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[39]   Development and Characterization of Proteasome Inhibitors

By Kyung Bo Kim, Fabiana N. Fonseca, and Craig M. Crews

Abstract

Although many proteasome inhibitors have been either synthesized or identified from natural sources, the development of more sophisticated, selective proteasome inhibitors is important for a detailed understanding of proteasome function. We have found that antitumor natural product epoxomicin and eponemycin, both of which are linear peptides containing a α,β-epoxyketone pharmacophore, target proteasome for their antitumor activity. Structural studies of the proteasome–epoxomicin complex revealed that the unique specificity of the natural product toward proteasome is due to the α,β-epoxyketone pharmacophore, which forms an unusual six-membered morpholino ring with the amino terminal catalytic Thr-1 of the 20S proteasome. Thus, we believe that a facile synthetic approach for α,β-epoxyketone linear peptides provides a unique opportunity to develop

proteasome inhibitors with novel activities. In this chapter, we discuss the detailed synthetic procedure of the α',β'-epoxyketone natural product epoxomicin and its derivatives.

Introduction

Studies into the chemical synthesis of proteasome inhibitors were initiated during the early 1990s after standard serine/cysteine protease inhibitors were shown to inhibit the 20S proteasome (Figueiredo-Pereira et al., 1994; Orlowski, 1990). Early synthetic efforts were largely focused on the modification of the amino acid sequence of the serine/cysteine protease inhibitors. For example, MG115 and MG132, which were developed by Rock and colleagues (1994) and have been widely used in proteasome biology, are tripeptide aldehydes that share similar peptide backbones and aldehyde pharmacophores with known protease inhibitors such as calpain inhibitor 1 (Fig. 1). Other known peptide-based protease inhibitors possessing different pharmacophores such as vinylketones (Bogyo et al., 1997, 1998) and boronates (Adams, 2002; Adams et al., 1998; Iqbal et al., 1996) have also been developed as proteasome inhibitors (Fig. 1). The major advantage of these peptide backbone–based proteasome inhibitors is their ease of preparation and derivatization, potentially providing an easy access for the development of proteasome inhibitors with novel activities. Although these peptide inhibitors, in general, are cell-permeable potent inhibitors of the 20S proteasome and are still widely used in the study of the role of proteasome in many cellular processes, the cross-reactivity with other proteases remains a major concern for their use as
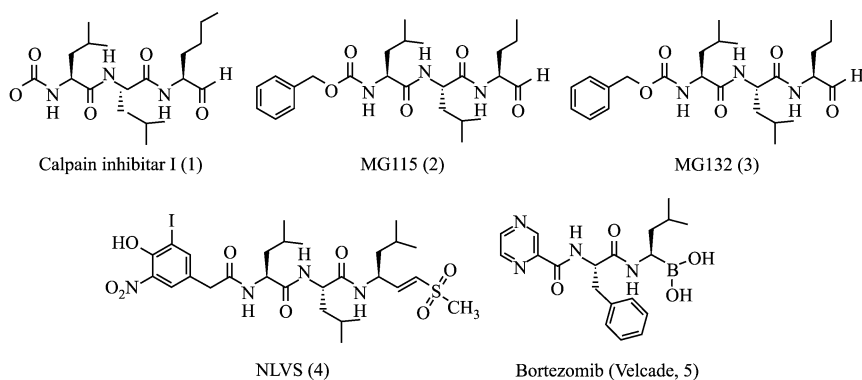

FIG. 1. Proteasome inhibitors derived from other known peptide-based protease inhibitors possessing common pharmacophores.

molecular probes in dissecting complex signaling pathways (Kim and Crews, 2003; Kisselev and Goldberg, 2001; Myung *et al.*, 2001a).

In addition to synthetic approaches, natural products have also provided both peptide and nonpeptide proteasome inhibitors (Kim and Crews, 2003). Examples include epoxomicin (Hanada *et al.*, 1992), eponemycin (Sugawara *et al.*, 1990), lactacystin (Fenteany *et al.*, 1995), TMC-95s (Koguchi *et al.*, 2000a), phepropeptins (Sekizawa *et al.*, 2001), and epigallocatechin-3-gallate (ECGC) (Nam *et al.*, 2001). Among these, epoxomicin and eponemycin are members of a growing family of α',β'-epoxyketone natural products having a linear peptide structure (Fig. 2) (Koguchi *et al.*, 1999, 2000b,c; Sugawara *et al.*, 1990; Tsuchiya *et al.*, 1997). We reported the first total synthesis of epoxomicin (Sin *et al.*, 1999), isolated from an unidentified actinomycete strain No.Q996–17, and showed that epoxomicin potently inhibits the 20S proteasome (Meng *et al.*, 1999b) using an affinity

Epoxomicin (6)

Eponemycin (7)

Epopromycin A (8)

Epopromycin B (9)

TMC-86A (10)

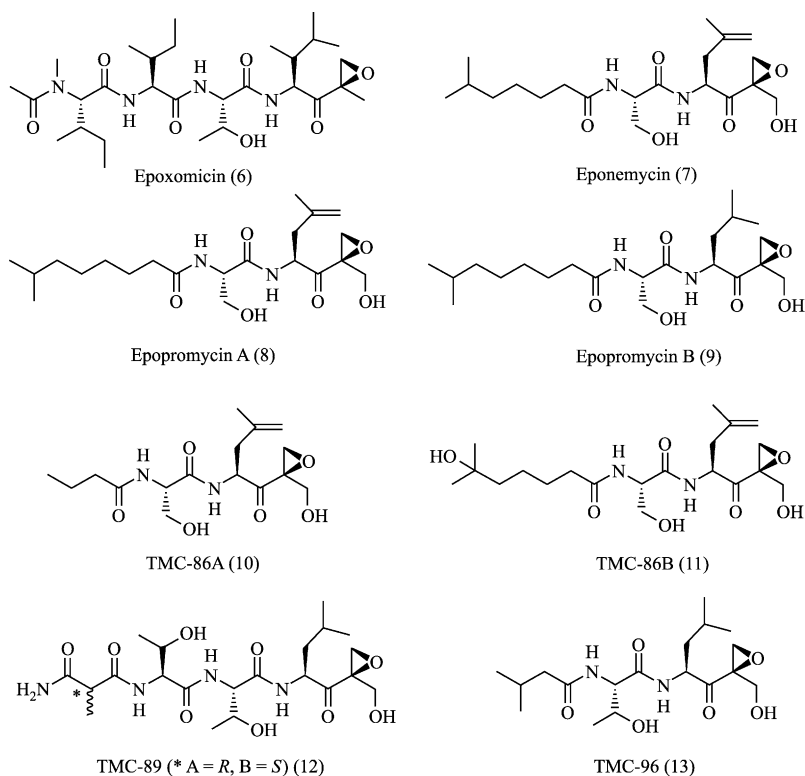TMC-86B (11)

TMC-89 (* A = *R*, B = *S*) (12)

TMC-96 (13)

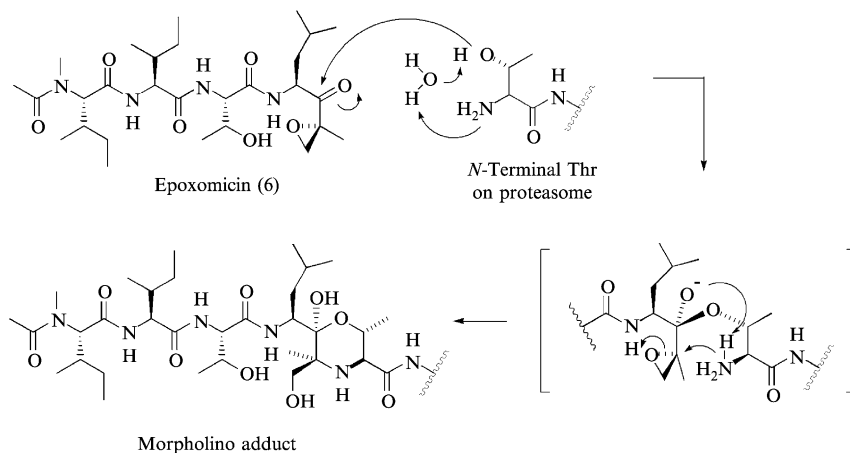FIG. 2. α',β'-Epoxyketone-containing proteasome inhibitors from natural sources.

FIG. 3. The mechanism of proteasome inhibition by epoxomicin is proposed on the basis of the x-ray structure of yeast 20S proteasome–epoxomicin complex. It is postulated that the unique specificity of epoxomicin is due to the formation of an unusual six-membered morpholino ring between Thr-1 of the catalytic subunit of 20S proteasome and the α',β'-epoxyketone pharmacophore of epoxomicin.

reagent–labeled epoxomicin (Meng *et al.*, 1999a; Sin *et al.*, 1998). Interestingly, antitumor natural products epoxomicin and eponemycin are shown to be specific for the proteasome despite their common peptide backbone that resembles structures of known serine/cysteine protease inhibitors (Meng *et al.*, 1999b; Sin *et al.*, 1999). Structural studies of the yeast 20S proteasome complexed with epoxomicin revealed that the unique specificity of epoxomicin is due to the formation of an unusual six-membered morpholino ring between the amino terminal catalytic Thr-1 of the 20S proteasome and the α',β'-epoxyketone pharmacophore of epoxomicin (Fig. 3) (Groll *et al.*, 2000). The facile synthetic strategy of α',β'-epoxyketone linear peptides developed through the total synthesis of epoxomicin and the unique specificity of its α',β'-epoxyketone pharmacophore for proteasome have prompted the development of proteasome inhibitors possessing higher potency or novel inhibitory specificities, such as YU101 (Elofsson *et al.*, 1999) and YU102 (Myung *et al.*, 2001b) (Fig. 4), respectively.

In this chapter, we discuss the synthesis and characterization of this important class of proteasome inhibitors, focusing on the α',β'-epoxyketone natural product epoxomicin and its derivatives. It should be noted that the synthetic strategy of epoxomicin described in this chapter can be applied easily to the development of proteasome inhibitors with novel activities.
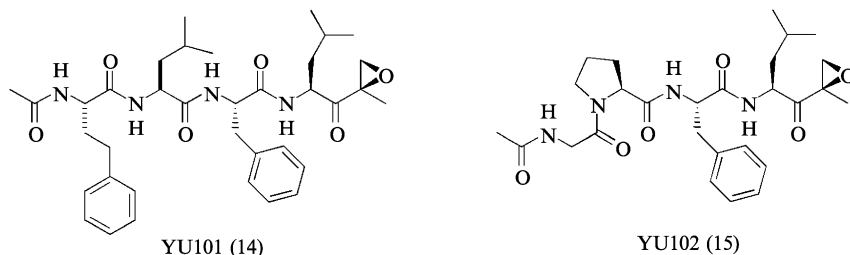
FIG. 4. Synthetic α',β'-epoxyketone proteasome inhibitors designed to target a certain proteasomal proteolytic activity/subunit with a high degree of specificity. YU101 is a chymotrypsin-like activity (CT-L)-selective inhibitor, whereas YU102 is shown to be specific for caspase-like activity.

Synthesis Strategy of α',β'-Epoxyketone Peptide Inhibitors: Total Synthesis of Epoxomicin

General

There are two main strategies for the chemical synthesis of linear peptides (Fields, 1997; Lloyd-Williams *et al.*, 1997). First, chain elongation in linear synthesis is carried out by repetitive N^α-amino group deprotection and protected amino acid coupling steps. Alternately, convergent synthesis involves the synthesis and coupling of protected peptide segments. Both strategies can be carried out in solution or on a solid support, although solution and solid-phase methods can coexist in convergent synthesis. For the synthesis of epoxomicin, chain elongation on solid support may be a challenging task because of the C-terminus epoxyketone pharmacophore, which lacks a handle for resin attachment. Solution phase chain elongation was also ruled out because of the possibility of decomposition of epoxyketone group during repetitive coupling and deprotection reactions. On the basis of these considerations, a convergent approach was chosen for the total synthesis of epoxomicin (Fig. 5).

The convergent approach in the synthesis of epoxomicin involves: (1) solution-phase synthesis of the right-hand fragment, (2) solution or solid-phase synthesis of left-hand fragment, (3) solution-phase assembly of complete peptide backbone of epoxomicin, and (4) purification and characterization of the coupled product. After the final coupling, HPLC purification is carried out to separate epoxomicin from the stereoisomeric counterpart generated during the final coupling reaction. Finally, the enzymatic inhibitory activity of epoxomicin was measured using purified bovine proteasome.
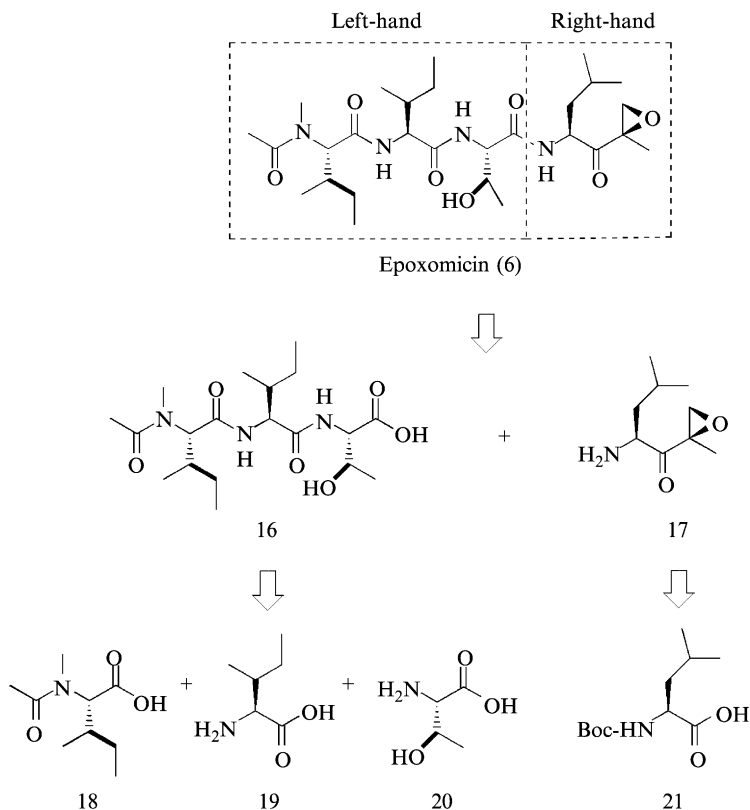
FIG. 5. A convergent approach for the total synthesis of epoxomicin.

Synthesis of the Right-Hand Fragment of Epoxomicin

There are two alternative approaches to introduce an epoxy group in the course of synthesis of epoxomicin. First, the epoxidation reaction can be performed after complete assembly of the peptide backbone of epoxomicin, and the resulting desired product is purified from the mixture of epoxomicin and *epi*-epoxomicin by HPLC (Schmidt and Schmidt, 1994) (Fig. 6A). The second approach relies on the preparation and isolation of stereochemically defined α',β'-epoxyketone leucine (Bennacer *et al.*, 2003; Dobler, 2001; Hoshi *et al.*, 1993; Iwabuchi *et al.*, 2001; Sin *et al.*, 1998), which is then coupled to the tripeptide left-hand fragment to yield the complete backbone of epoxomicin (Fig. 6B). Because the α',β'-epoxyketone leucine having the same configuration as that of epoxomicin can be

A

1. Epoxidation
2. HPLC

22

B

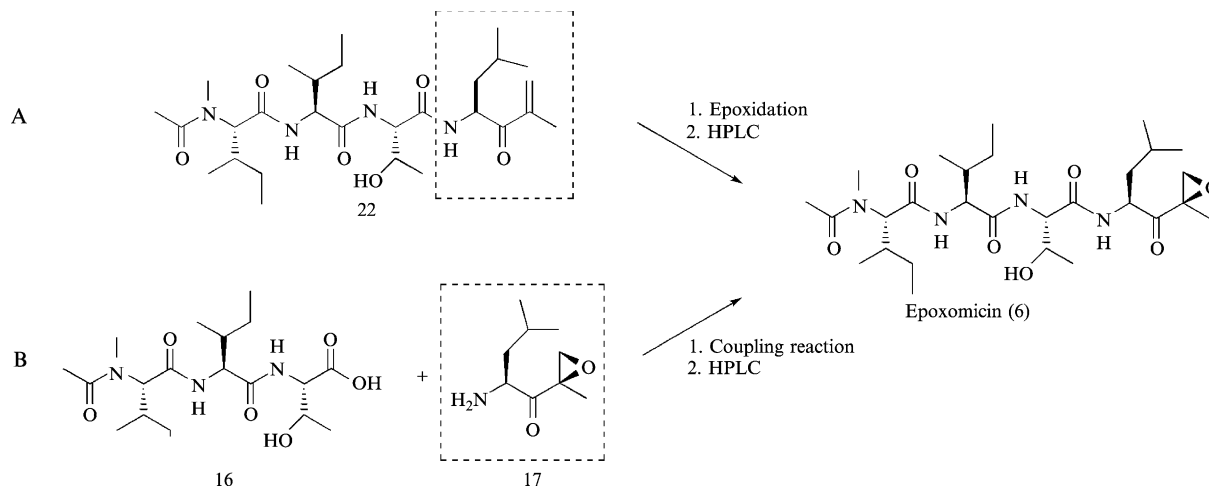16 + 17

1. Coupling reaction
2. HPLC

H₂N

Epoxomicin (6)

FIG. 6. Two potential strategies for the introduction of a α',β'-epoxyketone group.

easily chromatographically purified from its stereoisomeric counterpart, the second approach was initially chosen to prepare the right-hand fragment of epoxomicin (Fig. 7).

As the first step of the right-hand fragment synthesis, α-*tert*-butyloxy-carbonyl (Boc)-leucine (Boc-Leu-OH) was coupled to *N*-methoxy-*N*-methylamine with *O*-benzotriazo-1-yl-*N,N,N′, N′*-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) to yield the Boc-leucine Weinreb amide 23. Readily preparable Weinreb amides (*N*-methoxy-*N*-methylamides) (Nahm and Weinreb, 1981) are known to couple in good yields with Grignard and organolithium reagents to produce ketones, and to be reduced with hydrides to afford aldehydes. Fluoren-9-ylmethoxycarbonyl-leucine (Fmoc-Leu-OH), however, cannot be used, because the Fmoc group is unstable under Grignard or organolithium reaction conditions, which are used to introduce the $\alpha′,\beta′$-unsaturated ketone 24 in the following step.

Reaction of α-Boc-leucine-Weinreb amide 23 with commercially available propen-2-yl magnesium bromide (Grignard reagent) led to the formation of α-Boc-amino-$\alpha′,\beta′$-unsaturated ketone 24 without racemization at low temperature ($-78°$ to room temperature) in tetrahydrofuran (THF). The nucleophilic addition proceeds through stable metal-chelate intermediates that block over-addition. The resulting α-Boc-amino-$\alpha′,\beta′$-unsaturated ketone 24 was readily purified by flash column chromatography using hexanes-ethyl acetate system (10:1, v/v) as eluant. Subsequent epoxidation of α-Boc-leucine-$\alpha′,\beta′$-unsaturated ketone with alkaline hydrogen peroxide (Schmidt and Schmidt, 1994) in methanol yielded a mixture of epoxide stereoisomers; the ratio of 2-(*R*)-epoxide 25a to 2-(*S*)-epoxide 25b was 1.7. The two isomers of leucine epoxyketone were readily separated by
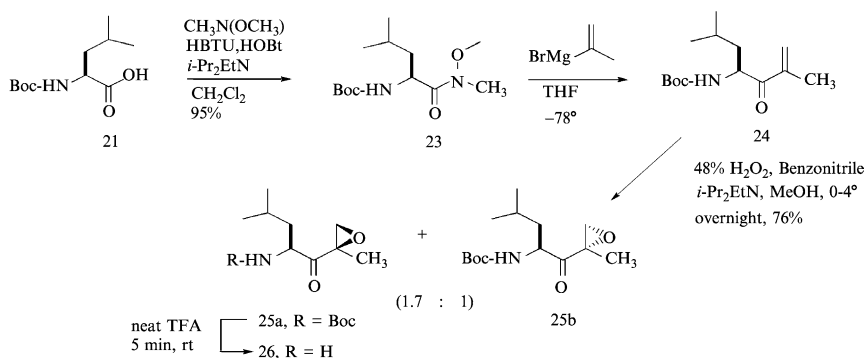

FIG. 7. Synthesis of the right-hand fragment (leucine epoxyketone).

flash column chromatography using hexanes-ethyl acetate (5:1, v/v) system. The isomer (2-(R)-epoxide) 25a, which migrates faster than the 2-(S)-epoxide 25b in thin-layer chromatography (TLC, hexanes-ethyl acetate = 5:1, v/v), was found to have the same configuration as that of epoxomicin (Sin *et al.*, 1999). Finally, the Boc group of Boc-Leu-α′,β′-epoxyketone 25a was deprotected by brief treatment (∼5 min) with neat trifluoroacetic acid (TFA) without concomitant opening of the epoxide ring. Excess TFA was removed under high vacuum. The resulting TFA salt of leucine-α′,β′-epoxyketone was used without further purification for the final coupling reaction with the left-hand fragment.

Synthesis of the Left-Hand Fragment

The tripeptide left-hand fragment of epoxomicin can be readily prepared either in solution or on a solid support (Sin *et al.*, 1999). Given that peptide synthesis on a solid support has been a subject of many extensive reviews (Fields, 1997), in this chapter we discuss only the solution-phase synthesis.

In the first step (Fig. 8), Fmoc-isoleucine-OH was coupled to threonine benzylester 27 with HBTU and HOBt. Although there are many coupling reagents that may be used for peptide bond formation, HBTU/HOBt coupling system was found to be very effective for reducing loss of configuration at the carboxylic acid residue in this coupling reaction. The resulting dipeptide 28 was purified by flash column chromatography using hexanes-ethyl acetate system (1:1, v/v) as eluant. The solubility of the dipeptide 28 was so poor that it was readily precipitated during flash column chromatography, requiring a large volume of elution solvent to redissolve and collect the dipeptide. The protection of threonine hydroxyl group of the dipeptide 28 was then accomplished with *tert*-butyldiphenylsilylchloride (TBDPSCl) in methylene chloride (CH_2Cl_2) at room temperature. The same TBDPSCl-protection reaction, however, was less effective in tetrahydrofuran (THF). The advantages of using TBDPS protecting group compared with other commonly used protecting groups such as *tert*-butyldimethylsilyl (TBDMS) and trimethylsilyl (TMSCl) are: (1) enhanced solubility of TBDPS protected peptides in organic solvents facilitating the following solution-phase reactions and purification process; (2) better stability under acid and base conditions; (3) easy UV detection provided by the two phenyl rings of TBDPS group, thus aiding the HPLC purification of completely assembled TBDPS protected epoxomicin from the stereoisomeric counterpart generated during the final coupling of the left- and right-hand fragments.

The Fmoc group of TBDPS-protected dipeptide 29 was deprotected using standard protocol (20% piperidine in dimethylformamide, v/v)
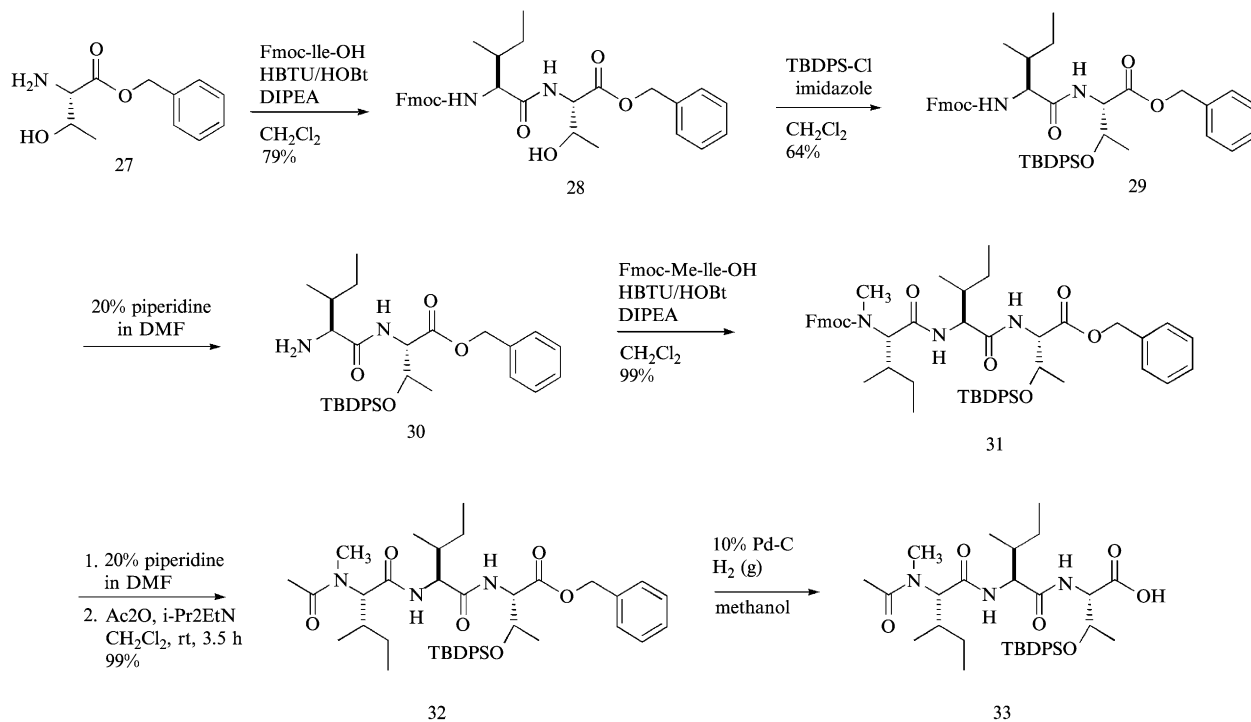
Fig. 8. Preparation of the tripeptide left-hand fragment.

(Fig. 8). The resulting Fmoc-deprotected dipeptide 30 was purified by flash column chromatography. In this type of Fmoc deprotection reaction, only one fourth or one fifth of the normal quantity of dried silica (SiO_2) that is used for routine column chromatography was packed for the purification of the Fmoc-deprotected product. Specifically, after directly loading the Fmoc-deprotected crude product into the silica gel column, fast-migrating Fmoc-adducts, which are shown as bright fluorescent spots under UV, were eluted away using a solvent system (hexanes-ethyl acetate, 1:1, v/v); under this elution condition, the Fmoc-deprotected product 30 was retained in the silica gel column. The retained deprotected dipeptide 30 was then eluted and collected using a different solvent system (CH_2Cl_2-MeOH, 9:1, v/v).

Next, Fmoc-N-methyl-isoleucine was coupled to the deprotected dipeptide 30 with HBTU/HOBt to yield the tripeptide 31, which was easily purified by flash column chromatography (hexanes-ethyl acetate, 1:1, v/v). After the Fmoc-protected tripeptide 31 was treated with 20% piperidine in DMF (v/v) for 20 min at room temperature, DMF and piperidine were removed under high vacuum. Without further purification, the crude product was mixed with excess acetic anhydride in pyridine for 1 h at room temperature. The resulting N-acetylated tripeptide 32 was purified by flash column chromatography (hexanes-ethyl acetate, 1:1, v/v). Finally, the C-terminus benzyl group of the left-hand fragment 32 was deprotected by catalytic hydrogenolysis mediated by 10% activated palladium-charcoal in methanol under hydrogen gas atmosphere. In this reaction, instead of performing hydrogenolysis under high pressure, a stream of hydrogen gas was directly applied to the reaction mixture providing hydrogen bubbles into the solution (Fig. 9). The benzyl protecting group was readily removed in nearly quantitative yield, and the reaction mixture was filtered through a coarse, Celite-packed, fritted-glass filter to afford the TBDPS-protected left-hand fragment 33, which was coupled to the right-hand fragment without further purification for the final coupling with the right-hand fragment.

Assembly of Complete Epoxomicin Peptide Backbone

The final coupling reaction between the right- and left-hand fragments of epoxomicin was performed with O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and 1-hydroxy-7-azabenzotriazol (HOAt) to give TBDPS protected epoxomicin 34b (Fig. 10). Unlike the coupling reactions performed during the synthesis of left-hand fragment, HATU was used for the final assembly of epoxomicin peptide backbone to enhance the efficiency of coupling reaction. HATU was developed by Carpino (Albericio and Carpino, 1997; Carpino, 1993) and
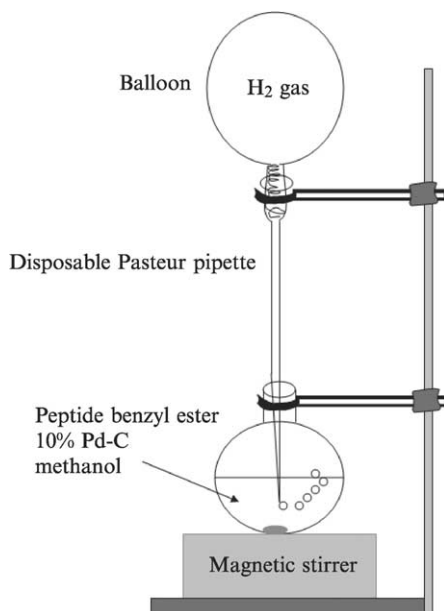
FIG. 9. Schematic of hydrogenation reaction, in which hydrogen gas is provided to the reaction mixture through a stream of hydrogen gas.

shown to be particularly effective with hindered couplings. After overnight stirring at room temperature, the resulting TBDPS protected epoxomicin 34b was initially purified by flash column chromatography (hexanes-ethyl acetate, 1:1, v/v) to verify the successful assembly of complete peptide backbone of epoxomicin by 1H NMR. 1H NMR spectra analysis showed the complete assembly of epoxomicin peptide backbone and the presence of minor stereoisomer 34a (5–15% of the assembled product), the formation of which during the amide bond formation is well documented (Carpino, 1993, 1997). It is assumed that the loss of configuration occurs at the carboxylic acid residue of P2 amino acid during the final coupling reaction (Fig. 10). It was difficult to purify the TBDPS protected epoxomicin 34b from the isomer 34a using standard column chromatography, but it was readily separated by normal-phase HPLC (hexanes-isopropanol, linear gradient, hexanes 100% to 50%). In normal-phase HPLC, the TBDPS-protected epoxomicin 34b displayed a longer retention time compared with its stereoisomeric counterpart 34a. Finally, treatment of the TBDPS protected epoxomicin with tetrabutylammonium fluoride (TBAF) gave epoxomicin (Fig. 10), which was purified by silica gel column chromatography (CH_2Cl_2-MeOH, 98:2, v/v), followed by reverse-phase HPLC.
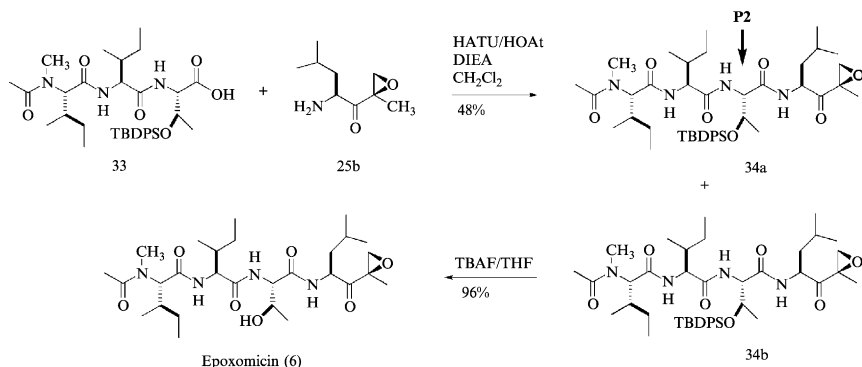
FIG. 10. The final assembly of epoxomicin peptide backbone and preparation of epoxomicin.

Purification of Epoxomicin by HPLC

Epoxomicin was purified by RP-HPLC (Rainin Dynamax) system composed of two solvent delivery pumps (Model SD200) and variable wavelength detector (Model UV-1) set at 214 nm. The column used was an YMC-Pack ODS-AM, 250 mm × 20 mm, 5-μm particles size and 120 Å pore size (Waters, Milford, MA) coupled to a guard column ODS-AM (10 mm × 10 mm) with same stationary phase specifications. The separation was carried out with a linear gradient of solvent A (water) into solvent B (MeOH): 65% of B to 80% of B over 90 min, run at 5.0 ml/min, room temperature. Epoxomicin was eluted at 49 min and the peak was collected as seven fractions of nearly 1 ml each.

Chemical Characterization of Epoxomicin

Analytical HPLC traces of collected fractions were carried out in a Waters Separation Module 2795 coupled to a Waters 2795 Photodiode Array Detector (set at 214 nm) and to a Micromass ZQ 2000 Electrospray Mass detector (cone voltage = +20V). A linear gradient of solvent A (water) in solvent B (MeOH), 30% of B to 90% of B in 30 min was run at 0.2 ml/min, at 25°. The analytical column used was an XTerra MS C18 (4.6 mm × 50 mm, 2.5-μm particle size, and 80 Å pore size).

Epoxomicin eluted at 19.8 min [observed $m/z = 555.2$ $(M + H)^+$, 577.6 $(M + Na)^+$]. Fractions showing purity ≥98% were grouped together and vacuum-dried with no heat until only water remained. Samples were then lyophilized. Epoxomicin was further characterized by ^{1}H NMR and ^{13}C NMR (Meng et al., 1999b; Sin et al., 1999).

Development Strategy of Proteasome Inhibitors with Novel Activities

General

The emergence of the ubiquitin-proteasome pathway as a major player in many important biological processes has prompted many synthetic efforts in proteasome inhibitor development (Kim and Crews, 2003; Myung *et al.*, 2001a). In addition, systematic natural product screening has also provided a number of novel proteasome inhibitors (Kim and Crews, 2003). Thus far, most of these proteasome inhibitors have been shown to target the chymotrypsin-like (CT-L) activity. Although the CT-L activity of the proteasome has been shown to be largely responsible for the proteolytic function of the proteasome *in vivo* and *in vitro* (Myung *et al.*, 2001b), the contribution of other major activities remains to be determined. Therefore, a novel class of inhibitors that target individual catalytic subunit/activity of proteasome may be required to dissect the role of each catalytic subunit.

Exposure of cells to stimuli such as IFN-γ, TNF-α, and LPS induces the synthesis of certain catalytic subunits (respectively, LMP2, MECL-1, and LMP7) that together are incorporated into alternative proteasome form (Kloetzel, 2001). This isoform, known as the immunoproteasome, has an enhanced capacity to generate peptides bearing hydrophobic and basic amino acids at their C-termini and a reduced capacity to produce peptides bearing acidic residues at their C-terminus (Kloetzel, 2001; Rock and Goldberg, 1999). Consequently, the spectrum of the produced peptides is shifted toward peptides that associate with MHC class I molecules with increased affinity (Fruh *et al.*, 1994), implicating a major role of immunoproteasome in antigen presentation. Furthermore, it has been suggested that the immunoproteasome may be involved in some pathological processes, such as diabetes and autoimmune diseases (Casp *et al.*, 2003). However, the exact role of the immunoproteasome remains unclear, caused in large part by the lack of appropriate molecular probes. Given that currently available proteasome inhibitors target both the constitutive proteasome and the immunoproteasome, development of immunoproteasome-specific inhibitors may be useful in the dissection of the role of immunoproteasomes.

Lessons from SAR Studies on the Natural Products
  Epoxomicin and Dihydroeponemycin

Although antitumor natural products epoxomicin (Meng *et al.*, 1999b; Sin *et al.*, 1999) and eponemycin (Meng *et al.*, 1999a) have been shown to target proteasome, they markedly differ in proteasome subunit binding specificity and rates of proteasome inhibition. To understand such differences and

develop proteasome inhibitors with novel activities, a combinatorial approach was taken to investigate the structure-activity relationship (SAR) of these compounds (Kim *et al.*, 1999). Although both are members of the α',β'-epoxyketone linear peptide natural product family, there are some structural differences in their left-, central-, and right-hand fragments (Fig. 11) that may cause the different subunit binding specificity and inhibitory potency between two natural products. To this end, epoxomicin/dihydroeponemycin chimerae and their biotinylated counterparts were synthesized to correlate these structural features with proteasome inhibitory activity and their specific subunit labeling (Fig. 11). Kinetic data of epoxomicin/dihydroeponemycin chimerae for proteasome activities showed that α',β'-epoxyketone tetrapeptide inhibitors (compounds 35–37) generally display 300–500 fold greater inhibition for the CT-L activity compared with that of isooctanoic-containing tripeptide inhibitors (compounds 38–40) (Kim *et al.*, 1999).

On the other hand, the α',β'-epoxyketone tetrapeptide inhibitors (compounds 35–37), regardless of changes in the central and right-hand fragments, displayed the same proteasome subunit binding pattern as epoxomicin, which predominantly binds LMP7/X and MECL1/Z (Meng *et al.*, 1999b), whereas tripeptide inhibitors (compounds 38–40) that possess isooctanoic residue at the *N*-terminus displayed the same labeling pattern as that of dihydroeponemycin, which predominantly labels immunoproteasome subunit LMP2 and, to a lesser extent, LMP7/X (Meng *et al.*, 1999a). Taken together, SAR studies on eponemycin/dihydroepoxomicin show that (1) α',β'-epoxyketone tetrapeptide inhibitors generally possess a higher inhibitory activity for the proteasome than tripeptide counterparts; (2) α',β'-epoxyketone tripeptide inhibitors containing the isooctanoic residue at the N-terminus, regardless of changes in the central and right-hand fragments, display greater specificity toward immunoproteasome over the constitutive proteasome. It is expected that the information on SAR studies will provide a basis for development of more potent proteasome inhibitors. In addition, the insights gained from SAR studies may facilitate the development of proteasome inhibitors with a high degree of immunoproteasome specificity.

Preparation of Epoxomicin/Dihydroeponemycin Chimerae

All the chimerae were prepared following the procedure used for the synthesis of epoxomicin with the exception of the right-hand fragment of dihydroeponemycin. The right-hand fragment of dihydroeponemycin was synthesized by a slight modification of a previously reported procedure (Hoshi *et al.*, 1993; Schmidt and Schmidt, 1994). Recently, similar procedures for the preparation of the right-hand fragment have
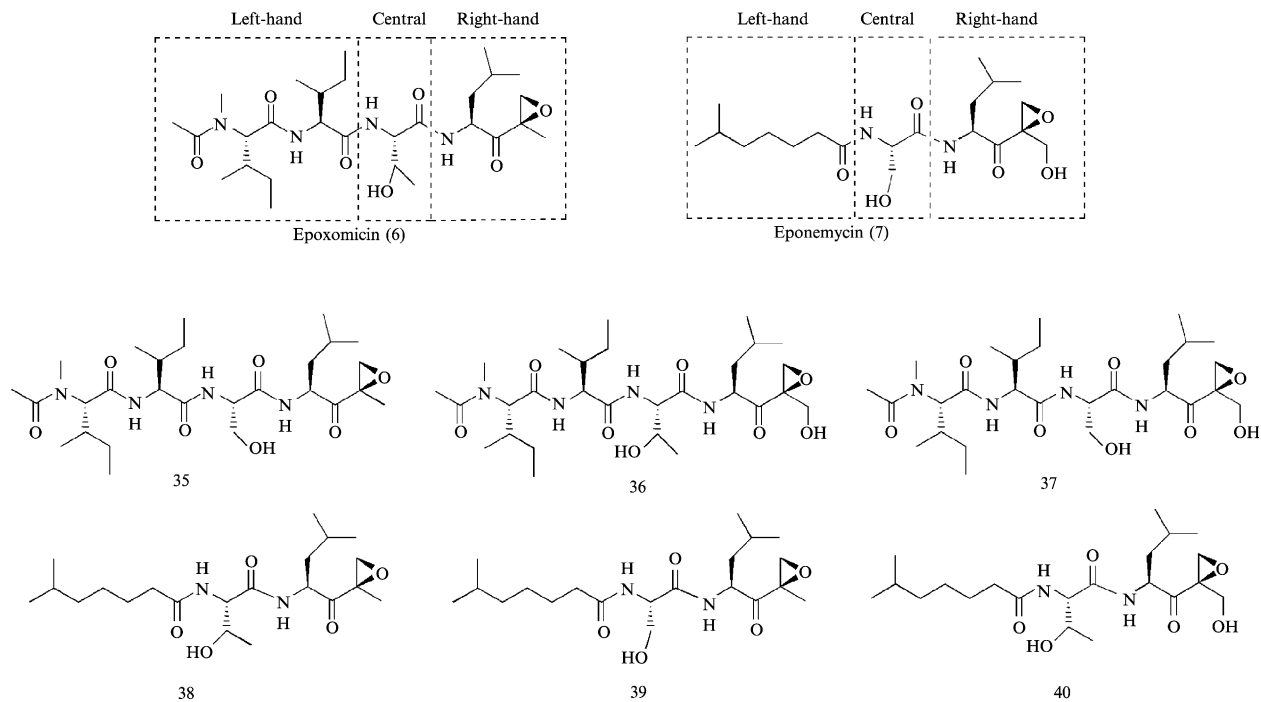
FIG. 11. Epoxomicin/eponemycin chimerae were prepared by a random combination of left- and right-hand and central fragments.

also been reported (Bennacer *et al.*, 2003; Dobler, 2001; Iwabuchi *et al.*, 2001). Given that a convergent approach that is applied for the preparation of epoxomicin/dihydroeponemycin chimerae is essentially the same as that described for epoxomicin, we only describe the total synthesis of dihydroeponemycin.

The synthesis of the right-hand fragment of dihydroeponemycin began with the addition of vinyl lithium derived from treatment of 2-bromo-1-hydroxy-2-propene (Hoshi *et al.*, 1993) with *t*-butyl lithium (Corey and Widiger, 1975) to leucine aldehyde 41, which afforded a 1:1 inseparable mixture of diol 42 (Fig. 12). The leucine aldehyde was prepared by the reduction of leucine Weinreb amide with LiAlH$_4$ in anhydrous ether. Selective protection of the primary alcohol of 42 with *t*-butyldimethylsilyl (TBS) group and a Swern oxidation of the remaining secondary alcohol yielded the α,β-unsaturated ketone 43. Epoxidation of 43 with hydrogen peroxide (Schmidt and Schmidt, 1994) afforded two epoxyketone isomers 44a and 44b as a 1.2:1 mixture that were readily separated by flash column chromatography using an elution system (hexanes-ethyl acetate = 5:1, v/v). The isomer (2-(*R*)-epoxide) 44a, which migrates faster than the 2-(*S*)-epoxide 44b in thin-layer chromatography (TLC), was found to have the same configuration as that of eponemycin epoxide. Final removal of the benzyloxycarbonyl (Z) protecting group of 44a through a catalytic hydrogenation reaction (Hoshi *et al.*, 1993) gave leucine epoxyketone 45 in nearly quantitative yield. The left-hand fragment was prepared by coupling of isooctanoic acid with serine benzylester using HBTU, followed by protection of the hydroxyl side chain of serine residue with TBDPS group and hydrogenolysis of the benzyl protecting group yielded TBDPS-protected left-hand fragment 49 (Fig. 13). The final coupling reaction between epoxyketone 45 and dipeptide 49 was performed with HATU, followed by
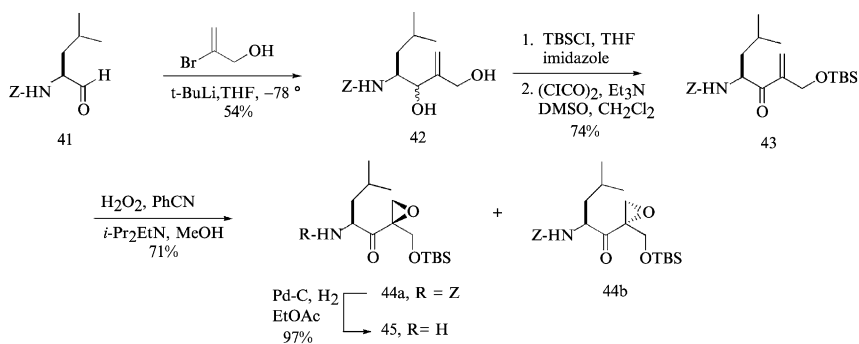

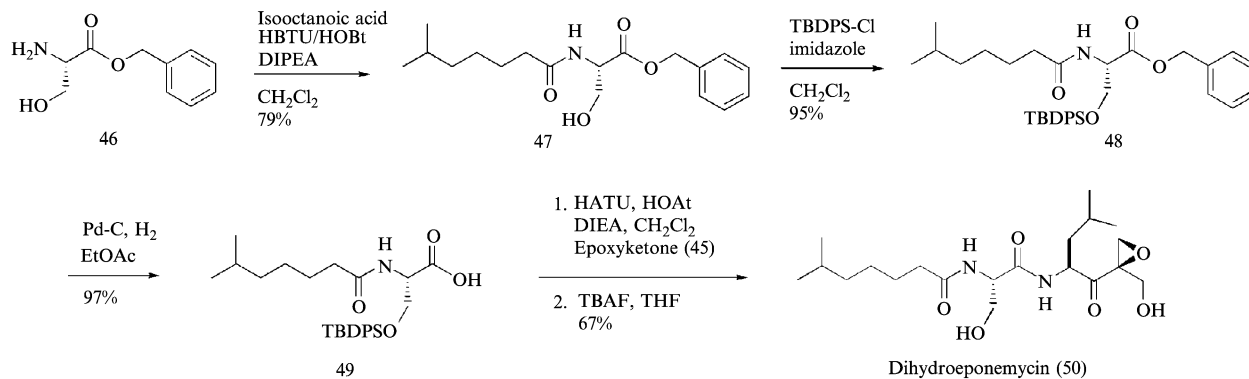FIG. 12. Preparation of the epoxyketone residue of eponemycin.

FIG. 13. The final assembly of eponemycin peptide backbone, which yielded dihydroeponemycin by TBDPS deprotection from serine side chain.

removal of the TBDPS group and normal-phase HPLC (hexanes-isopropanol, linear gradient, hexanes 100–50%) to yield dihydroeponemycin 50.

Development of Activity-Specific α',β'-Epoxyketone Proteasome Inhibitors

CT-L Activity-Specific α′,β′-Epoxyketone Inhibitors

On the basis of the information gathered from SAR studies on epoxomicin/dihydroeponemycin, α',β'-epoxyketone tetrapeptide was chosen as a lead compound in the development of CT-L activity-specific proteasome inhibitors. To develop highly potent CT-L specific α',β'-epoxyketone proteasome inhibitors, a combinatorial positional scanning approach with a variety of hydrophobic amino acids, such as alanine, leucine, phenylalanine, homophenylalanine, 3-(1-naphthyl)-alanine, and *p*-benzoylphenylalanine, was applied to find the optimal amino acids at each position (Elofsson *et al.*, 1999). After the synthetic strategy of epoxomicin described previously, peptide α',β'-epoxyketones were assembled from left-hand fragments, prepared by standard solid-phase synthesis, and the leucine α',β'-epoxyketone. The coupling reactions were carried out with HATU/HOAt and diisopropylethylamine (DIEA) in DMF. Unlike the final coupling in epoxomicin synthesis, in which CH_2Cl_2 was used as a solvent, DMF was used because of the poor solubility of the left-hand fragments. It seems that a slightly higher level of stereoisomer (5–30%) is formed in DMF conditions compared with CH_2Cl_2 environment. After reverse-phase HPLC purification, the resulting α',β'-epoxyketone tetrapeptides were tested for inhibition of three catalytic activities of the 20S proteasome. Once amino acids that display the best CT-L activity inhibition at each position were identified, the optimized inhibitor with optimized amino acids at each position was prepared, tested for CT-L activity inhibition to yield the most potent CT-L activity selective inhibitor (YU101, Fig. 4) to date (Elofsson *et al.*, 1999).

Purification of YU101. Impure YU101 was purified by RP-HPLC (for HPLC system and column specifications see the purification procedure for epoxomicin). A linear gradient of solvent A into B (water and methanol, respectively) consisting of 70% of B to 85% of B in 90 min, run at 5 ml/min, room temperature. YU101, which was eluted at 62.5 min, was collected as seven fractions of nearly 2 ml each.

Chemical Characterization. LC-MS analysis of collected fractions were performed in a Waters Separation Module 2795 coupled to a Waters 2795 Photodiode Array Detector (set at 214 nm), and to a Micromass ZQ 2000

Electrospray Mass detector (cone voltage = +20V). A linear gradient of solvent A (water) in solvent B (MeOH), 5% of B to 98% of B in 30 min was run at 0.2 ml/min, 25°. The analytical column used was the same as the one described for epoxomicin.

YU101 was eluted at 10.3 min [observed m/z = 635.5 (M + H)$^+$, 658.4 (M + Na)$^+$]. Fractions showing purity ≥98% were grouped together and vacuum-dried with no heat. YU101 was also confirmed by ^{1}H NMR.

Development of Caspase-Like Activity-Specific α′,β′-Epoxyketone Peptide Inhibitors

α′,β′-Epoxyketone-based caspase-like activity-specific proteasome inhibitors were also developed using a combinatorial positional scanning approach (Myung *et al.*, 2001b). Initial efforts involved peptidyl α′,β′-epoxyketones with glutamic acid at the P1 position. It was reasoned that the presence of Glu at the P1 position of peptide inhibitors alone would suffice to render higher selectivity toward the caspase-like activity. However, α′,β′-epoxyketone peptide inhibitors with Glu at the P1 position did not display any significant selectivity for the caspase-like activity (Myung *et al.*, 2001b). To screen an optimum P1 amino acid residue for the caspase-like activity inhibition, α′,β′-epoxyketones with various natural and unnatural amino acid residues (i.e., Leu, Ala, Val, Phe, norleucine, cyclohexyl, and *tert*-butyl) in place of Glu at the P1 position were prepared, which were then coupled to dipeptides containing the serine at the C-terminus and isooctanoic group at the *N*-terminus. Among these, α′,β′-epoxyketone tetrapeptide inhibitors with Leu at the P1 position provided the best caspase-like selectivity. In this regard, incorporation of leucine at the P1 position in an N-terminus protected peptidyl aldehyde proteasome inhibitor (i.e., Z-GPFL-CHO) has previously been shown to inhibit competitively the CASPASE-LIKE activity with a ∼13-fold selectivity over the CT-L activity (Vinitsky *et al.*, 1994).

Interestingly, α′,β′-epoxyketone inhibitors with Pro-Phe-Leu at the P3-P1 positions display no significant inhibition for the T-L activity, regardless of the nature of residues at the P4 position. Expanding on this finding, therefore, it was decided to derivatize the N-terminus of Pro-Phe-Leu-α′,β′-epoxyketone inhibitors to explore the importance of length, steric bulk, and protecting group at the P4 position for the caspase-like activity inhibition. The results from these studies showed that the presence of a bulky aromatic protecting group in place of an acetyl group at the amino terminus provides a rather stronger inhibition toward the CT-L activity, thus making them less caspase-like selective. On the basis of these findings, several α′,β′-epoxyketone peptides possessing a smaller amino-terminal

group (i.e., acetyl) instead of a bigger group (i.e., benzyloxycarbonyl group) were prepared and tested for their inhibitory activity.

To this end, one compound (YU102, Fig. 4) showed the highest selectivity toward the caspase-like activity with ∼50-fold higher values of $k_{obs}/[I]$ for inhibition of the caspase-like activity than the CT-L activity (Myung *et al.*, 2001b); 8 μM YU102 was found to inhibit only the caspase-like activity but not the CT-L activity at 8 μM concentration. Up to 90% of the caspase-like activity was found to be inhibited under the assay conditions. Against T-L activity, YU102 is a very poor inhibitor; even at concentrations of 100–150 μM, it displayed no significant inhibition of the T-L activity. Moreover, YU102 showed a time-dependent inhibition, indicating the irreversible modification of the catalytic Thr-1 of the proteasome. Therefore, a greater than 90% inhibition of the caspase-like activity under these conditions suggests that the caspase-like subunits were at least 90% saturated irreversibly with YU102. The CT-L activity of the 20S proteasome, however, was not affected. This results show that near quantitative occupancy of the caspase-like sites with YU102 did not trigger inhibition off the CT-L activity. YU102 was applied to probe the role of different catalytic subunits in cell-based protein degradation assays in living cells (Myung *et al.*, 2001b), revealing that selective caspase-like activity inhibition is not sufficient to inhibit total protein degradation.

Enzyme Kinetic Assays

Purification of 20S Proteasome

20S proteasome was purified from bovine reticulocyte lysates by batch DE-52 binding, DEAE Sephacel chromatography, gel filtration on Sephacryl S-300, and chromatography on hydroxyapatite. This procedure is as previously described (Elofsson *et al.*, 1999; Myung *et al.*, 2001b), with the exception that bovine reticulocytes were used as a starting source.

To evaluate the rates of proteolytic inactivation by epoxomicin and YU101, $K_{association}(K_{obs}/[I])$, values were determined by use of fluorogenic peptide substrates over a range of inhibitor concentrations.

Epoxomicin and YU101 inhibition of the chymotrypsin-like catalytic activity of the 20S proteasome complex was determined as follows: Inhibitors solubilized in DMSO were mixed with a final concentration of 5 μM of the fluorogenic peptide substrate Suc-LLVY-AMC and assay buffer (20 mM Tris·HCl, pH 8.0, 0.5 mM EDTA/0.035% SDS) in a 96-well plate. Inhibitors concentrations were adjusted so that the final DMSO concentration would not exceed 1%. Hydrolysis was initiated by the addition of bovine red blood cell 20S proteasome to a final volume of 100 μl/well,

and the reaction was followed by fluorescence (360 nm excitation/460 nm detection) using a multilable plate-reader Wallac Victor[2], set at 25°. Reactions were allowed to proceed for 50 min, and fluorescence data were collected every 10 sec. Fluorescence was quantified as arbitrary units, and progression curves were plotted for each reaction as a function of time. $K_{obs}/[I]$ values were obtained using KALEIDOGRAPH software by non-linear least-squares fit of the data to the following equation: fluorescence $= \nu_S t + [(\nu_O - \nu_S)/K_{obs}][1 - \exp(-K_{obs}t)]$, where ν_O and ν_S are the initial and final velocities respectively, and K_{obs} is the reaction rate constant. $K_{association} = K_{obs}/[I] \ (M^{-1}s^{-1})$.

The range of inhibitors final concentrations tested were 40–100 nM for epoxomicin and 5–12 nM for YU101. Bovine erythrocyte 20S proteasome (2.5 mg/ml) was diluted 1:500.

YU101 most potently inhibits the chymotrypsin-like activity of the 20S proteasome with a $K_{association} = 310,000 \pm 19,000 \ (M^{-1}s^{-1})$, CV = 60%. The chymotrypsin-like inhibitory activity of epoxomicin was $K_{association} = 20,000 \pm 4,600 \ (M^{-1}s^{-1})$, CV = 24%.

For YU102, peptide-AMC (10 μM Suc-LLVY-AMC, 10 μM Z-LLE-AMC, or 20 μM Boc LRR-AMC) and 20S proteasome were added to assay buffer (20 mM Tris·HCl, pH 8.0, and 0.5 mM EDTA). For Suc-LLVY and Z-LLE-AMC assays, 0.035% (w/v) SDS was added to the assay buffer. After the steady state of hydrolysis for each substrate was established, an inhibitor was added to the assay buffer containing substrate and enzyme in a Dynex™ 96-well plate at room temperature. Release of fluorescent 7-amino-4-methylcoumarin (AMC) was measured using a Cytofluor spectrofluorometer with an excitation wavelength of 360 nm, and kinetic data were processed as described previously.

Summary

Given the complex proteolytic activities associated with the proteasome and poorly understood biological role of each catalytic subunit in many important signaling pathways, there are unmet needs for more sophisticated, selective proteasome inhibitors to dissect proteasome function. Here we describe strategies for developing α',β'-epoxyketone peptide-based proteasome inhibitors: (1) a general approach for the synthesis of α',β'-epoxyketone peptides that was developed through the total synthesis of epoxomicin; (2) SAR studies on epoxomicin/dihydroeponemycin, potentially shedding light on a means to design catalytic subunit- or immuno-proteasome-specific α',β'-epoxyketone peptides; (3) development of highly potent, CT-L activity-specific α',β'-epoxyketone peptides; and (4) development of caspase-like activity-specific α',β'-epoxyketone inhibitors. Fortunately, all of the reactions for the synthesis of α',β'-epoxyketone peptides

can readily be carried out in solutions (CH_2Cl_2 or DMF solvents) with good to excellent yields and easily repeatable. In conclusion, our studies have shown that derivatization of α',β'-epoxyketone peptide proteasome inhibitors at positions P1–P4 can be easily accomplished to provide novel proteasome-specific, subunit-selective small molecule inhibitors.

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[40]   Screening for Selective Small Molecule Inhibitors of the Proteasome Using Activity-Based Probes

By MATTHEW BOGYO

Abstract

The proteasome's role in fundamental biological processes ranging from control of the cell cycle to production of peptides for display to immune cells has been uncovered with the help of small molecule inhibitors. Most of the commonly used inhibitors have been designed and synthesized by organic chemists or by Nature. To continue to develop new inhibitors and reagents for the proteasome, a rapid screening method is required that allows not only assessment of potency but also selectivity of inhibitors for each of the primary catalytic sites in the complex. This chapter outlines methods for the solid-phase synthesis of diverse peptide