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An RNA polymerase inhibitor, cyclothiazomycin B1, and its isomer

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Abstract—Novel cyclic thiopeptides, cyclothiazomycins B1 (**1**) and B2 (**2**), were isolated from *Streptomyces* sp. A307 as potent hyphal swelling inducing substances. They are stable in the solid state but slowly isomerize with one another in solution. Degradation experiments and spectroscopic analyses disclosed that they comprise unique tricyclic structures each containing a dehydroalanine, and two dehydrohomoalanine residues, along with three thiazolines, three thiazoles, and a trisubstituted pyridine. Cyclothiazomycin B1 (**1**) is expected to be a powerful tool for DNA–RNA transcription studies, because this cyclopeptide inhibits DNA-dependent RNA synthesis by bacteriophage RNA polymerases.

1. Introduction

Since Waksman discovered streptomycin in 1944,[1] a tremendous number of structurally distinct antibiotics have been isolated from *Streptomyces* spp. Of these, the thiopeptide antibiotics have attracted considerable attention in recent times,[2] because this family exhibits various biological properties such as inhibitions of ribosomal protein synthesis,[3] and elongation factor Tu,[4] renin activity,[5] and promotion of *TipA*.[6] Herein, we report the isolation of an RNA polymerase inhibitor cyclothiazomycin B1 (**1**) and its stereoisomer cyclothiazomycin B2 (**2**) from *Streptomyces* sp. A307. The ESI-MS provided the molecular weight to be 1527. The number of exchangeable protons was established by the increased mass number in the ESI-MS spectrum measured in the presence of D_2O. Since the high resolution MS spectrum was not sufficient to unambiguously establish the molecular formula, it was estimated by combined analysis with subsequent NMR studies and from the structures of the hydrolysates. The ^{1}H and ^{13}C NMR spectroscopic analysis of **1** detected 53 non-exchangeable protons and 60 carbons. Consideration of the original amino acids suggested one more carbon as well as the number of other heteroatoms, including nitrogen, oxygen, and sulfur to establish the molecular formula to be $C_{61}H_{69}N_{21}O_{13}S_7$ which is in good accordance with that obtained by HIESI-MS. The sequence of the amino acid residues was examined by field gradient (FG)HMBC and phase sensitive ROESY, and was further confirmed by MALDI-TOF/TOF. The Marfey's method revealed L-configurations for the arginine, proline, and cysteine residues but the absolute configuration of the aspartic acid residue could not be determined. The detailed ^{1}H NMR spectral comparison between those of **1** and **2** disclosed that the latter is an (*E*)-isomer of the dehydrohomoalanine moiety adjacent to the proline residue. These studies led to the conclusion that these compounds are arginine analogues of cyclothiazomycin (**3**), a thiopeptide antibiotic isolated by Aoki et al. in 1991.[5,7,8] The subsequent biological investigation disclosed that pure samples of these cyclic thiopeptides induced a potent hyphal swelling against *Cochliobolus*

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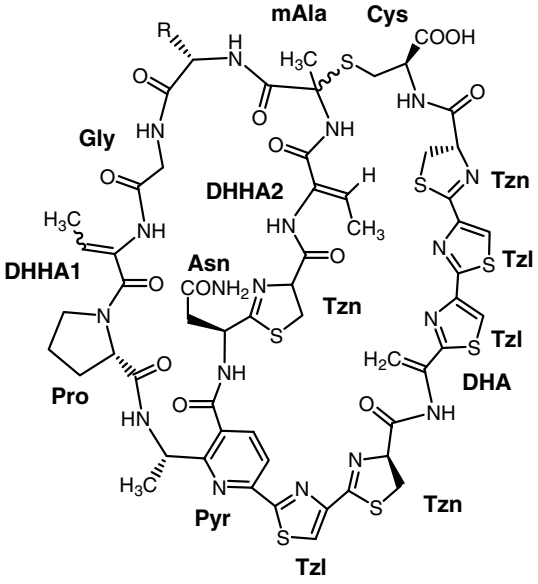
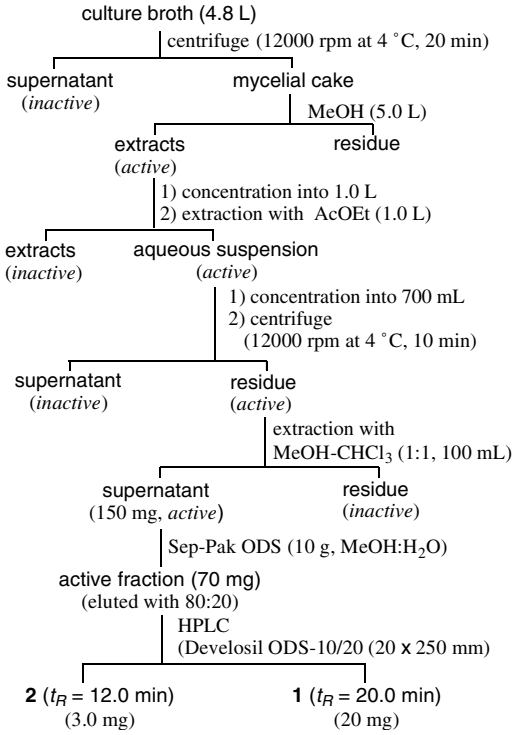
miyabeanus[9] (both IC_{50} = 0.5 μg/mL) which are more effective than the commercial agrochemical polyoxin,[10] and showed potent antifungal activities against several plant pathogenic fungi. Notably, **1** showed inhibitory activity for DNA-dependent RNA synthesis by bacteriophage RNA polymerase. Several transcription inhibitors, such as rifampicin for bacterial RNA polymerase[11] and α-amanitin for eukaryotic RNA polymerase,[12] have greatly contributed to the recent progress of molecular biology as well as cell biology. In contrast, there are only a few inhibitors for bacteriophage RNA polymerase, which have a simplified structure comprised of only a single polypeptide chain. Thus, **1** might be a new type of inhibitor for bacteriophage RNA polymerase and is expected to serve as a good tool for clarifying the molecular basis of DNA-dependent RNA synthesis. In this paper, we describe in detail our isolation, structure determination, and biological studies of these antibiotics.



2. Results and discussion

2.1. Isolation

Streptomyces sp. A307 was cultured with Fortimicin medium[13] on a rotary shaker at 27 °C for 12 days (Scheme 1). The mycelial cake obtained after centrifuge was extracted with methanol. This extract remarkably induced hyphal swelling of *Cochliobolus miyabeanus*. Concentration under reduced pressure resulted in an aqueous suspension. After removing hydrophobic inactive materials by washing the suspension with ethyl acetate, the active precipitate was collected by centrifuge. It was found that the active substance could be dissolved



Scheme 1. Isolations of **1** and **2**.

in a 1:1 mixture of $CHCl_3$ and methanol. Filtration followed by concentration afforded the crude material (150 mg).

Chromatographic purification of the resulting crude material was difficult because of its poor solubility in most solvent systems. Fortunately, it was subsequently found that aqueous pyridine can dissolve the material well. Thus, reverse-phase chromatography could be performed by loading the sample on ODS Sep-Pak® as an aqueous pyridine solution. After pyridine was washed off with water, stepwise elution of the loaded material was performed with methanol–water. Further purification by preparative HPLC provided two active fractions at t_R 12.0 and 20.0 min. These fractions were immediately concentrated in vacuo below 15 °C until the total volume was reduced to one-third. The resulting aqueous solution was lyophilized to provide cyclothiazomycins B2 (**2**, 3.0 mg) and B1 (**1**, 20.0 mg) as amorphous solids. Here it was noted the immediate concentration at low temperature was critical for obtaining pure samples. Standing these fractions at room temperature for a couple of hours resulted in considerable decomposition which involved isomerization between **1** and **2**, as discussed later.

2.2. Physical properties and degradation products

Physical properties of **1** and **2** are summarized in Table 1. The ESI-MS spectrum of **1** displayed the pseudo-molecular ion signal ($[M+H]^+$) at *m*/*z* 1528 along with a doubly charged molecular ion ($[M+2H]^{2+}$) at *m*/*z* 765. Thus, the molecular weight was established to be 1527, which was further confirmed by observing the adduct

Table 1. Physical properties of **1** and **2**

	Cyclothiazomycin B1	Cyclothiazomycin B2
ESI-MS		
With $CH_3CN–H_2O$	*m/z* 1528 $[M+H]^+$	*m/z* 1528 $[M+H]^+$
With $CH_3CN–H_2O$–NaCl	*m/z* 1550 $[M+Na]^+$	nd
With $CH_3CN–D_2O$	*m/z* 1545 $[M'+D]^+$	*m/z* 1545 $[M+D]^+$
HRESI-MS	*m/z* 1528.3448 ($[M+H]^+C_{61}H_{69}N_{21}O_{13}S_7$)	nd
UV (PDA)	271, 300, 315	271, 300, 315
IR (KBr)	3400, 1675, 1516 cm^{-1}	3400, 1675, 1516 cm^{-1}
Ninhydrin test	Positive	Positive
Constituent amino acids	Gly × 1, L-Arg × 1, L-Pro × 1, Asp × 1 (= Asn × 1), (Cys, not quantitative)	Nd
Soluble solvents	Pyridine/H_2O (3:1), DMF, $CHCl_3$/MeOH (1:1) AcOH[a], DMSO[a]	Pyridine/H_2O (3:1)[b] DMSO[a]

nd, not determined; [a]gradually decomposed; [b]isomerized, but very slowly.

ion ($[M+Na]^+$) at 1550 by injecting the sample with sodium chloride. The HRESI-MS spectrum provided the ion at *m/z* 1528.3448, but this was not sufficient to unambiguously deduce the molecular formula at this stage because of many candidates.

The molecular formula $C_{61}H_{69}N_{21}O_{13}S_7$ was established after much experimentation. The ESI-MS spectrum in $CH_3CN–D_2O$ provided the pseudo-molecular ion $[M'+D]^+$ at *m/z* 1545 which indicated the weight of M′ to be 1543 and disclosed the existence of 16 exchangeable protons in the molecule.

The ninhydrin test suggested the existence of amino acid residues. Acidic hydrolysis, followed by dansylation, revealed Arg, Asp, Gly, and Pro residues in almost equal intensities. This analysis also suggested the presence of Cys residue, but its intensity varied in experiments probably due to its instability. The Cys residue was observed as a dimmer, bis(*N*-dansyl)cysteine, based on the observation of the pseudo-molecular ion signal at *m/z* 707 in the ESI-MS. Later, we established that the aspartic acid was derived from asparagine residue in **1** (vide infra). The Marfey's protocol revealed the absolute configurations of some of these amino acid residues as L-Arg, L-Pro, and L-Cys, which will be discussed later in Section 3.3.

Acidic hydrolysis also provided two chromophores **4** and **5** (Fig. 1). The ESI-MS of **4** indicated a pseudo-molecular ion signal at *m/z* 254. The FGHMQC and FGHMBC spectra suggested that **4** is saramycetic acid,[14] however, the ^1H NMR spectral data of this sample in DMSO-d_6 were not consistent with those reported (max $\Delta\delta$ 0.47) as shown in Table 2, so it was converted into the methyl ester **6**. The ^1H NMR spectrum of **6** was in good accordance with those in the literature[15] The acetyl group in **4** was assumed to arise from the hydrolysis of the 2-(1-aminovinyl)thiazole moiety in **1**.

The molecular weight of another hydrolysate **5** was established to be 293 by observing the pseudo-molecular ion signal at *m/z* 294 $(M+H)^+$ in the ESI-MS. The FGHMQC and FGHMBC spectra of **5** suggested a dicarboxylic acid as depicted below. In order to study the substitution pattern, **5** was also converted into the corresponding dimethyl ester.[16] The obtained **7** provided sufficient FGHMBC cross peaks. Irradiation of the singlet at δ 3.99 (3′-CO_2Me) induced NOEs at C4′-H and C1″-H methine signals. Enhancement of C5-H signal was observed when another OMe (δ 3.97) group was irradiated. These experiments suggested that **1** and **2** are polypeptides containing several thiazoles and also implied substructures **I** and **II** in **1**.

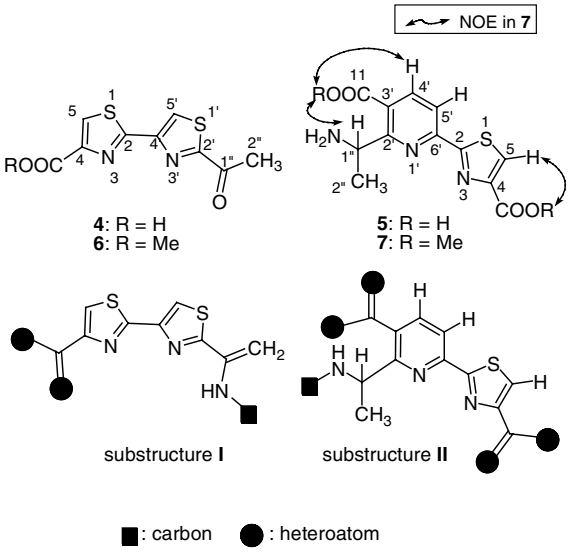


Figure 1. Structures of **4**–**7** and the proposed substructures.

3. Structural study of 1

3.1. NMR study

The NMR measurements of cyclothiazomycin B1 (**1**) were initially hampered by the poor solubility in common NMR solvents, such as deuterated methanol, chloroform, acetonitrile, and water. Although modest solubility was attained in $CDCl_3/CD_3OD$ (1:1), it was still not sufficient for the ^{13}C NMR spectra. Deuterated acetic acid and dimethylsulfoxide dissolved **1** well and provided ^1H NMR spectra in good resolution. However, the use of these solvents was limited to the ^1H NMR (1D, COSY, and ROESY) measurements because these slowly decomposed **1**. We found that aqueous pyridine

Table 2. The NMR spectral data of degradation products **4**, **5**, and **7**

4 (in dimethylsulfoxide-d_6)				**5** (in CD_3OD)				**7** (in CD_3OD)			
Position	^{13}C	^{1}H (Lit[12])	HMBC	Position	^{13}C	^{1}H	HMBC	Position	^{13}C	^{1}H	HMBC
2	165.10			2-	169.45		5	2	169.46		5, 5′
4	157.61		5	4	150.74		5	4	149.86		5
4-*C*O_2-	160.30		5	4-*C*O_2-	164.45			4-*C*O_2-	163.55		5,4-CO_2*Me*
5	124.23	8.06 (1H, s) *8.53 (1H, s)*		5	133.01	8.57 (1H, s)		5	133.29	8.62 (1H, s)	
2′	167.30		5′, 2″	2′	160.27		4′	2′	160.20		4′, 1″, 2″
4′	150.26		5′	3′	127.15		5′	3′	126.51		5′
5′	125.45	8.91 (1H, s) *7.75 (1H, s)*		3′-*C*O_2-	167.77		4′	4′	143.26	8.60 (1H, d, 8.2)	
1″	191.43		2″	4′	143.55	8.62 (1H, d, 8.3)		3′-*C*O_2-	166.72		4′,3′-CO_2Me
2″	26.28	2.67 (3H, s) *2.71 (3H, s)*		5′	121.22	8.39 (1H, d, 8.3)		5″	121.23	8.39 (1H, d, 8.2)	5′
				6′	153.88		4′	6′	154.01		4′, 5′
				1″	50.88	5.57 (1H, q, 6.6)	2″	1″	50.84	5.51 (1H, q, 6.8)	2″
				2″	20.74	1.65 (3H, d, 6.6)	1′	2″	20.67	1.67 (3H, d, 6.8)	1″
								4-CO_2*Me*	53.51	3.97 (3H, s)	
								3′-CO_2Me	54.00	3.99 (3H, s)	

(pyridine-d_5/D_2O 3:1) dissolved **1** well without substantial decomposition, providing ^{1}H NMR spectra with sufficient resolution as shown in Figure 2. Thus, this solvent system was predominantly adopted for structural studies. A mixture of pyridine-d_5 and light water was also employed for studying the exchangeable protons. However, higher concentration conditions resulted in serious signal broadening probably due to intermolecular hydrogen bondings, which brought difficulties in obtaining FGHMBC spectra in sufficient quality.

The final assignments are summarized in Figure 3. The HOHAHA and COSY experiments revealed 13 proton spin systems involving 53 non-exchangeable protons, these are highlighted in bold in the structure. Accounting for the 16 exchangeable protons suggested by MS experiments, the number of hydrogens in **1** was estimated to be 69.

The proton decoupled ^{13}C NMR spectrum displayed 59 signals. The FGHMQC spectrum revealed one more resonance at δ 57.27 which evidently showed a FGHMQC correlation with the proton signal at δ 5.33 as shown in Figure 4. Combined analysis of the DEPT and FGHMQC experiments succeeded in distinguishing the ^{13}C resonances into 4 methyl, 13 methylene, 15 methine, and 28 quaternary carbons. Actually **1** involves 61 carbons (vide infra), and one carbon resonance is missing in these assignments.

The degradation experiments have predicted Arg, Asx, Gly, Pro, and Cys units. Almost all NMR signals including amide protons and carbon signals for these amino acid units in **1** were successfully assigned by COSY, FGHMQC, and FGHMBC experiments. The carbonyl carbons for Pro and Cys could not be assigned by FGHMBC despite several experiments with different mixing times.[17] The signal being assigned as the α-carbonyl carbon (δ 177.66) of Asx was masked in the form of a thiazoline ring (Tzn1 for convenience) based on correlations with the methylene protons (δ 3.62 and 3.75) and methine proton (δ 5.40) in the FGHMBC spectrum. The COSY spectrum displayed two more sets of similar ABX systems. None of these protons showed a long range spin coupling with any amide proton in the COSY spectrum. The FGHMBC spectrum proved these constitute the other thiazoline rings Tzn2 and Tzn3 in a similar manner.

The ^{1}H NMR spectrum displayed three sets of doublet methyl resonances at δ 1.93, 2.02, and 2.14. These were coupled with methine protons at δ 6.08, 6.68, and 6.42, respectively. The methine at δ 6.08 was attached to the pyridine ring, since a quaternary carbon (δ 162.71) in the pyridine ring showed the FGHMBC correlations with both this methine proton and an aromatic doublet at δ 8.23. The other two methine protons (δ 6.68 and 6.42) were assigned to dehydrohomoalanines DHHA1 and DHHA2. For example, DHHA1 was established by (i) the COSY spectrum suggested a long range coupling between the methyne (δ 6.68) and an amide proton at δ 10.48, respectively, (ii) the FGHMQC and FGHMBC spectra displayed correlations δ 6.68 (H) ↔ δ 127.11 (C) and δ 6.68 (H) ↔ δ 131.99. (C), respectively. The other dehydrohomoalanine DHHA2 was assigned in a similar manner.

The singlet signals at δ 8.38, 8.73, and 9.08 belonged to the thiazole rings (Tzl1, Tzl2, and Tzl3, respectively) based on the hydrolysates **4** and **5** in the preceding experiments. The quaternary carbons in these rings could be assigned by the FGHMBC spectrum. The AB doublets at δ 8.11 and 8.23 were due to the pyridine ring. The FGHMBC spectrum revealed all of the other pyridine carbon resonances (δ 151.97, 132.81, and 162.71) and the attached carbonyl carbon (δ 170.42).

The degradation experiments have predicted the presence of a dehydroalanine unit (DHA) as described, which was verified by the FGHMQC and FGHMBC experiments (δ 5.80 and 6.85 for *exo*-methylene protons, δ 134.23 and 104.98 for the α- and β-carbons, respective-

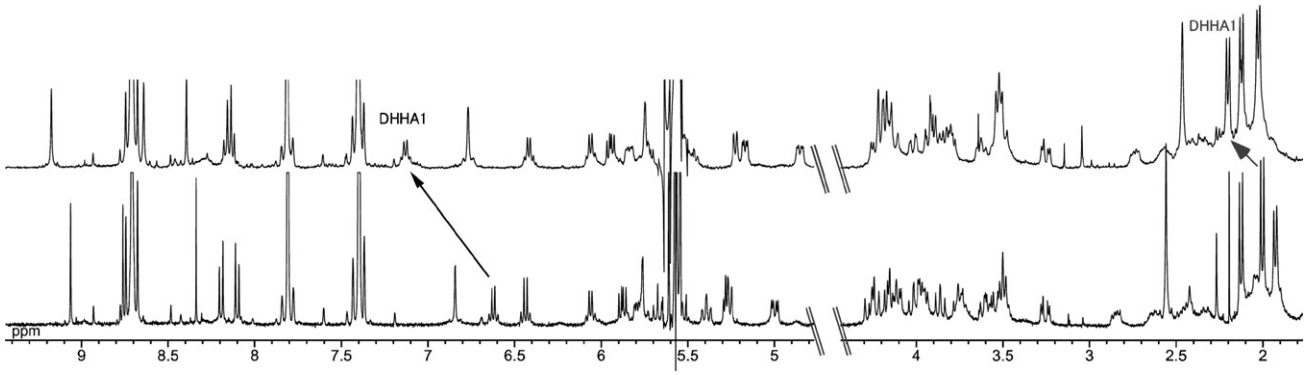


Figure 2. ^{1}H NMR spectra of cyclothiazomycin B1 (**1**, lower) and B2 (**2**, upper) in 3:1 pyridine-d_5/D_2O.

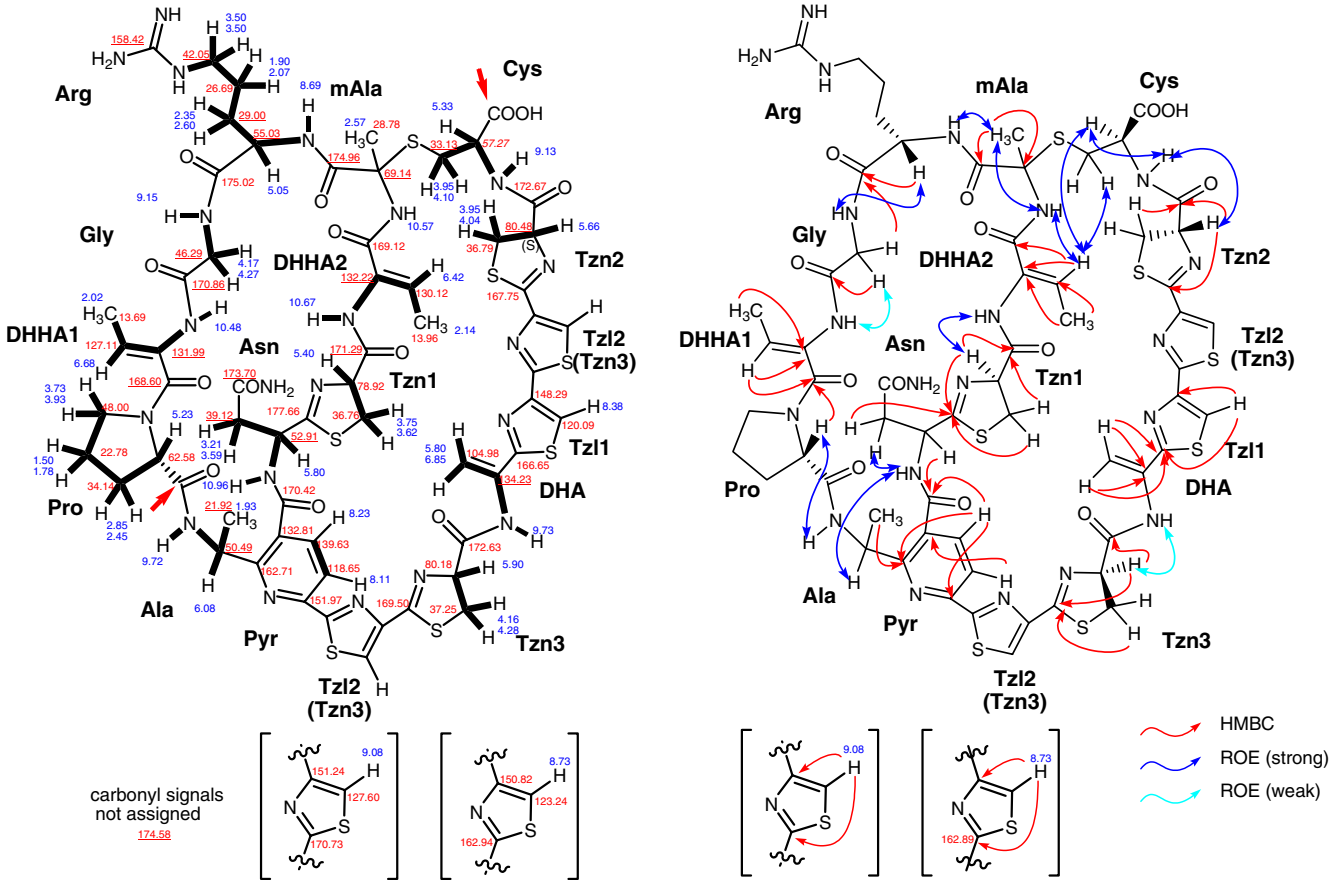


Figure 3. ^{1}H (blue numbers in the left structure), ^{13}C NMR chemical shifts (red numbers in the left structure), the HMBC (red arrows in the right structure), and ROE correlations (blue arrows in the right structure) of **1** in pyridine-d_5/H_2O (and pyridine-d_5/D_2O, right). The ^{13}C resonances, which indicated isotope shift more than 0.10 ppm by exchanging the proton with deuterium, were underlined in the left structure. The substructures revealed by COSY were highlighted with bold lines in the left structure. Configurations for amino acid residues were determined based on the Marfey's method after acidic hydrolysis.

ly). The *exo*-methylene protons showed COSY correlation to an amide proton at δ 9.73.

One ^{13}C signal for the quaternary carbon appeared at δ 69.14 which showed a FGHMBC correlation with singlet methyl protons at δ 2.57. We assumed that this carbon constituted substructure **III** as shown in Figure 5. The FGHMBC spectrum indicated a carbonyl group (δ 174.96) was attached to this quaternary carbon. Since the phase sensitive (PS) ROESY spectrum displayed correlation between the methyl singlet protons and the amide proton, the unknown function X in substructure **III** was assumed to be 'NH'. This fragment was named 'modified alanine', being abbreviated as 'mAla'. This substructure was supposed to be biosynthetically derived from alanine through an oxidation of the α-carbon. The function Y in the substructure **III** was later established to be 'S'.

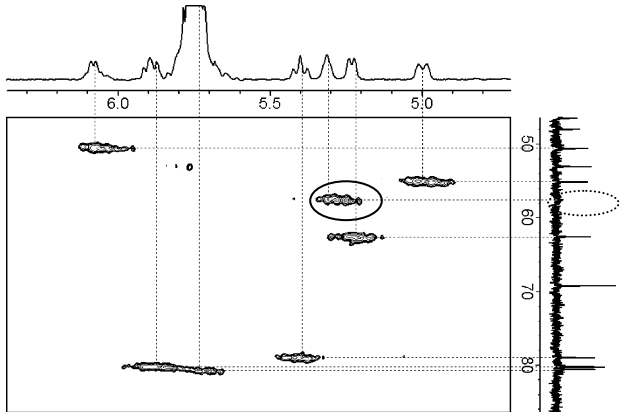
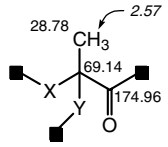


Figure 4. Region of FGHSQC spectrum of **1**.



substructure **III** (mAla)
(X = NH, Y = S)

Figure 5. ^{1}H and ^{13}C Chemical shifts for substructure **III**.

Regarding the stereochemistry, ROE experiments disclosed the (*Z*)-stereochemistry for DHHA2. However that of DHHA1 could not be assigned because of the lack of useful ROE, but it was established to be (*Z*)-configuration as described in Section 4. The absolute configurations of amino acid residues will be discussed in Section 3.

These NMR studies also led us to conclude the molecular formula of **1**. The number of nitrogens in **1** was considered based upon its biosynthetic components. The substructure **I** was supposed to be derived by cyclizations of a DHA-Cys-Cys fragment as the original component.[18] Other thiazole rings might be formed in a similar manner.[19] Floss et al. proposed a hetero-Diels–Alder cyclization pathway for biosynthesis of the 2,3,5,6-tetrasubstituted pyridine in noshiheptide,[18] following the similar hypothesis for the other thiopeptide antibiotics.[2]. In our case, hetero-Diels–Alder cyclization of Ala-DHA-Cys residue (diene) with another DHA (dienophile) is plausible.

Thus, we have evidenced all of the amino acid components of **1** as summarized in Table 3. This led to the number of carbons being 61, even though there were only 60 detectable signals in the ^{13}C NMR spectrum. The last carbon not assigned was the carbonyl carbon of 'Cys' or 'Pro'. This study also indicated that the number of sulfur atoms was seven, which could be the number of cysteines in the biosynthetic precursor. Although we could not assign whether the amino acid 'Asx' was whether 'Asn' or 'Asp', these considerations suggested that **1** contained at least 20 nitrogen atoms. Taking account of the odd molecular weight (MW = 1527), and the so-called 'nitorogen rule', the number of nitrogens in the molecule should be odd, suggesting 21. There was still one nitrogen not assigned at this stage.

The number of carbon, hydrogen, nitrogen, and sulfur atoms are thus established to be 61, 69, 21, and 7, respectively. The molecular formula so far was understood to be $C_{61}H_{69}N_{21}S_7$ (M_r 1319). Subtracting M_r from the observed molecular weight 1527 indicated the number of oxygens to be 13. Accordingly, the molecular formula thus obtained is $C_{61}H_{69}N_{21}S_7O_{13}$. The molecular weight for the ion $[M+H]^+$ is calculated to be 1528.3501, which is in good agreement with that observed by HRESI-MS (*m*/*z* 1528.3448). The index of hydrogen deficiency is 38, indicating that **1** has two macrocyclic peptide loops.

The sequence of these fragments was next studied. The amide linkage between 'DHHA1' and 'Pro' was found by detecting an FGHMBC correlation between α-*H*(Pro) and *C*O(DHHA1). The 'Arg' was adjacent to 'Gly' as judged from a long range C–H coupling between α-H_2(Gly) and *C*O(Arg). The α-proton of 'Asx' (δ 5.80) showed an FGHMBC cross peak with the carbonyl carbon (δ 170.42) of 'Pyr', which proved a connectivity 'Asx-Pyr'. Further linkage of the 'Asx' with 'Tzn1' has been established during the assignment of 'Tzn1'.

Since the amide protons for 'Ala' and 'DHA' (δ 9.72 and 9.73, respectively), and for 'Cys' and 'Gly' (δ 9.13 and 9.15, respectively) appeared very closely in pyridine-d_5/H_2O, we measured the PSROESY spectrum also in dimethylsulfoxide-d_6. This solvent system separated them well (Ala: δ 8.87, DHA: δ 9.41, Cys: δ 8.57, and Gly: δ 8.09). Prior to the PSROESY studies, almost all of the proton signals in dimethylsulfoxide-d_6 were assigned by the COSY spectrum (see supplemental data). ROEs were found at α-H(Tzn1) ↔ NH(DHHA2), β-H(DHHA2) ↔ > NH (mAla), β-H(mAla) ↔ NH(Arg), α-H(Gly) ↔ NH-(DHHA1), α-H(Pro) ↔ NH(Ala), α-H(Tzn3) ↔ NH-(DHA), and α-H(Tzn2) ↔ NH(Cys) to elucidate other peptide bonds. In order to satisfy the molecular formula, the sulfur atom of 'Cys' should be linked to the α-quaternary carbon of 'mAla' in a sulfide bond (function Y in Figure 5 is 'S'). The chemical shift for the quaternary carbon (δ 69.14) and ROE signals α-H(Cys) ↔ β-H(DHHA2) were consistent with the above assumption and allowed the total framework of **1** to be as depicted except for the last nitrogen.

Isotope shifts of more than $\Delta\delta$ 0.10 were observed in 20 resonances by comparison of ^{13}C spectrum in pyridine-d_5/H_2O (3:1) with that in pyridine-d_5/D_2O (3:1). They were expected to be located at the proximity of the exchangeable protons such as N–H and O–H.[20] The chemical shifts for these carbons with the isotope shifts are underlined in Figure 1. These were successfully found close to the exchangeable protons, supporting the proposed structure.

Table 3. The estimated component of **1**

Fragments	Original amino acids	Number of '*N*'	Number of '*C*'	Number of '*S*'
Asn (Asp)		2 or 1	4	
Pro		1	5	
Gly		1	2	
Arg		4	6	
Cys		1	3	1
DHHA1		1	4	
DHHA2		1	4	
mAla	Ala	1	3	
Tzn1	Cys + (neighboring CO)	1	3	1
Tzn2	Cys + (neighboring CO)	1	3	1
Tzn3	Cys + (neighboring CO)	1	3	1
Substructure A (Tzl-Tzl-DHA)	DHA+Cys+Cys	3	9	2
Substructure B (Ala-Py-Tzl)	[(Ala+DHA+Cys) + DHA]-NH_2	3	12	1
C-terminal		0 or 1		
Total number		21	61	7

3.2. Confirmation of the Asn residue and the sequence by MSMS experiments: the expected structure

The above NMR experiments succeeded in constructing the framework of **1**. However our assignments mainly relied on ROEs. We could not neglect a possibility completely that some of these were caused by transannular ROEs. There was another unsolved issue, which is the position of the last nitrogen, that is, the introduction of an amide (–$CONH_2$) function into one of two possible carbonyl groups, the *C*-terminal of Cys or the side chain of Asx (which means replacing that with Asn). These were investigated by MALDI-TOF/TOF experiments. The MALDI CID spectrum of the molecular related ion *m*/*z* 1528 provided dominant product ions at *m*/*z* 1413, 1380, 1355, 1130, 1104, 1060, 1035, 366, 293, 283, 141, 87, and 70. Assignments for those signals are summarized in Figure 6. The MS spectrum also showed signals at *m*/*z* 1381 and 1060, which suggested a ring opening at the aminothioacetal moiety of mAla during ionization, generating the monocyclic compound **1′**.

The product ion at *m*/*z* 87 could be estimated to be the immonium ion of the asparagine side chain. Another immonium typed product ion related to [Asn-Tzn-DHHA]$^+$ was also observed at *m*/*z* 283. These finally revealed the location of the last nitrogen, attached to the

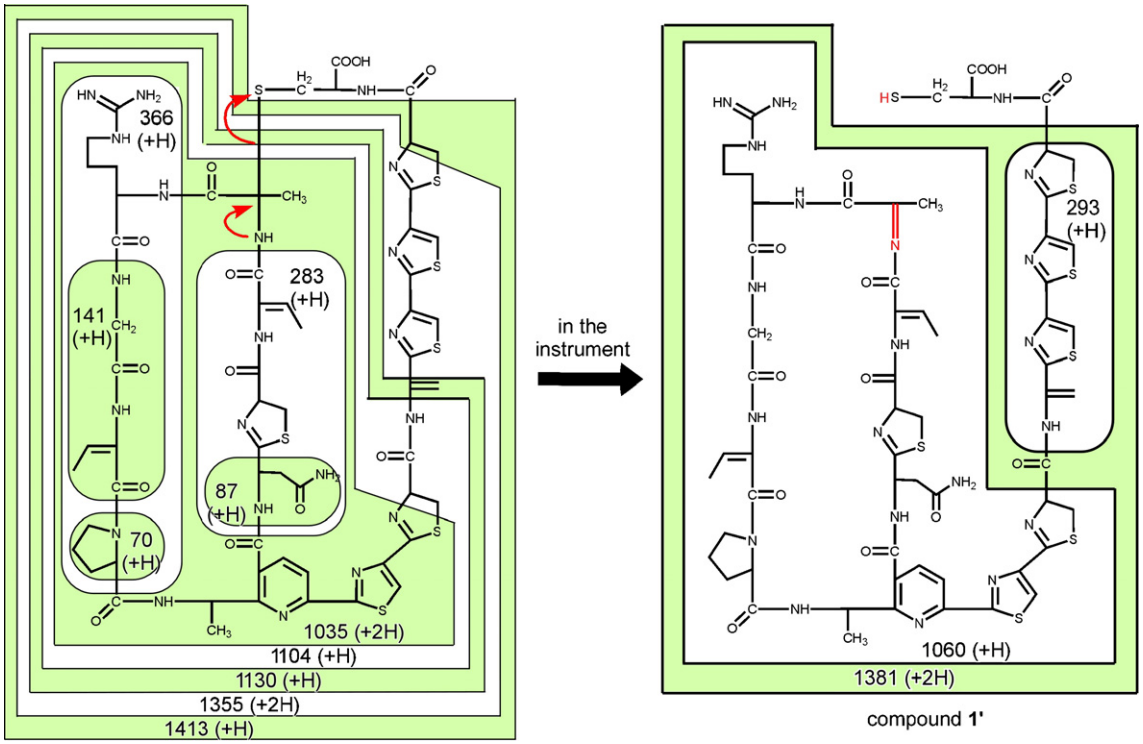


Figure 6. Product ions observed by MALDI-MSMS spectra.

β-carbonyl of Asx. The aspartic acid which we isolated by acidic degradation was derived from this asparagine residue. The product ion at *m*/*z* 1413 also supports this notion, because this ion can be explained by elimination of 'cysteine + CO' from the precursor ion *m*/*z* 1528. These observations led to the conclusion of the sequence 'Asn-Tzn-DHHA'.

The sequence 'Arg-Gly-DHHA-Pro' was also confirmed by *m*/*z* 70 (Pro^+), 141 ($[Gly\text{-}DHHA]^+$), and 366. The product ion at *m*/*z* 293 indicated the fragment 'DHA-Tzl-Tzl-Tzn' which produced saramycetic acid **4** by acidic degradation.[21]

3.3. Absolute configurations of amino acid residues

The absolute stereochemistry of the amino acid residues was then determined. After acidic hydrolysis, the mixture of hydrolysates was treated with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alaninamide: L-FDAA, the products by reaction with L-FDAA are abbreviated as DAA derivatives for convenience)[22] under the standard conditions. The reaction mixture was directly subjected to ODS HPLC. The chromatograms are shown in Figure 7. The signals were assigned by co-injections with the standard samples. The DAA derivatization accompanied oxidation of cysteine giving *N*,*N*′-bis(DAA)cystines regardless of the mixture of hydrolysates or pure cysteines, which was established by the ion chromatogram at *m*/*z* 745.

The experiments disclosed L-configuration for the Arg and Pro residues. In contrast, the HPLC chromatogram displayed bis(DAA)-L,L-cystine and the corresponding *meso*-isomer in 4:1 ratio based on these UV intensities. The derivative due to D,D-cystine was not detected. These indicated the ratio between L- and D-cysteines in **1** is 9:1. In contrast, **1** contains only four residues possible to provide cysteine by acidic degradation. Acidic hydrolysis usually accompanies racemization of cysteine residues by way of thiazoline ring formation,[23] and three thiazoline rings have already been involved in **1**. Taking predominant detection of the L,L-isomer into account, we assumed that the 'Cys' moiety and all the thiazoline units in **1** were derived from L-cysteine.

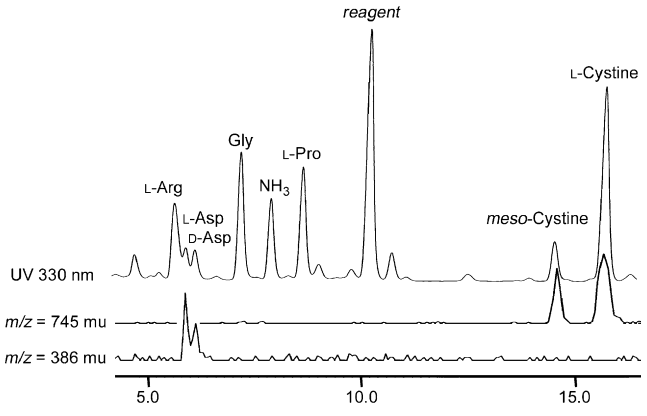


Figure 7. HPLC chromatograms of the DAA-amino acids derived from **1**.

The asparagine residue was more troublesome. We could not establish its configuration, because these experiments provided a 1:1 mixture of (DAA)-L-aspartic acid and the corresponding D-isomer. Since the DAA derivatization of standard L-aspartic acid accompanied no racemization, this unit clearly racemized during acidic hydrolysis. The special environment of the Asn unit in **1** (the α-carbonyl of Asn was masked as a thiazoline) may promote this racemization. Because of the L-configuration of other amino acids, we would tentatively assume L-configuration of the 'Asn' moiety in **1** without direct evidence.

The stereochemistry of 'Ala' remains unclear. But its biogenesis implies L-configuration despite a lack of experimental evidence. For discussion about this moiety, we may need to establish the stereochemistry of **5**, for example, by chemical synthesis.[24] There is still one more asymmetric carbon at the 'mAla' moiety, of which the stereochemistry remains unclear. So far, we do not have any information about that.

4. Structure of cyclothiazomycin B2

The minor component cyclothiazomycin B2 (**2**) was more labile than **1**. Standing the HPLC eluent of **2** for 30 min at room temperature generated a considerable amount of **1**. We also observed backward isomerization under the same conditions, but it was much slower. The PDA and ESI-MS spectrum of **2** was almost identical to that of **1**. These results implied that **1** and **2** constitute a pair of conformational or diastereomeric isomers.

Fortunately, this isomerization was not practical in aqueous pyridine. These conditions allowed the ^1H NMR and COSY spectra of **2** to be measured. However slow isomerization still existed, so that the ^{13}C NMR and FGHMBC spectra were not obtainable because these required longer accumulation times. Higher concentrations only resulted in the serious signal broadening and necessitated the re-purification of samples after every experiment.

The ^1H NMR spectrum of **2** looked quite similar to that of **1** except for some signals. An olefinic proton for one of 'DHHA' [δ 7.12 (q)] in **2** was significantly shifted to lower field as shown in Figure 2. The COSY spectrum revealed moderate changes in the chemical shifts only around proline [$\Delta\delta$ αH 0.05, βH_2: −0.16 and −0.35, γH_2: −0.29 and −0.45, and δH_2: +0.13 and +0.18] and glycine [$\Delta\delta$ αH_2: −0.09 and −0.25] residues. Other signals moved less (see supplemental data), suggesting an occurrence of some regional structural change around the 'Pro-DHHA1-Gly' moiety. This allowed us to assign the quartet at δ 7.13 to the olefinic proton for DHHA1.

We assumed that **2** is a diaestereomer due to the DHHA1 moiety by considering the significant chemical shift changes ($\Delta\delta$ −0.44) for the olefinic proton of this moiety. There are other possibilities; (i) conformational isomers at the peptide bond, (ii) diastereomers relating to the absolute configuration of the Asn unit, and (iii)

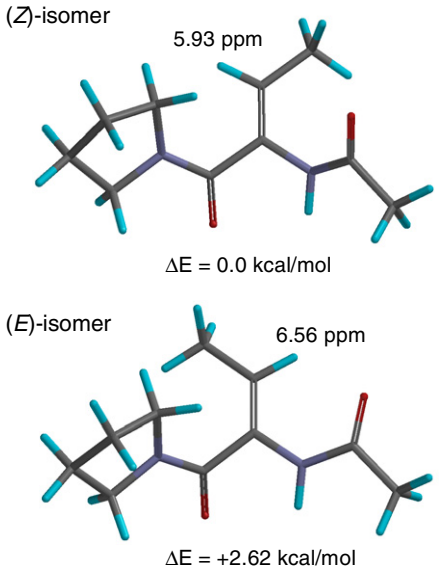


Figure 8. Estimated chemical shifts and relative steric energies for model compounds.

diastereomers relating to the absolute configuration of the mAla unit. However, the former two candidates were discounted because these must result in remarkable conformational alterations over the whole cyclic molecule. Thus, the chemical shift changing must not be localized in these cases. The last possibility can be eliminated by an entropic factor, because that requires opening and the following closure of the large peptide ring in vitro.

The stereochemistry of the DHHA1 moieties was estimated by calculations employing the (*E*)- and (*Z*)-model compounds as shown in Figure 8. Stable conformations of both isomers were subjected to the theoretical chemical shift predictions employing HF-631G* basis set, which indicated that the olefinic proton in (*E*)-DHHA unit appears in a significantly lower field than that of the corresponding (*Z*)-isomer.[25] The $\Delta\delta$ value was also in agreement with that observed. Accordingly, the stereochemistries for the DHHA1 moieties in **1** and **2** were proposed to be (*Z*)- and (*E*)-configurations, respectively.[26] This isomerization can be explained by the 'imine–enamine equilibration'.[27] Since isomerization from **1** to **2** was slower than the backward isomerization, **1** should be thermodynamically more stable than **2**. This was also supported by the calculations. The steric energy of the (*Z*)-model was estimated to be 2.62 kcal/mol more stable than the (*E*)-isomer.

5. Biological and biochemical activities of cyclothiazomycin B1

The biological properties of **1** are discussed. As described, **1** was isolated by screening for hyphyal swelling formation against *C. miyabeanus*. The IC_{50} (inhibition concentration 50%) value of pure **1** was estimated to be 0.3 nmol/mL in vitro. Cyclothiazomycin B2 (**2**) also induced the hyphal swelling at a similar level. Given the ready equilibration between **1** and **2** under mild conditions, only the major and more thermodynamically stable isomer **1** was subjected to further biological assay. It was found that **1** induced antibiotic activities against other plant pathogenic fungi, *Botrytis cinerea Persoon* (cucumber) and *Septoria nodorium* (wheat). The IC_{50} values for these fungi were 0.2 and 4.1 nmol/mL in vitro, respectively.

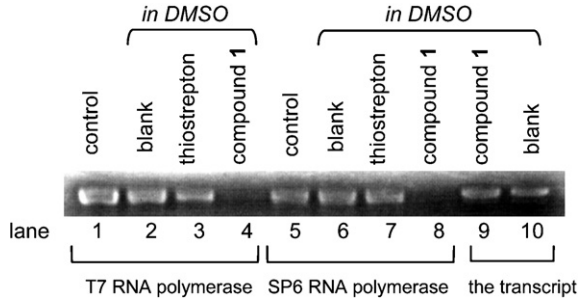


Figure 9. Inhibition of DNA-dependent RNA synthesis. RNA was synthesized from 1 μg template DNA for DHFR by T7 (lanes 1–4) or SP6 (lanes 5–8) RNA polymerase in the presence of 500 μM thiostrepton (lanes 3 and 7) or **1** (lanes 4 and 8). One microgram of transcript RNA was incubated for 4 h at 37 °C in the presence (lane 10) or absence (lane 9) of 500 μM **1**. Five percentage of DMSO was contained in the reaction (lanes 2, 3, 4, 6, 7, 8, 9, and 10).

We found that cyclothiazomycin B1 (**1**) inhibited DNA-dependent RNA synthesis. As shown in Figure 9, in vitro transcription from DNA for dihydrofolate reductase (DHFR) by bacteriophage T7[28] or SP6[29] RNA polymerase was inhibited by 500 μM of **1**, while not by another thiopeptide antibiotic, thiostrepton, under the same conditions.[2] No degradation occurred when the transcript was incubated with **1**, confirming that **1** had no ribonuclease activity. One hundred micromolar, but not 20 μM, of **1** was sufficient to inhibit transcription. It has been shown that the elongation process of transcription by T7 RNA polymerase is inhibited by T7 lysozyme,[30] while no inhibitor has been reported for SP6 RNA polymerase. As the promoter sequence, T7 and SP6 RNA polymerases prefer TAATACGACTCAC TATAGGG and GATTTAGGTGACACTATAG, respectively, which are apparently dissimilar from each other, thereby **1** is likely to inhibit any process common to transcription after the recognition of the promoter sequence.

It is well known that a thiopeptide antibiotic, thiostrepton,[2] binds to the ribosome to inhibit the GTPase activity of a translation factor. Our preliminary experiments revealed that a derivative of cyclothiazomycin B1 (**1**) also binds to the ribosome, but to a different region, to inhibit a ribosome-dependent GTPase activity.[31]

6. Conclusion

We disclosed cyclothiazomycins B1 (**1**) and B2 (**2**). The database search showed that these compounds are analogues of cyclothiazomycin (**3**), which contains a threonine residue in place of the 'Arg', reported by Aoki et al. in 1991.[5,7,8,32] Although **1**, **2**, and **3** are structural ana-

logues, the physical properties of **1** and **2** are remarkably different from those of **3**. For example, while we obtained aspartic acid in racemic form by acid hydrolysis of **1**, they isolated only the L-isomer from **3**. Aoki et al. proved the structure of **3** by measuring NMR spectra in aqueous methanol. However this solvent system hardly dissolved **1** or **2**. In fact, we took advantage of this insolubility in the purification of the natural products. The ROE patterns of these compounds were not identical, suggesting slightly different conformations from each other.

Our research also disclosed a characteristic biological property that **1** inhibited transcription by bacteriophage RNA polymerase. It might serve as a powerful tool for clarifying the molecular basis of transcription. The effect on the transcription by eukaryotic or eubacterial RNA polymerase as well as on the RNA-dependent RNA synthesis has yet to be examined. The detail of these activities and determination of the absolute configuration of the 'Asn', 'mAla', and the 1-aminoethyl group of the pyridine unit are under investigation in our laboratories.

7. Experimental

7.1. General

The ^{1}H, ^{13}C, and the 2D NMR spectra were recorded on a JEOL ALPHA 400 spectrometer. The remained proton signals in deuteriosolvents CHD_2SOCD_3 (δ 2.49), CHD_2OD (δ 3.30), or pyridine (for the C2-H, δ 7.19) for ^{1}H MMR spectra and isotope ^{13}C signals in $^{13}CD_3SOCD_3$ (δ 39.5), $^{13}CD_3OD$ (δ 49.0), or pyridine (for the $^{12}C2$, δ 123.59) for ^{13}C NMR spectra were used as the internal standards. Splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). IR spectra were obtained with HORIBA FT-720 Fourier transform infrared spectrometer on a KBr cell. HPLC was carried out with Waters 600 pump controller system with Waters 996 photodiode array detector. LCESI–MS was performed on a PerkinElmer API300 LC/MS/MS system equipped with Waters 600S HPLC pump controller system. Measurements of electron ionization, field desorption, fast atom bombardment, or electrospray ionization mass spectra (EI-MS, FD-MS, FAB-MS, or ESI-MS, respectively) were performed on a JEOL JMS AX500 spectrometer or a JEOL JMS AX102A spectrometer. MALDI-TOF MS/MS experiments were performed by using positive ion linear and reflector modes on a 4700 Proteomics Analyzer (Applied Biosystems, USA). The sample was dissolved in 20 μl of distilled water and 0.5 μl aliquots were loaded with DHB (2,5-dihydroxybenzoic acid) matrix onto the target. The accelerating voltage was 20 kV, the collision voltage was 1 kV for MS/MS experiments using argon gas, and the sum of 1000 shots was collected for each spectrum.

7.2. Fermentation and purification

In 500 mL Erlenmeyer flasks with baffles, *Streptomyces* sp. A307 provided from Mitsubishi Kasei Co. Ltd was inoculated on a medium established for preparation of Fortimicin[13] (soluble starch 6.0 g, dried yeast 6.0 g, KH_2PO_4 0.60 g, NaCl 0.50 g, $CaCO_3$ 0.20 g, and $MgSO_4 7{\cdot}H_2O$ 0.10 g in distilled H_2O 200 mL) and it was cultured at 27 °C on a rotary shaker (110 rpm) for 4 days. The cultured suspension (ca. 5.0 mL) was pipetted into 24 Erlenmeyer flasks (500 mL) with baffles, each containing 200 mL of the same medium. After shaking these flasks at 110 rpm at 27 °C for 12 days, the mycelial cake was collected by centrifuge (12,000 g, 20 min at 4 °C). The cake thus obtained was suspended with methanol (5.0 L) and stood at room temperature for 12 h. After filtration under reduced pressure, the filtrate was concentrated in vacuo below 20 °C until the whole volume became ca. 1.0 L. The obtained suspension was washed by shaking with AcOEt (400 mL) and the aqueous suspension was concentrated again in vacuo until the whole volume became 700 mL. After the insoluble material was collected by centrifuge (12,000*g*, 10 min at 4 °C), the residue was suspended again with a 1:1 mixture of $CHCl_3$ and MeOH (200 mL). After filtration, the filtrate was concentrated in vacuo to give a cure material (150 mg). The material was dissolved in aqueous pyridine (50 mL, pyridine/H_2O 1:9) and then loaded on ODS Sep-Pak® (10 g). After pyridine was eluted off by water, the mixture on Sep-Pack was developed stepwise with 20:80, 40:60, 60:40. 80:20, and 100:0 MeOH/H_2O. The fraction eluted with 80:20 MeOH/H_2O was collected and concentrated in vacuo. The residue (70 mg) was dissolved again with minimum amount of aqueous pyridine (1.0 mL) and then subjected to HPLC [Develosil ODS (20 × 250 mm), with 38:62 CH_3CN/H_2O containing 0.1% TFA, 10.0 mL/min flow]. The fractions eluted at 12.0 and 20.0 min were collected with cooling. The acetonitrile in each fraction was immediately removed in vacuo keeping the water bath below 15 °C and the resulting each aqueous solution was lyophilized to provide cyclothiazomycins B2 (**2**, 3.0 mg, t_R = 12.0 min under above conditions) and B1 (**1**, 20.0 mg t_R = 20.0 min under above conditions), both as amorphous powder.

7.3. Acid degradation

Cyclothiazomycin B1 **1** (30.0 mg, 19.6 μmol) was hydrolyzed by heating in 6 M aqueous HCl solution (15.0 mL) at 110 °C for 24 h. The residue obtained after lyophilization was diluted with H_2O and loaded on ODS Sep-Pak® (1.0 g). Elution with H_2O gave a mixture of amino acids. Further elution with 60% and 80% H_2O/MeOH gave pyridine derivative **5** (4.8 mg, 16 μmol) and saramycetic acid **4** (4.6 mg, 18 μmol), respectively. The ^{1}H and ^{13}C NMR data for **4** and **5** are summarized in Table 2.

The fraction eluted with H_2O was concentrated and a half of the residue was treated with *N*,*N*-5-dimethylaminonaphthalenesulfonyl chloride (5.0 mg) in 0.1 M aqueous $NaHCO_3$ solution (1.0 mL) at room temperature for 12 h in the dark. The HPLC analysis (Capcell Pack C18 4.0 mm (ID) × 150 mm, 0–100% CH_3CN/H_2O for 40 min, 1.0 mL/min flow, detected at UV 220 nm, also monitored with ESI-MS) indicated the dansyl derivatives of Arg (13.9 min), Asp (14.5 min),

Gly (15.2 min), Pro (18.3 min), and the cystine (19.2 min). Those except for cystine derivatives were confirmed by co-injections with the authentic samples prepared by the same procedure. The ESI-MS spectrum of the peak at 19.2 min displayed a pseudo-molecular ion at *m/z* 707 to reveal that it was detected in the form of bis(dansyl)cystine.

7.4. Determination of absolute configuration for hydrolytic amino acids

The mixture of the amino acids not used for the dansylation was treated with L-FDAA (2.0 mg/mL acetone, 0.3 mL) in 0.5 M aqueous $NaHCO_3$ solution (500 μL) at 40 °C for 90 min. The HPLC analysis of the reaction mixture (Merck Lichrosphere RP18e 120 Å, 4.0 × 150 mm, 30–50% CH_3CN/H_2O for 20 min, 1.0 mL/min flow, detected at UV 330 nm, also monitored with ESI-MS) indicated DAA derivatives of L-Arg (5.6 min), L-Asp (5.8 min), D-Asp (6.0 min), Gly (7.0 min), L-Pro (8.5 min), *meso*-cystine (14.5 min), and L-cystine (15.4 min). Those peaks were confirmed by co-injection with the authentic sample independently prepared from corresponding commercial L- and D-amino acid as well as the ion chromatograms.

7.5. Methyl saramycetate

Saramycetic acid **4** (3.0 mg, 12.0 μmol) was heated in methanolic hydrochloric acid [prepared by 3.0 mg of AcCl and methanol (0.5 mL)] at 80 °C for 6 h. After concentration the residue was purified by silica gel column chromatography (20:80 AcOEt/hexane) to give methyl saramycetate **6** (2.3 mg, 8.5 mmol, 70%). EI-MS (rel. int.) m/z = 268 (100, M^+), 237(22, $[M-CH_3O]^+$), 210 (43, $[M-CO_2]^+$), 195 (13), 168 (73), 136 (25), 57(23). ^1H NMR ($CDCl_3$) δ 2.76 (3H, s), 3.97 (3H, s), 8.23 (1H, s) 8.44 (1H, s). ^{13}C NMR ($CDCl_3$) δ 25.95, 52.62, 124.11, 128.52, 147.76, 150.65, 161.71, 162.48, 167.21, 191.16. IR (KBr) 3130, 3090, 1725, 1690, 1410, 1215 cm^{-1}. The mass and ^1H NMR spectra had good accordance with those reported.[15]

7.6. Esterification of 5

The pyridine derivative **5** (4.6 mg, 15.7 μmol) was heated in methanolic hydrochloric acid under the same conditions as above. After concentration, the residue was loaded on ODS Sep-Pak® (100 mg) and eluted with 50:50 MeOH/H_2O to give **7** (4.1 mg) $[\alpha]_D^{25}$ −8.1° (*c* 0.3, MeOH). EIMS (rel. int.) *m/z* 321 (27, M^+), 306 (9, $[M-CH_3]^+$), 289 (63, $[M-CH_3OH]^+$), 274 (100, $[M-CH_3OH\ Me]^+$). EI-HRMS found *m/z* 321.0744, calcd for $C_{14}H_{15}N_3O_4S$: M^+, 321.0783. The NMR spectral data are shown in Table 2.

7.7. Inhibition of DNA-dependent RNA synthesis

Template DNA for transcription was prepared by polymerase chain reaction (PCR) using the 5′ primer having a promoter sequence for T7 or SP6 RNA polymerase immediately adjacent to a portion of the upstream sequence of the *folA* gene encoding DHFR, the 3′ primer having a portion of the downstream sequence in the reverse direction and the genome DNA from *Escherichia coli* strain W3110 as the template. 1 μg of template DNA was incubated for 4 h at 37 °C in a reaction mixture (40 μl) containing 2.5 mM each of NTP, 0% or 5% DMSO, and 100 U T7 or SP6 RNA polymerase in the presence of 500 μM thiostrepton or **1**. To check the ribonuclease activity in **1**, 1 μg of transcript was incubated for 4 h at 37 °C in the presence or absence of 500 μM **1**. Each sample (4 μl) was electrophoresed on a 2% agarose gel containing 17% formaldehyde after treatment with 5 U RNase-free DNase I (Promega) for 1 h at 37 °C and the gel was stained with ethidium bromide.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.09.006.

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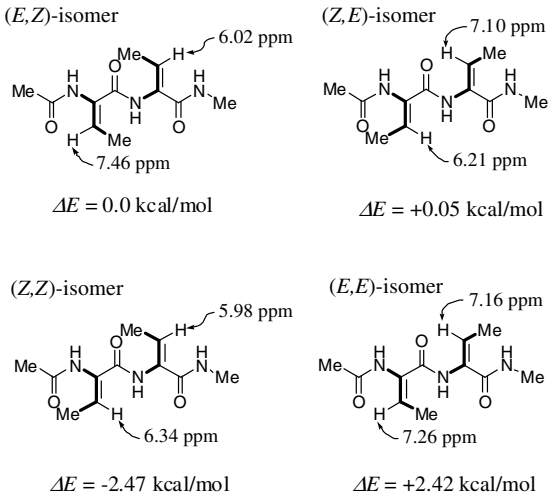
25. Prior to the chemical shift predictions, structural optimization was performed employing ab initio 6-31G* for each model compound. We used Spartan '04 program by Wavefunction Inc. (18401 Von Karman Avenue, Suite 370 Irvine, CA 92612).
26. The theoretical chemical shifts predictions suggested the olefinic protons of (*E*)-DHHA residues appear ca. 1.0 ppm lower field than that of the corresponding (*Z*)-DHHA residues for other model compounds as shown below. These calculations also indicated that (*Z*)-DHHA residues are ca. 2.5 kcal/mol sterically more stable than the (*E*)-DHHA residues. These results also are consisent with our assumption for the stereochemistry of DHHA2 moiety in **1** and **2**.



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32. In 1990, Cooper et al. disclosed antibiotic Sch 43057 isolated by them was identical to saramycetin. The constitutive amino acids (Thr, Cys, Gly, and Pro) found by acidic hydrolysis were identical to those involved in Aoki's cyclothiazomycin **3**. Both Cooper and Aoki also isolated saramycetic acid by acidic hydrolysis. The mass number of Cooper's saramycetin was *m/z* 1473.3395 $[M+H]^+$, while that of Aoki's **3** was *m/z* 1495.2733 $[M+Na]^+$. These indicated the same mass number 1472, whereas the mass formula by Cooper was one carbon smaller than that of Aoki's **3**. Cooper also disclosed existences of a dehydroalanine, two dehydrohomoalamnines, three thiazolines, and three thiazoles, which are the same residues as those involved in Aoki's **3**. Physical properties of our **1** and **2** were rather similar to those in Copper's report: Cooper, R.; Truumees, I.; Barrett, T.; Patel, M.; Schwartz, J.; Puar, M.; Das, P.; Pramanik, B. *J. Antibiot.* **1990**, *43*, 897.