Towards Immunoproteasome-Specific Inhibitors: An Improved Synthesis of Dihydroeponemycin

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Eponemycin, an antitumor and antiangiogenic epoxy ketone natural product, is previously shown to target proteasome for its activity. Although there have been many synthetic approaches developed, practical and efficient synthetic strategy for eponemycin has yet to be accomplished. Here, we report an efficient new route for the preparation of dihydroeponemycin, an active eponemycin derivative. This will aid the design of proteasome inhibitors with novel activity.

Introduction

Intracellular protein degradation is a highly regulated process in which proteins are first targeted for degradation by conjugation to ubiquitin, a 76 amino acid polypeptide. Ubiquitinated proteins are then recognized by the 19S regulatory domain of the 26S proteasome. Through a series of ATP hydrolysis-dependent processes, targeted proteins are deubiquitinated and threaded into the core proteolytic complex, the 20S proteasome, where they are degraded into small peptides. Interestingly, exposure of cells to stimuli, such as interferon (IFN)-γ, tumor necrosis factor (TNF)-γ and lipopolysaccharide (LPS), induces the synthesis of certain catalytic subunits (LMP2, MECL-1, and LMP7) that together are incorporated into alternative proteasome form, known as the immunoproteasome.[1]

The immunoproteasome, as compared to the constitutive proteasome, 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.[1,2] Consequently, the spectrum of the produced peptides is shifted towards peptides which associate with MHC class I molecules with increased affinity,[3] implicating a major role in antigen presentation. Furthermore, it has been suggested that the immunoproteasome may be involved in some pathological processes, such as diabetes and autoimmune diseases.[4] However, the exact role of the immunoproteasome is not clearly defined at this time, due in large part to the lack of appropriate molecular probes. As a result, it is yet to be determined whether immunoproteasome-specific inhibitors have potential as a pharmaceutical target. It should be noted that the proteasome inhibitor Velcade™ (bortezomib) has recently been approved for multiple myeloma that has not responded to other treatments. However, currently available proteasome inhibitors including Velcade™ randomly target both the constitutive proteasome and the immunoproteasome. Thus, development of immunoproteasome-specific inhibitors may be useful to investigate the role of immunoproteasome and to determine whether immunoproteasome is a potential target for development of pharmaceutical agents.

Antitumor natural products epoxomicin (**1**) and eponemycin (**2**) (Figure 1) are members of linear peptides containing α′,β′-epoxy ketone pharmacophore[5,6] and have been shown to exert their anticancer activity through proteasome inhibition.[7–10] Of particular interest was the finding that, despite structural similarities, epoxomicin (**1**) and dihydroeponemycin (**3**), an active derivative of eponemycin, differ in their proteasome subunit binding specificity.[8,10]

Moreover, unlike other classes of proteasome inhibitors that show non-target specificity, epoxy ketone proteasome inhibitor is shown to be highly specific for the 20S proteasome. The crystal structure of the yeast 20S proteasome complexed with epoxomicin revealed that the unique specificity of epoxy ketone pharmacophore is contributed 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′-epoxy ketone pharmacophore of epoxomicin (Figure 2).[11]

In addition, it has been shown that dihydroeponemycin (**3**) targets the subunits of both constitutive proteasome and immunoproteasome, whereas epoxomicin (**1**) preferentially labels the catalytic subunits of the constitutive proteasome.[8,10] Recent studies indicated that the ability of dihydroeponemycin to bind immunoproteasome subunits is attributed to the P3 isooctanoic moiety of dihydroeponemycin but not the hydroxy groups in the P2 and P1′ positions (see Figure 1).[12] Therefore, isooctanoic-based dihydroeponemycin analogue **4** may provide an opportunity for the development of immunoproteasome-specific inhibitors.

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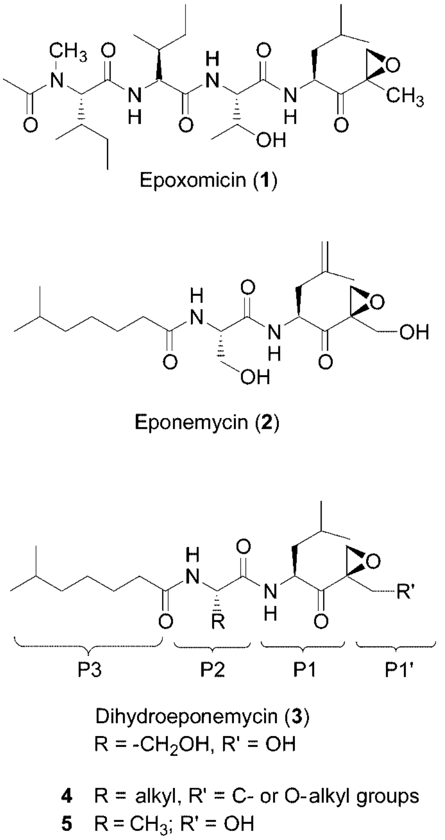


Figure 1. α′,β′-Epoxy ketone linear peptide natural products, epoxomicin and eponemycin, and eponemycin analogues.

However, a simple and practical approach for the synthesis of dihydroeponemycin has yet to be developed. Particularly, the lack of the efficient synthetic approach for the hydroxymethyl-substituted enone motif has been a major obstacle for efficient synthesis of dihydroeponemycin and their P1′ derivatives **4**. Of note, careful analysis of proteasome subunit labeling by inhibitors strongly indicate that P1′ site may be an important determinant for selective immunoproteasome subunit binding.[13]

Results and Discussion

Herein, we report a new and improved route to the synthesis of the hydroxymethyl-substituted enone and dihydroeponemycin (**3**). In addition, we show that the hydroxy group, commonly present in both eponemycin and epoxomicin at the P2 position, is not required for immunoproteasome binding.

Over the years, a number of elegant synthetic strategies for the synthesis of eponemycin and dihydroeponemycin have been developed. A key step in the synthesis is the preparation of hydroxymethyl-substituted enone **10**. In several earlier approaches, the enone **10** was prepared from the reaction of dilithio reagent **8** with the corresponding aldehyde (Scheme 1).[7,12,14–16] However, low yields and extra steps involving protection, oxidation and deprotection of OH groups prevented large scale preparation. In similar approaches, the Weinreb-type amide derivatives treated with dilithio reagent **8** did not yield the desired hydroxymethyl-substituted enone **10**.[17] More recently, new synthetic approaches have been developed based on the cinchona alkaloid-catalyzed Baylis–Hillman-type reactions that yield the intermediate **7**[18] or Stille coupling of Fmoc-Leu-Cl with *n*-tributylvinyltin followed by modified Baylis–Hillman reaction (Scheme 1).[17] However, multiple steps and low yields associated with these approaches may not be ideal for efficient derivatization or construction of small library of dihydroeponemycin analogues for screening immunoproteasome-specific inhibitors.

As an alternative approach, we set out to develop an efficient, practical synthetic strategy for the synthesis of hydroxymethyl-substituted enone and dihydroeponemycin. First, we started out with readily available Boc–Leu–OMe, which was prepared from the reaction of Boc–Leu–OH with iodomethane in DMF. The reaction of Boc–Leu–OMe with dimethyl methylphosphonate treated with *tert*-butyllithium yielded compound **13**. Finally, the combination of Wittig–Horner- and Baylis–Hillman-type one-pot reactions yielded the hydroxymethyl-substituted enone **14** in high yield (Scheme 2). The resulting hydroxymethyl-substituted enone

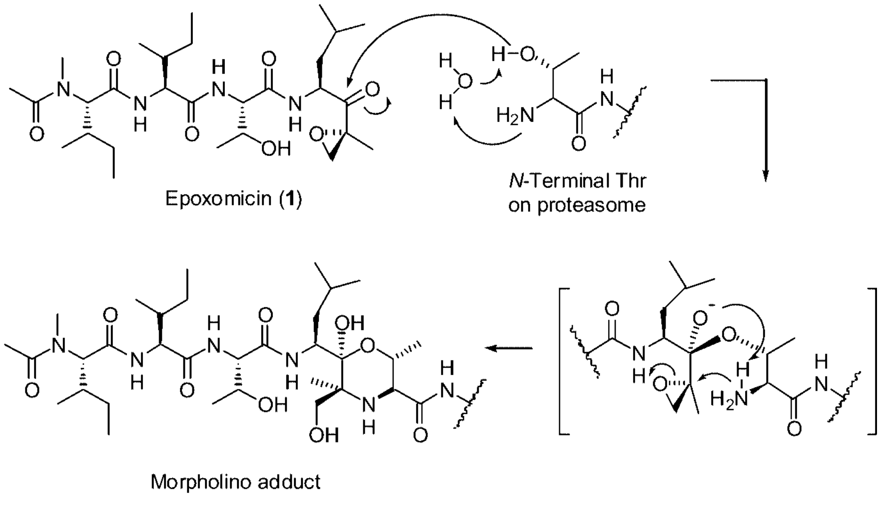
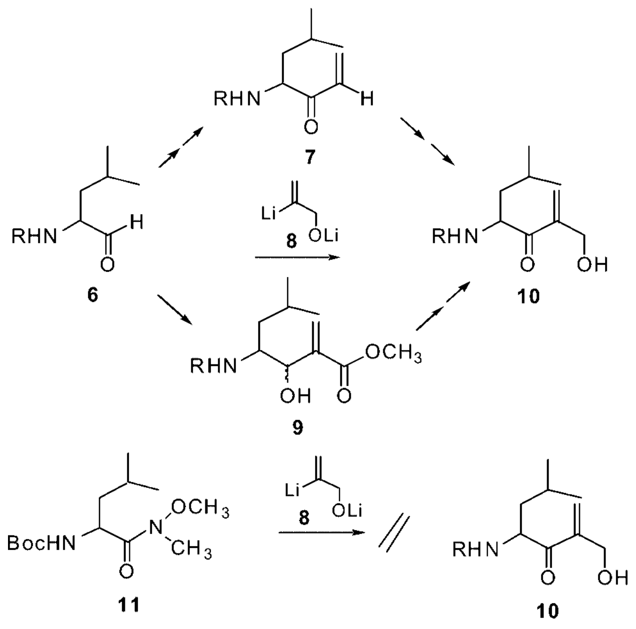


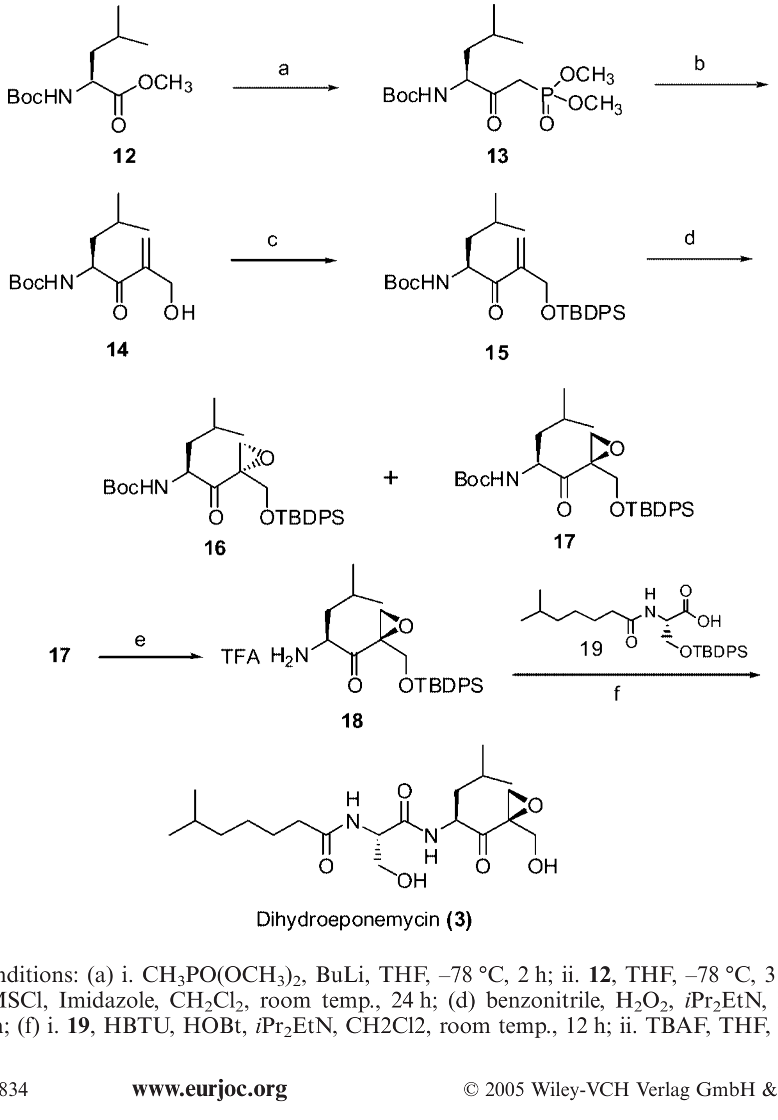
Figure 2. Proposed mechanism of proteasome inhibition by an epoxy ketone natural product epoxomicin.



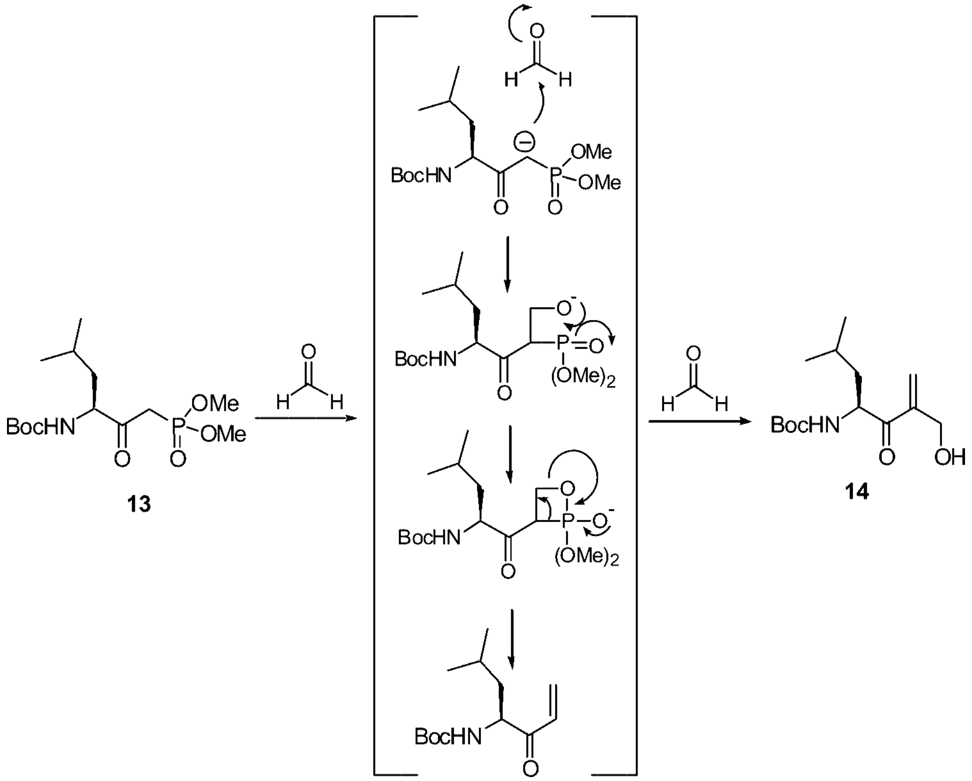
Scheme 1. Synthetic strategies for the preparation of α-hydroxymethyl-substituted enone.

was treated with *tert*-butyldimethylsilyl chloride (TBDMSCl) to yield compound **15**. Epoxidation of **15** with hydrogen peroxide[16] afforded two epoxy ketone isomers **16** and **17** as a 1:1.5 mixture, which were readily separated by flash column chromatography using an elution system (hexanes/ethyl acetate, 10:1, v/v). The isomer (2*R*)-epoxide **17**, which migrates faster than the (2*S*)-epoxide **16** in thin-layer chromatography (TLC), was found to have the same configuration as that of eponemycin epoxide.[7] Although epoxidation could occur at a higher stereoselectivity employing the Sharpless epoxidation,[19] it is of great interest to use both isomers for biological testing in the near future. The final coupling reaction between epoxy ketone **18** and dipeptide **19**[7,12] was performed with *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), followed by removal of the *tert*-butyldiphenylsilyl (TBDPS) and TBDMS groups and normal phase HPLC (hexanes/2-propanol, linear gradient, hexanes 100% to 50%) to yield dihydroeponemycin (**3**).

As compared to previously reported synthetic strategies, the combination of Wittig–Horner- and Baylis–Hillman-type two-step "one-pot" reaction presented here is efficient



Scheme 2. Reagents and conditions: (a) i. $CH_3PO(OCH_3)_2$, BuLi, THF, –78 °C, 2 h; ii. **12**, THF, –78 °C, 3 h; (b) CH_2O, K_2CO_3, H_2O, room temp., 4 h; (c) TBDMSCl, Imidazole, CH_2Cl_2, room temp., 24 h; (d) benzonitrile, H_2O_2, *i*Pr_2EtN, MeOH, 0 °C, 3 h; (e) TFA, CH_2Cl_2, room temp., 15 min; (f) i. **19**, HBTU, HOBt, *i*Pr_2EtN, CH2Cl2, room temp., 12 h; ii. TBAF, THF, room temp., 10 min.



Scheme 3. Mechanistic consideration for the one-pot reaction that may occur sequentially through the Wittig–Horner- and Baylis–Hillman-type reactions.

and practical for a large quantity preparation, easily providing the key intermediate, hydroxymethyl-substituted enone, in a multi-gram scale. The one-pot reaction may be rationalized by the following mechanistic consideration (Scheme 3).

Next, we wanted to determine whether or not the OH group of the serine residue that is commonly present in both epoxomicin and eponemycin plays a role in proteasome inhibition and also whether or not it is important for preferential immunoproteasome binding by dihydroeponemycin. To further investigate this, we have prepared a dihydroeponemycin derivative, in which the serine residue at the P2 site was replaced with alanine. To test its ability to label catalytic subunits of both the constitutive- and immunoproteasome, murine thymoma EL4 cells were first treated with biotinylated dihydroeponemycin.[12] Increasing concentration of either dihydroeponemycin analogue **5** or dihydroeponemycin (**3**) was co-treated in molar excess of 0, 1, 5 and 10. The cells were then incubated at room temperature for 30 minutes. Cell lysates were analyzed by denaturing gel electrophoresis (with sodium dodecyl sulfate polyacrylamide, SDS-PAGE) followed by protein immobilization on PVDF membrane and detection of biotinylated proteins by avidin-horseradish peroxidase (HRP). Since SDS denatures proteins, the presence of biotinylated proteins indicates the presence of covalent protein adduct formation.[16] As seen in Figure 3, when only biotinylated dihydroeponemycin was added, a major 23 kDa protein and a minor 25 kDa was observed. These two bands have been previously identified as LMP7/X and LMP2, in which LMP7 and LMP2 are the immunoproteasome catalytic subunits.[8,12] Not surprisingly, these protein bands were efficiently competed away by excess dihydroeponemycin. In addition, the bands were also efficiently competed away by compound **5**. Both **3** and **5** compete with biotinylated dihydroeponemycin at a similar rate. This result suggested that the serine residue of dihydroeponemycin is not crucial for the immunoproteasome catalytic subunits binding.

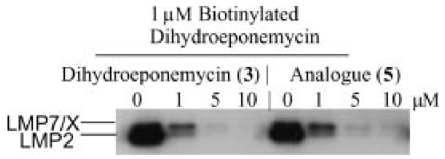


Figure 3. Competition assay to analyze the importance of serine residue at the P2 site of dihydroeponemycin in the binding of both constitutive- and immunoproteasome catalytic subunits. EL4 cells were treated concurrently with 1 μM of biotinylated dihydroeponemycin and 0, 1, 5, 10 molar excess of 3 or 5. The dihydroeponemycin analogue with alanine at the P2 site efficiently competes with biotinylated dihydroeponemycin for labeling both immuno- and constitutive proteasome subunits.

In conclusion, we have shown an efficient and practical synthesis of the antiangiogenic compound, dihydroeponemycin through a two-step "one-pot" procedure that applies Wittig–Horner-and Baylis–Hillman-type reactions. In addition, we have shown that the OH group present at the P2 site of dihydroeponemycin does not play a role in directing the preferential binding towards immunoproteasome catalytic subunits. The synthesis steps for dihydroeponemycin derivatives can be further reduced as the serine residue of dihydroeponemycin needs to be protected and the alanine residue of its analogue does not. The synthetic strategy presented here and SAR information will aid to design immunoproteasome-specific inhibitors.

Experimental Section

General Remarks: Unless otherwise stated, all reactions were carried out under nitrogen with dry freshly distilled solvents, oven-dried glassware and magnetic stirring. All solvents were reagent graded. Tetrahydrofuran (THF) was distilled from sodium/benzophenone. Methylene chloride (CH_2Cl_2) was distilled from calcium hydride. Diethyl ether anhydrous was purchased from EMD Chemicals and used without further purification. All reagents were purchased from Sigma–Aldrich and used without further purification. All reactions were monitored by thin layer chromatography (TLC) using E. Merk $60F_{254}$ pre-coated silica gel plates. Flash column chromatography was performed using E. Merk silica gel 60 (particle size 0.040–0.063 mm) and with the indicated solvents. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ using a Varian 300 MHz spectrometer at ambient temperature using an internal deuterium lock. Chemical shift are referenced to residual chloroform (δ = 7.27 ppm for ^{1}H and δ = 77.0 ppm for ^{13}C). High- and low-resolution mass spectra were carried out by the University of Kentucky Mass Spectrometry Facility.

Dimethyl (4*S*)-3-(*tert*-Butoxycarbonyl)amino-5-methylhexa-2-oxophosphonate (13): To a solution of dimethyl methylphosphonate (9 mL, 82.3 mmol) in THF (50 mL) at –78 °C, *t*BuLi (2.5 M in hexane, 33 mL, 82.3 mmol) was added dropwise. The solution was stirred at –78 °C for 2 h. A solution of Boc–Leu–OCH_3 (**12**) (5.05 g, 20.6 mmol) in THF (30 mL), was then added to the mixture at –78 °C. After stirring for 3 h, the resulting mixture was poured into water (100 mL) and extracted with diethyl ether (3 × 80 mL). The organic layers were combined, washed with brine, dried with Na_2SO_4, filtered and concentrated under reduced pressure. The product was then subjected to flash column chromatography (hexane/EtOAc, 1:1) to give a soft white solid **13** (11.5 g, 93%): ^{1}H NMR: δ = 5.22 (d, 1 H, NH), 4.34 (m, 1 H, 3-H), 3.81 [d, $^3J_{H,P}$ = 3.3 Hz, 3 H, $CH_3O(PO)OCH_3$], 3.77 [d, $^3J_{H,P}$ = 3.3 Hz, 3 H, $CH_3O(PO)OCH_3$], 3.22 (dd, $^2J_{H,P}$ = 94.8 Hz, $^2J_{H,H}$ = 13.8 Hz, 1 H, 1-H^a), 3.22 (dd, $^2J_{H,P}$ = 50.1 Hz, 1 H, 1-H^b), 1.67 (m, 2 H, 6-H, 5-H^a), 1.45 (s, 9 H, H_{Boc}), 1.38 (m, 1 H, 5-H^b), 0.96 (d, ^3J = 3.6 Hz, 3 H, CH_3CHCH_3), 0.94 (d, ^3J = 3.6 Hz, 3 H, CH_3CHCH_3) ppm. ^{13}C NMR: δ = 202.41 (C-2), 155.62 (CO_{Boc}), 76.80 (Boc), 59.09 (C-1), 53.40 (C-3), 40.14 (C-4), 39.15 [$CH_3O(PO)OCH_3$], 37.43 [$CH_3O(PO)OCH_3$], 28.62 (Boc), 25.14 (C-5), 23.59 (5-CH_3), 21.85 (C-6) ppm. HRMS (EI): *m*/*z* = 338.1709, calcd. for $C_{14}H_{27}DNO_6P$: *m*/*z* = 338.1712.

(4*S*)-4-(*tert*-Butoxycarbonyl)amino-2-hydroxymethyl-6-methylhept-1-en-3-one (14): K_2CO_3 solution (1.51 g, 10.2 mmol, in 33.3 mL H_2O) was added dropwise using a dropping funnel over a period of 15 min to a vigorously stirring solution of **13** in formaldehyde (10 mL, 360 mmol). The solution was then stirred vigorously at room temperature for 4 h. The resulting mixture was poured into water (80 mL), and the crude product was extracted with diethyl ether (3 × 50 mL). The combined organic layers were washed with brine, dried with Na_2SO_4, filtered and concentrated under reduced pressure. Flash column chromatography (hexane/EtOAc, 3:1) afforded **14** as a yellowish oil (5.15 g, 55.6%). ^{1}H NMR: δ = 6.24 (s, 1 H, 1-H^a), 6.12 (s, 1 H, 1-H^b), 5.13 (br., 1 H, NH), 5.03 (m, 1 H, 4-H), 4.34 (dd, ^2J = 15.3 Hz, ^2J = 15.6 Hz, 2 H, (2-CH_2), 2.38 (br., 1 H, OH), 1.74 (m, 1 H, 6-H), 1.50 (m, 1 H, 5-H^a), 1.43 (s, 9 H, H_{Boc}), 1.38 (m, 1 H, 5-H^b), 1.01 (d, ^3J = 6.6 Hz, 3 H, CH_3CHCH_3), 0.92 (d, ^3J = 6.6 Hz, 3 H, CH_3CHCH_3) ppm. ^{13}C NMR: δ = 201.86 (C-3), 155.69 (CO_{Boc}), 145.01 (C-2), 126.44 (C-1), 76.84 (Boc), 62.66 (2-CH_2), 53.31 (C-4), 43.06 (C-5), 28.70 (Boc), 25.37 (C-6), 23.74 (C-7), 22.08 (6-CH_3) ppm. HRMS (EI): *m*/*z* = 272.1844, calcd. for $C_{14}H_{24}DNO_4$: *m*/*z* = 272.1846.

(4*S*)-4-(*tert*-Butoxycarbonyl)amino-2-(*tert*-butyldimethylsiloxymethyl)-6-methylhept-1-en-3-one (15): To a solution of **14** (137 mg, 0.50 mmol) in CH_2Cl_2 (5 mL), imidazole (100 mg, 1.46 mmol) and *tert*-butyldimethylsilyl chloride (228 mg, 1.51 mmol) was added. After stirring at room temperature for 24 h, the resulting mixture was concentrated under reduced pressure and was subjected to flash column chromatography (hexane/EtOAc, 10:1) giving **15** (158 mg, 81%) as a yellowish oil. ^{1}H NMR: δ = 6.20 (d, ^2J = 10.2 Hz, 2 H, 1-H), 5.07 (m, 2 H, NH, 4-H), 4.36 (dd, ^2J = 30.90 Hz, ^2J = 14.70 Hz, 2 H, 2-CH_2), 1.72 (m, 1 H, 6-H), 1.48 (m, 1 H, 5-H^a), 1.42 (s, 9 H, H_{Boc}), 1.31 (m, 1 H, 5-H^b), 1.01 (d, ^3J = 6.6 Hz, 3 H, CH_3CHCH_3), 0.91 (s, 9 H, 2-*t*Bu), 0.90 (d, ^3J = 6.4 Hz, 3 H, CH_3CHCH_3), 0.07 (d, ^4J = 5.6 Hz, 6 H, 2-CH_3SiCH_3) ppm.

(2*RS*,4*S*)-4-(*tert*-Butoxycarbonyl)amino-2-(*tert*-butyldimethylsiloxymethyl)-6-methyl-1,2-oxiranylheptane (16, 17): To a solution of **15** (158 mg, 0.40 mmol) in MeOH (5 mL) at 0 °C was added benzonitrile (0.3 mL, 3.0 mmol), H_2O_2 (0.45 mL, 50% solution in H_2O, 7.8 mmol), and diisopropylethylamine (0.5 mL, 3.0 mmol). The reaction was stirred at 0 °C for 3 h. The resulting mixture was concentrated under reduced pressure and subjected to flash column chromatography to yield **16** and **17** with a ratio of 1:1.5 (107 mg, 65%). **17:** ^{1}H NMR: δ = 4.80 (br., 1 H, NH), 4.43 (d, ^2J = 11.7 Hz, 1 H, 2-CH^a_2), 4.34 (m, 1 H, 4-H), 3.57 (d, ^2J = 11.4 Hz, 1 H, 2-CH^b_2), 3.18 (d, ^2J = 4.3 Hz, 1 H, 1-H^a), 3.01 (d, ^2J = 4.3 Hz, 1 H, 1-H^b), 1.74 (m, 1 H, 6-H), 1.62 (m, 1 H, 5-H^a), 1.41 (s, 9 H, H_{Boc}), 1.07 (m, 1 H, 5-H^b), 0.99 (d, ^3J = 6.3 Hz, 3 H, CH_3CHCH_3), 0.94 (d, ^3J = 6.3 Hz, 3 H, CH_3CHCH_3), 0.87 (s, 9 H, 2-*t*Bu), 0.06 (d, ^4J = 5.2 Hz, 6 H, 2-CH_3SiCH_3) ppm. MS (ESI): *m*/*z* = 526, calcd. for $C_{30}H_{42}DNO_5Si$: *m*/*z* = 526.

Dihydroeponemycin (3): To a solution of **17** (66 mg, 0.16 mmol) in CH_2Cl_2 (1 mL), trifluoroacetic acid (100 µL, 0.87 mmol) was added. The reaction mixture was stirred at room temperature for 15 min. The resulting crude product was then concentrated under reduced pressure and subsequently dried under high vacuum to remove trifluoroacetic acid. The resulting crude product **18** (50 mg, ca. 100%) was used in the final coupling reaction without further purification. To a solution of the epoxy-β-amino ketone **18** (50 mg, 0.16 mmol) and **19**[12] (75 mg, 0.16 mmol) in CH_2Cl_2 (5 mL), HBTU (91 mg, 0.24 mmol), HOBt (37 mg, 0.24 mmol) and diisopropylethylamine (0.14 mL, 0.8 mmol) was added. The reaction solution was stirred at room temperature for 12 h. The resulting mixture was then concentrated under reduced pressure and purified by flash column chromatography (hexane/EtOAc, 5:1) to afford TBDPS-TBDMS-protected dihydroeponemycin as a white solid (92 mg, 78%). ^{1}H NMR: δ = 7.71 (d, ^3J = 7.8 Hz, 2 H, Ar-H), 7.63 (dd, ^3J = 16.9 Hz, ^3J = 6.7 Hz, 2 H, Ar-H), 7.41 (m, 6 H, Ar-H), 7.02 (d, ^3J = 8.4 Hz, 1 H, 4-NH), 6.18 (d, ^3J = 6.6 Hz, 1 H, 2′-NH), 4.63 (m, 2 H, 2′,4-H), 4.45 (dd, ^2J = 19, ^2J = 11.6 Hz, 1 H, 2-CH^a_2), 4.02 (m, 1 H, 3′-H^a), 3.71 (m, 1 H, 3′-H^b), 3.55 (dd, ^2J = 11.4, ^2J = 8.8 Hz, 1 H, 2-CH^b_2), 3.17 (d, ^2J = 5.1 Hz, 1 H, 1-H^a), 3.01 (d, ^2J = 4.8 Hz, 1 H, 1-H^b), 2.13 (t, ^3J = 7.5 Hz, 2 H, 2′′-H), 1.63 (m, 4 H, 3′′-H^a, 5-H^a, 6-H and 6′′-H), 1.27 (m, 4 H, 4′′-H, 5-H^{b}, 3′′-H^{b}), 1.16 (m, 2 H, 5′′-H), 1.06 (s, 9 H, 3′′-*t*Bu), 0.96 (d, ^{3}J = 6.2 Hz, 3 H, CH_3CHCH$_3$), 0.93 (d, ^{3}J = 6.1 Hz, 3 H, CH$_3$CHCH_3), 0.87 (s, 9 H, 2-*t*Bu), 0.06 (d, ^{4}J = 5.6 Hz, 6 H, 2-CH_3SiCH_3) ppm. To a solution of TBDPS-TBDMS-protected dihydroeponemycin (40 mg, 0.054 mmol) in THF (1 mL), tetrabutylammonium fluoride (1.0 M in THF, 32 µL, 0.11 mmol) was added. The reaction solution was stirred at room temperature for 10 min. The resulting mixture was concentrated under reduced pressure and subjected to flash column chromatography (hexane/EtOAc, 1:3) to give dihydroeponemycin as an oil (18 mg, 86%). ^{1}H NMR: δ = 7.13 (d, ^{3}J = 7.1 Hz, 1 H, 4-N*H*), 6.50 (d, ^{3}J = 7.1 Hz, 1 H, 2′-N*H*), 4.49 (m, 2 H, 2′, 4-H), 4.19 (d, ^{2}J = 12.6 Hz, 1 H, 2-CH^{a}_2), 4.00 (dd, ^{2}J = 11.4 Hz, ^{3}J = 3.4 Hz, 1 H, 3′-H^{a}), 3.70 (d, ^{2}J = 12.6 Hz, 1 H, 2-CH^{b}_2), 3.55 (dd, ^{2}J = 11.4 Hz, ^{3}J = 5.8 Hz, 1 H, 3′-Hb), 3.29 (d, ^{2}J = 4.9 Hz, 1 H, 1-H^{a}), 3.07 (d, ^{2}J = 4.9 Hz, 1 H, 1-H^{b}), 2.20 (s and t, ^{3}J = 7.6 Hz, 3 H, 2′′- H and OH), 1.62 (s, 1 H, OH), 1.57 (m, 4 H, 3′′-H^{a}, 5-H^{a}, 6-H and 6′′-H), 1.27 (m, 4 H, 4′′-H, 5-H^{b}, 3′′-H^{b}), 1.16 (m, 2 H, 5′′-H), 0.92 (d, ^{3}J = 6.2 Hz, 3 H, CH_3CHCH$_3$), 0.91 (d, ^{3}J = 6.1 Hz, 3 H, CH$_3$CHCH_3) ppm. ^{13}C NMR: δ = 208.64 (C-3), 174.65 (C-1′′), 172.12 (C-1′), 63.43 (C-3′), 62.99 (C-2), 62.23 (C-9), 54.13 (C-2′), 25.22 (C-4), 50.08 (C-1), 39.25 (C-5 and C-5′′), 37.19 (C-2′′), 28.46 (C-6′′), 27.68 (C-4′′), 26.53 (C-3′′), 25.96 (C-6), 23.97 (C-8), 23.24 (C-7′′ and C-8′′), 21.74 (C-7) ppm. MS (ES, Na): *m*/*z* = 423.2 [M + Na]$^{+}$, calcd. for $C_{20}H_{36}N_2O_6Na$: *m*/*z* = 423.2.

EL4 Cell Culture and Competition Assay: Murine lymphoma EL4 cells were purchased from ATCC and grown in RPMI Medium (Gibco), 10% Fetal Bovine Serum and 1% of penicillin and streptomycin at 37 °C in a 5% CO$_2$ incubator. Cells were pretreated with 1 µM biotinylated dihydroeponemycin and then treated with increasing concentrations of either dihydroeponemycin analogue **5** or dihydroeponemycin **3**. The cells were then incubated at room temperature for 30 minutes. Cells lysates were analyzed by 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. Biotinylated proteins were visualized by enhanced chemiluminescence (ECL) using streptavidin-conjugated horseradish peroxidase and Biomax X-ray film (Kodak).[15]

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[1] P. M. Kloetzel, *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 179–187.

[2] K. L. Rock, A. L. Goldberg, *Annu. Rev. Immunol.* **1999**, *17*, 739–779.

[3] K. Fruh, M. Gossen, K. Wang, H. Bujard, P. A. Peterson, Y. Yang, *Embo. J.* **1994**, *13*, 3236–3244.

[4] C. B. Casp, J. X. She, W. T. McCormack, *Genes. Immun.* **2003**, *4*, 492–499.

[5] M. Hanada, K. Sugawara, K. Kaneta, S. Toda, Y. Nishiyama, K. Tomita, H. Yamamoto, M. Konishi, T. Oki, *J. Antibiot. (Tokyo)* **1992**, *45*, 1746–1752.

[6] K. Sugawara, M. Hatori, Y. Nishiyama, K. Tomita, H. Kamei, M. Konishi, T. Oki, *J. Antibiot. (Tokyo)* **1990**, *43*, 8–18.

[7] N. Sin, L. Meng, H. Auth, C. M. Crews, *Bioorg. Med. Chem.* **1998**, *6*, 1209–1217.

[8] L. Meng, B. H. Kwok, N. Sin, C. M. Crews, *Cancer Res.* **1999**, *59*, 2798–2801.

[9] N. Sin, K. B. Kim, M. Elofsson, L. Meng, H. Auth, B. H. Kwok, C. M. Crews, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2283–2288.

[10] L. Meng, R. Mohan, B. H. Kwok, M. Elofsson, N. Sin, C. M. Crews, *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 10403–10408.

[11] M. Groll, K. B. Kim, N. Kairies, R. Huber, C. M. Crews, *J. Am. Chem. Soc.* **2000**, *122*, 1237–1238.

[12] K. B. Kim, J. Myung, N. Sin, C. M. Crews, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3335–3340.

[13] M. Bogyo, S. Shin, J. S. McMaster, H. L. Ploegh, *Chem. Biol.* **1998**, *5*, 307–320.

[14] H. Hoshi, T. Ohnuma, S. Aburaki, M. Konishi, T. Oki, *Tetrahedron Lett.* **1993**, *34*, 1047–1050.

[15] M. R. Dobler, *Tetrahedron Lett.* **2001**, *42*, 215–218.

[16] U. Schmidt, U. Schmidt, *Synthesis* **1994**, 300–304.

[17] B. Bennacer, C. Rivalle, D. Grierson, *Eur. J. Org. Chem.* **2003**, 4569–4574.

[18] Y. Iwabuchi, T. Sugihara, T. Esumi, S. Hatakeyama, *Tetrahedron Lett.* **2001**, *42*, 7867–7871.

[19] T. Katsuki, K. B. Sharpless, *J. Am. Chem. Soc.* **1980**, *102*, 5974–5976.

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