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Design, Synthesis, and Evaluation of Cystargolide-based β -lactones as Potent Proteasome Inhibitors

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Abstract

The peptidic β -lactone proteasome inhibitors (PIs) cystargolides A and B were used to conduct structure-activity relationship (SAR) studies in order to assess their anticancer potential. A total of 24 different analogs were designed, synthesized and evaluated for proteasome inhibition, for cytotoxicity towards several cancer cell lines, and for their ability to enter intact cells. X-ray crystallographic analysis and subunit selectivity was used to determine the specific subunit binding associated with the structural modification of the β -lactone (P_1), peptidic core, (P_x and P_y), and end-cap (P_z) of our scaffold. The cystargolide derivative **5k**, structurally unique at both P_y and P_1 , exhibited the most promising inhibitory activity for the $\beta 5$ subunit of human proteasomes ($IC_{50} = 3.1$ nM) and significant cytotoxicity towards MCF-7 ($IC_{50} = 416$ nM), MDA-MB-231 ($IC_{50} = 74$ nM) and RPMI 8226 ($IC_{50} = 41$ nM) cancer cell lines. Cellular infiltration assays revealed that minor structural modifications have significant effects on the ability of our PIs to inhibit intracellular proteasomes, and we identified **5k** as a promising candidate for continued therapeutic studies. Our novel drug lead **5k** is a more potent proteasome inhibitor than carfilzomib with mid-to-low nanomolar IC_{50} measurements and it is cytotoxic against multiple cancer cell lines at levels approaching those of carfilzomib.

Graphical Abstract

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Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Analytical data of intermediates, supplementary figures, methods for biological evaluation, and 1H and ^{13}C NMR spectra are available.

Keywords

Proteasome inhibitors; Cystargolides; Structure-activity relationship; Beta-lactones; Cytotoxicity

1. Introduction

Cellular protein composition in living organisms is homeostatically regulated by both synthesis and proteolysis. The latter pathway is primarily carried out by the Ubiquitin-Proteasome System (UPS) which contributes to many physiological processes such as cell cycle progression, cellular differentiation, signal transduction, and gene expression [1]. In order to survive the stress on protein homeostasis associated with rapid cell division, the activity of the UPS is upregulated in neoplastic cells. This increased activity leads to the degradation of proteins associated with tumor suppression, cell-cycle dysregulation, and NF κ B activation [2, 3]. Limiting the proteolytic activity of the UPS in these neoplastic cells with targeted therapeutics causes an accumulation of ubiquitinated proteins, which, in turn, arrests the cells in the G2/M phase of cell cycle and causes apoptosis [4].

The 20S core particle (CP) of the 26S proteasome is the proteolytic core of the UPS. Proteins targeted for degradation are broken down in the CP by the three β subunits that are threonine proteases [5]. The protein cleavage specificity is determined both by the class of proteasome as well as the chemical environment of the proteolytic pocket [4, 6]. In humans, there exist three isoforms of proteasomes, the constitutive proteasome (cCP), the immunoproteasome (iCP), and the thymoproteasome (tCP). [7] While each CP type is associated with distinct cellular responses, the substrate specificities of the three CPs exhibit moderate overlap [8]. The structure and sequence of cCP is evolutionarily conserved across eukaryotic 20S proteasomes, so the yeast proteasome (yCP) is often used as a model for human cCP (hcCP) [9]. We surmised that first focusing on the inhibitory activity for the cCP and mixtures of human proteasomes (hCP) may lead to identification of novel scaffolds that can be subsequently modified for subunit selectivity following guidelines proposed in previous studies [10, 11].

The three catalytic β subunits in the CP influence substrate specificity by restricting proteolysis to characteristic amino acid sequences. In agreement with inhibitor-docking experiments to the yeast core particle (yCP), which demonstrate the importance of the side-chain of the C-terminal amino acid directly next to the yCP active site (P_1) [9], proteasomal

cleavage specificities were assigned according to the preferred P₁ amino acid. The caspase-like (C-L), trypsin-like (T-L), and chymotrypsin-like (ChT-L) activities correspond to the proteasome subunits β 1, β 2, and β 5, respectively [3]. Preferential inhibition of the β 5 subunit is the primary mode of anticancer cytotoxicity of current proteasome inhibitors (PIs) while the additional blockage of subunits β 1 and β 2 seems to sensitize malignant cancer cells to the β 5 inhibitors [12, 13].

All of the clinically used PIs - the epoxyketone carfilzomib (Cfz), and boronic acid derivatives bortezomib and ixazomib - preferentially inhibit the ChT-L β 5 activity within the proteasome [14]. These PIs are currently the only FDA-approved drugs for treatment of multiple myeloma (MM). In spite of the promising activity of these PIs in anticancer therapy, bortezomib has been shown to cause peripheral neuropathy and induce thrombocytopenia [15]. Carfilzomib does not induce the same degree of peripheral neuropathy, but it has been associated with cardiovascular complications such as hypertension and heart failure [16]. In an attempt to minimize these side effects, novel PI scaffolds are being investigated for clinical use.

The clinical evaluations of PIs have focused on three classes of compounds: the boronic acids, the epoxy ketones, and the β -lactone- γ -lactams (clasto-lactacystin- β -lactone and salinosporamides) [4, 17]. Clasto-lactacystin β -lactone (omuralide), isolated from *Saccharomyces*, was the first PI identified and developed as a potential therapeutic [18, 19]. Even though the β -lactone- γ -lactam PIs have received much attention [20], the peptidic β -lactones (belactosins and cystargolides) represent a promising class of PI's since they are structurally simpler than the bicyclic β -lactone- γ -lactam natural products [21–24]. Cystargolides A and B (CysA and CysB, Scheme 1a), were isolated from the actinomycete *Kitasatospora cystarginea*, and these compounds have been described to block human proteasome activity in the μ M range [21]. Recently, our group accomplished the first total synthesis and determined the absolute stereochemistry for both CysA and CysB [22]. This work identified the benzyl ester **1** (Scheme 1a) as a potent analog that inhibited the proteasome at low nanomolar concentrations. Interestingly, **1** also displayed low micromolar cytotoxicity against MCF7 breast cancer cells [22], warranting further investigations of the cystargolide scaffold as a peptidic PI like the belactosins.

As a continuation of our interest in optimizing the anticancer activity of natural products [22, 25], and desiring to discover PIs with novel modes of action [26], we designed and synthesized an array of analogs based on **1** to develop comprehensive SAR for the cystargolide scaffold. Structural modification focused on three major components of the scaffold (Scheme 1b): substitution at the β -lactone side chain (P₁), the composition of the internal dipeptide (P_X and P_Y), and the functionality of the acid derivative cap at the end of our scaffold (P_Z). Guided by the x-ray structures of various analogs in complex with the γ CP, the structural importance of each of these positions was evaluated in terms of direct proteasome inhibition activity, subunit selectivity associated with the proteasome, cancer cell line cytotoxicity, and successful inhibition of intracellular proteasomes relative to free proteasomes (cellular infiltration). Although β -lactones were identified as the first natural ligands for the proteasome, these compounds still have not managed to find their home in

the clinics. This work provides useful insight into the design of novel peptidic β -lactone PIs for future therapeutic applications.

2. Results and discussion

2.1. Crystallographic analysis of the cystargolide scaffold

In our ongoing efforts to discover proteasome inhibitors with novel modes of action, we determined the crystal structure of the yeast 20S proteasome (yCP) in complex with CysB at 2.7 Å resolution ($R_{\text{free}}=21.4\%$, PDB ID 6G7F, see Table ST1 in the Supplementary Information). The experimental $2F_O-F_C$ electron density map unambiguously depicts the entire inhibitor bound to all proteasomal active sites (Fig. 1c-e). As anticipated, the carbonyl carbon atom of the β -lactone is covalently bound to the catalytic N-terminal threonines (Fig. 2a). Structural superposition of CysB with Omuralide (Omu) [9] and the belactosin derivative ESY [30] complexed with yCP reveal a uniform H-bond interaction of the acyl-oxygen with the oxyanion hole Gly47NH, even though the sp^2 -hybridized ester does not mimic a tetrahedral intermediate as observed with peptide aldehydes (Fig. 1f-h) [9, 27]. Moreover, this crystallographic study unveils that CysB adopts distinct arrangements in each substrate binding channel, which to date have not been observed in any natural or synthetic peptide ligand complexed with the CP (Fig. 2). Presumably, the unique configurations of CysB are induced by the unusual stereochemistry of its P_1 pseudo-valine residue and the generated α -hydroxy-ketone group that is different from the amide-bond of conventional proteasome peptide substrates. In the chymotrypsin- and caspase-like active sites ($\gamma\beta 5$ and $\gamma\beta 1$ respectively) CysB solely interacts with the nonprimed specificity pockets (Fig. 1c, d). Notably, the subunits $\gamma\beta 1$ and $\gamma\beta 5$ structurally align to each other, but the CysB molecules adopt singular conformations with most striking differences in the interactions that occur at the C-terminal moieties (Fig 2a). For instance, in the caspase-like substrate binding channel of $\beta 1$ the carboxylate of CysB protrudes into the S3 specificity pocket and is H-bonded to $\beta 1\text{Thr}22$ as well as to $\beta 2\text{His}114$, while it is solvent exposed in the $\beta 5$ subunit. These structural discrepancies might contribute to the 50-fold decreased IC_{50} value for $\gamma\beta 5$ (0.224 μM) versus $\gamma\beta 1$ (10.1 μM) (Table ST2). In contrast, in subunit $\gamma\beta 2$, the natural product predominantly occupies the primed site, but the peptide mimetic and P_1 -residue perfectly match the configuration of CysB bound to $\gamma\beta 1$ (Fig. 2). This exceptional mode of binding is feasible, because upon β -lactone ring opening the formed acyloin is able to rotate around its C3-C4-bond. Hence, the peptide moiety of CysB covering the primed site mimics a CP substrate bound in standard orientation ($N \rightarrow C$), but the generated C4-OH group only weakly interacts with $\gamma\beta 2\text{Arg}190$ located in a hydrophilic pocket. Yet, the main reason for this particular binding profile is enigmatic from a structural point of view. Most likely, the low affinity of CysB for $\gamma\beta 2$ ($IC_{50} = 61.6 \mu\text{M}$) results from the lack of defined interactions of the P_1 site with protein residues in the S_1 specificity pocket and the presence of only one H-bond of the ligand's carbonyl oxygen atom of its final amide bond with $\text{Ser}129\text{NH}$. On the other hand, the conspicuous arrangement of the natural product in $\gamma\beta 2$ is identical to that of the non-peptide belactosin C derivative ESY [30], despite the fact that both molecules significantly differ in their chemical composition except for the β -lactone warhead (Fig 1h). Though CysB adopts individual conformations in each active site, all residues forming the various specificity pockets look identical as in the yCP apo structure. As a consensus, the

slow-hydrolysis of CysB bound to the β_5 subunit of the proteasome (Fig. S1) is caused by the coordinative displacement of the nucleophilic water molecule from the Bürgi-Dunitz trajectory [31] and the suppression of ester bond hydrolysis either by the C4-OH group (subunits $\gamma\beta 1$ and $\gamma\beta 5$) or the 4-aminocarbonyl side chain (subunit $\gamma\beta 2$).

Interestingly, the benzyl ester of CysB (compound **1**) was described to be up to two orders of magnitude more potent than the natural product (IC_{50} values of **1** versus CysB: $\gamma\beta 1 = 4.18 / 10.1 \mu M$, $\gamma\beta 2 = 14.6 / 61.6 \mu M$, $\gamma\beta 5 = 0.0372 / 0.224 \mu M$, respectively). (Table ST2)[22] Therefore, we crystallographically analyzed **1** in complex with γCP at 2.7 Å resolution ($R_{free}=21.4\%$, PDB ID 6G8M, see Table ST1 in the Supplementary Information). Inspection of the $2F_O-F_C$ electron density map revealed that the benzyl ester derivative is bound in active site. Surprisingly, the introduced protective group is solvent exposed and thus, only partially resolved in all substrate binding channels. Moreover, at the $\gamma\beta 1$ and $\gamma\beta 2$ active sites, the conformation of **1** and CysB perfectly match (see supplementary info Fig. S2). These findings are unexpected because, compared to CysB, **1** owns a two-fold and four-fold increased IC_{50} value for $\gamma\beta 1$ and $\gamma\beta 2$, respectively. Furthermore, the binding mode of **1** to $\gamma\beta 5$ is similar to the conformation found for CysB bound to $\gamma\beta 2$ (see supplementary info Fig. S2). Remarkably, despite the ligands adopting similar arrangements, their IC_{50} values markedly differ by a factor of 6. These striking structural and functional insights led us to conclude that the free carboxylate group in CysB takes the main responsibility for the high IC_{50} values of the natural product, and may contribute to kinetic factors that affect drug infiltration rather than the interactions of the scaffold within the catalytic enzyme pockets. Notably, enhanced potency has also been observed for other proteasome inhibitors with protecting groups at their carboxy terminus versus the unmodified versions, exemplified in belactosin A versus bis-protected belactosin A [29] or syringolin A versus esterified glidobactin A (Fig. S3) [32]. Considering these results associated with the C-terminus, we demonstrated that the dipeptide moiety of CysB, which is linked to the β -lactone moiety in reverse order ($N \rightarrow C$), only plays a minor role in ligand stabilization and subtle alterations are sufficient to induce major structural rearrangements.

2.2. Biological characterization of the cystargolide scaffold

The crystallographic analyses have identified the peptide mimetic component of CysB as the minimal scaffold required to block the CP activity (Fig. 1b). Interestingly, our previous work showed that CysB exhibited minimal toxicity against MCF7 cancer cells ($IC_{50} = 84.7 \mu M$) whereas its benzyl ester derivative **1** was over 10 times more cytotoxic against MCF7 cells ($IC_{50} = 7.0 \mu M$) [22]. For this reason, we decided to focus our SAR analysis of proteasome inhibition and cytotoxicity on cystargolide derivatives bearing substitutions at the end cap (Pz) in order to improve the anticancer activity of our scaffold. Recently, it was shown that the presence of an isoleucine mimetic at the P1 side chain of a simplified β -lactone PIs can cause a displacement of Met45 which forms the bottom of the S1 pocket in proteasomal subunit β_5 by ca. 1 Å [30]. Since the difference of only one methyl group in the P1-site of β -lactones can be crucial for the potency of CP inhibition, we synthesized compound **2** (Scheme 2) which consists of a sec-butyl P1 residue (pseudo-isoleucine) compared to the pseudo-valine site in CysB and **1**. Compound **2** was synthesized coupling the known benzyl ester valine dipeptide [22] and sec-Bu β -lactone acid [23]. Expecting enhanced β_5

selectivity, we tested the biological activity of **2** in purified yeast proteasomes, human cell lysates, and intact human cells (Fig. 3).

We started by evaluating proteasome inhibition and subunit selectivity for our compounds. Interestingly, compared to **1**, the extra methyl group of the pseudo-isoleucine lactone for **2** did not improve preference for $\gamma\beta 5$ over both of the other subunits as expected. The ratio of inhibition of $\gamma\beta 1:\gamma\beta 5$ decreased 2-fold, whereas $\gamma\beta 2:\gamma\beta 5$ increased 2-fold (Fig. 3a, b). Since $\gamma\beta 5$ inhibition remained similar (IC_{50} values of **1** versus **2**: $\gamma\beta 5$ 0.0372 / 0.0368 μM) (see Table ST2), the difference in subunit selectivity suggests that there may be a shift in the binding mode. Once we tested the compounds on cell line models for MM (RPMI 8226) and breast cancer (MCF7 and MDA-MB-231), derivative **2** presented a much improved cytotoxicity profile (Fig. 3c). However, the change in the profile of subunit inhibition in yCP which is a reliable model for hcCP did not correlate with this improvement. Therefore, we performed Western blot and flow cytometry analysis which confirmed that treatment of cancer cells with **1** and **2** led to an accumulation of ubiquitinated proteins [34], induced apoptosis [35], and arrested cells in the G2/M phase [36] (Figure S4). Hence, given the literature precedents, similar results for both compounds indicate that proteasome inhibition remains the primary mode of toxicity. This led us to evaluate the difference between proteasome inhibition within lysed versus intact cells (Fig. 3d, e) as a representative measure of PIs ability to access intracellular proteasomes. Treatment of intact MCF7 (Michigan Cancer Foundation-7) breast cancer cells with inhibitors **1** or **2**, followed by cell washing, cell lysis, and evaluation of resulting proteasome activity revealed that **2** was approximately 10 times more capable of crossing the cell membrane in one hour than **1**. This result correlates directly with the observed differences in cytotoxicity.

Even though activity assays showed that **2** $\gamma\beta 1 = 2.48 \mu M$, $\gamma\beta 2 = 26.1 \mu M$, $\gamma\beta 5 = 0.0248 \mu M$ and **1** have comparable IC_{50} values on the yCP, the crystallographic analysis of the yCP:**2** complex at 3.0 Å resolution again depict surprising insights ($R_{free}=23.8\%$, PDB ID 6G8N, see Table ST1 in the supplementary Information). While **2** properly aligns with **1** bound to subunits $\gamma\beta 1$ and $\gamma\beta 2$ (Fig. S1), the expansion of the P1 site via a single methyl-group induces a flip in the ligand's configuration and reverts the mode of binding upon $\gamma\beta 5$ docking (Fig. 4). These unexpected findings point out that the binding probabilities for CysB and its derivatives are exceptional. In addition, depending on the ligand's conformation, blockage of ester-bond hydrolysis can be prevented by distinct functional groups leading to unique inhibition mechanisms.

2.3. Design and synthesis of analog library

The results from the crystallographic analysis along with the substantial increase in anticancer activity of **2** indicated that minor changes in the structure of the CysB scaffold significantly affect the efficacy of our compounds. Therefore, we synthesized a library of 23 analogs of compound **2** in an effort to determine the functional importance of the substitution at four structural handles (P_1 , P_x , P_y , and P_z , Scheme 2) and ultimately improve anticancer activity (Table 1).

The synthesis of our library of derivatives was achieved using a convergent approach that involved coupling P_1 substituted β - lactones with various dipeptide moieties. The dipeptide fragments were designed to be differentially substituted at the P_x , P_y , and P_z positions. Using chemistry described by Barlaam [37], Armstrong [23], and ourselves [22], the synthesis of β -lactone warheads **9a-e** (Scheme 2a) commenced by acylation of commercially available (*R*)-4-benzyl-2-oxazolidinone with P_1 - containing carboxylic acids to deliver imides **6a-e** (Scheme 2a). Diastereoselective alkylation of **6a-e** with *t*-butyl bromoacetate under Evans' conditions [38, 39] afforded intermediates **7a-e** which were isolated as a single diastereomer (see supplementary information). Next, cleavage of the chiral auxiliary under standard conditions [39] with LiOH/H₂O₂ effectively yielded acid derivatives **8a-e** (see supplementary information), which in turn were subjected to one-pot chlorination-lactonization [23,37] sequences to afford trans- β -lactones **9a-e**. Interestingly, we observed a decrease in the yield for the β -lactone formation as P_1 became progressively more strained.

The dipeptide fragments **11a-t** were assembled by esterification of L-amino acids with aliphatic or aromatic alcohols followed by coupling with either *N*-Boc or Fmoc protected amino acids under standard conditions [40–43]. Assembly of the final compounds was then carried out by cleavage of the protective groups on both the dipeptides **11a-t** and the β -lactones **9a-e**, followed by coupling using EDCI/HOBt [40] (Scheme 2, see supplementary information).

2.4. P_z analogs

P_z derivatives **3a-h** were prepared because our studies on the natural cystargolides showed that the benzyl ester **1** improved the activity of the natural product CysB by two orders of magnitude [22]. The observed change in subunit specificity indicated that P_z substitution may also contribute more to drug binding than previously expected. Systematic alteration of the P_z position was used to determine structure-activity relationships of the steric and electronic characteristics at this position (Table 2). Measuring the activity against a mixture of hiCP and hcCP contained in T- lymphocyte (Jurkat) cell lysate (hCP) [44], all of the compounds remained within 3 times the proteasome inhibition activity of compound **2**. The benzyl amide **3e** was the most potent agent, but it was not the most cytotoxic one. Allyl ester **3a** displayed the best cytotoxicity profile, but **3b**, **3d**, and **3f** exhibited similar effects. Compounds **2**, **3a**, **3b**, and **3f** share a common allylic π - system as a common feature. This may indicate that the π -system chemical structure is beneficial for promoting cytotoxicity. Further evaluation of proteasome subunit selectivity in purified yCP revealed that the benzyl amide **3e** improved selectivity against $\gamma\beta 1$ but not $\gamma\beta 2$ (Fig. S5). Based on the crystal structure of CysB (Fig. 1e), the presence of the H-bond donor potentially disrupts the interaction of P_z with $\gamma\beta 2$ His114 in the $\gamma\beta 1$ binding pocket. Furthermore, the decrease in selectivity against $\gamma\beta 1$ for **3a** (Fig. S5a) suggests that steric bulkiness contributes to $\gamma\beta 5$ selectivity of the cystargolide scaffold. Nonetheless, the results from this evaluation indicate that there is some structural flexibility at P_z with regards to cytotoxicity.

2.5. P_1 analog:

Simultaneously we decided to probe the potential steric interactions at the P_1 position. We synthesized alicyclic and isobutyl derivatives **4a-d** (Table 3). The modifications were chosen

to minimize both the chain length and the rotational freedom of the P1 moieties in an effort to improve $\beta 5$ selectivity via an induced fit [45]. As observed in Table 3, increasing the size of the alicyclic side chains indeed led to improved inhibitory selectivity of h $\beta 5$ over h $\beta 2$ activity by a factor of 4 when comparing **2** to **4c**. The increased selectivity correlates with the total number of carbons in the alicyclic chain at P₁. Notably, compound **4c** with the largest P1 side chain exhibited the greatest selectivity, which presumably is due to optimized interaction with the S₁ site. Each of these modifications, however, led to a substantial decrease in cytotoxicity with respect to **2**. Since we did not observe a positive correlation between subunit selectivity and cytotoxicity in the three cell lines that were evaluated, we concluded that the cellular infiltration was limiting cytotoxicity.

In order to evaluate our findings, we examined the ratio of IC₅₀ concentrations for proteasome inhibition within intact MCF7 cells compared to lysed cells (Fig. 5). Analogs **4a-d** each exhibited moderate decreases in the ability to inhibit intracellular proteasomes. For the compounds containing alicyclic P₁ side chains (**4a-c**), we observed that increased structural tension appears to prevent successful inhibition of the proteasomes contained in intact cells. Our results suggest that the successful inhibition of intracellular proteasomes with our PIs may be highly influenced by steric factors such as branching and structural rigidity as well as the molecular strains associated with the β -lactone. By restricting the rotational freedom of the alkyl substituent at P₁ yet maintaining a similar number of carbon atoms, we were able to improve the general selectivity of the drug scaffold for the $\beta 5$ subunit. This improvement in subunit selectivity came at the cost of these compounds' ability to infiltrate the cell and inhibit intracellular proteasomes.

2.6. P_x/P_y analog:

Finally, we evaluated peptide modifications (P_x/P_y analogs) to assess the role of these moieties with respect to $\beta 5$ selectivity and cytotoxicity (Table 4). Analogs **5a-k** were therefore designed to assess the role of sterics at P_x (analogs **5a**, **5b**, **5e**, **5h**, and **5j**), P_y (analogs **5c**, **5f**, **5i**, and **5k**), or combinations of both (**5d** and **5g**). Change in the steric profile of P_x substitutions appeared to have the most noticeable impact on the targeted inhibition of h $\beta 5$. Analogs **5b** and **5e**, bearing bulky hydrophobic P_x substituents, enhanced preference for the h $\beta 5$ subunit, whereas the flexible glycine analog, **5a**, reduced this selectivity.

While P_y substitutions did not notably affect proteasome subunit selectivity in Jurkat lysate h $\beta 5$, the selectivity of **5k** was substantially improved for y $\beta 5$ in the purified yeast proteasomes (Table ST2). Also, these P_y substitutions exhibited noteworthy effects on cytotoxicity. Analogs **5i** and **5k** exhibited midrange nanomolar IC₅₀ values for all three cell lines tested, and **5k** displayed an exceptionally low IC₅₀ (3.1 nM) for direct $\beta 5$ proteasome inhibition in human cell lysate. Since **5k**'s two fold increase in h $\beta 5$ proteasome inhibition did not correlate with the four fold increase in cytotoxicity, we measured the ability of our PIs to inhibit intracellular proteasomes relative to lysed cells.

As observed in Figure 6, P_x analogs **5a**, **5b**, **5e**, **5h**, and **5j** either maintain or limit the inhibition of intracellular proteasomes. The ability to block intracellular proteasomes was not as affected by increases in the steric profile at the P_y position. At this site, substitution of

the β carbon of the amino acid appears to decrease both infiltration and cytotoxicity associated with **5f**. Alternatively, enhanced infiltration of **5i** and **5k** directly correlated with cytotoxicity. Together with its improved proteasome inhibition profile, the IC₅₀ of **5k** cytotoxicity in the MM cell line, RPMI 8226 is only 6 times less than the clinically-used drug carfilzomib. Therefore, we decided to characterize the proteasomal activity of **5k** in purified hiCP in order to complete our comprehensive panel of biological activity.

We measured the inhibition of the $\beta 5$ subunit in the human immunoproteasome (hi $\beta 5$) for our most noteworthy compounds (**1**, **2**, **3a**, and **5k**) as a means of evaluating isoform selectivity (Fig. 7). The addition of the extra methyl group at P1 from the pseudo-valine **1** to pseudo-isoleucine **2** led to an increase in affinity for hi $\beta 5$. Presumably, the increase in hi $\beta 5$ inhibition is a result of the larger S1 pocket compared to hc $\beta 5$ [6]. Interestingly, measuring the inhibition of y $\beta 5$ as a model for hc $\beta 5$ and hi $\beta 5$, (Table ST2), the larger steric profile of P_z benzyl esters **1** and **2** appears to benefit drug binding to hi $\beta 5$ compared to the allyl ester **3a**. Ultimately, compounds **2** and **5k** exhibited slight preference for hi $\beta 5$, which is experimentally supported by carfilzomib's display of its characteristic specificity for the yCP [8, 46]. Strikingly, while P₁ and P_z impact hi $\beta 5$ selectivity, compared to **2**, the Py analog **5k** expresses a higher affinity for hi $\beta 5$, but the preference against y $\beta 5$ remains relatively unchanged.

3. Conclusion

In summary, we synthesized a library of 24 peptidic β -lactone PIs based on the cystargolide scaffold. We developed structure- activity relationships by evaluating proteasome inhibition, cytotoxicity, subunit selectivity, cellular infiltration, and yCP-PI co-crystallization. Structural variations focused on the side chains of the β -lactone (P₁), the dipeptide unit (P_x/P_y), and the acid derivative (P_z). Analog **2**, bearing a sec-butyl group at P1 displayed ~10 fold increase in cytotoxicity with respect to our original lead **1**. Evaluation of the subunit selectivity for the subsequent analogs revealed that increasing the steric profile of P1 and P_x improved $\beta 5$ selectivity in both human and yeast proteasomes. While, P1 and P_z substitution contributed to the preferential inhibition of hi $\beta 5$ over y $\beta 5$, there was a negligible change associated with the Py substituted analog **5k**. Even though cell lines may present different amounts of the proteasome isoforms and the clinically used PIs target the $\beta 5$ subunit, neither subunit or isoform selectivity correlated with cytotoxicity. Instead, improvement in the ability of our compounds to inhibit intracellular versus free proteasomes was directly associated with the increase in cytotoxicity for analogs **5i** and **5k**. As the most potent drug lead of this entire study, **5k** exhibited nanomolar IC₅₀ values against cancer cells. The efficacy against the MM cell line is within the same order of magnitude as that of the commercially available PI, carfilzomib. Cellular infiltration remains the primary barrier to further improvement of anticancer cytotoxicity for our PI. Analog **5k** is a more potent PI than carfilzomib, and possesses several drug-like properties: molecular weight close to 500, presence of 2 hydrogen bond donors, 9 hydrogen bond acceptors, as well as a LogP value of 1.52 (see supplementary information). In addition, the half-life ($t_{1/2}$) of **5k** in human AB serum and TEAA buffer was found to be 16.1 min and 4.32 min respectively. (see supplementary information for details). These findings suggest that the structure of **5k** could be further

modified to improve the pharmacokinetics of this lead PI. Overall, the results from the SAR studies described here are being used to identify and address the kinetic limitations of our drugs that limit toxicity against cancer cells. The results of these investigations will be disclosed in due course.

4. Experimental

4.1. Chemistry

4.1.1. General Considerations: All moisture sensitive reactions were conducted in oven-dried glassware under an atmosphere of dry nitrogen or argon. Reaction solvents (CH_2Cl_2 , Et_2O , THF) were dried and degassed by passing through a column of activated alumina in a solvent purification system. *N*-methyl morpholine was distilled from CaH_2 . Dimethyl formamide (DMF) was dried over activated 4Å molecular sieves and distilled under high vacuum. All other solvents and reagents were purchased from commercial suppliers and used as received, unless otherwise specified. Thin layer chromatography (TLC) was performed with glass plates precoated with silica 60 Å F-254 (250 µm) and visualized by UV light, KMnO_4 , phosphomolybdic acid, anisaldehyde or Cerium Ammonium Molybdate stains. ^1H and ^{13}C NMR spectra were recorded using a 400 MHz Bruker instrument working at a frequency of 400 MHz for ^1H and at 100 MHz for ^{13}C . Chemical shifts are reported in ppm using residual solvent resonances as internal reference (δ 7.26 and δ 77.0 for ^1H and ^{13}C in CDCl_3 , δ 2.50 and δ 39.51 for ^1H and ^{13}C in DMSO-d_6). ^1H NMR data are reported as follows: b = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Coupling constants are given in hertz. Purity of all compounds and synthetic intermediates were judged to be 95% or better based on ^1H NMR or RP-HPLC. IR measurements were performed in a Nicolet FTIR as thin films. Optical rotations were measured on a Rudolph Autopol III polarimeter. High-resolution mass spectrometry analyses were conducted at the Montana State University Mass Spectrometry facility.

4.1.2 Representative Synthesis of β -lactone PIs (2–5k): A solution of A-Boc peptide **11a** (40 mg, 0.11 mmol) in 0.75 mL of dry CH_2Cl_2 under nitrogen atmosphere was treated with 0.75 mL of TFA at room temperature. The reaction was stirred for about 30 minutes before carefully concentrating under high vacuum. (In case of Fmoc protected dipeptides, the free amine was used after the removal of Fmoc group using 1:1 (v/v) CH_2Cl_2 : Et_2NH). The trifluoroacetate salt (or free amine) thus obtained was immediately used for the next step without extensive characterization. The cleavage of *t*-butyl group of **9a** was accomplished following the previously reported procedure [22] resulting in a free β -lactone acid. To a solution of the lactone acid derived from **9a** (28.93 mg, 0.16 mmol) at 0 °C in 0.6 mL of dry THF, freshly distilled *N*-methyl morpholine (74 µL, 0.67 mmol) was added dropwise under nitrogen atmosphere. The mixture was stirred at the same temperature and added to a flask containing a stirred solution of the trifluoroacetate salt derived from **11a** (40.74 mg, 0.11 mmol) in 0.6 mL of dry THF held at 0 °C. HOBt (32.42 mg, 0.24 mmol) and EDCI (46.0 mg, 0.24 mmol) were simultaneously added in one portion. The reaction mixture was stirred and allowed to reach room temperature overnight before concentrating in rotavapor. The residue was taken in ethyl acetate and washed with 1M HCl solution, NaHCO_3 saturated solution, and brine. The solution was then dried over MgSO_4 , filtered

and the solvent was evaporated under reduced pressure. The residue was purified via flash column chromatography using hexane- EtOAc (3:1) to afford 26.0 mg (0.063 mmol) of the final compound **3a**.

4.1.2.1. benzyl((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane-2- carbonyl)-L-valyl-L-valinate(2): White amorphous solid; Yield: 41%; R_f : 0.4 (Hexanes: EtOAc = 7:3); $[\alpha]_D^{25} = -16.4$ (c 0.2, CHCl_3); IR (γ , cm^{-1}): 2948, 2387, 1914, 1762, 1713, 1614, 1518, 1416; ^1H NMR (400 MHz, CDCl_3): δ 7.35–7.32 (m, 5H), 6.99 (d, $J = 8.3$ Hz, 1H), 6.44 (d, $J = 8.9$ Hz, 1H), 5.22 (d, $J = 12.2$ Hz, 1H), 5.13 (d, $J = 12.0$ Hz, 1H), 4.66 (d, $J = 4.5$ Hz, 1H), 4.60 (dd, $J = 8.7, 4.7$ Hz, 1H), 4.30 (m, 1H), 3.65 (dd, $J = 7.9, 4.7$ Hz, 1H), 2.22–2.10 (m, 2H), 2.01–1.91 (m, 1H), 1.68–1.60 (m, 1H), 1.35–1.24 (m, 1H), 1.05 (d, $J = 6.7$ Hz, 3H), 0.95–0.88 (m, 12H), 0.85 (d, $J = 6.7$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.5, 170.1, 168.8, 168.1, 135.1, 128.6, 128.5, 128.4, 70.4, 67.2, 62.8, 58.6, 57.1, 33.6, 31.1, 30.8, 26.6, 19.1, 18.9, 18.1, 17.5, 16.3, 10.9; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{25}\text{H}_{37}\text{N}_2\text{O}_6$; 461.2646; Found 461.2668.

4.1.2.2. allyl((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane-2-carbonyl)- L-valyl-L-valinate (3a): White amorphous solid; Yield: 58%; R_f : 0.6 (Hexanes: EtOAc = 2:1); $[\alpha]_D^{24} = -3.6$ (c 0.025, CHCl_3); IR (γ , cm^{-1}): 3276, 3075, 1841, 1743, 1614, 1550, 1465; ^1H NMR (400 MHz, CDCl_3): δ 6.94 (d, $J = 8.3$ Hz, 1H), 6.34 (d, $J = 8.5$ Hz, 1H), 5.94–5.84 (m, 1H), 5.35 (d, $J = 17.1$ Hz, 1H), 5.27 (d, $J = 10.3$ Hz, 1H), 4.68 (m, 3H), 4.56 (dd, $J = 4.6, 4.6$ Hz, 1H), 4.29 (dd, $J = 8.3, 8.3$ Hz, 1H), 3.65 (dd, $J = 4.6, 4.6$ Hz, 1H), 2.24 (m, 2H), 2.03 (m, 1H), 1.69 (m, 1H), 1.38 (m, 2H), 1.06 (d, $J = 6.8$ Hz, 3H), 0.98 (m, 15H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.3, 170.0, 168.8, 168.1, 131.4, 119.2, 70.5, 66.0, 62.9, 58.7, 57.1, 33.7, 31.0, 30.8, 26.6, 19.1, 19.0, 18.1, 17.6, 16.3, 10.9; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{21}\text{H}_{35}\text{N}_2\text{O}_6$ 411.2490; Found 411.2502.

4.1.2.3. 4-chlorobenzyl((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane-2- carbonyl)-L-valyl-L-valinate (3b): White amorphous solid; Yield: 45%; R_f : 0.5 (Hexanes: EtOAc = 3:1); $[\alpha]_D^{24} = -12.5$ (c 0.025, CHCl_3); IR (γ , cm^{-1}): 3272, 1840, 1744, 1644, 1553; ^1H NMR (400 MHz, CDCl_3): δ 7.36–7.28 (m, 4H), 6.93 (d, $J = 8.9$ Hz, 1H), 6.32 (d, $J = 8.9$ Hz, 1H), 5.20 (d, $J = 12.4$ Hz, 1H), 5.11 (d, $J = 12.2$ Hz, 1H), 4.67 (d, $J = 4.5$ Hz, 1H), 4.59 (dd, $J = 4.7, 4.7$ Hz, 1H), 4.29 (dd, $J = 6.9, 6.8$ Hz, 1H), 3.66 (dd, $J = 4.6, 4.5$ Hz, 1H), 2.24–2.12 (m, 2H), 2.06–1.95 (m, 1H), 1.71–1.61 (m, 1H), 1.38–1.21 (m, 2H), 1.08 (d, $J = 6.9$ Hz, 3H), 0.98 (m, 12H), 0.87 (d, $J = 7.0$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.4, 170.1, 168.8, 168.1, 134.5, 133.6, 129.8, 128.8, 70.5, 66.3, 62.9, 58.7, 57.1, 33.7, 31.0, 30.8, 26.7, 19.1, 19.0, 18.1, 17.6, 16.3, 11.0; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{25}\text{H}_{36}\text{ClN}_2\text{O}_6$ 495.2256; Found 495.2260.

4.1.2.4. cyclohexylmethyl((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane- 2-carbonyl)-L-valyl-L-valinate (3c): White amorphous solid; Yield: 31%; R_f : 0.4 (Hexanes: EtOAc = 5:1); $[\alpha]_D^{22} = -4.4$ (c 0.08, CHCl_3); IR (γ , cm^{-1}): 3276, 2360, 1843, 1740, 1646, 1558, 1457; ^1H NMR (400 MHz, CDCl_3): δ 7.0 (d, $J = 8.2$ Hz, 1H), 6.33 (d, $J = 8.2$ Hz, 1H), 4.66 (d, $J = 4.6$ Hz, 1H), 4.65 (dd, $J = 4.6, 4.6$ Hz, 1H), 4.29 (dd, $J = 7.2, 6.9$ Hz, 1H), 3.98–3.90 (m, 2H), 3.66 (dd, $J = 7.5, 7.4$ Hz, 1H), 2.23–2.12 (m, 2H), 2.04–1.93 (m, 1H), 1.73–1.60

(m, 6H), 1.35–1.14 (m, 7H), 1.07 (d, J = 6.9 Hz, 3H), 0.98 (m, 15H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.7, 170.2, 168.8, 168.2, 70.6, 70.4, 62.9, 58.7, 57.2, 36.9, 33.6, 31.9, 31.2, 30.9, 29.7, 29.6, 26.7, 26.2, 25.6, 25.5, 19.1, 19.0, 18.1, 17.6, 16.3, 11.0; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{25}\text{H}_{43}\text{N}_2\text{O}_6$ 467.3116; Found 467.3115.

4.1.2.5. isobutyl((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane-2-carbonyl)-L-valyl-L-

valinate (3d): White amorphous solid; Yield: 70%; R_f : 0.5 (Hexanes: EtOAc = 5:1); $[\alpha]_D^{23}$ = -2.8 (c 0.25, CHCl_3); IR (γ , cm^{-1}): 3290, 2351, 1843, 1645, 1557; ^1H NMR (400 MHz, CDCl_3): δ 6.96 (d, J = 8.5 Hz, 1H), 6.38 (d, J = 8.5 Hz, 1H), 4.66 (d, J = 4.77 Hz, 1H), 4.55 (dd, J = 4.8, 4.8 Hz, 1H), 4.29 (dd, J = 8.1, 6.9 Hz, 1H), 3.95 (m, 2H), 3.65 (dd, J = 7.6, 7.6 Hz, 1H), 2.23–2.11 (m, 2H), 2.01–1.88 (m, 3H), 1.67–1.59 (m, 1H), 1.34–1.23 (m, 2H), 1.06 (d, J = 6.8 Hz, 3H), 0.98 (m, 19H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.7, 170.0, 168.8, 168.0, 71.5, 70.5, 62.8, 58.7, 57.1, 33.6, 31.2, 30.8, 27.6, 26.6, 19.1, 19.0, 18.9, 18.1, 17.6, 16.3, 10.9; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{22}\text{H}_{39}\text{N}_2\text{O}_6$ 427.2803; Found 427.2823.

4.1.2.6. (2R,3S)-N-((S)-1-(((S)-1-(benzylamino)-3-methyl-1-oxobutan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)-3-((S)-sec-butyl)-4-oxooxetane-2-carboxamide (3e): White amorphous solid; Yield: 52%; R_f : 0.3 (Hexanes: EtOAc = 2:1); $[\alpha]_D^{22}$ = -74.0 (c 0.25, MeOH); IR (γ , cm^{-1}): 3269, 3085, 2324, 1842, 1635, 1551; ^1H NMR (400 MHz, DMSO): δ 8.59 (d, J = 8.8 Hz, 1H), 8.53 (m, 1H), 8.08 (d, J = 8.7 Hz, 1H), 7.31–7.20 (m, 5H), 5.04 (d, J = 4.0 Hz, 1H), 4.36–4.30 (m, 2H), 4.26–4.13 (m, 2H), 3.66 (dd, J = 7.6, 7.6 Hz, 1H), 2.50 (m, 1H), 2.02–1.89 (m, 3H), 1.56–1.45 (m, 1H), 1.29–1.20 (m, 1H), 0.95 (d, J = 6.7 Hz, 3H), 0.86–0.80 (m, 15H); ^{13}C NMR (100 MHz, DMSO): δ 170.8, 170.4, 167.4, 139.4, 128.2, 127.1, 126.7, 69.6, 61.1, 58.1, 57.6, 42.0, 32.6, 30.7, 30.4, 26.0, 19.2, 19.2, 18.4, 17.9, 15.8, 10.9; HRMS analysis of 3e was not successful using ESI.

4.1.2.7. 4-methoxybenzyl((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane-2-carbonyl)-L-valyl-L-

valinate (3f): White amorphous solid; Yield: 39%; R_f : 0.3 (Hexanes: EtOAc = 5:1); $[\alpha]_D^{22}$ = -11.2 (c 0.24, CHCl_3); IR (γ , cm^{-1}): 3278, 2964, 1837, 1739, 1646, 1614, 1515; ^1H NMR (400 MHz, CDCl_3): δ 7.31 (d, J = 9.0 Hz, 2H), 6.91 (d, J = 8.7 Hz, 2H), 6.33 (d, J = 8.9 Hz, 1H), 5.20 (d, J = 11.2 Hz, 1H), 5.08 (d, J = 11.6 Hz, 1H), 4.68 (d, J = 4.6 Hz, 1H), 4.58 (dd, J = 4.6, 4.6 Hz, 1H), 4.30 (dd, J = 8.3, 8.3 Hz, 1H), 3.83 (s, 3H), 3.67 (dd, J = 7.6, 7.6 Hz, 1H), 2.26–2.12 (m, 2H), 2.06–1.96 (m, 1H), 1.72–1.61 (m, 1H), 1.39–1.30 (m, 2H), 1.09 (d, J = 6.8 Hz, 3H), 0.99 (m, 9H), 0.91 (d, J = 6.9 Hz, 3H), 0.86 (d, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.5, 170.0, 168.8, 168.1, 159.8, 130.3, 127.2, 113.9, 70.5, 67.0, 62.9, 58.7, 157.1, 55.3, 33.7, 31.1, 30.8, 26.7, 19.1, 18.9, 18.1, 17.5, 16.3, 11.0; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{26}\text{H}_{39}\text{N}_2\text{O}_7$ 491.2752; Found 491.2758.

4.1.2.8. but-3-en-1-yl((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane-2-carbonyl)-L-valyl-L-

valinate (3g): Colorless amorphous solid; Yield: 44%; R_f : 0.5 (Hexanes: EtOAc = 4:1); $[\alpha]_D^{25}$ = -5.8 (c 0.1, CHCl_3); IR (γ , cm^{-1}): 3284, 2966, 1842, 1732, 1638, 1556, 1540; ^1H NMR (400 MHz, CDCl_3): δ 6.87 (d, J = 8.4 Hz, 1H), 6.17 (d, J = 8.9 Hz, 1H), 5.81–5.71 (m, 1H), 5.14–5.08 (m, 2H), 4.66 (d, J = 4.7 Hz, 1H), 4.55 (dd, J = 8.8, 4.5 Hz, 1H), 4.27–4.22 (m, 2H), 4.21–4.14 (m, 1H), 3.66 (dd, J = 7.5, 4.7 Hz, 1H), 2.43–2.38 (m, 2H), 2.23–

2.14 (m, 2H), 2.04–1.96 (m, 1H), 1.70–1.62 (m, 1H), 1.38–1.27 (m, 1H), 1.08 (d, $J = 6.6$ Hz, 3H), 1.0–0.95 (m, 6H), 0.94–0.88 (m, 9H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.6, 169.9, 168.8, 168.1, 133.5, 117.7, 70.5, 64.5, 62.9, 58.7, 57.1, 33.7, 33.0, 31.1, 30.9, 26.7, 19.2, 19.0, 18.1, 17.6, 16.3, 11.0; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{22}\text{H}_{37}\text{N}_2\text{O}_6$ 425.2646; Found 425.2670.

4.1.2.9. methyl((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane-2-carbonyl)-L-valyl-L-valinate (3h): Colorless amorphous solid; Yield: 43%; R_f : 0.3 (Hexanes: EtOAc = 4:1); $[\alpha]_D^{23} = -6.2$ (c 0.003, CHCl_3); IR (γ , cm^{-1}): 2964, 1836, 1739, 1647, 1538, 1467; ^1H NMR (400 MHz, CDCl_3): δ 6.87 (d, $J = 8.5$ Hz, 1H), 6.18 (d, $J = 8.4$ Hz, 1H), 4.86 (d, $J = 4.6$ Hz, 1H), 4.55 (dd, $J = 8.8, 4.7$ Hz, 1H), 4.28 (dd, $J = 8.4, 6.6$ Hz, 1H), 3.75 (s, 3H), 3.66 (dd, $J = 7.7, 4.6$ Hz, 1H), 2.23–2.13 (m, 2H), 2.05–1.96 (m, 1H), 1.69–1.60 (m, 1H), 1.37–1.27 (m, 1H), 1.08 (d, $J = 6.7$ Hz, 3H), 1.00–0.89 (m, 15H); ^{13}C NMR (100 MHz, CDCl_3): δ 172.1, 169.9, 168.7, 168.1, 70.5, 82.9, 58.6, 57.1, 52.3, 33.7, 31.1, 30.9, 26.7, 19.1, 18.9, 18.1, 17.7, 16.3, 11.0; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{19}\text{H}_{33}\text{N}_2\text{O}_6$ 385.2333; Found 385.2347.

4.1.2.10. benzyl((2R,3S)-3-cyclopropyl-4-oxooxetane-2-carbonyl)-L-valyl-L-valinate (4a): Pale yellow oil; Yield: 53%; R_f : 0.5 (Hexanes: EtOAc = 2:1); $[\alpha]_D^{21} = -5.2$ (c 0.23, CHCl_3); IR (γ , cm^{-1}): 3270, 2360, 1843, 1740, 1646, 1558, 1457, 1205; ^1H NMR (400 MHz, CDCl_3): δ 7.37–7.32 (m, 5H), 6.83 (d, $J = 8.5$ Hz, 1H), 6.2 (d, $J = 8.4$ Hz, 1H), 5.23–5.11 (m, 2H), 4.65 (d, $J = 4.6$ Hz, 1H), 4.59–4.55 (m, 1H), 4.26 (dd, $J = 8.42, 8.25$ Hz, 1H), 3.53 (dd, $J = 4.9, 4.5$ Hz, 1H), 2.22–2.11 (m, 2H), 1.25–1.20 (m, 2H), 0.97–0.84 (m, 12H), 0.74–0.65 (m, 2H), 0.54–0.51 (m, 1H), 0.46–0.42 (m, 1H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.5, 169.9, 167.8, 167.7, 135.1, 128.6, 128.5, 77.2, 67.3, 61.3, 58.7, 57.2, 31.1, 30.9, 19.1, 18.9, 18.1, 17.5, 8.6, 3.4, 2.9; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{24}\text{H}_{33}\text{N}_2\text{O}_6$ 445.2333; Found 445.2323.

4.1.2.11. benzyl((2R,3S)-3-cyclobutyl-4-oxooxetane-2-carbonyl)-L-valyl-L-valinate (4b): Pale yellow oil; Yield: 54%; R_f : 0.3 (Hexanes: EtOAc : 2 : 1); $[\alpha]_D^{21} = -8.0$ (c 0.26, CHCl_3); IR (γ , cm^{-1}): 3011, 2994, 2369, 1739, 1725, 1691; ^1H NMR (400 MHz, CHCl_3): δ 7.39–7.32 (m, 5H), 6.86 (d, $J = 8.3$ Hz, 1H), 6.19 (d, $J = 8.7$ Hz, 1H), 5.23 (d, $J = 12.1$ Hz, 1H), 5.14 (d, $J = 12.2$ Hz, 1H), 4.60 (d, $J = 4.6$ Hz, 2H), 4.27 (dd, $J = 8.3, 6.8$ Hz, 1H), 3.78 (dd, $J = 7.0, 4.6$ Hz, 1H), 2.90–2.81 (m, 1H), 2.24–2.09 (m, 4H), 2.06–1.89 (m, 4H), 0.97–0.94 (m, 6H), 0.92 (d, $J = 6.7$ Hz, 3H), 0.87 (d, $J = 6.9$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.4, 170.0, 168.2, 168.0, 135.1, 128.6, 128.5, 128.4, 70.8, 67.2, 61.9, 58.7, 51.2, 32.8, 31.6, 31.1, 30.9, 25.2, 24.8, 22.6, 19.1, 18.9, 18.1, 18.1, 17.6; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{25}\text{H}_{35}\text{N}_2\text{O}_6$ 459.2490; Found 459.2439.

4.1.2.12. benzyl((2R,3S)-3-cyclopentyl-4-oxooxetane-2-carbonyl)-L-valyl-L-valinate (4c): Colorless oil; Yield: 67%; R_f : 0.6 (Hexanes: EtOAc = 3:1); $[\alpha]_D^{22} = -10.6$ (c 0.4, CHCl_3); IR (γ , cm^{-1}): 3175, 2323, 1642, 1599, 1471; ^1H NMR (400 MHz, CDCl_3): δ 7.38–7.31 (m, 5H), 6.91 (d, $J = 8.3$ Hz, 1H), 6.30 (d, $J = 8.7$ Hz, 1H), 5.23 (d, $J = 11.9$ Hz, 1H), 5.13 (d, $J = 11.9$ Hz, 1H), 4.614.56 (m, 2H), 4.29 (dd, $J = 8.4, 6.7$ Hz, 1H), 3.67 (dd, $J = 8.4, 4.5$ Hz, 1H), 2.42–2.33 (m, 1H), 2.24–2.09 (m, 2H), 1.95–1.82 (m, 2H), 1.70–1.58 (m, 4H),

1.47–1.39 (m, 2H), 0.96 (dd, J = 6.5, 6.0 Hz, 6H), 0.91 (d, J = 6.9 Hz, 3H), 0.86 (d, J = 7.0 Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.5, 170.0, 168.8, 168.0, 135.1, 128.6, 128.5, 128.4, 71.8, 67.2, 62.0, 58.6, 57.1, 38.1, 31.1, 30.9, 29.9, 29.7, 25.1, 24.8, 19.1, 18.9, 18.1, 17.5; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{26}\text{H}_{37}\text{N}_2\text{O}_6$ 473.2646; Found 473.2636.

4.1.2.13. benzyl((2R,3S)-3-iso-butyl-4-oxooxetane-2-carbonyl)-L-valyl-L-valinate

(4d): White amorphous solid; Yield: 31%; R_f : 0.6 (Hexanes: EtOAc = 2:1); $[\alpha]_D^{21} = -12.0$ (c 0.1, CHCl_3); IR (γ , cm^{-1}): 3027, 2353, 1744, 1739, 1764, 1454, 1376; ^1H NMR (400 MHz, CDCl_3): δ 7.37–7.35 (m, 5H), 6.87 (d, J = 8.8 Hz, 1H), 6.19 (d, J = 8.8 Hz, 1H), 5.23 (d, J = 12.0 Hz, 1H), 5.14 (d, J = 12.2 Hz, 1H), 4.60–4.57 (m, 2H), 4.26 (m, 1H), 3.77–3.72 (m, 1H), 2.23–2.11 (m, 2H), 1.86–1.76 (m, 3H), 0.98 (m, 15H), 0.86 (d, J = 6.9 Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.4, 170.0, 169.4, 167.8, 135.1, 128.6, 128.5, 128.4, 73.3, 67.2, 58.7, 57.1, 56.2, 37.1, 31.1, 30.8, 26.3, 22.4, 21.8, 19.1, 18.9, 18.1, 17.5; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{25}\text{H}_{37}\text{N}_2\text{O}_6$ 461.2646; Found 461.2644.

4.1.2.14. benzyl((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane-2-carbonyl)glycyl-L-valinate

(5a): Colorless amorphous solid; Yield: 84%; R_f : 0.4 (Hexanes: EtOAc = 7:3); $[\alpha]_D^{25} = +18.3$ (c 0.08, CHCl_3); IR (γ , cm^{-1}): 2956, 2806, 1692, 1646, 1536, 1425; ^1H NMR (400 MHz, CDCl_3): δ 7.39–7.32 (m, 5H), 7.10 (bs, 1H), 6.46–6.42 (m, 1H), 5.22 (d, J = 12.3 Hz, 1H), 5.16 (d, J = 12.1 Hz, 1H), 4.65–4.57 (m, 2H), 4.04 (d, J = 5.2 Hz, 1H), 3.67 (dd, J = 7.8, 4.6 Hz, 1H), 2.24–2.16 (m, 1H), 2.03–1.96 (m, 1H), 1.71–1.61 (m, 2H), 1.39–1.29 (m, 1H), 1.08 (d, J = 6.6 Hz, 3H), 0.96–0.91 (m, 6H), 0.87–0.84 (m, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.5, 168.8, 168.6, 167.7, 135.1, 128.6, 128.6, 128.4, 70.5, 67.3, 63.1, 57.3, 42.7, 33.8, 31.3, 31.2, 29.7, 26.6, 18.9, 17.6, 16.3, 11.0; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{22}\text{H}_{31}\text{N}_2\text{O}_6$ 419.2177; Found 419.2162.

4.1.2.15. benzyl((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane-2-carbonyl)-L-phenylalanyl-L-valinate (5b):

White amorphous solid; Yield: 46%; R_f : 0.3 (Hexanes: EtOAc = 3:1); $[\alpha]_D^{21} = -5.5$ (c 0.18, CHCl_3); IR (γ , cm^{-1}): 3082, 2947, 1804, 1743, 1638, 1556; ^1H NMR (400 MHz, CDCl_3): δ 7.39–7.33 (m, 5H), 7.30–7.26 (m, 2H), 7.24–7.20 (m, 3H), 6.93 (d, J = 7.5 Hz, 1H), 6.11 (d, J = 8.5 Hz, 1H), 5.14 (m, 2H), 4.65 (dd, J = 14.4, 7.4 Hz, 1H), 4.57 (d, J = 4.5 Hz, 1H), 4.49 (dd, J = 8.34, 4.7 Hz, 1H), 3.62 (dd, J = 7.5, 4.8 Hz, 1H), 3.10 (dd, J = 7.1, 4.3 Hz, 2H), 2.16–2.8 (m, 1H), 2.01–1.92 (m, 1H), 1.68–1.60 (m, 1H), 1.35–1.27 (m, 2H), 1.05 (d, J = 6.7 Hz, 3H), 0.95 (m, 3H), 0.84 (d, J = 6.8 Hz, 3H), 0.78 (d, J = 7.0 Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.1, 169.7, 168.6, 168.0, 135.8, 135.2, 129.2, 128.9, 128.6, 128.6, 128.4, 127.3, 70.4, 67.1, 63.0, 57.4, 54.6, 37.9, 33.7, 31.1, 26.7, 18.9, 17.5, 16.3, 10.9; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{29}\text{H}_{37}\text{N}_2\text{O}_6$ 509.2646; Found 509.2637.

4.1.2.16. benzyl((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane-2-carbonyl)-L-valyl-phenylalaninate(5c):

White amorphous solid; Yield: 63%; R_f : 0.3 (Hexanes: EtOAc = 3:1); $[\alpha]_D^{21} = +2.5$ (c 0.2, CHCl_3); IR (γ , cm^{-1}): 3282, 2965, 2360, 1838, 1739, 1645; ^1H NMR (400 MHz, CDCl_3): δ 7.39–7.36 (m, 3H), 7.31–7.29 (m, 2H), 7.23–7.22 (m, 3H), 7.01–6.99 (m, 2H), 6.82 (d, J = 8.5, 1H), 6.08 (d, J = 7.5 Hz, 1H), 5.20 (d, J = 12.2 Hz, 1H), 5.12 (d, J = 12.1 Hz, 1H), 4.95–4.90 (m, 1H), 4.63 (d, J = 4.8 Hz, 1H), 4.21 (dd, J = 8.5, 6.3

Hz, 1H), 3.58 (dd, $J = 7.6, 4.6$ Hz, 1H), 3.13 (dd, $J = 6.0, 2.3$ Hz, 2H), 2.13–2.04 (m, 1H), 2.02–1.93 (m, 1H), 1.70–1.60 (m, 1H), 1.36–1.28 (m, 1H), 1.08 (d, $J = 6.7$ Hz, 3H), 0.96 (m, 9H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.0, 169.5, 168.7, 168.0, 135.2, 134.8, 129.2, 128.7, 128.7, 128.6, 127.3, 70.4, 67.5, 62.9, 58.3, 52.9, 37.7, 33.7, 31.0, 26.7, 19.0, 17.9, 16.3, 11.0; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{29}\text{H}_{37}\text{N}_2\text{O}_6$ 509.2646; Found 509.2650.

4.1.2.17. benzyl((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane-2- carbonyl)-L-phenylalanyl-L-phenylalaninate (5d): White amorphous solid; Yield: 51%; R_f : 0.3 (Hexanes: EtOAc = 7:3); $[\alpha]_D^{25} = -1.8$ (c 0.2, CHCl_3); IR (γ , cm^{-1}): 3082, 2947, 1840, 1743, 1638, 1556; ^1H NMR (400 MHz, CDCl_3): δ 7.40–7.37 (m, 3H), 7.31–7.24 (m, 5H), 7.21–7.16 (m, 5H), 6.92–6.87 (m, 3H), 6.11 (d, $J = 7.6$ Hz, 1H), 5.16–5.09 (m, 2H), 4.83 (dd, $J = 13.4, 5.9$ Hz, 1H), 4.62 (dd, $J = 14.1, 7.2$ Hz, 1H), 4.54 (d, $J = 4.8$ Hz, 1H), 3.50 (dd, $J = 7.4, 4.4$ Hz, 1H), 3.12–2.98 (m, 4H), 2.0–1.89 (m, 1H), 1.68–1.57 (m, 1H), 1.33–1.23 (m, 1H), 1.05 (d, $J = 6.8$ Hz, 3H), 0.95–0.89 (m, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 170.6, 169.3, 168.6, 168.0, 135.8, 135.2, 134.9, 129.2, 129.1, 128.8, 128.6, 128.6, 128.5, 127.3, 127.2, 70.3, 67.3, 62.9, 54.4, 53.3, 37.9, 37.7, 33.6, 26.6, 24.7, 16.2, 11.0; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{33}\text{H}_{37}\text{N}_2\text{O}_6$ 557.2646; Found 557.2642.

4.1.2.18. benzyl((S)-2-((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane-2- carboxamido)-3,3-dimethylbutanoyl)-L-valinate(5e): White amorphous solid; Yield: 82%; R_f : 0.5 (Hexanes: EtOAc = 4:1); $[\alpha]_D^{25} = -6.3$ (c 0.4, CHCl_3); IR (γ , cm^{-1}): 2995, 2933, 1771, 1734, 1716, 1443, 1373, 1368; ^1H NMR (400 MHz, CDCl_3): δ 7.38–7.31 (m, 5H), 6.98 (d, $J = 8.7$ Hz, 1H), 6.22 (d, $J = 7.6$ Hz, 1H), 5.22 (d, $J = 12.1$ Hz, 1H), 5.13 (d, $J = 12.2$ Hz, 1H), 4.66 (d, $J = 4.5$ Hz, 1H), 4.58 (dd, $J = 8.5, 4.9$ Hz, 1H), 4.29 (d, $J = 9.1$ Hz, 1H), 3.63 (dd, $J = 7.8, 4.6$ Hz, 1H), 2.22–2.14 (m, 1H), 2.01–1.93 (m, 1H), 1.69–1.58 (m, 1H), 1.36–1.21 (m, 1H), 1.06 (d, $J = 6.8$ Hz, 3H), 0.99 (s, 9H), 0.93–0.88 (m, 6H), 0.86 (d, $J = 6.9$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.4, 169.4, 168.8, 167.8, 135.0, 128.6, 128.5, 128.4, 70.4, 67.1, 62.7, 60.8, 57.2, 34.6, 33.6, 31.1, 26.6, 26.5, 18.9, 17.6, 16.3, 11.0; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{26}\text{H}_{39}\text{N}_2\text{O}_6$ 475.2803; Found 475.2814.

4.1.2.19. benzyl(S)-2-((S)-2-((2R,3S)-3-((S)-sec-butyl)-4- oxooxetane-2- carboxamido)-3-methylbutanamido)-3,3- dimethylbutanoate (5f): White amorphous solid; Yield: 42%; R_f : 0.4 (Hexanes/ EtOAc = 4:1); $[\alpha]_D^{26} = -2.4$ (c 0.3, CHCl_3); IR (γ , cm^{-1}): 2957, 1740, 1706, 1476, 1436, 1266; ^1H NMR (400 MHz, CDCl_3): δ 7.37–7.33 (m, 5H), 6.88 (d, $J = 8.2$ Hz, 1H), 6.31 (d, $J = 9.1$ Hz, 1H), 5.21 (d, $J = 12.1$ Hz, 1H), 5.14 (d, $J = 12.2$ Hz, 1H), 4.65 (d, $J = 4.5$ Hz, 1H), 4.47 (d, $J = 9.1$ Hz, 1H), 4.24 (dd, $J = 8.3, 7.3$ Hz, 1H), 3.63 (dd, $J = 7.6, 4.5$ Hz, 1H), 2.17–2.08 (m, 1H), 2.04–1.94 (m, 1H), 1.69–1.58 (m, 1H), 1.37–1.28 (m, 1H), 1.06 (d, $J = 6.7$ Hz, 3H), 0.94–0.91 (m, 18H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.0, 169.8, 168.8, 168.1, 135.0, 128.6, 128.5, 128.5, 70.4, 67.1, 62.9, 60.2, 58.8, 34.8, 33.6, 30.7, 26.7, 26.5, 19.1, 18.1, 16.3, 11.0; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{26}\text{H}_{39}\text{N}_2\text{O}_6$ 475.2803; Found 475.2813.

4.1.2.20. benzyl(S)-2-((S)-2-((2R,3S)-3-((S)-sec-butyl)-4- oxooxetane-2- carboxamido)-3,3-dimethylbutanamido)-3,3- dimethylbutanoate (5g): White amorphous solid; Yield: 94%; R_f : 0.6 (Hexanes: EtOAc = 3:1); $[\alpha]_D^{25} = -9.7$ (c 0.4, CHCl_3); IR (γ , cm^{-1}): 2957, 1740, 1706, 1476, 1436, 1266; ^1H NMR (400 MHz, CDCl_3): δ 7.37–7.33 (m, 5H), 6.88 (d, $J = 8.2$ Hz, 1H), 6.31 (d, $J = 9.1$ Hz, 1H), 5.21 (d, $J = 12.1$ Hz, 1H), 5.14 (d, $J = 12.2$ Hz, 1H), 4.65 (d, $J = 4.5$ Hz, 1H), 4.47 (d, $J = 9.1$ Hz, 1H), 4.24 (dd, $J = 8.3, 7.3$ Hz, 1H), 3.63 (dd, $J = 7.6, 4.5$ Hz, 1H), 2.17–2.08 (m, 1H), 2.04–1.94 (m, 1H), 1.69–1.58 (m, 1H), 1.37–1.28 (m, 1H), 1.06 (d, $J = 6.7$ Hz, 3H), 0.94–0.91 (m, 18H); ^{13}C NMR (100 MHz, CDCl_3): δ 171.0, 169.8, 168.8, 168.1, 135.0, 128.6, 128.5, 128.5, 70.4, 67.1, 62.9, 60.2, 58.8, 34.8, 33.6, 30.7, 26.7, 26.5, 19.1, 18.1, 16.3, 11.0; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{26}\text{H}_{39}\text{N}_2\text{O}_6$ 475.2803; Found 475.2813.

⁻¹): 2954, 1837, 1738, 1640, 1527, 1443, 1323; ¹H NMR (400 MHz, CDCl₃): δ 7.37–7.32 (m, 5H), 6.96 (d, *J* = 9.1 Hz, 1H), 6.21 (d, *J* = 9.1 Hz, 1H), 5.2 (d, *J* = 12.2 Hz, 1H), 5.13 (d, *J* = 12.1 Hz, 1H), 4.66 (d, *J* = 4.7 Hz, 1H), 4.44 (d, *J* = 8.7 Hz, 1H), 4.27 (d, *J* = 8.8 Hz, 1H), 3.61 (dd, *J* = 7.6, 4.6 Hz, 1H), 2.01–1.92 (m, 1H), 1.68–1.58 (m, 1H); 1.35–1.24 (m, 1H), 1.05 (d, *J* = 6.8 Hz, 3H), 0.97 (s, 9H), 0.93 (s, 9H), 0.91–0.89 (m, 3H) ¹³C NMR (100 MHz, CDCl₃): δ 170.9, 169.2, 168.8, 167.9, 135.0, 128.5, 128.5, 70.4, 67.0, 62.8, 60.8, 60.3, 34.7, 34.7, 33.6, 26.6, 26.5, 16.3, 11.0; HRMS (ESI+) *m/z*: [M+H]⁺ Calc'd for C₂₇H₄₁N₂O₆ 489.2959; Found 489.2954.

4.1.2.21. benzyl((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane-2- carbonyl)-L-leucyl-L-

valinate (5h): White amorphous solid; Yield: 77%; *R*_f: 0.4 (Hexanes: EtOAc = 3:1); [α]_D²³ = - 97.0 (c 0.4, CHCl₃) IR (γ, cm⁻¹): 3018, 2941, 1764, 1735, 1719, 1433, 1392; ¹H NMR (400 MHz, CDCl₃): δ 7.38–7.33 (m, 5H), 6.8 (d, *J* = 8.1 Hz, 1H), 6.43 (d, *J* = 8.9 Hz, 1H), 5.22 (d, *J* = 12.1 Hz, 1H), 5.13 (d, *J* = 12.1 Hz, 1H), 4.63 (d, *J* = 4.6 Hz, 1H), 4.58 (dd, *J* = 8.8, 4.8 Hz, 1H), 4.48–4.43 (m, 1H), 3.64 (dd, *J* = 7.6, 4.6 Hz, 1H), 2.23–2.15 (m, 1H), 2.04–1.93 (m, 1H), 1.73–1.58 (m, 4H), 1.37–1.27 (m, 1H), 1.06 (d, *J* = 6.8 Hz, 3H), 0.94–0.88 (m, 12H), 0.85 (d, *J* = 6.9 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 171.5, 170.8, 168.8, 168.1, 135.1, 128.6, 128.5, 128.4, 70.4, 67.2, 62.9, 57.0, 51.8, 40.6, 33.6, 31.2, 26.6, 24.7, 22.7, 21.9, 19.0, 17.5, 16.3, 11.0; HRMS (ESI+) *m/z*: [M+H]⁺ Calc'd for C₂₆H₃₉N₂O₆ 475.2803; Found 475.2810.

4.1.2.22. benzyl((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane-2- carbonyl)-L-valyl-L-

leucinate (5i): White amorphous solid; Yield: 98%; *R*_f: 0.5 (Hexanes/ EtOAc = 3:1); [α]_D²³ = - 9.6 (c 0.5, CHCl₃); IR (γ, cm⁻¹): 3015, 2932, 2350, 1836, 1769, 1736, 1704, 1452, 1369; ¹H NMR (400 MHz, CDCl₃): δ 7.38–7.31 (m, 5H), 6.97 (d, *J* = 8.5 Hz, 1H), 6.32 (d, *J* = 8.3 Hz, 1H), 5.19 (d, *J* = 12.3 Hz, 1H), 5.14 (d, *J* = 12.1 Hz, 1H), 4.67–4.62 (m, 2H), 4.27 (dd, *J* = 8.4, 7.0 Hz, 1H), 3.62 (dd, *J* = 7.8, 4.5 Hz, 1H), 2.16–2.08 (m, 1H), 2.01–1.94 (m, 1H), 1.70–1.51 (m, 4H), 1.35–1.23 (m, 1H), 1.06 (d, *J* = 6.7 Hz, 3H), 0.95–0.89 (m, 15H); ¹³C NMR (100 MHz, CDCl₃): δ 172.4, 169.9, 168.8, 168.1, 135.1, 128.6, 128.4, 128.3, 70.4, 67.2, 62.9, 58.5, 50.9, 41.2, 33.7, 30.9, 26.6, 24.8, 22.7, 21.8, 19.0, 18.1, 16.3, 11.0; HRMS (ESI+) *m/z*: [M+H]⁺ Calc'd for C₂₆H₃₉N₂O₆ 475.2803; Found 475.2813.

4.1.2.23. benzylO-benzyl-N-((2R,3S)-3-((S)-sec-butyl)-4- oxooxetane-2-carbonyl)-L-

seryl-L-valinate(5j): White amorphous solid; Yield: 67%; *R*_f: 0.6 (Hexanes/ EtOAc = 3:1); [α]_D²⁴ = + 5.1 (c 0.5, CHCl₃); IR (γ, cm⁻¹): 2960, 2925, 1839, 1734, 1667, 1640, 1533, 1454; ¹H NMR (400 MHz, CDCl₃): δ 7.39–7.27 (m, 10H), 7.24 (d, *J* = 7.2 Hz, 1H), 7.07 (d, *J* = 8.8 Hz, 1H), 5.22 (d, *J* = 12.2 Hz, 1H), 5.12 (d, *J* = 12.2 Hz, 1H), 4.62–4.51 (m, 5H), 3.90 (dd, *J* = 9.1, 4.3 Hz, 1H), 3.73 (dd, *J* = 7.8, 4.8 Hz, 1H), 3.55 (dd, *J* = 9.1, 7.9 Hz, 1H), 2.23–2.14 (m, 1H), 2.04–1.95 (m, 1H), 1.71–1.60 (m, 1H), 1.38–1.26 (m, 1H), 1.07 (d, *J* = 6.8 Hz, 3H), 0.96–0.92 (m, 3H), 0.89 (d, *J* = 6.8 Hz, 3H), 0.77 (d, *J* = 6.9 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 171.4, 169.2, 168.7, 168.1, 136.8, 135.2, 128.6, 128.5, 128.4, 128.1, 128.0, 73.6, 70.4, 68.6, 67.1, 62.9, 57.4, 52.1, 33.7, 30.9, 26.6, 19.0, 17.3, 16.3, 10.9; HRMS (ESI+) *m/z*: [M+H]⁺ Calc'd for C₃₀H₃₉N₂O₇ 539.2752; Found 539.2731.

4.1.2.24. benzylO-benzyl-N-(((2R,3S)-3-((S)-sec-butyl)-4-oxooxetane-2-carbonyl)-L-valyl)-L-serinate(5k): White amorphous solid; Yield: 69%; R_f : 0.5 (Hexanes/ EtOAc = 2:1); $[\alpha]_D^{24} = +21.1$ (c 0.5, CHCl_3); IR (γ , cm^{-1}): 2958, 1857, 1848, 1743, 1637, 1197; ^1H NMR (400 MHz, CDCl_3): δ 7.34–7.28 (m, 8H), 7.21–7.19 (m, 2H), 6.93 (d, $J = 8.6$ Hz, 1H), 6.49 (d, $J = 8.0$ Hz, 1H), 5.23 (d, $J = 12.1$ Hz, 1H), 5.15 (d, $J = 12.1$ Hz, 1H), 4.80–4.76 (m, 1H), 4.65 (d, $J = 4.7$ Hz, 1H), 4.53 (d, $J = 12.0$ Hz, 1H), 4.43 (d, $J = 12.1$ Hz, 1H), 4.35 (dd, $J = 8.6, 5.8$ Hz, 1H), 3.97 (dd, $J = 9.6, 3.1$ Hz, 1H), 3.68–3.62 (m, 2H), 2.19–2.10 (m, 1H), 2.01–1.94 (m, 1H), 1.70–1.60 (m, 1H), 1.36–1.25 (m, 1H), 1.07 (d, $J = 6.7$ Hz, 3H), 0.98 (d, $J = 6.8$ Hz, 3H), 0.95–0.91 (m, 6H); ^{13}C NMR (100 MHz, CDCl_3): δ 169.8, 169.7, 168.8, 167.9, 137.1, 135.0, 128.5, 128.5, 128.4, 128.3, 127.9, 127.7, 73.3, 70.5, 69.4, 67.4, 62.8, 58.0, 52.6, 33.7, 31.5, 26.6, 19.0, 17.8, 16.2, 11.0; HRMS (ESI+) m/z : $[\text{M}+\text{H}]^+$ Calc'd for $\text{C}_{30}\text{H}_{39}\text{N}_2\text{O}_7$ 539.2752; Found 539.2762.

4.2. Cell Culture:

The human plasmacytoma cell line RPMI 8226 (ATCC: CCL- 155, Lot: 62014926), human T-cell leukemia cell line Jurkat (ATCC TIB-152, E6–1 clone), human mammary carcinoma MCF7 (ATCC® HTB-22™), and human breast adenocarcinoma cell line MDA-MB-231 (ATCC: HTB-26) were cultured using media composed of RPMI-1640 with glutamine (Corning) supplemented with 10% FBS (Invitrogen) as well as 100 mg/L penicillin G and 100 mg/L streptomycin (PenStrep; Invitrogen). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 .

4.3. Cell Lysate Preparation:

Following the previously reported procedure [22], human T-cell leukemia cell line, Jurkat (ATCC TIB-152, E6–1 clone), was cultured until the cell culture reached a density of approximately 2×10^6 cells/mL. The cells were pelleted, washed twice with HEPES saline solution (pH 7.37), and lysed by resuspending the cells in Lysis Buffer (150 mM NaCl, 50 mM HEPES pH 7.6, 5 mM EDTA, and 1% Triton X-100) at a cell density of 2×10^6 cells/mL. The lysate was then agitated on ice for 60 minutes. Using the BioRad protein assay, the final protein concentration of the lysed cells was ~0.5 mg/mL. The lysate was stored at -80°C until used for the proteasome assay. The procedure was repeated for MCF7 cells at a final density of 5×10^5 cells/mL.

4.4. Proteasome Assay:

Using the previously reported procedure [22], and adapting the protocol from a commercially available kit (Cayman Chemical), proteasome activity was determined by measuring the degree of peptide cleavage in 0.5 mg/mL Jurkat cell lysate, 8 $\mu\text{g/mL}$ purified yeast proteasome [47], or 8 $\mu\text{g/mL}$ purified immunoproteasomes (Enzo Life Sciences). Thawed cell lysate was centrifuged at ~10,000G for 5 minutes at room temperature in order to remove precipitated proteins and remaining cell fragments. 90 μL of Jurkat lysate was added to each of the wells on a 96 well plate. 100 mM stock solutions of the compounds were prepared in DMSO and 10 mM stock solutions of the fluorescent substrates were prepared in DMSO. Diluting the test compounds in Lysis Buffer, 10 μL of the drug at ten times the final concentration was added to each of the test wells, 10 μL of Lysis Buffer was

added for each negative control, and 10 μ L of 1 mM **2** was added to the remaining wells as an internal positive control. The plate was incubated at 37 °C for 30 minutes in order to allow binding. Following the incubation, 10 μ L of fluorescent substrate [100 μ M Suc-LLVY-AMC (Enzo Life Sciences) for Chymotrypsin-like activity and 100 μ M SUC-RLR-AMC (Enzo Life Sciences) for Trypsin-like activity] was added to each well. The plate was incubated for 60 minutes at 37 °C, and the proteasomal activity was determined by measuring the fluorescence of the cleaved substrate using the WALLAC Victor² 1420 multilabel counter set at an excitation wavelength of 360 nm and emission wavelength of 480 nm. Each technical replicate was performed in biological quadruplicate, selecting the three best values at each concentration. Data presented is the IC₅₀ values that were subsequently calculated using nonlinear regression on the normalized data in GraphPad Prism 6.0. The values were confirmed in at least technical triplicate and are reported with the corresponding standard deviation.

4.5. Cellular Viability

Cellular viability after 48 hours of treatment was measured using an MTT Assay [48]. 100 μ L of cells were added to each well of a 96 well microtiter plate at a concentration of 4×10^4 cells/mL and incubated overnight to allow proper adhesion. (RPMI 8226 cells were plated at 4×10^5 cells/mL and treated immediately). Cells were treated with the panel of test compounds at a series of concentrations, along with vehicle control DMSO and untreated cells as controls. After 48h incubation, 20 μ L of MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Alfa Aesar) in PBS (5 mg/mL) was added to each well of the plates. The plate was incubated for 2 h at 37 °C. For adherent cell lines (MCF7 and MDA-MB-231), the media was removed from each well of the plate and the resulting formazan crystals dissolved in 100 μ L of DMSO. For RPMI 8226 cells, 200 μ L of DMF/SDS mix (50% DMF, 10% w/v SDS pH<4) was used to solubilize the formazan crystals. Optical density (OD) at 490 nm was measured using a ThermoMAX microplate reader. Cells treated with 100 μ M phenylarsine oxide (PAO) served as a positive cellkilling control. Technical replicates of these assays were performed in biological quadruplicate, and the three best values were used for analysis in order to eliminate outliers. Data presented is the IC₅₀ values that were subsequently calculated using nonlinear regression on the normalized data in GraphPad Prism 6.0. The values were confirmed in at least technical triplicate and are reported with the corresponding standard deviation.

4.6. Cellular infiltration of PIs:

Infiltration was measured based on the amount of proteasome- inhibitory activity exhibited when whole cells were treated with proteasome inhibitors. 100 μ L MCF7 cells at a concentration of 500,000 cells/mL were added to a 96 well microtiter plate and incubated overnight at 37 °C and 5% CO₂ to allow cells to adhere at ~100% confluency. The cells were treated and then incubated for at 37 °C and 5% CO₂ for 1 hour as a standard time to allow for inhibitor uptake. Following incubation, wells were aspirated, washed 2 times with 100 μ L of PBS, and then resuspended in 110 μ L lysis buffer (150 mM NaCl, 50 mM HEPES pH 7.6, 5 mM EDTA, and 1% Triton X-100). The plate was rocked on ice for 1 hr and then 90 μ L of cell lysate was transferred to a black 96 well microtiter plate. 10 μ L of lysis buffer was added to the top of all wells containing treated cells and one set of control wells. 10 μ L of 1

mM of **2** was added to the second set of control wells as a positive control for proteasome inhibition. The plate was then incubated at 37 °C for 15 minutes to allow for control drug binding. Chymotrypsin-like activity was then measured by adding 10 µL of 100 µM Suc-LLVY-AMC in lysis buffer to each well and then incubating for 1 h at 37 °C. The change in fluorescence was measured using an excitation wavelength of 360 nm and emission wavelength of 480 nm. The three best values from biological quadruplicate at each concentration were used to determine the IC₅₀ after 1 h of treatment. Cellular infiltration was then represented as the ratio of IC₅₀ values for intact MCF7 cells compared to direct inhibition of the proteasome in 0.5 mg/mL MCF7 lysate. Standard deviation for was calculated by propagating the error from the two IC₅₀ values.

4.7. X-ray Crystallography:

Crystals of yCP were grown in hanging drops at 20 °C as described previously [47, 49]. The protein concentration used for crystallization was 40 mg/mL in Tris / HCl (20 mM, pH 7.5). The drops contained 1 µL of protein and 1 µL of the reservoir solution (30 mM MgAc₂, 100 mM morpholino-ethane-sulfonic acid (MES, pH 6.8) and 10% (wt/vol) 2-methyl-2,4-pentanediol (MPD)). Crystals appeared after two days and were then soaked with inhibitors in dimethyl sulfoxide at final concentrations of 3 mM for 12 h. Droplets were complemented with a cryoprotecting buffer (30% (wt/vol) MPD, 20 mM MgAc₂, 100 mM MES, pH 6.8) and vitrified in a stream of liquid nitrogen gas at 100 K (Oxford Cryo Systems). Datasets were collected at the X06SA- beamline (Swiss Light Source, Villigen, Switzerland, Table ST1). X-ray intensities were assessed with the software XDS, while data reduction was carried out with XSCAEE [50]. Molecular replacement started with coordinates of the yeast apo proteasome (PDB ID 5CZ4 [5]) using REFMAC5 in the CCP4 suite [51]. Rigid body calculations were followed by translation/libration/screw motion refinements that accounted for the 2-fold NCS symmetry of the particle. Small molecule energy minimizations and parametrizations were performed with SYBYL, while model building was carried out with MAIN [52] and COOT [53]. The final coordinates yielded excellent R factors, as well as r.m.s.d. bond and angle values. Coordinates were confirmed to fulfill the Ramachandran plot and have been deposited in the RCSB (Table ST1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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Highlights

The work presented here discusses the following highlights:

- Design and synthesis of 24 cystargolide-based inhibitors.
- Crystallographic studies revealed unique and intriguing binding modes.
- SAR studies revealed potent proteasome inhibitors with remarkable anticancer activity.

Figure 1.

Crystallographic analysis of Cystargolide B (CysB) complexed with the yeast 20S proteasome (yCP). (a) Schematic overview of proteasomal substrate-binding channels with a peptide substrate bound in standard orientation (N→C) [3]. The nonprimed specificity (S) pockets and the interacting P-sites of the ligand are shown in black; the primed specificity (S') pockets are colored in gray and the active site nucleophile Thr1 is depicted in red. (b) Chemical structure of the natural product CysB. The β-lactone moiety acts as the electrophile and is presented in red. Notably, CysB possesses three unique structural features

which significantly differ from prevalent natural proteasome substrates: i) the β -lactone is a peptide mimic harboring a C4-O1 instead of an NH group; ii) the P1- isopropyl side chain (pseudo-valine) has inverted stereochemistry; iii) the peptidic scaffold is attached to the β -lactone in reverse order (C $\rightarrow$ N). (c-e) Illustration of the 2FO-FC electron density map (gray mesh; contoured to 1σ) of CysB (green) bound to the active site nucleophile Thr1O γ of subunit $\gamma\beta 5$ (gold), $\gamma\beta 1$ (cyan) and $\gamma\beta 2$ (tan), respectively (PDB ID 6G7F). The chemical structure for the covalent adduct of CysB with Thr1 is provided. Notably, in the caspase-like $\beta 1$ substrate binding channel the carboxy-terminus of CysB is stabilized by H-bonds with $\gamma\beta 2$ -His114 and $\gamma\beta 1$ -Thr22 (d), explaining the high affinity of CysB for this active site. In the trypsin-like active site CysB displays a peculiar conformation and the majority of its peptide moiety is directed towards the primed sites. (f- g) Structural superposition of CysB (green) and the peptide aldehyde MG132 (PDB ID 4NNN [27]) or Omu (PDB ID 3DY4 [9, 28]) bound to the active site Thr1O γ in subunit $\gamma\beta 5$. The overlay depicts a so far unobserved binding mode of CysB. The yellow ellipse highlights the carbonyl oxygen atom of the ester bond pointing towards Gly47NH, while the ellipses in grey indicate individual H-bonds of CysB with protein main chain atoms. h) The conspicuous configuration of CysB at the trypsin-like site resembles that of homobelactosin C and its derivative ESY (PDB ID 4Z1L [29, 30]).

Figure 2.

a-b) Structural overlay of the subunits yβ1, yβ2 and yβ5 in complex with CysB and the peptide aldehyde MG132 (PDB ID 4NNN [27]), respectively. Unexpectedly, CysB adopts distinct conformations at the three active sites. The yellow and grey ellipses hallmark the carbonyl oxygen atom of the ester bond and H-bond interactions with protein main chain atoms, respectively.

Figure 3.

(a-b) Selective inhibition of $\beta5$ subunit of purified yeast proteasomes reported as ratio of IC_{50} values comparing either (a) $\gamma\beta1:\gamma\beta5$ or (b) $\gamma\beta2:\gamma\beta5$. (c) Cytotoxicity EC_{50} profile for compounds **1** and **2** on MCF7, MDA-MB-231, and RPMI 8226 cancer cell lines. (d-e) Dose response curves of hcCP $\beta5$ (hcP5) inhibition in (d) lysed and (e) intact MCF7 cells which are known to primarily express human constitutive proteasomes [33]. Assays and IC_{50} determination were performed in biological triplicate ($n=3$) and the data points are reported with the corresponding SD.

Figure 4.

(a-b) $2F_o-F_c$ electron density maps for the CysB derivatives **1** (teal, PDB ID 6G8M) and **2** (cyan, PDB ID 6G8N) bound to the active site nucleophile Thr1O γ of subunit y β 5. Color coding and labeling are according to Fig. 1c. Masking the negative charge of the carboxy-group of Cys B by a benzyl group (**1**) significantly affects the conformation of the ligand. In subunit y β 5, **1** adopts a configuration similar to that observed for CysB of the trypsin-like active site. Prolonging the P1-site by adding a methyl group (pseudo-isoleucine) in **2** reverts the mode of binding and targets the compound to the non-primed sites. Thus, the difference of one methyl group (highlighted in tan and emphasized by a black arrow) is crucial for the ligand's arrangement. Notably, the benzyl-moieties in both crystal structures are only partially resolved in the electron density maps. (c-d) Structural superposition of y β 5 complexed with **2** and CysB, respectively, shows a perfect match, whereas the overlay of this subunit bound to **1** and **2** highlights significant variations of the peptide moieties in the primed and nonprimed substrate binding channels.

Figure 5. Cellular infiltration of P_1 analogs reported as the ratio of the IC_{50} values of hc β 5 proteasome inhibition when treating MCF7 cells that are intact versus lysed MCF7 cells. Each value is reported with the corresponding SD determined from propagating the error of both of the IC_{50} values that were determined from a serial dilution in biological triplicate.

Figure 6. Cellular infiltration of P_x/P_y analogs reported as the ratio of the IC_{50} values of $\beta 5$ proteasome inhibition when treating MCF7 cells that are intact versus lysed MCF7 cells. Each value is reported with the corresponding SD determined from propagating the error of both of the IC_{50} values that were determined from a serial dilution in biological triplicate. Analogs are grouped based on the position of variation.

Figure 7.

Inhibition of the hiβ5 subunit of the immunoproteasome by selected analogs. IC₅₀ values for proteasome inhibition measured in purified human immunoproteasomes at a concentration of 8 μg/mL. IC₅₀ values were calculated from dose-response curves generated from assays performed in biological triplicate using β5 substrate Suc-LLVY-AMC. The values were calculated using a nonlinear dose response model in GraphPad Prism 6.0 and are reported with the corresponding SD.

Scheme 1.

(**a**) cystargolides and cystargolide-derived Drag Lead **1** (**b**) cystargolide scaffold

Scheme 2.

Synthesis of β -lactone PIs. Reagents and conditions: (i) NaHMDS, THF, -78°C then *t*-Bu bromoacetate (ii) LiOH/H₂O₂, THF:H₂O (3:2), 0°C to RT. (iii) LHMDs, CCl₄, THF, -78°C (iv) aq. 5% NHCO₃, ether, RT. (v) Pz-OH, *p*-TSA H₂O, toluene, reflux (vi) *N*-Boc/Fmoc amino acid, EDCI, HOBT, NMM, THF, 0°C to RT. (vii) TFA, CH₂Cl₂, RT, for **11a-r**, Et₂NH, CH₂Cl₂, 0°C , for **11s-11t** (viii) **9a-e**, TFA, CH₂Cl₂, then TFA salt of **11a-r** or free amine **11s-t**, then EDCI, HOBT, NMM, THF, 0°C to RT.

Table 1.

Chemical structures of synthesized compounds.

Compound	P _z	P _y	P _x	P ₁
2
3a
3b
3c

Compound	P _z	P _y	P _x	P ₁
3d
3e
3f
3g
3h
4a
4b

Compound	P _z	P _y	P _x	P ₁
4c				
4d				
5a				
5b				
5c				
5d				

Compound	P _z	P _y	P _x	P ₁
5e
5f
5g
5h
5i
5j

Compound	P _z	P _y	P _x	P ₁
5k

Table 2:

Cytotoxicity and inhibition of human proteasomes (hCP) measured in Jurkat lysate for P_z analogs. Assays were performed in biological triplicate (n=3) and confirmed in technical triplicate. The IC₅₀ values were calculated using a nonlinear dose response model in GraphPad Prism 6.0 and are reported with the corresponding SD. ND: Not determined.

Compound	Substitution P _z	IC ₅₀ (μM) ± SD			
		hCP Inhibition		Cytotoxicity	
		hβ5	MCF7	MDA-MB-231	RPMI 8226
2	BnO	0.0070 ± 0.0003	1.853 ± 0.261	0.287 ± 0.008	0.191 ± 0.050
3a	<i>allyl</i> -O	0.0094 ± 0.0009	0.353 ± 0.022	0.275 ± 0.008	0.088 ± 0.004
3b	<i>p</i> -Cl-BnO	0.0082 ± 0.0007	0.918 ± 0.129	0.312 ± 0.019	0.085 ± 0.006
3c	<i>cyc</i> -Hex-CH ₂ -O	0.0187 ± 0.0003	4.617 ± 0.654	3.217 ± 0.213	0.303 ± 0.027
3d	<i>i</i> -Bu-O	0.0088 ± 0.0008	0.664 ± 0.047	0.317 ± 0.009	0.095 ± 0.003
3e	BnNH	0.0036 ± 0.0003	0.892 ± 0.078	0.885 ± 0.025	0.320 ± 0.020
3f	<i>p</i> -MeO-BnO	0.0102 ± 0.0005	0.854 ± 0.131	0.280 ± 0.013	0.114 ± 0.009
3g	<i>homo-allyl</i> -O	0.0066 ± 0.0007	0.523 ± 0.018	0.341 ± 0.008	0.272 ± 0.009
3h	MeO	0.0159 ± 0.0008	0.427 ± 0.019	0.312 ± 0.009	0.268 ± 0.003
Carfilzomib (Cfz)		0.0096 ± 0.0006	0.0041 ± 0.0001	0.0044 ± 0.0001	0.0067 ± 0.0001
Cystargolide B		0.90 ± 0.11	84.7 ± 18.6	ND	ND

Table 3:

Cytotoxicity and inhibition of human proteasomes (hCP) measured in Jurkat lysate for P₁ analogs. Assays were performed in biological triplicate (n=3) and confirmed in technical triplicate. The IC₅₀ values were calculated using a nonlinear dose response model in GraphPad Prism 6.0 and are reported with the corresponding SD. ND: Not determined

Compound	Substitution P _z	IC ₅₀ (μM) ± SD				
		hCP Inhibition		Cytotoxicity		
		hβ5	hβ2	MCF7	MDA-MB-231	RPMI 8226
2	<i>sec</i> -Bu	0.0070 ± 0.0003	1.430 ± 0.089	1.853 ± 0.261	0.287 ± 0.008	0.191 ± 0.015
4a	<i>cyc</i> -Pr	0.0076 ± 0.0006	3.188 ± 0.164	>100	>100	73.86 ± 2.65
4b	<i>cyc</i> - Bu	0.0132 ± 0.0005	4.070 ± 0.461	>50	>50	37.22 ± 3.19
4c	<i>cyc</i> -Pent	0.0129 ± 0.0007	7.468 ± 0.748	50.62 ± 4.20	28.17 ± 3.88	2.346 ± 0.334
4d	<i>i</i> -Bu	0.0197 ± 0.0010	4.580 ± 0.161	55.32 ± 4.94	30.32 ± 3.03	33.52 ± 1.31
Carfilzomib (Cfz)		0.0096 ± 0.0006	0.203 ± 0.005	0.0041 ± 0.0001	0.0044 ± 0.0001	0.0067 ± 0.0002
Cystargolide B		0.90 ± 0.11	ND	84.7 ± 18.6	ND	ND

Table 4:

Cytotoxicity and inhibition of human proteasomes (hCP) measured in Jurkat lysate for P_x/P_y analogs. Assays were performed in biological triplicate (n=3) and confirmed in technical triplicate. The IC₅₀ values were calculated using a nonlinear dose response model in GraphPad Prism 6.0 and are reported with the corresponding SD. ND: Not determined.

Compound	Substitution		IC ₅₀ (μM) ± SD				
	P _x	P _y	hCP Inhibition		Cytotoxicity		
			hβ5	hβ2	MCF7	MDA-MB-231	RPMI 8226
2	<i>i</i> -Pr	<i>i</i> -Pr	0.0070 ± 0.0003	1.430 ± 0.089	1.853 ± 0.261	0.287 ± 0.008	0.191 ± 0.015
5a	H	<i>i</i> -Pr	0.0027 ± 0.0001	0.261 ± 0.008	2.085 ± 0.872	0.678 ± 0.045	0.762 ± 0.106
5b	Bn	<i>i</i> -Pr	0.0078 ± 0.0004	3.716 ± 0.294	6.945 ± 1.031	3.756 ± 0.435	2.608 ± 0.206
5c	<i>i</i> -Pr	Bn	0.0057 ± 0.0005	1.777 ± 0.222	2.748 ± 0.229	3.349 ± 0.263	1.103 ± 0.067
5d	Bn	Bn	0.0116 ± 0.0007	2.713 ± 0.130	6.249 ± 0.636	5.211 ± 0.187	1.176 ± 0.091
5e	<i>t</i> -Bu	<i>i</i> -Pr	0.0228 ± 0.0009	5.053 ± 0.688	2.288 ± 0.141	0.699 ± 0.021	0.748 ± 0.035
5f	<i>i</i> -Pr	<i>t</i> -Bu	0.0098 ± 0.0005	3.749 ± 0.263	1.137 ± 0.078	1.910 ± 0.119	1.748 ± 0.094
5g	<i>t</i> -Bu	<i>t</i> -Bu	0.0359 ± 0.0053	3.517 ± 0.109	1.905 ± 0.130	0.673 ± 0.031	1.217 ± 0.059
5h	<i>i</i> -Bu	<i>i</i> -Pr	0.0050 ± 0.0004	2.953 ± 0.135	2.465 ± 0.328	1.453 ± 0.183	2.243 ± 0.188
5i	<i>i</i> -Pr	<i>i</i> -Bu	0.0077 ± 0.0004	1.509 ± 0.160	0.332 ± 0.012	0.219 ± 0.008	0.286 ± 0.011
5j	CH ₂ O-Bn	<i>i</i> -Pr	0.0077 ± 0.0008	0.759 ± 0.020	3.452 ± 0.236	2.859 ± 0.056	1.706 ± 0.131
5k	<i>i</i> -Pr	CH ₂ O-Bn	0.0031 ± 0.0002	1.558 ± 0.062	0.416 ± 0.025	0.074 ± 0.006	0.041 ± 0.002
Carfilzomib (Cfz)			0.0096 ± 0.0006	0.203 ± 0.005	0.0041 ± 0.0001	0.0044 ± 0.0001	0.0067 ± 0.0002
Cystargolide B			0.90 ± 0.11	ND	84.7 ± 18.6	ND	ND