Recent Advances in the Identification and Development of 20S Proteasome Inhibitors

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Abstract: The involvement of the 20S proteasome in the degradation of critical intracellular regulatory proteins has suggested the potential use of proteasome inhibitors as novel anti-inflammatory agents and for the treatment of cancer and auto-immune diseases. Early inhibitors of the 20S proteasome were relatively non-specific compounds and used for *in vitro* studies of the ubiquitin/proteasome-dependent degradation pathway. The inherent drawbacks of these inhibitors (e.g., non-target specific, too reactive or unstable) has prompted medicinal chemists to search for alternative subunit-specific proteasome inhibitors. This manuscript summarises recent salient medicinal chemistry achievements in this area of research.

1. INTRODUCTION

The proteasome is an intracellular multicatalytic protease complex that is involved in the degradation of a variety of cytosolic proteins (e.g. misfolded proteins, transcriptional factors, cyclins and metabolic enzymes) [1-6]. The proteolytic activity of this protease occurs in a 700-kDa barrel-shaped core structure known as the 20S proteasome, which consists of four stacked rings arrayed in an $\alpha_7\beta_7\beta_7\alpha_7$ manner [7-8]. Three β-subunits in each β-ring are catalytically active and have an *N*-terminal threonine as the active nucleophile involved in proteolyis [9]. The substrate specificity of the catalytic components of eukaryotic proteasomes has been defined with the use of fluorogenic synthetic substrates, natural peptides and proteins (for a review see, [10]). These studies have revealed at least three distinct peptidase activities: chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolytic (PGPH) activities [11]. The 20S proteasome is implicated in the ubiquitin (Ub)-dependent and Ub-independent degradation of proteins involved in critical intracellular regulatory cascades (e.g. mitotic cycle, cell growth and viability, antigen presentation or inflammatory response) [12-15]. Inhibitors of this enzyme are currently being explored for use as potential anti-inflammatory agents and for the treatment of cancer and auto-immune diseases (for recent reviews on this target, see [16-24]). The lack of selectivity of early proteasome inhibitors boosted the identification and development of more selective, potent and drug like compounds. This manuscript reviews recent salient medicinal chemistry achievements in the design, synthesis and biological characterization of a variety of inhibitors of the 20S proteasome. Examples have been selected to illustrate the impact of structure-based design and natural product screening on this area of research.

2. 20S PROTEASOME INHIBITORS

2.1 Peptide Aldehydes, α-Keto Aldehydes, α-Keto Amides and Vinyl Sulfones

The use of modified peptides that contain an active site-directed inhibitory functional group at the *C*-terminus has proven to be a valuable method for studying proteasome-substrate interactions and for the identification of potent and selective inhibitors. This is the case for compounds containing an aldehyde moiety that forms an adduct with the hydroxyl group of the threonine involved in proteasome proteolysis [8-9]. Ever since the initial identification of a series of peptide aldehydes as reversible proteasome inhibitors (e.g. the calpain inhibitors Ac-Leu-Leu-norleucinal and Ac-Leu-Leu-methional) [25-26], different groups have designed peptide aldehydes with increased selectivity for the 20S proteasome [27-30]. Compound **1** (CEP-1612, Fig. **1**) is a potent inhibitor of the chymotrypsin-like activity of the 20S proteasome (IC_{50}= 2 nM), but it was unable to inhibit the trypsin-like activity of this enzyme at concentrations up to 1 μM [28]. This compound induces apoptosis in human Jurkat T cells overexpressing Bcl-2 and also in a panel of human tumor cell lines. Induction of apoptosis is p53-independent and is associated with accumulation of the cyclin-dependent kinase inhibitors p21 and p27 [31].[a] Other peptide aldehydes have been described in several patent applications for disease treatment in a whole range of indications (e.g. cancer, inflammation, autoimmune disorders, neurological disease, cachexia, osteopenia, osteolytic lesions, osteopetrosis or bone fracture): compound **2** (MG-167; K_i= 0.015 nM; Fig. **1**) [33]; compound **3** (IC_{50}= 0.011 μM; Fig. **1**) [34], and compound **4** (PSI; Fig. **1**) [35] are active against the 20S proteasome. Additional modifications of the carbonyl head group have also been reported. Di- and tri-peptide α-keto aldehydes (glyoxals) were synthesised as putative inhibitors of the chymotryptic-

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[a] J. L. Herrmann *et al.* also showed that the proteasome inhibitor Ac-Leu-Leu-norleucinal signals death in prostate carcinoma cells with elevated Bcl-2 and independent of functional p53 [32].

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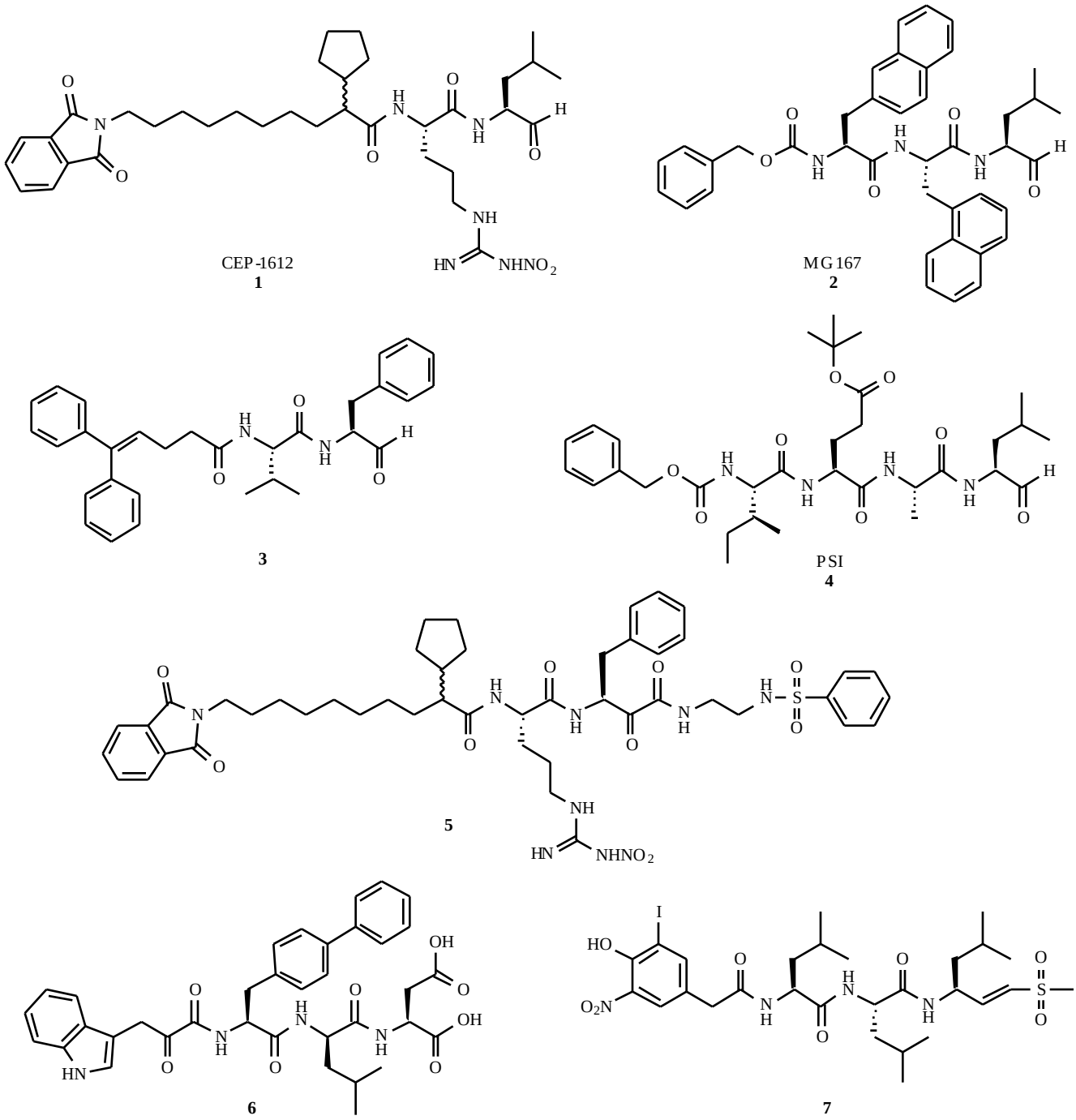
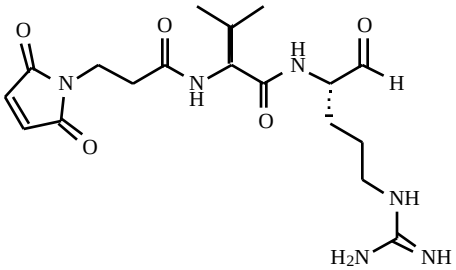


Fig. (1). Peptide aldehydes, α-keto aldehydes, α-keto amides and vinyl sulfones.

like activity of proteasome. Cbz-Leu-Leu-Tyr-COCHO (K_i= 3.1 nM) and Bz-Leu-Leu-Leu-COCHO (K_i= 3.7 nM), which were prepared by a combination of solid-phase and classical solution methodologies, are representative examples of such slow-binding reversible inhibitors [36]. Along the same theme of using an α-keto group, compound **5** (Fig. **1**) emerged as the most potent chymotrypsin-like activity inhibitor (K_i= 1.1 nM) of a series of P'-extended α-keto amide derivatives. Compound **5**, which is an analogue of CEP-1612 (compound **1**) [28], was found to be more than 150-fold less active against calpain 1 and the trypsin-like activity of the 20S proteasome enzyme complex [37]. This class of compounds was shown to be effective in reducing tumor volume in female mice with B16-F0 murine melanoma tumors [38]. The α-keto amide moiety has also been introduced at the *N*-terminus of peptide derivatives. For example, compound **6** (Fig. **1**) inhibited in an *in vitro* assay the 20S proteasome purified from bovine brain [26] with an IC_{50} value of 1 μg/ml. Additionally these new α-keto amide derivatives may be used in the treatment of cancer, autoimmune disorders, lupus, multiple sclerosis, rheumatoid arthritis, infectious diseases and inflammation [39].

Although originally introduced as inhibitors of cysteine proteases [40], peptide vinyl sulfones (e.g. Cbz-Leu-Leu-Leu-VS; VS, vinyl sulfone) were found to covalently inhibit



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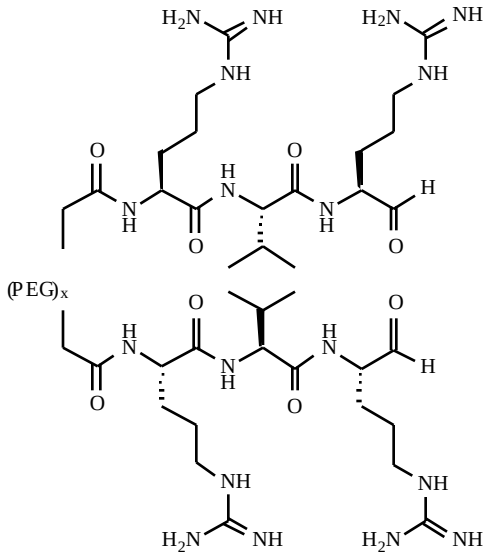

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Fig. (2). Bifunctional and bivalent inhibitors.

the chymotrypsin-like, trypsin-like, and post-glutamyl-peptide hydrolytic activities of the proteasome both *in vitro* and *in vivo* [41]. This unexpected reactivity of the vinyl sulfone moiety, which was initially designed to react as a Michael acceptor with soft nucleophiles such as thiols, towards the *N*-terminal threonine of this enzyme prompted further investigation. To this end, a series of tri- and tetra-peptide vinyl sulfones (e.g. compound **7**, Fig. **1**; chymotrypsin-like activity, IC_{50}= 0.1-0.5 μM, trypsin-like activity, IC_{50}= 50-100 μM; and PGPH activity, IC_{50}= 50-100 μM) were used to study substrate binding to the proteaosme [42]. Importantly, this work identified the amino acid at position P4 as an important recognition motif for defining substrate processing by the proteasome.

Recently, Ellman's modification [43-45] of Kenner's safety catch protocol [46] has been applied to the solid-phase synthesis of peptide vinyl sulfone proteasome inhibitors [47]. This approach opens the way to the automated construction of combinatorial libraries containing this reactive group.

2.2 Bifunctional Inhibitors

The basic principle of multivalency [48-49] has been applied to selectively target the trypsin-like activity of the 20S proteasome. The X-ray structure of the yeast 20S proteasome revealed that cysteine 118, which is highly conserved among the known primary structures of mammalian proteasomes, protrudes with its side chain into the S3 subsite of the trypsin-like active site of this enzyme [8]. The unique location of this residue was exploited for the structure-based design of bidentate inhibitors containing a maleimido moiety at the P3 position for covalent linkage to the thiol group of cysteine 118 and a *C*-terminal aldehyde group for hemiacetal formation with the hydroxyl group of threonine 1 [50]. This approach provided highly selective inhibitors for the trypsin-like activity of yeast proteasome [e.g., compound **8** (Fig. **2**), IC_{50}= 0.5 μM, trypsin-like activity; IC_{50}> 100 μM chymotrypsin-like and PGPH activities],[b] but the general reactiviy of the maleimido group towards thiols seriously limit the utility of this class of compounds.

2.3 Homo- and Heterobivalent Inhibitors

The principle of multivalency has been exploited in another example of a structure-based design approach. Homo- and heterobivalent inhibitors based on peptide aldehyde head groups were linked at the *N*-terminus with a polyoxyethylene (PEG)[c] spacer [51-53] to access simultaneously two different active sites from the non-primed subsites [54]. The bivalent compounds were two orders of magnitude more potent inhibitors than the monovalent compounds [e.g. $IC_{50}^{\text{trypsin-like}}$= 0.071 μM for compound **9** (Fig. **2**) *versus* $IC_{50}^{\text{trypsin-like}}$= 6.4 μM for Ac-Arg-Val-Arg-CHO]. Analysis of the crystal structures of the proteasome/bivalent inhibitors complexes confirmed independent recognition and binding of the inhibitory aldehyde groups. Although PEG is known to improve cell permeability [51-53], it remains to be confirmed if these compounds are active in cell based assays.

2.4 5-Methoxy-1-Indanone Dipeptide Benzamides

The 5-methoxy-1-indanone moiety was considered as a less reactive, but potentially hydratable, head group to target

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[b] The binding mode of compound **8** was confirmed by solving the X-ray crystal structure of the yeast proteasome-inhibitor **8** adduct (2.5 Å resolution) [50].

[c] Polyoxyethylene (PEG) was chosen to mimic random coil polypeptide chains and the number of monomers was determined by molecular modeling. For example, a PEG with a statistical distribution of 19-25 monomers was selected to span a distance of around 50 Å.

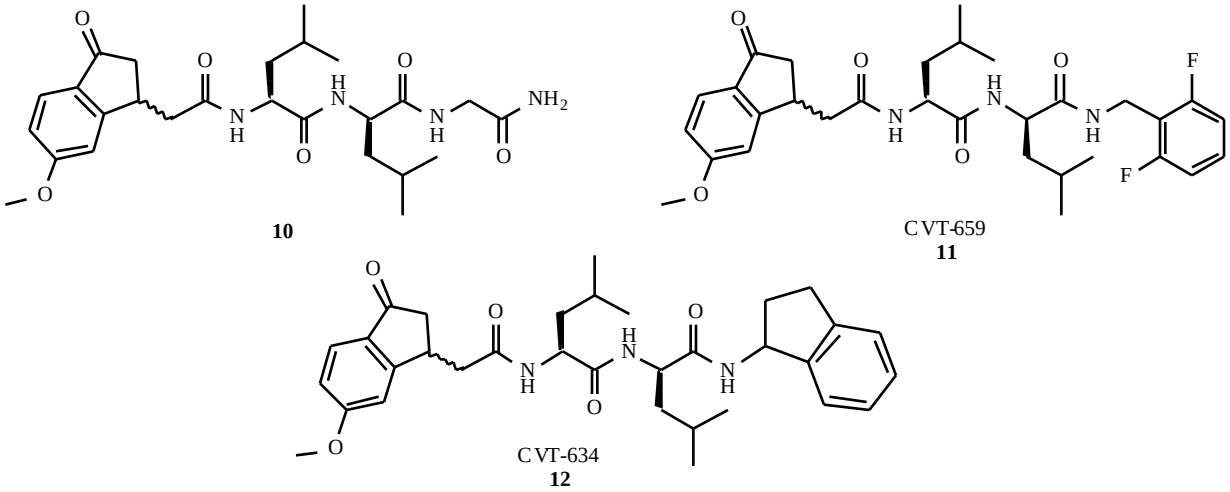


Fig. (3). 5-Methoxy-1-indanone dipeptide benzamides.

the chymotrypsin-like activity of the 20S proteasome [55]. The most potent compounds identified by screening different peptide libraries containing this head group were compound **10** (IC_{50}= 0.20 μM, Fig. **3**) and compound **11** (CVT-659, IC_{50}= 0.14 μM; Fig. **3**), which are selective for the chymotrypsin-like activity of the 20S proteasome and do not inhibit calpain 1. CVT-659 displayed antiproliferative activity (IC_{50}= 8 μM) against RAW cells (murine macrophage cell line), but compound **10** proved inactive in this cellular assay. In another study [56], compound **12** (CVT-634; Fig. **3**) prevented activation of nuclear transcriptional factor-κB (NF-κB) *in vitro* and lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF) synthesis in RAW cells (IC_{50}= 7 μg/mL). In addition, serum TNF levels were significantly lower in Female Swiss Webster mice pretreated with CVT-634 (i.p.) than in those mice that were treated only with LPS (865 pg/mL *versus* 225 and 83 pg/mL for 25 and 50 mg/kg of CVT-634, respectively) [56].

2.5 Peptide Boronic Acids

Peptide boronic acids that are close structural analogs of enzyme substrates have been extensively used as serine protease inhibitors [57-60]. In these enzymes, the active-site serine forms a covalent, nearly tetrahedral adduct with the boronic acid moiety of the inhibitor [61-62]. By analogy, it was presumed that boronic acid derivatives could also form stable tetrahedral intermediates with the *N*-terminal threonine residues of the proteasome active sites. On the basis of this approach and due to the lack of activity of such derivatives against cysteine proteases [63], peptide boronic acids and esters were synthesised to target the 20S proteasome [64-68]. A representative example of this class of compounds is compound **13** (LDP-341, former PS-341; Fig. **4**), which is currently in multiple Phase I clinical trials by Millennium in patients with advanced cancers and in a Phase II clinical trial for multiple myeloma.[d] This compound is a slow, tight-binding and selective inhibitor of the chymotryptic activity of the 20S proteasome (K_i= 0.6 nM) [67], [69].[e] LDP-341 has shown broad-spectrum cytotoxic activity (average GI_{50} value was 7 nM) in the National Cancer Institute *in vitro* screen (sixty cell lines derived from multiple human tumors). In athymic nude mice bearing PC-3M xenografts, 60% reduction in tumor volume was observed after intravenous administration of 1.0 mg/kg/week for four weeks [69]. Antitumor activity has also been reported against the Lewis lung carcinoma, both primary and metastatic disease, and in combination regimens with cytotoxic agents [70]. Furthermore, this compound had anti-inflammatory effects in a model of Streptococcal cell wall-induced polyarthritis and liver inflammation in rats after oral administration. This therapeutic effect was attributed to inhibition of IκBα degradation and NF-κB-dependent gene expression [71].

A new series of proteasome inhibitors and inducers of apoptosis have been made that are based on a boronic electrophile. Significant inhibition in B16 tumor growth was observerd in female C57BL mice after i.p administration of compound **14** (Fig. **4**) at 10 mg/Kg/day [66]. This compound is an analogue of CEP-1612 (compound **1**, Fig. **1**).

Recently, several peptidyl boronic acids and their pinacol esters have been characterized against the mammalian 20S and 26S proteasomes [65]. This study demonstrates significant differences in the characteristics of inhibition of these proteasomes in cultured cells.

2.6 Peptide α',β'-Epoxyketone

Peptide α',β'-epoxyketones have emerged in the past years as an interesting class of proteasome inhibitors and have been extensively used as molecular probes for understanding proteasome biology. The antitumor compounds dihydroeponemycin (compound **15**, Fig. **5**) and

[d] In a Phase I clinical trial, LDP-341 is being administered intravenously weekly for four weeks in a six week cycle at doses up to 0.75 mg/m^2.

[e] LDP-341 is at least 500-fold selective for the proteasome over a series of enzymes: e.g. Ki= 2.3 μM, human leukocyte elastase; Ki= 630 nM, human cathepsin G; Ki= 320 nM, human chymotrypsin; and Ki= 13 μM, thrombin) [67].

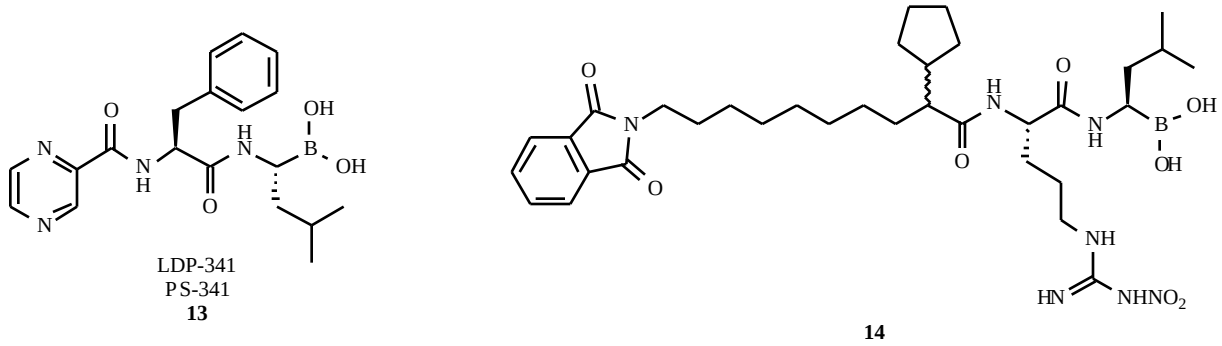


Fig. (4). Peptide boronic acids.

epoxomicin (compound **16**, Fig. **5**), which share an α',β'-epoxyketone pharmacophore as well as a linear peptide backbone, are representative examples of this class of compounds.

Dihydroeponemycin (compound **15**, Fig. **5**) [72-73] inhibits competitively and irreversibly the enzymatic activity of all three major activities of the 20S proteasome but at different rates [74]. Inhibition of the chymotrypsin-like activity ($k_{association}$= 66.4 ± 8.9 $M^{-1}\cdot s^{-1}$) and PGPH activity ($k_{association}$= 60.5 ± 8.8 $M^{-1}\cdot s^{-1}$) proceeded more than 10-fold faster than inhibition of the trypsin-like activity ($k_{association}$= 4.4 ± 0.4 $M^{-1}\cdot s^{-1}$). Moreover, this natural product does not inhibit calpain and trypsin, although minor inhibition is observed against cathepsin B and chymotrypsin at higher concentration. In addition, dihydroeponemycin-mediated proteasome inhibition induces a spindle-like morphological change in bovine aortic endothelial cells (BAECs) and apoptosis in BAECs and EL4 cells.

Epoxomicin (compound **16**, Fig. **5**) is a peptide α',β'-epoxyketone isolated from the actinomycete strain No. Q996-17 that exhibits *in vivo* antitumor activity against B16 melanoma [75]. Given its high structural similarity to dihydroeponemycin, it was hypothesised that the cellular target of this natural product could also be proteasome.

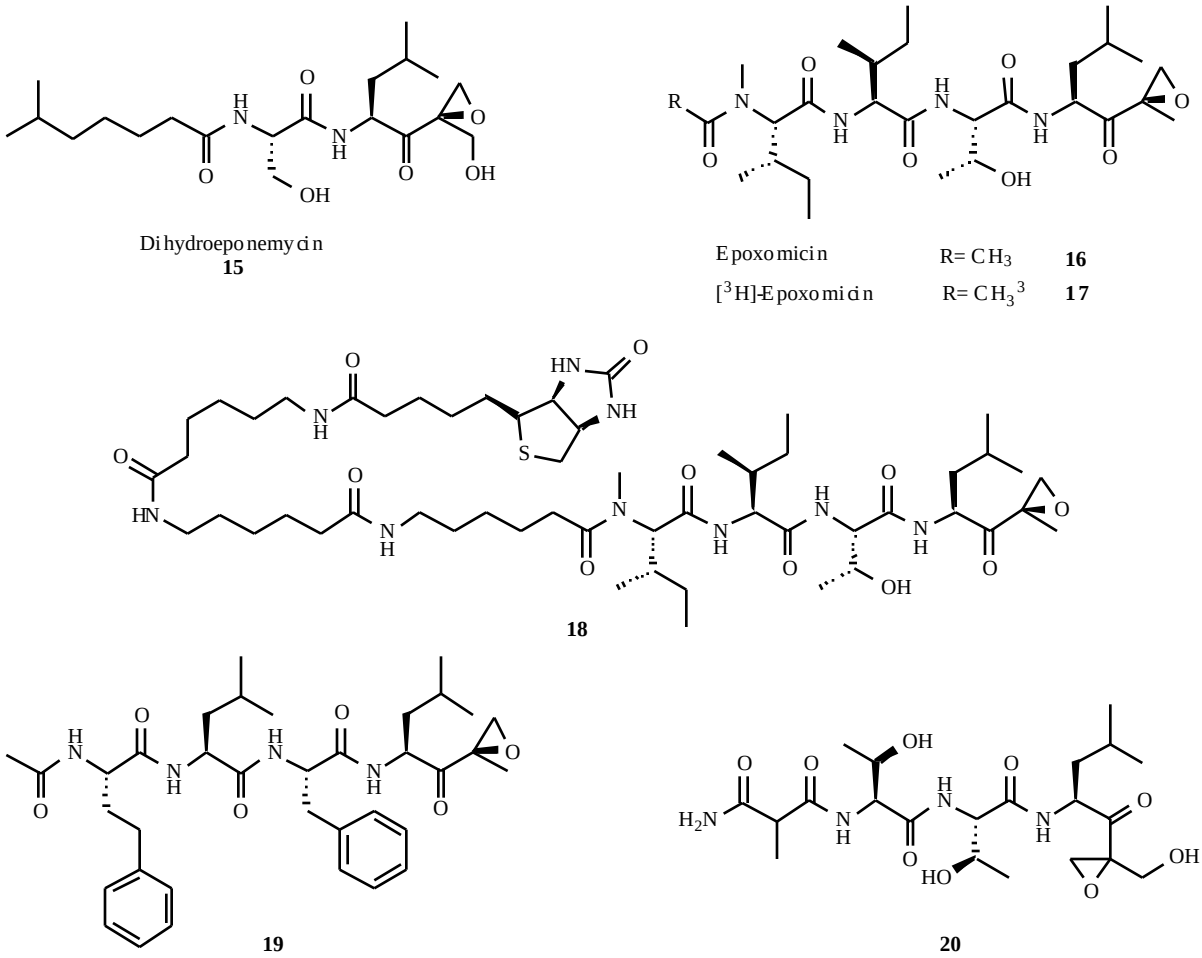


Fig. (5). Peptide α',β'-epoxyketones.

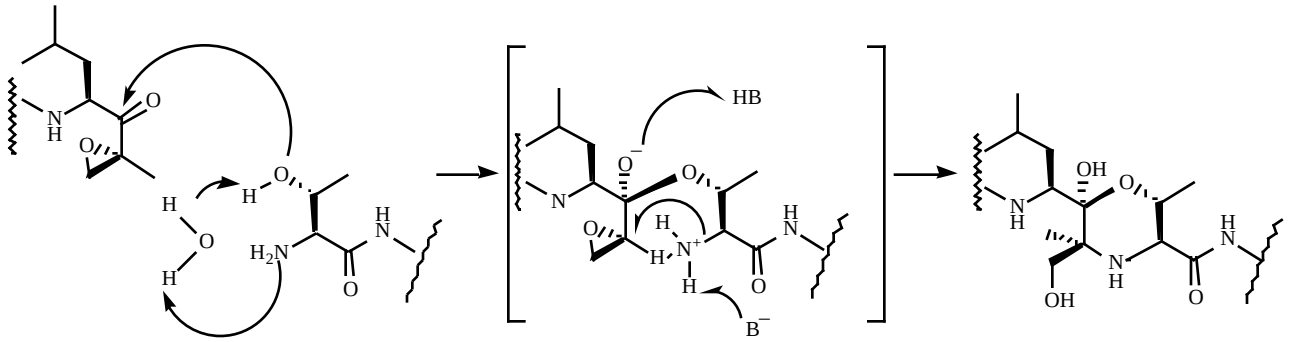


Fig. (6). Proposed mechanism for the formation of a morpholine derivative adduct between the epoxyketone group of epoxomicin and the active site *N*-terminal threonine of proteasome.

Confirmation of this hypothesis was achieved by total synthesis of epoxomicin (compound **16**, Fig. **5**), [^{3}H]-epoxomicin (compound **17**, Fig. **5**), and biotinylated epoxomicin (compound **18**, Fig. **5**) and using affinity chromatography techniques with murine EL4 cell lysates [76]. Epoxomicin potently and irreversibly inhibits the chymotrypsin-like ($k_{obs}/[I]$= 37,200 $M^{-1}s^{-1}$) activity of the 20S proteasome, and to a lesser extent the trypsin-like ($k_{obs}/[I]$= 287 $M^{-1}s^{-1}$) and PGPH activities ($k_{obs}/[I]$= 34 $M^{-1}s^{-1}$) of this protease complex. Enzyme kinetic analysis showed that epoxomicin did not inhibit papain, chymotrypsin, trypsin, cathepsin B or calpain at concentrations up to 50 μM. Furthermore, this compound was shown to stabilize p53 levels in human umbilical vein enthodelial (HUVEC) cells, to inhibit NF-κB activation in HeLa and HEK 293 cells, and to have antiinflammatory activity in a mouse model of cutaneous inflammation at nontoxic doses (0.58 mg/Kg/day, i.p.) [77].

The first insights into the intriguing selectivity of epoxomicin for the proteosome were obtained by solving the X-ray crystal structure of a complex between the natural product and the yeast *S. cerevisiae* 20S proteasome [78]. The structure revealed the presence of a morpholino ring deriving from the interaction of the α',β'-epoxyketone pharmacophore of epoxomicin with the *N*-terminal threonyl Oγ and N atoms. The morpholine ring was derived by a two-step process (Fig. **6**): i) formation of a hemiacetal by nucleophilic attack of Thr 1Oγ on the carbonyl group of the epoxyketone pharmacophore; and ii) nucleophilic attack of Thr 1N at the more hindered C2 epoxy methylene resulting in an inversion of configuration at this carbon and formation of the morpholino adduct (6 *Exo-Tet* ring closure; see Baldwin's rules in [79-80]). The occurrence of this adduct is also supported by mass spectrometric analysis [78].

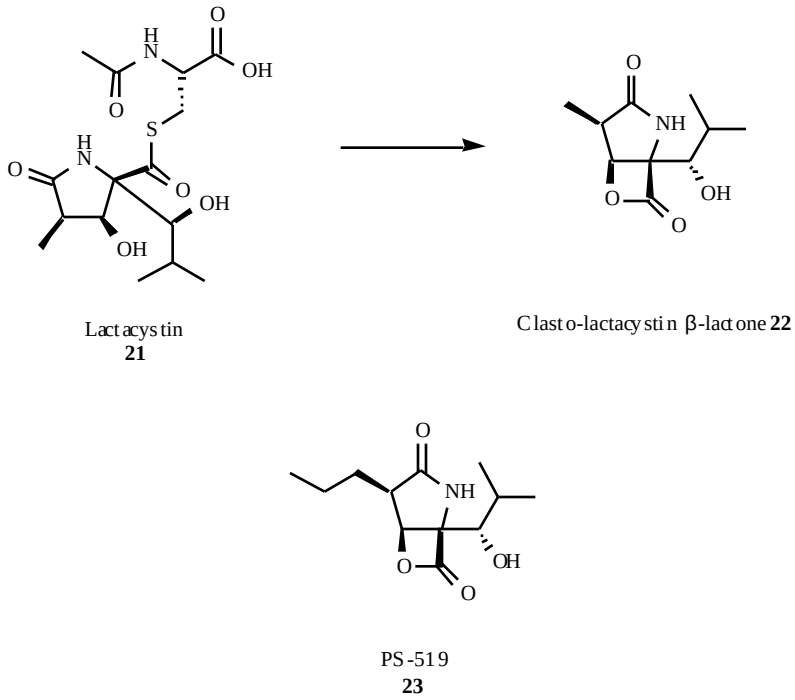
Recently, a series of α',β'-epoxyketone derivatives was obtained by a solution phase coupling of a panel of peptides of varied length and amino acid sequence with α',β'-epoxyleucine [81]. All the peptide α',β'-epoxyketones were found to be good inhibitors of the chymotrypsin-like activity and poor inhibitors of the trypsin-like and PGPH-activities. Optimization of the P2-P4 sites furnished compound **19** (Fig. **5**), which showed a remarkable selectivity and potency for inhibition of the chymotrypsin-like activity (IC_{50}= 5-12 nM, chymotrypsin-like activity; IC_{50}= 80-130 μM, trypsin-like activity; and IC_{50}= 80-150 μM, PGPH activity). In a different approach, biochemical studies of epoxomicin/dihydroeponemycin chimeraerevealed that the length of the left-hand peptide moiety (P4-P3) of these derivatives is important for maximal inhibition of the chymotrypsin-like and trypsin-like activities [82]. No strong preference for peptide inhibitor length of the epoxomicin/dihydroeponemycin chimerae was observed for the PGPH activity.

Additional proteasome inhibitors containing an α',β'-epoxyketone moiety have been obtained by culturing Streptomyces (e.g. compound **20**, Fig. **5**). These natural products may be used in the treatment of autoimmune diseases, inflammatory enteritis, asthma, and Alzheimer disease [83].

2.7 Lactacystin and Synthetic Analogs

Lactacystin (compound **21**, Fig. **7**), which is a *Streptomyces* metabolite first isolated by Omura *et al.* [84-85] (for representative articles on the total synthesis of this natural product, see [86-94]), is an irreversible, covalent inhibitor of the chymotrypsin-like and trypsin-like activities and a weak, reversible inhibitor of the PGPH activity of the 20S proteasome [95-99; for a recent review on lactacystin, see [100]]. This natural product does not react with the proteasome *in vitro*; rather, it undergoes a spontaneous conversion (lactonization) to the active proteasome inhibitor, clasto-lactacystin β-lactone (compound **22**, Fig. **7**), that deactivates the 20S proteasome at a much faster rate [95, 101]. When the β-lactone is added to cells in culture, it rapidly enters the cells and possibly reacts with the thiol group of glutathione to form a thioester adduct that is both structurally and functionally analogous to lactacystin. This adduct (lactathione) does not react with the proteasome, but can undergo lactonization to yield back the active β-lactone. The formation of lactathione inside the cells may provide a reservoir for prolonged release of the active β-lactone [101-102].

The acylation of the *N*-terminal threonine subunit of the 20S proteasome by lactacystin was confirmed by X-ray crystallography studies at 2.4 Å resolution [41]. Furthermore, *in vitro* experiments with radiolabelled lactacystin in whole cells have shown that almost all the radioactivity becomes associated with the proteasome, demonstrating a remarkable specificity for that entity over a very large number of other proteins. In spite of this



Lactacystin
21

Clasto-lactacystin β-lactone **22**

PS-519
23

Fig. (7). Lactacystin and synthetic analogs.

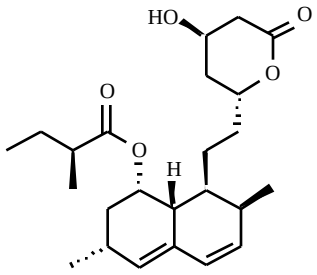
experimental result, lactacystin is not absolutely specific for the proteasome, as it has been reported to inhibit cathepsin A activity from platelets and purified tripeptidyl peptidase II (TPP II) [103].[f]

Studies on the molecular basis of the selectivity and potency of the lactacystin-derived-β-lactone have been performed by varying the substituents at C5, C7 and C9 [105]. The SAR data revealed that the original groups at C5 and C9 seem to be optimal for proteasome inhibition and only the replacement of the 7-methyl substituent by ethyl, *n*-butyl or isopropyl improved 2- to 2.8-fold the chymotrypsin-like peptidase inhibitory activity of the parent compound.

A related compound from this class is compound **23** (PS-519, Fig. **7**), which is a C-7 propyl analog of lactacystin [106-107]. This compound is currently in preclinical development for the treatment of ischemia-reperfusion injury in stroke and myocardial infarction.[g]

2.8 Lovastatin

Lovastatin (compound **24**, Fig. **8**) is an example of proteasome modulator coming from a completely different area of research. This compound inhibits hydroxymethyl-glutaryl (HMG)-CoA reductase, the rate-limiting enzyme in cholesterol synthesis [111-112], and is used for the treatment of hypercholesterolemia [113]. The pro-drug form of



Lovastatin
24

Fig. (8). Lovastatin.

lovastatin (β-lactone ring), which does not block HMG-CoA reductase activity, inhibits the proteasome complex in MDA-MB-157 cell extracts in a dose-dependent manner with half-maximal inhibition occurring at 40 μM [114]. Treatment of the same tumor cells with the preceding compound resulted in inhibition of cell proliferation, pronounced induction of p21 and p27, and increased half-life of these cyclin-dependent kinase inhibitors (0.13 *versus* 1.5 h for both p21 and p27). The lovastatin mixture, which contains 20% of the pro-drug form, inhibited the proteasome activity at much higher concentration, and pravastatin (a lovastatin analogue that resembles only the active form of lovastatin) was inactive. From these results, it seems that the β-lactone is the key functional moiety for the proteasome inhibitory activity of lovastatin.

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[f] Recently, Princiotta et al. [104] have shown that purified TPP II was inhibited by less than 50% at c= 10 μM of lactacystin. Furthermore, 10 μM lactacystin has a small effect on the ability of cells to hydrolyze the TPP substratre H-Ala-Ala-Phe-7-amido-4-methylcoumarin (AAF-amc).

[g] For biological information on the cardioprotective effects of this proteasome inhibitor in a rat heart model of ischemia and reperfusion, see [108]. This compound has also been characterised in models for multiple sclerosis [109] and asthma [110].

2.9 HIV-I Protease Inhibitors as Proteasome Inhibitors

In addition to their antiviral activity, inhibitors of the human immunodeficiency virus type-1 (HIV-1) protease have been described as modulators of proteasome activity [115-117]. Ritonavir (compound **25**, Fig. **9**) inhibits *in vitro* the chymotrypsin-like activity of the 20S proteasome isolated from murine B8 fibroblast cells (IC_{50} value not provided) and causes a marked increase in ubiquitin conjugates when murine B cells where treated with this compound at 100 μM [118].[h] In addition to ritonavir, a series of HIV-1 protease inhibitors containing the 2-aminobenzylstatine template [119-121] have shown proteasome-inhibitory capacity. A representative example of this new class of non-covalent 20S proteasome inhibitors is compound **26** (Fig. **9**) [120], which inhibits the chymotrypsin-like activity of the 20S proteasome with an IC_{50} value of 0.9 μM and shows good selectivity over the trypsin-like and PGPH activities (IC_{50}> 20 μM) [115]. Using the crystal structure of the yeast proteasome [8-9], a structural model of compound **26** bound to the chymotrypsin-like active site of the human 20S proteasome was developed to guide the optimization process [116]. A large number of complementary hydrophobic and hydrogen bond interactions between the inhibitor and the binding site were achieved in this model. These interactions could account for the ability of compound **26** to inhibit the chymotrypsin-like active site of the human 20S proteasome without possessing a reactive group capable of forming a covalent bond with the catalytic threonine. The model was also consistent with preliminary SAR data. The decrease in binding activity (> 20-fold) observed by removal of the methoxy group or methylation of the phenol group in the *C*-terminal benzylamide ring can be due to the involvement of these groups in hydrogen bond interactions with the side chains of serine-53 and threonine-1, respectively. Initial medicinal chemistry efforts were focused on the *N*-terminal part of the 2-aminobenzylstatine template. In this modular approach, the *N*-terminal part of the molecule was modified while the other remained unchanged. To this end, a series of

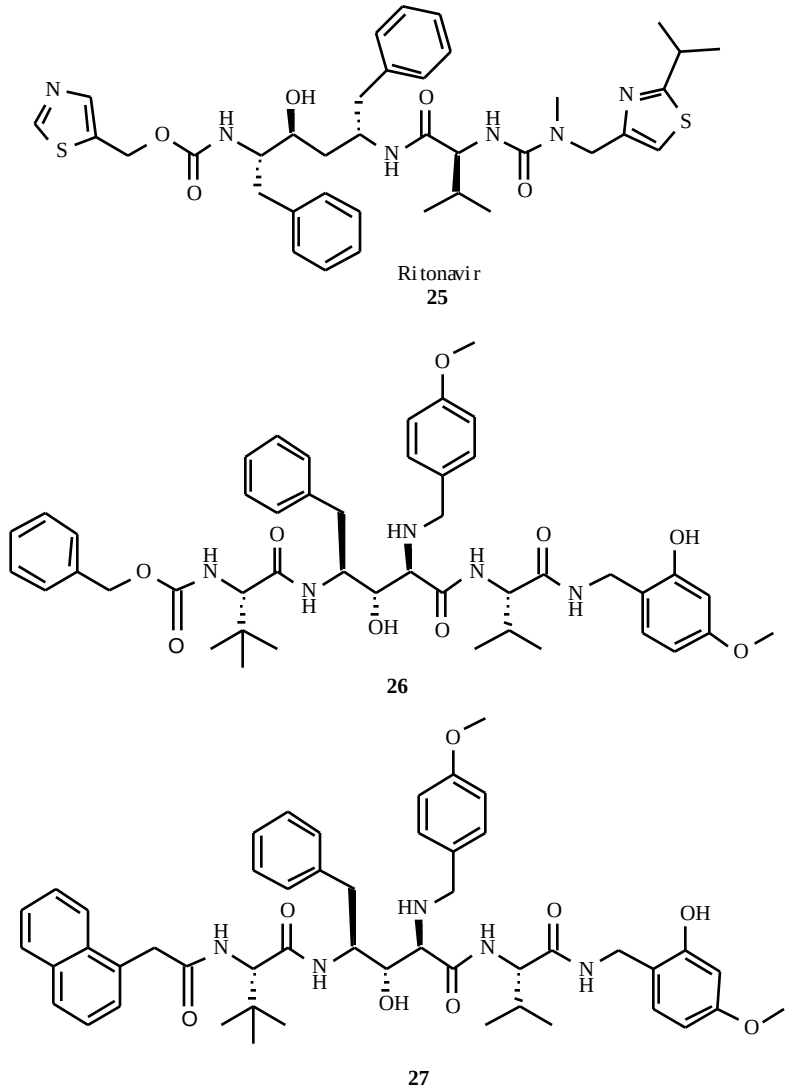
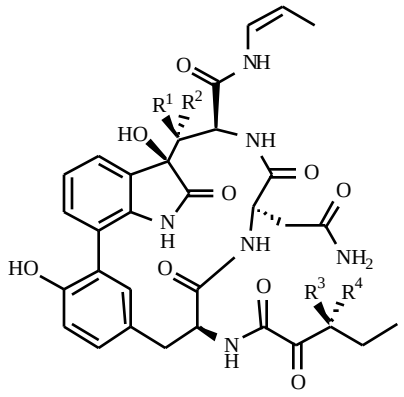
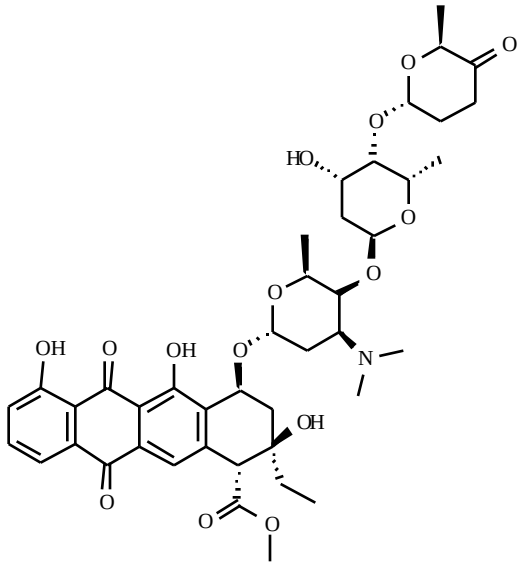


Fig. (9). HIV-1 protease inhibitors as proteasome inhibitors.

[h] Apart from ritonavir, saquinavir also inhibits the chymotrpysin-like activity of the 20S proteasome, although much less efficiently. No inhibition at all was observed for other commercialy available HIV-1 protease inhibitor (e.g., indinavir and nelfinavir)) [118].



TMC-95	R_1	R_2	R_3	R_4	
A	H	OH	CH_3	H	**28**
B	H	OH	H	CH_3	**29**
C	OH	H	CH_3	H	**30**
D	OH	H	H	CH_3	**31**



Aclacinomycin A
32

Fig. (10). TMC-95s and aclacinomycin A.

derivatives determined by molecular modeling were prepared to examine the effects of *N*-terminal substitution on potency. The possibility to increase potency by establishing additional hydrophobic interactions with the accessory pocket formed by the side chains of Tyr-33, Tyr-133 and Pro-131 motivated the synthesis of compound **27** (Fig. **9**). This new derivative turned out to be almost one order of magnitude more potent than compound **26** (IC_{50}= 0.1 μM for compound **27** *versus* IC_{50}= 0.9 μM for compound **26**) confirming the validity of the model and its utility in the design of new proteasome inhibitors. Compound **27** is not only a potent inhibitor of the chymotrypsin-like activity of the 20S proteasome, but also very selective for this catalytic site. This compound shows at least 200-fold selectivity over the trypsin-like and PGPH activities [115-116].

2.10 Varia

The fermentation broths of *Apiospora montagnei* Sacc. TC 1093, *Streptomyces* sp. TC 1087 and 1084, and *Saccharothrix* sp. TC 1094 have been the source of novel proteasome inhibitors [122-128]. TMC-95A-D (compounds **28**-**31**, Fig. **10**) [123, 125], which were isolated from the fermentation broth of *Apiospora montagnei* Sacc. TC 1093, can be considered representative examples of these natural products.[i] TMC-95A (compound **28**, Fig. **10**) inhibited the chymotrypsin like, trpysin-like, and PGPH activities of the 20S proteasome with IC_{50} values of 5.4 nM, 200 nM, and 60 nM, respectively. Similar IC_{50} values were obtained for TMC-95B (compound **29**, Fig. **10**), while the inhibitory activities of TMC-95C (compound **30**, Fig. **10**) and D (compound **31**, Fig. **10**) were 20 to 150-fold lower than that of TMC-95A (compound **28**) and B (compound **29**). These compounds did not inhibit m-calpain, cathepsin L and trypsin at 30 μM. The enzyme inhibitory activity of TMC-95s can be ascribed to the α-keto group present in the 3-methyl-2-oxopentanoic acid moiety.[j]

Modest *in vitro* inhibition of proteasome proteolytic activity has been reported for several antitumor agents (e.g. aclacinomycin A, compound **32**, Fig. **10**; IC_{50}= 50 μM, chymotrypsin-like activity) [131]. The aglycone and sugar moieties of aclacinomycin A are essential for inhibition.

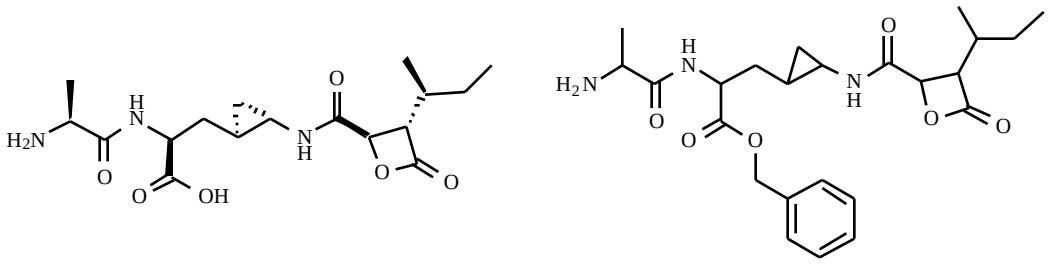
Belactosin A (compound **33**, Fig. **11**), which is a Streptomyces metabolite (KY11780) identified in a yeast-based assay [132], has been shown to inhibit the 20S proteasome activity *in vitro* (IC_{50}= 0.4 μM, chymotrypsin-like activity). This finding has prompted the synthesis of a series of derivatives containing the β-lactone and 3-(2-aminocyclopropyl)-alanine moieties of this novel antitumor antibiotic (e.g. compound **34**, Fig. **11**; IC_{50}= 0.04 μM, chymotrypsin-like activity) [133]. *In vivo* activity has been claimed for compound **34** (10 mg/Kg/day): T/C of 49% in BALB/c-nu/nu mice implanted with WiDr tumor cells.

3. CONCLUSIONS

Early inhibitors of the 20S proteasome proved to be invaluable tools for improving our understanding of the

[i] TMC-86A, B and TMC-96, which were isolated from the fermentation broth of *Streptomyces* and *Saccharothrix*, contain an epoxy-β-aminoketone moiety and are less potent than the TMC-95s [126].

[j] The α-keto moiety plays the most important role on the inhibitory mechanism of the naturally occurring serine protease inhibitors eurysatatin A and B [129], and poststatin [130].



Belactosin A
33

KF33955
34

Fig. (11). Belactosin A and a derivative.

ubiquitin/proteasome-dependent degradation pathway *in vitro*. New classes of potential therapeutic agents that target the 20S proteasome have emerged in the last few years by combining traditional drug discovery approaches with new methods to find or optimize lead structures. These compounds are capable of modulating the subunit-specific proteolytic activities of the 20S proteasome in ways not previously possible. The increased potency and selectivity of these new inhibitors suggests that some of the promising findings observed in *in vitro* and animal models may eventually come to be realized in clinical settings. However, to the best of our knowledge, only one 20S proteasome inhibitor (LDP-341; compound **13** in Fig. **4**) is currently in clinical trials for the treatment of a variety of cancers. Additional studies are needed to further establish the potential therapeutic application of this approach.

One major concern in this area of research is that most of the potent and selective 20S proteasome inhibitors reported until now are peptide-like molecules. These compounds can be susceptible to proteolytic degradation and can suffer from other major delivery problems such as low membrane permeability, rapid elimination from plasma, high-first pass metabolism, and low oral bioavailability. Structure-based design approaches that build upon the knowledge accumulated in the last few years and new methods to increase compound diversity will have the greatest opportunity to identify and optimize new lead structures.

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ABBREVIATIONS

AAF-amc = H-Ala-Ala-Phe-7-amido-4-methylcoumarin

AMC = 7-Amido-4-methyl-coumarin

BAECs = Bovine aortic endothelial cells

HIV-1 = Human immnunodeficiency virus type-1

HMG = Hydroxymethyl-glutaryl

HUVEC = Human umbilical vein endothelial cells

LPS = Lipopolysaccharide

NF-κB = Nuclear transcription factor-κB

PARP = Poly(ADP-ribose) polymerase

PEG = Polyoxyethylene

PGPH = Peptidylglutamyl-peptide hydrolytic

TNF = Tumor necrosis factor

TPP II = Tripeptidyl peptidase II

Ub = Ubiquitin

VS = Vinyl sulfone

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