Cyclothialidine Analogs, Novel DNA Gyrase Inhibitors

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DNA gyrase inhibitors, cyclothialidines B, C, D and E were isolated from four Streptomycete strains (NR 0659, NR 0660, NR 0661 and NR 0662). Their structures have been elucidated based on the amino acid analysis of the hydrolysates, NMR and HRFAB-MS experiments and shown to be cyclothialidine analogs. The absolute stereochemistry has been determined by the chiral HPLC analysis of the hydrolysates. Cyclothialidines B, D and E are novel and potent inhibitors of DNA gyrase.

DNA gyrase is a bacterial enzyme that introduces superherical twists into covalently closed circular DNA *in vitro*[1]. It is needed for transcription, DNA replication, and cell division *in vivo*[2]. Therefore, an inhibitor of this enzyme could be used as an antibacterial agent with selective toxicity against bacteria. Novobiocin and coumermycins of microbial origin, and synthetic quinolones are known antibacterial agents that inhibit DNA gyrase[3,4]. Discovery of cyclothialidine (**5**)[5~9] from *Streptomyces filipinensis* NR 0484 in 1993 introduced a new class of DNA gyrase inhibitors having a unique twelve membered lactone structure. Cyclothialidine is a very potent inhibitor of the DNA gyrase B subunit ATPase activity. In the course of our continuous screening program to find more selective and potent inhibitors of DNA gyrase, we identified cyclothialidines B (**1**), C (**2**), D (**3**), and E (**4**), as shown in Fig. 1, that were isolated from four Streptomycete strains. In this paper, we report on the production, isolation, structure determination and biological activities of cyclothialidines B, C, D and E.

Results

Production

The organism producing **1** and **2**, strain NR 0659, was isolated from a soil sample collected at Mito city, Ibaraki Prefecture, Japan, and was identified as *Streptomyces* sp. based on its morphological and physiological properties and cell wall type. The stock culture of *Streptomyces* sp. NR 0659 stored at −80°C was thawed, and 200 μl of the culture was inoculated into a 500-ml baffled Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 2.0%, yeast extract 0.5%, Toast soya 2.0%, NaCl 0.25%, $ZnSO_4 \cdot 7H_2O$ 0.005%, $CuSO_4 \cdot 5H_2O$ 0.0005%, $MnCl_2 \cdot 4H_2O$ 0.0005%, Nissan disfoam CA-115 0.05%. The pH of the medium was adjusted to 7.0 with 5 N NaOH before sterilization and then autoclaved. The flask was shaken at 220 rpm on a rotary shaker at 27°C for 3 days. Then, 2 ml of the resultant vegetative inoculum was transferred into 500-ml baffled Erlenmeyer flasks containing 100 ml of the same medium as described above, followed by incubation on a rotary shaker at 27°C for 4 days at 220 rpm.

Streptomyces sp. NR 0660 isolated from a soil sample collected at Aguiha city, Bolivia also produced **2** under the same conditions described above. The organisms producing **3**, *Streptomyces* sp. NR 0661, and producing **4**, *Streptomyces* sp. NR 0662 were isolated from a soil sample collected at Mt. Kenya, Kenya and Aso city, Kumamoto Prefecture, Japan, respectively. They produced **3** and **4** individually under the same conditions described above.

Isolation

The isolation was carried out by monitoring the inhibition of DNA gyrase. The isolation procedure of cyclothialidines B (**1**) and C (**2**) is outlined in Fig. 2. **1** (23 mg) and **2** (5 mg) were isolated as white powders from the broth filtrate (90 liters) of *Streptomyces* sp. NR 0659 by activated charcoal adsorption followed by chromatography with Diaion HP-21, DEAE Toyopearl and Sephadex LH-20. Cyclothialidine C (23 mg) was also isolated from the broth filtrate (43 liters) of *Streptomyces* sp. NR 0660 according to the similar methods.

Details of the isolation procedures of cyclothialidines

Fig. 1. Structures of cyclothialidines B~E (**1**~**4**) and cyclothialidine (**5**).

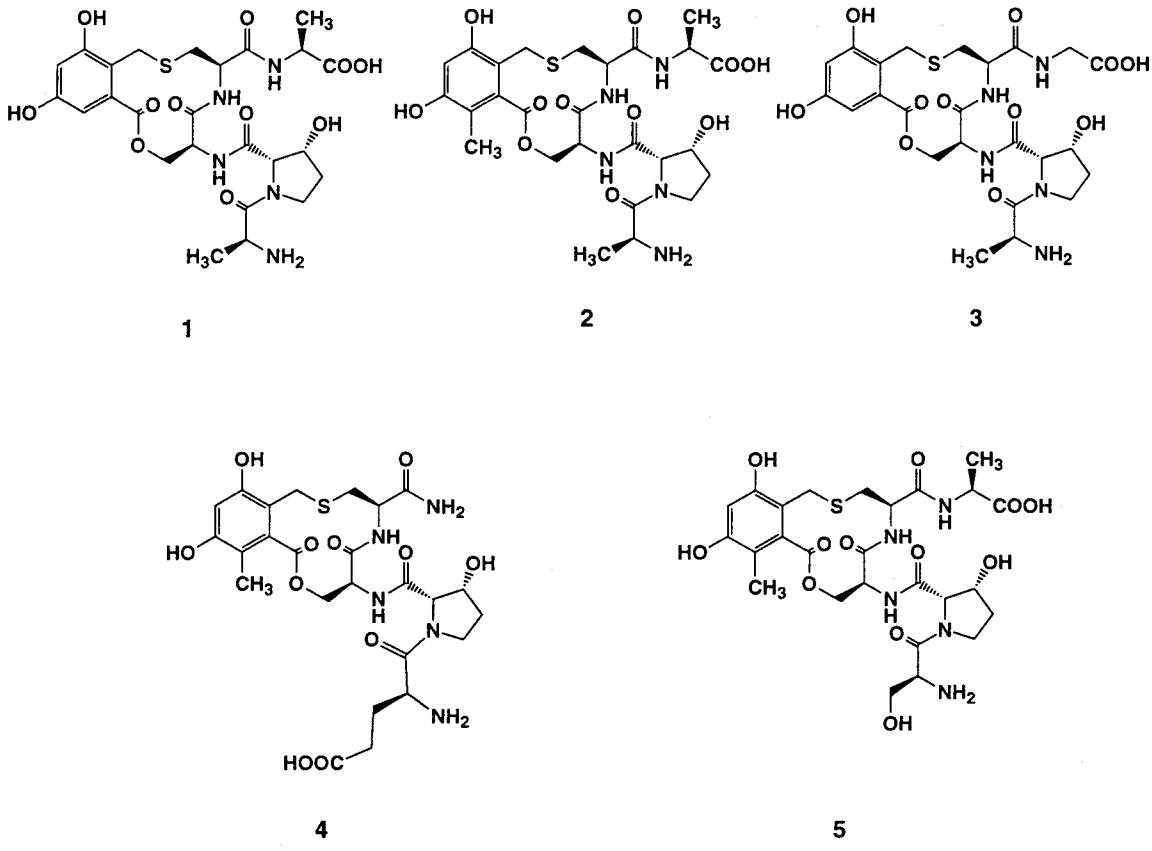


Table 1. Physico-chemical properties of cyclothialidines B~E (**1**~**4**) and cyclothialidine (**5**).

	Cyclothialidine B (**1**)	Cyclothialidine C (**2**)	Cyclothialidine D (**3**)	Cyclothialidine E (**4**)	Cyclothialidine (**5**)
Molecular weight	611	625	597	611	641
Molecular formula	$C_{25}H_{33}N_5O_{11}S$	$C_{26}H_{35}N_5O_{11}S$	$C_{24}H_{31}N_5O_{11}S$	$C_{25}H_{33}N_5O_{11}S$	$C_{26}H_{35}N_5O_{12}S$
HRFAB-MS (*m/z*)					
	$(M-H)^-$	$(M-H)^-$	$(M-H)^-$	$(M-H)^-$	$(M+H)^+$
Calcd:	610.1819	624.1976	596.1663	610.1819	642.2081
Found:	610.1845	624.1956	596.1689	610.1812	642.2064
UV $\lambda_{max}^{H_2O}$ nm (ε)	313 (2500)	294 (3200)	313 (1800)	294 (2400)	293 (1600)
	256 (sh) (3300)		258 (sh) (2300)		
$\lambda_{max}^{0.01N\ NaOH}$ nm (ε)	346 (2500)	315 (3700)	346 (1800)	315 (2500)	309 (2000)
	243 (sh) (12000)		243 (sh) (11000)		
$[\alpha]_D^{21}$	-36°	-15°	-26°	+11°	-13°
	(*c* 0.25, H_2O)	(*c* 0.25, H_2O)	(*c* 0.10, H_2O)	(*c* 0.28, H_2O)	(*c* 1.0, H_2O)
IR ν_{max}^{KBr} cm^{-1}	3290, 1735, 1650	3350, 1730, 1650	3390, 1720, 1650	3400, 1730, 1640	3350, 1720, 1640
Retention time (R_t) *	11.8 min	15.7 min	16.1 min	24.5 min	14.0 min

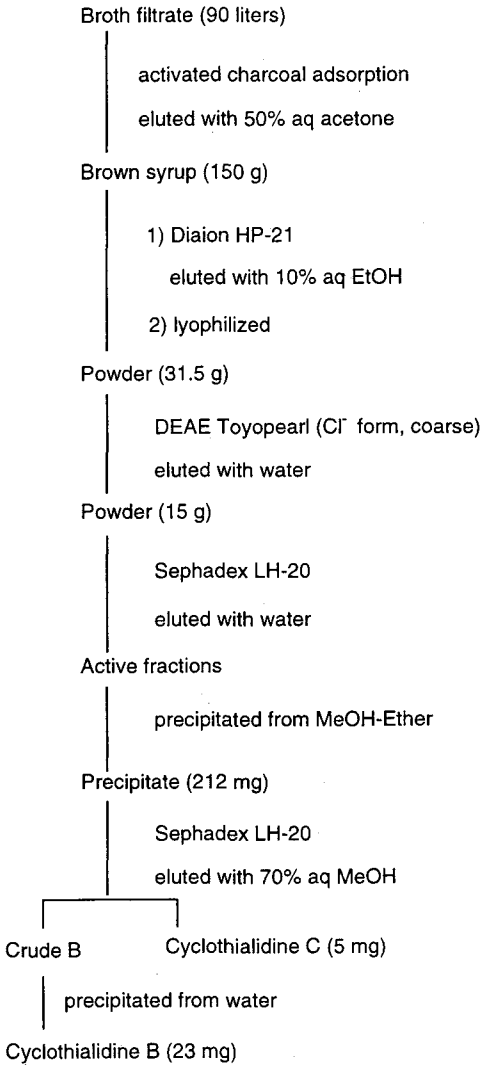
* HPLC conditions : column, YMC-pack R&D-ODS-10 (4.6 mm i.d. x 250 mm) ;
mobile phase, MeOH-0.1M NaCl = 5 : 95 ; flow rate, 3.0 ml/minute ; detection, UV 210nm

B, C, D and E are described in the experimental section.

Physico-chemical Properties

The physico-chemical properties of the cyclothialidines are summarized in Table 1. They are soluble in water, MeOH and DMSO, but insoluble in EtOAc and *n*-hexane. The positive color obtained from their reaction to $FeCl_3$ suggests the presence of a phenolic group. The IR spectra of **1**, **2**, **3**, **4** and cyclothialidine (**5**) were almost identical and suggested the presence of ester (1730 cm^{-1}) and amide (1640 cm^{-1}) functional groups. The UV spectra of **2** and **4** in water showed absorption maxima at 294 nm and the bathochromic effect was observed in 0.01 N NaOH solution. This result indicated that the same chromophore is contained in **2**, **4** and cyclothialidine[6]. Those of **1** and **3** showed absorption maxima at 243 and 346 nm, suggesting that they contain a different chromophore in their structures. The molecular formulae of **1**, **2**, **3** and **4** were determined to be $C_{25}H_{33}N_5O_{11}S$, $C_{26}H_{35}N_5O_{11}S$, $C_{24}H_{31}N_5O_{11}S$ and $C_{25}H_{33}N_5O_{11}S$, respectively, from their high resolution FAB-MS data. These molecular formulae were supported by the analyses of the ^{13}C NMR spectra summarized in Table 3. These physico-chemical properties indicated that **1**, **2**, **3** and **4** were analogs of cyclothialidine.

Fig. 2. Isolation procedure of cyclothialidines B (**1**) and C (**2**).



Amino Acid Composition

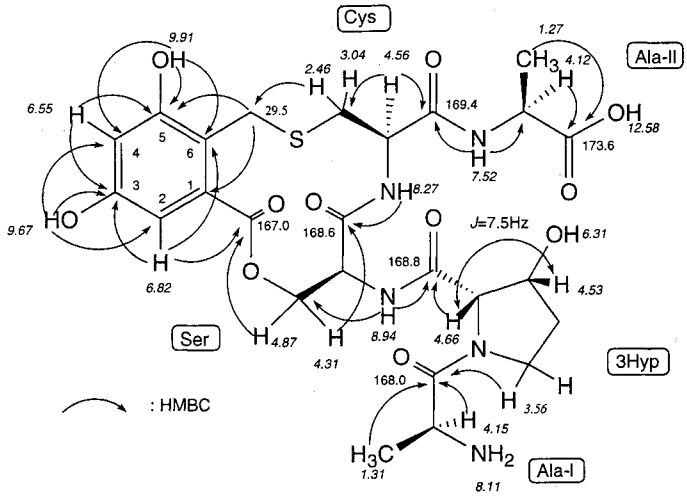
The results of amino acid analyses of the cyclothialidines are summarized in Table 2. Cyclothialidines were hydrolysed with hydrochloric acid under standard conditions for peptides. Amino acid analyses of the hydrolyzates of cyclothialidines revealed the presence of 1 mol each of serine, cysteine and 3-hydroxyproline as common residues and the presence of 2 mol of alanine both in **1** and **2**, the presence of 1 mol each of alanine and glycine in **3**, and the presence of 1 mol of glutamic acid or glutamine in **4**. The absolute configurations of all amino acids in the cyclothialidines 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. The details of analytical conditions are described in the experimental section.

Table 2. Amino acid composition of cyclothialidine analogs (**1**~**5**).

Compound	Amino acid composition				
Cyclothialidine B (**1**)	L-Ser	L-Cys	L-*cis*-3Hyp	L-Ala x 2	
Cyclothialidine C (**2**)	L-Ser	L-Cys	L-*cis*-3Hyp	L-Ala x 2	
Cyclothialidine D (**3**)	L-Ser	L-Cys	L-*cis*-3Hyp	L-Ala	Gly
Cyclothialidine E (**4**)	L-Ser	L-Cys	L-*cis*-3Hyp	L-Glx	
Cyclothialidine (**5**)	L-Ser x 2	L-Cys	L-*cis*-3Hyp	L-Ala	

L-*cis*-3Hyp : *cis*-3-hydroxy-L-proline

Fig. 3. HMBC experiments of cyclothialidine B (1) in DMSO-d_6.



Structural Elucidation

Structural Elucidation of Cyclothialidine B (1)

The amino acid analyses of hydrolysate of **1** indicated 2 mol of alanine, 1 mol each of cysteine, 3-hydroxyproline and serine. The full assignments of all protons and carbons of **1** were obtained by the analyses of the ^{1}H-^{1}H COSY, DEPT and HMBC experiments in DMSO-d_6 (Fig. 3). The presence of the 3-hydroxyproline residue was confirmed by the comparison of ^{1}H NMR spectral data of **1** with those of **5** in Table 3. The *cis* configuration of the 3-hydroxyproline residue was supported by the large coupling constant of 2″-H ($J_{2'',3''}$ = 7.5 Hz)[6,10,11] in the residue (Fig. 3) obtained by the analysis of ^{1}H NMR spectra. The sequence of amino acids in **1** was determined by the HMBC experiments: ^{13}C-^{1}H long range couplings were observed between the NH proton (δ_H 7.52) of the alanine-II residue and the carbonyl carbon (δ_C 169.4) of the cysteine residue, between the NH proton (δ_H 8.27) of the cysteine residue and the carbonyl carbon (δ_C 168.6) of the serine residue, and between the NH proton (δ_H 8.94) of the serine residue and the carbonyl carbon (δ_C 168.8) of the 3-hydroxyproline residue, and the δ-CH_2 proton (Ha) of the 3-hydroxyproline residue (δ_H 3.56) and carbonyl carbon (δ_C 168.0) of the alanine-I residue. Thus, the sequence of all amino acid residues in **1** was established as Ala(II)-Cys-Ser-3Hyp (3-hydroxyproline)-Ala(I).

The comparison of NMR spectral data revealed that aromatic methyl signals (δ_H 1.98, δ_C 14.7) for **5** were not found for **1** and an additional aromatic proton signal (δ_H 6.85) was observed for **1** (Table 3). This means that the chromophore for **1** was a demethyl one of that for cyclothialidine. The structure of the chromophore was also confirmed by the HMBC experiments of **1** as shown in Fig. 3. The linkage between this chromophore and the pentapeptide residues was determined by the analysis of the HMBC experiments of **1**. ^{13}C-^{1}H long range coupling observed between the β-CH_2 proton (Ha) of the serine residue (δ_H 4.87) and the 1-C=O of the chromophore moiety (δ_C 167.0) indicated the connection between the chromophore and serine residue through an ester bond. The ^{13}C-^{1}H long range coupling between β-CH_2 (δ_H 2.46) of the cysteine and 6-CH_2 carbon (δ_C 29.5) of the chromophore moieties indicated the connection between the cysteine residue and the benzylic methylene carbon through a thioether bond. The structure of **1** was, thus, established as shown in Fig. 3.

Structures of Cyclothialidines C (2), D (3) and E (4)

The structures of **2**, **3**, and **4** were determined by comparing their MS and ^{13}C NMR spectral data with those of **1** and cyclothialidine.

Considering **2**, ^{13}C NMR signals observed at δ_C 21.0 (CH_3), δ_C 50.6 (α-CH) and δ_C 172.3 (C=O) indicated that alanine in **2** has been substituted for serine in **5**. The remaining parts of the ^{1}H and ^{13}C NMR spectral data of **2** and **5** were practically identical (Table 3). The result of amino acid analyses and similar UV spectra of **2** and **5** supported this assignment. The difference of molecular formulae between **2** ($C_{26}H_{35}N_5O_{11}S$) and **5** ($C_{26}H_{35}N_5O_{12}S$) was ascribed to the difference of amino acid residue (alanine in **2** and serine in **5**). Thus, the structure of cyclothialidine C was determined to be **2** shown in Fig. 1 and identical with that of

Table 3-1. ^{1}H and ^{13}C NMR chemical shifts of cyclothialidines B~D (**1**~**3**) in D_2O.

		Cyclothialidine B (**1**)		Cyclothialidine C (**2**)		Cyclothialidine D (**3**)	
Moiety	Position	δC	δH (*J* in Hz)	δC	δH (*J* in Hz)	δC	δH (*J* in Hz)
Chromophore	1	134.2	-	137.3	-	134.3	-
	2	113.3	6.85 (d, 2.4)	116.5	-	113.5	6.95 (d, 2.4)
	3	158.6	-	160.5	-	158.6	-
	4	109.9	6.57 (d, 2.4)	108.5	6.37 (s)	110.2	6.68 (d, 2.4)
	5	159.1	-	158.6	-	159.1	-
	6	117.5	-	112.4	-	117.8	-
	2-CH_3	-	-	14.7	1.93 (s)	-	-
	6-CH_2	31.0	4.13 (d, 10.0)	30.5	3.80 (d, 10.8)	31.2	4.22 (d, 10.0)
			3.80 (d, 10.0)		3.42 (d, 10.8)		3.87 (d, 10.0)
	1-C=O	171.4		173.4		171.7	
cis -3Hyp	5	48.0	3.72 (m)	47.8	3.71 (m)	48.2	3.77 (m)
	4	35.8	2.21 (m)	35.5	2.17 (m)	35.7	2.28 (m)
			2.04 (m)		2.00 (m)		2.11 (m)
	3	73.5	4.73 (m)	73.4	4.67 (m)	74.0	4.79 (m)
	2	66.2	4.76 (m)	66.7	4.67 (m)	66.6	4.87 (d, 7.0)
	1	173.5		174.0		173.9	
Ser-1	3	68.2	5.05 (dd, 12.0, 2.0)	66.6	5.59 (br d, 10.0)	68.2	5.12 (dd, 12.0, 2.4)
			4.56 (dd, 12.0, 2.0)		4.32 (br d, 10.0)		4.62 (br dd, 12.0, 2.4)
	2	57.2	4.65 (t, 2.0)	58.0	4.67 (m)	57.2	4.72 (m)
	1	174.0		173.6		174.3	
Cys	3	36.3	3.26 (dd, 14.8, 4.4)	35.6	3.26 (dd, 14.8, 4.8)	36.4	3.34 (dd, 16.0, 5.6)
			2.62 (dd, 14.8, 11.2)		2.63 (dd, 14.8, 11.2)		2.72 (dd, 16.0, 12.2)
	2	56.3	4.74 (dd, 11.2, 4.4)	55.5	4.67 (m)	56.6	4.73 (m)
	1	174.2		173.7		174.3	
Ala-1	3	17.9	1.49 (d, 7.6)	21.0	1.32 (d, 7.2)	18.0	1.65 (d, 8.0)
	2	50.6	4.35 (q, 7.6)	50.0	3.81 (q, 7.2)	50.7	4.42 (q, 8.0)
	1	172.3		178.3		172.6	
Ala-2	3	19.0	1.40 (d, 7.6)	20.2	1.23 (d, 7.2)	-	
	2	51.5	4.31 (q, 7.6)	54.1	4.08 (q, 7.2)	-	
	1	178.7		182.5		-	
Gly	2	-		-		46.3	3.81 (d, 18.0)
							3.74 (d, 18.0)
	1	-		-		179.1	
Glu	5	-		-		-	
	4	-		-		-	
	3	-		-		-	
	2	-		-		-	
	1	-		-		-	
Ser-2	3	-		-		-	
	2	-		-		-	
	1	-		-		-	

GR122222X[12~14].

Considering **3**, ^{13}C NMR signals observed at δ_C 46.3 (α-CH_2), δ_C 179.1 (C=O) indicated that glycine in **3** has been substituted for alanine in **1**. The remaining parts of the ^{1}H and ^{13}C NMR spectral data of **1** and **3** were practically identical (Table 3). To confirm which alanine residue at the *N*-terminal or *C*-terminal was replaced by the glycine residue, ^{13}C-^{1}H long range couplings obtained by HMBC experiment in D_2O were utilized. ^{13}C-^{1}H long range coupling obtained between α-CH_2 protons of the glycine residue (δ_H 3.74, δ_H 3.81) and the carbonyl carbon (δ_C 174.3) of the cysteine residue

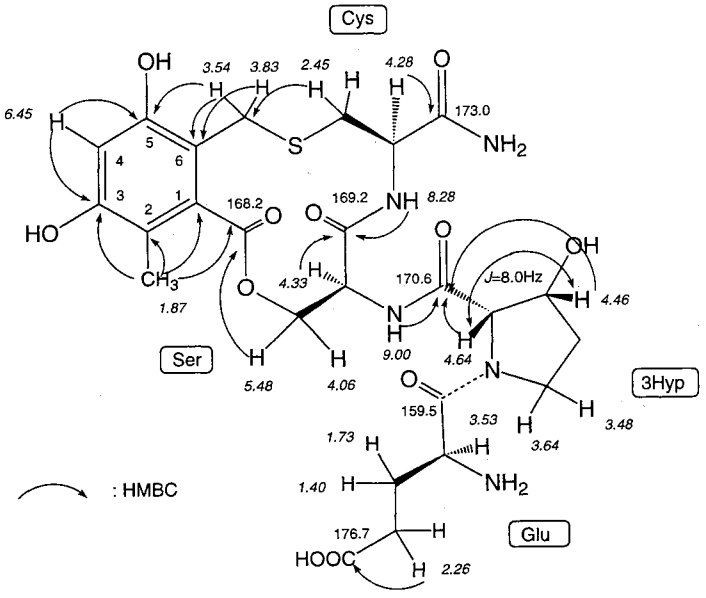
Table 3-2. ^{1}H and ^{13}C NMR chemical shifts of cyclothialidine E (**4**) and cyclothialidine (**5**) in D_2O.

		Cyclothialidine E (**4**)		Cyclothialidine (**5**)	
Moiety	Position	δC	δH (*J* in Hz)	δC	δH (*J* in Hz)
Chromophore	1	137.4	-	137.5	-
	2	117.5	-	117.0	-
	3	157.5	-	159.2	-
	4	107.6	6.50 (s)	108.0	6.43 (s)
	5	156.4	-	157.6	-
	6	114.0	-	113.0	-
	2-CH_3	14.5	1.97 (s)	14.7	1.98 (s)
	6-CH_2	29.2	3.84 (d, 10.5)	30.4	3.86 (d, 10.7)
			3.36 (d, 10.5)		3.46 (d, 10.7)
	1-C=O	172.7		173.1	
cis -3Hyp	5	48.2	3.77 (m)	48.2	3.80 (m)
	4	35.6	2.20 (m)	35.3	2.22 (m)
			2.03 (m)		2.05 (m)
	3	73.5	4.69 (m)	73.4	4.72 (m)
	2	67.1	4.69 (m)	66.6	4.72 (m)
	1	174.1		176.1	
Ser-1	3	66.4	5.60 (dd, 12.4, 3.0)	66.6	5.66 (dd, 12.0, 2.4)
			4.35 (dd, 12.4, 3.0)		4.36 (dd, 12.0, 2.2)
	2	58.1	4.62 (br t, 3.0)	58.0	4.72 (m)
	1	173.8		174.1	
Cys	3	35.1	3.33 (dd, 14.0, 6.0)	35.3	3.32 (dd, 15.0, 4.6)
			2.63 (dd, 14.0, 11.0)		2.66 (dd, 15.0, 11.5)
	2	54.7	4.56 (dd, 11.0, 6.0)	55.5	4.72 (m)
	1	177.7		173.4	
Ala-1	3	-		-	
	2	-		-	
	1	-		-	
Ala-2	3	-		20.2	1.35 (d, 7.3)
	2	-		54.1	4.10 (q, 7.3)
	1	-		182.5	
Gly	2	-		-	
	1	-		-	
Glu	5	183.6		-	
	4	35.8	2.20 (m)	-	
	3	30.4	1.80 (m)	-	
	2	54.3	4.10 (m)	-	
	1	174.1		-	
Ser-2	3	-		65.8	3.70 (dd, 11.5, 5.5)
	2	-		56.3	3.58 (dd, 11.5, 6.5)
	1	-		173.4	3.88 (dd, 6.5, 5.5)

indicated the linkage between the glycine and the cysteine residues. Thus, the structure of cyclothialidine D was determined to be **3** as shown in Fig. 1.

The amino acid analysis of the hydrolysate of **4** indicated the presence of 1 mol each of serine, cysteine, 3-hydroxyproline and glutamic acid or glutamine. The structure of **4** was determined by analyses of ^{1}H-^{1}H COSY, HSQC, HMBC, HOHAHA experiments. The sequence of the three amino acids was established as Cys-Ser-3Hyp and linkage between chromophore and peptides were determined by HMBC experiments (Fig. 4). However, no direct evidence for the connectivity of

Fig. 4. HMBC experiments of cyclothialidine E (**4**) in DMSO-d_6.



the remaining Glx residue could be obtained from the analyses of the HMBC experiments. When the ^{1}H NMR spectrum of **4** was taken in D_2O and CF_3COOD, the chemical shifts of α-CH and γ-CH_2 of the glutamic acid or glutamine residue showed large down field shifts from δ_H 4.10 to δ_H 4.40 and from δ_H 2.20 to δ_H 2.55, respectively, while the signals of other amino acid residues didn't change. This means that the nitrogen atoms of the 3-hydroxyproline residue must be amide nitrogen and *C*-terminal of cysteine residue must be protected by an amino group, and α-amino and γ-carboxyl groups of Glu must be free. Therefore, we concluded that the Glu residue is bound to *cis*-3-hydroxyproline *via* an amide bond. The structure of **4** was, thus, established as shown in Fig. 4. Furthermore, the structure of cyclothialidine E (**4**) was confirmed by the total synthesis of the compound (unpublished data).

Biological Activity

The inhibitory activities of the cyclothialidines against *E. coli* DNA gyrase are shown in Table 4. Cyclothialidine analogs inhibit *E. coli* DNA gyrase with IC_{100}s ranging between 0.3 μM and 1.0 μM. Cyclothialidines were found to be ones of the most potent inhibitors of known gyrase inhibitors tested.

Table 4. Inhibition of *E. coli* DNA gyrase.

Compound	IC_{100} (μM)
Cyclothialidine B (**1**)	0.7
Cyclothialidine C (**2**)	0.3
Cyclothialidine D (**3**)	0.5
Cyclothialidine E (**4**)	1.0
Cyclothialidine (**5**)	0.3
Novobiocin	1.2
Coumermycin A_1	1.8

Discussion

Cyclothialidines were produced by many Streptomycete strains isolated from soil samples collected at the different places in the world.

In spite of its potent enzyme inhibition, cyclothialidine only showed weak *in vitro* antibacterial activity against *Eubacterium* species.[7] It was suggested that poor penetration through the cytoplasmic membrane might be one of the reasons for cyclothialidine's lack of antibacterial activity. The *in vitro* antibacterial testing has identified congeners of cyclothialidine exhibiting broad-spectrum antibacterial activity against Gram-positive bacteria[15].

The structural studies revealed that cyclothialidines shared the same units of L-cysteine, L-serine and *cis*-3-hydroxy-L-proline, and **2**, **4** and cyclothialidine had the same chromophore, while **1** and **3** had the same chromophore that differed from cyclothialidine. Cyclothialidines show potent inhibitory activity against

E. coli DNA gyrase with IC_{100}s ranging between 0.3 μM and 1.0 μM. Replacement of an amino acid residue at the *N*-terminal from serine in **5** to alanine in **1**, **2**, and **3**, and glutamic acid in **4** didn't affect their inhibitory activities. Replacement of an amino acid residue at the *C*-terminal from alanine in **5** to glycine in **3** and just to an amide in **4** didn't affect it either. This meant that the core part of the cyclothialidine, 12-membered ring created from the aromatic chromophore, and the cysteine and serine moieties, must be essential for their inhibitory activities. GÖTSCHI *et al.*[15] studied the structure-activity relationship on synthetic analogs of cyclothialidine. They reported that the phenolic hydroxy group at position 5 in the aromatic chromophore is also essential for the activity.

The cyclothialidine analogs in this study could provide useful information for the synthesis of new antibacterial agents.

Experimental

General Procedures

UV and IR spectra were recorded on a SHIMAZU UV-2200 spectrometer and on a PERKIN ELMER 1600 series FTIR infrared spectrometer, respectively. Fast atom bombardment mass spectra (FAB-MS) were obtained with JEOL-HX-110 spectrometers using *m*-nitrobenzyl alcohol as a matrix. Optical rotations were measured on a JASCO DIP-140 digital polarimeter. ^1H and ^{13}C NMR spectra were recorded on a JEOL-GSX-400 NMR spectrometer.

Isolation of **1** and **2**

Activated charcoal (900 g) was added to 90 liters of the broth filtrate (*Streptomyces* sp. NR 0659). The mixture was stirred at room temperature for 30 minutes and then filtered. The carbon cake was suspended in 50 liters of 50% aqueous acetone. After stirring for 30 minutes, the mixture was filtered and the filtrate was concentrated under reduced pressure. The resulting brown syrup (150 g) was dissolved in 4 liters of water and applied to a column (8 liters) of Diaion HP-21 (Mitsubishi Chemical Industries, Tokyo) which was successively washed with water (24 liters) and eluted with 10% aqueous EtOH (40 liters). The active fractions were combined and concentrated under reduced pressure and lyophilized to give 31.5 g of crude powder. This powder was then dissolved in 200 ml of water and applied to a column (1 liter) of DEAE Toyopearl (Cl^- form), coarse type (Tosoh, Tokyo), which was then washed with water and eluted with water. The active fractions were combined and concentrated. The resulting powder (15 g) was dissolved in 100 ml of water and applied to a column (4.5 liters) of Sephadex LH-20 (Pharmacia, Sweden) and eluted with water. Combined active fractions were concentrated under reduced pressure and lyophilized to give 1.8 g of cyclothialidine complex and then precipitated from MeOH-Ether. The precipitates (212 mg) were dissolved in 5 ml of water, and applied to a column (1 liter) of Sephadex LH-20 and eluted with 70% aqueous MeOH. Active fractions were combined and concentrated under reduced pressure to give crude **1** (49 mg) and **2** (5 mg). Then crude **1** was precipitated from water to give **1** (23 mg).

Isolation of **3**

18 liters of the broth filtrate (*Streptomyces* sp. NR 0661) was applied to a Diaion HP-21 column (5.6 liters), washed with water (18 liters) and eluted with 10% aqueous EtOH (14 liters). The active fractions were combined and concentrated under reduced pressure to give 18 g of the syrup. The brown syrup was dissolved in 40 ml of water, applied to a Sephadex LH-20 column (4.5 liters) and eluted with water. The active fractions were combined, concentrated under reduced pressure and lyophilized to give 660 mg of crude powder. This powder was dissolved in 500 ml of water, applied to a DEAE Toyopearl column (100 ml), and eluted with water. The active fractions were collected and concentrated under reduced pressure to give a syrup. The syrup was separated by preparative HPLC in a C_{18} reversed-phase silica gel column (YMC pack R&D-ODS-10, 10 mm i.d. × 250 mm, YMC, Kyoto) with 0.1 M NaCl - MeOH (95 : 5). The active fractions were concentrated under reduced pressure to give a syrup, and the syrup was applied to a Diaion HP-21 column (50 ml), washed with water, and eluted with 10% aqueous EtOH. The active fractions were concentrated under reduced pressure and lyophilized to give a yellow powder. Then the powder was dissolved in 1 ml of water, applied to a DEAE Toyopearl column (80 ml) and eluted with water. The active fractions were collected and lyophilized to give pure **3** (3 mg).

Isolation of **4**

Activated charcoal (1.5 kg) was added to 100 liters of the broth filtrate (*Streptomyces* sp. NR 0662), and the mixture was stirred and then filtered. The carbon cake was extracted with 36 liters of 50% aqueous acetone. The extract was concentrated to give a brown residue

(282 g). The residue was washed with MeOH (2.4 liters) and dried to give 206 g of a brown powder. The powder was divided in half and run in 2 batches on the same Sephadex LH-20 column (5 liters) and eluted with water. The active fractions were collected and concentrated to give 23.8 g of powder. This powder was dissolved in water and rechromatographed in a Sephadex LH-20 column (5 liters). The active fractions were combined and concentrated to give 2.4 g of crude **4**. This powder was dissolved in water (2.2 liters), applied to a DEAE Toyopearl column (1.5 liters), washed with water, and eluted with 10 mM NaCl. The active fractions were combined (1 liter), applied to a Diaion HP-21 column (200 ml), successively washed with water and eluted with 10% aqueous EtOH. The active fractions were concentrated under reduced pressure to give 24 mg of powder. This powder was dissolved in water, applied to a Sephadex LH-20 column (30 ml), and eluted with water. The active fractions were collected and lyophilized to give **4** (5 mg).

Analysis of Amino Acids

The amino acid analysis was done according to the method of CHANG *et al.*[16,17]. Each compound (200 pmol) was completely hydrolyzed with 6 N HCl vapor for 24 hours. The hydrolysates were reacted with dimethylaminoazobenzensulfonyl chloride (DABSYL), to form DABSYL-amino acids. The amino acid analysis was carried out under the following conditions: column, Capcell pak C_{18} (4.6 mm i.d. × 250 mm, Shiseido, Tokyo); flow rate, 1.2 ml/minute; mobile phase, solvent A: 25 mM NaOAc (pH 6.5) containing 4% (v/v) of dimethylformamide, solvent B: 100% CH_3CN; detection, UV 436 nm. The gradient curve was modified to separate 3Hyp and other amino acids, using the synthetic 3Hyp as an authentic sample. Gradient curve I: The gradient was kept at 27% solvent B/0~20 minutes, 27~48% solvent B/20~26 minutes, 48~90% solvent B/26~29 minutes, kept at 90% solvent B/29~30 minutes and then 90~27% solvent B/30~31 minutes. On this HPLC analysis, Glu, Ser, 3Hyp, Ala and Cys were eluted at 3.8, 9.3, 12.0, 12.9 and 29.1 minutes, respectively. Gradient curve II: The gradient was kept at 20% solvent B/0~65 minutes, 20~48% solvent B/65~70 minutes, kept at 48% solvent B/70~77 minutes, 48~64% solvent B/77~79 minutes, 64~90% solvent B/79~80 minutes, kept at 90% solvent B/80~81 minutes and then 90~20% solvent B/81~82 minutes. On this HPLC analysis, Ser, Gly, 3Hyp, Ala and Cys were eluted at 42.5, 58.6, 59.6, 62.7 and 74.1 minutes, respectively.

Absolute Configuration of the Amino Acids

Each compound was completely hydrolyzed in the same manner as previously reported[6]. The chiral HPLC analysis was carried out as follows[6,18]. Conditions I for Ser, Cys, *cis*-3Hyp, and Ala are: two columns of YMC-Pack R&D ODS (4.6 mm i.d. × 250 mm); flow rate, 0.7 ml/minute; mobile phase, 1.0 mM cupric acetate + 2.0 mM *N*,*N*-di-*n*-propyl-L-alanine (pH 6.2); detection, UV 230 nm. For this HPLC analysis, D-Ser and L-Ser were eluted at 23.7 and 28.0 minutes, D-Cys(O_3H) and L-Cys(O_3H) were eluted at 30.3 and 32.7 minutes, *cis*-3-hydroxy-D-proline and *cis*-3-hydroxy-L-proline were eluted at 27.8 and 41.0 minutes, D-Ala and L-Ala were eluted at 25.6 and 36.2 minutes, respectively. Conditions II for Glu are: two columns of TSK-gel Enantio L1 (4.6 mm i.d. × 250 mm, Tosoh, Tokyo); mobile phase, 1.0 mM $CuSO_4$ (pH 5.3); flow rate, 0.5 ml/minute; detection, UV 254 nm for Glu. For these conditions, D-Glu and L-Glu were eluted at 44.9 and 46.8 minutes respectively.

DNA Supercoiling Assay[7]

Supercoiled pUC18 plasmid, purified by caesium chloride density gradient centrifugation[19], was relaxed with calf thymus topoisomerase I (LIFE TECHNOLOGIES INC. MD) in 50 mM Tris (pH 7.6), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA, 30 μg/ml BSA, 0.2 μg/ml RNAse for 1 hour at 37°C. The relaxed topoisomerase was purified by a phenol/chloroform extraction and an ethanol precipitation. *E. coli* DNA gyrase A and B proteins were purified separately by Heparin-Sepharose (Pharmacia) column chromatography and FPLC Mono Q HR 5/5 (Pharmacia) column chromatography[20]. 20 μl of the reaction mixture contained 40 mM Tris-HCl (pH 8.0), 25 mM KCl, 4 mM $MgCl_2$, 2.5 mM spermidine·HCl, 50 μg of bovine serum albumin per ml, 2 mM dithiothreitol, 1.4 mM ATP, 0.1 μg of relaxed pUC18 DNA, and DNA gyrase. The mixture was incubated for 30 minutes at 37°C. The production of supercoiled pUC18 DNA was monitored by agarose gel electrophoresis. The IC_{100} was defined as the minimum inhibitory concentration at which the supercoiling band of DNA completely disappeared on the agarose gel.

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