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Eponemycin Analogues: Syntheses and use as Probes of Angiogenesis

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Abstract—Derivatives of the epoxy-β-aminoketone containing natural product eponemycin have been prepared in order to study the molecular mode of action of this anti-angiogenic compound. Synthesis and use of a biotinylated dihydroeponemycin analogue demonstrated that dihydroeponemycin forms a covalent adduct with at least two intracellular proteins in human endothelial cells. Pretreatment of cells with a five equivalent excess of dihydroeponemycin precluded biotin-dihydroeponemycin binding indicating a specific interaction between natural product and the target proteins. This biotin-dihydroeponemycin derivative will prove useful in the purification and identification of eponemycin receptors.

Introduction

Tumor-induced new blood vessel formation (angiogenesis or neovascularization) plays a crucial role in both tumor progression and metastases by supplying essential nutrients to tumor cells within the cell mass and by providing conduits for dislodged cancerous cells.[1] Given the critical role of angiogenesis in tumor progression, significant pharmaceutical interest has focused on the identification of novel anti-angiogenic compounds.[2] Clearly, such compounds hold much clinical promise as a means of limiting both the size and spread of primary tumors. Despite the identification and development of a number of potential anti-angiogenic therapeutics, little is known of the mechanisms of action of these compounds at the molecular level. For example, TNP-470, derived from the biologically active natural product fumagillin,[3] is the leading anti-angiogenic drug in clinical trials.[4,5] However, until recently, investigation of this compound was limited primarily to its potential clinical applications. Because of its potency and endothelial cell selectivity, TNP-470/fumagillin has proven useful in our efforts to use biologically active natural products as molecular probes in the study of angiogenesis. As part of this endeavor, a semi-synthetic fumagillin biotinylated derivative was constructed and demonstrated to bind covalently to the metalloprotease methionine aminopeptidase 2 (MetAP-2).[6] We and others have further shown that fumagillin inhibits the in vivo and in vitro enzymatic activity of MetAP-2,[6,7] providing strong evidence that MetAP-2 is the physiological target of fumagillin.

In our continuing development of angiogenic molecular probes, we have recently focused on one of the most promising angiostatic natural products, eponemycin (**1**). Isolated from *Streptomyces hygroscopicus* No. P247-71,[8] this microbial metabolite has proven to be very potent in the inhibition of endothelial cell growth and migration in vitro and in the inhibition of neovascularization in an animal model system.[9] Specifically, eponemycin was found to exhibit in vivo anti-angiogenic activity ($ID_{50} = 0.1$ ng/egg) in the chorioallantoic membrane (CAM) of growing chick embryos.[9] Despite this potent activity and potential clinical use, the mechanism of action for eponemycin remains unknown.

In designing molecular probes based on biologically active natural products, it is important not to compromise the active pharmacophore of the original

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compound. Fortunately, comparison of eponemycin with a related natural product, epoxomicin, provides insights into the critical structural determinants that comprise eponemycin's pharmacophore. Epoxomicin (**2**), isolated from the actinomycete strain, No. Q996-17,[10] also possesses an epoxy-β-aminoketone moiety. Moreover, like eponemycin, epoxomicin is more active against solid tumors in animals than leukemias, suggesting that it too mediates its antitumor activity via an anti-angiogenic mechanism.[10] Since the two compounds share several structure elements, we propose that the common structural motifs may represent an anti-angiogenic pharmacophore (**3**) (Figure 1).

Given the potency of eponemycin and the requirement for the epoxide for biological activity,[8] we hypothesized that eponemycin may mediate its cytostatic activity via a covalently interaction with a protein receptor. As the first step towards the purification and identification of an eponemycin receptor(s), an affinity reagent, dihydroeponemycin-biotin (**5**), has been synthesized and used to probe endothelial cellular proteins. Because dihydroeponemycin (**4**) has comparable biological activities to eponemycin,[8] we also synthesized it for use as a positive control for binding specificity assays (Fig. 2).

Results and Discussion

Affinity reagent synthesis

Given the similar biological activities between eponemycin and dihydroeponemycin,[8] the latter was chosen as the scaffolding for the design of an eponemycin-based molecular probe. Our syntheses of **4** and **5** were influenced by the previously reported strategies of Schmidt[11] and Hoshi.[12] Our approach to **4** and **5** began with the addition of a vinyl lithium derived from treatment of 2-bromo-1-hydroxy-2-propene (**7**)[12,13] with *t*-butyl lithium,[14] to aldehyde **6**, which afforded a 1:1 inseparable mixture of diol **8** (Scheme 1). Selective protection of the primary alcohol of **8** as a *t*-butyldimethylsilyl (TBS) ether and a Swern oxidation of the remaining secondary alcohol yielded the α,β-unsaturated ketone **9**. Epoxidation of **9** with hydrogen peroxide[11] afforded the epoxyketone **10a** and **10b** as a 1.2:1 mixture which was readily separated by flash column chromatography. Final removal of the benzyloxycarbonyl protecting group of **10a** through a catalytic hydrogenation[12] reaction gave epoxy-β-aminoketone **11** in excellent yield.

We next turned to the preparation of carboxylic acid **13** (Scheme 2), the left-hand fragment of **4**. Coupling of isooctanoic acid **12** (a generous gift from Exxon) with

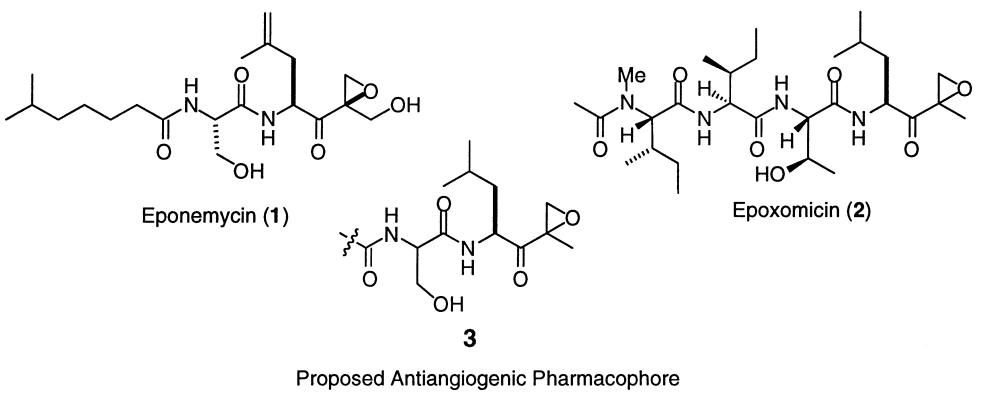


Figure 1.

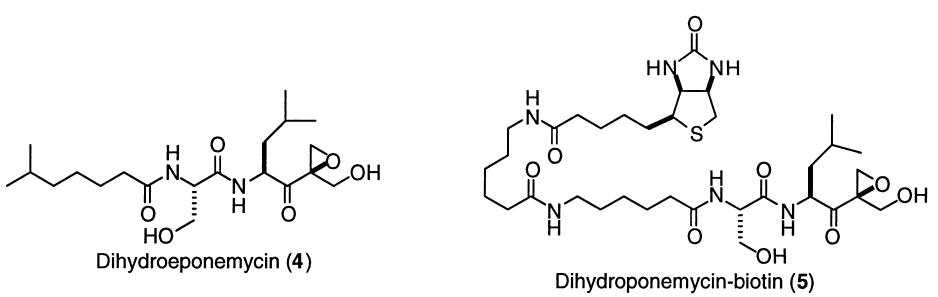
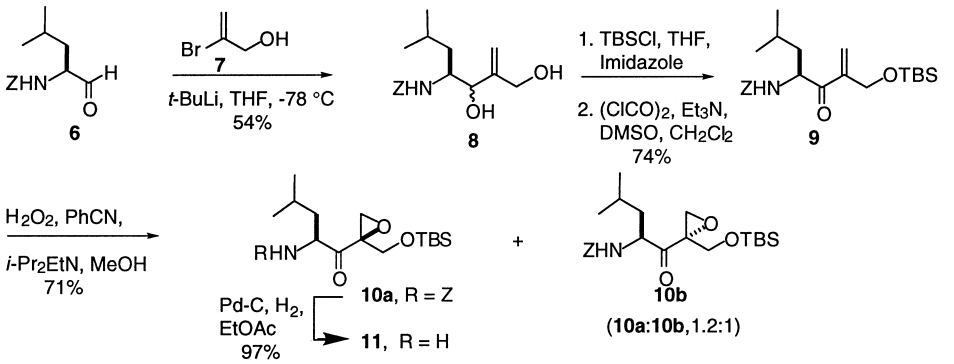
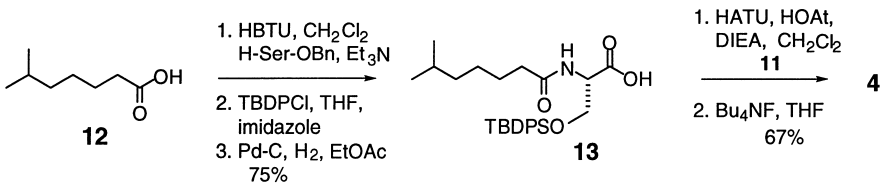


Figure 2.



Scheme 1.



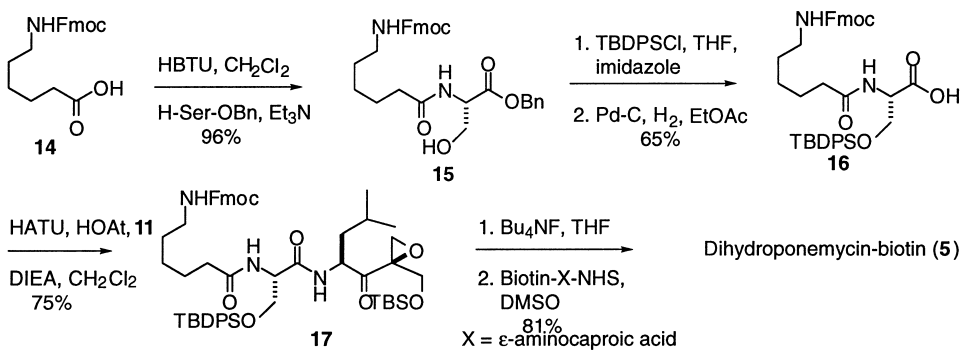
Scheme 2.

serine benzylester using HBTU, followed by protection of the serine moiety side chain as a TBDPS ether and hydrogenalysis of the benzyl protecting group afforded **13**. An HATU and HAOt initiated coupling of carboxylic acid **13** with epoxy-β-aminoketone **11**, followed by removal of the silyl protecting groups accomplished the synthesis of dihydroeponemycin **4**. Correlation of the spectroscopic data of **4** with the published data established the stereochemistry of **4** and epoxy-β-aminoketone **11**.

After the synthesis of **4**, our attention focused on the synthesis **5** (Scheme 3). Our approach to **5** began with coupling of *N*-Fmoc-ε-aminocaproic acid **14** to the serine benzylester which gave hydroxyester **15** in excellent yield. The hydroxyl group of **15** was protected as a TBDPS ether and subsequently the benzyl protecting group was removed to give the carboxylic acid left hand fragment **16**. Coupling of the epoxy-β-amino-ketone **11** to **16** afforded the tripeptide **17**. The Fmoc and silyl protecting groups of **17** were removed in a single step using tetrabutylammonium fluoride. Finally, coupling of the resulting amine with *N*-hydroxysuccinimide-biotin reagent (Calbiochem) accomplished the synthesis of the biotinylated dihydroeponemycin affinity reagent (**5**).

Covalent and selective adduct formation with eponemycin protein receptors

Given the importance of the epoxide moiety for biological activity,[8] we hypothesized that eponemycin may form a covalent adduct with a protein receptor(s) via nucleophilic attack on the epoxide. In order to test this hypothesis, the biotinylated dihydroeponemycin affinity



Scheme 3.

reagent **5** was incubated with cultured bovine aortic endothelial cells (BAECs) in increasing concentrations before examination of protein lysates for newly biotinylated proteins. Lysates from treated cells were analyzed by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), protein immobilization onto polyvinylidene difluoride (PVDF) membrane followed by biotinylated protein detection via avidin conjugated horseradish peroxidase (HRP). As shown in Figure 3, in the absence of added dihydroeponemycin-biotin, only higher molecular weight biotinylated proteins were detected. However, upon addition of increasing concentrations of **5** to intact BAECs, a major 23 kDa protein and minor 25 kDa protein were biotinylated at concentration of **5** equal to the IC_{50} value for BAEC proliferation inhibition (data not shown). A much weaker biotinylated protein of approximately 30 kDa, which correlates with the addition of **5**, was detected upon overexposure of the enhanced chemiluminescence blot (data not shown).

Given the high degree of reactivity of the epoxide moiety, it was necessary to rule out the possibility that the protein–natural product adduct formation is simply due to non-specific association. To discount this possibility, a five molar excess of non-biotinylated dihydroeponemycin was added to BAECs for 30 min before the cells were challenged with biotinylated dihydroeponemycin **5**. As seen in Figure 3, such pretreatment of the cells with **4** abolishes subsequent covalent interaction with **5**. This result demonstrates that **5** is specifically and selectively interacting with at least two protein receptors with molecular weights of 23 and 25 kDa. These proteins are presently being purified in sufficient quantities for protein sequencing.

Conclusions

Neovascularization (angiogenesis) is an attractive target for pharmaceutical intervention since it plays an important role in a variety of human pathologies. In addition to tumor growth and metastatic control, diabetic retinopathy and rheumatoid arthritis are also examples of diseases currently being targeted using an anti-angiogenic pharmacological approach. Despite the many clinical applications of several of the leading anti-angiogenic compounds, little is known of the molecular mechanisms by which they act. In order to gain possible insights into endothelial cell cycle control, we have synthesized derivatives of the potent anti-angiogenic natural product eponemycin. Addition of the cell-permeable dihydroeponemycin-biotin affinity reagent **5** to human endothelial cell cultures results in covalent adduct formation between this natural product derivative and two 23–25 kDa proteins. Moreover, these protein–ligand interactions were shown to be specific through the pretreatment of endothelial cells with excess non-biotinylated compound **4** before challenging with the biotinylated natural product derivative. Covalent attachment to these protein receptors will greatly facilitate the purification and identification of these proteins that are likely to mediate the anti-angiogenic activity of eponemycin.

Experimental

General experimental methods

Solvent and reagent abbreviations used are Bn = benzyl, Boc = *tert*-butyloxycarbonyl, DIEA = diisopropylethylamine, DMF = dimethylformamide, DMSO =

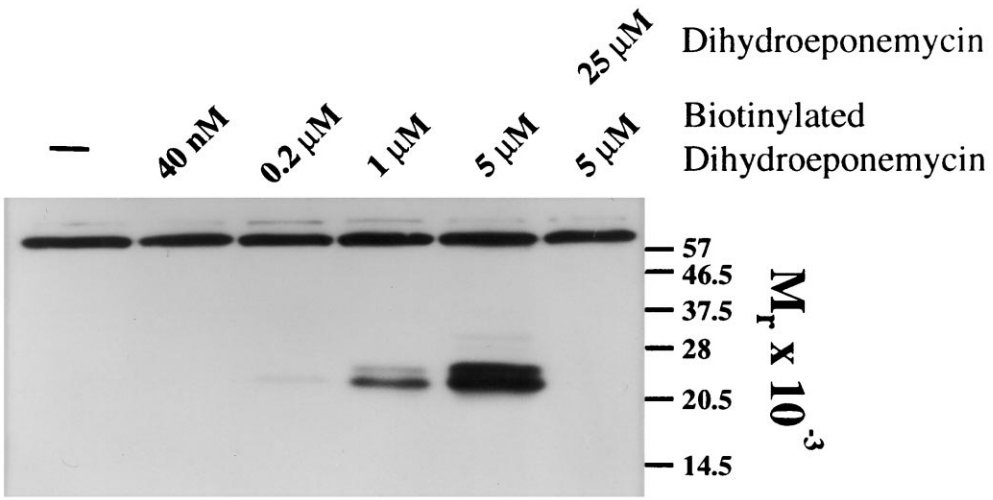


Figure 3. Biotinylated protein analysis from 12.5% SDS–PAGE of dihydroeponemycin-biotin (**4**) treated HUVEC lysates visualised with ECL and avidin-HRP.

dimethylsulfoxide, ECL = enhanced chemiluminescence, ECGS = endothelial cell growth supplement, Fmoc = 9-fluorenylmethoxycarbonyl, HBTU = *O*-benzotriazo-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HRP = horseradish peroxidase, HUVEC = human umbilical cord venous endothelial cells, NHS = *N*-hydroxyl succinimide, PMA = phosphomolybdic acid, PVDF = polyvinylidene difluoride, SDS–PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis, TBS = *tert*-butyldimethylsilyl, TBDPS = *tert*-butyldiphenylsilyl, THF = tetrahydrofuran, TFA = trifluoroacetic acid, Z = benzyloxycarbonyl.

Optical rotations were determined at the sodium D line (589 nm) and recorded on a Perkin–Elmer 241 polarimeter. IR spectra were recorded from neat samples evaporated on a sodium chloride plate using a Perkin–Elmer Paragon 1000 FT-IR instrument. NMR spectra were recorded in $CDCl_3$ using a Bruker AM-500 (500 MHz), except as noted. High and low resolution mass spectra were obtained at the Mass Spectrometry Laboratory of the University of Illinois at Urbana-Champaign. High-pressure liquid chromatography (HPLC) separations were performed on a Ranin Preparative Chromatography System using an 8×100 mm or 25×100 mm Prep Nova-Pak HR silica (60 Å, 6 μm) column for analytical or preparative scale, respectively. Flash column chromatography was performed following the method of Still with Merck silica gel 60, 230–400 mesh and monitored by thin-layer chromatography (TLC) using Merck silica gel 60 F254 precoated 0.25 mm layer thickness plates. Hexanes:EtOAc or CH_2Cl_2:MeOH solvent systems were used for elution and the TLC plates were stained with anisaldehyde or PMA.

Solvents were purified before each reaction as described below unless otherwise noted. THF and dichloromethane were distilled from sodium and benzophenone ketyl and calcium hydride respectively. DMF was distilled from calcium hydride under reduced pressure and stored over 4 Å molecular sieves. DIEA and piperidine were purchased from Aldrich and were used without further purification.

All anhydrous reactions were performed under a nitrogen atmosphere except as noted. The glassware for these anhydrous reactions was flame-dried and cooled under nitrogen atmosphere prior to use. The progress of the reactions was monitored by TLC unless otherwise noted. Reactions were dried over $MgSO_4$ or Na_2SO_4 before concentration which was performed under reduced pressure using a Buchi roto-evaporator.

(3*RS*,4*S*)-4-Benzyloxycarbonylamino-3-hydroxy-2-hydroxymethyl-6-methyl-1-heptene (8). To a solution of *t*-BuLi (35.5 mL, 49.8 mmol) in Et_2O (30 mL) at −78 °C, 2-bromo-3-propen-1-ol (**7**) (2.73 g, 19.9 mmol) solution in Et_2O (10 mL) was added dropwise. The solution was warm to 0 °C immediately. After stirring for 4 h, the solution was re-cooled to −78 °C and was treated with *Z*-leucinal (**6**) (1.22 g, 4.89 mmol) solution in Et_2O (10 mL) dropwise. After stirring at −78 °C for 2.5 h, the reaction was stirred at 0 °C for an additional 1.5 h; subsequently, it was quenched with brine (30 mL). The organic layer was separated and the aqueous layer was extracted with Et_2O (3×50 mL). The combined organic layer was dried and concentrated. The product was purified by flash column chromatography (hexanes:EtOAc, 1:1) to give a mixture of diol **8** (807.4 mg, 54%): IR 3336, 2956, 1693 cm^{-1}; ^1H NMR: δ 7.36–7.33 (m, 5H), 5.16–5.06 (m, 5H), 4.25–4.24 (d, J = 5.0 Hz, 1H), 4.20–4.12 (m, 1H), 4.0–3.95 (m, 1H), 3.91–3.84 (m, 1H), 3.68–3.61 (d, J = 6.1 Hz, 1H), 3.38–3.37 (d, J = 5.1 Hz, 1H), 3.09–3.07 (d, J = 5.7 Hz, 1H), 1.71–1.65 (m, 2H), 1.47–1.41 (m, 1H), 1.39–1.37 (m, 1H), 1.35–1.25 (m, 1H), 0.97–0.89 (m, 6H); ^{13}C NMR: δ 156.9, 156.8, 148.7, 147.3, 136.4, 136.3, 128.4, 128.4, 128.1, 128.0, 127.9, 127.8, 113.9, 113.1, 74.6, 66.8, 66.7, 64.5, 63.7, 52.7, 52.1, 41.3, 38.3, 24.9, 24.6, 23.1, 23.0, 21.9, 21.4; HRFABMS calcd for $C_{17}H_{26}NO_4$ 308.1862, found 308.1863.

(3*RS*,4*S*,)-4-Benzyloxycarbonylamino-2-*tert*-butyldimethylsiloxymethyl-3-hydroxy-6-methyl-1-heptene. To a solution of **8** (807.4 mg, 2.63 mmol) was added imidazole (196.7 mg, 2.89 mmol), and *t*-butyldimethylsilyl chloride (407.8 mg, 2.71 mmol). After stirring at rt for 12 h, the reaction was vacuum filtered. The mother liquor was concentrated and purified by flash column chromatography (hexanes:EtOAc, 5;1) to give (3*RS*,4*S*,)-4-benzyloxycarbonylamino-2-*tert*-butyldimethylsiloxymethyl-3-hydroxy-6-methyl-1-heptene (880.7 mg, 80%): IR 3434, 2955, 1704 cm^{-1}; ^1H NMR δ: 7.32–7.28 (m, 5H), 5.28–5.26 (d, J = 9.7, 1H), 5.19–5.03 (m, 4H), 4.29–4.2 (m, 3H), 3.93–3.90 (p, J = 4.7 Hz, 1H), 3.27–3.26 (d, J = 4.27 Hz, 1H), 1.70–1.65 (m, 1H), 1.56–1.51 (m, 1H), 1.38–1.32 (m, 1H), 0.97–0.91 (m, 15H), 0.11–0.09 (d, J = 5.9 Hz, 6H); ^{13}C NMR: δ 156.9, 148.3, 136.8, 128.5, 128.0, 128.0, 127.9, 111.8, 74.4, 66.6, 64.9, 51.8, 41.9, 26.0, 24.9, 23.4, 22.2, 18.3.

(4*S*)-4-Benzyloxycarbonylamino-2-*tert*-butyldimethylsiloxymethyl-3-oxo-6-methyl-1-heptene (9). To a −78 °C solution of oxalyl chloride (0.11 mL, 1.30 mmol) in CH_2Cl_2 (1 mL) was added, in 8 min interval, DMSO (0.18 mL, 2.59 mmol) in CH_2Cl_2 (1 mL), (3*RS*,4*S*,)-4-benzyloxycarbonylamino-2-*tert*-butyldimethylsiloxymethyl-3-hydroxy-6-methyl-1-heptene (182.1 mg, 0.432 mmol), and triethylamine (0.36 mL, 2.59 mmol). The cold bath was removed and the reaction mixture was stirred at ambient temperature for additional 10 min. The reaction mixture was poured into aqueous

HCl (1.5 mL, 2 N) and shaken; the organic layer was separated and the aqueous layer was extrated with CH_2Cl_2 (2×5 mL). The combined organic layer was washed with saturated $NaHCO_3$ (3 mL), brine (5 mL), dried and concentrated. The crude product was purified by flash column chromatography (hexanes:EtAOc, 10:1) to give **9** (168.4 mg, 93%): ^{1}H NMR: δ 7.36–7.31 (m, 5H), 6.24–6.22 (d, J = 6.6 Hz, 2H), 5.42–5.41 (d, J = 8.7 Hz, 1H), 5.15–5.13 (m, 1H), 5.10 (s, 2H), 4.43–4.31 (dd, J = 44.7 Hz, 15.6, 2H), 1.78–1.67 (m, 1H), 1.61–1.52 (m, 1H), 1.43–1.35 (m, 1H), 1.04–1.02 (d, J = 6.5 Hz, 3H), 0.93 (s, 9H), 0.92–0.91 (d, J = 6.8 Hz, 3H); ^{13}C NMR: δ 200.3, 156.1, 145.3, 128.5, 128.1, 128.0, 124.8, 66.9, 61.1, 53.7, 43.1, 25.9, 24.9, 23.4, 21.7, 18.3; HRFABMS calcd for $C_{23}H_{38}NO_4Si$ 420.2570, found 420.2569.

(2*RS*,4*S*)-4-Benzyloxycarbonylamino-2-*tert*-butyldimethylsiloxymethyl-6-methyl-1,2-oxiranylheptane (10a, 10b). To a 0 °C solution of **9** (146.3 mg, 0.35 mmol) was added benzonitrile (0.18 mL, 1.74 mmol), H_2O_2 (0.10 mL, 50% solution in H_2O, 1.74 mmol) and diisopropylethylamine (0.30 mL, 1.74 mmol). The reaction was stirred at 4 °C for 15 h. The solvent was removed and the crude oil product was purified by flash column chromatography (hexanes:EtOAc, 10:1) to give a 1.2:1 of **10a**:**10b** (107.2 mg, 71%).

10a. $[\alpha]_D^{20}$ +34.84° (*c* 0.31, $CHCl_3$); IR 3346, 2957, 1713, 1517, 1257, 838 cm^{-1}; ^{1}H NMR: δ 7.38–7.32 (m, 5H), 5.10–5.04 (m, 3H), 4.45–4.43 (d, J = 11.5 Hz, 2H), 3.59–3.57 (d, J = 11.6 Hz, 1H), 3.18–3.17 (d, J = 5.0 Hz, 1H), 3.03–3.02 (d, J = 4.9 Hz, 1H), 1.78–1.74 (m, 1H), 1.74–1.60 (m, 2H), 1.16–1.10 (m, 1H), 1.00–0.99 (d, J = 6.4 Hz, 3H), 0.95–0.94 (d, J = 6.5 Hz, 3H), 0.88 (s, 9H), 0.07–0.06 (d, J = 7.2 Hz, 6H); ^{13}C NMR: δ 156.2, 136.2, 128.5, 128.1, 128.0, 66.9, 62.0, 52.7, 48.3, 39.5, 25.8, 25.1, 23.4, 21.1, 18.3.

10b. $[\alpha]_D^{20}$ −13.33° (*c* 0.15, $CHCl_3$); IR 3350, 2956, 1715, 1513, 1256, 838 cm^{-1}; ^{1}H NMR: δ 7.36–7.32 (m, 5H), 5.23–5.22 (d, J = 8.3 Hz, 1H), 5.13–5.06 (m, 3H), 4.68–4.65 (t, J = 7.2 Hz, 1H), 4.29–4.27 (d, J = 11.9 Hz, 1H), 3.79–3.77 (d, J = 11.9 Hz, 1H), 3.01–3.00 (d, J = 4.9 Hz, 1H), 2.91–2.90 (d, J = 4.8 Hz, 1H), 1.74–1.67 (m, 1H), 1.51–1.47 (m, 1H), 1.42–1.37 (m, 1H), 0.99–0.98 (d, J = 6.1 Hz, 3H), 0.93–0.92 (d, J = 6.6 Hz, 3H), 0.88 (s, 9H), 0.07–0.06 (d, J = 4.6 Hz, 6H); ^{13}C NMR: δ 206.2, 155.9, 136.3, 128.5, 128.1, 128.0, 67.0, 62.6, 62.5, 54.8, 49.0, 40.5, 29.7, 25.8, 24.7, 23.3, 21.5, 18.3.

(2*R*,4*S*)-4-Amino-2-*tert*-butyldimethylsiloxymethyl-6-methyl-1,2-oxiranylheptane (11). To a solution of **10a** (27.3 mg, 0.063 mmol) in MeOH (1.5 mL) was added palladium on activated carbon catalyst (7.0 mg). After stirring at rt under a hydrogen atmosphere for 15 min, the catalyst was removed by vacuum filtration through a celite pad. The crude product was stirred in vacuo for 0.5 h (18.4 mg, 97%) and was used immediately without further purification: IR 3378, 2955, 1715, 1103, 838 cm^{-1}; ^{1}H NMR: δ 4.43–4.41 (d, J = 10.4 Hz, 1H), 3.89–3.75 (m, 1H), 3.63–3.61 (dd, J = 11.7, 1.3 Hz, 2H), 3.58–3.57 (d, J = 8.8 Hz, 1H), 3.00–2.99 (d, J = 5.0 Hz, 1H), 2.83–2.82 (d, J = 5.1 Hz, 1H), 1.87–1.83 (m, 1H), 1.69–1.64 (m, 1H), 1.33–1.22 (m, 2H), 0.97–0.88 (m, 15H), 0.07–0.06 (d, J = 6.0 Hz, 6H); ^{13}C NMR: δ 210.8, 62.6, 53.3, 48.3, 42.7, 29.7, 25.9, 25.8, 25.0, 23.6, 21.0, 18.2.

(*S*)-*N*-(6-Methylheptanoyl)serine benzyl ester. To a solution *S*-serine benzyl ester hydrochloride (0.50 g, 2.16 mmol) in CH_2Cl_2 (15 mL), was added isooctanoic acid (0.374 g, 2.59 mmol), HBTU (1.07 g, 2.81 mmol) and triethylamine (1.3 mL, 8.64 mmol). The reaction was stirred at rt for 8 h, after which it was poured into aqueous HCl (3 mL, 2 N). The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (2×10 mL). The combined organic layer was washed with saturated aqueous $NaHCO_3$ (3 mL), brine (5 mL), dried and concentrated. The crude product was purified by flash column chromatography (hexanes:EtOAc, 1:1) to give (*S*)-*N*-(6-methylheptanoyl)serine benzyl ester (0.569 g, 82%): $[\alpha]_D^{20}$ +8.91° (*c* 1.75, $CHCl_3$); IR 3364, 2958, 1744, 1648 cm^{-1}; ^{1}H NMR: δ 7.38–7.32 (m, 5H), 6.50–6.49 (d, J = 6.9 Hz, 1H), 5.25–5.18 (dd, J = 16.3, 12.4 Hz, 2H), 4.75–4.71 (m, 1H), 4.0–3.97 (q, J = 5.5 Hz, 1H), 3.92–3.91 (q, J = 2.6 Hz, 1H), 2.85–2.84 (d, J = 3.1 Hz, 1H), 2.33–2.17 (m, 1H), 2.05–1.96 (m, 2H), 1.37–1.23 (m, 4H), 1.17–1.08 (m, 2H), 0.92–0.79 (m, 6H); ^{13}C NMR: δ 170.4, 135.1, 128.6, 128.5, 128.2, 67.5, 63.6, 63.5, 54.7, 46.3, 44.5, 28.4, 25.2, 23.3, 22.0, 19.6, 16.7.

(*S*)-*O*-*tert*-Butyldiphenylsiloxymethyl-*N*-(6-methylheptanoyl)serine benzyl ester. To a solution of (*S*)-*N*-(6-methylheptanoyl)serine benzyl ester (0.77 g, 2.40 mmol) was added imidazole (0.212 g, 2.88 mmol), and *t*-butyldiphenylsilyl chloride (0.75 mL, 2.88 mmol). After stirring at rt for 24 h, the reaction mixture was vacuum filtered and the mother liquor was washed with brine. The aqueous layer was extracted with EtOAc:Et_2O (1:1, 50 mL). The combined organic layer was dried and concentrated. The crude oily product was purified by flash column chromatography (hexanes:EtOAc, 5:1) to give (*S*)-*O*-*tert*-butyldiphenylsiloxymethyl-*N*-(6-methylheptanoyl)serine benzyl ester (1.23 g, 92%): $[\alpha]_D^{20}$ +17.36° (*c* 1.10, $CHCl_3$); IR 3319, 2956, 1746, 1660 cm^{-1}; ^{1}H NMR: δ 7.59–7.55 (dd, J = 13.5, 7.31 Hz, 4H), 7.45–7.41 (m, 2H), 7.40–7.32 (m, 9H), 6.31–6.30 (d, J = 4.9 Hz, 1H), 5.23–5.17 (dd, J = 14.9, 12.5 Hz, 2H), 4.79–4.76 (t, J = 7.4 Hz, 1H), 4.19–4.17 (dd, J = 10.3, 2.4 Hz, 1H), 3.91–3.90 (dd, J = 10.3, 2.7 Hz, 1H), 2.23–2.14 (m, 2H), 1.63–1.58 (m, 2H), 1.37–1.31

(m, 3H), 1.18–1.14 (m, 2H), 1.03 (s, 9H), 0.90–0.83 (m, 6H); ^{13}C NMR: δ 170.5, 135.5, 132.6, 129.9, 128.6, 128.4, 128.3, 127.8, 127.8, 67.3, 64.5, 54.1, 34.7, 32.5, 29.5, 29.3, 26.7, 22.6, 20.1, 19.3, 15.1.

(*S*)-*O*-*tert*-Butyldiphenylsiloxymethyl-*N*-(6-methylheptanoyl)serine (13). To a solution of (*S*)-*O*-*tert*-butyldiphenylsiloxymethyl-*N*-(6-methylheptanoyl)serine benzyl ester (1.23 g, 2.17 mmol) in MeOH (10 mL) was added palladium on activated carbon catalyst (0.308 g). After stirring at rt under a hydrogen atmosphere for 2 h, the catalyst was removed by vacuum filtration through a celite pad. The crude product was stirred in vacuo for 12 h to give **13** (1.018 g, 99%) which was used without further purification: $[\alpha]_D^{20}$ +16.76° (*c* 1.05, $CHCl_3$); IR 3335, 2957, 1736, 1629 cm^{-1}; ^{1}H NMR: δ 7.63–7.61 (dd, *J* = 6.4, 1.4 Hz, 4H), 7.46–7.27 (m, 6H), 6.32–6.31 (d, *J* = 3.5 Hz, 1H), 4.71–4.70 (d, *J* = 3.6 Hz, 1H), 4.20–4.17 (dd, *J* = 10.4, 3.0 Hz, 1H), 3.93–3.91 (dd, *J* = 10.3, 2.4 Hz, 1H), 2.28–2.14 (m, 2H), 1.71–1.51 (m, 2H), 1.45–1.23 (m, 3H), 1.19–1.10 (m, 2H), 1.05 (s, 9H), 0.89–0.88 (d, *J* = 4.4 Hz, 3H), 0.87–0.86 (d, *J* = 7.4 Hz, 3H); ^{13}C NMR: δ 174.0, 135.5, 135.4, 132.8, 132.4, 64.0, 54.0, 44.5, 34.6, 31.9, 29.3, 26.8, 22.6, 19.3, 17.9, 15.0; HRFABMS calcd for $C_{27}H_{40}NO_4Si$ 470.2727, found 470.2722.

(4*S*)-2-*tert*-Butyldimethylsiloxymethyl-4-[(*S*)-*O*-*tert*-butyldiphenylsiloxymethyl-*N*-(6-methylheptanoyl)serylamino]-6-methyl-1,2-oxiranylheptane. To a solution of (*S*)-*O*-*tert*-butyldiphenylsiloxymethyl-*N*-(6-methylheptanoyl) serine (58.2 mg, 0.124 mmol) in CH_2Cl_2 (1 mL), was added HATU (49.5 mg, 0.130 mmol), HOAt (17.7 mg, 0.130 mmol) and diisopropylethylamine (70.2 μL, 0.40 mmol). After 15 min, a solution of epoxy-β-amino-ketone **11** (18.7 mg, 0.062 mmol) in CH_2Cl_2 (2 mL) was added and stirred at rt for 10 h. The reaction mixture was concentrated and purified by flash column chromatography (CH_2Cl_2:MeOH, 97:3) to give (4*S*)-2-*tert*-butyldimethylsiloxymethyl-4-[(*S*)-*O*-*tert*-butyldiphenylsiloxymethyl-*N*-(6-methylheptanoyl)serylamino]-6-methyl-1,2-oxiranylheptane (36.2 mg, 78%): $[\alpha]_D^{20}$ +13.25° (*c* 0.15, $CHCl_3$); IR 3291, 3072, 2957, 1829, 1727, 1641, 1112 cm^{-1}; ^{1}H NMR: δ 7.73–7.72 (d, *J* = 6.7 Hz, 1H), 7.67–7.62 (dd, *J* = 16.9, 6.7 Hz, 2H), 7.45–7.37 (m, 8H), 6.16 (br, 1H), 4.72–4.69 (t, *J* = 8.8 Hz, 1H), 4.64–4.59 (m, 1H), 4.48–4.42 (dd, *J* = 19.0, 11.6 Hz, 1H), 4.07–4.02 (m, 2H), 3.74–3.70 (q, *J* = 7.6 Hz, 1H), 3.59–3.55 (dd, *J* = 11.4, 8.8 Hz, 1H), 3.20–3.19 (dd, *J* = 5.0 Hz, 1H), 3.16–3.15 (d, *J* = 5.1 Hz, 1H), 3.01 (d, *J* = 2.6 Hz, 1H), 2.17–2.11 (m, 2H), 1.71–1.55 (m, 2H), 1.33–1.21 (m, 2H), 1.08 (s, 18H), 0.98–0.82 (m, 12H), 0.08–0.06 (d, *J* = 11.3 Hz, 6H); ^{13}C NMR: δ 206.5, 171.0, 170.5, 135.5, 135.4, 130.0, 129.9, 127.9, 127.9, 63.7, 62.7, 54.0, 50.9, 48.4, 39.6, 28.6, 26.8, 26.8, 25.8, 25.2, 25.1, 23.3, 22.0, 21.0, 19.6, 19.2, 19.1, 18.2.

Dihydroeponemycin (4). To a solution of (4*S*)-2-*tert*-butyldimethylsiloxymethyl-4-[(*S*)-*O*-*tert*-butyldiphenylsiloxymethyl-*N*-(6-methylheptanoyl)serylamino]-6-methyl-1,2-oxiranylheptane (22.9 mg, 0.0304 mmol) in THF (1 mL) was added tetrabutylammonium fluoride (0.129 mL, 0.5 M in THF, 0.0638 mmol). After 1 h, the solvent was removed and the crude product was purified by flash column chromatography (CH_2Cl_2:MeOH, 97:3) to give dihydroeponemycin **4** (10.5 mg, 86%): $[\alpha]_D^{20}$ +35.76° (*c* 0.425, $CHCl_3$); IR 3304, 2958, 1721, 1644, 1536, 1048 cm^{-1}; ^{1}H NMR: δ 7.18 (br, 1H), 6.60 (br, 1H), 4.53–4.49 (m, 2H), 4.24–4.22 (d, *J* = 12.7 Hz, 1H), 4.20–4.18 (d, *J* = 12.6 Hz, 1H), 4.12–4.10 (d, *J* = 10.6 Hz, 1H), 4.05–4.04 (d, *J* = 5.4 Hz, 1H), 3.75–3.72 (d, *J* = 5.81 Hz, 1H), 3.62–3.60 (m, 2H), 3.32 (br, 1H), 3.10–3.09 (d, *J* = 4.8 Hz, 1H), 2.35–2.19 (m, 1H), 2.11–1.95 (m, 1H), 1.37–1.23 (m, 2H), 1.40–1.10 (m, 4H), 0.97–0.96 (d, *J* = 6.6 Hz, 3H), 0.96–0.94 (d, *J* = 6.6 Hz, 3H), 0.90–0.88 (d, *J* = 6.4 Hz, 3H), 0.84–0.83 (d, *J* = 7.1 Hz, 3H); ^{13}C NMR: δ 207.6, 171.5, 62.6, 61.5, 53.5, 51.5, 49.4, 46.3, 38.7, 29.7, 28.4, 25.8, 25.2, 25.1, 23.3, 21.9, 21.1, 19.5, 14.2, 12.1; HRFABMS calcd for $C_{20}H_{37}N_2O_6$ 401.2652, found 401.2652.

(*S*)-*N*-[6-*N*-Fluorenylmethoxycarbonylamino)hexanoyl] serine benzyl ester (15). To a solution *S*-serine benzyl ester hydrochloride (0.50 g, 2.16 mmol) in CH_2Cl_2 (20 mL), was added *N*-Fmoc-ε-aminocaproic acid (0.84 g, 2.38 mmol), HBTU (1.20 g, 3.23 mmol) and triethylamine (1.2 mL, 8.64 mmol). The reaction was stirred at rt overnight, after which it was poured into aqueous HCl (3 mL, 2 N). The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (2×10 mL). The combined organic layer was washed with saturated aqueous $NaHCO_3$ (3 mL), brine (5 mL), dried and concentrated. The crude product was purified by flash column chromatography (1:3, hexanes:EtOAc) to give **15** (1.10 g, 96%): $[\alpha]_D^{20}$ +5.91° (*c* 1.10, $CHCl_3$); IR 3314, 2936, 1721, 1644, 1527, 844 cm^{-1}; ^{1}H NMR: δ 7.77–7.75 (d, *J* = 7.5 Hz, 2H), 7.60–7.58 (d, *J* = 7.4 Hz, 2H), 7.41–7.30 (m, 9H), 6.60–6.58 (d, *J* = 7.5 Hz, 1H), 5.20 (s, 2H), 4.96 (br, 1H), 4.73–4.70 (m, 1H), 4.40–4.38 (d, *J* = 6.8 Hz, 2H), 4.22–4.19 (t, *J* = 6.6 Hz, 1H), 4.01–3.98 (t, *J* = 6.3 Hz, 1H), 3.92–3.89 (t, *J* = 10.7 Hz, 1H), 3.23–3.10 (m, 3H), 2.28–2.25 (t, *J* = 6.8 Hz, 2H), 1.93 (s, 1H), 1.67–1.66 (m, 2H), 1.52–1.35 (q, *J* = 7.20 Hz, 2H); ^{13}C NMR: δ 173.3, 170.4, 165.6, 143.9, 141.3, 135.1, 128.6, 128.5, 128.1, 127.6, 127.0, 125.0, 119.9, 67.4, 66.5, 63.3, 54.7, 47.4, 47.2, 40.7, 38.6, 36.1, 29.5, 26.0, 24.9, 8.7; HRFABMS calcd for $C_{31}H_{35}N_2O_6$ 531.2495, found 531.2495.

(*S*)-[*O*-*tert*-Butyldiphenylsiloxymethyl-*N*-(6-*N*-fluorenylcarbonylamino)hexanoyl]serine benzyl ester. To a solution of **15** (427.8 mg, 0.806 mmol) was added imidazole

(78.8 mg, 0.97 mmol), and *t*-butyldiphenylsilyl chloride (0.30 mL, 0.97 mmol). After stirring at rt for 15 h, the reaction mixture was poured into brine (10 mL) and extracted with EtOAc:Et_2O (1:1, 3×50 mL). The combined organic layer was dried and concentrated. The crude oily product was purified by flash column chromatography (hexanes:EtOAc, 1:1) to give (*S*)-[*O*-*tert*-butyldiphenylsiloxymethyl-*N*-(6-*N*-fluorenylcarbonylamino)hexanoyl]serine benzyl ester (0.429 g, 69%): $[\alpha]_D^{20}$ +11.5° (*c* 1.05, $CHCl_3$); IR 3332, 3068, 2932, 1719, 1663, 1520, 1112 cm^{-1}; ^1H NMR: δ 7.78–7.52 (d, *J* = 7.5 Hz, 2H), 7.60–7.54 (m, 6H), 7.45–7.31 (m, 10H), 6.29–6.28 (d, *J* = 7.9 Hz, 1H), 5.19 (s, 2H), 4.87 (br, 1H), 4.77–4.74 (m, 1H), 4.71–4.00 (d, *J* = 6.9 Hz, 2H), 4.24–4.22 (d, *J* = 6.7 Hz, 1H), 4.20–4.17 (dd, *J* = 10.3, 2.6 Hz, 1H), 3.22–3.18 (q, *J* = 6.5 Hz, 2H), 2.20–2.18 (q, *J* = 3.5 Hz, 2H), 1.67–1.64 (m, 3H), 1.54–1.51 (m, 2H), 1.39–1.36 (m, 2H), 1.03 (s, 9H); ^{13}C NMR: δ 172.4, 170.4, 156.4, 144.0, 141.3, 135.5, 128.6, 128.4, 128.3, 127.8, 127.8, 127.6, 127.0, 125.0, 119.9, 67.4, 66.5, 64.4, 54.1, 47.3, 40.7, 36.2, 29.6, 26.7, 26.2, 25.0, 19.3.

(*S*)-[*O*-*tert*-Butyldiphenylsiloxymethyl-*N*-(6-*N*-fluorenylmethoxycarbonylamino)hexanoyl]serine (16). To a solution of (*S*)-[*O*-*tert*-butyldiphenylsiloxymethyl-*N*-(6-*N*-fluorenylcarbonylamino)hexanoyl]serine benzyl ester (0.407 mg, 0.529 mmol) in MeOH (5 mL) was added palladium on activated carbon catalyst (0.102 g). After stirring at rt under a hydrogen atmosphere for 1 h and 15 min, the catalyst was removed by vacuum filtration through a celite pad. The solvent was removed and the crude product was stirred in vacuo to give **16** (0.336 g, 94%) as a white foam material and was used without further purification: $[\alpha]_D^{20}$ +5.36° (*c* 1.18, $CHCl_3$); IR 3320, 2932, 1722, 1656 cm^{-1}; ^1H NMR: δ 7.77–7.76 (d, *J* = 7.5 Hz, 2H), 7.62–7.56 (m, 8H), 7.43–7.29 (m, 8H), 6.37–6.36 (d, *J* = 7.7 Hz, 1H), 4.89 (br s, 1H), 4.72–4.69 (dd, *J* = 11.2, 3.4 Hz, 2H), 4.41–4.17 (m, 2H), 3.93–3.91 (d, *J* = 10.3 Hz, 1H), 3.18–3.15 (t, *J* = 6.4 Hz, 2H), 2.22–2.19 (t, *J* = 7.4 Hz, 2H), 1.66–1.62 (m, 2H), 1.52–1.49 (m, 2H), 1.36–1.33 (m, 2H), 1.05 (s, 9H); ^{13}C NMR: δ 173.3, 173.1, 144.0, 141.3, 135.5, 135.4, 130.0, 127.8, 127.6, 127.0, 125.0, 119.9, 66.5, 63.9, 53.9, 47.3, 40.8, 36.1, 29.6, 26.8, 26.1, 25.0, 19.3; HRFABMS calcd for $C_{40}H_{47}N_2O_6Si$ 679.3203, found 679.3201.

(2*R*,4*S*)-2-*tert*-Butyldimethylsiloxymethyl-4-[(*S*)-*O*-*tert*-butyldiphenylsiloxymethyl-*N*-(6-*N*-fluorenylmethoxycarbonylserylamino]-6-methyl-1,2-oxiranylheptane (17). To a solution of **16** (63.8 mg, 0.094 mmol) in CH_2Cl_2 (2 mL), was added HATU (50.0 mg, 0.130 mmol), HOAt (18.0 mg, 0.130 mmol) and diisopropylethylamine (71.0 μL, 0.41 mmol). After 12 min, a solution of epoxy-β-aminoketone **11** (18.9 mg, 0.063 mmol) in CH_2Cl_2 (2 mL) was added and stirred at rt for 8 h. The reaction mixture was concentrated and purified by flash column chromatography (CH_2Cl_2:MeOH, 97:3) to give **17** (45.3 mg, 75%): $[\alpha]_D^{20}$ +12.35° (*c* 0.58, $CHCl_3$); IR 3304, 2930, 1723, 1649, 1514, 1113 cm^{-1}; ^1H NMR (300 MHz): δ 7.77–7.71 (m, 2H), 7.67–7.62 (m, 2H), 7.56–7.28 (m, 14H), 7.05–7.03 (d, *J* = 9 Hz, 1H) 6.80–6.77 (d, *J* = 8.2 Hz, 1H), 6.21–6.19 (d, *J* = 6.6 Hz, 1H), 4.87 (br, 1H), 4.70–4.64 (t, *J* = 9.8 Hz, 1H), 4.61–4.56 (m, 1H), 4.50–4.41 (t, *J* = 12.2 Hz, 1H), 4.40–4.38 (d, *J* = 6.9 Hz, 1H), 4.23–4.19 (t, *J* = 6.6 Hz, 1H), 4.06–3.99 (m, 2H), 3.75–3.67 (m, 1H), 3.56–3.55 (d, *J* = 4.5 Hz, 1H), 3.53–3.49 (t, *J* = 4.6 Hz, 1H), 3.19–3.13 (q, *J* = 5.2 Hz, 2H), 3.01–2.99 (d, *J* = 4.8 Hz, 1H), 2.16–2.12 (t, *J* = 7.0 Hz, 2H), 1.71–1.60 (m, 8H), 1.58–1.48 (m, 2H), 1.39–1.28 (m, 4H), 1.22–1.20 (d, *J* = 6.0 Hz, 6H), 1.06 (s, 9H), 1.0–0.98 (d, *J* = 6.0 Hz, 3H), 0.98–0.94 (t, *J* = 5.5, 6H), 0.87 (s, 9H), 0.06–0.04 (d, *J* = 3.8 Hz, 6H); ^{13}C NMR (75 MHz): δ 206.4, 172.8, 170.3, 156.5, 144.1, 141.4, 135.6, 135.5, 130.1, 128.0, 127.9, 127.9, 127.7, 127.1, 125.1, 120.0, 66.5, 63.7, 63.0, 62.0, 54.1, 51.1, 48.5, 47.3, 40.8, 39.6, 38.7, 36.3, 29.7, 26.9, 25.8, 25.1, 23.4, 21.1, 19.3; HRFABMS calcd for $C_{55}H_{76}N_3O_8Si_2$ 962.5171, found 962.5171.

(2*R*,4*S*)-2-Hydroxymethyl-4-[(*S*)-*N*-(6-aminohexanoyl)serylamino]-6-methyl-1,2-oxiranylheptane. To a solution of **17** (11.8 mg, 0.0122 mmol) in THF (0.5 mL) was added tetrabutylamonium fluoride (76.0 μL, 0.5 M in THF, 0.0378 mmol). After 1 h, the solvent was removed and crude product was stirred in vacuo for 2 h to give (2*R*,4*S*)-2-hydroxymethyl-4-[(*S*)-*N*-(6-aminohexanoyl)serylamino]-6-methyl-1,2-oxiranylheptane and was used without further purification.

Dihydroeponemycin-biotin (5). To a solution of (2*R*,4*S*)-2-hydroxymethyl-4-[(*S*)-*N*-(6-aminohexanoyl)serylamino]-6-methyl-1,2-oxiranylheptane (0.0122 mmol) in DMSO (0.5 mL) and CH_2Cl_2 (0.1 mL) was added *N*-hydroxysuccinimide-biotin reagent (8.8 mg, 0.018 mmol). After stirring at rt for 8 h, the solvent was removed in vacuo and the crude product was purified by flash column chromatography (CH_2Cl_2:MeOH, 80:20) to give **5** (7.20 mg, 81% over two steps): $[\alpha]_D^{20}$ +17.2° (*c* 0.25, $CHCl_3$); IR 3307, 2925, 2479, 1717, 1684, 1653, 1646, 1457, 1260 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$ with 7% CD_3OD): δ 7.11–7.10 (br, 1H), 4.22–4.38 (t, *J* = 5.9, 1H), 4.37–4.38 (m, 1H), 4.23–4.18 (t, *J* = 5.7 Hz, 1H), 3.76–3.69 (m, 1H), 3.53–3.46 (t, *J* = 10.9 Hz, 1H), 3.28–3.24 (d, *J* = 12.0 Hz, 1H), 3.14–3.04 (m, 2H), 2.96–2.94 (d, *J* = 4.8 Hz, 1H), 2.84–2.78 (dd, *J* = 13.0, 4.8 Hz, 1H), 2.64–2.60 (d, *J* = 12.4 Hz, 1H), 2.22–2.11 (m, 1H), 2.09–2.03 (t, *J* = 6.1 Hz, 1H), 1.53–1.50 (m, 6H), 1.41–1.22 (m, 4H), 1.20-1.10 (m, 4H), 0.84–0.82 (d, *J* = 5.9 Hz, 3H), 0.76–0.74 (d, *J* = 6.9 Hz, 3H); HRFABMS calcd for $C_{34}H_{59}N_6O_9S$ 727.4064, found 727.4042.

HUVEC cell culture and dihydroeponemycin-biotin addition. Human umbilical cord venous endothelial cells (HUVECs) were kindly supplied by J. Pober, (Yale Boyer Center for Molecular Medicine) and grown on collagen-coated plastic culture plates in Dulbecco's Modified Eagle's Medium (DMEM) plus 75 μg/mL Endothelial Cell Growth Supplement (ECGS, Sigma Chemicals), 50 Units/L of penicillin and 50 Units/L streptamicin at 5% CO_2, 3 °C. Confluent cells were routinely split 1:3 every three days. HUVECs grown to 70% confluency were treated for 6 h with different concentrations of dihydroeponemycin-biotin. In parallel HUVECs were preincubated for 1 h with 25 μM of dihydroeponemycin **4** before a subsequent 6 h incubation with **5**. Treated and control cells were lysed in sodium dodecyl sulfate (SDS) polyacrylamide gel sample buffer and boiled before separation by 12% SDS–PAGE. Gels were electrophoretically transferred to PVDF membrane and probed using enhanced chemiluminescence (ECL, Amersham) and streptavidin-conjugated horseradish peroxidase (Sigma).

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