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MAN-DESIGNED BLEOMYCINS. SYNTHESIS OF DIOXYGEN ACTIVATING MOLECULES AND A DNA CLEAVING MOLECULE BASED ON BLEOMYCIN-Fe(II)-O_2 COMPLEX[1]

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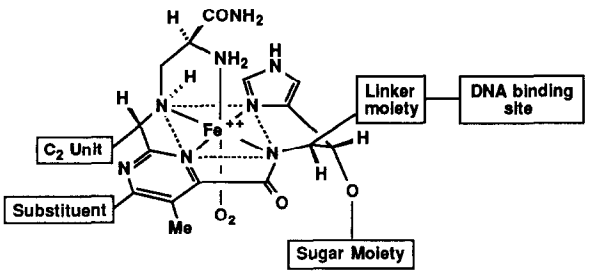
Abstract - A synthetic model for the metal binding site of bleomycin with a 4-methoxypyridine nucleus and a tert-butyl group is shown to be comparable to bleomycin in terms of dioxygen activation. This model ligand is coupled with a DNA affinity moiety, tetrapeptide S, to afford a man-designed bleomycin which exhibits potent DNA cleaving activity in vitro.

Bleomycins (BLMs) are a family of antitumor antibiotics used in the clinical treatment of Hodgkin's lymphoma, carcinomas of the skin, head and neck, and tumors of testis.[2-5] BLM comprises a linear hexapeptide and a disaccharide and each part of the molecule appears to play its specified role co-operatively to exert the antitumor activity. While the β-aminoalanine-pyrimidine-β-hydroxy-histidine region contributes to the iron chelation and subsequent dioxygen activation, the bithiazole-terminal amine moiety recognizes and binds to DNA, inducing the base sequence specific cleavage of DNA (Figure 1).[6] The

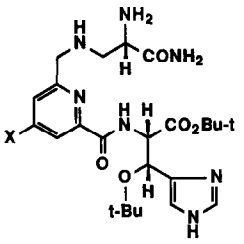
Figure 1. Implication for the role of each functional moiety of BLM.



disaccharide moiety seems to be significant not only for the formation of a molecular cavity to accommodate dioxygen but also for the membrane permeability at the cell surface. The BLM-Fe(II)-O_2 complex system cleaves isolated DNA specifically at G-C (5'→3') and G-T (5'→3') sequences[7] and also can preferentially degrades the DNA sequence in open chromatin within isolated nuclei.[8] In our model study on the metal binding site of BLM, we demonstrated that the pyrimidine ring of BLM can be replaced by a pyridine and dioxygen activating capability of the model ligand is markedly enhanced by the introduction of a tert-butyl group in place of the disaccharide (i. e., PYML-4,

Figure 2). We now investigated the structural optimization of model ligands for dioxygen activating capability and exploited sequence specific cleaving agents of DNA based on BLM.[10]

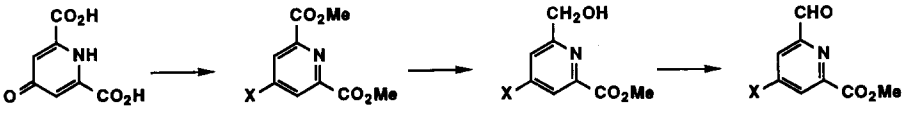
Figure 2. Synthetic models for the metal binding site of BLM.



In the BLM-Fe(II)-O_2 complex, the two-electron reduction of dioxygen to generate "activated BLM" has been well documented, suggesting a co-operative electron transfer from the ligand (donor) through iron to oxygen (acceptor).[6,11] Electronic property of the ligand around the iron center seemed to exert a profound effect in oxygen activation. We assumed that the increased π-electron density on the N-atom of the pyridine ring would increase the capacity to activate dioxygen for the Fe(II) complex, and vice versa. In fact, Hückel molecular orbital calculation indicated that frontier electron density of 4-methoxypyridine is close to that of 4-amino-3-methylpyrimidine of BLM and that there are no π-electron density at N-1 of 4-chloropyridine.[10a] Thus, we designed models having an electron donating methoxyl substituent and an electron withdrawing chloro substituent (PYML-6 and -7, Figure 2).

Methoxy- and chloropyridines were prepared as shown in Scheme 1. Chelidamic acid **1** was converted into methoxy diester **2** in 79% yield by treatment with diazomethane. Methoxypyridine aldehyde **6** was obtained by partial reduction of **2** with sodium tetrahydroborate (85%) followed by the Swern oxidation (78%). Chloropyridine aldehyde **7** was also obtained from the known **3**[12] via the similar tetrahydroborate reduction (89%) - the Swern oxidation (99%) sequence. PYML-6 and -7 were prepared as shown in Scheme 2. Aldehyde **6** was treated with amine **8**[13] and the resulting Schiff base was hydrogenated over Pd-C, affording amine **9** in 89% yield. The Boc protective group of **9** was then replaced by a o-nitrophenylthio (Nps) group by treatment with TFA followed by Nps-Cl[14] to give bis(Nps) derivative **11** in 86% overall yield. The acid **13**, obtained by hydrolysis of **11** in 62% yield, was treated with amine **15** in the presence of

Scheme 1. Synthesis of 4-substituted pyridinealdehydes.



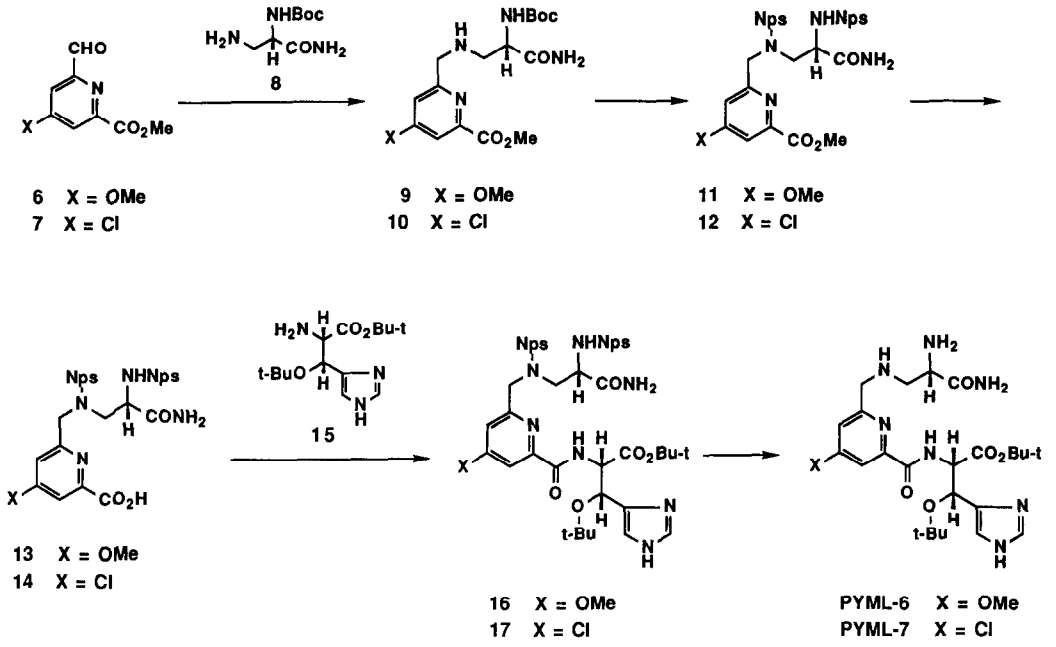
1

2 X = OMe
3 X = Cl

4 X = OMe
5 X = Cl

6 X = OMe
7 X = Cl

Scheme 2. Synthesis of PYML-6 and -7.

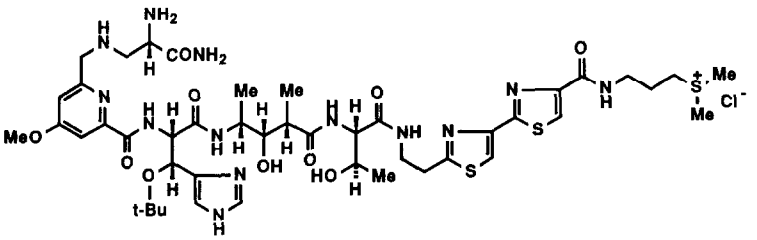


DCC-HOBt to afford dipeptide **16** in 36% yield based on **15**. Treatment of **16** with aqueous acid gave PYML-6 in 57% yield. PYML-7 was obtained by the similar procedure starting with chloropyridine **7**.

Oxygen activation of the model ligands were monitored by ESR spin trapping experiments using N-tert-butyl-α-phenylnitrone. It was gratifying to find that PYML-6, with a methoxyl substituent, showed oxygen activating capability virtually equivalent (97%) to that of BLM.[15] On the other hand, an electron withdrawing chloro group induced a reverse effect in PYML-7, less active (55%) than the unsubstituted PYML-4, suggesting a significant influence of the π-electron density at the N atom of the pyridine ring on the dioxygen activation.[15]

It was considered that the overall effect of the sterically bulky tert-butyl group and the electron donating methoxyl substituent in PYML-6 induced a metal binding and oxygen activating property comparable to BLM.[16] This finding provided a basis for the exploitation of sequence specific cleaving agents of DNA and new anticancer compounds if the sugar moiety is not critical

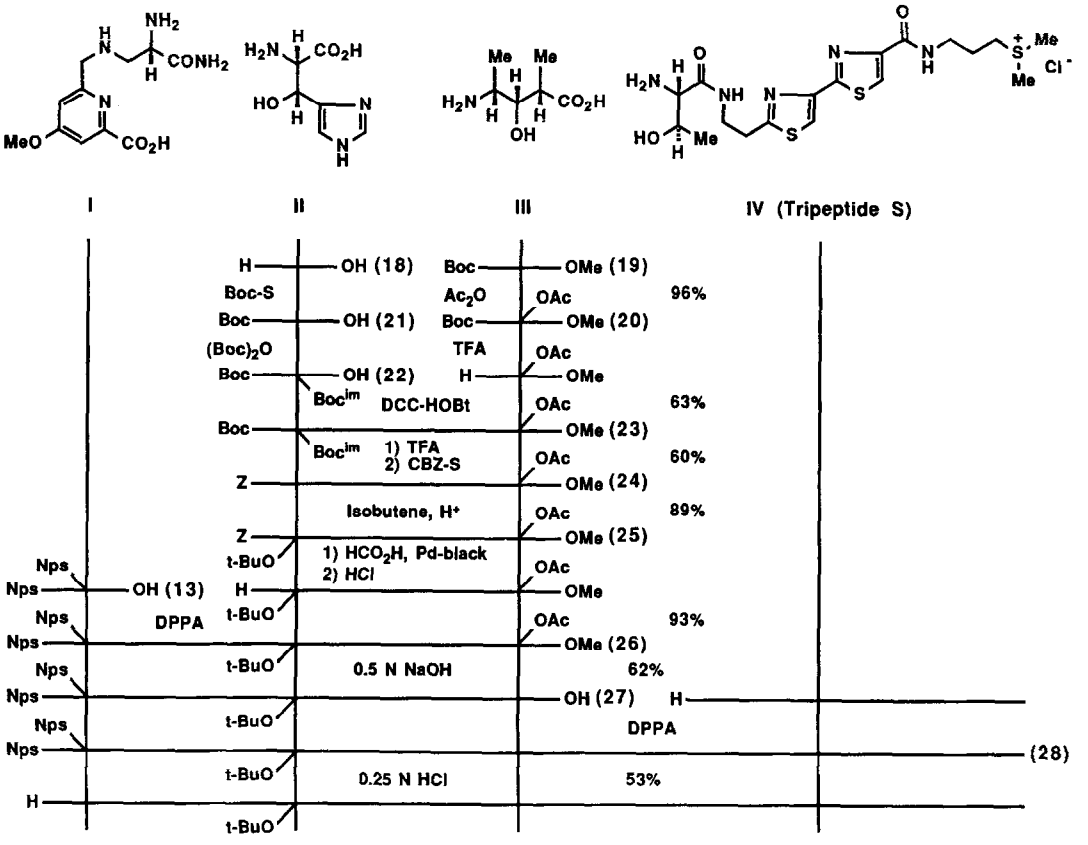
Figure 3. PYML(6)-bleomycin.



for the permeability through the cell membrane. Thus, we designed PYML(6)-bleomycin (Figure 3) wherein the DNA binding site, tetrapeptide S, was introduced into the PYML-6 skeleton.

PYML(6)-bleomycin was synthesized as shown in Scheme 3. The strategy of the synthesis was based on the coupling of three fragments, I, II-III and IV. The fragment I, methoxypyridine derivative **13**, was already prepared (see above). For the synthesis of the fragment **II-III**, the amino group and the imidazole nitrogen of erythro-β-hydroxy-L-histidine **18** were succesively treated with BOC-S[17] and $(Boc)_2O$[18] to afford **22**. On the other hand, methyl (2S,3S,4R)-4-[(tert- butoxycarbonyl)aminc]-3-hydroxy-2-methylpentanoate **19**[19] was reacted with acetic anhydride to give the corresponding acetate **20** (96%), which was treated with TFA followed by a coupling with the acid component **22** (DCC-HOBt). Thus, dipeptide **23** was obtained in 63% yield based on **18**. The tert-butoxycarbonyl amino protection of **23** was replaced by a benzyloxycarbonyl group (TFA followed by CBZ-S, 60%), before introducing the O-tert-butyl group under an acidic condition (isobutene, H^+, 89%). The fragment **II-III** obtained by the removal of the amino protective group (20% HCO_2H, Pd-black) was coupled with the fragment **I** acid, **13** (DPPA,[20] 93%). The resulting tripeptide **I-II-III**, **26**, was hydrolyzed to the acid **27** (0.5 M NaOH, 62%), which was then coupled with the fragment **IV** (tripeptide S) (DPPA). The two Nps groups of the hexapeptide **28** were carefully removed with 0.25 M HCl-EtOAc in the presence of 3-methylindole. To facilitate an efficient purification, the crude PYML(6)-bleomycin thus

Scheme 3. Synthesis of PYML(6)-bleomycin.



obtained was converted into the Cu(II) complex and subjected to liquid chromatography on a column of CM-Sephadex C-25. The blue eluant was treated with an excess of EDTA at pH 2-4.5 and purified on a column of Amberlite XAD-2, affording the PYML(6)-bleomycin (53% from **27**).

PYML(6)-bleomycin exhibited metal binding properties closed to those of BLM and showed an evident DNA cleaving activity (about 10% of that of BLM) in the presence of iron, molecular oxygen and reducing agent.[15] These results shows that, in order to cause an effective DNA strand scission, a DNA affinity site is essential for the PYML ligands to deliver a metal center to an appropriate site of the DNA helix.[21] Whereas the BLM-Fe(II) complex system preferentially attacks guanine-pyrimidine (5'→3') such as G-C and G-T sequences, PYML(6)-bleomycin reveals a somewhat random cleavage pattern.[15] Some of the structural changes might induce a slight deviation with respect to DNA base recognition. Although the membrane permeability must be very critical for the anticancer activity, the present study is believed to provide a basis for design and development of sequence-specific DNA cleaving molecules and some useful BLM analogues.[22]

EXPERIMENTAL

Melting points were measured on a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP 140 instrument. ^1H NMR (100 MHz) spectra were recorded on a JEOL FX-100 spectrometer. Abbreviations are as follows; s (singlet), d (doublet), t (triplet) and q (quartet). IR spectra were recorded on a JASCO DS-402G or JASCO A-102 spectrometer. Low resolution and fast atom bombardment mass spectra (FABMS) were recorded on a JEOL JMS DX-300 spectrometer. HPLC was carried on a JASCO TRI ROTAR SR model equipped with a 254 nm fixed-wavelength detector. The reagents and solvents were purified by standard procedures.

Dimethyl 4-Methoxypyridine-2,6-dicarboxylate **2**.

A solution of CH_2N_2 (ca 2.5 g) in Et_2O was added to a stirred suspension of **1**·H_2O (4.0 g, 19.9 mmol) in Et_2O (300 ml) at room temperature. The mixture was stirred for 30 min then additional CH_2N_2 (ca 2.5 g) in Et_2O and BF_3·OEt_2 (0.5 ml) was added. After being stirred for 1h at room temperature, excess CH_2N_2 was removed by N_2 bubbling and CH_2Cl_2 (200 ml) was added. The resulting solution was filtered and concentrated <u>in vacuo</u> to yield a white solid, which was recrystallized from hot water to give **2** as colorless needles (3.54 g, 79%).

Mp 126.2-127.1°C; ^1H NMR ($CDCl_3$, TMS) δ 3.98 (3H, s), 4.08 (6H, s), 7.80 (2H,s); IR (KBr) 2950, 1720, 1708, 1601, 1584, 1383 cm^{-1}; MS m/e 225 (M^+).

Dimethyl 4-Chloropyridine-2,6-dicarboxylate **3**.

Obtained by a known procedure[12] (81%).

Mp 142.2-142.4°; ^1H MNR ($CDCl_3$, TMS) δ 4.03 (6H, s), 8.27 (2H, s); IR (KBr) 1738, 1722, 1576, 1339 cm^{-1}; MS m/e 230 (M^+), 232 (M^++2).

Methyl 6-Hydroxymethyl-4-methoxypyridine-2-carboxylate **4**.

$NaBH_4$ (400 mg, 10.6 mmol) was added to a stirred solution of **2** (1.53 g, 6.79 mmol) in MeOH (150 ml) at 0°C. After being stirred for 3 h at room temperature, the solution was neutralized with HCl (0.5 M) at 0°C. This was concentrated <u>in vacuo</u> and purified by flash chromatography eluted with AcOEt to give **4** as colorless needles (1.10 g, 85%).

Mp 127.3-127.5°C; ^{1}H NMR ($CDCl_3$, TMS) δ3.66 (1H, br s), 3.92 (3H, s), 3.98 (3H, s), 4.80 (2H, s), 7.04 (1H, d, J = 3 Hz), 7.55 (1H, d, J = 3 Hz); IR (KBr) 3370, 3300, 1738, 1600, 1564, 1482 cm^{-1}; MS m/e 197 (M^+).

Methyl 4-Chloro-6-hydroxymethylpyridine-2-carboxylate **5**.

Obtained by the same procedure as that for **4** starting with **3** (814.9 mg, 3.55 mmol). The final purification by flash chromatography eluted with 5% MeOH/CH_2Cl_2 gave **5** as white crystals (636.2 mg, 89%).

Mp 118.2-119.1°C; ^{1}H NMR ($CDCl_3$, TMS) δ3.41 (1H, br s), 4.00 (3H, s), 4.86 (2H, s), 7.57 (1H, d, J = 4 Hz), 7.98 (1H, d, J = 4 Hz); IR (KBr) 3460, 1732, 1718, 1578 cm^{-1}; MS m/e 201 (M^+), 203 (M^++2).

Methyl 6-Formyl-4-methoxypyridine-2-carboxylate **6**.

DMSO (0.95 ml, 13.4 mmol) was added to a stirred solution of $(COCl)_2$ (0.59 ml, 6.72 mmol) in CH_2Cl_2 (15 ml) at -30°C over 5 min under argon. After 15 min the solution was cooled to -60°C, then a solution of **4** (687.7 mg, 3.49 mmol) in CH_2Cl_2 (10 ml) was added over 5 min. The mixture was stirred at -60°C for 25 min and Et_3N (3.7 ml, 26.9 mmol) was added. The mixture was allowed to warm to room temperature and partitioned betweed CH_2Cl_2 and water. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash chromatography eluted with 30% AcOEt/hexane to give **6** as pale yellow needles (532.4 mg, 78%).

Mp 116.5-117.1°C; ^{1}H NMR ($CDCl_3$, TMS) δ 4.00 (3H, s), 4.05 (3H, s), 7.59 (1H, d, J = 3 Hz), 7.82 (1H, d, J = 3 Hz), 10.15 (1H, s); IR (KBr) 2975, 1728, 1702, 1695, 1478, 1382 cm^{-1}; MS m/e 195 (M^+).

Methyl 4-Chloro-6-formylpyridine-2-carboxylate **7**.

Obtained by the same procedure as that for **6** starting with **5** (616.5 mg, 3.06 mmol). The final purification by flash chromatography eluted with 5% MeOH/CH_2Cl_2 gave **7** as pale yellow crystals (606.6 mg, 99%).

Mp 126.8-127.2°C; ^{1}H NMR ($CDCl_3$, TMS) δ 4.05 (3H, s), 8.08 (1H, d, J = 3 Hz), 8.28 (1H, d, J = 3 Hz), 10.07 (1H, s); IR (KBr) 3070, 1725, 1702, 1571, 1448 cm^{-1}; MS m/e 199 (M^+), 201 (M^++2).

Methyl 6-[[N-[(S)-2-Carbamoyl-2-[(*tert*-butoxycarbonyl)amino]ethyl]amino]-methyl]-4-methoxypyridine-2-carboxylate **9**.

Obtained according to the reported procedure[23] starting with **6** (132.1 mg, 0.677 mmol). The final purification by flash chromatography eluted with 5% MeOH/CH_2Cl_2 gave **9** as a colorless oil (230.1 mg, 89%).

$[\alpha]_D^{22}$ +12.1° (c 0.60, MeOH); ^{1}H NMR ($CDCl_3$, TMS) δ1.44 (9H, s), 2.76 (1H, dd, J = 7, 12 Hz), 3.15 (1H, dd, J = 5, 12 Hz), 3.92 (3H, s), 3.98 (3H, s), 3.99 (2H, s), 4.18 (1H, ddd, J = 7, 7, 5 Hz), 5.84 (1H, d, J = 7 Hz), 6.08 (1H, br s), 7.03 (1H, d, J = 3 Hz), 7.54 (1H, d, J = 3 Hz); IR (neat) 2960, 1730, 1710, 1678, 1600, 1489 cm^{-1}; FABMS m/e 383 (MH^+).

Methyl 6-[[N-[(S)-2-Carbamoyl-2-[(*tert*-butoxycarbonyl)amino]ethyl]amino]-methyl]-4-chloropyridine-2-carboxylate **10**.

A solution of **7** (100.0 mg, 0.501 mmol) and amine **8**[13] (154 mg, 0.758 mmol) in CH_3CN (6 ml) was vigorously stirred in the presence of an activated molecular sieve 3A (1 g) at room temperature for 12 h under argon. The mixture was filtered through celite and the filter cake was washed with CH_3CN (6 ml). The combined CH_3CN solutions was concentrated *in vacuo* below 30°C and the resulting

Schiff base was dissolved in MeOH (8 ml). $NaBH_4$ (11.4 mg, 0.305 mmol) was added to the solution at 0°C. The mixture was stirred at room temperature for 20 min, neutralized with citric acid (1 M) at 0°C, and the MeOH was removed in vacuo. The residual aqueous solution was partitioned between AcOEt and water. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by flash chromatography eluted with 5% MeOH/CH_2Cl_2 to give **10** as a clorless oil (191.6 mg, 99% based on **7**).

$[\alpha]_D^{22}$ +11.1° (c 0.94, MeOH); ^{1}H NMR ($CDCl_3$, TMS) δ 1.46 (9H, s), 2.78 (1H, dd, J = 7, 12 Hz), 3.24 (1H, dd, J = 12, 4.5 Hz), 4.01 (3H, s), 4.04 (2H, s), 4.24 (1H, ddd, J = 7, 6, 4.5 Hz), 5.76 (1H, d, J = 6 Hz), 5.80 (1H, s), 7.55 (1H, d, J = 3 Hz), 7.98 (1H, d, J = 3 Hz); IR (neat) 2970, 1723, 1670, 1575, 1440 cm^{-1}; FABMS m/e 388 (MH^+), 390 (MH^++2).

Methyl 6-[[N-[(S)-2-Carbamoyl-2-[(o-nitrophenylthio)amino] ethyl] -N-(o-nitrophenylthio)amino] methyl] -4-methoxypyridine-2-carboxylate **11**.

Trifluoroacetic acid (1 ml) was added to a solution of **9** (61.6 mg, 0.161 mmol) in CH_2Cl_2 (1 ml) at 0°C under argon. The solution was stirred at 0°C for 30 min then at room temperature for 1 h. The mixture was concentrated in vacuo below 25°C and the residual oil was dissolved in CH_2Cl_2 (2 ml). Nps-Cl[14] (79 mg, 0.419 mmol) and Et_3N (0.15 ml, 1.05 mmol) were successively added to the solution at 0°C under argon. After being stirred at 0°C for 30 min then at room temperature for 6 h, the mixture was partitioned between AcOEt and water. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by flash chromatography eluted with 5% MeOH/CH_2Cl_2 to give **11** as a yellow solid (81.6 mg, 86%).

$[\alpha]_D^{22}$ +53.3° (c 0.73, MeOH); ^{1}H NMR ($CDCl_3$, TMS) δ 3.60-3.91 (3H, m), 3.88 (3H, s), 3.92 (3H, s), 4.49 (2H, s), 5.77 (1H, br s), 6.75-8.36 (10H, m); IR (KBr) 3450, 1723, 1676, 1595, 1508 cm^{-1}; FABMS m/e 589 (MH^+).

Methyl 6-[[N- [(S)-2-Carbamoyl-2-[(o-nitrophenylthio)amino] ethyl] -N-(o-nitrophenylthio)amino] methyl] -4-chloropyridine-2-carboxylate **12**.

Obtained by the same procedure as that for **11** starting with **10** (191.6 mg, 0.495 mmol) and Nps-Cl (282 mg, 1.49 mmol) in 90% yield (yellow solid, 264.8 mg).

^{1}H NMR ($CDCl_3$, TMS) δ 3.54-3.88 (3H, m), 3.96 (3H, s), 4.54 (2H, s), 5.77 (1H, br s), 7.16-8.36 (10H, m); IR (KBr) 1732, 1678, 1579, 1526, 1458 cm^{-1}.

6-[[N- [(S)-2-Carbamoyl-2- [(o-nitrophenylthio)amino] ethyl] -N-(o-nitrophenylthio)amino] methyl] -4-methoxypyridine-2-carboxylic Acid **13**.

LiOH (0.1 M, 6.8 ml) was added to a stirred solution of **11** (198.0 mg, 0.337 mmol) in THF (4.2 ml) at 0°C. After being stirred for 3 h at room temperature, water (10 ml) was added to the solution and the THF was removed in vacuo. The resulting aqueous solution was acidified with citric acid (0.1 M) to pH 4.5 at 0°C. Yellow precipitate **13** was collected, washed with a small amount of water and dried (119.5 mg, 62%). It was used for the next step without further purification.

N^{α} - [6- [[N- [(S)-2-Carbamoyl-2- [(o-nitrophenylthio)amino] ethyl] -N-(o-nitrophenylthio)amino] methyl] -4-methoxypyridine-2-carbonyl] -erythro-β -tert-butoxy-L-histidine tert-Butyl Ester **16**.

HOBt·H_2O (63.7 mg, 0.416 mmol), DCC (51.5 mg, 0.249 mmol), N-methylmorpholine (45.7 μ l, 0.416 mmol) and a solution of **15** (69.1 mg, ca 0.194

mmol) in DMF (5 ml) were successively added to a solution of **13** in DMF (5 ml) at 0°C under argon. After being stirred at 0°C for 1 h then at room temperature for 12 h, the mixture was concentrated in vacuo. AcOEt (10 ml) was added to the residue and insoluble materials were filtered off. The filtrate was successively washed with water, aq $NaHCO_3$ and water, and dried over Na_2SO_4. The solution was concentrated in vacuo. The residue was purified by flash chromatography eluted with 3% MeOH/$CHCl_3$ followed by liquid chromatography on alumina column eluted with 10% MeOH/$CHCl_3$ to give **16** as yellow powder (57.9 mg, 36% based on **15**).

$[\alpha]_D^{22}$ +29.7° (c 1.0, MeOH); ^{1}H NMR ($CDCl_3$, TMS) δ 1.25 (9H, s), 1.42 (9H, s), 3.83 (3H, s), 3.34-3.96 (3H, m), 4.38 (1H, d, J = 14 Hz), 4.54 (1H, d, J = 14 Hz), 5.05 (1H, dd, J = 4, 9 Hz), 5.24 (1H, d, J = 4 Hz), 6.14 (1H, br s), 6.76-8.32 (13H, m); IR (KBr) 1670, 1596, 1564, 1511, 1338 cm^{-1}; FABMS m/e 840 (MH^+).

N^{α}-[6-[[N-[(S)-2-Carbamoyl-2-[(o-nitrophenylthio)amino]ethyl]-N-(o-nitrophenylthio)amino]methyl]-4-chloropyridine-2-carbonyl]-erythro-β-tert-butoxy-L-histidine tert-Butyl Ester **17**.

Obtained by the same procedure as that for **16** starting with **12** (403.0 mg, 0.680 mmol) and **15** (83.8 mg, ca 0.235 mmol) in 63% yield (yellow powder, 139.2 mg).

$[\alpha]_D^{23}$ +39.9° (c 1.0, MeOH); ^{1}H NMR ($CDCl_3$, TMS) δ 1.25 (9H, s), 1.39 (9H, s), 3.34-4.04 (3H, m), 4.46 (1H, d, J = 15 Hz), 4.59 (1H, d, J = 15 Hz), 5.10 (1H, dd, J = 4, 9 Hz), 5.25 (1H, d, J = 4 Hz), 5.95 (1H, s), 6.82-8.39 (13H, m); IR (KBr) 1670, 1588, 1564, 1508, 1338 cm^{-1}; FABMS m/e 844 (M^+).

PYML-6.

Water (3 ml) and HBr (0.88 M, 0.8 ml) were added to a vigorously stirred solution of **16** (31.7 mg, 0.0377 mmol) in AcOEt (5 ml) at 0°C under argon. The resulting biphasic system was stirred at room temperature for 90 min. The aqueous layer was separated, washed three times with AcOEt, and charged on a column of Amberlite IRA 93 (OH^- form, 7 ml). The column was elued with water (15 ml). The eluant was concentrated in vacuo and freeze-dried to give PYML-6 as white powder (10.5 mg, 57%).

Rf 0.21 (AcOEt : EtOH : water = 3 : 1 : 1); $[\alpha]_D^{22}$ +1.0° (c 0.53, MeOH); ^{1}H NMR (D_2O) δ 1.32 (9H, s), 1.56 (9H, s), 3.13 (3H, m), 3.51 (1H, m), 3.73 (1H, m), 4.07 (3H, s), 4.17 (2H, s), 7.27 (1H, d, J = 2 Hz), 7.37 (1H, s), 7.61 (1H, d, J = 2 Hz), 7.96 (1H, s); IR (KBr) 2920, 1730, 1698, 1605, 1363 cm^{-1}; FABMS m/e 534 (MH^+).

PYML-7.

Obtained by the same procedure as that for PYML-6 starting with **17** (31.7 mg, 0.0375 mmol) in 78% yield (white powder 15.8 mg).

Rf 0.18 (AcOEt : EtOH : water = 3 : 1 : 1); $[\alpha]_D^{22}$ +11.4° (c 0.78, MeOH); ^{1}H NMR (D_2O) δ 1.37 (9H, s), 1.56 (9H, s), 2.93-3.21 (3H, m), 3.85 (2H, s), 4.17 (2H, s), 7.34 (1H, s), 7.78 (1H, d, J = 2 Hz), 7.87 (1H, s), 8.07 (1H, d, J = 2 Hz); IR (KBr) 2950, 1725, 1670, 1576, 1380 cm^{-1}; FABMS m/e 538 (M^+), 540 (M^++2).

Methyl (2S,3S,4R)-3-Acetoxy-4-[(tert-butoxycarbonyl)amino]-2-methylpentanoate **20**.

Acetic anhydride (0.25 ml, 2.63 mmol), pyridine (0.71 ml, 8.75 mmol), and a spatula tip of dimethylaminopyridine were successively added to a solution of

19[19] (456.6 mg, 1.75 mmol) in CH_2Cl_2 (7 ml) under argon. The solution was stirred overnight at room temperature and then partitioned between cold AcOEt and aq $NaHCO_3$. The organic layer was washed with cold HCl (1 M), water and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by flash chromatography eluted with 15% AcOEt/hexane to give **20** as a colorless oil (507.1 mg, 96%).

$[\alpha]_D^{23}$ +17.8° (c 1.6, $CHCl_3$); ^1H NMR ($CDCl_3$, TMS) δ 1.12 and 1.19 (6H, 2d, J = 7 Hz), 1.44 (9H, s), 2.10 (3H, s), 2.76 (1H, dq, J = 6, 7 Hz), 3.69 (3H, s), 3.86 (1H, m), 4.31 (1H, br d, J = 9.5 Hz), 5.11 (1H, dd, J = 6, 6.5 Hz); IR ($CHCl_3$) 3425, 2975, 1725, 1495, 1450, 1368, 1220 cm^{-1}; MS m/e 304 (MH^+).

Methyl (2S,3S,4R)-3-Acetoxy-4-[[N^{α},N^{im}-bis(tert-butoxycarbonyl)-erythro-β-hydroxy-L-histidyl]amino]-2-methylpentanoate **23**.

A solution of di-tert-butyl dicarbonate[18] (312.1 mg, 1.43mmol) in THF (2 ml) and N-methylmorpholine (0.172 ml, 1.56 mmol) were succesively added to a solution of **21**[24] (352.0 mg, 1.30 mmol) in DMF (2 ml) and THF (4 ml) at 0°C under argon. The solution was stirred at room temperature for 2 h to afford a solution containing **22**.

On the other hand, trifluoroaceic acid (5 ml) was added to a solution of **20** (456.6 mg, 1.75 mmol) in CH_2Cl_2 (5 ml) at 0°C under argon. The solution was stirred at room temperature for 30 min and concentrated in vacuo to give the TFA salt of the free amine. A solution of this amine in THF (9 ml), N-methylmorpholine (0.215 ml, 1.95 mmol), HOBt·H_2O (304.6 mg, 1.99 mmol) and DCC (423.8 mg, 2.05 mmol) were successively added to the solution of **22**, prepared above, at 0°C under argon. After being stirred at 0°C for 30 min and then at room temperature for 2 h, the mixture was diluted with Et_2O (15 ml) and filtered through celite. The filter cake was washed with Et_2O(10 ml). The combined organic solutions was concentrated in vacuo and the residue was partitioned between Et_2O and water. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by flash chromatography eluted with 50-60% AcOEt/hexane to give **23** as a white foam (452.8 mg, 63%). Regenerated **20** was also recovered (128.7 mg, 24%).

$[\alpha]_D^{21.5}$ +16.1° (c 1.25, $CHCl_3$); ^1H NMR ($CDCl_3$, TMS) δ1.02 and 1.12 (6H, 2d, J = 7 Hz), 1.45 (9H, s), 1.61 (9H, s), 2.05 (3H, s), 2.48 (1H, m), 3.65 (3H, s), 4.12 (2H, m), 4.55 (1H, dd, J = 5, 8 Hz), 5.02 (1H, m, contains 5Hz), 5.12 (1H, m), 6.07 (1H, br d, J = 8 Hz), 6.77 (1H, br d, J = 9.5 Hz), 7.35 (1H, s), 8.03 (1H, d, J = 1.2 Hz); IR ($CHCl_3$) 3310, 2985, 1757, 1740, 1668, 1490, 1372, 1253, 1151 cm^{-1}; FABMS m/e 557 (MH^+).

Methyl (2S,3S,4R)-3-Acetoxy-4-[(N^{α}-benzyloxycarbonyl-erythro-β-hydroxy-L-histidyl)amino]-2-methylpentanoate **24**.

Dipeptide **23** (362.9 mg, 0.652 mmol) was dissolved in trifluoroacetic acid (5 ml) at 0°C under argon. The solution was stirred at room temperature for 25 min, then concentrated in vacuo to afford the corresponding amine. To a suspension of this amine in CH_2Cl_2 (5 ml) were added Et_3N (0.318 ml, 2.28 mmol) and CBZ-S (214.6 mg, 0.782 mmol) at 0°C under argon. After 10 min an additional Et_3N (0.182 ml, 1.30 mmol) was added at 0°C. The mixture was stirred at room temperature for 3 h and diluted with CH_2Cl_2 (30 ml). The solution was washed with aq $NaHCO_3$ and brine, and dried over Na_2SO_4. The solution was concentrated in vacuo and the residue was purified by flash chromatography eluted with 5% MeOH/CH_2Cl_2 to give **24** as white wax (190.5 mg, 60%).

$[\alpha]_D^{22.9}$ +19.9° (c 0.725, $CHCl_3$); ^1H NMR ($CDCl_3$, TMS) δ 0.94 (3H, d, J =

6.8 Hz), 1.06 (3H, d, J = 7 Hz), 1.99 (3H, s), 2.51 (1H, m), 3.55 (3H, s), 4.09 (1H, m), 4.63 (1H, dd, J = 6, 8 Hz), 4.96-5.20 (2H, m), 5.04 (2H, s), 6.47 (1H, br d, J = 8 Hz), 6.80 (1H, s), 7.28 (5H, s), 7.36 (1H, s); IR ($CHCl_3$) 3330, 2910, 1737, 1666, 1508, 1372, 1235, 1210 cm^{-1}; FABMS m/e 491 (MH^+).

Methyl (2S,3S,4R)-3-Acetoxy-4-[(N^{α}-benzyloxycarbonyl-erythro-β-tert-butoxy-L-histidyl)amino]-2-methylpentanoate **25**.

A solution of **24** (132.8 mg, 0.271 mmol) in CH_2Cl_2 (40 ml), isobutene (15 ml) and c H_2SO_4 (0.3 ml) was stirred at room temperature for 3 days in a sealed bulb. The bulb was cooled to -78°C and opened. Excess Et_3N (5 ml) was immediately added and the solution was concentrated in vacuo to ca 10 ml. This was partitioned between AcOEt and aq $NaHCO_3$. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by chromatography on silica gel with AcOEt to give **25** as a white foam (132.1 mg, 89%).

$[\alpha]_D^{21}$ +43.9° (c 1.76, $CHCl_3$); ^1H NMR ($CDCl_3$, TMS) δ 1.08 (9H, s), 1.10 and 1.17 (6H, 2d, J = 7 Hz), 2.06 (3H, s), 2.09 (1H, dq, J = 5.3, 7 Hz), 3.62 (3H, s), 4.18 (1H, dq, J = 7, 7 Hz), 4.56 (1H, m), 4.88 (1H, d, J = 8 Hz), 4.96 (2H, s), 5.16 (1H, dd, J = 7, 5.3 Hz), 6.14 (1H, d, J = 8 Hz), 7.09 (1H, s), 7.27 (5H, s), 8.33 (1H, s); IR ($CHCl_3$) 3415, 3270, 2980, 1737, 1673, 1500, 1370, 1233, 1210 cm^{-1}; FABMS m/e 547 (MH^+).

Methyl (2S,3S,4R)-3-Acetoxy-4-[[N^{α}-[6-[[N-[(S)-2-carbamoyl-2-[(o-nitrophenylthio)amino]ethyl]-N-(o-nitrophenylthio)amino]methyl]-4-methoxypyridine-2-carbonyl]-erythro-β-tert-butoxy-L-histidyl]amino]-2-methylpentanoate **26**.

Formic acid (99%, 2 ml) and Pd-black (30 mg) were added to a solution of **25** (117.9 mg, 0.216 mmol) in MeOH (8 ml). After being stirred effectively at room temperature for 30 min under argon, the suspension was filtered. HCl (0.2 M, 3 ml) was added to the filtrate and the resulting solution was concentrated in vacuo. The residue was dissolved in 50% MeOH/toluene (2 ml) and the solution was concentrated azeotropically in vacuo to afford the amine component as white powder. To a solution of this amine in DMF (2 ml) were added a solution of the acid **13** (113.4 mg, 0.197 mmol) in DMF (5 ml), $DPPA^{20}$ (50.9 μl, 0.236 mmol) and Et_3N (99.4 μl, 0.713 mmol) successively at 0°C under argon. After being stirred at 0°C for 1 h then at room temperature for 12 h, the solution was concentrated in vacuo and the residue was partitioned between AcOEt and aq $NaHCO_3$. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by chromatography on silica gel eluted with 5% $MeOH/CH_2Cl_2$ to give **26** as a yellow wax (177.2 mg, 93%).

$[\alpha]_D^{21.7}$ +53.6° (c 0.74, $CHCl_3$); ^1H NMR ($CDCl_3$, TMS) δ 1.08 (3H, s), 1.14 (3H, s), 1.18 (9H, s), 2.02 (3H, s), 2.50 (1H, m), 3.63 (3H, s), 3.77 (2H, m), 3.86 (3H, s), 4.12 (2H, m), 4.49 (2H, br s), 4.87 (1H, dd, J = 7.2, 3.8 Hz), 5.12 (1H, dd, J = 5, 6.2 Hz), 5.26 (1H, d, J = 3.8 Hz), 6.22 (1H, br s), 6.86 (1H, m), 7.01 (1H, s), 7.11-7.96 (8H, m), 8.23 (2H, m); IR ($CHCl_3$) 3340, 2980, 1738, 1675, 1602, 1594, 1512, 1435, 1370, 1340, 1308, 1235, 1215 cm^{-1}; FABMS m/e 969 (MH^+).

(2S,3S,4R)-4-[[N^{α}-[6-[[N-[(S)-2-Carbamoyl-2-[(o-nitrophenylthio)-amino]-ethyl]-N-(o-nitrophenylthio)amino]methyl]-4-methoxypyridine-2-carbonyl]-erythro-β-tert-butoxy-L-histidyl]amino]-3-hydroxy-2-methylpentanoic Acid **27**.

NaOH (0.5 M, 1.06 ml, 0.53 mmol) was added to an ice cooled solution of **26** (102.9 mg, 0.106 mmol) in MeOH (4 ml) over 30 min. The solution was stirred at

10°C for 10 h, and then cooled again to 0°C, diluted with water (12 ml), and neutralized with citric acid (1 M, 0.5 ml). After evaporation of the MeOH in vacuo, the solution was acidified with citric acid (1 M) to pH 3.5 at 0°C and extracted twice with AcOEt. The combined AcOEt extracts was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by chromatography on silica gel eluted with 15% MeOH/CH_2Cl_2 to give **27** as yellow powder (59.7 mg, 62%) which was used for the next step without further purification.

[3-[[2'-[2-[[N-[(2S,3S,4R)-4-[[N^{α}-[6-[[N-[(S)-2-Carbamoyl-2-[(o-nitrophenylthio)amino]ethyl]-N-(o-nitrophenylthio)amino]methyl]-4-methoxy-pyridine-2-carbonyl]-erythro-β-tert-butoxy-L-histidyl]amino]-3-hydroxy-2-methyl-pentanoyl]-L-threonyl]amino]ethyl]-2,4'-bithiazole-4-carbonyl]amino]propyl]-dimethylsulfonium Chloride **28**.

Tripeptide S·HCl[24] (41.6 mg, 0.0785 mmol), DPPA (21.1 μl, 0.0981 mmol) and Et_3N (20.1 μl, 0.144 mmol) were successively addded to an ice-cooled solution of **27** (59.7 mg, 0.0654 mmol) in DMF (2 ml) under argon. After being stirred at 0°C for 1 h then at room temperature for 18 h, the mixture was concentrated in vacuo. The residue was partially purified by chromatography on silica gel eluted with AcOEt : EtOH : AcOH : water = 6 : 1 : 1 : 2 to give **28** as a yellow wax which was used for the next step without further purification.

PYML(6)-Bleomycin.

To a solution of the above **28** in water (2 ml) were added a solution of 3-methylindole (30 mg, 0.229 mmol) in AcOEt (5 ml) and HCl (0.5 M, 2 ml) successively at 0°C. The resulting biphasic system was efficiently stirred at room temperature for 1 h. The aqueous layer was collected and washed with AcOEt (5 x 5 ml). $Cu(OAc)_2 \cdot H_2O$ (20 mg, 0.100 mmol) was added to the aqueous layer and the solution was adjusted to pH 6.8 with NaOH (0.5 M) at 0°C. The resulting blue solution was allowed to warm to room temperature and stirred for a further 2 h, and then purified by chromatography on CM-Sephadex C-25 (200 ml; conditioned by 0.05 M sodium phosphate buffer, pH 6.8) eluted with a linear gradient of 0.05 M sodium phosphate (pH 6.8) and 1.05 M NaCl. Purity of each fraction was checked by HPLC (reverse phase SSC-ODS-171 column, 6 mm x 200 mm; 4% NH_4OAc : MeOH = 6 : 4; flow rate, 1 ml/min; retention time, 10.5 min) and homogeneous blue fractions were collected. $Na_2EDTA \cdot 2H_2O$ (600 mg, 1.61 mmol) was added to the solution. The resulting suspension was acidified with HCl (1 M) to pH 2 at 0°C and then adjusted to pH 4.5 with 1M NaOH. The clear solution was stirred at room temperature for further 30 min and charged on an Amberlite XAD-2 column (40 ml). The column was successively washed with 3% EDTA-2% NaCl solution (100 ml), 2% NaCl (100 ml) and water (100 ml), and then eluted with 20% HCl (0.002 M)/MeOH to give PYML(6)-bleomycin as white powder (36.6 mg, 53% based on **27**).

Rf 0.31 (10% NH_4OAc : MeOH = 1 : 1); $[\alpha]_D^{21}$ +33.5° (c 0.97, H_2O); ^1H NMR (D_2O) δ 1.68 (9H, s), 1.73 (9H, m), 2.70 (2H, m), 3.18 (1H, m), 3.49 (6H, s), 3.70 (3H, m), 3.95 (2H, br s), 3.99-4.92 (12H, m), 4.28 (3H, s), 5.30 (1H, m), 5.60 (1H, m), 7.41 (1H, s), 7.68 (1H, br s), 7.72 (1H, br s), 8.30 (1H, s), 8.33 (1H, br s), 8.62 (1H, s); IR (KBr) 3240, 2950, 2300, 1610, 1510, 1430 cm^{-1}; FABMS m/e 1047 (M^+).

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