Synthesis of 2,2′-Bis(3,6,9-triazanonyl)-4,4′-bithiazole and Related Compounds as New DNA Cleavage Agents

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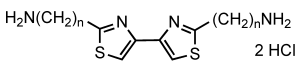
Two new bithiazole derivatives, 2,2′-bis(3,6,9-triazanonyl)- and 2,2′-bis(3,7,11-triazaundecyl)-4,4′-bithiazoles (3a, b), were readily synthesized in six steps using the corresponding dialkylenetriamine as starting materials. Under physiological conditions, 5.0 μM 3a exhibited significant DNA cleavage activity in the presence of Co(II), whereas even at 50 μM, 3b exhibited no DNA cleavage activity. Furthermore, it was demonstrated that 3a forms a 1 : 2 complex with Co(II) ions, whereas 3b does not. These conclusions were based on measurements of stoichiometries of the bithiazole–cobalt complexes obtained by the Job continuous variation method. In contrast, 3a, which contains diethylenetriamine moieties, showed decreased affinity for *Calf Thymus* (CT) DNA compared with that of 3b, which contains dipropylenetriamine moieties. These findings indicate that the structure of the two aminoalkyl side chains attached at the 2- and 2′-positions of the 4,4′-bithiazole ring significantly influence the formation of cobalt complexes, and affects the compound's ability to cleave DNA as well as its affinity for double-stranded DNA.

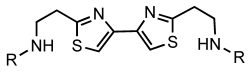
Key words 4,4′-bithiazole; DNA cleavage; cobalt complex; polyamine; thiazole

Recently, the synthesis and properties of many metal complexes of amines and cyclic amines used as DNA cleavage agents have been reported.[1—4] These compounds generally contain heterocycles or polycyclic aromatic rings tethered to one or more aminoalkyl side chains, and are complexed with transition metals known to play important roles in DNA cleavage reactions. Many studies on the role of 2,4′-bithiazole, which constitutes a part of the C-terminus of Bleomycin, a naturally occurring anticancer agent, have also been reported.[5—7] Previously, we reported the synthesis and metal-dependent DNA cleavage activities of 4,4′-bithiazoles (**1a**, **b**) linked to two aminoalkyl groups at the side chain,[8—10] since the synthesis of substituted 4,4′-bithiazoles is simpler than the synthesis of 2,4′-bithiazoles. Our studies on these 4,4′-bithiazole derivatives demonstrated that 2,2′-bis(2-aminoethyl)-4,4′-bithiazole (**1b**) could cleave DNA under physiological conditions only in the presence of Co(II), and that DNA cleavage efficiently progressed in the absence of reductants, oxygen and light.[9] It was also found that DNA cleavage by 2,2′-bis(aminomethyl)-4,4′-bithiazole (**1a**) required Cu(II) ions whereas 2,2′-bis(3-aminopropyl)-4,4′-bithiazole (**1c**) could not cleave DNA in the presence of any metal.[8,9] We also showed that 2,2′-bis(3,6-diazahexyl)- and 2,2′-bis(3,7-diazaheptyl)-4,4′-bithiazoles (**2a**, **b**) with additional aminoalkyl groups substituted on the amino group of each side chain of **1b** did not initiate detectable DNA cleavage in the presence of Co(II) at pH 7.0 after half an hour of incubation.[11] These results showed that the structure of the aminoalkyl side chains of the 4,4′-bithiazles determined the metal selectivity and DNA cleavage activities of these bithiazoles (**1**, **2**). However, Stewart demonstrated that naturally occurred polyamines such as spermine, spermidine, putrescine and their derivatives strongly bind double-stranded DNA.[12] Moreover, several authors have reported that many compound containing polyaminoalkyl moieties, such as spermine, spermidine and ethylenediamine, interact efficiently with DNA.[13,14] These studies prompted us to synthesize new 4,4′-bihiazoles substituted with polyaminoalkyl groups at each side chain, and to expect that these new compounds would interact efficiently with DNA and exhibit enhanced DNA cleavage activity. We report herein the synthesis, affinities for double-stranded DNA, and plasmid DNA cleavage abilities of new 4,4′-bithiazole derivatives (**3**). These new compounds possess longer side chains, such as 3,6,9-triazanonyl- and 3,7,11-triazaundecyl groups, than those of **1** and **2**, as shown in Fig. 1. Furthermore, bithiazole derivatives (**4**), used as reference compounds for **3a** were prepared by acylating the amino groups of **1b** and **2a** with amino acids. This derivatization was conducted in order to elucidate the role of the amino groups at the 3- and 6-positions of the side chains of **3a** in binding to and cleaving double-stranded DNA. The structural features of the 4,4′-bithiazole derivatives (**3**, **4**) lead us to expect that they would be highly soluble in water and have strong affinities for DNA (an anionic biopolymer), as these compounds have six (compound **3**) and four (compound **4**) amino cationic moieties on both side chains of the bithiazole ring.

Synthesis of the New Bithiazoles (3, 4) *N*-[*tert*-butoxycarbonyl (Boc)] protected alkylenediamine-*N*-propionthio-



1a n=1
1b n=2
1c n=3



2a : R=$(CH_2)_2NH_2$ 4HCl
2b : R=$(CH_2)_3NH_2$ 4HCl

3a : R=$(CH_2)_2NH(CH_2)_2NH_2$ 6HCl
3b : R=$(CH_2)_3NH(CH_2)_3NH_2$ 6HCl

4a : R=$COCH_2NH(CH_2)_2NH_2$ 4HCl
4b : R=$(CH_2)_2NHCOCH_2NH_2$ 4HCl

Fig. 1

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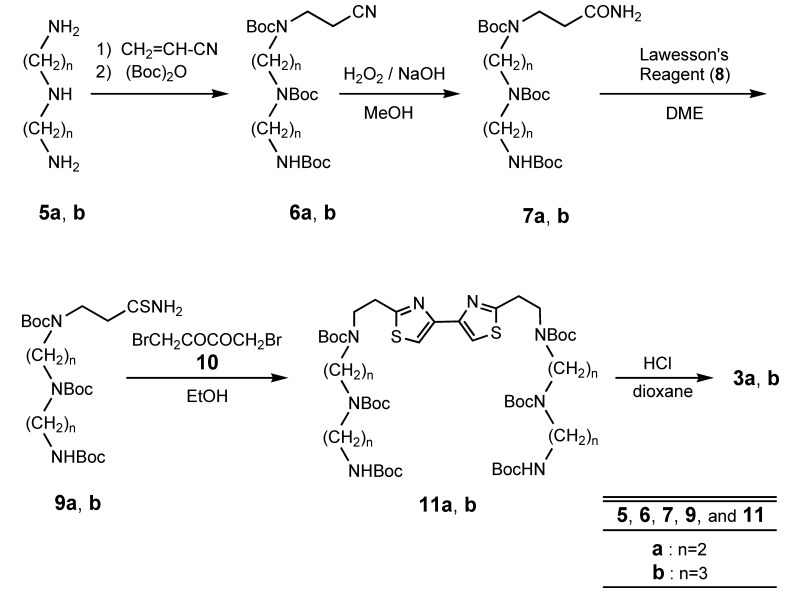

Chart 1

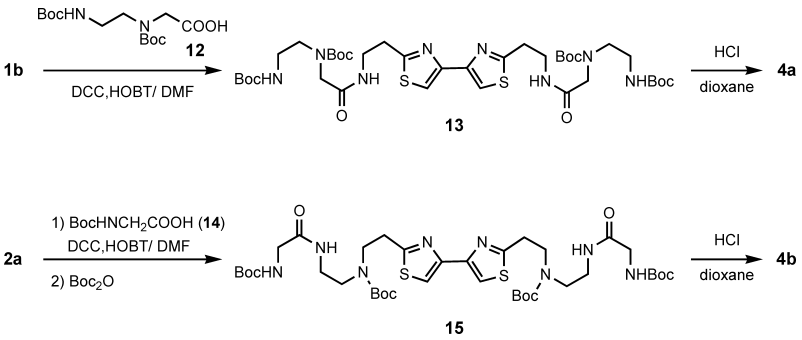

Chart 2

amides (**9a**, **b**), precursors of the 4,4′-bithiazoles (**3a**, **b**), were readily prepared from the corresponding triamines (**5a**, **b**). As shown in Chart 1, *N*,*N*′,*N*″-tri(Boc)-dialkylenetriamine-*N*-propionamides (**7a**, **b**) were obtained by the addition of **5a** and **5b** to acrylonitrile, then the amino groups were protected using di(*tert*-butyl) dicarbonate (Boc_2O) to afford *N*,*N*′,*N*″-tri(Boc)-*N*-(2-cyanoethyl)dialkylenetriamine (**6a**, **b**). Alkaline hydrolysis of the cyano groups with hydrogen peroxide provided amides (**7a**, **b**) in 56% and 51% yields, respectively. Furthermore, **9a** and **9b** were obtained by thiation of the amido carbonyl groups in **7a** and **7b** with 2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4-disulfide (Lawesson's reagent, **8**) in 70% and 61% yields, respectively. Subsequently, the bithiazole rings were constructed using Hantzsch's thiazole synthesis.[15,16] In brief, two equimolar amounts of **9a** and **9b** were reacted with 1,4-dibromobutane-2,3-dione (**10**) at 70 °C for 2 h to provide the corresponding 4,4′-bithiazoles, 2,2′-bis[3,6,9-tri(Boc)-3,6,9-triazanonyl]- and 2,2′-bis[3,7,11-tri(Boc)-3,7,11-triazaundecyl]-4,4′-bithiazoles (**11a**, **b**), respectively. Acidic deprotection of the amino groups in **11a** and **11b** afforded the bithiazoles (**3a**, **b**) in 26% and 17% overall yields from **5a** and **5b**, respectively. Next, as shown in Chart 2, 2,2′-bis[2-(3,6-diazahexanoyl)aminoethyl]-4,4′-bithiazole (**4a**) and 2,2′-bis(6-glycyl-3,6-diazahexyl)-4,4′-bithiazole (**4b**), used as reference compounds, were prepared by the condensation of **1b** with 3,6-di(Boc)-3,6-diazahexanoic acid (**12**) and **2a** with *N*-Boc glycine (**14**), followed by deprotection of the amino groups to provide **4a** and **4b** in 65 and 58% total yield, respectively. The structures of the new bithiazoles (**3**, **4**) were confirmed from their ^{1}H-NMR spectra, IR spectra, Matrix Assisted Laser Desorption Ionization (MALDI)-Time of Flight (TOF) MS, and by elemental analysis.

Table 1. C_{50} Values of the New Bithiazoles (**3**, **4**)

Compd. No.	**3a**	**3b**	**4a**	**4b**
C_{50} (μM)	1.5	0.16	260	1.3

The Affinities of the New Bithiazoles (3, 4) for Double-Stranded CT-DNA The affinities of the new bithiazoles (**3**, **4**) for double stranded CT-DNA were estimated by fluorescence spectroscopic assays at pH 7.0. The results are provided as C_{50} values, the micromolar concentration of bithiazole necessary to displace 50% of DNA-bound ethidium bromide (EtBr), a strong DNA intercalator.[17,18] As listed in Table 1, the C_{50} value of **3b** (0.16 μM), which possesses 3,7,11-triazaundecyl groups as the longest side chains, was the lowest, showing that the affinity of **3b** for CT-DNA was the highest amongst the bithiazoles tested (**3a**: 1.5 μM, **4a**:

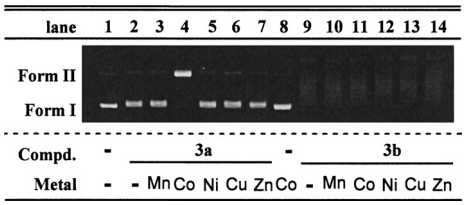

Fig. 2. DNA Cleavage Reactions by **3a** (5.0 μM) and **3b** (50 μM) in the Presence of Metal Ion (100 μM) at pH 7.0 and 37 °C for 30 min

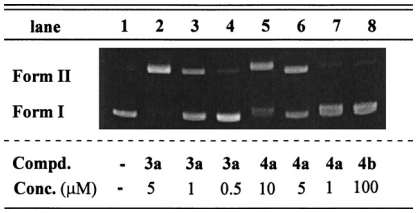

Fig. 3. DNA Cleavage Reactions by **3a**, **4a**, and **4b** in the Presence of Co(II) ion (100 μM) at pH 7.0 and 37 °C for 30 min

260 μM, and **4b**: 1.3 μM). Also, the C_{50} values of **3a** and **4b** were almost equal and significantly smaller than that of **4a**. These findings suggest that the affinities of the bithiazoles (**3**, **4**) for DNA at pH 7.0 depend on the number of protonated nitrogen atoms, which are affected by structural differences between the side chains. All nitrogen atoms in the side chains of **3b**, which has dipropylenetriamine moieties, were expected to be protonated at pH 7.0. However, it was comparatively difficult to protonate the two central nitrogen atoms in both side chains of **3a** (which have diethylenetriamine moieties) presumably due to electronic repulsion between positive charges on nitrogen atoms separated by an ethylene group.[19,20] In contrast, **4b** possesses amido nitrogen atoms at 6-position in both side chains, so remaining four amino groups were expected to be protonated; however, only one amino group in each side chain of **4a** was protonated because **4a** possesses ethylenediamine moieties in both side chains. Since **3a** and **4b** were expected to have a total of four positively-charged nitrogen atoms in the neutral condition, the affinity of **3a** for DNA would be almost the same as that of **4b**. Consequently, it was shown that the affinities of bithiazole derivatives **3** and **4** for DNA increased with increasing number of positively charged nitrogen atoms in the side chains of the 4,4′-bithiazoles. These results showed that the intensity of the interaction with DNA is determined by the structure and the length of the aminoalkyl group containing the side chains attached to the bithiazole ring.

DNA Cleavage Reactions of the New Bithiazoles (3, 4) The ability of the bithiazoles (**3**, **4**) to cleave plasmid pBR322 DNA[21] was examined by monitoring their effectiveness in converting circular supercoiled DNA (form I) to the corresponding circular nicked (form II) and linear forms (form III). First, as shown in Fig. 2, **3a** at 5 μM in the absence of any metals (lane 2) and only Co(II) at 100 μM (lane 8) showed no DNA cleavage at pH 7 and 37 °C, as compared with the DNA control (lane 1). As with **1b** in the presence of Co(II),[9] DNA strand cleavage by **3a** was significantly affected by the addition of Co(II) (lane 4), whereas the addition of Mn(II) (lane 3), Ni(II) (lane 5), Cu(II) (lane 6), or Zn(II) (lane 7) failed to initiate cleavage under the conditions tested. On the other hand, it was found that form I DNA incubated with **3b** was broadened and form II DNA was retarded (Fig. 2, lanes 9—14). Since **3b** exhibited the significant affinity for plasmid DNA as judged by the C_{50} value, it can be expected that broadening of form I DNA and delay in migration of form II DNA were attributed to the structural change of DNA by means of the binding of more molecules of **3b** to plasmid DNA. Therefore, **3b** indicated no DNA cleavage activity even at 50 μM in the presence or absence of metals. Next, the DNA cleavage activities of reference compounds (**4**) were examined and compared to that of **3a**. As shown in Fig. 3, **4a**, which has acylated amino groups at the 3-positions in both side chains**,** showed moderate cleavage activity at a concentration of 10 μM in the presence of Co(II) (lane 6). In contrast, little DNA cleavage by **4b**, which possesses acylated amino groups at the 6-positions, was observed even at 100 μM in the presence of Co(II) (lane 8). On the other hand, it was found that **3a**, even at 1.0 μM (lane 3), afforded a detectable amount of form II DNA under these reaction conditions, These findings strongly suggest that diethylenetriamine and ethylenediamine amino groups in both side chains of the bithiazole play an important role in DNA cleavage activity. In order to examine the effect of pH on these DNA cleavage reactions, DNA was incubated with **3a** in different buffer solutions (pH values 5.0—10.0). As listed in Table 2, **3a** effectively cleaved form I DNA to the corresponding form II DNA at pH 7.0 (91%, lane 4), 8.0 (95%, lane 5) and 9.0 (94%, lane 6) together with relatively small amounts of form III DNA at pH 7.0 (9%), pH 8.0 (5%) and pH 9.0 (4%), but not at pH 5.0 (lane 2), 6.0 (lane 3) or 10.0 (lane 7). Therefore, the optimal pH for DNA cleavage by **3a** is pH 7.0. In general, free amino groups at alkaline conditions play an important role in forming complexes with metal ions, whereas cationic ammonium groups at low pH range are important for interactions with polyanionic DNA. Interestingly, strand cleavage of plasmid DNA by **3a** and **4a**, which have ethylenediamine-type amino groups, was more effective than DNA cleavage by **3b**, which has propylenediamine-type amino groups, or by **4b**, in which the amino groups are separated by the amido moiety.

Table 2. pH Dependence of the DNA Cleavage Reaction by **3a**

Lane No.	1	2	3	4	5	6	7
3a+Co(II)	−	+	+	+	+	+	+
pH	7	5	6	7	8	9	10
Form I (%)	96	88	71	0	0	2	85
Form III (%)	0	0	0	9	5	4	0
Form II (%)	4	12	29	91	95	94	15

Concentrations of **3a** and Co(II) ion are 10 μM and 100 μM, respectively. All reactions were carried out at 37 °C for 30 min.

Since Co(II) ion was necessary for DNA cleavage by the bithiazoles (**3a**, **4a**), it was expected that these compounds would form Co(II)-complexes. The Job method of continuous variation[22] was used to determine the stoichiometries of the bithiazole (**3**, **4**) metal complexes with Co(II) ion. As shown in Fig. 4A, the Job plot strongly suggests that **3a** forms a 1 : 2 complex with Co(II) ion. Also, the UV spectroscopic method demonstrated that **4a**, which cleaves DNA as effectively as **3a**, forms a 1 : 2 complex with Co(II) ion (Fig. 4B), whereas **3b** and **4b**, which do not cleave DNA, do not form complexes with Co(II) ion. These findings demonstrate

that the formation of Co(II)-complexes of bithiazoles (**3a**, **4a**) are essential for DNA cleavage activity. Incidentally, the formation of 1 : 1 complexes of **1b** and **2a** with Co(II) ion were confirmed, however, it was found that **1c** and **2b**, which showed no DNA cleavage, do not form complexes with Co(II) ion (data not shown). Furthermore, Co(II)-dependent DNA cleavage activities of new bithiazoles (**3a**, **4a**) are found to be more efficient compared to those of **1b**[9] and **2a**[11] on the basis of both the concentration of bithiazoles examined and the reaction time. These findings suggested that 1 : 2 complexes of **3a** and **4a** with Co(II) ion could more advantageously cleave DNA as compared with 1 : 1 complexes of **1b** and **2a** with Co(II) ion.[23] On the other hand, the affinities of Co(II)-complexes of new bithiazoles (**3a**, **4a**) for CT-DNA were estimated at pH 7.0 by means of the same procedure mentioned above. It was found that the C_{50} values of Co(II)-complexes of **3a** (1.1 μM) and **4a** (4.3 μM) are slightly and significantly less than those of the corresponding bithiazoles, **3a** and **4a**, respectively. These data suggest that the total charge and the structure of Co(II)-complexes presumably affected the affinities of Co(II)-complexes of bithiazoles for CT-DNA.[24]

Although the structure of Co(II)-complexes of new bithiazoles (**3a**, **4a**) were still unknown. Xu and coworkers recently reported the synthesis and the structure of 1 : 1 Cu(II)-complex of 2,2′-diamino-4,4′-bithiazole.[25] Yang and coworkers also indicated the formation and the structural features of 2 : 1 Co(II)-complex of 2,2′-diamino-4,4′-bithiazole.[26,27] The formation of 3 : 1 complexes of 4,4′-bithiazole with iron and nickel were demonstrated by Baker and Goodwin.[28] These reports showed that two nitrogen atoms of bithiazole ring were used as coordinating atoms. However, as judged by the stoichiometry of Co(II)-complexes of **3a** and **4a**, it was assumed that cobalt atoms were coordinated by the amino groups on both side chains of the bithiazole ring.

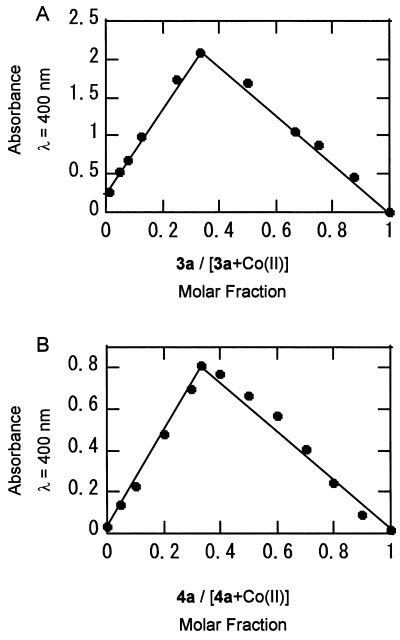

Fig. 4. Continuous Variation Plots for **3a** and Co(II) (A) and **4a** and Co(II) (B). The plots were obtained by Job's method at pH 7.0.

Previously, it was reported that DNA cleavage reactions with **1b** do not require any reductants, oxygen or light.[9] The mode of activation of DNA cleavage by **3a** was next examined by adding different inhibitors to the reaction mixture. As listed in Table 3, DNA cleavage by **3a** was unaffected by hydroxyl radical scavengers such as ethanol (EtOH), propanol (PrOH), D-mannitol or dimethyl sulfoxide (DMSO), but ethylendiamine tetraacetic acid (EDTA), a metal chelating agent, completely inhibited the cleavage reaction. In addition, no inhibition of DNA cleavage by **3a** was observed in the presence of catalase, superoxide dismutase (SOD), NaN_3 or 1,4-didazabicyclo[2.2.2]octane (DABCO). Furthermore, it was observed that DNA cleavage by **3a** is not affected by the presence or absence of O_2 or light, indicating that no oxidative process is involved in this DNA cleavage reaction.

In conclusion, it was demonstrated that 2,2′-bis(3,6,9-triazanonyl)-4,4′-bithiazole (**3a**), which has both DNA cleavage activity and significant affinity for double-stranded DNA, can be easily synthesized. Moreover, 2,2′-bis(3,7,11-triazaundecyl)-4,4′-bithiazole (**3b**) was also synthesized and shown to have strong affinity for double-stranded DNA but no DNA cleavage activity. On the other hand, **4a** with amido groups at 3-position of both side chain has DNA cleavage activity and a little affinity for double-stranded DNA, however, **4b** with amido groups at 6-position has no cleavage activity and considerable affinity for double-stranded DNA as well as that of **3a**. These findings indicate that the structure of the two alkylamino side chains attached at the 2- and 2′-positions of 4,4′-bithiazole significantly influence the formation of cobalt complexes, and thus in effect control the ability of the compound to bind to and cleave DNA.

Finally, the results of an anticancer screening study with **3a** were recently reported.[29] Although no significant activity of **3a** was observed, the synthesis of related compounds is ongoing in our laboratory.

Experimental

All melting points were taken on a Yanagimoto micro melting point determination apparatus and are uncorrected. IR and UV spectra were recorded on a Shimadzu R8000 infrared spectrophotometer and a Jasco U550 spectrophotometer, respectively. Fluorescence spectra were recorded on a Shi-

Table 3. Inhibitions of DNA Cleavage Reaction by **3a** in the Presence of Co(II) Ion

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12
3a+Co(II)	−	+	+	+	+	+	+	+	+	+	+	+
Inhibitors	—	—	EtOH	PrOH	DMSO	D-mannitol	NaN_3	DABCO	SOD	Catalase	Dark	Anaerobic
Conc.			0.2 M	0.2 M	0.1 M	0.2 M	5 mM	5 mM	20 mg/ml	20 mg/ml		
Form I (%)	90	0	0	0	0	0	11	14	0	0	5	6
Form III (%)	0	8	5	6	5	6	0	0	7	5	2	3
Form II (%)	10	92	95	94	95	94	89	86	93	95	93	91

Concentrations of **3a** and Co(II) ion are 10 μM and 100 μM, respectively. All reactions were carried out at 37 °C for 30 min.

madzu RF5300 spectrophotometer. ^{1}H-NMR spectra were obtained on a 400 MHz Bruker DPX-400 spectrometer using tetramethylsilane in $CDCl_3$, and 4,4-dimethyl-4-silapentane-sulfonic acid sodium salt in D_2O, as internal references. MALDI-TOF-MS analysis was conducted using a Kratos Kompact MALDI IV instrument. A saturated solution of 2,5-dihydroxybenzoic acid in a 1 : 1 mixture of water and acetonitrile containing 1% trifluoroacetic acid was used as the matrix. The instrument was calibrated externally with a C60/70 mixture.

***N*,*N*′,*N*″-Tris(*tert*-butoxycarbonyl)-*N*-(2-cyanoethyl)diethylenetriamine (6a) and *N*,*N*′,*N*″-Tris(*tert*-butoxycarbonyl)-*N*-(2-cyanoethyl)-dipropylenetriamine (6b)** General Procedure: Acrylonitrile (2.65 g, 50 mmol) was added dropwise to liquid dialkylenetriamine (**5**, 500 mmol) at 5 °C over a period of 30 min with stirring. The mixture was stirred for an additional 30 min and excess dialkylenetriamine was removed under reduced pressure to give crude *N*-(2-cyanoethyl)dialkylenetriamine, which was used in the next step without purification. Boc_2O (33 g, 150 mmol) was added in small portions to a stirred solution of the residue in a mixture of tetrahydrofuran (THF, 200 ml) and an aqueous NaOH (4.0 mol/l, 40 ml), and the mixture was stirred for 5 h at room temperature. After the organic solvent was removed under reduced pressure, ethyl acetate (EtOAc, 400 ml) was poured into the residual aqueous suspension. The organic layer was separated, washed twice with brine (50 ml), and then dried over anhydrous Na_2SO_4. The organic solvent was removed *in vacuo* to yield a colorless waxy solid, which was purified by silica gel flash column chromatography with EtOAc–hexane (1 : 1).

6a: Yield 20 g (87%), colorless prisms (ether), mp 99—100 °C. IR (KBr) cm^{-1}: 3360, 2244, 1682. ^{1}H-NMR ($CDCl_3$) δ: 1.43 (9H, s) 1.47 (18H, s), 2.53—2.67 (2H, m), 3.22—3.54 (10H, m), 4.65—5.15 (1H, m). MALDI-TOF-MS *m*/*z*: 479 (M+Na)$^+$. *Anal.* Calcd for $C_{22}H_{40}N_4O_6$: C, 57.87; H, 8.83; N, 12.27. Found: C, 57.63; H, 8.92; N, 12.23.

6b: Yield 22 g (92%), amorphous solid. IR (KBr) cm^{-1}: 3368, 2252, 1692. ^{1}H-NMR ($CDCl_3$) δ: 1.44, 1.46, 1.47 (27H, each s), 1.63—1.66 (4H, m), 1.78 (2H, q, *J*=7.3 Hz), 2.63 (2H, q, *J*=7.3 Hz), 3.09—3.29 (8H, m), 4.65—5.10 (2H, m). MALDI-TOF-MS *m*/*z*: 507 (M+Na)$^+$.

***N*,*N*′,*N*″-Tris(*tert*-butoxycarbonyl)diethylenetriamine-*N*-propionamide (7a) and *N*,*N*′,*N*″-Tris(*tert*-butoxycarbonyl)dipropylenetriamine-*N*-propionamide (7b)** General Procedure: An aqueous hydrogen peroxide solution (30%, 50 ml) was added dropwise to a stirred suspension of **6** (30 mmol) in a mixture of aqueous NaOH (2.0 mol/l, 50 ml) and EtOH (200 ml) over a period of 1 h while the temperature was maintained at 5 °C or less. The mixture was heated at 60 °C for 1 d with stirring, then the solution was concentrated to 1/3 the original volume *in vacuo*. The obtained precipitate was collected by suction filtration and washed with ice cold water, then purified by silica gel flash column chromatography with EtOAc–hexane (3 : 1).

7a: Yield: 9.1 g (64%), colorless microcrystals (EtOAc), mp 143—144 °C. IR (KBr) cm^{-1}: 3408, 1704, 1676. ^{1}H-NMR ($CDCl_3$) δ: 1.44, 1.46, 1.47 (27H, each s), 2.51 (2H, br s), 3.20—3.60 (10H, m), 4.65—5.15 (1H, m). MALDI-TOF-MS *m*/*z*: 497 (M+Na)$^+$. *Anal.* Calcd for $C_{22}H_{42}N_4O_7$: C, 55.68; H, 8.92; N, 11.81. Found: C, 55.64; H, 9.15; N, 11.64.

7b: Yield: 8.3 g (55%), amorphous solid. IR (KBr) cm^{-1}: 3356, 1676. ^{1}H-NMR ($CDCl_3$) δ: 1.46 (27H, s), 1.61—1.77 (4H, m), 2.51 (2H, br s), 3.09—3.21 (8H, m), 3.50 (2H, t, *J*=6.7 Hz), 5.31—5.51 (1H, br s). MALDI-TOF-MS *m*/*z*: 525 (M+Na)$^+$.

***N*,*N*′,*N*″-Tris(*tert*-butoxycarbonyl)diethylenetriamine-*N*-propionthioamide (9a) and *N*,*N*′,*N*″-Tris(*tert*-butoxycarbonyl)trimethylenetriamine-*N*-propionthioamide (9b)** General Procedure: To a stirred suspension of **7** (10 mmol) in dry 1,2-dimethoxyethane (DME, 100 ml), Lawesson's reagent (**8**, 2.6 g, 6.5 mmol) was added in portions at room temperature. After the reaction mixture was heated at 60 °C for 4 h with stirring, the organic solvent was removed under reduced pressure. A mixture of EtOAc (200 ml) and aqueous NaOH (2.0 mol/l, 50 ml) was poured onto the residue and the organic layer was separated, washed twice with brine (30 ml), then dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* to give a crude product, which was purified by silica gel flash column chromatography with EtOAc–hexane (2 : 1).

9a: Yield: 3.4 g (70%), colorless microcrystals (ether : hexane=5 : 1), mp 147—148 °C. IR (KBr) cm^{-1}: 3384, 1682. ^{1}H-NMR ($CDCl_3$) δ: 1.44, 1.46, 1.47 (27H, each s), 3.01 (2H, br s), 3.20—3.65 (10H, m), 4.65—5.15 (1H, m). MALDI-TOF-MS *m*/*z*: 513 (M+Na)$^+$. *Anal.* Calcd for $C_{22}H_{42}N_4O_6S$: C, 53.85; H, 8.63; N, 11.42. Found: C, 53.62; H, 8.69; N, 11.40.

9b: Yield: 3.2 g (61%), amorphous solid. IR (KBr) cm^{-1}: 3336, 1682. ^{1}H-NMR ($CDCl_3$) δ: 1.46 (27H, s), 1.59—1.83 (4H, m), 3.00—3.11 (10H, m), 3.58 (2H, t, *J*=6.6 Hz), 4.78—5.25 (1H, br s). MALDI-TOF-MS *m*/*z*: 541 (M+Na)$^+$.

2,2′-Bis[3,6,9-tris(*tert*-butoxycarbonyl)-3,6,9-triazanonyl]-4,4′-bithiazole (11a) and 2,2′-bis[3,7,11-tris(*tert*-butoxycarbonyl)-3,7,11-triazaundecyl]-4,4′-bithiazole (11b) General Procedure: To a stirred solution of **9** (10 mmol) in dry EtOH (100 ml), 1,4-dibromobutane-2,3-dione (**10**, 1.22 g, 5.0 mmol) was added in one portion at room temperature. The mixture was heated at 60 °C for 5 h with stirring, then the organic solvent was removed under reduced pressure. A mixture of EtOAc (100 ml) and aqueous NaOH (2.0 mol/l, 30 ml) was poured onto the residue and the organic layer was separated, washed twice with brine (30 ml), and dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* to give a crude product, which was purified by silica gel flash column chromatography with EtOAc–hexane (1 : 1).

11a: Yield: 3.7 g (73%), colorless microcrystals (acetone), mp 123—124 °C. IR (KBr) cm^{-1}: 3388, 3192, 1184. ^{1}H-NMR ($CDCl_3$) δ: 1.43 (18H, s), 1.46 (36H, s), 3.15—3.70 (24H, m), 7.55, 7.70 (2H, each s). MALDI-TOF-MS *m*/*z*: 1050 (M+Na)$^+$. *Anal.* Calcd for $C_{48}H_{82}N_8O_{12}S_2$: C, 56.12; H, 8.05; N, 10.91. Found: C, 56.08; H, 7.98; N, 10.94.

11b: Yield: 3.3 g (60%), amorphous solid. IR (KBr) cm^{-1}: 3368, 3212, 1692. ^{1}H-NMR ($CDCl_3$) δ: 1.43 (18H, each s), 1.45 (36H, each s), 3.09—3.61 (32H, m), 5.30 (1H, br s), 7.63 (2H, s). MALDI-TOF-MS *m*/*z*: 1106 (M+Na)$^+$.

2,2′-Bis(3,6,9-triazanonyl)-4,4′-bithiazole Hexahydrochloride (3a) and 2,2′-Bis(3,7,11-triazaundecyl)-4,4′-bithiazole Hexahydrochloride (3b) General Procedure: To a solution of **11** (2.0 mmol) in dry dioxane (10 ml), HCl in dioxane (4.0 mol/l, 10 ml) was added at room temperature and the mixture was stirred overnight. The obtained precipitate was collected by suction filtration and purified by recrystallization.

3a: Yield: 1.2 g (93%), colorless plates (MeOH : H_2O=4 : 1), mp 257—262 °C (dec.). IR (KBr) cm^{-1}: 3488—2944. ^{1}H-NMR (D_2O) δ: 3.42—3.72 (16H, m), 3.61 (8H, m), 7.90 (2H, s). MALDI-TOF-MS *m*/*z*: 449 (M+Na)$^+$. *Anal.* Calcd for $C_{18}H_{34}N_8S_2\cdot 6HCl$: C, 33.50; H, 6.25; N, 17.36. Found: C, 33.67; H, 6.30; N, 17.30.

3b: Yield; 1.26 g (90%), colorless plates (MeOH : H_2O=4 : 1), mp 263—268 °C (dec.). IR (KBr) cm^{-1}: 3200—2952. ^{1}H-NMR (D_2O) δ: 2.11—2.21 (4H, m), 3.11—3.62 (8H, m), 3.55—3.62 (8H, m), 7.88 (2H, s). MALDI-TOF-MS *m*/*z*: 505 (M+Na)$^+$. *Anal.* Calcd for $C_{22}H_{42}N_8S_2\cdot 6HCl$: C, 37.67; H, 6.90; N, 15.97. Found: C, 37.50; H, 6.87; N, 15.73.

2,2′-Bis{2-[3,6-bis(*tert*-butoxycarbonyl)-3,6-diazahexanoyl]aminoethyl}-4,4′-bithiazole (13) *N*,*N*′-Dicyclohexylcarbodiimide (DCC, 1.03 g, 5.0 mmol) was added to a stirred solution of 3,6-di(Boc)-3,6-diazahexanoic acid (**12**, 1.6 g, 5.0 mmol) and 1-hydroxybenzotriazole (HOBT, 0.68 g, 5.0 mmol) in dry *N*,*N*′-dimethylformamide (DMF, 20 ml) at room temperature, the mixture was stirred for 1 h, then **1b** (0.65 g, 2.0 mmol) and diisopropylethylamine (0.65 g, 5.0 mmol) at room temperature were added with stirring. After the mixture was stirred overnight at room temperature, the obtained precipitate was filtered and the filtrate was concentrated *in vacuo* to give an oily residue. A mixture of EtOAc (100 ml) and aqueous NaOH (1.0 mol/l, 20 ml) was poured onto the residue and the organic layer was separated, washed twice with 10% citric acid solution (30 ml) and brine (30 ml), and dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* to give a crude product, which was purified by silica gel flash column chromatography with acetone–hexane (1 : 1). Yield: 1.3 g (76%), colorless microcrystals (acetone : hexane=5 : 1), mp 139—140 °C. IR (KBr) cm^{-1}: 3388, 1702, 1680. ^{1}H-NMR ($CDCl_3$) δ: 1.42 (36H, s), 3.20—3.30 (8H, m), 3.33—3.40 (4H, m), 3.76 (4H, q, *J*=6.0 Hz), 5.35 (2H, br s), 7.00 (2H, br s), 7.69 (2H, s). MALDI-TOF-MS *m*/*z*: 877 (M+Na)$^+$. *Anal.* Calcd for $C_{38}H_{62}N_8O_{10}S_2$: C, 53.38; H, 7.31; N, 13.10. Found: C, 53.47; H, 7.24; N, 13.13.

2,2′-Bis[2-(3,6-diazahexanoyl)aminoethyl]-4,4′-bithiazole Tetrahydrochloride (4a) Following the general procedure described above for the preparation of **3b**, HCl in dioxane (4.0 mol/l, 10 ml) was added dropwise to a solution of **13** (855 mg, 1.0 mmol) in dioxane (10 ml) at room temperature and the mixture was stirred overnight to give a crude product, which was purified by recrystallization. Yield 509 mg (85%), colorless microcrystals (MeOH : H_2O=9 : 1), mp 248—250 °C (dec.). IR (KBr) cm^{-1}: 3456—2940, 1670. ^{1}H-NMR (D_2O) δ: 3.33 (4H, t, *J*=6.6 Hz), 3.40—3.55 (8H, m), 3.72 (4H, t, *J*=6.6 Hz), 3.98 (4H, s), 7.79 (2H, s). MALDI-TOF-MS *m*/*z*: 455 (M+H)$^+$, 477 (M+Na)$^+$. *Anal.* Calcd for $C_{18}H_{30}N_8O_2S_2\cdot 4HCl$: C, 36.01; H, 5.71; N, 18.66. Found: C, 35.85; H, 5.60; N, 18.66.

2,2′-Bis{3-(*tert*-butoxycarbonyl)-6-[*N*-(*tert*-butoxycarbonyl)glycyl]-3,6-diazahexyl}-4,4′-bithiazole (15) Following the procedure described above for the preparation of **13**, the condensation of **2a** (1.94 g, 4.0 mmol) with *N*-Boc glycine (**14**, 1.4 g, 8 mmol) using DCC (2.0 g, 9.0 mmol) as a dehydration agent were carried out in the presence of HOBT (1.35 g, 10 mmol) and diisopropylethylamine (2.58 g, 20 mmol) in dry DMF (30 ml) at room

temperature. After 10 h, Boc_2O (1.74 g, 8 mmol) was added in one portion and the resulting mixture was stirred for 5 h at room temperature. The obtained precipitate was filtered and the filtrate was concentrated *in vacuo* to give an oily residue. A mixture of EtOAc (100 ml) and aqueous NaOH (1.0 mol/l, 20 ml) was poured onto the residue and the organic layer was separated, washed twice with 10% citric acid solution (30 ml) and brine (30 ml), and dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* to give a crude product, which was purified by silica gel flash column chromatography with acetone–hexane (2 : 1).

Yield 2.2 g (64%) amorphous solid. IR (neat) cm^{-1}: 3420, 1710, 1664. ^{1}H-NMR ($CDCl_3$) δ: 1.41 (36H, s), 3.22—3.70 (16H, m), 3.79 (4H, d, J=6.4 Hz), 5.22 (2H, br s), 7.61 (2H, s). MALDI-TOF-MS m/z: 877 $(M+Na)^+$.

2,2′-Bis[6-glycyl-3,6-diazahexyl]-4,4′-bithiazole Tetrahydrochloride (4b) Following the general procedure described above for the preparation of **3b**, HCl in dioxane (4.0 mol/l, 10 ml) was added dropwise to a solution of **15** (428 mg, 0.50 mmol) in dioxane (10 ml) at room temperature and the mixture was stirred overnight to give a crude product, which was purified by recrystallization. Yield 273 mg (91%), colorless microcrystals (MeOH : H_2O=9 : 1), mp 258—261 °C (dec.). IR (KBr) cm^{-1}: 3470—2960, 1667. ^{1}H-NMR (D_2O) δ: 3.41 (4H, t, J=6.2 Hz), 3.60—3.85 (12H, m), 3.89 (4H, s), 7.90 (2H, s). MALDI-TOF-MS m/z: 455 $(M+H)^+$, 477 $(M+Na)^+$. *Anal.* Calcd for $C_{18}H_{30}N_8O_2S_2 \cdot 4HCl$: C, 36.01; H, 5.71; N, 18.66. Found: C, 35.88; H, 5.64; N, 18.50.

Fluorescence Spectroscopy Experiments to Measure the C_{50} Values of the Bithiazoles (3, 4) EtBr displacement assays were carried out as described in reference 18. Measurements were done in quartz cuvettes (1 cm pathlength) on a Shimadzu R5300 spectrofluorimeter. Experiments were performed by adding increasing amounts of the bithiazoles to preformed EtBr/CT-DNA complex. The concentration of EtBr and CT-DNA (purchased from Sigma-Aldrich Japan Co., Ltd.) was 2.56 μM and 2.59 μM, respectively.

DNA Cleavage Reactions of Bithiazoles (3, 4) Plasmid pBR322 DNA purchased from Toyobo Co., Ltd. as supercoiled DNA was used. Each reaction solution contained 0.1 μg of supercoiled plasmid pBR322 DNA in 10 mM MOPS (pH 7.0) buffer. All cleavage reactions were run for 30 min at 37 °C, and electrophoresis was carried out at 100 V (40 min) in 1.2% agarose gels in 40 mM TAE (pH 8.1) buffer. The gel patterns were developed by soaking the gels in EtBr buffer (1 μg/1 ml TAE buffer) and photographed with a CCD camera under UV irradiation.

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