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.65, 171.76, 166.79, 135.22, 131.90, 129.08 (2C), 128.76 (2C), 128.66 (2C), 128.36 (2C), 128.32, 126.79, 67.51, 52.60, 49.83, 39.57, 30.67, 26.83, 22.24, 18.11. FT-IR (neat, cm^{-1}): 3275, 3068, 1740, 1675, 1541, 1204. HRMS (ESI+) Calcd. For $\text{C}_{23}\text{H}_{29}\text{BrN}_3\text{O}_4^+$ ($[\text{M}]^+$): 490.1336. Found: 490.1320.

4.1.25. BnO-Ala-Cbz-Lys dipeptide (**16e**)

BnO-Ala-Cbz-Lys(Boc) dipeptide (**15e**) (1.0 g, 1.85 mmol) was dissolved in dry CH_2Cl_2 (10 mL) in a dry round-bottomed flask under a positive atmosphere of nitrogen at 22 °C and then TFA was added (1.41 mL, 18.46 mmol). The reaction mixture was stirred for 8 h at 22 °C and then concentrated under reduced pressure and diluted with toluene (2×10 mL) and concentrated to provide the desired BnO-Ala-Cbz-Lys dipeptide **16e** (1.015 g, 99% yield) as a viscous colorless oil. ^1H NMR (500 MHz, CDCl_3) δ 8.04 (bs, 3H), 7.61 (d, $J = 6.5$ Hz, 1H), 7.32–7.27 (m, 10H), 6.08 (d, $J = 8.0$ Hz, 1H), 5.13 (d, $J = 12.5$ Hz, 1H), 5.05 (d, $J = 12.0$ Hz, 1H), 5.03 (d, $J = 12.0$ Hz, 1H), 5.01 (d, $J = 12.0$ Hz, 1H), 4.51 (q, $J = 7.0$ Hz, 1H), 4.23 (t, $J = 6.0$ Hz, 1H), 2.82 (bs, 2H), 1.75–1.67 (m, 1H), 1.63–1.54 (m, 3H), 1.42–1.29 (m, 2H), 1.35 (d, $J = 7.0$ Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.85, 172.01, 162.04 (q, $J = 35.75$ Hz, CF_3CO_2^-), 156.65, 136.24, 135.44, 128.75 (2C), 128.67, 128.56 (2C), 128.33 (2C), 128.21(2C), 127.99, 116.56 (q, $J = 289.5$ Hz, CF_3CO_2^-), 67.30, 67.21, 54.10, 48.39, 39.30, 32.20, 26.49, 21.95, 17.31. FT-IR (neat, cm^{-1}): 3280, 2939, 1681, 1538. HRMS (ESI+) Calcd. for $\text{C}_{24}\text{H}_{32}\text{N}_3\text{O}_5^+$ ($[\text{M}]^+$): 442.2336. Found: 442.2341.

4.1.26. Hydantoin (**16f**)

Boc-hydantoin dipeptide (**15ff**) (100 mg, 0.230 mmol) was dissolved in dry CH_2Cl_2 (3 mL) in a dry, round-bottomed flask under a positive atmosphere of nitrogen at 22 °C and then TFA was added (0.176 mL, 2.31 mmol). The reaction mixture was stirred for 8 h at 22 °C and then concentrated under reduced pressure and diluted with toluene (2×3 mL) and concentrated to provide the desired hydantoin dipeptide (103 mg, 99% yield) as a viscous colorless oil. ^1H NMR (500 MHz, CDCl_3) δ 7.70 (bs, 3H), 7.40 (bs, 1H), 7.36–7.29 (m, 5H), 5.18 (d, $J = 12.5$ Hz, 1H), 5.12 (d, $J = 12.5$ Hz, 1H), 4.76 (q, $J = 7.5$ Hz, 1H), 4.04 (dd, $J = 7.0$, 4.5 Hz, 1H), 2.93 (bs, 2H),

1.87–1.80 (m, 1H), 1.63–1.56 (m, 3H), 1.62 (d, $J = 7.5$ Hz, 3H), 1.47–1.38 (m, 2H). ^{13}C NMR (125 MHz, CDCl_3) δ 173.64, 169.72, 161.61 (q, $J = 38.50$ Hz, CF_3CO_2^-), 157.57, 135.25, 128.77 (2C), 128.67, 128.30 (2C), 116.05 (q, $J = 286.12$ Hz, $\text{CF}_3\text{CCO}_2^-$), 67.84, 56.84, 48.43, 39.78, 30.57, 26.60, 21.07, 14.76. FT-IR (neat, cm^{-1}): 3179, 2948, 1776, 1713, 1673, 1530. HRMS (ESI+): Calcd. for $\text{C}_{17}\text{H}_{24}\text{N}_3\text{O}_4^+$ ($[\text{M}]^+$), 334.1761. Found: 334.1750.

4.1.27. 4-Ethynylbenzyl N-Cbz-L-lysyl-L-alaninate (**16g**)

To the solution of **15g** (280 mg, 0.495 mmol) in 10 mL of DCM was slowly added TFA (1.2 mL) at 0 °C. The solution was kept in a 4 °C refrigerator for 15 h. Toluene (10 mL) was added and most of the DCM and TFA were evaporated in vacuo. The residue was purified on silica gel chromatography (DCM:MeOH = 20:1) to give the product **16g** as a yellow oil (130 mg, 62%) together with unseparable methyl ketone (~40%) derived from hydration of the alkyne. ^1H NMR (500 MHz, CDCl_3) δ 7.83 (brs, 3H), 7.43 (d, $J = 8.1$ Hz, 2H), 7.29–7.22 (m, 5H), 7.22 (d, $J = 8.1$ Hz, 2H), 6.02 (brs, 1H), 5.40 (brs, 1H), 5.11 (d, $J = 12.5$ Hz, 1H), 5.03–4.99 (m, 3H), 4.57–4.51 (m, 1H), 4.28–4.23 (m, 1H), 3.08 (s, 1H), 2.85 (brs, 2H), 1.71–1.52 (m, 4H), 1.38–1.32 (m, 5H). HRMS (ESI+) Calcd. for $\text{C}_{29}\text{H}_{32}\text{N}_3\text{O}_5^+$ ($[\text{M}]^+$): 466.2336. Found: 466.2338.

4.1.28. Representative procedure for coupling of dipeptides and carboxylic acids: synthesis of β -lactone dipeptide **17**

To a round-bottomed flask was added dipeptide **16a** (13.0 mg, 0.023 mmol, 1 equiv), β -lactone acid **12a'** (4.7 mg, 0.023 mmol, 1 equiv), EDCI (8.9 mg, 0.047 mmol, 2.0 equiv), HOBt (3.1 mg, 0.023 mmol, 1 equiv), DMF (0.1 mL), and DCM (0.4 mL), followed by TMP (3.0 μL , 0.023 mmol, 1.0 equiv). The reaction mixture was stirred at 0 °C under N_2 for 4 h, transferred to a separatory funnel, and diluted with EtOAc (10 mL) and MTBE (10 mL). The organic layer was washed with aq. NaHSO_4 soln. (1 M), water and brine. The solution was dried over MgSO_4 and concentrated in vacuo. The residue was purified by a flash chromatography (hexanes/acetone) to afford **17** as a viscous colorless oil (11.3 mg, 77%). $[\alpha]_D^{20} - 7.0$ ($c = 2.4$, CHCl_3). ^1H NMR (500 MHz, CDCl_3) δ 7.39–7.30 (m, 10 H), 6.70 (d, $J = 6.3$ Hz, 1H), 6.58 (bs, 1H), 5.49 (d, $J = 6.3$ Hz, 1H), 5.20 (d, $J = 12.4$ Hz, 1H), 5.13 (d, $J = 12.4$ Hz, 1H), 5.09 (s, 2H), 4.59–4.52 (m, 2H), 4.30–4.23 (m, 1H), 3.64–3.61 (m, 1H), 3.36–3.29 (m, 1H), 3.16–3.11 (m, 1H), 1.90–1.79 (m, 3H), 1.69–1.64 (m, 1H), 1.57–1.43 (m, 4H), 1.37 (d, $J = 7.0$ Hz, 3H), 1.34–1.25 (m, 8H), 0.88 (t, $J = 6.7$ Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.69, 172.07, 169.83, 168.42, 156.23, 136.49, 135.45, 128.90 (2C), 128.80, 128.78 (2C), 128.60 (2C), 128.42, 128.25 (2C), 73.10, 67.51, 67.20, 57.99, 52.14, 50.72, 45.20, 38.70, 31.76/31.67 (rotamers), 28.98, 28.67, 28.32, 26.71, 22.74, 22.31, 18.63, 14.25. HRMS (ESI+): Calcd. For $\text{C}_{34}\text{H}_{45}\text{N}_3\text{O}_8\text{Li}^+$ ($[\text{M}+\text{Li}]^+$), 630.3367. Found: 630.3334.

4.1.29. β -lactone dipeptide **18**

Prepared according to the representative procedure from β -lactone acid **12a'** (5.0 mg, 0.025 mmol) and dipeptide amine **16b** (13.5 mg, 0.025 mmol) and obtained as a viscous colorless oil (9.0 mg, 59%). ^1H NMR (500 MHz, CDCl_3) δ 7.39–7.31 (m, 10H), 6.66 (d, $J = 7.1$ Hz, 1H), 6.53 (bs, 1H), 5.55 (bs, 1H), 5.20 (d, $J = 12.1$ Hz, 1H), 5.15 (d, $J = 12.1$ Hz, 1H), 5.14 (s, 2H), 4.66–4.62 (m, 1H), 4.54 (d, $J = 4.5$ Hz, 1H), 3.91 (d, $J = 5.7$ Hz, 2H), 3.64 (td, $J = 7.6$ Hz, $J = 4.4$ Hz, 1H), 3.29–3.18 (m, 2H), 1.93–1.80 (m, 3H), 1.72–1.64 (m, 1H), 1.58–1.45 (m, 4H), 1.37–1.26 (m, 8H), 0.89 (t, $J = 6.5$ Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.09, 169.89, 169.27, 168.27, 156.87, 136.36, 135.33, 128.89 (2C), 128.82, 128.77 (2C), 128.56 (2C), 128.44, 128.23 (2C), 73.07, 67.56, 67.43, 57.98, 52.02, 44.70, 38.83, 31.97, 31.63, 28.95, 28.61, 28.29,

26.66, 22.70, 22.31, 14.23. HRMS (ESI⁺) Calcd. For C₃₃H₄₄N₃O₈ ([M+H]⁺): 610.3128. Found: 610.3114. (~85% purity by HPLC).

4.1.30. β -lactone dipeptide 19

Prepared according to the representative procedure from β -lactone acid **12a'** (5.0 mg, 0.025 mmol) and dipeptide amine **16c** (13.5 mg, 0.025 mmol) and obtained as a viscous colorless oil (9.5 mg, 62%). ¹H NMR (500 MHz, CDCl₃) δ 7.40–7.30 (m, 10 H), 6.78 (d, J = 7.7 Hz, 1H), 6.62 (bs, 1H), 5.429 (d, J = 7.2 Hz, 1H), 5.21 (d, J = 12.3 Hz, 1H), 5.14 (d, J = 12.3 Hz, 1H), 5.11 (s, 2H), 4.62 (td, J = 7.6 Hz, J = 5.1 Hz, 1H), 4.49 (d, J = 4.4 Hz, 1H), 4.27 (pent, J = 7.0 Hz, 1H), 3.62 (ddd, J = 8.3 Hz, J = 6.9 Hz, J = 4.4 Hz, 1H), 3.32–3.25 (m, 1H), 3.24–3.17 (m, 1H), 1.92–1.78 (m, 3H), 1.71–1.61 (m, 2H), 1.54–1.42 (m, 3H), 1.38 (d, J = 7.2 Hz, 3H), 1.35–1.27 (m, 6H), 0.89 (t, J = 6.8 Hz, 3H). ¹³C NMR (125 MHz, CDCl₃) δ 172.51, 171.80, 169.74, 168.21, 156.31, 136.39, 135.28, 128.91 (2C), 128.86, 128.76 (2C), 128.62 (2C), 128.43, 128.25 (2C), 73.03, 67.63, 67.27, 57.98, 51.98, 50.73, 38.58, 31.63, 29.91/29.50 (rotamers), 28.96, 28.93, 26.63, 25.33, 22.70, 18.50, 14.23. HRMS (ESI⁺) Calcd. For C₃₃H₄₄N₃O₈ ([M+H]⁺): 610.3128. Found: 610.3105.

4.1.31. β -lactone dipeptide 20

Prepared according to the representative procedure from β -lactone acid **12a'** (5.0 mg, 0.025 mmol) and dipeptide amine **16b** (15.1 mg, 0.025 mmol) and obtained as a viscous colorless oil (5.6 mg, 51%). ¹H NMR (500 MHz, CDCl₃) δ 7.69–7.65 (m, 2H), 7.58–7.56 (m, 2H), 7.38–7.20 (m, 5H), 6.86 (d, J = 7.5 Hz, 1H), 6.73–6.72 (m, 1H), 6.58–6.55 (m, 1H), 5.22–5.16 (m, 1H), 5.12 (d, J = 12.3 Hz, 1H), 4.76–4.67 (m, 1H), 4.54 (d, J = 4.5 Hz, 1H), 3.70–3.62 (m, 1H), 3.34–3.26 (m, 2H), 1.92–1.83 (m, 4H), 1.76–1.71 (m, 1H), 1.66–1.60 (m, 4H), 1.50 (d, J = 7.0 Hz, 3H), 1.37–1.27 (m, 8H), 0.88 (t, J = 6.7 Hz, 3H). ¹³C NMR (125 MHz, CDCl₃) δ 172.4, 171.9, 170.0, 168.2, 166.6, 135.3, 132.7, 132.0 (2C), 129.0, 128.9 (2C), 128.84 (2C), 128.79, 128.5 (2C), 73.2, 67.6, 57.9, 52.1, 49.5, 39.0, 32.2, 31.6, 29.9, 28.6, 28.3, 26.6, 22.6, 22.5, 18.6, 14.2. HRMS (ESI⁺) Calcd. For C₃₃H₄₁BrN₃O₇ ([M+H]⁺): 670.2128. Found: 670.2113.

4.1.32. β -lactone dipeptide 21

Prepared according to the representative procedure from β -lactone acid **12a'** (5.0 mg, 0.025 mmol) and dipeptide amine **16e** (13.9 mg, 0.025 mmol) and obtained as a viscous, colorless oil (7.0 mg, 47%). [α]_D²⁰ – 4.0 (c = 0.8, CHCl₃). ¹H NMR (500 MHz, CDCl₃) δ 7.39–7.30 (m, 10H), 6.54–6.50 (m, 2H), 5.46 (d, J = 7.7 Hz, 1H), 5.22 (d, J = 12.2 Hz, 1H), 5.15 (d, J = 12.2 Hz, 1H), 5.11 (s, 2H), 4.62 (pent, J = 7.2 Hz, 1H), 4.49 (d, J = 4.1 Hz, 1H), 4.21–4.15 (m, 1H), 3.65 (ddd, J = 8.3 Hz, J = 7.1 Hz, J = 4.7 Hz, 1H), 3.36 (dq, J = 13.6 Hz, J = 7.0 Hz, 1H), 3.28–3.21 (m, 1H), 1.90–1.80 (m, 3H), 1.71–1.63 (m, 1H), 1.58–1.45 (m, 4H), 1.42 (d, J = 7.0 Hz, 3H), 1.39–1.26 (m, 8H), 0.89 (t, J = 6.8 Hz, 3H). ¹³C NMR (125 MHz, CDCl₃) δ 172.76, 171.34, 169.74, 168.27, 156.41, 136.37, 135.48, 128.85 (2C), 128.77 (2C), 128.68, 128.45, 128.37 (2C), 128.32 (2C), 73.03, 67.48, 67.31, 57.98, 54.64, 48.40, 38.65, 32.18, 31.63, 29.92, 28.95/28.85 (rotamers), 28.32, 26.66, 22.71, 22.26, 18.25, 14.21. HRMS (ESI⁺) Calcd. For C₃₄H₄₆N₃O₈ ([M+H]⁺): 624.3285. Found: 624.3303.

4.1.33. β -lactone dipeptide 22

Prepared according to the representative procedure from β -lactone acid **12a'** (5.0 mg, 0.025 mmol) and dipeptide amine **16f** (11.2 mg, 0.025 mmol) and obtained as a viscous, colorless oil (7.4 mg, 57%). [α]_D²⁰ – 25 (c = 0.8, CHCl₃). ¹H NMR (500 MHz, CDCl₃) δ 7.38–7.31 (m, 5H), 6.49 (t, J = 6.3 Hz, 1H), 5.97 (bs, 1H), 5.21 (d, J = 12.4 Hz, 1H), 5.15 (d, J = 12.4 Hz, 1H), 4.79 (q, J = 7.2 Hz, 1H), 4.55 (d, J = 4.3 Hz, 1H), 4.04 (t, J = 5.4 Hz, 1H), 3.68 (ddd, J = 8.1 Hz,

J = 6.8 Hz, J = 4.4 Hz, 1H), 3.33–3.23 (m, 2H), 1.96–1.83 (m, 3H), 1.75–1.69 (m, 1H), 1.66 (d, J = 7.2 Hz, 3H), 1.56–1.46 (m, 4H), 1.40–1.26 (m, 8H), 0.89 (t, J = 7.2 Hz, 3H). ¹³C NMR (125 MHz, CDCl₃) δ 173.29, 169.69, 169.49, 168.42, 156.53, 135.45, 128.79 (2C), 128.61, 128.45 (2C), 73.01, 67.82, 58.13, 56.78, 48.49, 38.55, 31.65, 30.61, 29.92, 29.00/28.95 (rotamers), 28.40, 26.63, 22.72, 21.02, 14.94, 14.23. HRMS (ESI⁺) Calcd. For C₂₇H₃₆N₃O₇ ([M+H]⁺): 514.2553. Found: 514.2576. (~85% purity by HPLC).

4.1.34. β -lactone dipeptide 23

Prepared according to the representative procedure from β -lactone acid **12a** (10.0 mg, 0.050 mmol) and dipeptide amine **16e** (27.8 mg, 0.050 mmol) and obtained as a viscous colorless oil (9.3 mg, 60%). [α]_D²⁰ – 13 (c = 3.1, CHCl₃). ¹H NMR (500 MHz, CDCl₃) δ 7.39–7.30 (m, 10H), 6.65 (d, J = 6.7 Hz, 1H), 6.60 (t, J = 5.6 Hz, 1H), 5.50 (d, J = 8.1 Hz, 1H), 5.21 (d, J = 12.3 Hz, 1H), 5.15 (d, J = 12.3 Hz, 1H), 5.10 (s, 2H), 4.60 (pent, J = 7.4 Hz, 1H), 4.53 (d, J = 4.4 Hz, 1H), 4.20 (q, J = 7.3 Hz, 1H), 3.65 (ddd, J = 8.1 Hz, J = 6.9 Hz, J = 4.3 Hz, 1H), 3.33 (dq, J = 13.3 Hz, J = 6.6 Hz, 1H), 3.24 (dq, J = 13.3 Hz, J = 6.6 Hz, 1H), 1.93–1.79 (m, 3H), 1.69–1.62 (m, 1H), 1.58–1.44 (m, 4H), 1.41 (d, J = 7.4 Hz, 3H), 1.37–1.28 (m, 8H), 0.88 (t, J = 6.8 Hz, 3H). ¹³C NMR (125 MHz, CDCl₃) δ 172.74, 171.42, 169.86, 168.12, 156.38, 136.37, 135.50, 128.82 (2C), 128.73 (2C), 128.64, 128.39, 128.31 (2C), 128.23 (2C), 73.04, 67.45, 67.27, 57.90, 54.55, 48.39, 38.91, 32.44, 31.61, 29.89, 28.95/28.83 (rotamers), 28.29, 26.63, 22.60, 22.43, 18.14, 14.20. HRMS (ESI⁺) Calcd. For C₃₄H₄₆N₃O₈ ([M+H]⁺): 624.3285. Found: 624.3304.

4.1.35. β -Lactone dipeptide 24

Prepared according to the representative procedure from β -lactone acid **12a** (10.0 mg, 0.050 mmol) and dipeptide amine **16b** (27.0 mg, 0.050 mmol) and obtained as a viscous, colorless oil (16.0 mg, 52%). ¹H NMR (500 MHz, CDCl₃) δ 7.40–7.31 (m, 10H), 6.67 (d, J = 6.8 Hz, 1H), 6.53 (bs, 1H), 5.56 (bs, 1H), 5.20 (d, J = 12.1 Hz, 1H), 5.15 (d, J = 12.1 Hz, 1H), 5.14 (s, 2H), 4.68–4.64 (m, 1H), 4.54 (d, J = 4.2 Hz, 1H), 3.91 (d, J = 5.9 Hz, 2H), 3.64 (td, J = 7.6 Hz, J = 4.2 Hz, 1H), 3.31–3.24 (m, 1H), 3.22–3.16 (m, 1H), 1.95–1.82 (m, 3H), 1.73–1.65 (m, 1H), 1.59–1.44 (m, 4H), 1.38–1.24 (m, 8H), 0.90 (t, J = 6.7 Hz, 3H). ¹³C NMR (125 MHz, CDCl₃) δ 172.05, 169.94, 169.33, 168.18, 156.88, 136.36, 135.34, 128.87 (2C), 128.79, 128.75 (2C), 128.53 (2C), 128.42 (2C), 128.20, 73.06, 67.53, 67.43, 57.86, 52.00, 44.73, 38.93, 31.98, 31.61, 28.93, 28.59, 28.27, 26.65, 22.68, 22.27, 14.20. HRMS (ESI⁺) Calcd. For C₃₃H₄₄N₃O₈ ([M+H]⁺): 610.3128. Found: 610.3114.

4.1.36. α -Methyl- β -lactone dipeptide 25

Prepared according to the representative procedure from β -lactone acid **12b** (10.0 mg, 0.045 mmol) and dipeptide amine **16a** (24 mg, 0.045 mmol) and obtained as a viscous colorless oil (15 mg, 55%). ¹H NMR (500 MHz, CDCl₃) δ 7.38–7.29 (m, 10H), 6.76–6.70 (m, 1H), 6.58 (bs, 1H), 5.51/5.38 (d, J = 6.4 Hz, 1H), 5.19 (d, J = 12.2 Hz, 1H), 5.13 (d, J = 12.2 Hz, 1H), 5.10 (s, 2H), 4.62–4.56 (m, 1H), 4.59 (s, 1H), 4.30–4.23 (m, 1H), 3.26–3.16 (m, 2H), 1.90–1.82 (m, 1H), 1.76–1.73 (m, 2H), 1.72–1.63 (m, 1H), 1.54–1.42 (m, 3H), 1.37 (d, J = 7.1 Hz, 3H), 1.36–1.25 (m, 9H), 1.23 (s, 3H), 0.88 (t, J = 6.8 Hz, 3H). ¹³C NMR (125 MHz, CDCl₃) δ 173.5, 172.4, 171.9, 167.2, 156.2, 136.4, 135.3, 128.8 (2C), 128.74 (2C), 128.70 (2C), 128.6 (2C), 128.4, 128.2, 76.8, 67.4, 67.2, 61.4, 52.1, 50.7, 38.7, 35.4, 31.8, 31.7, 29.4, 28.9, 24.4, 22.7, 22.3, 18.4, 15.9, 14.2. HRMS (ESI⁺) Calcd. For C₃₅H₄₇ClN₃O₈ ([M+Cl]⁺): 672.3057. Found: 672.3026.

4.1.37. α -Methyl- β -lactone dipeptide 26

Prepared according to the representative procedure from β -lactone acid **12b'** (8.0 mg, 0.035 mmol) and dipeptide amine **16a** (19.5 mg, 0.035 mmol) and obtained as a viscous colorless oil

(11.5 mg, 51%). ^1H NMR (500 MHz, CDCl_3) δ 7.38–7.30 (m, 10H), 6.67 (d, J = 7.2 Hz, 1H), 6.53 (brs, 1H), 5.47 (d, J = 7.3 Hz, 1H), 5.19 (d, J = 12.2 Hz, 1H), 5.13 (d, J = 12.2 Hz, 1H), 5.10 (s, 2H), 4.64 (s, 1H), 4.60–4.56 (m, 1H), 4.29–4.23 (m, 1H), 3.33–3.27 (m, 1H), 3.21–3.15 (m, 1H), 1.91–1.84 (m, 1H), 1.76–1.68 (m, 3H), 1.56–1.44 (m, 3H), 1.37 (d, J = 7.1 Hz, 3H), 1.34–1.26 (m, 9H), 1.24 (s, 3H), 0.88 (t, J = 6.8 Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 173.3, 172.5, 172.0, 167.4, 156.1, 136.5, 135.4, 128.8 (2C), 128.74, 128.70 (2C), 128.6 (2C), 128.4 (2C), 128.2, 76.9, 67.5, 67.2, 61.4, 52.1, 50.7, 38.4, 35.4, 31.7, 29.9, 29.4, 28.8, 24.4, 22.7, 22.2, 18.5, 15.9, 14.2. HRMS (ESI⁺) Calcd. For $\text{C}_{35}\text{H}_{48}\text{N}_3\text{O}_8$ ($[\text{M}+\text{H}]^+$): 638.3441. Found: 638.3455.

4.1.38. α -Methyl- β -lactone dipeptide **27**

Prepared according to the representative procedure from β -lactone acid **12b'** (10 mg, 0.045 mmol) and dipeptide amine **16a** (24 mg, 0.045 mmol) and obtained as a viscous colorless oil (18 mg, 63%). ^1H NMR (500 MHz, CDCl_3) δ 7.39–7.30 (m, 10H), 6.67 (d, J = 7.3 Hz, 1H), 6.56 (brs, 1H), 5.51 (d, J = 6.9 Hz, 1H), 5.20 (d, J = 12.2 Hz, 1H), 5.13 (d, J = 12.2 Hz, 1H), 5.09 (s, 2H), 4.60–4.56 (m, 1H), 4.56 (s, 1H), 4.26 (pent, J = 7.1 Hz, 1H), 3.38–3.31 (m, 1H), 3.17–3.11 (m, 1H), 1.90–1.83 (m, 1H), 1.73–1.66 (m, 1H), 1.62–1.59 (m, 2H), 1.51–1.45 (m, 1H), 1.49 (s, 3H), 1.40–1.36 (m, 2H), 1.37 (d, J = 7.1 Hz, 3H), 1.28–1.23 (m, 9H), 0.85 (t, J = 6.8 Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 173.1, 172.5, 172.0, 167.1, 156.1, 136.4, 135.3, 128.8 (2C), 128.74, 128.71 (2C), 128.5 (2C), 128.4, 128.2 (2C), 79.0, 67.5, 67.2, 61.0, 52.0, 50.7, 38.4, 31.8, 31.6, 29.9, 29.7, 28.7, 24.1, 22.7, 22.2, 20.0, 18.7, 14.2. HRMS (ESI⁺) Calcd. For $\text{C}_{35}\text{H}_{47}\text{ClN}_3\text{O}_8$ ($[\text{M}+\text{Cl}]^+$): 672.3057. Found: 672.3058.

4.1.39. ABPP probe **28**

Prepared according to the representative procedure from β -lactone acid **12a'** (6.8 mg, 0.034 mmol) and dipeptide amine **16g** (19 mg, 0.034 mmol) and obtained as a viscous colorless oil (11 mg, 50%). ^1H NMR (500 MHz, CDCl_3) δ 7.48 (d, J = 8.2 Hz, 2H), 7.36–7.32 (m, 5H), 7.30 (d, J = 8.2 Hz, 2H), 6.60 (d, J = 6.8 Hz, 1H), 6.65–6.53 (m, 1H), 5.49 (d, J = 7.8 Hz, 1H), 5.19 (d, J = 12.4 Hz, 1H), 5.12 (d, J = 12.4 Hz, 1H), 5.11 (d, J = 12.5 Hz, 1H), 5.08 (d, J = 12.5 Hz, 1H), 4.59 (pent, J = 7.2 Hz, 1H), 4.48 (d, J = 4.2 Hz, 1H), 4.17 (q, J = 6.1 Hz, 1H), 3.64 (td, J = 7.7 Hz, J = 4.6 Hz, 1H), 3.37–3.31 (m, 1H), 3.26–3.20 (m, 1H), 3.10 (s, 1H), 1.90–1.25 (m, 16H), 1.41 (d, J = 7.2 Hz, 3H), 0.88 (t, J = 6.7 Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 172.6, 171.3, 169.6, 168.2, 156.1, 136.3, 136.2, 132.5 (2C), 128.7 (2C), 128.4, 128.2 (2C), 128.1 (2C), 122.4, 83.3, 78.0, 73.0, 67.3, 66.8, 58.0, 54.7, 48.4, 38.7, 32.2, 31.6, 29.0, 28.9, 28.3, 26.6, 22.7, 22.3, 18.1, 14.2. HRMS (ESI⁺) Calcd. For $\text{C}_{36}\text{H}_{46}\text{N}_3\text{O}_8$ ($[\text{M}+\text{H}]^+$): 648.3285. Found: 648.3278. (~80% purity by HPLC).

4.1.40. Fluorogenic assay for detection of 20S human proteasome inhibition

For determination of IC_{50} values, 50 ng of 20S proteasome, purified from human red blood cells,⁵ was incubated in assay buffer (20 mM Tris, pH 7.5) at 37 °C with varying concentrations of each inhibitor. After twenty minutes, the reaction was initiated by the addition of fluorogenic substrate for the chymotrypsin-like (Suc-Leu-Leu-Val-Tyr-AMC), trypsin-like (Boc-Leu-Arg-Arg-AMC), or caspase-like (Z-Leu-Leu-Glu-AMC) activity of the proteasome, with final substrate concentrations of 10 μM , 20 μM , and 20 μM respectively. The rates of hydrolysis of the substrates were monitored using a POLARstar Omega microplate reader, with excitation/emission wavelengths of 360 nm/460 nm. The rates of hydrolysis can be directly correlated to the enzymatic activity of the proteasome. The reaction was monitored for 600 s, and the linear portion for each curve was used in calculating the IC_{50} values (inhibitor concentration to cause 50% inhibition of the enzyme active site). This was accomplished by plotting the residual proteasomal activity against

the applied inhibitor concentration using GraphPad Prism version 6 for Windows, and fitting the experimental data to the equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(\text{LogIC}_{50} - X) * \text{HillSlope}})$ where X is the logarithm of inhibitor concentration and Y is the residual activity. Results are average of triplicate measurements. IC_{50} values were deduced from the fitted data. They depend on enzyme concentration and are comparable only within the same experimental settings.

4.1.41. Fluorogenic assay for detection of human FAS-TE inhibition

The synthetic fluorogenic substrate, 4-methylumbelliferyl heptanoate (4-MUH), was purchased from Sigma (St. Louis, MO). The reaction mixture consisted of 500 nM human FAS-TE in buffer (100 mM Tris-HCl, 50 mM NaCl at pH 7.4) which was pre-incubated with 2.5 μL test compounds dissolved in DMSO at final concentrations of 0.32–100 μM and/or 0.08–10 μM at 37 °C for 30 min. The reaction was initiated by addition of 5 μL of 1.25 mM 4-MUH in 1:1 DMSO:buffer A. The resulting fluorescence from liberated 4-methylumbelliferone was measured every five minutes at 350/450 nm for 60 min. 4-MUH incubated without enzyme served as a background control. Results are the average of triplicate time points. IC_{50} values were determined in similar fashion as described for the proteasome using GraphPad Prism 6 for Windows. IC_{50} values were deduced from the fitted data. They depend on enzyme concentration and are comparable only within the same experimental settings.

4.1.42. Cell cytotoxicity assays

HeLa cells were grown in DMEM containing 10% fetal bovine serum (FBS). Cells were seeded at the density of 5000 cells per well in a 96-well plate (Thermo) and were incubated overnight at 37 °C with 5% CO_2 . The medium was removed and the cells were treated with 100 μL of fresh culture medium with **17**, **18**, **21**, **25** and **26** (1–100 μM) or vehicle in duplicate. After 24 h, a stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 10 μL , 5 mM) was added to each well. As a negative control 10 μL of the MTT stock solution was added to 100 μL of medium alone. The plate was incubated at 37 °C for 4 h. All the medium was removed from the wells and 50 μL of DMSO was added to each well and the solution was mixed thoroughly with a pipette. Absorbance was read at 570 nm with a microplate reader (BioTek, Winooski, VT). The data are shown as mean $\pm$ SEM based on three independently repeated experiments. IC_{50} values were derived from dose-response curves. All statistical analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). IC_{50} values were deduced from the fitted data. They depend on enzyme concentration and are comparable only within the same experimental settings.

4.2. Stability studies of β -lactones **21** and **25**

4.2.1. In DMEM without FBS

A stock solution of **21** or **25** in DMSO (1 mM, 5 μL) was added to 995 μL of DMEM (pH = 7.5) to reach the final concentration of 5 μM . The vial was loosely capped and placed in an incubator at 37 °C with circulating 5% CO_2 . At regular time intervals 25 μL of the solution was transferred to another vial and mixed with 50 μL of phosphate buffer (pH = 2.0, 1 M). The solution was submitted to HPLC analysis (Phenomenex® Gemini-NX 3 μm C18, 110A. Size: 150 $\times$ 4.60 mm; Mobile Phase A: water; mobile phase B: acetonitrile) with a constant injection volume (20 μL). The β -lactone remaining was based on integration in comparison to the initial pure β -lactone. All compounds were assayed in triplicate.

4.2.2. In DMEM with 5% FBS

A stock solution of **21** or **25** in DMSO (1 mM, 5 μL) was added to the pre-mixed 5% fetal bovine serum (FBS) in DMEM (995 μL) to

reach the final concentration of 5 μM . The vial was loosely capped and placed in an incubator at 37 °C with circulating 5% CO_2 . At regular time intervals 100 μL of the solution was transferred to another vial and mixed with 100 μL of phosphate buffer (pH = 2.0, 1 M) and 150 μL of CH_3CN . The vial was vortexed for 1 min to precipitate out the serum and 80 μL of the clear solution was transferred to another vial for HPLC analysis with a constant injection volume (20 μL) and analyzed by HPLC in a manner identical to that described above. The 'time vs% β -lactone remaining' curves are provided in the SI.

4.2.3. Activity-Based *in situ* proteome labeling

For labeling experiments, cells were grown to ca. 80–90% confluency in a complete medium in a 6-well plate. Then the medium was aspirated, cells were washed with pre-warmed PBS (1.0 mL), followed by removal of PBS by suction. Then, 1.0 mL serum-free fresh medium containing either DMSO (as blank control) or probe at the appropriate concentration (10–50 μM) were added. DMSO content in the medium did not exceed 0.1% (1000 $\times$ dilution from DMSO stock). Cells were incubated for 1 h with varying concentrations of alkyne probe **28** at 37 °C and 5% CO_2 . Subsequently, the medium was carefully aspirated, cells were washed with cold PBS (2 $\times$ 1.0 mL) to remove the excess of the probe and then harvested in 1.0 mL fresh cold PBS by scraping. Cell pellets were isolated by centrifugation (800g, 3 min, 4 °C), resuspended in 100 μL lysis buffer (1% NP-40, 1% sodium deoxycholate in PBS, supplemented with protease inhibitor cocktail tablets (Roche)) and lysed for 15 min on ice. Soluble and insoluble fractions were separated by centrifugation at 14,800 rpm for 45 min at 4 °C. Protein concentration (soluble fraction) was determined using a BCA protein concentration assay (Carl Roth) and adjusted to the same concentration in PBS (typically 1.0 mg/mL).

4.2.4. Activity-based *in situ* competitive labeling

For labeling experiments, cells were grown to ca. 80–90% confluency in a complete medium in a 6-well plate. Then the medium was aspirated, cells were washed with pre-warmed PBS (1.0 mL), followed by removal of PBS by suction. Then, 1.0 mL serum-free fresh medium containing either DMSO (as blank control), 10 μM competitive dual inhibitor **21** or **25** (for competitive labeling) at the appropriate concentration (0 $\rightarrow$ 100 μM) were added. DMSO content in the medium did not exceed 0.1% (1000 $\times$ dilution from DMSO stock). Cells were incubated for 30 min with varying concentration of the competitive inhibitor (10 $\rightarrow$ 100 μM corresponding to 1, 2.5, 5, 10-fold excess compared to the alkyne probe **28**) at 37 °C and 5% CO_2 . Then, 1 μL 100 mM alkyne probe **28** was added directly to the medium to achieve a 10 μM final concentration. Cells were incubated for another 30 min at 37 °C and 5% CO_2 . Subsequently, the medium was carefully aspirated, cells were washed with cold PBS (2 $\times$ 1.0 mL) to remove the excess of the probe and then harvested in 1.0 mL fresh cold PBS by scraping. Cell pellets were isolated by centrifugation (800g, 3 min, 4 °C), resuspended in 100 μL lysis buffer (1% NP-40, 1% sodium deoxycholate in PBS, supplemented with protease inhibitor cocktail tablets (Roche)) and lysed for 15 min on ice. Soluble and insoluble fractions were separated by centrifugation at 14,800 rpm for 45 min at 4 °C. Protein concentration (soluble fraction) was determined using BCA protein concentration assay (Carl Roth) and adjusted to the same concentration in PBS (typically 1.0 mg/mL).

4.2.5. Sharpless-Huisgen (Click) reactions, protein electrophoresis (SDS-PAGE) and in-gel fluorescence scanning (IG-FS)

To 88 μL probe **28**-bound cell lysate, 10 μL of a freshly prepared master mix containing 2 μL TAMRA- N_3 fluorescent tag (5 mM stock in DMSO), 2 μL TCEP (50 mM stock in water) and 6 μL TBTA ligand (1.7 mM stock in DMSO:tert-BuOH 1:4 (v/v)) were added.

The samples were gently vortexed and 2 μL CuSO_4 (50 mM stock in water) was added to initiate the Sharpless-Huisgen cycloaddition reaction, giving a total reaction volume of 100 μL . The final concentrations were as follows: 100 μM TAMRA- N_3 , 1 mM TCEP, 100 μM TBTA and 1 mM CuSO_4 . Samples were incubated at RT (25 °C) for 1–1.5 h in the dark. After click chemistry reaction, 400 μL pre-chilled acetone were added, the samples were gently vortexed and left overnight at -20 °C. Then, samples were centrifuged at 14,800 rpm for 30 min at 4 °C. Supernatant was discarded and the protein pellets were washed with 200 μL pre-chilled MeOH, resuspended by sonication, centrifuged at 14,800 rpm for 30 min at 4 °C and supernatant removed (repeated twice if necessary). After removal of MeOH, the samples were left to warm up to RT and dried in air for ca. 5 min. Then, samples were redissolved in 0.2% SDS in PBS, an equal volume of 2 $\times$ SDS-PAGE loading buffer (reducing) was added, samples were vortexed and denatured at 95 °C for 5 min. Proteins were separated by 1D SDS-PAGE on 10% polyacrylamide gels (ca. 25 μg of protein/gel lane) applying 300 V and then visualized by in-gel fluorescence scanning. Fluorescence was recorded using Fujifilm LAS-4000 Luminescent Image Analyzer with a Fujinon VRF43LMD3 Lens and a 575DF20 filter. Gels were then subjected to Coomassie Brilliant Blue staining to verify equivalent protein loading.

4.2.6. Western blot

Proteins were separated by 1D SDS-PAGE on 10% polyacrylamide gels and visualized by in-gel fluorescence scanning as described above. Then, the proteins were transferred to a PVDF membrane (VWR) with a semi-dry blotter for 70 min at 14 kV. Prior to the transfer, gel was incubated for ca. 5 min in the transfer buffer (48 mM Tris, 39 mM glycine, 0.38% w/v SDS, 20% v/v MeOH) while the membrane in 100% MeOH. After the transfer, the membrane was saturated with 5% BSA in 0.1% TBS-T pH 8.0 for 1 h at RT. Then, the blot was cut into three parts for probing with three different antibodies. The upper part was incubated with rabbit anti-FAS primary antibody (1:1000 in 5% BSA in 0.1% TBS-T pH 8.0 (Cell Signaling C20G5)) overnight at 4 °C. Middle part was probed with goat anti-actin primary antibody (1:1000 in 5% BSA in 0.1% TBS-T pH 8.0 (SantaCruz Biotechnology sc-1616)) overnight at 4 °C. Lower part was incubated with rabbit anti-proteasome beta-5 (anti-PSB-5) primary antibody (1:1000 in 5% BSA in 0.1% TBS-T pH 8.0 (Enzo BML-PW8895)) overnight at 4 °C. The blots were then washed three times 15 min with TBS-T followed by the probing with the secondary antibody. Goat anti-rabbit IgG HRP-conjugated antibody (1:10,000 dilution in 5% BSA in TBS-T pH 8.0 (Pierce 32260)) and donkey anti-goat IgG HRP-conjugated antibody (1:10,000 dilution in 5% BSA in TBS-T pH 8.0 (SantaCruz Biotechnology sc-2020)) were incubated for 1 h at RT (25 °C) with anti-FAS, anti-PSB-5 and anti-actin probed blots, respectively. Signals were detected using AmershamTM ECLTM Prime Western Blotting Detection Reagent (GE HealthCare RPN2232). Chemiluminescence was recorded for 10–60 s using CCD camera of Fujifilm LAS-4000 Luminescent Image Analyzer.

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A. Supplementary material

Synthetic procedures and characterization data for thioester **S3** required to prepare ketene acetal **10b**. Details of relative and absolute configuration assignment of β -lactone acids **12a**, **12a'**, **11b**, **11b'**, **11b''**, **11b'''**. Details of buffer/serum stability assays of β -lactones **21** and **25**. ^1H and ^{13}C NMR spectra for all new compounds, and LC-MS data for β -lactone inhibitors **17–28**. Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmc.2017.01.020>.

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