

## CYCLOTHIALIDINE, A NOVEL DNA GYRASE INHIBITOR

## II. ISOLATION, CHARACTERIZATION AND STRUCTURE ELUCIDATION

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Cyclothialidine is a novel DNA gyrase inhibitor produced by *Streptomyces filipinensis* NR 0484. It was isolated from the culture broth by charcoal adsorption, Diaion HP-21, Amberlite CG-50, DEAE Toyopearl, and Toyopearl HW-40 SF column chromatography. The structure of cyclothialidine was determined to be a unique twelve membered lactone by amino acid analysis and various 2D-NMR experiments. Cyclothialidine inhibited *Escherichia coli* DNA gyrase with an  $IC_{50}$  of 30 ng/ml.

DNA gyrase is a bacterial enzyme that introduces superherical twists into covalently closed circular DNA *in vitro*<sup>1)</sup>. It is needed for transcription, DNA replication, and cell division *in vivo*<sup>2)</sup>. Therefore, an inhibitor of this enzyme could be used as an antibacterial agent with selective toxicity against bacteria. Novobiocin and coumermycins, which have a microbial origin and synthetic quinolones are known antibacterial agents that inhibit DNA gyrase<sup>3,4)</sup>. Recently, cinodine<sup>5)</sup> and clerocidin<sup>6)</sup> were found to inhibit the bacterial DNA gyrase.

In the course of our screening program for new DNA gyrase inhibitors of microbial origin, we have discovered a novel DNA gyrase inhibitor, cyclothialidine (**1**), produced by *Streptomyces filipinensis* NR 0484. In this paper, we describe the isolation, characterization and structural elucidation of cyclothialidine. The taxonomical studies, production and biological activity are described in the preceding paper<sup>7)</sup>.

## Results

### Isolation

The isolation procedure of cyclothialidine is outlined in Fig. 2. Each fraction was monitored by the DNA gyrase inhibition assay described in the preceding paper<sup>7)</sup>.

Activated charcoal (1,100 g) was added to 110 liters of the broth filtrate. The mixture was stirred at room temperature for 30 minutes and then filtered. The carbon cake was then washed with water and suspended in 100 liters (50 liters  $\times$  2) of 50% aqueous acetone. After stirring at 60°C for 30 minutes, the mixture was filtered and the filtrate was concentrated under reduced pressure. The resulting brown syrup

Fig. 1. Structure of cyclothialidine.

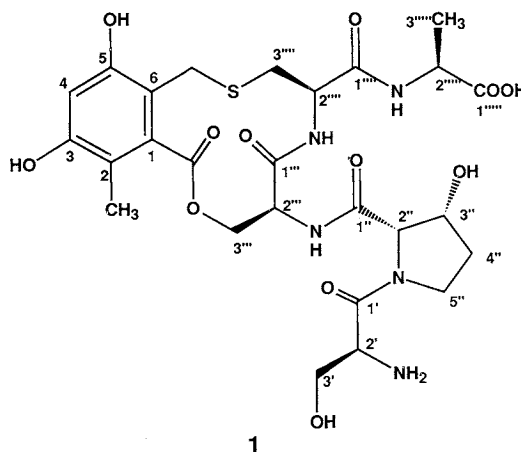


Fig. 2. Isolation procedure for cyclothialidine.

Broth filtrate (110 liters)
activated charcoal adsorption eluted with 50% aq acetone
Brown syrup (247.5 g)
1) Diaion HP-21 eluted with 10% aq EtOH
2) lyophilized
Yellow powder (78.6 g)
1) Amberlite CG-50 (1 part NH <sub>4</sub> <sup>+</sup> , 5 parts H <sup>+</sup> ) eluted with water
Active fraction
DEAE Toyopearl (Cl <sup>-</sup> form, coarse) eluted with 20 mM aq NaCl
Active fraction
1) Diaion HP-21 eluted with 50% aq acetone
2) washed with <i>n</i> -BuOH
3) lyophilized
Crude powder (303 mg)
1) Toyopearl HW-40 (super fine) eluted with water
2) lyophilized
Cyclothialidine (76 mg)

Table 1. Physico-chemical properties of cyclothialidine.

Appearance	Pale yellow amorphous powder
Molecular weight	641
Molecular formula	C <sub>26</sub> H <sub>35</sub> N <sub>5</sub> O <sub>12</sub> S
HRFAB-MS ( <i>m/z</i> )	
Calcd:	642.2081 for C <sub>26</sub> H <sub>36</sub> N <sub>5</sub> O <sub>12</sub> S
Found:	642.2064 (M+H) <sup>+</sup>
UV λ <sub>max</sub> <sup>H<sub>2</sub>O</sup> nm (log ε)	293 (3.20)
λ <sub>max</sub> <sup>H<sub>2</sub>O+NaOH</sup> nm (log ε)	309 (3.30)
[α] <sub>D</sub>	13.2° ( <i>c</i> 1.0, H <sub>2</sub> O)
IR ν <sub>max</sub> <sup>KBr</sup> cm <sup>-1</sup>	3350 (NH, OH), 1720 (ester C=O), 1640 (amido C=O)
Rf <sup>a</sup>	0.17
Color reaction	Ninhydrin (+), FeCl <sub>3</sub> (+)

<sup>a</sup> TLC: Silica gel TLC plate F<sub>254</sub> Art. 5715 (MERCCK); Solvent system: *n*-BuOH - AcOH - H<sub>2</sub>O = 4 : 1 : 2.

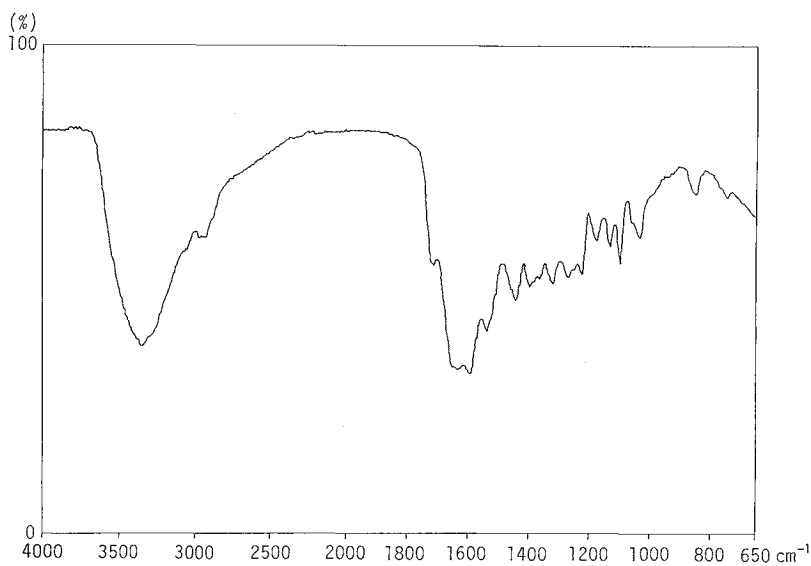
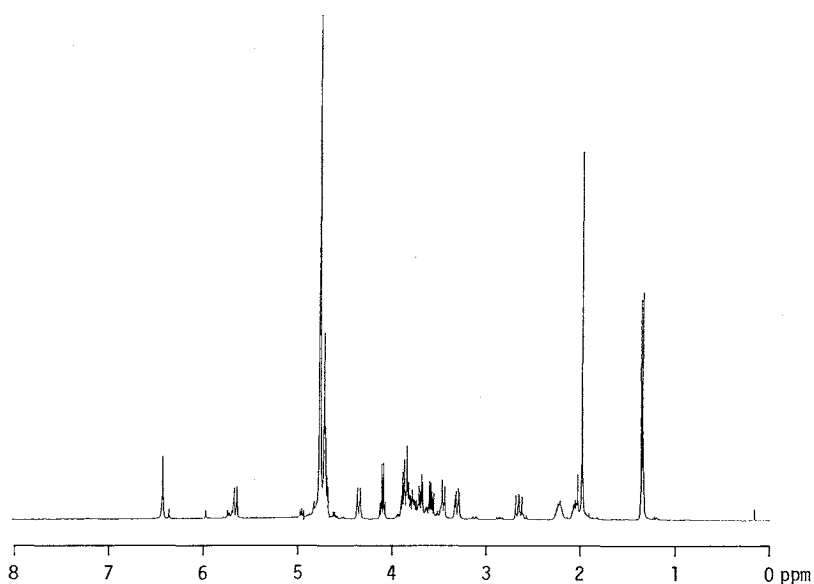
(247.5 g) was dissolved in 500 ml of water and put on a column (10 liters) of Diaion HP-21 (Mitsubishi Chemical Industries, Tokyo) which was then eluted successively with water (20 liters) and 10% aqueous EtOH (50 liters). The active fractions eluted with 10% aqueous EtOH were pooled. The pooled sample was concentrated under reduced pressure and lyophilized to give 78.6 g of a yellow powder. This lyophilized powder was then dissolved in 180 ml of water and put on an Amberlite CG-50 column (3.6

liters of a mixed bed consisting of one part of ammonium form and five parts of H<sup>+</sup> form, type 1). The active fractions (1.8 liters) eluted with water were combined and put on a column (2.5 liters) of DEAE Toyopearl (Cl<sup>-</sup> form), coarse type (Tosoh, Tokyo), which was first eluted with water (12 liters) and then with 0.02 M aqueous NaCl (80 liters). The fractions eluted with 0.02 M aqueous NaCl were monitored by HPLC. The active fractions were combined (30 liters) and put on a column (2.5 liters) of Diaion HP-21, and then successively eluted with water (5 liters) and with 50% aqueous acetone (15 liters). The active fractions eluted with 50% aqueous acetone were combined (10 liters) and concentrated to 100 ml under reduced pressure and partitioned between water and *n*-BuOH. The aqueous layer was concentrated under reduced pressure and lyophilized to give crude cyclothialidine as a pale yellow powder. This material (303 mg) was dissolved in water (3 ml) and applied onto a column (1.5 liters) of Toyopearl HW-40 SF (Tosoh, Tokyo), and eluted with water. Lyophilization of the active fractions gave 76 mg of cyclothialidine as a pale yellow amorphous powder.

#### Characterization

The physico-chemical properties of cyclothialidine (1) are summarized in Table 1. Cyclothialidine is an amphoteric water soluble compound. The molecular formula (C<sub>26</sub>H<sub>35</sub>N<sub>5</sub>O<sub>12</sub>S) was determined from the HRFAB-MS data and <sup>1</sup>H and <sup>13</sup>C NMR spectral data. The characteristic UV absorption maxima (λ<sub>max</sub> 293 nm in neutral and acidic solutions, and λ<sub>max</sub> 309 nm in alkaline solution) and the positive color

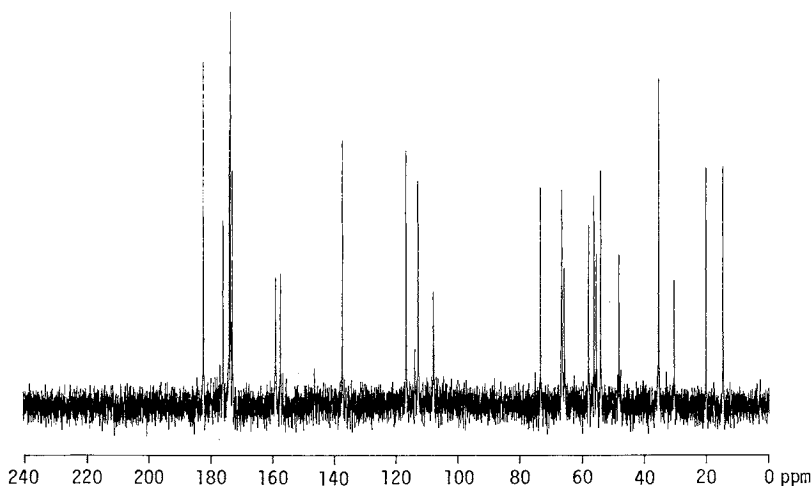
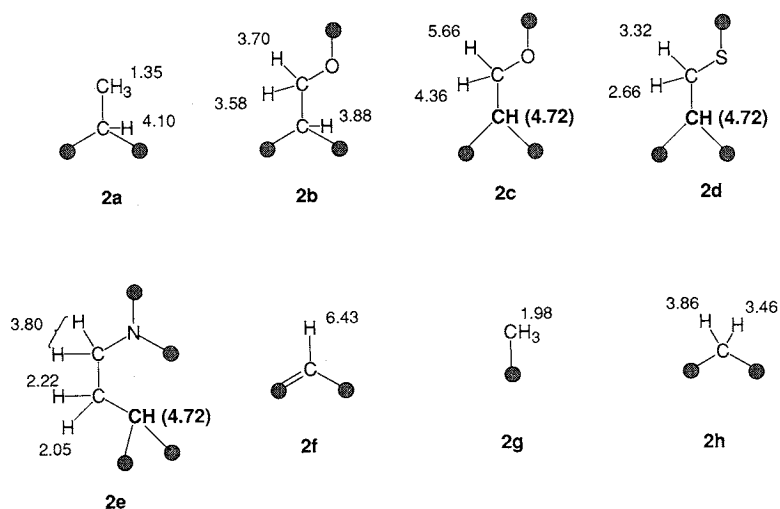
Fig. 3. IR spectrum of cyclothialidine in KBr.

Fig. 4.  $^1\text{H}$  NMR spectrum of cyclothialidine (400 MHz, in  $\text{D}_2\text{O}$ ).

reaction to ferric chloride suggested the presence of phenolic hydroxy group(s) in the structure. The IR spectrum of **1** (Fig. 3) showed an intense amide carbonyl band at  $1640\text{ cm}^{-1}$  and an ester carbonyl band at  $1720\text{ cm}^{-1}$ .  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra indicated that **1** is a peptide (Figs. 4 and 5).

#### Structure Elucidation

The structure elucidation of **1** was mainly carried out with various NMR experiments.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data in  $\text{D}_2\text{O}$  are shown in Table 2. Partial structures (**2a**~**2h**) were elucidated from the analyses of the  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR,  $^1\text{H}$ - $^1\text{H}$  COSY, and  $^{13}\text{C}$ - $^1\text{H}$  COSY experiments on **1** (Fig. 6). It was

Fig. 5.  $^{13}\text{C}$  NMR spectrum of cyclothialidine (100 MHz, in  $\text{D}_2\text{O}$ ).Fig. 6. Partial structures deduced from  $^1\text{H}$ - $^1\text{H}$  COSY and  $^{13}\text{C}$ - $^1\text{H}$  COSY experiments.

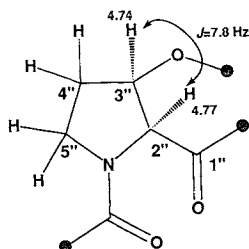
apparent that partial structures (2a ~ 2e) would be derived from amino acids and others from a chromophore. As four methine signals were overlapped at 4.72 ppm in the  $^1\text{H}$  NMR, however, we could not clarify the remaining connectivities. Thus, we carried out acid hydrolysis to confirm the presence of the constituent amino acids of 1.

#### Amino Acid Composition

The amino acid analysis of the hydrolyzate of 1 (6 N HCl,  $115^\circ\text{C}$ , 16 hours) indicated the presence of 2 mol of serine, 1 mol each of alanine and cysteine, and an unusual amino acid. This unusual amino acid was thought to be 3-hydroxyproline, based on the analysis of 1 by  $^1\text{H}$ - $^1\text{H}$  COSY (in  $\text{D}_2\text{O}$ ) and  $\text{D}_2\text{O} + \text{CF}_3\text{COOD}$ ) and  $^1\text{H}$ - $^{13}\text{C}$  COSY (in  $\text{D}_2\text{O}$ ) experiments (Fig. 7, Table 2). The large coupling constant

of 2''-H ( $J_{2'',3''}=7.8$  Hz) of the 3-hydroxyproline moiety suggested a *cis* configuration<sup>8</sup>). The presence of

Fig. 7. Partial structure of the unusual amino acid moiety of cyclothialidine.



*cis*-3-hydroxyproline was further confirmed by the amino acid analysis; the chromatogram of the hydrolyzate was compared with that of an authentic specimen which was prepared according to the method reported by MORITA *et al.*<sup>9</sup>). The absolute configurations of all amino acids in **1** were determined to be L when the chromatogram of the hydrolyzate was compared with that of authentic optically active amino acids under the same HPLC conditions<sup>†</sup>.

Table 2. <sup>1</sup>H and <sup>13</sup>C NMR chemical shifts and coupling constants for cyclothialidine.

	Position No.	In D <sub>2</sub> O		In D <sub>2</sub> O + <i>d</i> -TFA
		$\delta_C$	$\delta_H^a$	$\delta_H^a$
Chromophore moiety	1	137.5	—	—
	2	117.0	—	—
	3	159.2	—	—
	4	108.0	6.43 (1H, s)	6.52 (1H, s)
	5	157.6	—	—
	6	113.0	—	—
	2-CH <sub>3</sub>	14.7	1.98 (3H, s)	1.98 (3H, s)
	6-CH <sub>2</sub>	30.4	3.86 (1H, d, 10.7), 3.46 (1H, d, 10.7)	3.86 (1H, d, 10.7), 3.42 (1H, d, 10.7)
1-C=O	173.1	—	—	
Serine-I moiety	3	65.8	3.70 (1H, dd, 11.5, 5.5), 3.58 (1H, dd, 11.5, 6.5)	3.98 (1H, dd, 11.5, 5.5), 3.85 (1H, dd, 11.5, 6.5)
	2	56.3	3.88 (1H, dd, 6.5, 5.5)	4.41 (1H, dd, 6.5, 5.5)
<i>cis</i> -3-Hydroxyproline moiety	1	173.4 <sup>b</sup>	—	—
	5	48.2	3.80 (2H, m)	3.75 (2H, t, 7)
	4	35.3	2.22 (1H, m), 2.05 (1H, m)	2.20 (1H, m), 2.04 (1H, m)
	3	73.4	4.72 (1H, m)	4.74 (1H, dt, 7.8, 5.5)
	2	66.6	4.72 (1H, m)	4.77 (1H, d, 7.8)
	1	176.1	—	—
Serine-II moiety	3	66.6	5.66 (1H, dd, 12.0, 2.4), 4.36 (1H, dd, 12.0, 2.2)	5.63 (1H, dd, 12.0, 2.5), 4.30 (1H, dd, 12.0, 2.5)
	2	58.0	4.72 (1H, m)	4.63 (1H, t, 2.5)
Cysteine moiety	1	174.1 <sup>b</sup>	—	—
	3	35.3	3.32 (1H, dd, 15.0, 4.6), 2.66 (1H, dd, 15.0, 11.5)	3.28 (1H, dd, 15.0, 4.6), 2.60 (1H, dd, 15.0, 11.5)
	2	55.5	4.72 (1H, m)	4.63 (1H, dd, 11.5, 4.6)
Alanine moiety	1	173.4 <sup>b</sup>	—	—
	2	54.1	4.10 (1H, q, 7.3)	4.29 (1H, q, 7.3)
	2-CH <sub>3</sub>	20.2	1.35 (3H, d, 7.3)	1.40 (3H, d, 7.3)
	1	182.5	—	—

<sup>a</sup> The *J* values are in parenthesis (Hz).

<sup>b</sup> Interchangeable.

<sup>†</sup> The HPLC analyses were carried out under the following conditions: column; YMC R-ODS-5-A 4.6mm i.d. × 250mm, flow rate; 0.7ml/minute, mobile phase; 1.0mM Cu(OAc)<sub>2</sub> + 2.0mM *N,N*-dipropyl-L-alanine (pH 6.2), detection; UV 230nm<sup>10</sup>).

### The Structure of the Chromophore of Cyclothialidine

The partial structures (**2f**~**2h** in Fig. 6) were connected by  $^{13}\text{C}$ - $^1\text{H}$  long range couplings obtained by the HMBC experiment in  $\text{D}_2\text{O}$ .  $^{13}\text{C}$ - $^1\text{H}$  long range couplings were observed between the 6- $\text{CH}_2$  protons ( $\delta_{\text{H}}$  3.46 and 3.86, **2h** in Fig. 6) and the three aromatic quaternary carbons, C-6 ( $\delta_{\text{C}}$  113.0), C-1 ( $\delta_{\text{C}}$  137.5), and C-5 ( $\delta_{\text{C}}$  157.6), and also between the 2- $\text{CH}_3$  protons ( $\delta_{\text{H}}$  1.98, **2g** in Fig. 6) and the three aromatic quaternary carbons, C-2 ( $\delta_{\text{C}}$  117.0), C-1 ( $\delta_{\text{C}}$  137.5), and C-3 ( $\delta_{\text{C}}$  159.2). The remaining aromatic methine (**2f** in Fig. 6) is coupled to C-3 and C-5. Furthermore, the  $^{13}\text{C}$ - $^1\text{H}$  long range coupling between 3''''-H ( $\delta_{\text{H}}$  2.66 and 3.32) of the cysteine moiety and 6- $\text{CH}_2$  carbon ( $\delta_{\text{C}}$  30.4) indicated that the cysteine moiety forms a thioether bond to the benzylic methylene carbon. Thus, the partial structure **3a** was established, as shown in Fig. 8. This structure was corroborated by mild acid hydrolysis of **1** (1 N HCl,  $100^\circ\text{C}$ , 48 hours) which gave 4,6-dihydroxy-7-methyl-1-(3*H*)-isobenzofuranone (**3b**), isolated from the hydrolyzate by extraction with ethyl acetate.

### Amino Acid Sequence

In general, amino acid sequences have been determined by HMBC experiments using  $^{13}\text{C}$ - $^1\text{H}$  long range couplings either between the  $\alpha$ -methine proton of the amino acid and the carbonyl carbon of the adjacent amino acid, or between the amide proton of the amino acid and the carbonyl carbon of the adjacent amino acid. However, both approaches were unsuccessful in the case of **1**, because 1) the signals of the  $\alpha$ -methine protons of cysteine, the *cis*-3-hydroxyprolines, and the serine-II moieties all overlap at 4.72 ppm in  $\text{D}_2\text{O}$  and, 2) the  $^1\text{H}$  NMR spectrum of **1** in  $\text{DMSO}-d_6$  is so complicated that definite assignment of each amide proton could not be made. Cyclothialidine exists as a 2:1 equilibrium mixture of two conformers in  $\text{DMSO}-d_6$ .

Among the derivatives of **1** prepared for HMBC experiments, the *tert*-butoxycarbonyl (Boc) derivative **4**, obtained by the treatment of **1** with 2-Boc-thio-4,6-dimethylpyridine/triethylamine in aqueous MeOH, existed as a single conformer in  $\text{DMSO}-d_6$ . The  $^1\text{H}$  NMR spectrum indicated that the *tert*-butoxycarbonyl group of **4** is attached to the amino group of the serine-I moiety, since the signal of the  $\alpha$ -methine proton of this serine moiety showed a downfield shift to 4.30 ppm as a result of derivatization, compared to the 3.88 ppm resonance for this proton in cyclothialidine. The signal of two phenolic hydroxy groups was observed at 9.68 ppm. All amide protons and carbonyl carbons of **4** could be assigned by the  $^1\text{H}$ - $^1\text{H}$  COSY and HMBC experiments (Fig. 9). Thus, the sequence of amino acids in **4** was determined by the HMBC experiments:  $^{13}\text{C}$ - $^1\text{H}$  long range couplings were observed between the NH proton ( $\delta_{\text{H}}$  7.30) of the alanine moiety and the carbonyl carbon ( $\delta_{\text{C}}$  168.3) of the cysteine moiety, between the NH proton ( $\delta_{\text{H}}$  8.30) of the cysteine moiety and the carbonyl carbon ( $\delta_{\text{C}}$  168.6) of the serine-II moiety, and between the NH proton ( $\delta_{\text{H}}$  8.77) of the serine-II moiety and the carbonyl

Fig. 8. Partial structure of the chromophore moiety of cyclothialidine.

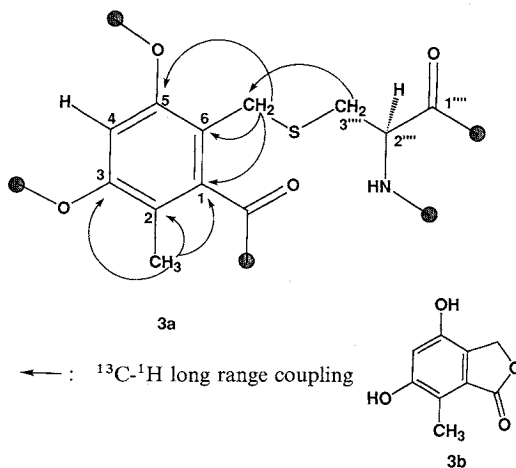
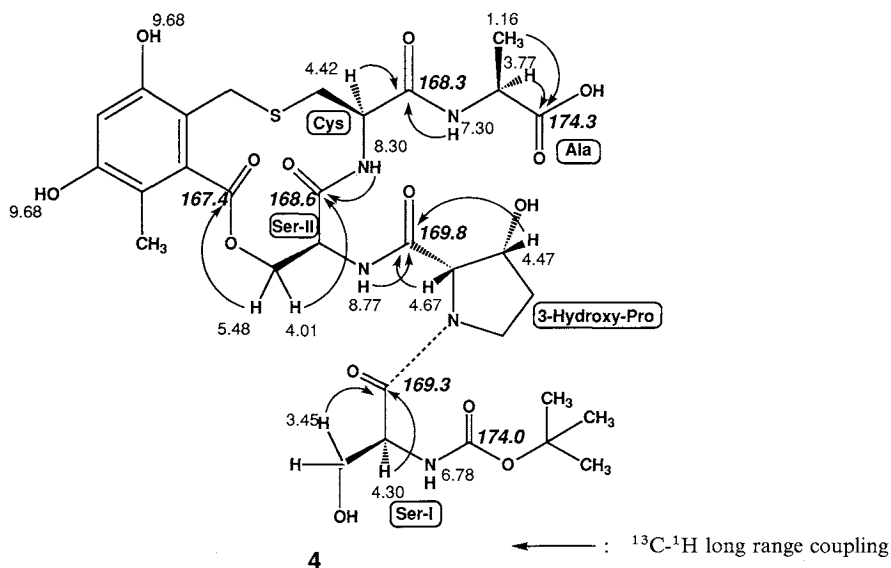


Fig. 9.  $^{13}\text{C}$ - $^1\text{H}$  long range coupling of the Boc-derivative of cyclothialidine (partial, in  $\text{DMSO}-d_6$ ).

carbon ( $\delta_{\text{C}}$  169.8) of the *cis*-3-hydroxyproline moiety (Fig. 9). Thus, the sequence of the four amino acids was established as Ala-Cys-Ser(II)-3-Hydroxypro. Additionally, the hydroxyl group of the serine-II moiety was shown to form a lactone with the carbonyl group of the chromophore:  $^{13}\text{C}$ - $^1\text{H}$  long range couplings were observed between the  $\beta$ - $\text{CH}_2$  proton (Ha) of the serine-II moiety ( $\delta_{\text{H}}$  5.48) and the 1-C=O of the chromophore moiety ( $\delta_{\text{C}}$  167.4), and between the  $\beta$ - $\text{CH}_2$  proton (Hb) of the serine-II moiety ( $\delta_{\text{H}}$  4.01) and the carbonyl carbon of the serine-II moiety ( $\delta_{\text{C}}$  168.6). These results are shown in the partial structure of **4** (Fig. 9). However, no direct evidence for the connectivity of the remaining serine-I moiety could be obtained from the analysis of the HMBC experiments. When the  $^1\text{H}$  NMR spectrum of **1** was taken in  $\text{D}_2\text{O}$  and  $\text{CF}_3\text{COOD}$ , only the methine proton of the serine-I moiety showed a large down field shift from 3.88 to 4.41 ppm due to protonation of the free amino group; whereas the signals of other amino acid moieties retained at almost the same chemical shifts. This indicates that the nitrogen atom of the *cis*-3-hydroxyproline must be an amide nitrogen. Therefore, we concluded that the serine-I moiety is bound to *cis*-3-hydroxyproline *via* an amide bond. The hydroxy groups of the *cis*-3-hydroxyproline and serine-I moieties and the carboxyl group of the alanine moiety must be in their free form to be consistent with the empirical formula. The molecular formula of cyclothialidine was thus determined to be **1** as shown in Fig. 1. The structure of cyclothialidine was confirmed by the total synthesis of this compound<sup>11)</sup>. All physico-chemical properties of cyclothialidine were identical with those of the synthetic sample.

### Discussion

Cyclothialidine is a new DNA gyrase inhibitor isolated from the culture broth of *Streptomyces filipinensis* NR 0484. The structure of **1** was found to belong to a new class of natural products containing a 12-membered lactone ring, which is partly integrated into a pentapeptide chain, and is completely different from those of the known DNA gyrase inhibitors. Cyclothialidine showed potent inhibitory activity against DNA gyrase and, therefore, could be a lead structure for new antibacterial agents.

## Experimental

### General

UV spectra were recorded on a Kontron Uvikon 860 UV spectrometer, and IR spectra on a Hitachi 260-10 IR spectrometer. FAB- and EI-MS were measured on a JEOL JMS-DX-300/system 3500 mass spectrometer.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra recorded on a JEOL JNM-GSX-400 NMR spectrometer at 400 and 100 MHz, respectively, using TMS or sodium 3-(trimethylsilyl)-propionate- $d_4$  (in  $\text{D}_2\text{O}$ ) as an internal standard. The various 2D-NMR techniques utilized were  $^1\text{H}$ - $^1\text{H}$  COSY,  $^1\text{H}$ - $^{13}\text{C}$  COSY and HMBC. Carbon multiplicities were determined by DEPT experiments. Optical rotation was measured on a Jasco FIP-140 digital polarimeter. Amino acid analysis was carried out on a Kontron Chromakon 500 automatic amino acid analyzer.

### Acid Hydrolysis of Cyclothialidine

A) A solution of cyclothialidine (10 mg) in 1 N hydrochloric acid (10 ml) was refluxed for 48 hours. After cooling, the reaction mixture was diluted with water (10 ml) and extracted with ethyl acetate (20 ml  $\times$  3). The combined extract was concentrated under reduced pressure. The yellow residue was purified by a sep-pack silica gel short column (Millipore) using  $\text{CHCl}_3$ -MeOH (10:1) as an eluent. Two mg of 4,6-dihydroxy-7-methyl-1(3*H*)-isobenzofuranone (**3b**) was obtained as an amorphous powder: HREI-MS ( $m/z$ ), Calcd for  $\text{C}_9\text{H}_8\text{O}_4$  180.042, Found 180.042 ( $\text{M}^+$ ); UV  $\lambda_{\text{max}}$  (MeOH) 251, 320 nm,  $\lambda_{\text{max}}$  (MeOH +  $\text{OH}^-$ ) 227, 295, 342 nm; IR  $\nu_{\text{max}}$  (KBr) 3400 (OH), 1720 (ester C=O)  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$  9.72 (2H, br s, OH  $\times$  2), 6.65 (1H, s, arom-H), 5.07 (2H, s, benzylic  $\text{CH}_2$ ), 2.26 (3H, s, arom- $\text{CH}_3$ );  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  174 (ester C=O), 159 and 152 (arom-C-OH  $\times$  2), 127, 126 and 117 (arom-C  $\times$  3), 109 (arom-CH), 68 (benzylic  $\text{CH}_2$ ), 9 ( $\text{CH}_3$ ).

B) A solution of cyclothialidine (2.7 mg) in freshly prepared performic acid (2 ml) was stirred at  $0^\circ\text{C}$  for 4 hours. After excess performic acid was decomposed with 48% hydrobromic acid (0.8 ml), the reaction mixture was evaporated to dryness under reduced pressure. The brown residue was dissolved in 20% hydrochloric acid (1.4 ml) and heated in a sealed tube at  $115^\circ\text{C}$  for 16 hours. The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in water (0.5 ml) and subjected to amino acid analysis.

### Preparation of *tert*-Boc Derivative (**4**)

Triethylamine (17 ml) and 2-Boc-thio-4,6-dimethylpyridine (11 mg) were added to a solution of cyclothialidine (26 mg) in a mixed solution of water (0.2 ml) and MeOH (0.9 ml). The mixture was stirred at room temperature for 8.5 hours. The reaction was monitored by silica gel TLC, which was developed with 80% *n*-propanol. After removal of the solvent under reduced pressure, the residue was dissolved in water (5 ml) and washed with methylene chloride (5 ml  $\times$  3). The aqueous solution was concentrated under reduced pressure and purified by preparative HPLC using a YMC R-ODS-5-A column with 70% aqueous MeOH. The *tert*-Boc derivative **4**, 15 mg, was obtained after removal of the solvent under reduced pressure: FAB-MS  $m/z$  740 ( $\text{M} + \text{H}^+$ );  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ ) alanine moiety:  $\delta$  1.16 (3H, d,  $J=7$  Hz,  $\beta$ - $\text{CH}_3$ ), 3.77 (1H, dq,  $J_1=J_2=7$  Hz,  $\alpha$ -CH), 7.30 (1H, d,  $J=7$  Hz, NH), cysteine moiety: 3.10 (1H, dd,  $J_1=15$ ,  $J_2=4$  Hz,  $\beta$ - $\text{CH}_2$  (Ha)), 3.45 (1H,  $\beta$ - $\text{CH}_2$  (Hb)), 4.42 (1H, m,  $\alpha$ -CH), 8.30 (1H, d,  $J=9$  Hz, NH), serine-I moiety: 3.45 (1H,  $\beta$ - $\text{CH}_2$  (Ha)), 3.56 (1H, dd,  $J_1=11$ ,  $J_2=5.5$  Hz,  $\beta$ - $\text{CH}_2$  (Hb)), 4.30 (1H, m,  $\alpha$ -CH), 6.78 (1H, d,  $J=7.5$  Hz, NH), 1.36 (9H, s, Boc), serine-II moiety: 4.01 (1H, dd,  $J_1=12$ ,  $J_2=2.5$  Hz,  $\beta$ - $\text{CH}_2$  (Hb)), 4.52 (1H, m,  $\alpha$ -CH), 5.48 (1H, dd,  $J_1=12$ ,  $J_2=2.5$  Hz,  $\beta$ - $\text{CH}_2$  (Ha)), 8.77 (1H, d,  $J=7$  Hz, NH), *cis*-3-hydroxyproline moiety: 2.03 (2H, m,  $\gamma$ - $\text{CH}_2$ ), 3.58 (1H, m,  $\delta$ - $\text{CH}_2$  (Ha)), 3.72 (1H, m,  $\delta$ - $\text{CH}_2$  (Hb)), 4.47 (1H, m,  $\beta$ -CH), 4.67 (1H, d,  $J=7.5$  Hz,  $\alpha$ -CH), chromophore part: 1.92 (3H, s, arom- $\text{CH}_3$ ), 3.45 (1H, benzyl- $\text{CH}_2$  (Ha)), 3.81 (1H, d,  $J=11$  Hz, benzyl- $\text{CH}_2$  (Hb)), 6.48 (1H, s, arom-CH), 9.68 (2H, br s, OH  $\times$  2).

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