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# TOTAL SYNTHESIS OF THE POTENT PROTEASOME INHIBITOR EPOXOMICIN: A USEFUL TOOL FOR UNDERSTANDING PROTEASOME BIOLOGY

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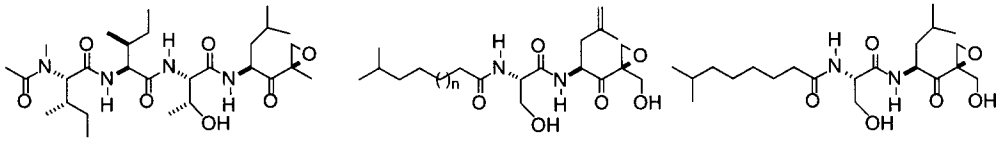
**Abstract:** Epoxomicin (**1**), a peptide α',β'-epoxyketone isolated from the actinomycete strain No.Q996-17, possesses potent *in vivo* anti-tumor and anti-inflammatory activities. In this paper, we report the first syntheses of epoxomicin, [$^3$H]-epoxomicin, and a biotinylated epoxomicin analog as well as the absolute configuration of the epoxide stereocenter. The natural product and derivatives have permitted the first identification of the proteasome as the specific cellular target of epoxomicin. 

Numerous biologically active natural products that mediate their activities via inhibition of specific intracellular protein targets have been isolated. Several of these compounds have been directly employed as pharmaceuticals (e.g., the antitumor compounds paclitaxol, Taxol®, and vincristine, Oncovin®). Recently, however, natural products are increasingly serving two additional purposes in the identification of novel proteins for therapeutic intervention, namely 'target validation' and in the exploration of cell biology ('chemical genetics'). Both applications involve the investigation of a natural product's mode of action using a variety of chemical and biochemical techniques.[1] This strategy has proven useful with a number of biologically active natural products such as immunosuppressants[2,3] and anti-angiogenic agents.[4,5]

Despite recent interest in novel cellular targets for anti-tumor chemotherapy, many anti-tumor natural products have been identified for which no mode of action is known. One example is the microbial metabolite epoxomicin, which was isolated based on its potent *in vivo* antitumor activity against solid tumors derived from B16 melanoma.[6] Epoxomicin belongs to a small family of linear peptides, all of which share two structural features: a threonine or serine residue and an α',β'-epoxyketone derived from leucine or γ,δ-dehydroleucine. Other members of this family include the antitumor agent eponemycin (**2**)[7] and epopromycins A (**3**) and B (**4**), which were identified based on their ability to inhibit plant cell wall synthesis.[8] These α',β'-epoxyketone containing peptides most likely exert their biological activities via adduct formation with intracellular target proteins since reduction of the reactive epoxide in eponemycin results in loss of biological activity.[7]



Epoxomicin (**1**)

n = 1 Eponemycin (**2**)
n = 2 Epopromycin A (**3**)

Epopromycin B (**4**)

**Figure 1.** The epoxomicin family of peptide α',β'-epoxyketone natural products.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Following the publication of epoxomicin's structure and anti-tumor activity, Spaltenstein and coworkers demonstrated that a tripeptide α',β'-epoxyketone potently inhibited the chymotrypsin-like activity of the proteasome,[9] a cytoplasmic high molecular weight (ca.700 kDa) multiple protein complex. The proteasome has several proteolytic activities defined by different substrate specificities and is responsible for the degradation of most intracellular proteins. Therefore, it plays an important role in a number of cellular processes such as cell growth regulation, antigen processing, and the transduction of pro-inflammatory signals.[10] Despite the growing evidence of the proteasome's role in these processes, the complexity of the proteasome has complicated efforts to understand at the molecular level the determinants underlying the proteasome's different proteolytic specificities.

Given the structural similarity to epoxomicin, the Spaltenstein findings suggested that epoxomicin could also be a member of the growing class of small molecules targeting the proteasome. Additional compounds that have been reported to inactivate the proteasome include the natural product lactacystin[11] and short peptides with aldehyde, vinyl sulfone, boronic acid and glyoxal functional groups.[12] Many of these compounds share with epoxomicin the ability to induce a spindle-like cell morphology and cellular apoptosis.[12] However, as molecular probes of proteasome biology, many of these compounds lack the specificity, stability, or potency necessary to explore the roles of the proteasome at the cellular and molecular level.

Given the similarities in structure and in cellular effects between epoxomicin and known proteasome inhibitors, we have aimed to identify and characterize epoxomicin's molecular target. The strategy employed is based on the use of affinity chromatographic techniques. We report herein the first total syntheses of epoxomicin, [$^3$H]-epoxomicin, and a biotinylated epoxomicin analog, in which biotin is coupled to the amino terminus of epoxomicin. We have used these reagents to identify and characterize four biological targets of epoxomicin, the proteasomal catalytic subunits, X, LMP-7, MECL1, and Z. These reagents have allowed us to show that epoxomicin potently and irreversibly inhibits the chymotrypsin-like (CT-L), trypsin-like (T-L) and peptidylglutamyl peptide hydrolyzing (PGPH) activities of the proteasome as well as show that epoxomicin has potent anti-inflammatory activity *in vivo*.[13] Moreover, we demonstrate here that epoxomicin readily lends itself to derivativization via solid-phase synthesis suggesting that additional proteasome inhibitors with novel specificities could be easily generated.

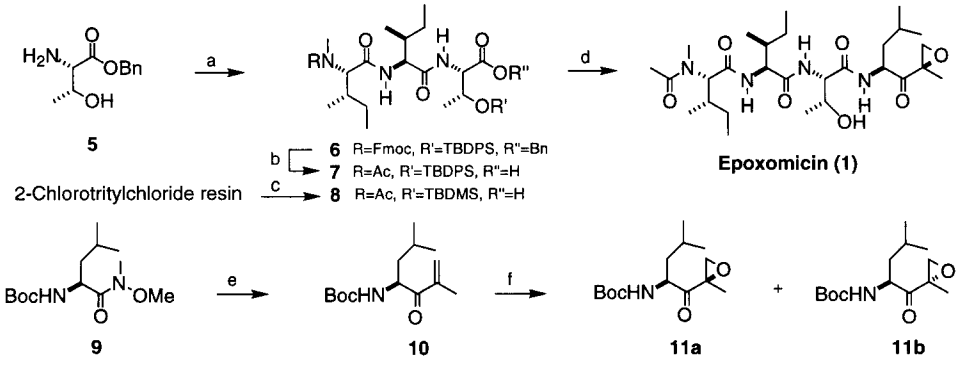
## Results and Discussion

**Total Synthesis of Epoxomicin.** As a first step in the elucidation of epoxomicin's mode of action, we developed a synthetic strategy for the generation of the natural product and its derivatives. A few total syntheses of the related α',β'-epoxyketone containing natural product eponemycin[14,15] and it derivative dihydroeponemycin[16,17] have been reported. In the Schmidt syntheses, epoxidation was performed after complete assembly of the peptide backbone and the resulting eponemycin diastereomers were separated by HPLC.[14,15] Our synthesis[17] and the synthesis of Hoshi et al.,[16] on the other hand, relied on the preparation and isolation of stereochemically defined α',β'-epoxy leucine right-hand fragments, which were coupled to protected *N*-isooctanoyl-serine left-hand fragments to yield the complete backbone of the target compound. A similar convergent approach was applied in this study to the total syntheses of epoxomicin **1**, biotinylated epoxomicin **13**, and [$^3$H]-epoxomicin **14**.

In the first step of the left-hand fragment synthesis (Scheme 1), fluoren-9-ylmethoxycarbonyl-isoleucine (Fmoc-Ile-OH) was coupled to threonine benzyl ester **5** with *O*-benzotriazo-1-yl-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt). The threonine hydroxyl group of the resulting

dipeptide was protected with *t*-butyldiphenylsilylchloride (TBDPSCl). Removal of the Fmoc group followed by coupling of Fmoc-*N*-methyl-isoleucine with HBTU/HOBt gave the fully protected left-hand fragment **6**. Removal of the Fmoc group and subsequent acetylation followed by catalytic hydrogenolysis gave the target left-hand fragment **7**. Synthesis of the right-hand fragment (Scheme 1) was initiated with the addition of propen-2-yl lithium to the Boc-leucine Weinreb amide **9**[18] which resulted in formation of the α',β'-unsaturated ketone **10**.

**Scheme 1**

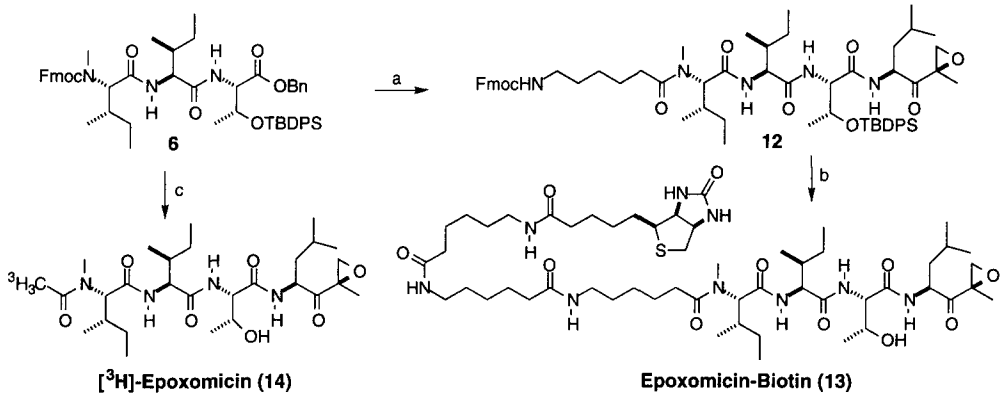



*Reagents and conditions*: (a) i. Fmoc-Ile-OH, HBTU, HOBt, *i*-$Pr_2EtN$, $CH_2Cl_2$, rt, 23 h, 79%; ii. TBDPSCl, Imidazole, THF, rt, 48 h, 64%; iii. Piperidine, DMF, rt, 20 min; iv. Fmoc-MeIle-OH, HBTU, HOBt, *i*-$Pr_2EtN$, $CH_2Cl_2$, rt, 18 h, 99%; (b) i. Piperidine, DMF, rt, 20 min; ii. Ac2O, *i*-$Pr_2EtN$, $CH_2Cl_2$, rt, 3.5 h, 99%; iii. 10% Pd-C, $H_2$ (g), MeOH, rt, 1.5 h, 88%; (c) i. standard Fmoc solid-phase peptide synthesis; ii. Acetic acid:trifluoroethanol:dichloromethane, 1:1:3, rt, 2 h, 55% based on initial resin capacity; (d) i. TFA.**11a**, HATU, HOAt, *i*-$Pr_2EtN$, $CH_2Cl_2$, rt, 18 h, 48%; ii. TBAF, THF, rt, 1h, 96%; (e) 2-Bromopropene, *t*-BuLi, $Et_2O$, –78 °C, 2.5 h, 92%; (f) $H_2O_2$, $H_2O$, Benzonitrile, *i*-$Pr_2EtN$, MeOH, 0–4 °C, 43 h, 76%, **11a:11b** 1.7:1.

Subsequent epoxidation with alkaline hydrogen peroxide furnished a mixture of the epoxides **11a** and **b** (1.7:1), which were readily separated by column chromatography. Both epoxides **11a** and **11b** were brought forward to give epoxomicin and its epoxide epimer. It was concluded that epoxide **11a** gave a final product identical to epoxomicin. The absolute stereochemistry of the epoxide ring in **11a** and **11b** was determined chemically according to the method previously described by Spaltenstein.[9] Compound **11a** was shown to have the same stereochemistry as eponemycin.[16] The final efforts towards epoxomicin started with the removal of the Boc group of **11a** by brief treatment with neat trifluoroacetic acid (TFA) without concomitant opening of the epoxide. The left-hand fragment **7** was coupled to the deprotected TFA salt of **11a** with *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)[19] and 1-hydroxy-7-azabenzotriazole (HOAt)[20] to give TBDPS protected epoxomicin. Treatment of the TBDPS protected epoxomicin with tetrabutylammonium fluoride (TBAF) gave epoxomicin **1**. Spectroscopic data and optical rotation for the synthetic material were identical to those reported for epoxomicin **1**.[6] In addition, the TBDMS-protected left hand fragment **8** was prepared on solid-phase by the treatment of a commercially available 2-chlorotrityl chloride resin with Fmoc-Thr(*O*-TBDMS)-OH and diisopropylethylamine in $CH_2Cl_2$ followed by standard solid-phase peptide synthesis.[22,23] The solid-phase syntheses of left hand fragments will facilitate the library generation of epoxomicin-based proteasome inhibitors possessing potentially novel inhibitory specificities.

**Synthesis of Biotinylated Epoxomicin.** Biotinylated derivatives of the epoxide containing natural products fumagillin and eponemycin have proven useful in the identification of their intracellular targets.[4,5] Considering the structural similarity between eponemycin **2** and epoxomicin **1** we decided to attach a biotin handle to the amino-terminus of epoxomicin (Scheme 2) using a similar strategy.[17] The fully protected left-hand fragment **6** was treated with piperidine and the resulting amine was acylated with *N*-Fmoc-6-aminohexanoic acid using bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBrOP),[21] giving the fully protected left-hand benzyl ester linker derivative. Hydrogenolysis of the benzyl ester followed by coupling to the right-hand fragment with HATU/HOAt furnished **12**. Simultaneous removal of the Fmoc and TBDPS groups and subsequent *N*-acylation with commercially available *N*-(*N*-biotinyl-6-aminohexanoyl)-6-aminohexanoic acid succinimidyl ester (Biotin-X-X-NHS) gave biotinylated epoxomicin **13**.

**Scheme 2**




*Reagents and conditions*: (a) i. Piperidine, THF, rt, 26 h, 91%; ii. *N*-Fmoc-6-aminohexanoic acid, PyBrOP, *i*-$Pr_2$EtN, $CH_2Cl_2$, rt, 2 h, 98%; iii. 10% Pd-C, $H_2$ (g), MeOH, rt, 2 h, 99%; iv. TFA.**11a**, HATU, HOAt, *i*-$Pr_2$EtN, $CH_2Cl_2$, rt, 22 h, 60%; (b) i. TBAF, THF, rt, 1 h; ii. Biotin-X-X-NHS, DMSO, rt, 24 h, 87%. (c) i. 10% Pd-C, $H_2$ (g), EtOAc, rt, 2 h, 98%; ii. TFA.**11a**, HATU, HOAt, *i*-$Pr_2$EtN, $CH_2Cl_2$, rt, 12 h, 43%; iii. Piperidine, DMF, rt, 20 min; iv. [$^3$H]-$Ac_2O$, *i*-$Pr_2$EtN, $CH_2Cl_2$, rt, 8 h; v. TBAF, THF, rt, 20 min, 56% over three steps.

**Isolation of Covalently Bound Epoxomicin Receptor.** Given our goal of understanding the mode of action of epoxomicin through the isolation and identification of putative binding proteins, the synthesis of an affinity reagent afforded the direct analysis of such proteins. Due to their structural similarities, we hypothesized that epoxomicin **1** and eponemycin **2** bind similar protein receptors. Our previous results using a biotinylated dihydroeponemycin derivative have shown that it binds specifically and covalently to two intracellular receptors with molecular weights of 22 kDa and 23 kDa,[17] which we have subsequently identified as the 20S proteasomal catalytic subunits LMP2 and LMP7, respectively.[5] Biotinylated epoxomicin **13** proved critical in demonstrating epoxomicin also targets the proteasome, albeit different catalytic subunits. Various concentrations of epoxomicin-biotin **13** were added to different sets of murine thymoma EL4 cells for 6 h.[24] Cell lysates were analyzed by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by protein immobilization on PVDF membrane and visualization of biotinylated proteins via avidin-horseradish peroxidase enhanced chemiluminescence. Since SDS denatures proteins, the presence of biotinylated proteins after addition of biotinylated epoxomicin **13** and SDS-PAGE indicates the presence of covalent protein adduct formation. As

shown in Figure 2, addition of increasing concentrations of **13** resulted in newly biotinylated proteins of 23 kDa and 30 kDa in size. In addition to these two major epoxomicin-binding proteins, a minor 28 kDa protein band was also observed. MALDI-MS analyses of peptides generated by trypsin digestion of 23, 30, and 28 kDa epoxomicin-binding proteins identified them as LMP7, Z and MECL1 catalytic subunits, respectively.[13] Since EL4 cells constitutively express the γ-interferon-inducible subunit LMP7, which comigrates with its reciprocal subunit X on SDS-PAGE, cells lacking LMP7 were tested for epoxomicin-biotin binding. In these cells, epoxomicin-biotin specifically labelled the Z subunit and a 23 kDa protein, which was identified by immunoblot analysis as the subunit X.[13] Adduct formation between epoxomicin-biotin and these binding proteins most likely occurs via nucleophilic attack on the α',β'-epoxyketone of epoxomicin by the proteasome subunits' catalytic amino-terminal threonine. This has been previously shown for lactacystin binding.[11] Incubation with a ten equivalent excess of epoxomicin **1** with cells for 30 min prior to challenge with epoxomicin-biotin **13** resulted in a significant decrease in biotin incorporation into all three protein targets (lane 5). This competition for epoxomicin-biotin binding confirms that the interaction is specific. Since the linker and biotin may artefactually influence epoxomicin binding to particular proteasome subunits, [$^3$H] epoxomicin (**14**) was synthesized (Scheme 2) and tested for binding to murine spleen 20S proteasome. The autoradiograph of [$^3$H] epoxomicin (**14**) binding proteins (lane 6) is identical to the binding pattern seen with biotin-epoxomicin, thus validating biotin-epoxomicin as an affinity reagent.

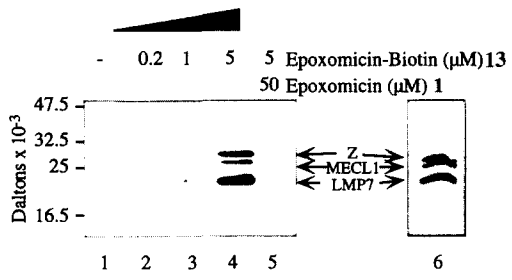



**Figure 2.** Epoxomicin targets the proteasome catalytic subunits.

Given the ability of many other proteasome inhibitors (i.e., peptide aldehydes, vinyl sulfones) to inhibit other proteases as well,[12] we tested epoxomicin for inhibition of several proteases. Enzyme kinetic assays (Table 1) show that epoxomicin potently inhibited the chymotrypsin-like activity of the 20S proteasome but did not inhibit papain, chymotrypsin, trypsin, cathepsin B or calpain at concentrations up to 50 μM. Interestingly, Roush and colleagues have recently shown that α',β'-epoxyketone containing peptides potently inhibit Cruzain, the cysteine protease isolated from *Trypanosoma cruzi*.[25] However, the stereochemistry of these α',β'-epoxyketone containing peptides differs from the natural products epoxomicin (**1**), eponemycin (**2**), and the previously reported α',β'-epoxyketone proteasome inhibitor described by Spaltenstein, et al.[9] Given the significantly lower rate of proteasome inhibition by 2-*epi*-epoxomicin (Table 1), the stereochemistry of C-2 of epoxomicin plays an important role in its inhibitory specificity. Additional studies regarding the mechanism of inhibition specificity are ongoing.

**Table 1.** Enzymatic Inhibitory Profile of Epoxomicin and 2-*epi*-epoxomicin.[a]

| Enzyme | Compound | $k_{obs}/[I]$ ($M^{-1}s^{-1}$) |
|---|---|---|
| 20S proteasome | Epoxomicin (**1**) | 37,200 |
| 20S proteasome | 2-*epi*- Epoxomicin | 246 |
| Papain | (**1**) | — |
| Chymotrypsin | (**1**) | — |
| Trypsin | (**1**) | — |
| Cathepsin B | (**1**) | — |
| Calpain | (**1**) | — |

[a]Kinetic assays and analyses were performed as previously described.[13]

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24. Murine EL4 cells were grown in RPMI Medium (Gibco), 10% Fetal Bovine Serum and 50 μg/L each of penicillin and streptomycin at 37 °C in a 5% $CO_2$ incubator. Different concentrations of **13** were added to 5 mL of cells ($10^6$ cells/mL) for 6 h. Control cells were pretreated with 50 μM of epoxomicin for 1 h before addition of 5 μM of **13**. Cells were lysed in sodium dodecyl sulfate (SDS) containing sample buffer (2% SDS, 10% glycerol, 0.02% bromophenol blue), analyzed by 12% SDS-PAGE, and transferred to polyvinylidene fluoride (PVDF) membrane. Biotinylated proteins were visualized by enhanced chemiluminescence (ECL) using streptavidin-horseradish peroxidase and Biomax X-ray film (Kodak). Purified murine spleen proteasome was incubated with [$^3$H]-epoxomicin **14** for 15 min at rt before SDS-PAGE and transfer to PVDF membrane, which was exposed to Kodak Biomax X-ray film for 21 days.
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