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Man-Designed Bleomycins: Significance of the Binding Sites as Enzyme Models and of the Stereochemistry of the Linker Moiety†

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Key words: bleomycin; DNA cleavage; metal binding site; DNA binding site; linker moiety.

Abstract: *Comparison of the DNA cleavage activity of man-designed bleomycins demonstrates that bleomycins are small enzymes comprised of a catalytic site and a binding site. The linker moiety is shown to be significant for DNA binding, and inversion of its stereochemistry results in a dramatic decrease in the DNA-cleaving efficiency. One of the man-designed BLMs shows excellent cytotoxicity against L1210.*

The bleomycins (BLMs) are a family of glycopeptide antitumor antibiotics isolated from cultures of *Streptomyces verticillus* by Umezawa *et al.* in 1966, and are presently used for the clinical treatment of Hodgkin's lymphoma, carcinomas of the skin, head, and neck, and tumors of the testis.[1] The drug, a combination of an unusual hexapeptide and a disaccharide,[2] cleaves DNA preferentially at G-C (5'→3') and G-T (5'→3') sequences in the presence of oxygen and ferrous ion.[3] The molecule can be regarded as an integrated entity made of components endowed with the following functions; i) iron binding and dioxygen activation by the amine-pyrimidine-imidazole region, ii) stabilization of the iron-oxygen complex and contribution to membrane transport by the disaccharide, iii) interaction with certain nucleotide sequences by the bithiazole-terminal amine region, and iv) holding of each part of the molecule at an appropriate distance and in a suitable orientation by the linker group (Figure 1).

It is intriguing that BLM binds to DNA specifically and breaks it. Mizuno first referred to BLM as "a minimum sized naturally occurring molecule showing minimum sized enzyme function.[4a] Indeed, BLM appears to be analogous to an enzyme in that the metal-oxygen moiety corresponds to the catalytic site of enzymes and the bithiazole can be regarded as the substrate binding site. Although BLM (a hexapeptide-disaccharide) is much smaller than real enzyme (e.g., Eco RI endonuclease contains 276 amino acids), it is comparable, in size, to the cleft of the active site of enzyme (i. e., Eco RI recognizes DNA by six amino acid residues).[4b] Therefore, the structure of BLM may provide a model system for the active site of DNA-cleaving enzymes, such as nuclease or restriction enzymes. We considered that this enzymic character of BLM could be most reasonably investigated by a synthetic model approach. Previously we have demonstrated that a synthetic model for the iron-binding site of BLM having a 4-methoxypyridine and a *tert*-butyl substituent, namely PYML-6 (Figure 2), was able to activate molecular oxygen as efficiently as BLM.[5] This finding allowed us to design DNA-cleaving molecules based on PYML-6. We reported a G-specific nucleotide cleavage by the first man-designed BLM, PYML(6)-bleomycin

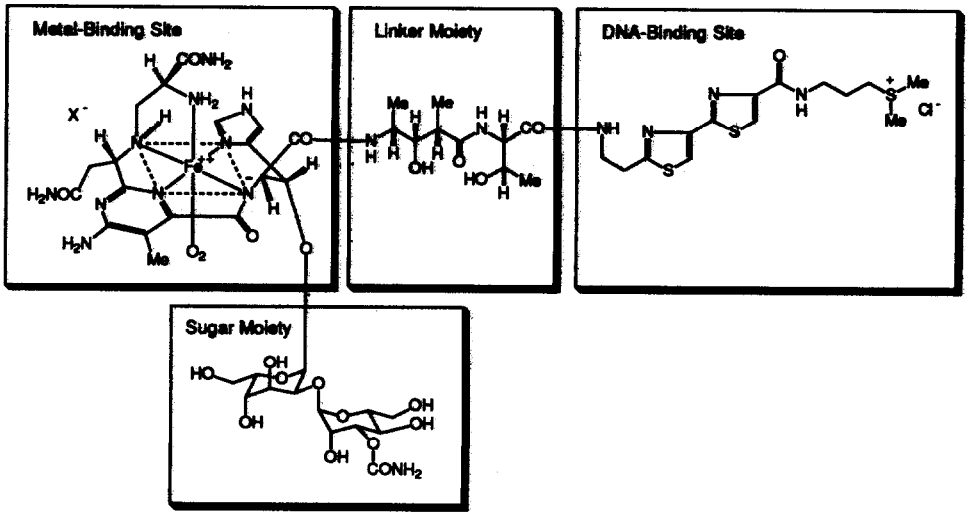


Figure 1. Proposed structure of BLM-Fe(II)-O_2 and assumed role of each functional moiety.

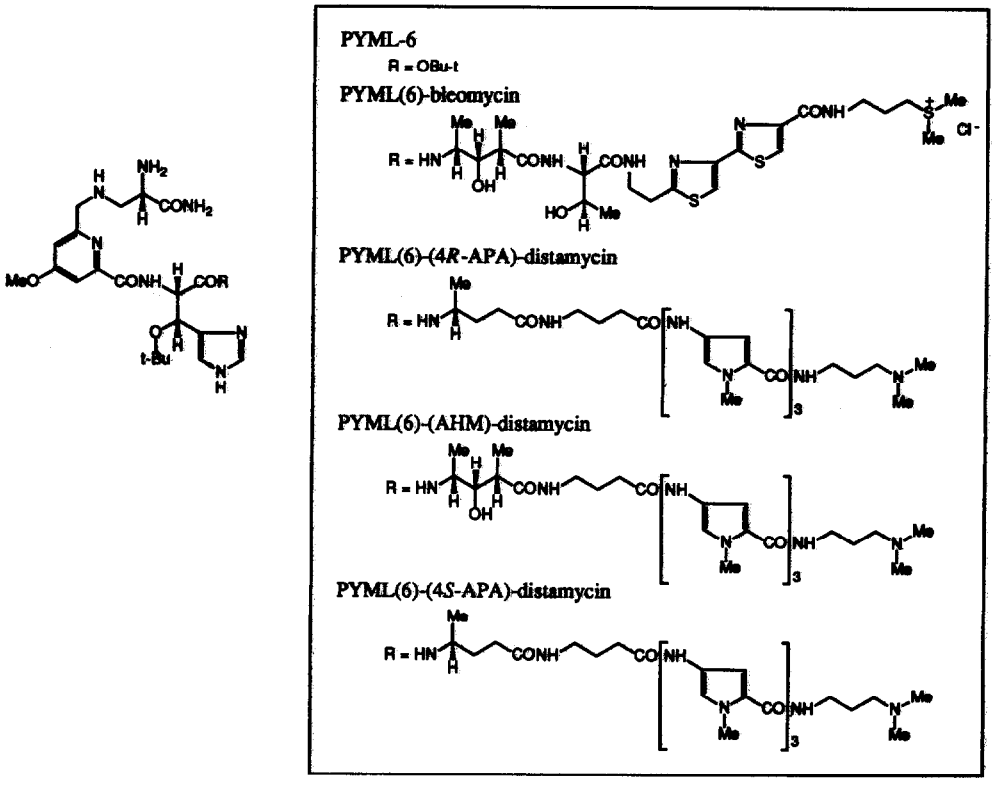
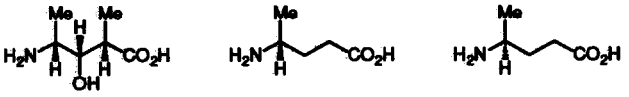


Figure 2. Man-designed bleomycins.

(Figure 2), in which the bithiazole-terminal amine fragment was coupled to PYML-6.[6] More recently, we have prepared the second man-designed BLM, PYML(6)-(4*R*-APA)-distamycin (Figure 2), which contains distamycin as the A,T-specific DNA affinity site and predominantly cleaves A, T rich regions of DNA.[7] This is in accordance with the results of Dervan's pioneering work on A,T-specific DNA-cleaving molecules consisting of a distamycin and an EDTA-Fe(II) group.[8]

Structurally, PYML(6)-bleomycin and PYML(6)-(4*R*-APA)-distamycin differ not only in the DNA binding site but also in the linker moiety. Whereas PYML(6)-bleomycin contains (2*S*,3*S*,4*R*)-4-amino-3-hydroxy-2-methylpentanoic acid (AHM, Figure 3), the same linker as found in natural BLM, PYML(6)-(4*R*-APA)-distamycin contains a simplified (*R*)-4-aminopentanoic acid (4*R*-APA, Figure 3). It should be noted that Umezawa *et al.* have made an important observation that the anti-HeLa and DNA-cleavage activities of BLM depend considerably on the stereochemistry and the structure of the linker moiety.[9,10] In particular, the 4*R*-APA derivative of BLM showed excellent DNA-cleaving activity, superior to that of natural BLM Therefore, it might be possible to control the DNA-cleavage activity of man-designed BLMs by varying the linker moiety as well as the DNA binding site. In order to clarify the relative roles of the linker moiety and the distamycin moiety, we have now designed PYML(6)-(AHM)-distamycin and PYML(6)-(4*S*-APA)-distamycin (Figure 2), containing AHM and (*S*)-4-aminopentanoic acid (4*S*-APA, Figure 3), the antipode of 4*R*-APA, respectively. Herein reported are the synthesis and the DNA cleavage experiments of these molecules.



AHM 4*R*-APA 4*S*-APA

Figure 3. The linker amino acids.

Synthetic Aspects of Man-Designed Bleomycins

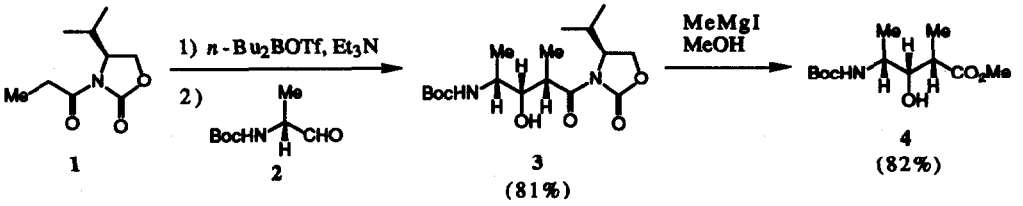
Synthesis of the Linker Amino Acids. AHM is a novel amino acid found in BLM and characterized by the 4*R*-amino, 3*S*-hydroxyl, and 2*S*-methyl arrangement on the three consecutive carbons 2-4. Efficient construction of these stereocenters is the major concern in the synthesis of AHM. In the first synthesis of AHM, Yoshioka *et al.* prepared all possible stereoisomers of AHM for the purpose of structure elucidation.[11] A synthesis of optically active AHM was carried out by Hecht *et al.* by a multistep procedure starting from L-rhamnose.[12] Previously we achieved a short and stereoselective synthesis of AHM by the Masamune-Mukaiyama aldol strategy.[13] More recently, Bock *et al.* reported an alternate synthesis by the Evans' acylation methodology using chiral oxazolidinone and (Boc-D-Ala)$_2$O.[14] This procedure still had several inconveniences, i. e., i) unnatural D-valine is required for the Evans' oxazolidinone to attain the desired (2*S*)-stereochemistry of the product, ii) half of the D-alanine constituent of (Boc-D-Ala)$_2$O is neither utilized nor recycled, and iii) an extra reduction step is required compared with the aldol strategy.

Herein we report the preparation of AHM by an improved procedure based on the Evans-Mukaiyama aldol strategy whereby the above drawbacks were overcome (Scheme 1). Thus, chiral *N*-acyloxazolidinone **1**, prepared from L-valine, was converted into the corresponding boron enolate and reacted with (*R*)-2-[(*tert*-butoxycarbonyl)amino]propanal **2** to afford aldol adduct **3** with the desired absolute stereochemistry as the sole product in 81% yield. Other stereoisomers were neither isolated nor detected by 400 MHz ^{1}H NMR. Treatment of aldol adduct **3** with methylmagnesium iodide gave methyl ester **4** (82% yield), suitably protected for further elaboration.

4*S*-APA was prepared according to the procedure used in our previous synthesis of the enantiomeric 4*R*-APA[6] and the specimen obtained was identical to that reported by Saito.[10]

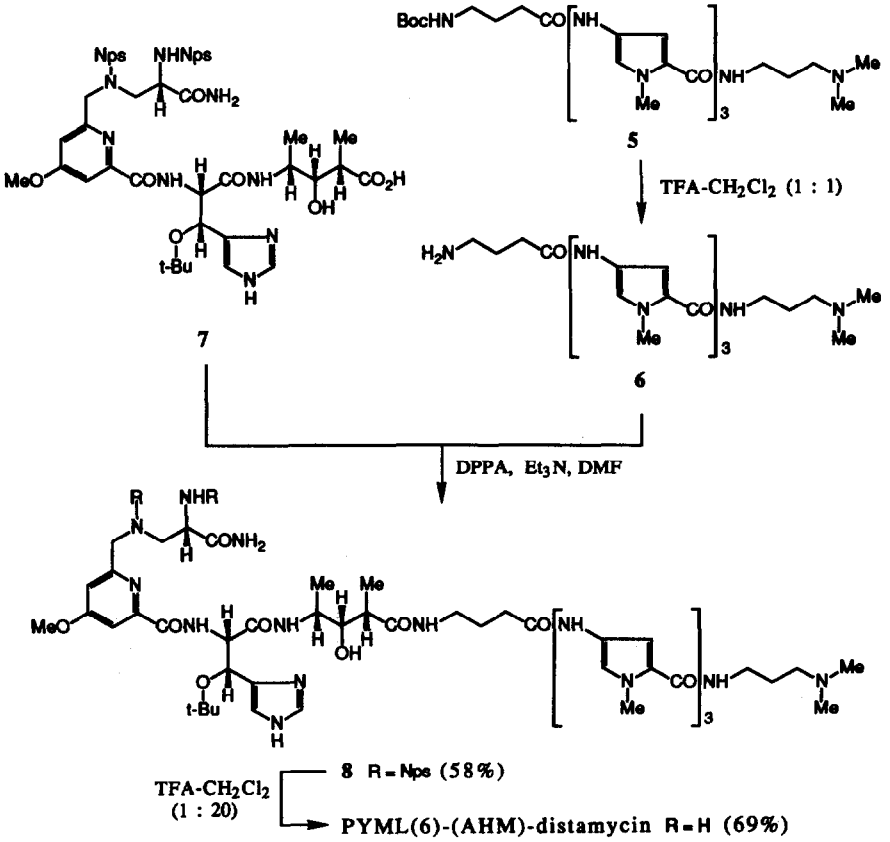
Thus, all of the linker amino acids, AHM, 4R-APA, and 4S-APA, are easily accessible.

Scheme 1



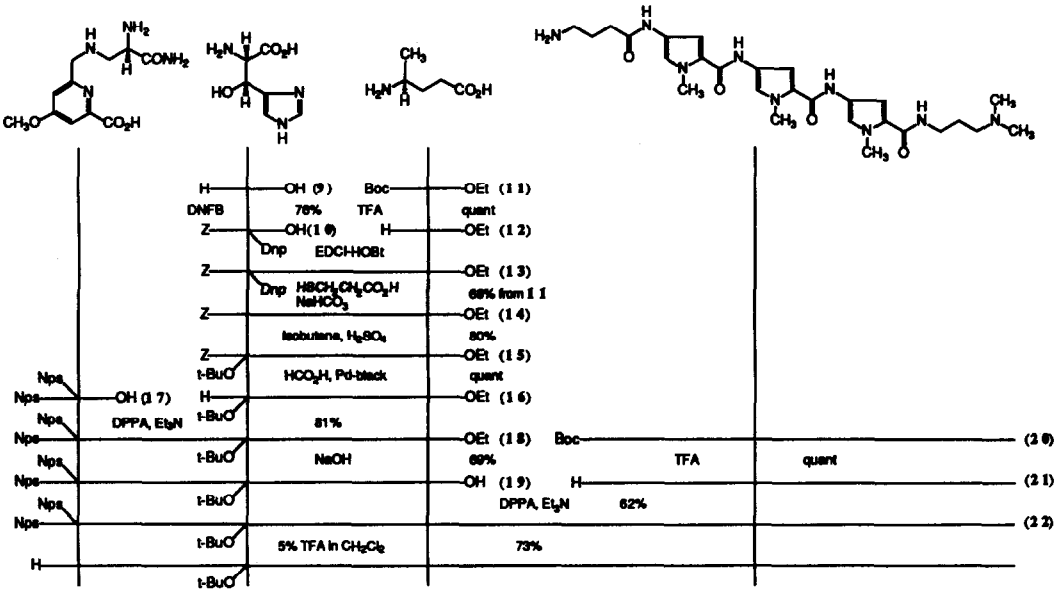
Synthesis of PYML(6)-(AHM)-distamycin. PYML(6)-(AHM)-distamycin was prepared by taking advantage of the previous syntheses of fragments and intermediates for man-designed BLMs. The distamycin moiety **5** has already been prepared for the synthesis of PYML(6)-(4*R*-APA)-distamycin and its Boc protective group was removed with TFA (Scheme 2).[7] The methoxypyridine-*tert*-butoxyhistidine-AHM fragment **7** has previously been synthesized during the preparation of PYML(6)-bleomycin.[6] The coupling of amine **6** and acid **7** was carried out by the DPPA procedure[15] furnishing bis(Nps) derivative **8** in 58% overall yield. The final deprotection of **8** was accomplished by careful treatment with 5% TFA in CH_2Cl_2, and PYML(6)-(AHM)-distamycin was obtained in 69% yield.

Scheme 2



Synthesis of PYML(6)-(4S-APA)-distamycin. Except for several modifications the synthetic strategy for PYML(6)-(4*S*-APA)-distamycin was basically the same as that for PYML(6)-(4*R*-APA)-distamycin[7] (Scheme 3). Thus, N^{α}-benzyloxycarbonyl-*erythro*-β-hydroxy-L-histidine **9**[5b] was treated with 2,4-dinitrofluorobenzene to give Dnp derivative **10** in 76% yield. Treatment of ethyl (*S*)-4-[(*tert*-butoxycarbonyl)amino]pentanoate **11**[7,10] with TFA gave amine **12**, which was coupled with acid **10** by the EDCI procedure[16] to give peptide **13**. The Dnp protective group of **13** was removed with 3-mercaptopropionic acid at pH~8 and compound **14** was obtained in 68% yield based on **11**. Subsequent introduction of a *tert*-butyl group with isobutene (80% yield) and deprotection of the Z group with formic acid and Pd-black (quantitative) afforded amine **16**. Coupling of the amine **16** with acid **17**,[6c] facilitated by the DPPA method,[15] afforded tripeptide **18** in 81% yield, and subsequent alkaline hydrolysis gave fragment **19** in 69% yield. The distamycin amine **21** was quantitatively prepared from the Boc derivative **20**[7] by treatment with TFA. The DPPA procedure was again applied to the condensation of acid **19** with amine **21** to afford bis(Nps) derivative **22** in 62% yield. The deprotection of **22** was accomplished with 5% TFA in CH_2Cl_2 to give PYML(6)-(4*S*-APA)-distamycin in 73% yield.

Scheme 3



Enzymic Character of Man-Designed Bleomycins: DNA Cleavage by PYML-6-Fe(II) and PYML(6)-(AHM)-distamycin-Fe(II)

Man-designed BLMs prepared as above were shown to possess the fundamental properties of an enzyme able to catalyze oxidative degradation of DNA. Sequence specific nucleotide cleavage was examined by using 5'-end ^{32}P-labeled G4 phage DNA fragment (100 base pairs) which contains a relatively high proportion of the base A and T. First of all, the DNA cleavage properties of PYML-6, the metal core of our man-designed BLMs, were examined. PYML-6-Fe(II) at 500 μM to 1 mM concentration was required for efficient DNA cleavage and the cleavage mode was found to be non-specific (Figure 4). In contrast, PYML(6)-(AHM)-distamycin-Fe(II) was shown to cleave DNA at 5 μM concentration in the presence of O_2 and dithiothreitol (1 mM) and A, T rich regions were found to be preferentially cleaved (Figure 5, lanes 3 and 6). However, peplomycin, a natural BLM derivative, cleaved DNA with G-specificity (lanes 4 and 5). This increased efficiency (about 100 to 200 times)

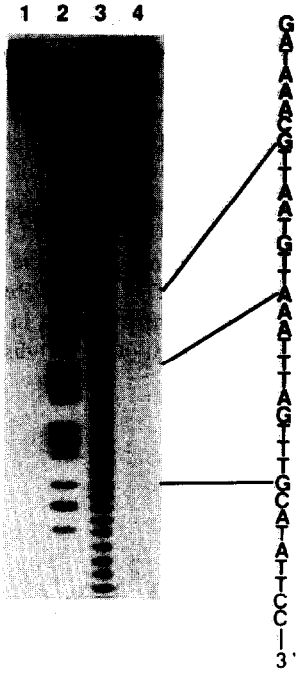


Figure 4. Autoradiogram of cleavage of 3'-end labeled G4 phage DNA fragment (100 base pairs) by PYML-6-Fe(II) in the presence of oxygen and dithiothreitol (5 mM). Lane 1, intact DNA; lane 2, the Maxam-Gilbert sequencing reaction of A + G; lane 3, PYML-6 at 1 mM; lane 4, PYML-6 at 500 μM.

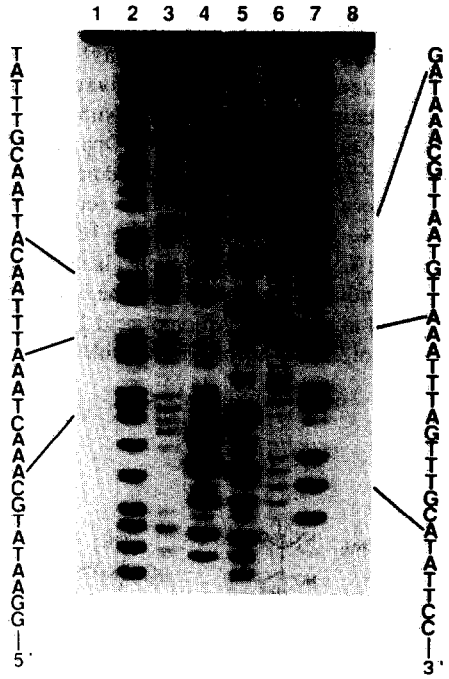


Figure 5. Autoradiogram of cleavage of 5'-end (lanes 1 - 4) and 3'-end (lanes 5-8) labeled G4 phage DNA fragment (100 base pairs) by PYML(6)-(AHM)-distamycin-Fe(II) in the presence of oxygen and dithiothreitol (1 mM). Lanes 1 and 8, intact DNA; lanes 2 and 7, the Maxam-Gilbert sequencing reaction of A + G; lanes 3 and 6, PYML(6)-(AHM)-distamycin-Fe(II) at 5 μM; lanes 4 and 5, peplomycin-Fe(II) at 5 μM.

in DNA cleavage is well understood by considering that the distamycin moiety actually contributes to the effective interaction with DNA, delivering the iron core to the vicinity of the cleavage site. The low efficiency and the lack of base specificity in DNA cleavage by PYML-6 alone are explained reasonably by the reaction initiated by random collision. The A,T-specific cleavage by PYML(6)-(AHM)-distamycin indicates that the distamycin moiety is responsible for the DNA-affinity and the base sequence recognition. Thus, the molecule PYML(6)-(AHM)-distamycin can be regarded as an assembly of a catalytic site (PYML-6 moiety) and a binding site (the distamycin moiety). In this sense, our man-designed BLM also deserves as a small sized and artificial molecule exhibiting the enzymic function of DNA degradation. A histogram taken from the autoradiogram (Figure 6) showed a symmetric cleavage pattern on opposite strands, indicating that PYML(6)-(AHM)-distamycin interacts with the minor groove of DNA. The DNA cleavage efficiency and pattern shown in Figures 5 and 6 were virtually identical to those of PYML(6)-(4*R*-APA)-distamycin previously reported, suggesting that the 2-methyl and 3-hydroxyl groups of the AHM moiety do not contribute to the DNA base interaction or recognition.[7] PYML(6)-(AHM)-distamycin clearly exhibited ESR signals of the transient catalytically active Fe(III) complex, presumably the ferric-peroxide species, at $g_1 = 2.250$, $g_2 = 2.171$, and $g_3 = 1.938$ (Figure 7), similar to those of

BLM (g_1 = 2.254, g_2 = 2.171, and g_3 = 1.937) and PYML(6)-bleomycin (g_1 = 2.249, g_2 = 2.173, and g_3 = 1.939).[6]

It was gratifying to find that PYML(6)-(AHM)-distamycin showed potent antitumor activity. In particular, its IC_{50} value against L1210 was 5.7 μg/ml whereas those of BLM and distamycin fragment **6** were 83.3 μg/ml and 35.6 μg/ml, respectively (Table 1).

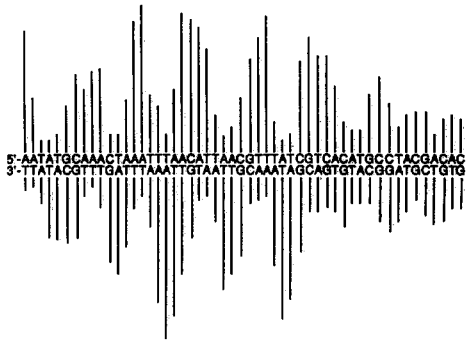


Figure 6. Histogram of cleavage sites by PYML-(6)-(AHM)-distamycin-Fe(II) for G4 phage DNA fragment (100 base pairs).

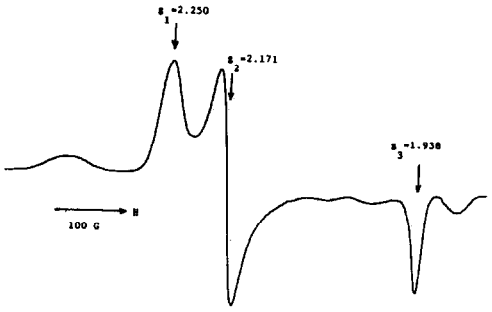


Figure 7. ESR spectrum of PYML(6)-(AHM)-distamycin-Fe(III)-O_2H^- complex at 77 K.

Table 1. Cytotoxicity of man-designed BLM against tumor cells in suspension cultures.

Tumor Cells	IC_{50} (μg/ml) PYML(6)-(AHM)-distamycin	BLM	Compound **6**
LX-1	>100	12.5	>100
SC-6	77.8	8.0	>100
L1210	5.7	83.3	35.6
EL 4	26.4	4.1	>100
$P388D_1$	29.2	0.8	>100
IMC Ca.	26.7	15.2	>100
Meth A	13.6	14.3	>100
Ehrlich Ca.	35.0	5.9	>100

Significance of the Stereochemistry of the Linker Moiety: DNA Cleavage by PYML(6)-(4S-APA)-distamycin-Fe(II)

Whereas DNA cleavage by PYML(6)-(AHM)-distamycin was found to be mostly the same as that by the previously synthesized PYML(6)-(4*R*-APA)-distamycin, a drastic influence due to a change in the stereochemistry of the linker moiety was observed in the case of PYML(6)-(4*S*-APA)-distamycin. Figure 8 shows the cleavage of G4 phage DNA fragment (100 base pairs) by PYML(6)-(4*S*-APA)-distamycin-Fe(II) (5 μM, lanes 4 and 5) in the presence of O_2 and dithiothreitol (1 mM) compared with that of PYML(6)-(4*R*-APA)-distamycin-Fe(II) (5 μM, lanes 3 and 6). The microdensitometric scans of the autoradiograms revealed that the cleavage activity of PYML(6)-(4*S*-APA)-distamycin is remarkably less than that of PYML(6)-(4*R*-APA)-

distamycin by a factor of about 10, whereas both showed virtually the same cleavage pattern. Thus, the DNA cleaving power was shown to be reduced by inverting the stereochemistry of the linker amino acid.

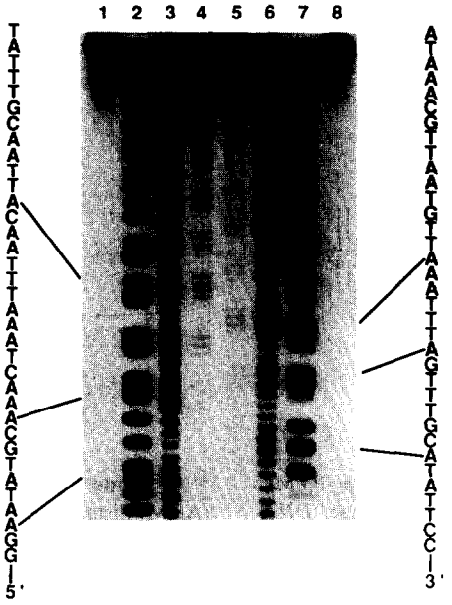


Figure 8. Autoradiogram of cleavage of 5'-end (lanes 1 - 4) and 3'-end (lane 5 - 8) labeled G4 phage DNA fragments (100 base pairs) by PYML(6)-(4*R*-APA)-distamycin-Fe(II) and PYML(6)-(4*S*-APA)-distamycin-Fe(II) in the presence of oxygen and dithiothreitol (1 mM). Lanes 1 and 8, intact DNA; lanes 2 and 7, the Maxam-Gilbert sequencing reaction of A + G; lanes 3 and 6, PYML(6)-(4*R*-APA)-distamycin-Fe(II) at 5 μM; lanes 4 and 5, PYML(6)-(4*S*-APA)-distamycin at 5 μM.

It appeared surprising that the stereochemical inversion of a single asymmetric center in a large molecule such as the man-designed BLM resulted in a dramatic decrease in DNA cleavage activity. In order to explain this phenomenon, we carried out a simulation study of the structure of the complexes of DNA and man-designed BLMs based on the X-ray structure of the distamycin-DNA complex (see EXPERIMENTAL SECTION). Figure 9 shows stereo pair computer drawings of the energy-minimized structures of the complexes of double helical DNA d(CGCAAATTTTTGCG-GCGTTTAAAAACGC) (which represents the actual A,T-rich cleaved sites shown in Figures 5 and 8) and PYML(6)-(AHM)-distamycin (A), PYML(6)-(4*R*-APA)-distamycin (B), and PYML(6)-(4*S*-APA)-distamycin (C). The iron-oxygen site, the linker moiety, and a part of the distamycin moiety are displayed. It appears that the base sequence specificity in DNA binding is determined solely by the distamycin moiety which lies in the minor groove, anchored to an AATT region. The linker, tethered to the distamycin moiety, appears to contribute to the delivery of the metal-oxygen moiety to the site of DNA cleavage. This interaction with DNA seems to be less favored in the case of PYML(6)-(4*S*-APA)-distamycin because of the steric repulsion between the C5-methyl group of the 4*S*-APA linker and the nucleic acid, and also because the iron-oxygen site is placed slightly further from the DNA, resulting in the lowered cleavage power. The 4*R*-amino, 3*S*-hydroxyl, and 2*S*-methyl groups of AHM and 4*R*-APA are located outside the minor groove and hence there is no steric repulsion between these groups and the DNA. In fact, the energy of the whole system of DNA-drug complex decreased in the order 4*S*-APA > AHM > 4*R*-APA, consistent with the experimental results.

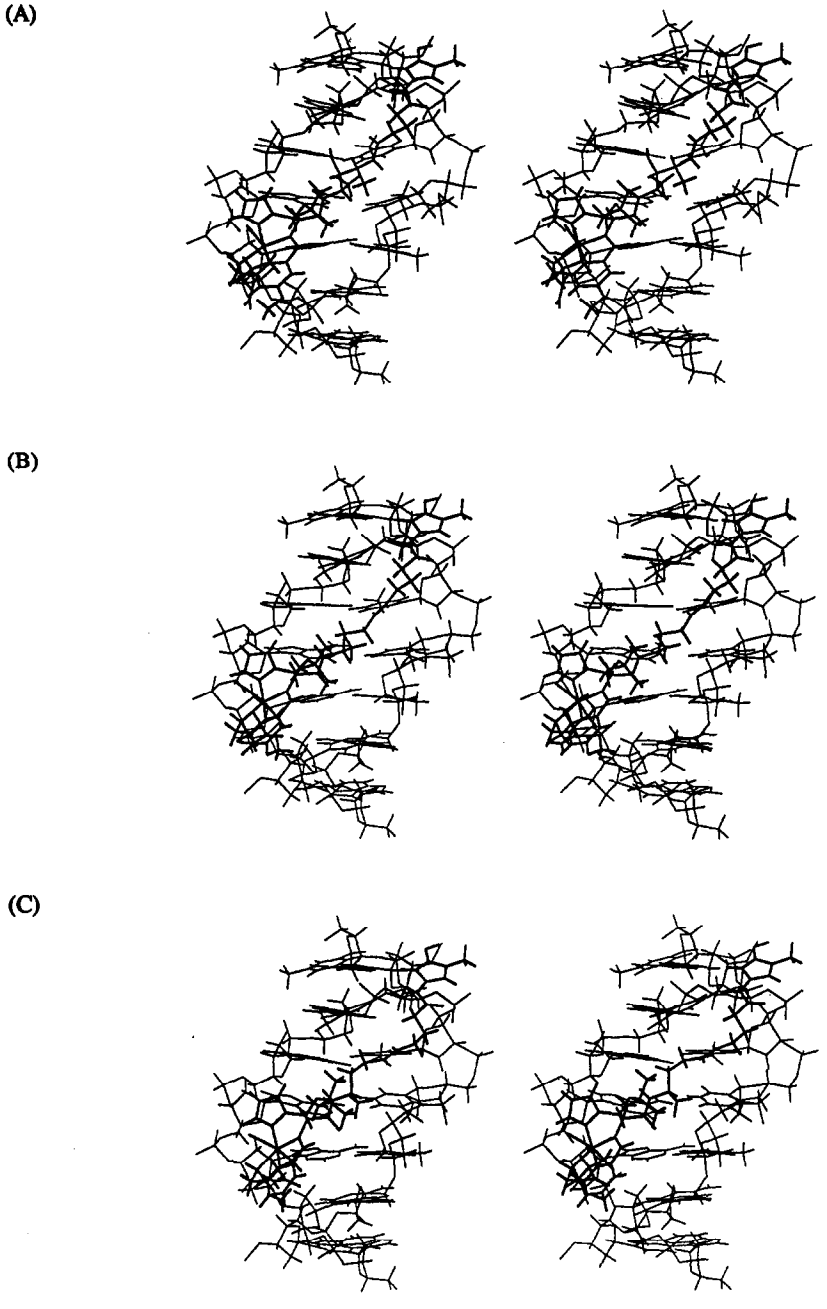


Figure 9. Energy-minimized structures of the complexes of double helical d(CGCAAATTTTTGCG-GCGTTTAAAAACGC) and PYML(6)-(AHM)-distamycin (A), PYML(6)-(4*R*-APA)-distamycin (B), and PYML(6)-(4*S*-APA)-distamycin (C).

Discussion

Since we have previously demonstrated the catalytic activation of molecular oxygen by the PYML-6-Fe(II) complex,[5] the PYML-6 site should be recognized as the *catalytic* site of man-designed BLM. Whereas 500 to 1000 μM of PYML-6 was required for DNA cleavage, PYML(6)-(AHM)-distamycin and PYML(6)-(4*R*-APA)-distamycin cleaved DNA at 5 μM concentration. Thus, introduction of the distamycin moiety to PYML-6 was shown to amplify the latter's DNA-cleaving power by 200 times. In addition, the DNA affinity site also furnished base sequence specificity to the DNA cleavage, i. e., PYML(6)-bleomycin cleaved DNA with G-specficity and PYML(6)-(AHM)-distamycin or PYML(6)-(4*R*-APA)-distamycin cleaved DNA with A,T-specificity. Therefore, the distamycin site is indeed the binding site for the specific base recognition having selective affinity.

The present study also demonstrated the significance of the stereochemistry of the linker moiety as shown by the lowered efficiency in the DNA cleavage by PYML(6)-(4*S*-APA)-distamycin. This is in accordance with Umezawa's finding that the modification of the linker moiety of BLM greatly influences the DNA cleavage activity.[9] This is comparable to the enzymatic discrimination of stereochemistry. That is, PYML(6)-(4*S*-APA)-distamycin-DNA is a worse-matched pair, whereas PYML(6)-(4*R*-APA)-distamycin or PYML(6)-(AHM)-distamycin forms a better-matched pair with DNA as indicated by the computer simulation study.

BLM and PYML(6)-bleomycin, both possessing the bithiazole moiety, caused pinpoint cleavage of DNA probably due to the action of iron-bound oxygen species, i. e., Fe(III)-O_2H^- or Fe(V)=O. In contrast, PYML(6)-(AHM)-distamycin, PYML(6)-(4*R*-APA)-distamycin, and PYML(6)-(4*S*-APA)-distamycin cleaved several bases both up- and down-stream from the place where the iron center is presumed to be located. Presumably this was because not only iron-bound oxygen, but also diffusible oxygen species generated and liberated from the iron site attacked the DNA. ESR measurement revealed that PYML(6)-(AHM)-distamycin produces the same iron-bound oxygen species as BLM or PYML(6)-bleomycin, suggesting the possibility that the observed DNA-cleavage mode is a superimposition of many pinpoint cuttings by iron-bound oxygen due to varied potential binding of the distamycin domain within the AT-rich region. However, it is more likely that the diffusible oxygen is the major DNA-attacking species, because the computer simulation suggests that the iron-bound oxygen can hardly reach the deoxyribose to be cleaved. On the other hand, it is thought that the metal-oxygen region of BLM is placed a short distance from the deoxyribose ring next to the recognized guanine.[17]

The DNA binding mode of the bithiazole-based DNA cleaver seems intrinsically different from that of distamycin derivatives. The whole molecule of BLM, spanning from the pyrimidine region to the bithiazole terminus, appears to be responsible for the specific recognition of DNA. In fact, Hecht demonstrated that even the pyrimidine region contributes remarkably to control of the site of cleavage.[18] On the other hand, it seems that the distamycin moiety alone dominates the site of DNA binding, and that DNA cleavage takes place near the iron which is tethered to the distamycin moiety by the linker.

In conclusion, we have demonstrated the enzyme-like character of man-designed BLMs and disclosed an important role of the linker amino acid in placing the metal center in a good orientation relative to the DNA binding moiety. Particularly interesting was the finding that our man-designed BLM, namely PYML(6)-(AHM)-distamycin, was shown to be more potent than natural BLM in cytotoxicity against L1210. The present results set the stage for the design of new man-designed BLM with tailored specificity in anticancer action.

EXPERIMENTAL SECTION

Melting points were measured on a Yanagimoto micro melting point apparatus and were uncorrected. Optical rotations were measured on a JASCO DIP 140 instrument. ^{1}H NMR (400 MHz) spectra were recorded on a JEOL GX-400 spectrometer. Abbreviations are as follows; s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). IR spectra were recorded on a PERKIN-ELMER 1600 Series FTIR spectrometer. Fast atom bombardment mass spectra (FABMS) and high resolution mass spectra (HRMS) were recorded on a JEOL JMS DX-300 spectrometer and a JEOL JMS AH-100 spectrometer. X-band ESR spectra were measured at

77K with a JES-FE-3X spectrometer. E. Merck silica gel (60PF-254 or 60PF-254 silanised) was used for preparative TLC. Reagents and solvents were purified by standard procedures.

(*S*)-3-[(2*S*,3*S*,4*R*)-4-[*N*-(*tert*-Butoxycarbonyl)amino]-3-hydroxy-2-methylpentanoyl]-4-isopropyl-1,3-oxazolidin-2-one (3). A 1.0 M solution of dibutylboryl triflate in CH_2Cl_2 (6.5 mL, 6.5 mmol) and triethylamine (1.2 mL, 8.3 mmol) were added to a solution of (*S*)-4-isopropyl-3-propionyl-1,3-oxazolidin-2-one (**1**)[19] (1.10 g, 5.95 mmol) in Et_2O (23 mL) under argon at -78°C. The solution was allowed to warm to room temperature, stirred for 1.5 hr, and again cooled to -78°C. A solution of (*R*)-2-[*N*-(*tert*-butoxycarbonyl)amino]propanal (**2**)[13] (1.03 g, 5.95 mmol) in CH_2Cl_2 (6 mL) was added dropwise over 10 min. After the solution was stirred at -78°C for 30 min, then at room temperature for 1.5 hr, pH 7 phosphate buffer (30 mL), MeOH (40 mL), and 30% H_2O_2 (6.5 mL) were added at 0°C. After 1 hr at 0°C, the organic solvents were evaporated and the remaining aqueous phase was extracted 5 times with Et_2O. The Et_2O extracts were combined, washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by chromatography on silica gel (eluted with hexane : AcOEt = 5 : 2 → 1 : 1) to give **3** as a colorless foam (1.72 g, 81%): Rf 0.18 (hexane : AcOEt = 2 : 1); $[\alpha]_D^{27.0}$ = +63.2° (c = 1.03, $CHCl_3$); IR (KBr) 3443, 2974, 2879, 1778, 1694, 1514, 1456, 1386, 1301, 1206, 1105, 1055, 1022 cm^{-1}; ^1H NMR (CD_3OD) δ 0.87 (3H, d, J = 7.0 Hz, $(CH_3)_2CH$), 0.93 (3H, d, J = 7.0 Hz, $(CH_3)_2CH$), 1.11 (3H, d, J = 6.6 Hz, CH_3CH), 1.23 (3H, d, J = 7.0 Hz, CH_3CH), 1.43 (9H, s, $(CH_3)_3C$), 2.31 (1H, dqq, J = 3.7, 7.0, 7.0 Hz, $(CH_3)_2CH$), 3.58 (1H, m, CH_3CH), 3.71 (1H, dd, J = 6.2, 6.6 Hz, $CHCHCH$), 3.92 (1H, dq, J = 6.6, 6.6 Hz, CH_3CH), 4.26~4.38 (2H, m, $CHCHCH_2$), 4.41~4.52 (1H, m, $CHCHCH_2$); FABMS m/z 359 (MH^+); HRMS (FAB) calcd for $C_{17}H_{31}N_2O_6$ (MH^+): 359.21824, found: 359.21852.

Methyl (2*S*,3S,4*R*)-4-[*N*-(*tert*-Butoxycarbonyl)amino]-3-hydroxy-2-methylpentanoate (4). A 0.93 M solution of methylmagnesium iodide in Et_2O (2.3 mL, 2.1 mmol) was added to anhydrous MeOH (9.4 mL). The resulting mixture was added to an ice-cooled solution of aldol adduct **3** (687 mg, 1.92 mmol) in anhydrous MeOH (9.4 mL) by cannula. After the reaction mixture was stirred for 10 min, it was quenched by addition of pH 7 phosphate buffer (9.4 mL). The organic solvents of the mixture were removed by evaporation. The remaining aqueous solution was acidified to pH 3 with 1N HCl and extracted 3 times with AcOEt. The AcOEt extracts were combined, washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by chromatography on silica gel (eluted with hexane : AcOEt = 5 : 2 → 1 : 1) to give **4** as a colorless, amorphous material (411 mg, 82%): Rf 0.27 (hexane : AcOEt = 2 : 1); $[\alpha]_D^{27.0}$ = +8.3° (c = 1.00, $CHCl_3$); IR (KBr) 3414, 3372, 2983, 1723, 1686, 1529, 1365, 1253, 1176 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.18 (3H, d, J = 6.6 Hz, CH_3CHNH), 1.26 (3H, d, J = 7.0 Hz, CH_3CHCO), 1.44 (9H, s, $(CH_3)_3C$), 2.66 (1H, dq, J = 5.5, 7.0 Hz, CH_3CHCO), 3.09 (1H, br s, OH), 3.63~3.79 (2H, m, CH_3CHNH and $CHCHCH$), 3.71 (3H, s, CO_2CH_3), 4.55~4.70 (1H, br m, OCONH); FABMS m/z 262 (MH^+); HRMS (FAB) calcd for $C_{12}H_{24}NO_5$ (MH^+): 262.16547, found: 262.16480.

bis(Nps)-PYML(6)-(AHM)-distamycin (8). Trifluoroacetic acid (1.0 mL, 13 mmol) was added dropwise to an ice-cooled solution of carbamate **5**[7] (58.8 mg, 8.99×10^{-2} mmol) in CH_2Cl_2 (1.0 mL) over 5 min under argon. After being stirred at 0°C for 30 min and then at room temperature for 2 hr, the solution was concentrated in vacuo. The residue was dried over KOH in vacuo to give amine TFA salt **6** as a pale brown amorphous material. Acid **7**[6c] (68.4 mg, 7.49×10^{-2} mmol), Et_3N (44 mL, 0.31 mmol) and DPPA[15] (24 mL, 0.11 mmol) were successively added to an ice-cooled solution of the amine TFA salt **6** in DMF (4.0 mL) under argon. The solution was allowed to warm to room temperature, stirred for 2 days, and concentrated in vacuo. The residue was purified by gel filtration on Sephadex LH-20 (eluted with MeOH) followed by preparative TLC on silica gel (developed with $CHCl_3$: MeOH : Et_3N = 16 : 4 : 1) to give **8** as a yellow, amorphous material (63.0 mg, 58%): Rf 0.21 ($CHCl_3$: MeOH : Et_3N = 16 : 4 : 1); $[\alpha]_D^{23.0}$ = +21.1° (c = 0.45, MeOH); IR (KBr) 3422, 1654, 1524, 1339 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.00 (3H, d, J = 6.8 Hz, CH_3CH), 1.01 (3H, d, J = 6.2 Hz, CH_3CH), 1.04 (9H, s, $(CH_3)_3C$), 1.63 (2H, m, $CH_2CH_2CH_2$), 1.68 (2H, m, $CH_2CH_2CH_2$), 2.14~2.21 (1H, br m, CH_3CHCO), 2.19 (6H, s, $N(CH_3)_2$), 2.23 (2H, t, J = 7.7 Hz, CH_2CONH or $CH_2N(CH_3)_2$), 2.30 (2H, t, J = 6.6 Hz, CH_2CONH or $CH_2N(CH_3)_2$), 3.00~3.11 (2H, m, $CONHCH_2$), 3.14~3.23 (2H, m, $CONHCH_2$), 3.42~3.75 (4H, m, $CH_2CHCONH_2$, $CH_2CHCONH_2$, and CH_3CHNH), 3.80 (6H, s, $PyOCH_3$

and/or NC*H*3), 3.82 and 3.84 (each 3H, s, PyOC*H*3 and/or NC*H*3), 4.40~4.62 (2H, br m, PyC*H*2), 4.62~4.73 (1H, m, CONHC*H*CONH), 4.90~4.98 (1H, br, CHC*H*CH), 4.94 (1H, d, J = 5.9 Hz, C*H*Im), 5.13~5.22 (1H, br d, N*H*Nps), 6.83 (1H, d, J = 1.8 Hz, Pyrrole), 6.88 (1H, d, J = 1.8 Hz, Pyrrole), 6.96~7.02 (1H, br s), 7.03 (1H, d, J = 1.8 Hz, Pyrrole), 7.13~7.25 (1H, br), 7.15 (1H, d, J = 1.8 Hz, Pyrrole), 7.18 (1H, d, J = 1.5 Hz, Pyrrole), 7.22 (1H, d, J = 1.5 Hz, Pyrrole), 7.29~7.42 (4H, m, Nps), 7.42~7.60 (2H, br), 7.60~7.78 (2H, br), 7.69 (1H, m, Nps), 7.78~8.02 (2H, br), 8.02~8.13 (3H, m), 8.25 (2H, dd, J = 1.2, 8.4 Hz, Nps), 9.03 (1H, br m, PyCON*H*), 9.80 (1H, s, CON*H*), 9.89 (1H, s, CON*H*), 9.90 (1H, s, CON*H*); FABMS m/z 1449 (MH^+), 1450 (MH^++1); HRMS (FAB) calcd for $C_{66}H_{86}N_{19}O_{15}S_2$ (MH^+): 1448.59922, found: 1448.60126.

PYML(6)-(AHM)-distamycin. Trifluoroacetic acid (0.29 mL, 3.9 mmol) was added dropwise to an ice-cooled solution of **8** (29.3 mg, 0.0202 mmol) and skatole (58.6 mg, 0.447 mmol) in CH_2Cl_2 (5.9 mL) over 3 min under argon. The solution was stirred for 2.5 hr at room temperature, treated with Et_3N (0.82 mL, 5.9 mmol) with ice cooling, and concentrated in vacuo. The residue was purified by repeated gel filtration on Sephadex LH-20 (eluted with MeOH) followed by reverse phase preparative TLC (developed with 4% NH_4OAc : MeOH = 3.5 : 6.5). The resulting colorless powder was further purified with Amberlite XAD-2 (30 mL), washed with water (100 mL) and eluted with MeOH to give PYML(6)-(AHM)-distamycin as a colorless amorphous material (15.9 mg, 69%): Rf 0.67 (*n*-PrOH : AcOH : Py : H_2O = 15 : 3 : 10 : 12); $[\alpha]_D^{25.0}$ = +15.4° (c = 0.60, MeOH); IR (KBr) 3422, 1676, 1654, 1648, 1204, 1132 cm^{-1}; ^1H NMR (CD_3OD) δ 1.13 and 1.14 (total 9H, each s, (C*H*3)3C), 1.17 (3H, d, J = 7.0 Hz, C*H*3CH), 1.23 (3H, d, J = 6.6 Hz, C*H*3CH), 1.85 and 1.99 (each 2H, m, $CH_2CH_2CH_2$), 2.28~2.43 (3H, m, C*H*2CONH and CH_3CHCO), 2.86~2.94 (1H, m, C*H*2CHCONH2), 2.91 (6H, s, N(C*H*3)2), 3.02 (1H, dd, J = 6.0, 13.0 Hz, C*H*2CHCONH2), 3.12~3.27 (4H, m, CONHC*H*2 and/or C*H*2N(CH3)2), 3.41 (2H, t, J = 6.6 Hz, CONHC*H*2 or C*H*2N(CH3)2), 3.56~3.69 (1H, m, CH2C*H*CONH2), 3.85, 3.88, 3.89, and 3.90 (each 3H, s, PyOC*H*3 and NC*H*3×3), 3.93~4.14 (1H, br m, CH3C*H*NH), 3.95~4.05 (2H, m, PyC*H*2), 4.70 (1H, d, J = 8.8 Hz, CONHC*H*CONH), 4.92~5.08 (1H, br m, CHC*H*CH), 5.11 (1H, d, J = 8.8 Hz, C*H*Im), 6.81 (1H, d, J = 1.8 Hz, Pyrrole), 6.88 (1H, d, J = 1.8 Hz, Pyrrole), 6.90 (1H, d, J = 1.8 Hz, Pyrrole), 7.04 (1H, d, J = 2.2 Hz, Py), 7.16 (1H, d, J = 1.8 Hz, Pyrrole), 7.18 (1H, distorted d), 7.24 (1H, distorted d), 7.34 (1H, d, J = 2.6 Hz), 7.48~7.58 (1H, br m), 8.07~8.20 (1H, br); FABMS m/z 1141 (M^+), 1143 (MH^++1); HRMS (FAB) calcd for $C_{54}H_{80}N_{17}O_{11}$ (MH^+): 1142.62231, found: 1142.62048.

***N*$^{\alpha}$-(Benzyloxycarbonyl)-*N*^{im}-(2,4-dinitrophenyl)-*erythro*-β-hydroxy-L-histidine (10).** Sodium bicarbonate (780 mg, 9.29 mmol) and a solution of 2,4-dinitrofluorobenzene (640 mg, 3.44 mmol) in MeOH (20 mL) were added successively to a solution of *N*$^{\alpha}$-(benzyloxycarbonyl)-*erythro*-β-hydroxy-L-histidine (9)[4b] in water (13 mL). The mixture was stirred in the dark at room temperature for 16 hr and concentrated in vacuo. The residue was partitioned between water and AcOEt. The aqueous layer was acidified to pH 3 with 1N HCl. The yellow precipitate deposited was collected by filtration, dried in vacuo, and crystallized from MeOH - Et2O to give **10** as a pale-yellow powder (1.23 g, 76%): mp 110.5~113.5°C (dec); $[\alpha]_D^{21.0}$ = +23.1° (c = 0.36, MeOH); IR (KBr) 3401, 3115, 2927, 1712, 1610, 1542, 1454, 1344, 1252, 1083, 1056 cm^{-1}; ^1H NMR (DMSO-*d*6) δ 4.47 (1H, dd, J = 5.5, 8.8 Hz, NHC*H*CO2H), 4.92 (1H, dd, J = 5.1, 5.5 Hz, C*H*Im), 5.03 (2H, s, PhC*H*2), 5.67 (1H, br d, J = 5.1 Hz, O*H*), 7.25 (1H, d, J = 8.8 Hz, OCON*H*), 7.27~7.38 (6H, m, Ph and Im), 7.94 (1H, d, J = 8.8 Hz, Dnp), 8.03 (1H, d, J = 1.1 Hz, Im), 8.66 (1H, dd, J = 2.6, 8.8 Hz, Dnp), 8.94 (1H, d, J = 2.6 Hz, Dnp); FABMS m/z 472 (MH^+); Anal calcd for $C_{20}H_{17}O_9N_5$, C50.96, H3.64, N14.86, found C50.49, H3.71, N14.90.

Ethyl (*S*)-4-[[*N*$^{\alpha}$-(Benzyloxycarbonyl)-*erythro*-β-hydroxy-L-histidyl]amino]pentanoate (14). Trifluoroacetic acid (7.0 mL) was slowly added to an ice-cooled solution of ethyl (*S*)-4-[*N*-(*tert*-butoxycarbonyl)amino]pentanoate (**11**)[20] (307 mg, 1.25 mmol) in CH_2Cl_2 (7.0 mL). After being stirred at room temperature for 1 hr, the solution was concentrated in vacuo and the residue was thoroughly dried to give amine TFA salt **12** as a pale brown wax. Acid **10** (589 mg, 1.25 mmol), 4-methylmorpholine (0.15 mL, 1.4 mmol), EDCI[16] (300 mg, 1.56 mmol), and HOBT•H_2O (211 mg, 1.38 mmol) were added successively to an ice-cooled solution of the amine TFA salt **12** obtained as above in DMF (7.4 mL) under argon. The solution was allowed to warm to room temperature, stirred in the dark for 14 hr, and concentrated in vacuo. The residue was dissolved in

AcOEt, washed with 5% aqueous $NaHSO_4$, saturated $NaHCO_3$, and brine, dried over Na_2SO_4, and concentrated in vacuo. Light sensitive **13** was used in the next step without further purification. Thus, 3-mercaptopropionic acid (0.87 mL, 10 mmol) was added to a solution of crude **13** in THF (7.5 mL). The resulting solution was adjusted to pH 8 with saturated $NaHCO_3$ and stirred at room temperature for 1 hr. After the completion of the deprotection of the Dnp group was confirmed by TLC, saturated $NaHCO_3$ and water were added to the solution and the mixture was extracted with AcOEt. The AcOEt solution was washed with saturated $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by chromatography on silica gel (eluted with CH_2Cl_2 : MeOH = 15 : 1) to give **14** as a colorless foam (368 mg, 68%): Rf 0.27 (CH_2Cl_2 : MeOH = 10 : 1); ($[\alpha]_D^{21.0}$ = +7.5° (c = 1.00, $CHCl_3$); IR (KBr) 3303, 2974, 2477, 2429, 1732, 1691, 1639, 1536, 1455, 1378, 1238, 1187, 1099, 1027 cm^{-1}; ^1H NMR (CD_3OD) δ 1.07 (3H, d, J = 6.6 Hz, C*H*$_3$CH), 1.23 (3H, t, J = 7.1 Hz, C*H*$_3$CH$_2$), 1.54~1.69 (1H, m, CHC*H*$_2$CH$_2$), 1.69~1.81 (1H, m, CHC*H*$_2$CH$_2$), 2.19~2.40 (2H, m, CH$_2$C*H*$_2$CO), 3.81~3.95 (1H, br m, CH$_3$C*H*), 4.10 (2H, q, J = 7.1 Hz, CH$_3$C*H*$_2$), 4.42 (1H, d, J = 7.3 Hz, NHC*H*CONH), 4.90 (1H, d, J = 7.3 Hz, C*H*Im), 5.01 (1H, d, J = 12.5 Hz, PhC*H*$_2$), 5.05 (1H, d, J = 12.5 Hz, PhCH$_2$), 7.00 (1H, br d, J = 1.1 Hz, Im), 7.24~7.36 (5H, m, Ph), 7.61 (1H, d, J = 1.1 Hz, Im); FABMS m/z 433 (MH^+); HRMS (FAB) calcd for $C_{21}H_{29}N_4O_6$ (MH^+): 433.20873, found: 433.20994.

Ethyl (*S*)-4-[[*N*$^\alpha$-(Benzyloxycarbonyl)-*erythro*-β-*tert*-butoxy-L-histidyl]amino]pentanoate (15). A solution of alcohol **14** (254 mg, 0.587 mmol) in CH_2Cl_2 (60 mL) in a sealable tube was cooled with dry ice-acetone under argon. Isobutene (ca 35 mL) and concentrated H_2SO_4 (0.35 mL) were added successively to the solution. The tube was sealed, allowed to warm to room temperature, and stirred for 3 days. The tube was again cooled with dry ice-acetone and opened. After addition of Et_3N (7.0 mL), the solution was stirred at room temperature to remove the excess isobutene. The resulting mixture was partitioned between AcOEt and saturated $NaHCO_3$. 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 $CHCl_3$: MeOH = 20 : 1) to give **15** as a colorless wax (230 mg, 80%): Rf 0.45 (CH_2Cl_2 : MeOH = 10 : 1); $[\alpha]_D^{21.0}$ = +39.3° (c = 0.95, $CHCl_3$); IR (KBr) 3317, 3090, 2979, 2479, 2427, 1736, 1643, 1544, 1456, 1368, 1296, 1277, 1182, 1047, 1024 cm^{-1}; ^1H NMR (CD_3OD) δ 1.07 (3H, d, J = 6.6 Hz, C*H*$_3$CH), 1.10 (9H, s, (C*H*$_3$)$_3$C), 1.23 (3H, t, J = 7.1 Hz, C*H*$_3$CH$_2$), 1.64~1.84 (2H, m, CHC*H*$_2$CH$_2$), 2.34 (1H, ddd, J = 7.3, 7.3, 16.5 Hz, CH$_2$C*H*$_2$CO), 2.40 (1H, ddd, J = 7.3, 7.3, 16.5 Hz, CH$_2$C*H*$_2$CO), 3.86 (1H, m, CH$_3$C*H*), 4.10 (2H, q, J = 7.1 Hz, CH$_3$C*H*$_2$), 4.29 (1H, d, J = 7.7 Hz, NHC*H*CONH), 4.88 (1H, d, J = 7.7 Hz, C*H*Im), 4.94 (1H, d, J = 12.8 Hz, PhC*H*$_2$), 5.01 (1H, d, J = 12.8 Hz, PhC*H*$_2$), 6.95 (1H, br d, J = 1.1 Hz, Im), 7.20~7.36 (5H, m, Ph), 7.58 (1H, d, J = 1.1 Hz, Im); FABMS m/z 489 (MH^+), 490 (MH^++1); HRMS (FAB) calcd for $C_{25}H_{37}N_4O_6$ (MH^+): 489.27133, found: 489.27140.

Ethyl (*S*)-4-[[*N*$^\alpha$-[[6-[[*N*-[(*S*)-2-Carbamoyl-2-[*N*-[(*o*-nitrophenyl)thio]amino]ethyl]-*N*-[(*o*-nitrophenyl)thio]amino]methyl]-4-methoxy-2-pyridyl]carbonyl]-*erythro*-β-*tert*-butoxy-L-histidyl]amino]pentanoate (18). Pd black (ca 60 mg) and 99% HCO_2H (3.6 mL, 94 mmol) were added to a solution of Z-derivative **15** (192 mg, 0.394 mmol) in EtOH (15 mL) under argon. After the solution was stirred for 1 hr at room temperature, the catalyst was removed by filtration and the filtered catalyst was washed with EtOH. The EtOH solutions were combined and concentrated in vacuo, and the residue was treated with 0.2N HCl (5.5 mL). Toluene and EtOH were added to the solution and the solvents were azeotropically removed in vacuo to give amine salt **16** as a colorless, amorphous material (quantitative). DPPA[15] (0.10 mL, 0.47 mmol) and Et_3N (0.17 mL, 1.2 mmol) were successively added to an ice-cooled solution of the amine salt **16** and acid **17**[6c] (226 mg, 0.394 mmol) in DMF (13.5 mL) under argon. The solution was allowed to warm to room temperature, stirred for 12 hr, and concentrated in vacuo. The residue was partitioned between AcOEt and aqueous $NaHCO_3$. 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 $CHCl_3$: MeOH = 25 : 1) followed by medium pressure liquid chromatography (Merck, LiChroprep Si60) to give **18** as a yellow, amorphous material (291 mg, 81%): Rf 0.27 ($CHCl_3$: MeOH = 20 : 1); $[\alpha]_D^{21.0}$ = +47.2° (c = 0.85, $CHCl_3$); IR (KBr) 3320, 2975, 2931, 1727, 1655, 1600, 1566, 1509, 1448, 1367, 1337, 1305, 1187, 1097, 1048 cm^{-1}; ^1H NMR (CD_3OD) δ 1.12 (3H, d, J = 7.0 Hz, C*H*$_3$CH), 1.14 (9H, s, (C*H*$_3$)$_3$C), 1.22 (3H, t, J = 7.0 Hz, C*H*$_3$CH$_2$), 1.70~1.82

(2H, m, CHC*H*$_2$CH$_2$), 2.36 (1H, ddd, J = 7.3, 7.3, 16.5 Hz, CH$_2$C*H*$_2$CO), 2.42 (1H, ddd, J = 7.3, 7.3, 16.5 Hz, CH$_2$C*H*$_2$CO), 3.60~3.86 (3H, br m, C*H*$_2$CHCONH$_2$ and CH$_2$C*H*CONH$_2$), 3.82 (3H, s, PyOC*H*$_3$), 3.90 (1H, m CH$_3$C*H*), 4.09 (2H, q, J = 7.0 Hz, CH$_3$C*H*$_2$), 4.40~4.65 (2H, br m, PyC*H*$_2$), 4.70 (1H, d, J = 7.3 Hz, CONHC*H*CONH), 5.11 (1H, br d, J = 7.3 Hz, C*H*Im), 6.99~7.12 (2H, br m, Py and Im), 7.26 (1H, ddd, J = 1.5, 7.0, 8.4 Hz, Nps), 7.29~7.39 (2H, m, Nps), 7.45~7.76 (3H, br m), 7.91~8.14 (1H, br), 7.99 (1H, d, J = 8.1 Hz, Nps), 8.21 (1H, dd, J = 1.5, 8.4 Hz, Nps), 8.28 (1H, d, J = 8.1 Hz, Nps); FABMS m/z 911 (MH$^+$), 912 (MH$^+$+1); HRMS (FAB) calcd for $C_{40}H_{51}N_{10}O_{11}S_2$ (MH$^+$): 911.31804, found: 911.31719.

(*S*)-4-[[*N*$^{\alpha}$-[[6-[[*N*-[(*S*)-2-Carbamoyl-2-[*N*-[(*o*-nitrophenyl)thio]amino]ethyl]-*N*-[(*o*-nitrophenyl)thio]amino]methyl]-4-methoxy-2-pyridyl]carbonyl]-*erythro*-β-*tert*-butoxy-L-histidyl]amino]pentanoic Acid (19). 0.5N NaOH (1.6 mL) was added dropwise over 10 min to an ice-cooled solution of ester **18** (195 mg, 0.214 mmol) in MeOH (7.5 mL). After being stirred for 28 hr at 0~4°C, the solution was acidified to pH 6~7 with citric acid at the same temperature. Toluene and MeOH were added to the solution and the solvents were azeotropically removed in vacuo. The residue was purified by preparative TLC (developed with CHCl$_3$: MeOH = 15 : 2) followed by gel filtration on Sephadex LH-20 (eluted with MeOH) to give **19** as a yellow, amorphous material (130 mg, 69%): Rf 0.23 (CHCl$_3$: MeOH = 10 : 1); $[\alpha]_D^{23.0}$ = +35.4° (c = 0.93, MeOH); IR (KBr) 3327, 2974, 2928, 1654, 1601, 1566, 1509, 1337, 1305 cm^{-1}; ^{1}H NMR (CD$_3$OD) δ 1.12 (3H, d, J = 7.0 Hz, C*H*$_3$CH), 1.14 (9H, s, (C*H*$_3$)$_3$C), 1.70~1.82 (2H, m, CHC*H*$_2$CH$_2$), 2.33 (1H, ddd, J = 7.7, 7.7, 16.5 Hz, CH$_2$C*H*$_2$CO), 2.40 (1H, ddd, J = 7.7, 7.7, 16.5 Hz, CH$_2$C*H*$_2$CO), 3.60~3.86 (3H, br m, C*H*$_2$CHCONH$_2$ and CH$_2$C*H*CONH$_2$), 3.83 (3H, s, PyOC*H*$_3$), 3.90 (1H, m CH$_3$C*H*), 4.36~4.64 (2H, br m, PyC*H*$_2$), 4.70 (1H, d, J = 7.3 Hz, CONHC*H*CONH), 5.04~5.22 (1H, br, C*H*Im), 7.04 (1H, d, J = 2.2 Hz, Py), 7.08 (1H, s, Im), 7.27 (1H, ddd, J = 1.5, 7.0, 8.4 Hz, Nps), 7.29~7.39 (2H, m, Nps), 7.46~7.82 (3H, br m), 7.93~8.15 (1H, br), 7.99 (1H, d, J = 8.1 Hz, Nps), 8.21 (1H, dd, J = 1.5, 8.4 Hz, Nps), 8.28 (1H, d, J = 8.4 Hz, Nps); FABMS m/z 883 (MH$^+$); HRMS (FAB) calcd for $C_{38}H_{47}N_{10}O_{11}S_2$ (MH$^+$): 883.28674, found: 883.28822.

bis(Nps)-PYML(6)-(4*S*-APA)-distamycin (22). Trifluoroacetic acid (1.2 mL, 16 mmol) was added dropwise to an ice-cooled solution of **20**[7] (77.4 mg, 0.118 mmol) in CH$_2$Cl$_2$ (1.2 mL) over 5 min under argon. After being stirred at the same temperature for 30 min and then at room temperature for 1.5 hr, the solution was concentrated in vacuo. The residue was dried over KOH in vacuo to give amine TFA salt **21** as a pale-brown, amorphous material. Acid **19** (95.0 mg, 0.108 mmol), Et$_3$N (75 mL, 0.54 mmol) and DPPA[14] (37 mL, 0.17 mmol) were added successively to an ice-cooled solution of the amine TFA salt **21** in DMF (4.0 mL) under argon. The solution was allowed to warm to room temperature, stirred for 2 days, and concentrated in vacuo. The residue was purified by chromatography on silica gel (eluted with CHCl$_3$: MeOH = 10 : 1 then CHCl$_3$: MeOH = 3 : 1) followed by preparative TLC (developed with CHCl$_3$: MeOH : Et$_3$N = 16 : 4 : 1) and gel filtration on Sephadex LH-20 (eluted with MeOH) to give **22** as a yellow, amorphous material (94.8 mg, 62%): Rf 0.32 (CHCl$_3$: MeOH : Et$_3$N = 25 : 5 : 1); $[\alpha]_D^{23.0}$ = +26.3° (c = 0.99, MeOH); IR (KBr) 3298, 2930, 1648, 1592, 1566, 1511, 1465, 1434, 1405, 1338, 1305, 1259, 1202 cm^{-1}; ^{1}H NMR (DMSO-*d*$_6$) δ 0.97 (3H, d, J = 6.2 Hz, C*H*$_3$CH), 1.05 (9H, s, (C*H*$_3$)$_3$C), 1.46~1.73 (6H, m, CH$_2$C*H*$_2$CH$_2$×2 and CHC*H*$_2$CH$_2$), 1.99~2.12 (2H, m, CHCH$_2$C*H*$_2$), 2.19 (6H, s, N(C*H*$_3$)$_2$), 2.23 (2H, t, J = 7.0 Hz, CH$_2$CH$_2$C*H*$_2$CO or C*H*$_2$N(CH$_3$)$_2$), 2.30 (2H, t, J = 7.0 Hz, CH$_2$CH$_2$C*H*$_2$CO or C*H*$_2$N(CH$_3$)$_2$), 3.06 (2H, m, CONHC*H*$_2$), 3.19 (2H, m, CONHC*H*$_2$), 3.57~3.90 (3H, br m, C*H*$_2$CHCONH$_2$ and CH$_2$C*H*CONH$_2$), 3.70 (1H, m, CH$_3$C*H*), 3.80, 3.81, 3.83, and 3.84 (each 3H, s, PyOC*H*$_3$ and NC*H*$_3$×3), 4.40~4.62 (2H, br m, PyC*H*$_2$), 4.58~4.68 (1H, m, CONHC*H*CONH), 4.97 (1H, d, J = 5.5 Hz, C*H*Im), 5.13~5.24 (1H, br m, N*H*Nps), 6.83 (1H, d, J = 1.5 Hz, Pyrrole), 6.87 (1H, d, J = 1.5 Hz, Pyrrole), 7.00 (1H, br s), 7.04 (1H, d, J = 1.5 Hz, Pyrrole), 7.11~7.26 (1H, br), 7.15 (1H, d, J = 1.8 Hz, Pyrrole), 7.18 (1H, d, J = 1.5 Hz, Pyrrole), 7.23 (1H, d, J = 1.8 Hz, Pyrrole), 7.28~7.43 (4H, m, Nps), 7.55 (2H, br s), 7.61~7.90 (2H, br), 7.68 (1H, m, Nps), 7.79 (1H, t, J = 5.5 Hz, CON*H*), 7.95~8.13 (3H, br m), 8.08 (1H, t, J = 5.5 Hz, CON*H*), 8.25 (2H, d, J = 8.1 Hz, Nps), 9.02~9.28 (1H, br, PyCON*H*), 9.82 (1H, s, CON*H*), 9.89 (1H, s, CON*H*), 9.90 (1H, s, CON*H*); FABMS m/z 1418 (M$^+$), 1419 (MH$^+$); HRMS (FAB) calcd for $C_{65}H_{84}N_{19}O_{14}S_2$ (MH$^+$): 1418.58865, found: 1418.59112.

PYML(6)-(4*S*-APA)-distamycin. Trifluoroacetic acid (0.30 mL, 4.0 mmol) was added dropwise to an ice-cooled solution of bis(Nps)-derivative 22 (30.0 mg, 2.11×10^{-2} mmol) and skatole (60.0 mg, 0.457 mmol) in CH_2Cl_2 (6.0 mL) over 3 min under argon. The solution was stirred for 2 hr at room temperature, treated with Et_3N (1.13 mL, 8.08 mmol) with ice cooling, and concentrated in vacuo. The residue was purified by repeated gel filtration on Sephadex LH-20 (eluted with MeOH) to give a pale-yellow, amorphous material, which was further charged on an Amberlite IRA 93 (OH^- form, 15 mL) and eluted with MeOH to give PYML(6)-(4*S*-APA)-distamycin as colorless powder (17.3 mg, 73%): Rf 0.61 (*n*-PrOH : AcOH : Py : H_2O = 15 : 3 : 10 : 12); $[\alpha]_D^{23.0}$ = +24.6° (c = 0.52, MeOH); IR (KBr) 3314, 2926, 1654, 1528, 1466, 1436, 1406, 1261, 1202, 1134 cm^{-1}; ^1H NMR (CD_3OD) d 1.09 (3H, d, J = 6.6 Hz, C*H*$_3$CH), 1.15 (9H, s, (C*H*$_3$)$_3$C), 1.66~1.80 (2H, m, CHC*H*$_2$CH$_2$), 1.86 (2H, m, CH$_2$C*H*$_2$CH$_2$), 1.98 (2H, m, CH$_2$C*H*$_2$CH$_2$), 2.23 (2H, t, J = 7.3 Hz, CH$_2$CH$_2$C*H*$_2$CO), 2.28~2.43 (2H, m, CHCH$_2$C*H*$_2$), 2.84~2.94 (1H, m, C*H*$_2$CHCONH$_2$), 2.88 (6H, s, N(C*H*$_3$)$_2$), 3.00 (1H, dd, J = 7.1, 13.0 Hz, C*H*$_2$CHCONH$_2$), 3.13 (2H, t, J = 7.3 Hz, CONHC*H*$_2$ or C*H*$_2$N(CH$_3$)$_2$), 3.23~3.34 (2H, m, CONHC*H*$_2$ or C*H*$_2$N(CH$_3$)$_2$), 3.41 (2H, t, J = 6.6 Hz, CONHC*H*$_2$ or C*H*$_2$N(CH$_3$)$_2$), 3.78~3.97 (2H, m, CH$_3$C*H* and CH$_2$C*H*CONH$_2$), 3.87, 3.88, 3.89, and 3.90 (each 3H, s, PyOC*H*$_3$ and NC*H*$_3$×3), 4.00 (2H, s, PyC*H*$_2$), 4.71 (1H, d, J = 7.3 Hz, CONHC*H*CONH), 5.14 (1H, d, J = 7.3 Hz, C*H*Im), 6.81 (1H, d, J = 1.8 Hz, Pyrrole), 6.88 (1H, d, J = 1.5 Hz, Pyrrole), 6.92 (1H, d, J = 1.5 Hz, Pyrrole), 7.06 (1H, d, J = 2.2 Hz, Py), 7.08 (1H, d, J = 1.1 Hz, Im), 7.156 (1H, d, J = 1.8 Hz, Pyrrole), 7.162 (1H, d, J = 1.8 Hz, Pyrrole), 7.17 (1H, d, J = 1.8 Hz, Pyrrole), 7.43 (1H, d, J = 2.2 Hz, Py), 7.65 (1H, d, J = 1.1 Hz, Im); FABMS m/z 1112 (M^+), 1113 (MH^+); HRMS (FAB) calcd for $C_{53}H_{78}N_{17}O_{10}$ (MH^+): 1112.61174, found: 1112.60970.

DNA cleavage experiment. Nucleotide sequence cleavage was investigated on the 5'- and 3'-end labeled strands of a 100 base pairs DNA restriction fragment (AluI-HaeIII) from the phage R199/G4ori. The reaction mixtures contained 10 mM Tris-HCl buffer (pH 7.4), 5'- or 3'-end ^{32}P-labeled G4 phage DNA fragment, 1 μg carrier calf thymus DNA, 1 mM dithiothreitol, and 5 μM PYML(6)-linker-distamycin (or natural BLM)-iron complex. After the reaction solutions were incubated at 37°C for 10 min, the DNA samples were subjected to electrophoresis on a 10% polyacrylamide/7M urea slab gel. The autoradiogram was scanned with a microdensitometer.

Computer simulation of the complex between double-stranded DNA and man-designed bleomycins. The three-dimensional structure of the double stranded DNA was prepared based on the structure of B-DNA (CGCAAATTTGCG)$_2$ in the DNA-distamycin complex (Protein Data Bank: 2DND).[21] As the molecules of PYML(6)-linker-distamycins were larger than distamycin, the DNA sequence was changed to CGCAAATTTTGCG-GCGAAAATTTCGC by inserting two A-T base pairs and optimizing by molecular mechanics calculation. Structures of PYML(6)-linker-distamycins were prepared by model building using the crystal structure of P-3A-Cu(II) complex, the biosynthetic intermediate of BLM.[22]

Binding of PYML(6)-linker-distamycins to DNA was simulated by quenched molecular dynamics simulation as follows. (The same procedure was applied to all three compounds.) An initial model for the calculation was determined considering intermolecular hydrogen bonds and the relative position of the active oxygen on PYML-Fe and the possible reaction site on DNA. Specific intermolecular hydrogen bonds observed in the DNA-distamycin complex were conserved in the model. In order to relax the model, the molecular dynamics trajectory was calculated at temperature 600 K for 25 ps allowing the PYML(6)-linker-distamycin molecule to move. Snapshots of the trajectory were stored every 0.5 ps and then optimized by molecular mechanics calculation allowing the whole model to move. A snapshot with the lowest energy was selected as the relevant structure of the complex. In order to take into account the highly solvated environment of DNA, the dielectric constant was set to the value for water (78.3) throughout these calculations. All molecular dynamics and molecular mechanics calculations were done with AMBER 3.0A.[23]

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