Novel Bleomycin Analogues: Synthesis, Antitumor Activity, and Interaction with DNA

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The novel analogues **11**–**16** of bleomycin A_6 (**3**) were obtained by selective protection of the primary-amine function of the β-aminoalaninamide moiety of **3** by means of coordination with Cu^{II} ions, condensation with an aliphatic or aromatic acid R′COOH in the presence of dicyclohexylcarbodiimide, and demetalization (*Scheme*). The antitumor activity against HeLa and BGC-823 *in vitro*, binding property with CT-DNA, and cleavage potency towards pBR322 DNA were also studied (*Tables 1–3*). All the compounds **11**–**16** displayed significant antitumor activity, which was enhanced as the hydrophobicity of the C-terminus substituent R′ increased, but decreased as the DNA-binding affinity increased. There was a negative relationship between DNA-cleavage potency and binding affinity to DNA in this series of compounds.

Introduction. – The bleomycins (BLMs) are glycopeptide-derived antitumor antibiotics originally isolated from *Streptomyces verticillus* by *Umezawa* and co-workers [1]. A number of BLMs, such as BLM A_2 (**1**), BLM A_5 (**2**), and peplomycin are now used routinely as antitumor agents for the treatment of several neoplastic diseases including squamous cell carcinomas, non-*Hodgkin*'s lymphomas, testicular carcinomas, and ovarian cancer [2–6]. The generally accepted basis for the clinical efficacy of BLMs is believed to derive from their ability to bind and oxidatively cleave DNA and possibly RNA, in the presence of a metal ion as cofactor [7–10]. The structure of BLMs (*Fig. 1*) is commonly divided into four functional domains: the N-terminus domain, which is responsible for metal binding, oxygen activation, and site selection of DNA cleavage, the C-terminus domain containing a bithiazole moiety and polyamine side chain that provides the majority of the DNA binding affinity, the methylvalerate-threonine linker domain connecting the N-terminus domain with the C-terminus domain, and the disaccharide domain, which is proposed to influence metal-ion binding and cell-surface recognition [11][12]. Naturally occurring BLMs differ only in the nature of the C-terminus substituent, such as a sulfonium moiety in BLM A_2 (**1**), a spermidine moiety in BLM A_5 (**2**) and a spermine moiety in BLM A_6 (**3**).

As a consequence of their clinical utility as well as of their mechanism of action and their interesting structures, BLMs have been the focus of considerable attention [13–16]. Some studies have established that the C-terminus properties are related to their antitumor activity and renal and pulmonary toxicity [17][18]. Hitherto, a number of interesting analogues of BLMs modified at the C-terminus have been prepared by means of a fermentation method in media containing a special amine or by means of a semi-synthetic method starting from bleomycinic acid, and some of the analogues have

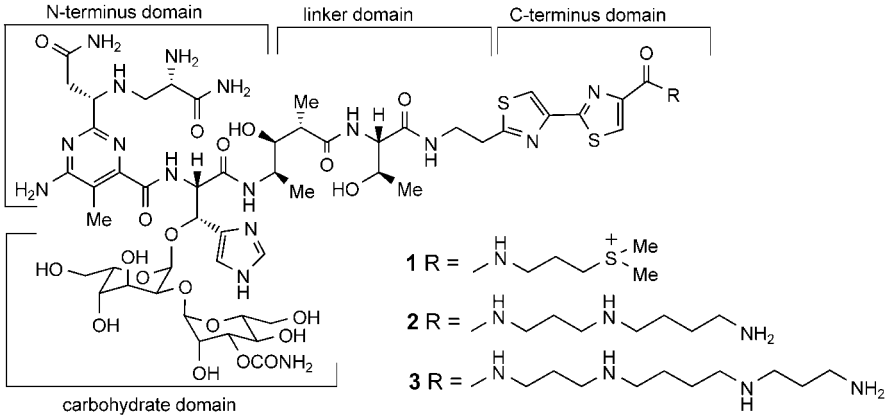


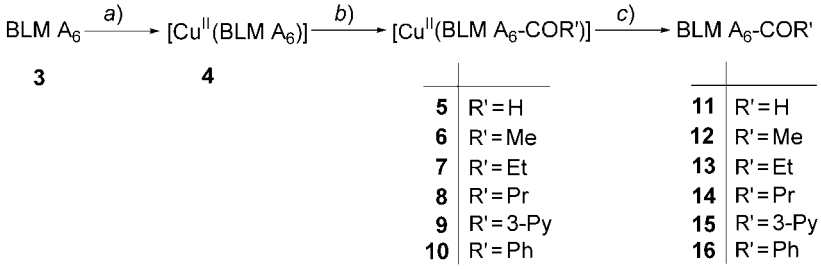
Fig. 1. *Structure of bleomycins* (BLMs) **1**–**3** *and functional domains*

been reported to diminish pulmonary toxicity as compared to blenoxane [19][20]. The development of a generally applicable route to modify the C-terminal amine moiety still represents a nice challenge in BLMs synthetic chemistry.

BLM A_6 (= *boanmycin*; **3**) is a naturally occurring BLM antibiotic isolated from the fermentation solution of *S. Verticillus var, Pingyangensis n. var* [21]. It has completed phase-III clinical trials as antitumor agents for treatment of malignant lymphoma and cancer of head and neck [22][23]. However, BLM A_6 (**3**) possesses a number of drawbacks as a member of BLMs, notably the side effect of pulmonary fibrosis. This prompted us to develop new BLMs with more effectiveness and fewer side effects. Herein we describe our facile synthetic method modifying the C-terminal amine moiety of BLM A_6 (**3**) by a carboxylic acid derivative, starting from commercially available **3**. In addition, the antitumor activity, DNA-binding property with calf thymus DNA (CT-DNA), and cleavage potency towards pBR 322 DNA in the presence of Fe^{II} ions were also studied.

Results and Discussions. – *Synthesis.* A general synthesis for BLM-A_6 derivatives **11**–**16** was performed starting from BLM A_6 (**3**) (*Scheme*). To selectively protect the primary amine function of the β-aminoalaninamide part in the N-terminus domain of BLM A_6 (**3**), the latter was converted to its copper complex [Cu^{II}·(BLM A_6)] (**4**) by reaction with $CuSO_4$ in aqueous solution. Compound **4**, a blue powder, was an ideal intermediate for further synthetic transformations, because it contains only two free NH_2 groups unemployed as metal ligands with different reactivities; one is the terminal NH_2 group in the C- terminus domain and the other the NH_2 group at the pyrimidine moiety. Thus, **4** was coupled with an aliphatic or aromatic acid R′COOH at $-5°$ for 12 h in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) in MeOH, to yield [Cu^{II}·(BLM-A_6-COR′)] complexes **5**–**10** as major products. Compounds **5**–**10** were treated with 15% EDTA solution to remove the Cu^{II} ion and then desalted on a *HP-20* column to afford BLM-A_6-COR′ **11**–**16** as colorless powders in 31–54% overall yield. The proposed structures of **11**–**16** were confirmed by FAB-MS and NMR data.

Scheme



a) $CuSO_4$, H_2O, 0°, 30 min. *b*) DCC, MeOH, −5°, 12 h. *c*) 15% aq. EDTA soln., 30°, 1 h.

Compared with **3**, the δ(H) of the terminal CH_2 group of the C-terminal spermine moiety of **11**–**16** were shifted downfield from δ(H) 2.99 to 3.22–3.49 in the ^{1}H-NMR, while its δ(C) was shifted upfield from δ(C) 40.8 to 36.6–37.6 in the ^{13}C-NMR, indicating that the acylation had occurred at the NH_2 group within the C-terminal spermine moiety.

Cytotoxicity Assay. Compounds **11**–**16** were evaluated for cytotoxity *in vitro* against human cervical carcinoma (HeLa) and human stomach carcinoma (BGC-823) cell lines by using the tetrazolium-salt (MTT) assay. The 50% inhibition concentrations (IC_{50}) of BLM-A_6-COR′ **11**–**16** are summarized in *Table 1* and compared with the positive control drugs BLM A_2 (**1**) and BLM A_6 (**3**). Thus **11**–**16** exhibited significant antitumor activity with IC_{50} values in the range of 1.28–19.30 μM. Furthermore, the antitumor activity of compounds **11**–**16** was moderately enhanced as the hydrophobicity of the C-terminus substituent R′ increased. Compound **16** was the most-promising agent in the series. This interesting observation suggests that the hydrophobicity of the C-terminus substituent R′ of BLM derivatives affects their binding and cleavage properties to nuclear DNA and their permeability to the cell membrane, thus resulting in various antitumor activities.

Table 1. In vitro *Antitumor Activities of Compounds* **1**, **3** *and* **11**–**16** *against Human Tumor Cell Lines*

	IC_{50} [μM] ± s.d.[a])	
	HeLa	BGC-823
11	1.28 ± 0.22	8.19 ± 1.05
12	1.95 ± 0.16	19.30 ± 3.12
13	1.40 ± 0.21	14.50 ± 2.35
14	2.41 ± 0.32	3.43 ± 0.39
15	1.70 ± 0.30	4.10 ± 0.76
16	1.36 ± 0.21	3.05 ± 0.64
1	1.92 ± 0.23	14.80 ± 2.25
3	1.61 ± 0.11	10.10 ± 2.15

[a]) The IC_{50} values of the test compounds were determined by using the MTT cytotoxicity assay as described in the *Exper. Part*; values are means ± standard deviations (s.d.) of triplicate determinations.

DNA Cleavage. BLMs are believed to mediate their therapeutic effects *via* degradation of chromosomal DNA. To further elucidate this issue, the abilities of

compounds **11**–**16** and of BLM A_6 (**3**) to cleave duplex DNA were tested by examination of single-strand and double-strand cleavage of supercoiled pBR322 DNA (form I) to produce relaxed DNA (Form II) in the presence of Fe^{II} ions [24][25]. The results of the densitometric analysis of the gel picture (*Fig. 2*) are shown in *Fig. 3*, indicating that **11**–**16** cleaved DNA with 1.01–1.44 fold greater potency than BLM A_6 (**3**) and that **14** was the most potent. Interestingly, there was an increase in the DNA-cleavage potency as the chain length of the substituent R′ increased from C_1 in **12** to C_3 in **14**, and in going from **15** to **16** where R′ is pyridin-3-yl and Ph, respectively. The trend of the cleavage potency in the series **11**–**16** was not completely consistent with that of their antitumor activities, which indicates that the DNA cleavage is a necessary but not sufficient condition for antitumor activity. The permeability to the cell membrane or the uptake by cells of BLMs seems to play a key role in their biological activity.

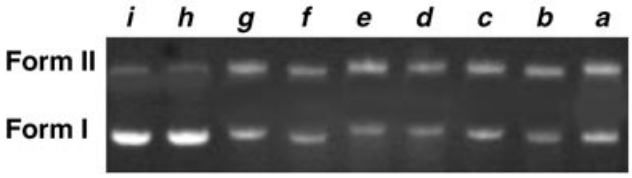


Fig. 2. *Agarose-gel picture illustrating the cleavage reaction of supercoiled pBR322 by* **3** *and* **11**–**16** (2.0 μM) *in the presence of* Fe^{II} *ions* (4.0 μM). *Lane a*, **3**/Fe^{II}; *Lane b*, **11**/Fe^{II}; *Lane c*, **12**/Fe^{II}; *Lane d*, **13**/Fe^{II}; *Lane e*, **14**/Fe^{II}; *Lane f*, **15**/Fe^{II}; *Lane g*, **16**/Fe^{II}; *Lane h*, 4.0 μM Fe^{II}; *Lane i*, DNA alone.

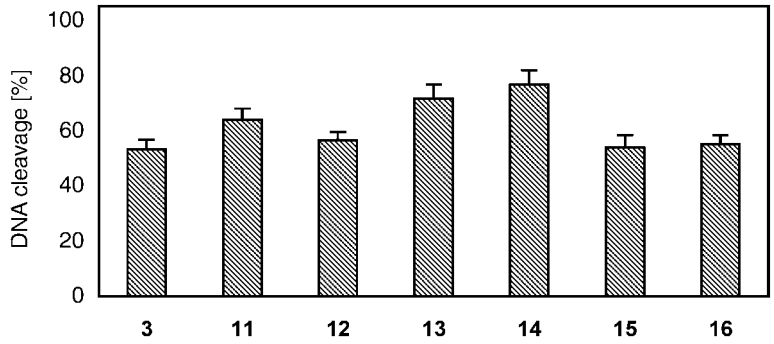


Fig. 3. *Cleavage efficiency of supercoiled pBR322 plasmid DNA to form-II DNA by* **3** *and* **11**–**16** *in the presence of* Fe^{II} *ions.* Error bars represent the standard error of the mean of three independent experiments.

DNA-Binding Studies. The C-terminus domain of BLMs provides the majority of the DNA-binding affinity and is important for their antitumor activities. To compare the effects on DNA-binding affinity and antitumor activity resulting from the C-terminus modification, the DNA-binding properties including thermal denaturization alteration (ΔT_m; see *Table 2*) and apparent binding constant (K_b; see *Table 3*) of the BLM-A_6 derivatives **11**–**16** bound to CT-DNA were studied and compared to the behavior of **3**. Compounds **11**–**16** elevated the helix melting temperature T_m of CT-DNA by 5.6–7.3° and, thus, are efficient stabilizing agents for double-stranded CT-

Table 2. *Thermal Denaturization Data* (ΔT_m) *for Compounds* **3** *and* **11**–**16**

	ΔT_m [°][a])		ΔT_m [°][a])
11	7.3 ± 0.06	**15**	8.2 ± 0.11
12	6.9 ± 0.06	**16**	7.2 ± 0.06
13	5.9 ± 0.11	**3**	13.7 ± 0.10
14	5.6 ± 0.06		

[a]) Values are means ± s.d. of triplicate determinations.

Table 3. *Apparent Binding Constants* (K_b) *of Compounds* **3** *and* **11**–**16** *to CT-DNA*

	$K_b \cdot 10^5$ [M^{-1}][a])		$K_b \cdot 10^5$ [M^{-1}][a])
11	2.19 ± 0.08	**15**	2.74 ± 0.11
12	1.50 ± 0.07	**16**	2.16 ± 0.07
13	1.28 ± 0.06	**3**	5.32 ± 0.12
14	1.19 ± 0.06		

[a]) Values are means ± s.d. of triplicate determinations.

DNA. However, BLM A_6 (**3**) gave a ΔT_m of 13.7° in the same experiment, illustrating that **11**–**16** and **3** bound to CT-DNA with a markedly different binding affinity.

Quantitative measurements of the DNA binding were determined by fluorimetric titration. The results (*Table 3*) showed that compounds **11**–**16** displayed high DNA-binding activity. However, the DNA-binding affinity decreased dramatically with the hydrophobicity of the C-terminus substituent R′ from **12** to **14** and from **15** to **16**. It could also be observed that BLM A_6 (**3**) still exhibited a significantly higher affinity for CT-DNA than **11**–**16**. This result can be explained by a decrease in positive charge of the C-terminus domain by the hydrophobic group R′, and, thus, a decrease of the electrostatic binding affinity to CT-DNA, and by the longer flexible side chain decreasing the intercalation stability of the bithiazol domain.

The trend of the DNA binding affected by the substituent R′ is opposite to that of the DNA-cleavage efficiency. The DNA-cleavage efficiency of compound **3** was weaker and its DNA-binding strength stronger than those of compounds **11**–**16**. This suggested that **3** was more positively charged at the C-terminal amine moiety under the testing conditions, thus decreasing its cleavage efficiency. This finding is in good agreement with our previous studies [25].

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Experimental Part

General. TLC: precoated silica-gel *60 F_{254}* plates; detection by UV light (254 nm). Column chromatography (CC): silica-gel (200–300 mesh; *Qingdao Chemical Co.*). M.p.: *Electrothermal XT-4* digital melting-point apparatus; uncorrected. NMR Spectra: *Varian Inova-500* instrument; δ in ppm with Me_4Si as an internal standard (= 0 ppm). FAB-MS: *ZAB-HS* instrument; in *m*/*z*.

{N^1-{3-[{4-[(3-Aminopropyl)amino]butyl}amino]propyl}bleomycinamide}copper(II) (**4**). An aq. soln. containing **3** (470 mg, 315 μmol) was treated with $CuSO_4$ (560 mg, 350 μmol), and the mixture was maintained

at 0° for 30 min. The resulting soln. was subjected to reversed-phase CC (*C-18*, washing with H_2O, then MeOH/2 mM HCl 4:1). The blue product-containing fraction was concentrated (removal of excess MeOH) and then lyophilized: **4** (438 mg, 89.4%) as a blue powder.

N^1-*{3-{[4-{[3-(Formylamino)propyl]amino}butyl]amino}propyl}bleomycinamide* (**11**). Formic acid (6.9 mg, 150 μmol) was added to a MeOH (6 ml) soln. containing DCC (62 mg, 300 μmol). The mixture was stirred at 0° for 30 min. A soln. of **4** (60 mg, 38 μmol) in MeOH (5 ml) was then added and stirred at −5° for 12 h (TLC monitoring). The DCU precipitate was filtered off and the filtrate evaporated. The crude product was purified by CC (silica gel, MeOH/10% NH_4Ac soln./10% NH_3 soln. 100:10:1). The MeOH of the product fraction was evaporated and the residue lyophilized: **5** as a blue powder.

Demetallation was accomplished by stirring **5** with 15% EDTA soln. (10 ml) at 30° for 1 h. The mixture was subjected to ion-exchange (*HP-20* column, washing with H_2O, then MeOH/2 mM HCl 4:1). The eluate was evaporated and the residue lyophilized: **11** (20 mg, 35%). White powder. M.p. 174.0–175.0°. ^{1}H-NMR (D_2O): 1.07 (*d*, 3 H); 1.10 (*d*, 3 H); 1.16 (*d*, 3 H); 1.69 (*m*, 4 H); 1.88 (*m*, 2 H); 1.98 (*s*, 3 H); 2.46 (*m*, 1 H); 2.62 (*m*, 2 H); 2.69 (*m*, 1 H); 2.98 (*m*, 2 H); 3.09 (*m*, 2 H); 3.12 (*m*, 2 H); 3.22 (*m*, 2 H); 3.27 (*m*, 2 H); 3.43 (br., 2 H); 3.56 (*m*, 2 H); 3.63 (*m*, 2 H); 3.71 (*m*, 1 H); 3.78 (*m*, 2 H); 3.84 (br., 2 H); 3.86 (br., 1 H); 3.92 (*m*, 1 H); 4.00 (*m*, 2 H); 4.05 (*m*, 3 H); 4.09 (br., 1 H); 4.21 (br., 1 H); 4.65 (br., 1 H); 5.00 (*s*, 1 H); 5.07 (*d*, 1 H); 5.28 (*m*, 2 H); 5.48 (*d*, 1 H); 7.29 (*s*, 1 H); 7.86 (*d*, 1 H); 7.98 (*s*, 1 H); 8.05 (*d*, 1 H); 8.16 (*s*, 1 H). ^{13}C-NMR (D_2O): 11.4; 12.9; 14.9; 19.5; 23.3; 24.1; 26.3; 26.9; 32.6; 37.0; 37.6; 37.9; 40.0; 40.8; 43.3; 45.7; 46.2; 47.6; 48.0; 48.3; 53.0; 56.9; 59.8; 60.2; 61.6; 61.8; 65.3; 67.7; 67.9; 68.4; 69.0; 69.7; 70.8; 73.6; 74.2; 75.0; 98.0; 98.6; 113.0; 118.9; 119.7; 125.5; 135.2; 137.5; 147.6; 149.6; 152.7; 158.6; 163.7; 165.3; 165.9; 166.2; 168.3; 169.6; 171.3; 171.7; 172.6; 176.9; 178.2; 180.8. FAB-MS: 1526.0 ($C_{61}H_{97}N_{20}O_{22}S_2^+$, $[M+H]^+$; calc. 1526.7).

N^1-*{3-{[4-{[3-(Acetylamino)propyl]amino}butyl]amino}propyl}bleomycinamide* (**12**). As described for **11**, with AcOH (9.0 mg, 150 μmol): **12** (18 mg, 31%). White powder. M.p. 174.0–175.0°. ^{1}H-NMR (D_2O): 1.05 (*d*, 3 H); 1.11 (*m*, 6 H); 1.75 (*m*, 4 H); 1.84 (*m*, 2 H); 1.94 (*s*, 3 H); 1.96 (*s*, 3 H); 2.00 (*m*, 2 H); 2.55 (*m*, 1 H); 2.70 (*m*, 3 H); 3.01 (*m*, 2 H); 3.03 (*m*, 2 H); 3.07 (*m*, 2 H); 3.11 (*m*, 2 H); 3.22 (*m*, 5 H); 3.48 (*m*, 2 H); 3.56 (*m*, 2 H); 3.62 (br., 2 H); 3.71 (br., 1 H); 3.72 (br., 2 H); 3.82 (br., 1 H); 3.86 (br., 2 H); 3.97 (br., 1 H); 4.00 (br., 2 H); 4.03 (br., 2 H); 4.07 (*s*, 1 H); 4.12 (br., 1 H); 4.18 (br., 1 H); 4.71 (br., 1 H); 4.99 (*s*, 1 H); 5.05 (*d*, 1 H); 5.23 (*s*, 1 H); 5.46 (*d*, 1 H); 7.56 (*s*, 1 H); 7.99 (*s*, 1 H); 8.15 (*s*, 1 H); 8.72 (*s*, 1 H). ^{13}C-NMR (D_2O): 10.2; 11.8; 13.4; 14.7; 19.5; 23.5; 26.3; 26.5; 28.3; 29.8; 36.6; 37.0; 38.9; 39.8; 43.6; 45.7; 45.9; 47.4; 47.6; 48.4; 52.3; 56.8; 59.8; 60.1; 61.3; 61.6; 65.2; 67.6; 67.8; 68.8; 69.0; 69.8; 70.3; 72.2; 74.2; 75.0; 98.1; 98.7; 113.2; 119.4; 119.7; 125.8; 130.1; 135.1; 147.6; 149.5; 152.4; 158.8; 163.5; 164.3; 165.0; 167.6; 168.4; 171.5; 172.6; 175.6; 178.1; 179.3. FAB-MS: 1541.0 ($C_{62}H_{99}N_{20}O_{22}S_2^+$, $[M+H]^+$; 1540.7).

N^1-*{3-{[4-{[3-[(1-Oxopropyl)amino]propyl}amino}butyl]amino}propyl}bleomycinamide* (**13**). As described for **11**, with propanoic acid (11.1 mg, 150 μmol): **13** (28 mg, 47%). White powder. M.p. 171.0–172.0°. ^{1}H-NMR (D_2O): 1.08 (*d*, 3 H); 1.10 (*d*, 3 H); 1.15 (*m*, 6 H); 1.78 (*m*, 4 H); 1.88 (*m*, 2 H); 2.02 (*s*, 3 H); 2.04 (*m*, 2 H); 2.25 (*m*, 2 H); 2.60 (*m*, 1 H); 2.86 (*m*, 2 H); 3.04 (*m*, 2 H); 3.07 (*m*, 2 H); 3.11 (*m*, 2 H); 3.15 (br., 2 H); 3.27 (*m*, 2 H); 3.31 (br., 1 H); 3.54 (*m*, 2 H); 3.63 (br., 4 H); 3.75 (*m*, 1 H); 3.80 (br., 2 H); 3.86 (br., 1 H); 3.91 (*m*, 2 H); 4.01 (*m*, 1 H); 4.06 (br., 2 H); 4.07 (br., 2 H); 4.16 (br., 1 H); 4.22 (br., 1 H); 4.28 (*d*, 1 H); 4.73 (br., 1 H); 5.03 (*s*, 1 H); 5.10 (*d*, 1 H); 5.27 (*d*, 1 H); 5.51 (*d*, 1 H); 7.61 (*s*, 1 H); 8.06 (*s*, 1 H); 8.22 (*d*, 1 H); 8.80 (*d*, 1 H). ^{13}C-NMR (D_2O): 11.7; 13.3; 14.8; 19.5; 22.5; 23.5; 26.2; 26.5; 32.1; 32.7; 36.7; 37.0; 37.6; 39.8; 40.0; 43.5; 45.7; 45.9; 47.5; 47.6; 48.4; 52.4; 56.8; 59.8; 60.3; 61.3; 61.6; 65.2; 67.6; 67.7; 68.7; 69.0; 69.8; 70.3; 72.3; 74.2; 75.0; 98.1; 98.7; 112.8; 119.3; 119.7; 125.7; 130.3; 135.7; 147.6; 149.5; 152.9; 158.7; 163.4; 164.1; 165.0; 165.6; 168.0; 168.5; 171.0; 171.3; 172.6; 175.3; 176.1; 178.1. FAB-MS: 1554.0 ($C_{63}H_{101}N_{20}O_{22}S_2^+$, $[M+H]^+$; 1554.7).

N^1-*{3-{[4-{[3-[(1-Oxobutyl)amino]propyl}amino}butyl]amino}propyl}bleomycinamide* (**14**). As described for **11**, with butanoic acid (13.2 mg, 150 μmol): **14** (32 mg, 54%). White powder. M.p. 176.0–177.0°. ^{1}H-NMR (D_2O): 0.87 (*m*, 3 H); 1.08 (*d*, 3 H); 1.14 (*m*, 6 H); 1.57 (*m*, 2 H); 1.78 (*m*, 4 H); 1.88 (br., 2 H); 1.99 (*s*, 3 H); 2.04 (*m*, 2 H); 2.21 (*m*, 2 H); 2.58 (*m*, 1 H); 2.70 (*m*, 2 H); 3.03 (*m*, 2 H); 3.06 (*m*, 2 H); 3.10 (*m*, 2 H); 3.14 (*m*, 2 H); 3.27 (*m*, 5 H); 3.52 (*m*, 2 H); 3.61 (*m*, 2 H); 3.66 (br., 2 H); 3.74 (*m*, 1 H); 3.79 (br., 2 H); 3.85 (br., 1 H); 3.89 (*m*, 3 H); 4.03 (*m*, 3 H); 4.06 (br., 2 H); 4.15 (br., 2 H); 4.21 (br., 1 H); 4.74 (br., 1 H); 5.02 (*s*, 1 H); 5.08 (*d*, 1 H); 5.26 (*d*, 1 H); 5.48 (*d*, 1 H); 7.57 (*s*, 1 H); 8.02 (*s*, 1 H); 8.18 (*d*, 1 H); 8.70 (*d*, 1 H). ^{13}C-NMR (D_2O): 11.6; 13.3; 13.4; 14.8; 19.5; 19.7; 23.5; 26.3; 26.5; 32.6; 36.6; 37.0; 38.3; 39.8; 40.4; 43.5; 45.8; 45.9; 47.5; 47.6; 48.4; 52.7; 56.9; 59.8; 60.4; 61.3; 61.6; 65.3; 67.7; 68.7; 69.0; 69.8; 70.3; 72.4; 74.2; 75.0; 98.1; 98.6; 112.6; 119.2; 119.7; 125.7; 130.7; 135.8; 147.6; 149.4; 153.1; 158.7; 163.3; 164.2; 165.0; 165.1; 168.1; 198.5; 171.3; 172.6; 176.5; 178.1; 178.3. FAB-MS: 1568.0 ($C_{64}H_{103}N_{20}O_{22}S_2^+$, $[M+H]^+$; calc. 1568.7).

N^1-*{3-{[4-{[3-[(Pyridin-3-ylcarbonyl)amino]propyl}amino}butyl]amino}propyl}bleomycinamide* (**15**). As described for **11**, with nicotinic acid (18.5 mg, 150 μmol): **15** (29 mg, 48%). White powder. M.p. 197–198°. ^{1}H-NMR (D_2O): 1.08 (*d*, 3 H); 1.13 (*m*, 6 H); 1.69 (*m*, 2 H); 1.76 (*m*, 2 H); 1.99 (*s*, 3 H); 2.04 (*m*, 2 H); 2.55 (*m*, 1 H); 2.65 (*m*, 2 H); 3.00 (*m*, 2 H); 3.10 (*m*, 2 H); 3.16 (*m*, 2 H); 3.22 (*m*, 2 H); 3.41 (*m*, 2 H); 3.521 (*m*, 2 H); 3.59 (*m*, 2 H); 3.66 (br., 2 H); 3.73 (*m*, 1 H); 3.79 (br., 2 H); 3.84 (br., 1 H); 3.91 (*m*, 3 H); 3.98 (*m*, 2 H); 4.03 (br., 1 H); 4.06 (br., 2 H); 4.09 (br., 1 H); 4.13 (br., 1 H); 4.22 (*d*, 1 H); 4.71 (br., 1 H); 5.01 (*s*, 1 H); 5.07 (*d*, 1 H); 5.26 (*d*, 1 H); 5.42 (*d*, 1 H); 7.49 (*s*, 1 H); 7.52 (*s*, 1 H); 7.97 (*d*, 1 H); 8.13 (*d*, 1 H); 8.15 (br., 1 H); 8.45 (*s*, 1 H); 8.62 (br., 1 H); 8.80 (br., 1 H). ^{13}C-NMR (D_2O): 11.6; 13.1; 15.0; 19.5; 23.6; 26.2; 26.4; 32.6; 37.0; 39.7; 39.8; 40.8; 43.5; 45.6; 47.5; 47.8; 48.3; 52.9; 57.1; 59.8; 60.4; 61.2; 61.6; 65.3; 67.7; 67.9; 68.4; 69.0; 69.8; 70.4; 72.8; 74.2; 75.0; 98.3; 98.5; 112.6; 119.0; 119.6; 125.0; 125.7; 130.9; 132.0; 136.3; 137.1; 147.6; 149.4; 151.7; 153.1; 158.7; 163.3; 164.1; 165.1; 165.9; 168.3; 168.9; 171.2; 171.5; 172.6; 176.8; 178.1. FAB-MS: 1603.0 ($C_{66}H_{100}N_{21}O_{22}S_2^+$, $[M+H]^+$; calc. 1603.7).

N^1-*{3-{[4-{[3-(Benzoylamino)propyl]amino}butyl]amino}propyl}bleomycinamide* (**16**). As described for **11**, with benzoic acid (18.3 mg, 150 μmol): **16** (23 mg, 38%). White powder. M.p. 171.0–173.0°. ^{1}H-NMR (D_2O): 1.08 (*d*, 3 H); 1.14 (*m*, 6 H); 1.80 (*m*, 4 H); 1.99 (*s*, 3 H); 2.01 (*m*, 4 H); 2.56 (*m*, 1 H); 2.62 (*m*, 1 H); 2.68 (*m*, 1 H); 3.00 (*m*, 2 H); 3.09 (*m*, 2 H); 3.11 (*m*, 2 H); 3.12 (*m*, 2 H); 3.16 (br., 2 H); 3.23 (br., 1 H); 3.48 (*m*, 2 H); 3.52 (br., 2 H); 3.60 (*m*, 2 H); 3.65 (br., 2 H); 3.74 (*m*, 1 H); 3.79 (br., 2 H); 3.88 (br., 1 H); 3.91 (br., 2 H); 3.98 (*m*, 1 H); 4.03 (br., 2 H); 4.05 (br., 2 H); 4.10 (*m*, 1 H); 4.14 (*m*, 1 H); 4.22 (*d*, 1 H); 4.71 (br., 1 H); 5.01 (*s*, 1 H); 5.07 (*d*, 1 H); 5.27 (*d*, 1 H); 5.44 (*d*, 1 H); 7.47 (*s*, 2 H); 7.51 (*s*, 1 H); 7.56 (*m*, 1 H); 7.71 (*d*, 2 H); 7.99 (*d*, 1 H); 8.17 (*d*, 1 H); 8.52 (*s*, 1 H). ^{13}C-NMR (D_2O): 11.6; 13.2; 14.9; 19.5; 23.4; 23.5; 26.4; 26.5; 32.6; 37.0; 37.2; 39.8; 40.7; 43.5; 45.7; 47.6; 48.4; 49.6; 52.9; 57.0; 57.8; 60.4; 61.2; 61.6; 65.3; 67.7; 67.8; 68.5; 69.0; 69.8; 70.4; 72.7; 74.2; 75.0; 98.2; 98.5; 112.6; 119.0; 119.7; 125.7; 127.7; 129.5; 131.7; 133.0; 133.8; 136.2; 147.6; 149.4; 153.1; 158.7; 163.4; 164.2; 165.1; 165.8; 168.3; 168.8; 171.5; 171.9; 172.6; 176.7; 178.1. FAB-MS: 1602.0 ($C_{67}H_{101}N_{20}O_{22}S_2^+$, $[M+H]^+$; calc. 1602.7).

Cytotoxicity Assay. Cytotoxicity *in vitro* was evaluated by using the MTT colorimetric assay. Human-derived cell lines (HeLa and BGC-823) were cultured in RPMI 1640 medium supplemented with 10% freshly inactivated fetal calf serum (FCS) and antibiotics. Cells were seeded into 96-well microtiter plates at a density of $4 \cdot 10^3$ per well. Following a 24 h incubation at 37°, 5% CO_2, 95% air, and 100% relative humidity, 100 μl of culture medium containing the test compound was added to the appropriate microtiter wells. The plates were incubated for an additional 48 h prior to the addition of 50 μl of MTT (1 mg/ml) reagent. After incubation for 4 h at 37°, 0.04N HCl/^{i}PrOH (100 μl) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. The absorbance measurement of the solns. related to the number of live cells were carried out on a *Bio-Rad-450* microplate reader at 570 nm [26]. IC_{50} Values were calculated from curves constructed by plotting suppression ratios [%] *vs.* compound concentration [M].

Cleavage of Relaxed Covalently Closed Circular DNA. A mixture (15 μl total volume) of relaxed covalently closed pBR 322 DNA (0.5 μg), TAE (= *Tris*/acetate/EDTA) buffer soln. (pH 8.0), and 2 μM [Fe^{II} (**11**)–(**16**)] or [Fe^{II}(BLM A_6)] was incubated at 37° for 40 min under agitation and then quenched with EDTA (5 μl) to a final concentration of 50 μM. A 15-μl aliquot was mixed with 40% glycerol loading soln. (containing 0.25% (*w*/*v*) of bromophenol blue) and loaded onto a 0.8% agarose gel. The gel was run at 3 V/cm for 120 min, and the spots were visualized by ethidium bromide (1 μg/ml). The percentage of net DNA cleavage was calculated by the following equation: $\{(\text{form II})_s/[(\text{form I})_s + (\text{form II})_s] \cdot 100\} - \{(\text{form II})_c/[(\text{form I})_c + (\text{form II})_c] \cdot 100\}$; the subscripts 's' and 'c' refer to the sample and control, resp.

Thermal Denaturation Studies. Thermal denaturation studies with the duplex form CT-DNA were carried out with a *WFZ800-D$_2$* spectrophotometer equipped with a thermoelectric cell temp. controller (±0.2°) and a stirrer unit, and a 1-cm quartz cuvette was used. The working soln. was an aq. buffer (1 mM NaH_2PO_4, 0.1 mM EDTA, pH 7.0) containing CT-DNA (50 μM) and the test compound (20 μM). A heating rate of 0.4°/min was used in the range of 25–90°, and data points were measured at 260 nm every minute. DNA Helix → coil transition temperatures (T_m) were obtained from the maxima in the $d(A_{260})/dT$ derivative plots. Drug-induced alterations in DNA melting behavior were given by $\Delta T_m = T_{m(\text{DNA+Drug})} - T_{m(\text{DNA alone})}$, where the T_m value for CT-DNA alone was 66.50°.

DNA Binding Constants. Quantitative measurements of the binding of the testing compounds to DNA were based on fluorescence studies performed with a *Shimadzu RF-5301* spectrofluorimeter. The fluorescence of bleomycin was partially quenched by DNA, thereby providing a sensitive method to determine apparent equilibrium constants and stoichiometry of binding. Two solns. were prepared, one containing 0.36 mM CT-DNA in 50 mM *Tris*·HCl buffer (pH 8.4); and the other containing only the buffer. Small amounts of the test compound, covering a wide range of concentrations, were added to both solns., and the fluorescence intensities

were measured at 352 nm following excitation at 293 nm. Measurements of the fluorescence quenching allowed the concentrations of free and bound test compound to be determined. Double-reciprocal plots of free and bound test compounds were used to determine equilibrium constants and the stoichiometry [27].

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