Synthesis of Some Spin-labeled Bithiazoles, Useful Probes for Studying Bleomycin-DNA Binding

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The antibiotic antitumor drug Bleomycin is known to bind to DNA by intercalation of its bithiazole chromophore into the DNA base-pairs. Four spin-labeled bithiazoles, bearing a 4-(2,2,6,6-tetramethyl-1-piperidinooxy) free radical, have been synthesized. These derivatives where the nitroxide group is linked to the end of spacers of various lengths, can be used as probes for the study, by esr spectroscopy, of the intercalation of the heterocyclic moiety of Bleomycin.

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Bleomycin is the generic name of closely related glycopeptidic antibiotics useful in the treatment of various malignant diseases, that differ only in the substituent on the bithiazole unit [1,2]. The drugs are believed to act as DNA binding-DNA cleaving molecules [2] with two well defined active parts in their structure: a metal ion-chelating portion able to form an oxygen dependent Fe(II)-complex [3] and a bithiazole containing moiety which has been suggested to be involved in an intercalative process [4].

The metal-binding properties of Bleomycin and the role of a Fe(II)-Bleomycin-oxygen complex in the induction of strand breaks in DNA is well documented [1,2,3,5,6]. Recently, we have described the design and the synthesis of a synthetic metal-complexing model which permit to define the metal binding sites of Bleomycin [7]. But little has been reported on the DNA binding part and particularly on the putative intercalation of the bithiazole rings into the DNA base-pairs [4,8,9]. On the basis of model studies

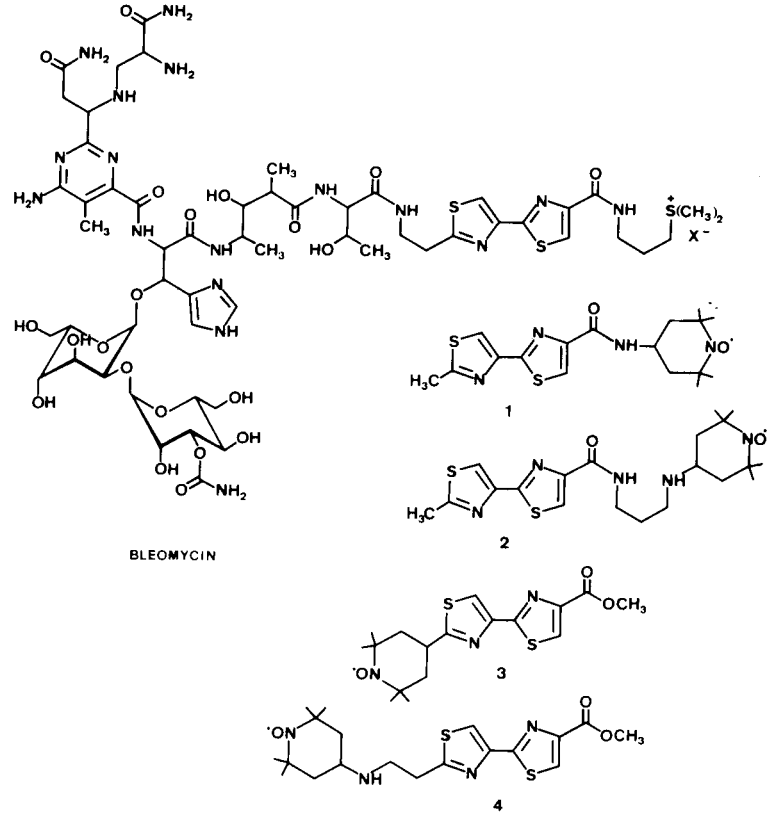
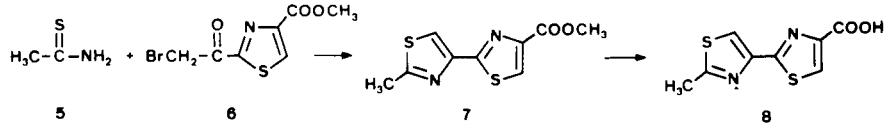
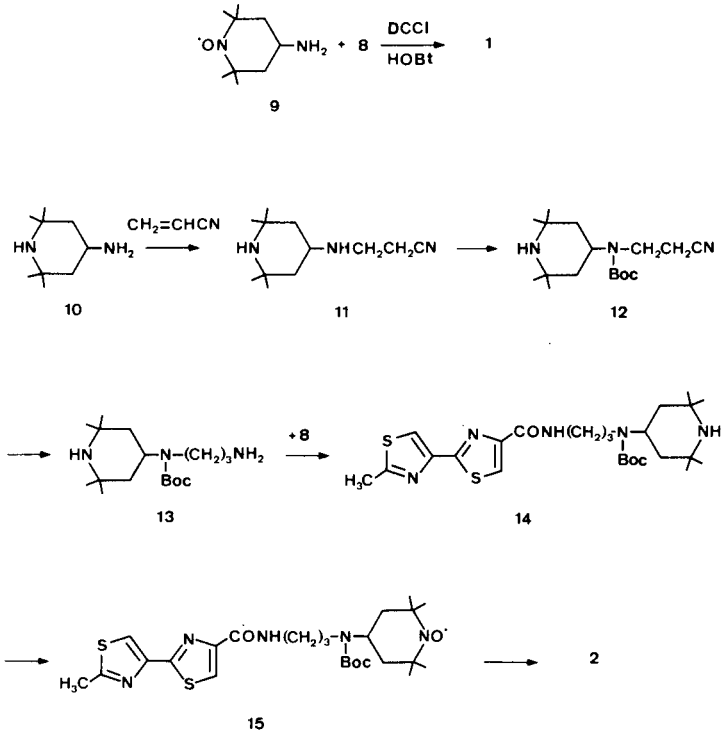


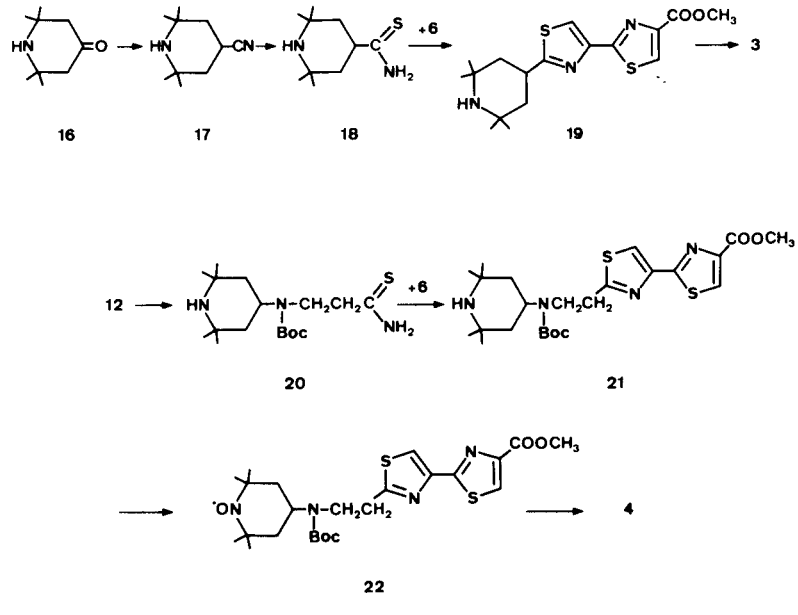
Figure 1.



Scheme 1



Scheme 2



Scheme 3

[10] and of X-ray data [11], it has been established that the two coplanar rings of simple derivatives of the bithiazole intercalate, but no accurate study has dealt with the question of the position of the bithiazole in its intercalation site. For this purpose, we have carried out the synthesis of some spin-labeled bithiazole derivatives.

The vital properties of a spin-label is that the shape of its electron spin resonance (esr) spectrum is sensitive to the polarity of the environment and to the type of motion [12]. When a stable paramagnetic nitroxide is chemically attached to a biological molecule, a "spy" is formed whose spectrum reveals certain properties of the labeled site [13]. Thus, the intercalation of spin-labeled intercalating drugs with DNA can be studied using these properties [14,17]. The radical moiety of the spin-labeled dye acts as a reporter group giving accounts of the rotational freedom of the local environment in which it resides and of the way by which the intercalative process occurs. Varying the distance with longer or shorter aliphatic chains between the nitroxide and the intercalating dye, the depth of the site can also be outlined. With this information, the size of the combining site can be mapped and the intercalation pointed out.

The spin-labeled probes here synthesized bear their nitroxide group either in the vicinity of the bithiazole rings, with the piperidinooxy moiety linked to the heterocycle itself (**1** and **3**) or introduced at the extremity of an aliphatic chain (**2** and **4**) (Figure 1).

Preparation of Amides **1** and **2**.

The two amides **1** and **2** were synthesized starting from 2'-methyl-2,4'-thiazole-4-carboxylic acid (**8**) obtained by cyclization of thioacetamide **5** with methyl 2-(2-bromoacetyl)thiazole-4-carboxylate (**6**) followed by the hydrolysis of the methyl ester group (see Scheme 1). The coupling reaction between the bithiazole carboxylic acid **8** and the amines was achieved using dicyclohexylcarbodiimide (DCC) in dichloromethane in the presence of hydroxybenzotriazole (HOBt). It is important to note that the use of HOBt was necessary to obtain the expected amides **1** and **2** *via* the hydroxybenzotriazole activated ester of **8**. In the absence of HOBt, the carboxylic salts of the amines inevitably precipitated in the reaction solvent.

For the synthesis of **1**, 4-amino-2,2,6,6-tetramethylpiperidinooxy **9** was coupled with compound **8** under these conditions.

The synthesis of **2** was carried out as depicted in Scheme 2. Because of the relative instability of the nitroxide radical, the oxidation was performed in the penultimate step. Thus, the cyanoethylation of 4-amino-2,2,6,6-tetramethylpiperidine (**10**) gave 4-(2'-cyanoethyl)amino-2,2,6,6-tetramethylpiperidine (**11**). Protection of the exocyclic secondary amine of **11** by a *t*-butyloxycarbonyl group (Boc) was followed by the reduction of the cyano group by hydrogen in the presence of Raney Ni [7] under such conditions that the protective group Boc was not cleaved.

Coupling of the primary amine **13** with the bithiazole carboxylic acid **8** in the presence of DCC-HOBt, gave the amide **14** in an excellent yield, and oxidation of **14** with hydrogen peroxide in the presence of sodium phosphotungstate followed by cleavage of the Boc protective groups afforded the desired spin-labeled amide **2**.

Preparation of Compounds **3** and **4**.

The synthesis of labeled compounds **3** and **4** consisted first of preparing appropriate thioamides to be condensed with the methyl 2-(2-bromoacetyl)thiazole-4-carboxylate (**6**). Thioamides were synthesized starting from the corresponding nitriles using hydrogen sulfide and it was the reason why the nitroxide group was introduced at the final steps as above.

The 4-thiocarboxamido-2,2,6,6-tetramethylpiperidine (**18**), the starting material for the preparation of **3**, was obtained by addition of hydrogen sulfide to the corresponding nitrile **17** synthesized by the action of tosylmethylisocyanide (TOSMIC) on 2,2,6,6-tetramethyl-4-piperidone (**16**).

Thioamide **20** was obtained from **12** and was condensed with the bromomethylketone **6** to give the bithiazole **21**. Oxidation by hydrogen peroxide and deprotection of secondary amide yielded the expected spin-label **4**.

The four spin-labeled bithiazoles here described have been assayed for their esr spectra, in dilute aqueous solution, in the absence of DNA. The spectra consist of three sharp lines characteristic of the freely rotating nitroxide group. These spin-labels will be useful probes for bithiazole-DNA interaction [18].

EXPERIMENTAL

Melting points were determined on a Tottoli melting point apparatus and are uncorrected. The ir spectra were obtained on a Beckmann Acculab 1 spectrophotometer. The ^{1}H nmr spectra were obtained on a JEOL-JNM-MH60 spectrometer. Chemical shifts are reported in ppm from TMS as an internal standard and are given in δ units. Mass spectra were obtained on a quadrupole mass spectrometer Ribermag R10-10 (combined with Riber 400 data system) at 70 eV. The esr spectra were recorded on a Varian E-109 X-band spectrometer. Elemental analyses were performed by the "Service Central d'Analyses", CNRS, Vernaison, France.

Methyl 2-(2-Bromoacetyl)thiazole-4-carboxylate (**6**).

This compound was prepared according to the procedure of Sakai *et al.* [19], starting from ethyl bromopyruvate and *O*-benzoylthiolactamide [20]. The ethanolic solution of *O*-benzoyllactonitrile **2** (60 g) containing a few drops of diethylamine was reacted in a sealed stainless steel bomb with hydrogen sulfide at 5 atmospheres and 20° for 8 hours.

The solvent was removed, and the residue solidified after cooling. The *O*-benzoylthiolactamide was washed with cyclohexane and dried, affording a white solid (70 g, 98%). This compound was then allowed to react with ethyl bromopyruvate as described before.

Methyl 2'-Methyl-2,4'-bithiazole-4-carboxylate (**7**).

A solution of **6** (1.0 g, 15 mmoles) and thioacetamide (1.10 g, 15 mmoles) in ethanol was stirred for 24 hours at 20°. The white precipitate was filtered (1.6 g, 66%). The product was recrystallized from an ethanol-water mixture to afford an analytical sample, mp 153°; ir (potassium bromide): 1695 cm^{-1} (C=O); nmr (DMSO-d_6): δ 2.69 (s, CH_3, 3 H), 3.98 (s, CH_3, 3 H), 8.17 (s, CH, 1 H), 8.50 (s, CH, 1 H).

2'-Methyl-2,4'-bithiazole-4-carboxylic Acid (**8**).

The ester **7** (1.2 g, 5 mmoles) was dissolved in 30 ml of methanol and sodium hydroxide (0.8 g in 5 ml of water) was added. The mixture was stirred at room temperature for 3 hours. The solution was neutralized by 10 *N* hydrochloric acid to *p*H 4.5. Removal of the solvents *in vacuo* was followed by the addition of boiling absolute ethanol to the residue. Sodium chloride was separated by filtration of the boiling suspension. After cooling, the desired carboxylic acid **8** precipitated as white needles (0.73 g, 65% yield). Evaporation of the solvent gave an additional amount of the compound (0.17 g, 15% yield). An analytical sample was obtained by recrystallization from absolute ethanol to give colorless needles, mp 239-240°; ir (potassium bromide): 1695 cm^{-1} (C=O); nmr (DMSO-d_6): δ 2.77 (s, CH_3, 3 H), 7.62 (s, OH, 1 H), 8.30 (s, CH, 1 H), 8.52 (s, CH, 1 H); ms: 228 ($M^+ + 2$, 4), 226 (M^+, 85), 126 ($M^+ - 100$, 67), 57 ($M^3 - 169$, 100).

Anal. Calcd. for $C_6H_6N_2O_2S_2$: C, 42.5; H, 2.7; N, 12.4. Found: C, 42.5; H, 2.7; N, 12.5.

N-[4-(2,2,6,6-Tetramethyl)-1-piperidinooxyl]-2'-methyl-2,4'-bithiazole-4-carboxamide (**1**).

To a solution of the acid **8** (0.680 g, 3 mmoles) in DMF (50 ml) were added dicyclohexylcarbodiimide (DCC) (0.640 g, 3.1 mmoles) and 1-hydroxybenzotriazole hydrate (HOBt) (0.48 g, 3.1 mmoles) in DMF (20 ml) at 0°, with stirring. After 30 minutes, a solution of 4-amino-2,2,6,6-tetramethylpiperidinooxy (0.514 g, 3 mmoles) in DMF (10 ml) cooled to 0° was added. The mixture was stirred at 0° for 2 hours and at room temperature overnight. Filtration of dicyclohexylurea (DCU) and removal of the solvent under reduced pressure afforded an oily residue which was dissolved in ethyl acetate. Filtration, washing with 1 *N* hydrochloric acid, 1 *M* sodium bicarbonate, water, and drying of the solution over sodium sulfate, followed by removal of the solvent under reduced pressure, gave a yellow-orange oil which solidified on standing. Crystallization from petroleum ether afforded compound **1** as deep orange crystals, 1.08 g (95%), mp 181-183°; ir (potassium bromide): 1670 (C=O), 1365, 1250, 1170 cm^{-1} (NO·); ms: 380 ($M^+ + 1$, 4), 379 (M^+, 5), 84 ($M^+ - 295$, 100).

Anal. Calcd. for $C_{17}H_{23}N_4O_2S_2$: C, 53.8; H, 6.1; N, 14.8. Found: C, 54.2; H, 5.8; N, 15.3.

4-[(2'-Cyanoethyl)amino]-2,2,6,6-tetramethylpiperidine (**11**).

To a solution of 4-amino-2,2,6,6-tetramethylpiperidine (6.24 g, 40 mmoles) in ethanol (30 ml) was added dropwise acrylonitrile (2.12 g, 40 mmoles). The mixture was refluxed for 3 hours. Removal of the solvent under reduced pressure gave the desired nitrile as a colorless liquid (8 g, 96%), $bp_{0.1}$ = 164°; ir (neat): 2230 cm^{-1} (C≡N).

N-Boc-*N*-[4-(2,2,6,6-tetramethyl-1-piperidinyl)]-3-aminopropionitrile (**12**).

The nitrile **11** (7.32 g, 35 mmoles) was dissolved in dichloromethane (40 ml) and a solution of di-*t*-butyldicarbonate (8.28 g, 38 mmoles) in dichloromethane (20 ml) was added dropwise with stirring. After a short induction period, the temperature rose to 40°. The reaction was complete after 3 hours. Evaporation of the solvent to 20 ml gave a white precipitate of the expected compound **12** (8.9 g, 82%); mp 117-118°; ir (potassium bromide): 2225 (C≡N), 1680 cm^{-1} (O-CO); ms: 312 ($M^+ + 1$, 5), 311 (M^+, 14); nmr (deuteriochloroform): δ 1.17 (s, CH_3, 6H), 1.27 (s, CH_3, 6H), 1.41 (m, CH_2, 2H), 1.54 (s, CH_3, 9H), 1.73 (m, CH_2, 2H), 2.65 (t, CH_2, 2H, J = 14 Hz), 3.46 (t, CH_2, 2H, J = 14 Hz), 4.45 (m, CH, 1H).

N-Boc-*N*-[4-(2,2,6,6-tetramethyl-1-piperidinyl)]-1,3-diaminopropane (**13**).

A high pressure autoclave was charged with a solution of **12** (3.5 g, 11 mmoles) in 50 ml of presaturated ethanolic ammonia, 0.5 g of Raney nickel and hydrogen at a pressure of 40 atmospheres. The mixture was stirred at 40° for 10 hours. The catalyst was filtered, and the filtrate was evaporated to dryness. The resulting oil was distilled *in vacuo*, yield 2.8 g (79%), bp 123-125° (0.2 mm). The colorless oil crystallized slowly upon standing; ir (neat): 1665 cm^{-1} (O-CO); nmr (deuterichloroform): δ 1.16 (s, CH_3, 6H), 1.24 (s, CH_3, 6H), 1.30 (s, NH_2, 2H), 1.50 (s, CH_3, 9H), 1.65 (m, CH_2, 2H), 2.77 (t, CH_2, 2H), 3.22 (t, CH_2, 2H), 4.38 (m, CH, 1H).

N-Boc-*N*-[4-(2,2,6,6-tetramethyl-1-piperidinyl)]-3-(2'-methyl-2,4'-bithiazole-4-carboxamido)]propylamine (**14**).

To a solution of the acid **8** (0.565 g, 2.5 mmoles) in DMF (40 ml) were added DCC (0.560 g, 2.7 mmoles) and HOBt (0.360 g, 2.7 mmoles) in DMF (15 ml) at 0°. The mixture was stirred for 30 minutes and **13** (0.780 g, 2.5 mmoles) in DMF (20 ml) was added. The mixture was stirred for 2 hours at 0° then overnight at room temperature. The solvent was evaporated under reduced pressure after filtration of DCU. The residue was dissolved in dichloromethane, remaining DCU was discarded, and the solution was washed with 1 *N* hydrochloric acid, 1 *M* sodium bicarbonate, and water and dried over sodium sulfate. Removal of the solvent afforded the amide **14** (0.96 g; 74%), mp 144-145°; ir (potassium bromide): 1695 (O-CO), 1630 cm^{-1} (CONH).

Anal. Calcd. for $C_{25}H_{39}N_5O_3S_2$: C, 57.6; H, 7.5; N, 13.4. Found: C, 57.4; H, 7.4; N, 13.4.

N-[4-(2,2,6,6-Tetramethyl-1-piperidinooxyl)]-3-[2'-methyl-2,4'-bithiazole-4-carboxamido]propylamine (**2**).

To **14** (0.780 g, 1.5 mmoles) in 10 ml of methanol, were added phosphotungstic acid (30 mg) sodium hydroxide (30 mg) and 5 ml of 30% hydrogen peroxide. The mixture was stirred for 48 hours. The reaction was considered complete when the amplitude of the esr signal became constant. The labeled compound was extracted with dichloromethane (2 × 25 ml). After drying over sodium sulfate and removal of the solvent, the nitroxide **15** was obtained as a red oil. Trifluoroacetic acid (10 ml) was added to the crude compound **15** and the mixture was allowed to stand at room temperature for 15 minutes. Excess trifluoroacetic acid was then evaporated under reduced pressure and the residue dissolved in water. The aqueous solution was neutralized with 1 *M* sodium bicarbonate, and extracted with dichloromethane. The organic layer was dried over sodium sulfate and the solvent evaporated to give **2** as a yellow-orange oil which crystallized on trituration in petroleum ether (0.4 g, 62%). The orange solid was recrystallized from ether-petroleum ether, mp 72-74°; ir (potassium bromide): 1640 (C=O), 1350, 1245, 1170 cm^{-1} (NO·); ms: 438 ($M^+ + 2$, 16), 437 ($M^+ + 1$, 16), 436 (M^+, 5), 58 ($M^+ - 378$, 100).

Anal. Calcd. for $C_{20}H_{30}N_5O_2S_2$: C, 55.0; H, 6.9; N, 16.0. Found: C, 55.4; H, 6.5; N, 15.7.

4-Cyano-2,2,6,6-tetramethylpiperidine (**17**).

To a stirred solution of 2,2,6,6-tetramethyl-4-piperidone hydrochloride (**16**) (3.83 g, 20 mmoles) and tosylmethyl isocyanide (3.88 g, 20 mmoles) in 80 ml of dimethoxyethane at 0° were added three equivalents (2.34 g) of potassium *t*-butoxide in *t*-butanol (80 ml). The mixture was stirred at 0° for 45 minutes and at room temperature for 1 hour. Solvents were evaporated under reduced pressure and the residue dissolved in water (20 ml). The aqueous solution was extracted by three times with 30 ml of dichloromethane. The combined organic layers were dried over sodium sulfate and the solvent removed *in vacuo* to give a red oil which crystallized from petroleum ether. Recrystallization from ether-petroleum ether gave red needles (2.55 g; 76%) mp 98°; ir (potassium bromide): 2240 cm^{-1} (C≡N).

4-Thiocarboxamido-2,2,6,6-tetramethylpiperidine (**18**).

The solution of **17** (1.5 g, 9 mmoles) in DMF containing a few drops of diethylamine was allowed to react in a sealed stainless steel bomb with hydrogen sulfide at 5 atmospheres and 20° for 12 hours. Evaporation of the solvent gave a grey-green solid (0.7 g, 39%); ir (potassium bromide): 1650 cm^{-1} (C=S); nmr (DMSO-d_6): δ 1.27 (s, CH_3, 12H), 1.56 (t, CH_2, 2H), 2.0 (m, CH_2, 2H), 3.40 (m, CH, 1H). The compound was satisfactory for use in subsequent reaction without further purification.

Methyl 2'-[4-(2,2,6,6-Tetramethyl-1-piperidinyl)]-2,4'-bithiazole-4-carboxylate (**19**).

A mixture of crude **18** (0.47 g, 2.3 mmoles) and **6** (0.2 g, 2.3 mmoles) in methanol (40 ml) was stirred at room temperature for 24 hours. The solvent was removed under reduced pressure. The residue crystallized upon standing and the white solid was recrystallized from ethanol, mp 55-57°; ir (potassium bromide): 1710 cm^{-1} (C=O).

Methyl 2'-[4-(2,2,6,6-Tetramethyl-1-piperidinooxyl)]-2,4'-bithiazole-4-carboxylate (**3**).

Oxidation of **19** was achieved by the procedure used for the preparation of **2** and gave a red oil which crystallized upon standing (0.53 g, 61% yield from **18**); ir (potassium bromide): 1690 (C=O), 1355, 1250, 1170 cm^{-1} ($NO^{\cdot}$); ms: 381 ($M^+ + 1$, 14), 380 (M^+, 7), 350 ($M^+ - 30$, 100).

Anal. Calcd. for $C_{17}H_{22}N_3O_3S_2$: C, 53.7; H, 5.8; N, 11.0. Found: C, 54.5; H, 6.1; N, 11.3.

N-Boc-*N*-[4-(2,2,6,6-tetramethyl-1-piperidinyl)]-3-amino-1-thiopropionamide (**20**).

This compound was prepared from **12** by a procedure similar to that described for **18**. The residue obtained after evaporation of DMF was triturated with absolute ethanol. The insoluble material was filtered off, and the filtrate was evaporated to dryness. The operation was repeated two times, the insoluble fractions were then collected and recrystallized from acetonitrile (0.8 g, 40%), mp = 174°; ir (potassium bromide): 1660 (O-C-O), 1650 cm^{-1} (shoulder, C=S); nmr (DMSO-d_6): δ 1.08 (s, CH_3, 6H), 1.16 (s, CH_3, 6H), 1.41 (s, CH_3, 9H), 2.67 (m, CH_2, 4H), 3.35 (m, CH_2, 4H), 4.22 (m, CH, 1H), 9.20 (m, NH_2, 1H), 9.35 (m, NH_2, 1H).

Methyl 2'-[*N*-Boc-*N*-[4-(2,2,6,6-tetramethyl-1-piperidinyl)]-2-aminoethyl]-2,4'-bithiazole-4-carboxylate (**21**).

A mixture of **20** (0.40 g, 1 mmole) and **6** (0.31 g, 1 mmole) in methanol was stirred for 24 hours at 20°. The solvent was removed *in vacuo* at room temperature. The paste solidified by trituration with isopropyl ether, (0.5 g, 83% yield); ir (potassium bromide): 1715 (C=O), 1675 cm^{-1} (O-CO); nmr (deuteriochloroform): δ 1.09 (s, CH_3, 6H), 1.17 (s, CH_3, 6H), 1.48 (s, CH_3, 9H), 2.7 (m, CH_2, 4H), 3.58 (m, CH_2, 4H), 4.0 (s, CH_3, 3H), 4.4 (m, CH, 1H), 8.04 (s, CH, 1H), 8.18 (s, CH, 1H). The product was used in the next reaction without purification.

2'-[*N*-[4-(2,2,6,6-Tetramethyl-1-piperidinooxyl)]-2-aminoethyl]-2,4'-bithiazole-4-carboxylate (**4**).

The oxidation of the piperidinyl group of **21** and deprotection of the exocyclic secondary amine of **22** have been achieved by the methods described above for the preparation of **2**. The compound was obtained as a yellow-orange solid (0.18 g, 43% yield from **20**), mp 72-75°; ir (potassium bromide): 1705 (CO), 1360, 1240, 1170 cm^{-1} ($NO^{\cdot}$); ms: 424 ($M^+ + 1$, 7), 423 (M^+, 16), 113 ($M^+ - 300$, 100).

Anal. Calcd. for $C_{19}H_{27}N_4O_3S_2$: C, 53.9; H, 6.4; N, 13.2. Found: C, 54.3; H, 6.2; N, 13.0.

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