Synthesis and DNA-Binding Study of A Thiazole-Containing Analog of Netropsin

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In order to study the influence of the nature of the heterocyclic rings of Netropsin (Nt) on the binding to specific nucleotide sequences in the minor groove of DNA, a thiazole-containing analogue (Thia-Nt) was designed. Optimized syntheses of the key compound 2-aminothiazole-4-carboxylic acid and Thia-Nt were reported together with the results of a preliminary study of Thia-Nt-DNA binding.

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DNA is the support of the genetic information; of its chemical structure depend normal or anarchic cell growth or cell death. Differences between normal and tumor cells take origin at the gene level. Beyond the always increasing frequency of cancer, it seems particularly important to develop an efficient strategy for the design of chemotherapeutic drugs designed as gene control agents. In this way, minor groove binding compounds [1], typified by netropsin and distamycin, appear to be a promising source of active compounds because of their ability to bind to large DNA sequences.

Oligopeptide xenobiotics including antibiotic, antineoplastic and antiviral drugs interfere with the replication and transcription by the recognition and preferential binding to specific double-stranded DNA sequences. Such is the case of antiviral and antitumor distamycin-A (Dst) and of antibiotic netropsin (Nt) (Figure 1), two *N*-methylpyrrole drugs able to block the template function of DNA by binding to specific nucleotide sequences in the minor groove of double strand DNA [2]. On the basis of X-ray data, it was found that the adenine-thymine base-specific binding of both drugs is due to electrostatic bonds between ends of side chains of the ligands and DNA phosphates, hydrogen-bonding interactions between amide NH and adenine N(3) and thymine O(2), and Van der Waals contacts between methylenes and heterocyclic CH of DNA bases [3,4]. In order to confirm these criteria, some structural modifications have been carried out including replacement of pyrrole ring by pyridine [5,6] and imidazole [7,8] heterocycles and/or lengthening of the chain [9,10].

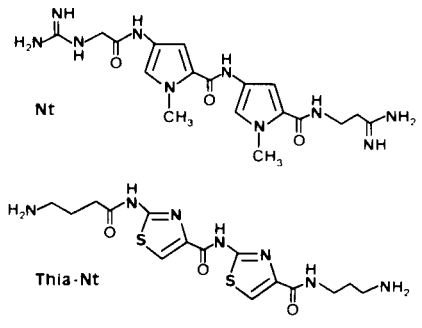

Figure 1. Structure of netropsin(A) and Thia-Nt (B).

In this attempt to study the influence of the nature of the heterocyclic parts and the linking groups on the curvature of the obtained compounds fitting well with DNA geometry, we proposed the design of thiazole containing Dst and Nt analogues [11]. The choice of this heterocycle was directed by chemical considerations concerning the ring size and the relative position of heterocyclic N atom in the whole peptide chain. Moreover the thiazole carboxylic acid, a cyclized cysteine, was found to play an important biological role in the cytotoxic activity of cyclopeptides isolated from marine animals [12-15] and in the DNA binding capacity of antitumor drugs such as bleomycin [16]. The chemistry of starting materials leading to the synthesis of thiazole-containing cyclopeptides [17,18] or bleomycin models [19,20] was extensively worked in our laboratory and prompted us to propose the synthesis of a new thiazole Nt analogue.

This report includes an efficient and easily reproducible synthesis of the 2-aminothiazole-4-carboxylic acid which was found to be a key compound in the strategic pathway leading to the synthesis of the Nt-analog, Thia-Nt (Figure 1), in the structure of which the pyrrole rings of Nt were replaced by thiazole heterocycles and amidine and guanidine ends simplified in primary amines. Together with details on the optimized synthesis, the results of a preliminary study of Thia-Nt—DNA binding are also reported.

Chemistry.

The synthetic method for the preparation of 2-amino-4-carboethoxythiazole **2** consists of the cyclising condensa-

tion of thiourea with ethyl bromopyruvate according to the Kuhn procedure [21]. Thiazole **2** was coupled with 4-*t*-butyloxycarbonylaminobutyric acid [22] in the presence of dicyclohexylcarbodiimide (DCC) and 1*H*-hydroxy-1,2,3-benzotriazole (HOBt). After saponification of the ester group of the "dipeptide" **3**, the acid **4** was coupled with 2-amino-4-carboethoxythiazole **2** in the above conditions (DCC, HOBt) to give the thiazole-containing "tripeptide" **5** which was saponified to give the acid **6**.

The introduction of the second side chain was achieved by a last coupling, in the presence of DCC and HOBt, with the mono-protected diamine **7** [23] to give the protected diamine **8**. The desired compound Thia-Nt **9** was obtained as a dihydrobromide by cleavage of the tert-butyloxycarbonyl (Boc) groups by dry hydrogen bromide in acetic acid (see Figure 2).

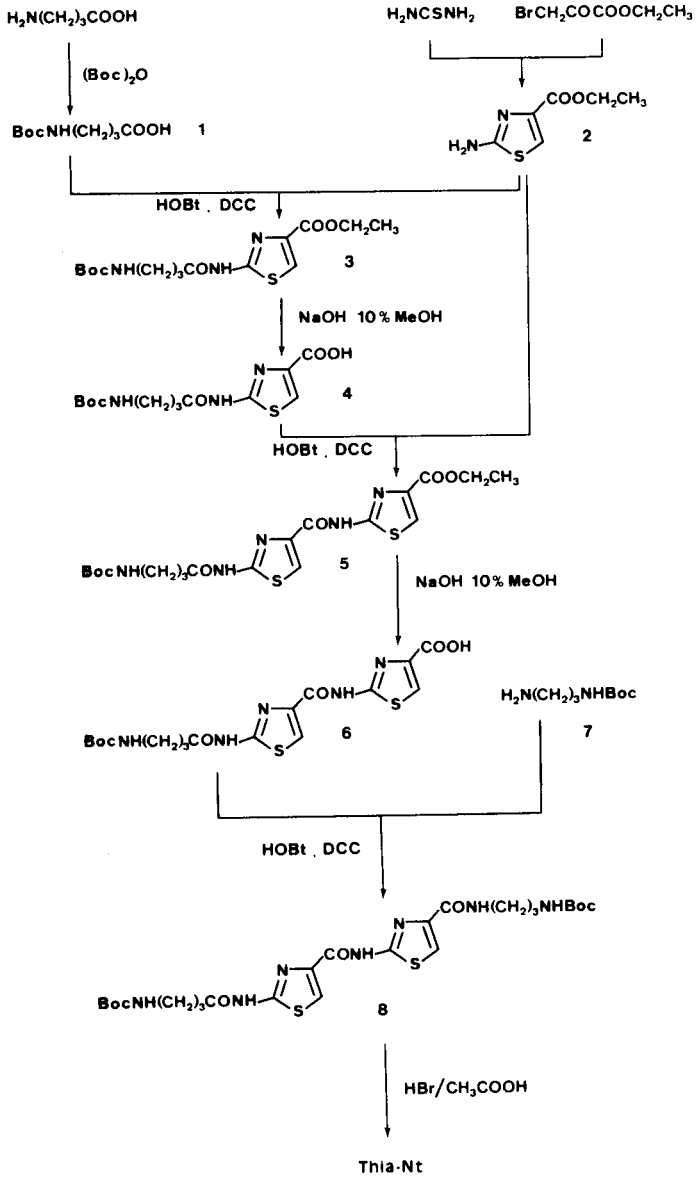

Figure 2. Synthesis of Thia-Nt.

DNA-Binding.

Binding of the synthetic ligand Thia-Nt to DNA was deduced from the hypochromic effects observed in the uv absorption spectra of this ligand when DNA was added (Figure 3). A red shift (4 nm), indicative of an increased delocalization of the π-electrons in the ligand, was noted. These spectral modifications were found upon addition of coliphage T_4 DNA in which the major groove is occulted by bulky glucose residues on the 5-(hydroxymethyl)cytidine bases [24]. The results were consistent with a binding in the minor groove as established for Nt [4]. In order to verify this result, linear electric dichroism studies have been undertaken.

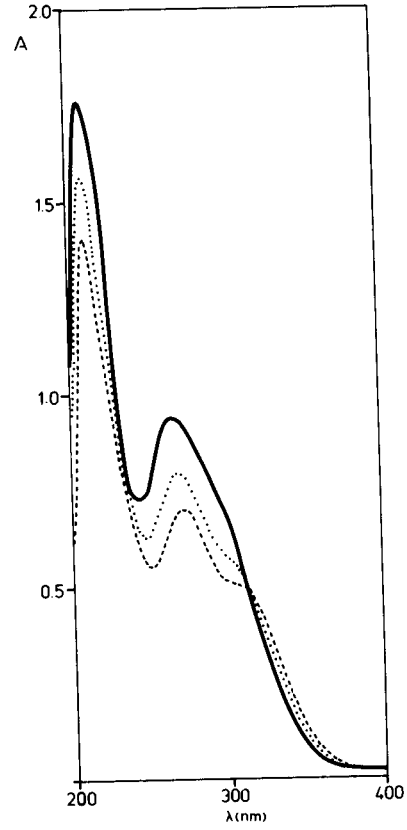

Figure 3. The uv absorption spectra of Thia-Nt at a concentration of 78 μM (—) and its complex with coliphage T_4 DNA at a DNA/Thia-Nt ratio of 0.35 (···) and 0.7 (-). The sample and reference cell contained equal concentrations of DNA[(···)27 μM and (-)55 μM].

The Thia-Nt-calf thymus DNA complex reduced linear dichroism spectrum (Figure 4) shows a positive part (295-330 nm) reflecting the position of the bis-thiazole chromophore in the DNA complex. At these wavelengths, a significative contribution from the DNA itself was observed which certainly minimizes the measured linear dichroism values for the complex.

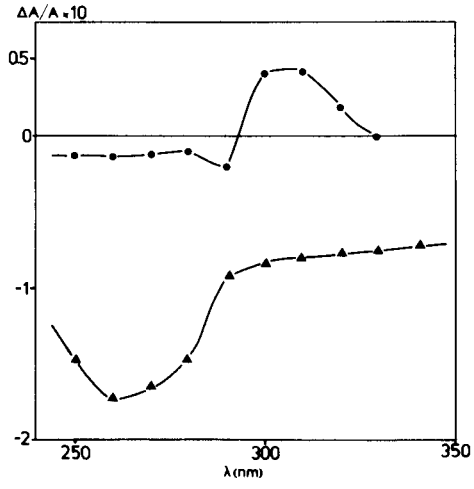

Figure 4. Reduced linear electric dichroism (ΔA/A) spectra of the calf thymus DNA (▲) and of the Thia-Nt—DNA complex (●) at a ligand/-DNA ratio of 0.1 and at 12.5 kV. cm^{-1}.

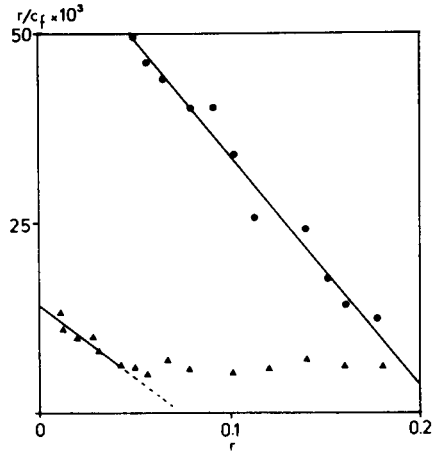

Figure 5. Representative Scatchard plot for the evaluation of the binding parameters for the interaction of Thia-Nt with calf thymus DNA (▲) and poly[d(A.T)-d(A.T)] (●).

Nevertheless, this positive curve clearly demonstrates that the ligand is preferentially oriented parallel to the DNA axis, a geometry which excludes intercalation. Thus, replacement of the *N*-methylpyrrole heterocycle in Nt by a thiazole ring does not perturb the minor groove binding.

The binding affinities were calculated by means of Scatchard plots [25] (Figure 5) and allowed us to precise the binding parameters (Table I). The Scatchard plots were done at 265 nm, a wavelength at which a large DNA absorbance is observed and it was necessary to substract the DNA contribution by adding an equal concentration of DNA to the reference cell. The apparent binding constant K_a, measured with two DNAs of different base composition, indicate that Thia-Nt binds DNA with a weaker affinity than Nt does [2] (Nt-calf thymus DNA, $K_a = 2.9 \times 10^5$ M^{-1}; Nt-poly[d(A.T)-d(A.T)], $K_a = 4 \times 10^5$ M^{-1}).

The ligand was found to cover 4-5 nucleotides per binding site (n) with the synthetic poly[d(A.T)-d(A.T)] DNA. This result is an argument in favor of a location of the ligand in the minor groove of DNA. The difference in the DNA binding affinity of the studied compound toward the two different DNA was also apparent considering the ΔTm values (Table I), meaning a marked preference of this drug for A-T sites as observed for Nt [2-4].

The spectroscopic measurements have provided information concerning the mode of binding of the thiazole-containing Nt analogue to DNA. Orientation of this drug in the minor groove of DNA can readily be postulated.

EXPERIMENTAL

Melting points were taken on a Tottoli Büchi 510 apparatus and are uncorrected. The ir spectra were recorded with a Perkin Elmer 297 spectrophotometer using potassium bromide pellets. The ^{1}H-nmr spectra were recorded on a Brücker WP 80 SY spectrophotometer. Chemical shifts are reported in ppm from tetramethylsilane as an internal standard and are given in δ units.The EI mass spectra were recorded on a Ribermag, R10.10 (combined with Riber 400 data system) mass spectrophotometer at 70 eV by using direct insertion. FAB mass spectra were determined on a Kratos MS-50 RF mass spectrometer. Thin layer chromatography (tlc) was carried out using silica gel 60F-254 Merck, in system solvent A(chloroform-methanol, 80/20, v:v in a saturated ammonia atmosphere). Elemental analyses were performed by the "Service Central d'Analyses", CNRS, Vernaison, France.

Table I

DNA-Binding Parameters of Thia-Net

	ΔTm [a]/(D/P) [b]			λ [c]	Ka(m^{-1}) [d]	n [e]
Poly[d(A.T) - d(A.T)]	3°4 (0.1)	9°3 (0.5)	12° (1.0)	265	6.4 10^4	4.8
Calf thymus DNA	0°7 (0.1)	3° (0.5)	5°7 (1.0)	265	1.2 10^4	---

[a] Elevation in thermal denaturation temperature (°C) [b] Drug to Phosphate DNA ratio. [c] Wavelength at which the absorbance change was measured. [d] Apparent binding constant. [e] Binding site size.

The uv absorption spectra and melting temperature studies were recorded with a Uvikon Kontron 810/820 spectrophotometer and realised in 0.1 *M* SSC buffer (0.15 *M* sodium chloride, 0.015 *M* sodium citrate, *p*H 7.0) as previously described [26]. Linear dichroism experiments were made using a well-established procedure [27]. Scatchard analysis and determination of the binding parameters were realized using a described method [28].

Amino-2-ethoxycarbonyl-4-thiazole (**2**).

A mixture of thiourea (5 g, 65.8 mmoles) and ethyl 2-bromopyruvate (12.8 g, 65.8 mmoles) was heated gradually to 70° under stirring until the mixture became highly viscous. On cooling, the hydrobromide crystallized and was recrystallized from ethanol-petroleum ether. The recrystallized hydrobromide was solubilized in water and the base was precipitated from addition of dilute ammonia. The crude product was filtered and recrystallized from methanol to give **2** in a 57.5% yield, mp 175-177°; Rf (A) 0.8; ir: 1600 (NH), 1680 (C=O), 3100-3400 cm^{-1} (NH), ^1H nmr (DMSO-d_6): δ 7.4 (s, 1H, CH thiazole), 7.3 (s, 2H, NH_2), 4.2 (q, 2H, CH_2), 1.2 (t, 3H, CH_3); ms: m/e (relative intensity) 172 (M^+, 44.8), 144 (9.2), 73 (98.8).

Anal. Calcd. for $C_6H_8N_2O_2S$: C, 41.9; H, 4.7; N, 16.3; O, 18.6; S, 18.6. Found: C, 41.5; H, 4.7; N, 16.1; O, 19.0; S, 18.6.

Ethyl 2-(4-*t*-Butyloxycarbonylaminobutyryl)aminothiazole-4-carboxylate (**3**).

Cold solutions of DCC (2.64 g, 12.7 mmoles) and HOBt (1.95 g, 12.7 mmoles) in dichloromethane/dimethylformamide (1:1, 20 ml) were added to a solution of 4-*t*-butyloxycarbonylaminobutyric acid **1** [22] (2.36 g, 11.6 mmoles) in 15 ml of dichloromethane/dimethylformamide (1:1) at 0° for 1 hour under stirring. A cold solution of **2** (2 g, 11.6 mmoles) in dichloromethane/dimethylformamide (1:1, 20 ml) was added and stirring was continued for 2 hours at 0° and 12 hours at room temperature. The solvent was evaporated and the residue taken up with dichloromethane provided dicyclohexylurea (DCU) which was filtered. The organic solution was washed with 1 *N* hydrochloric acid, water and finally with 1 *M* sodium bicarbonate. After drying over anhydrous sodium sulfate, the solvent was removed *in vacuo*. The remaining DCU was discarded by precipitation with ethyl acetate, the resulting material was recrystallized from acetone giving pure **3** (2.6 g, 63% yield), mp 155-157°; Rf (A) 0.95; ir: 1720 (CO amide), 1680 cm^{-1} (CO ester); ^1H nmr (deuteriochloroform): δ 11.6 (m, 1H, NH), 7.8 (s, 1H, CH thiazole), 4.9 (m, 1H, NHBoc), 4.4 (q, 2H, CH_2), 3.2 (m, 2H, CH_2NH), 2.6 (t, 2H, CH_2CO), 1.9 (m, 2H, CH_2), 1.4 (m, 12H, CH_3); ms: m/e (relative intensity) 357 (M^+, 1.8).

Anal. Calcd. for $C_{15}H_{23}N_3O_5S$: C, 50.5; H, 6.5; N, 11.8; O, 22.4; S, 9.0. Found: C, 50.5; H, 6.6; N, 11.5; O, 22.0; S, 8.6.

2-(4-*t*-Butyloxycarbonylaminobutyrylamino)thiazole-4-carboxylic Acid (**4**).

A solution of **3** (2 g, 5.6 mmoles) in methanol (11 ml) and sodium hydroxide (0.9 g in 9 ml of methanol/water, 10/1) was stirred at 65°. The progress of the reaction was monitored by tlc and was thereby judged to be complete after 3 hours. The solvent was evaporated and the residue was taken up in water. Impurities were extracted with ethyl acetate (2 × 20 ml). The aqueous solution was cautiously acidified to *p*H 4-5 with 1 *N* hydrochloric acid and extracted with ethyl acetate (3 × 20 ml). After drying over anhydrous sodium sulfate and evaporation of the organic layer, trituration in cyclohexane and evaporation of cyclohexane yield 1.6 g of **4**, (87% yield), mp 187-189°; Rf (A) 0.25; ir: 3000-2500 cm^{-1} (OH dimer); ^1H nmr (deuteriochloroform): δ 12.2 (m, 1H, NH), 7.8 (s, 1H, CH thiazole), 5.2 (m, 1H, NHBoc), 3.3 (m, 2H, CH_2NHBoc), 2.6 (t, 2H, CH_2CO), 2.0 (m, 2H, CH_2), 1.4 (s, 9H, CH_3); ms: (FAB) 330 ($M^+ + 1$).

Ethyl 2-[2'-[4-*t*-Butyloxycarbonylaminobutyrylamino]thiazole-4'-carboxamido]thiazole-4-carboxylate (**5**).

The acid **4** (2 g, 6.08 mmoles) was coupled to the amine **2** (1.045 g, 6.08 mmoles) using DCC (1.38 g, 6.66 mmoles) and HOBt (1.023 g, 6.68 mmoles) in dichloromethane/dimethylformamide (1:1) as described for the preparation of **3**. DCU was removed by precipitation with ethyl acetate and the filtrate washed with 1 *N* hydrochloric acid (20 ml), water (20 ml) and 1 *M* sodium bicarbonate (20 ml), then dried over anhydrous sodium sulfate. The solvent was removed *in vacuo* giving 1.17 g (38%) of **5** after recrystallization from a mixture of ethanol-ether, mp 131-135°; Rf (A) 0.87; ir: 1720 (CO amide), 1680 cm^{-1} (CO ester); ^1H nmr (deuteriochloroform): δ 11.2 (m, 1H, NH), 7.9 (s, 1H, CH thiazole), 7.8 (s, 1H, CH thiazole), 5.1 (m, 1H, NHBoc), 4.4 (q, 2H, CH_2), 3.3 (m, 2H, CH_2NH), 2.7 (t, 2H, CH_2CO), 2.0 (m, 2H, CH_2), 1.4 (m, 12H, CH_3); ms: (FAB) 484 ($M^+ + 1$).

Anal. Calcd. for $C_{19}H_{25}N_5O_6S_2$: C, 47.2; H, 5.2. Found: C, 47.4; H, 5.5.

2-[2'-(4-*t*-Butyloxycarbonylaminobutyrylamino)thiazole-4'-carboxamido]thiazole-4-carboxylic Acid (**6**).

The ethyl ester **5** (500 mg, 1.035 mmoles) was totally converted after 3 hours to the corresponding acid **6**, according to the method for preparation of **4**. Compound **6** was obtained pure after column chromatography with chloroform-methanol, 8/2, v:v, as eluent (410 mg, 87%), mp >250°; Rf (A) 0.6; ir: 3000-2500 cm^{-1} (OH dimer); ^1H nmr (DMSO-d_6): δ 15.5 (s, 1H, OH), 7.9 (s, 1H, CH thiazole), 7.5 (s, 1H, CH thiazole), 6.8 (m, 1H, NHBoc), 3.2 (m, 2H, CH_2NH), 2.7 (m, 2H, CH_2CO), 1.9 (m, 2H, CH_2), 1.4 (s, 9H, CH_3).

Anal. Calcd. for $C_{17}H_{21}N_5O_6S_2$: C, 44.8; H, 4.65; N, 15.4; O, 21.1; S, 14.1. Found: C, 44.6; H, 4.8; N, 15.1; O, 21.2; S, 13.8.

t-Butyl[2-[2'-(4-*t*-Butyloxycarbonylaminobutyrylamino)thiazole-4'-carboxamido]thiazole-4-carboxamido]propyl Carbamate (**8**).

The acid **6** (300 mg, 0.66 mmole) was coupled to 3-*t*-butyloxycarbonylaminopropylamine **7** [23] (115 mg, 0.66 mmole) using DCC (149 mg, 0.73 mmole) and HOBt (111 mg, 0.73 mmole) in a mixture of dichloromethane-dimethylformamide (1:1, 18 ml) according to the procedure described for **3**. Purification of the crude material was accomplished by column chromatography with chloroform-methanol, 8/2, v:v, as eluent. Collection and evaporation of the appropriate fractions give **8** with 52% yield, mp 58-63°; Rf (A) 0.95; ir: 1680 cm^{-1} (CO); ^1H nmr (deuteriochloroform): δ 11.5 (m, 1H, NH), 8.3 (m, 1H, NHCO), 7.8 (d, 2H, CH thiazoles), 5.1 (m, 1H, NHBoc), 3.4 (m, 6H, CH_2NH), 2.7 (m, 2H, CH_2CO), 2.0 (m, 4H, CH_2), 1.4 (s, 18H, CH_3); ms: m/e (relative intensity): 612 (M^+, 30.2).

Anal. Calcd. for $C_{25}H_{37}N_7O_7S_2$: C, 49.1; H, 6.1; N, 16.0; O, 18.3; S, 10.5. Found: C, 48.9; H, 6.0; N, 16.1; O, 18.1; S, 10.2.

(2-(2'-(4-Aminobutyrylamino)thiazole-4'-carboxamido)thiazole-4-carboxamido)propylamine Dihydrobromide (Thia-Nt) (**9**).

A solution of **8** (133 mg, 0.217 mmole) in acetic acid (30 ml) was flushed with dry hydrogen bromide for 10 minutes and stirring was maintained for 20 minutes. After evaporation of the solvent

under vacuum, the residue was washed with ethanol (4 × 30 ml) to remove acids, taken up with water, washed with chloroform and diethyl ether (15 ml). Lyophilisation of the aqueous layer gave **9** (90% yield), mp 197-202°; Rf (A) 0; ^{1}H nmr (DMSO-d_6): 400 MHz δ 12.4 (s, 2H, NHCO), 8.3 (s, 1H, CH thiazole), 8.2 (t, 1H, NH CH_2), 7.8 (s, 1H, CH thiazole), 7.75 (m, 6H, NH_3^+), 3.3 (m, 1H, CH_2NH), 2.8 (m, 4H, $CH_2NH_3^+$), 2.6 (m, 2H, CH_2CO), 1.8 (m, 4H, CH_2).

Anal. Calcd. for $C_{15}H_{23}N_7O_3S_7Br_2$: C, 31.4; H, 4.0; N, 17.1; O, 8.4; S, 11.2 Found: C, 31.2; H, 4.2; N, 17.5; O, 8.1; S, 10.9.

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