TETRONOTHIODIN, A NOVEL CHOLECYSTOKININ TYPE-B RECEPTOR ANTAGONIST PRODUCED BY *Streptomyces* sp. NR0489

III. STRUCTURAL ELUCIDATION

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Tetronothiodin (**1**) is a potent and selective cholecystokinin type B (CCK-B) receptor antagonist produced by *Streptomyces* sp. NR0489. Its structure was elucidated to be a macrocyclic compound comprising cyclohexene, α-acyltetronic acid and tetrahydrothiophene moieties based on various 2D NMR experiments on **1** and its dihydro derivative. The stereochemistries for the cyclohexene and tetrahydrothiophene rings were elucidated based on the analysis of NOEs obtained by NOESY experiments and NOE difference spectroscopy. The relative configuration of the cyclohexene moiety in **1** was revealed to be the same as that of the corresponding part in kijanimicin and chlorothricin, which can be structurally related to **1** in terms of their containing a cyclohexene ring with a spirotetronic acid in the molecule.

In our screening program for new cholecystokinin type-B (CCK-B) receptor antagonists, we discovered a novel and potent CCK-B receptor antagonist named tetronothiodin (**1**) from the culture broth of *Streptomyces* sp. NR0489. Preliminary communication of this work has been reported[1]. Details of the taxonomy, fermentation, isolation, characterization and biological activities of **1** are reported in the preceding papers[2,3]. In the present article we describe details of the structural elucidation including the stereochemical studies on **1**.

Results

Structural Elucidation

1 was obtained as a pale brown powder from the fermentation broth of the strain NR0489 by isolation procedures described in the preceding paper[3]. The molecular formula of **1** was determined to be $C_{31}H_{38}O_8S$ based on positive-ion FAB-MS (*m/z* 593 $(M+Na)^+$) and negative-ion high resolution FAB-MS data (569.2237, calcd for $(M-H, C_{31}H_{37}O_8S)^-$ 569.2210). This molecular formula was further supported by qualitative analysis for sulfur[4] and ^{13}C NMR spectral data which showed 31 signals. The IR spectral data suggested the presence of γ-lactone (1760 cm^{-1}) and carboxyl groups (3000~2300, 1728 cm^{-1}). The UV spectral data (λ_{max} (MeOH) 233 nm (ε 29,900), 271 (12,200); λ_{max} (MeOH - NaOH) 234 nm (ε 31,000), 271 (13,500); λ_{max} (MeOH - HCl) 234 nm (ε 25,000), 269 (sh, 9,800)) indicated the presence of an α-acyltetronic acid chromophore, also common to kijanimicin[5] and tetrocarcins[6].

Comparison of the ^{13}C NMR spectral data for the sodium salt of **1** with those for its free acid established the carbon signal assignment of the carboxylic acid suggested by the IR spectral data. The carbon signal δ 166.7 (in DMSO-d_6) of the sodium salt was assigned to a carboxylic acid because the chemical shift was 3 ppm lower than the corresponding signal of the free acid (δ 163.5), whereas the other $-CO_2-$ carbon signal (δ 173.9) of the sodium salt remained unaffected in the free acid (δ 174.4). Since the free acid of **1** was unstable in solution, its potassium and sodium salts were used for further NMR experiments. The ^1H and ^{13}C NMR spectral data for these salts in D_2O and DMSO-d_6 are shown in Table 1. The structural

Table 1. NMR spectral data[a] for the potassium salt of **1** in D_2O and the sodium salt of **1** in DMSO-d_6.

Position	D_2O[b]		DMSO-d_6[c]	
	δ_C	δ_H (J/Hz)	δ_C	δ_H (J/Hz)
1	177.2		173.9	
2	96.6		94.3	
3	202.4		199.1	
4	85.6		81.6	
5	38.6	a 1.58 (br d, J=13)	38.5	a 1.33 (dd, J=1.8, 14)
		b 2.10 (dd, J=7, 13)		b 1.88 (dd, J=7, 14)
6	32.0	2.32	31.7	2.16
7	139.5		136.3	
8	123.3	5.28 (br s)	124.7	5.08 (br s)
9	39.7	2.73	~40.3	2.45
10	34.3	2.23	33.5	a 1.96
				b 2.15
11	131.0	5.50 (m)	131.8	5.40 (ddd, J=5, 8.5, 15)
12	130.6	5.99 (m)	129.7	5.81 (dd, J=10, 15)
13	132.5	5.92 (m)	133.2	5.65 (dd, J=10, 15)
14	128.5	5.45 (m)	126.7	5.27 (ddd, J=6, 9, 15)
15	40.1[d]	a 2.30	40.7	a 2.06
		b 2.43		b 2.21
16	70.9	3.86 (m)	68.43	3.55 (m)
17	40.05[d]	a 2.25	40.7	a 2.00
		b 2.42		b 2.14
18	127.5	5.41	126.1	5.18 (dt, J=15, 7.5)
19	131.5	5.41	131.6	5.01 (ddd, J=5, 9, 15)
20	39.3	a 1.81	~40.0	a 1.55 (dt, J=13, 9)
		b 2.00 (m)		b 1.93
21	33.7	1.99 (m)	33.2	1.80
22	50.7	2.75 (m)	50.5	2.63 (q, J=~6.5)
23	54.1[e]	4.62 (d, J=6)	53.1	4.67 (d, J=7)
24	36.1	a 3.02 (br d, J=12)	35.6	a 2.83 (dd, J=4, 11.5)
		b 3.18 (dd, J=7, 12)		b 2.85 (dd, J=6.5, 11.5)
25	54.0[e]	4.50 (m)	53.1	4.40 (dt, J=4, 6.5)
26	195.5		192.5	
27	19.7	1.19 (d, J=7)	19.5	1.08 (d, J=6.5)
28	21.1	1.80 (br s)	21.5	1.66 (br s)
29	17.7	0.84 (d, J=7)	17.4	0.71 (d, J=6.5)
30	200.6		202.2	
31	169.0		166.7	
OH				4.57 (br s)

[a] Assignments were established based on DEPT, ^1H-^1H COSY, HMQC and HMBC experiments.

[b] ^{13}C and ^1H NMR spectra were recorded at 100 and 400 MHz, respectively.

[c] ^{13}C and ^1H NMR spectra were recorded at 125 and 500 MHz, respectively.

[d,e] Interchangeable.

Fig. 1. Partial structures of tetronothiodin (**1**) revealed by the interpretation of ^{1}H-^{1}H COSY spectral data.

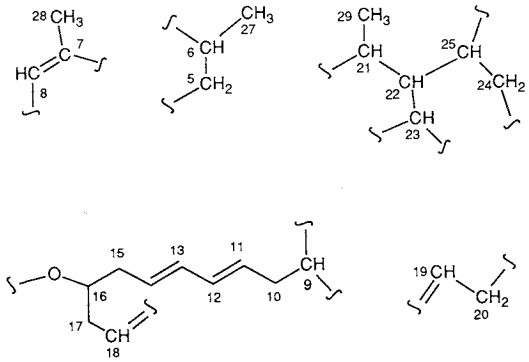


Fig. 2. Partial structure of tetronothiodin (**1**) elucidated by the analysis of ^{13}C-^{1}H long-range couplings (arrows).

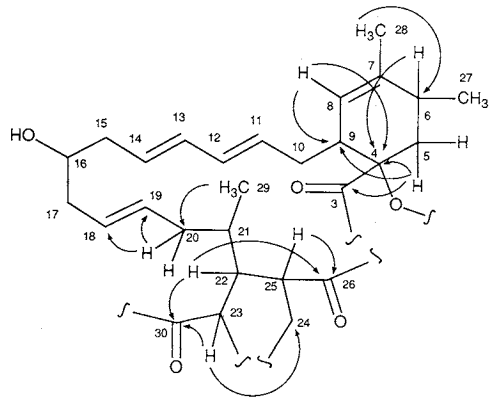
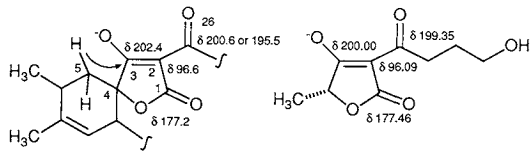


Fig. 3. ^{13}C NMR data and a ^{13}C-^{1}H long-range coupling (arrow) for α-acyltetronic acid moiety of tetronothiodin (**1**), and comparison of the ^{13}C NMR data with those of a carolic acid derivative (**3**).



1 **3**

elucidation was mainly carried out based on the NMR data obtained with the potassium salt of **1** in D_2O because all carbon signals were clearly observed, whereas two carbon signals were hidden by the large solvent signal in DMSO-d_6.

Partial structures in Fig. 1 were elucidated by interpretation of the ^{1}H-^{1}H COSY spectra (potassium salt of **1** in D_2O). An allylic coupling between 8-H and the methyl protons 28-H connected the olefinic carbon C-8 and the quaternary olefinic carbon C-7. These fragments and the remaining quaternary carbons, C-4 (δ 85.6), C-26 (δ 195.5), C-30 (δ 200.6) and C-3 (δ 202.4), were connected to form the partial structure in Fig. 2 based on the analysis of the ^{13}C-^{1}H long-range couplings obtained by HMBC experiments. An alternative cyclopentanone structure containing a ketone function (C-26) between C-23 and C-24, compatible with the relevant ^{13}C-^{1}H long-range couplings, was eliminated because of (1) the ^{13}C chemical shift (δ 195.5) of C-26, which was not in agreement with the general trend[7] that the ketones in cyclopentanones were observed at the region lower than 210 ppm, and (2) the structural analysis of a reduced derivative of **1** (*vide infra*). The geometries of the three disubstituted double bonds were determined to be all *E* because of the large coupling constants (15 Hz) observed with olefinic proton signals (sodium salt of **1** in DMSO-d_6). A hydroxy group was located at C-16 by a spin-coupling between the hydroxy proton (δ 4.57) and 16-H (δ 3.55) signals of the sodium salt of **1** taken in DMSO-d_6. The ^{1}H-^{1}H COSY experiments in DMSO-d_6 confirmed the spin-couplings 8-H–9-H, 18-H–19-H and 20-H–21-H.

The presence of an α-acyltetronic acid suggested by the UV and IR spectral data was confirmed by comparison of the ^{13}C NMR spectral data of **1** with those of a carolic acid derivative[8] (Fig. 3). Thus, the carbon signals at δ 96.6, 177.2, 195.5 (or 200.6) and 202.4 were assigned to C-2, C-1, C-26 and C-3, respectively. This moiety was attached to the cyclohexene ring at C-4 because of the ^{13}C-^{1}H long-range coupling between 5-Ha and C-3.

Although 23-H and 24-H were not coupled to each other, a ^{13}C-^{1}H long-range coupling was observed between 23-H and C-24, suggesting the linkage of C-23 and C-24 through a heteroatom or quaternary

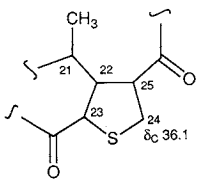
carbon. Taking the remaining units (one sulfur atom and one carboxylic acid) into consideration, a sulfur atom or the ketone function (C-30) substituted on C-23 must be inserted between C-23 and C-24 resulting in the formation of a 5-membered ring. It is most reasonable to locate a sulfur between these two carbons forming a tetrahydrothiophene ring because a cyclopentanone structure was again eliminated by the consideration of the ^{13}C chemical shift[7] (δ 200.6) of concern and the structural analysis of the reduced derivative (*vide infra*). This conclusion was corroborated by the chemical shift of C-24 (δ 36.1) which can be compared with that of position 5 (δ 33.1) in dimethyl (2α,3β,4α)-2-(trimethylsilyl)tetrahydrothiophene-3,4-dicarboxylate (**4**)[9] (Fig. 4).

The establishment of the tetrahydrothiophene structure left two possible structures A and B for **1** (Fig. 5). To allocate the only remaining carboxylic function to either C-26 or C-30, **1** was reduced by $NaBH_4$ to give epimeric alcohols **2a** and **2b** (Fig. 6). The molecular formulae of **2a** and **2b** were established to be $C_{31}H_{40}O_8S$ by their negative-ion HRFAB-MS data. The UV spectra of **2a** and **2b** were identical to that of **1**. These data indicated the α-acyltetronic acid moiety remained unchanged and one ketone which did not constitute the chromophore of **1** was reduced to give these dihydro derivatives. The newly observed proton signal (δ 3.95) in a ^1H NMR spectrum of **2a** was assigned to 30-H by ^1H-^1H COSY experiments which revealed the proton sequence 30-H–23-H–22-H–25-H–24-H. Since it was spin-coupled to only 23-H, the reduced ketone could not be incorporated into the cyclopentanone formed by C-23, C-22, C-25 and C-24. These results established the structure of this moiety to be represented by A in Fig. 5. The planar structure of tetronothiodin was thus elucidated to be **1** in Fig. 6.

Stereochemistry

The NOE between 9-H and 5-Hb (δ 1.88) observed in the NOESY spectrum of the sodium salt of **1** in DMSO-d_6 indicated the *cis* diaxial relation between these protons (Fig. 7). The small coupling constant (2 Hz) between 9-H and 8-H determined by a homodecoupling experiment further supported the pseudoaxial orientation of 9-H because the value was consistent with the dihedral angle (70~80°) between 9-H and the plane of the double bond, C-7=C-8. The *cis* relation between the methyl group on C-6 and 5-Ha was established based on a strong NOE between 5-Ha and 27-H and a very weak NOE between 5-Hb and 27-H observed in the NOE difference spectrum.

Fig. 4. Comparison of the ^{13}C chemical shift of the methylene carbon (C-24) in tetronothiodin (**1**) with that of the corresponding carbon of dimethyl (2α,3β,4α)-2-(trimethylsilyl)tetrahydrothiophene-3,4-dicarboxylate (**4**).



1

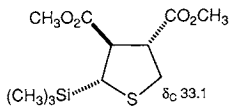


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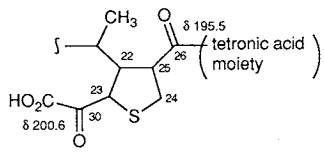
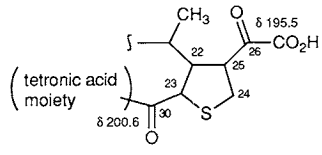
Fig. 5. Possible structures for tetronothiodin (**1**).

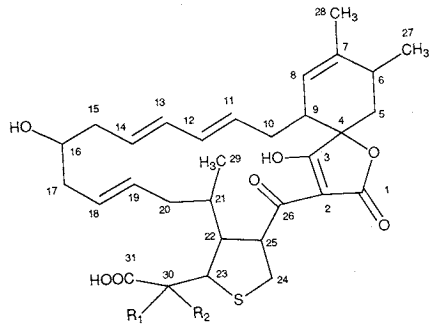


A



B

Fig. 6. Structures of tetronothiodin (**1**) and its dihydro derivatives (**2a** and **2b**).



1 (Tetronothiodin) $R_1 = R_2 = =O$
2a, **2b** $R_1 = H$ $R_2 = OH$

Fig. 7. NOEs (arrows), ^{13}C-^{1}H long-range couplings (solid lines) and ^{1}H-^{1}H couplings (dashed lines) for the cyclohexene moiety of tetronothiodin (**1**).

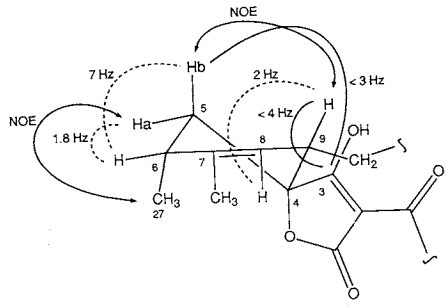
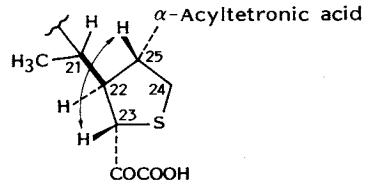


Fig. 8. An NOE (arrow) for the tetrahydrothiophene moiety of tetronothiodin (**1**).



To determine the coupling constants between C-3 and proton signals, 5-Hb and 9-H, long range selective proton decoupling (LSPD) experiments using a double irradiation technique were carried out on the sodium salt of **1** in DMSO-d_6. Coupling constants $J_{C\text{-}3\text{-}H\text{-}5b}$ and $J_{C\text{-}3\text{-}H\text{-}9}$ were determined to be less than 4 Hz from the respective signals C-3 by irradiating both 9-H and 5-Ha for the former and 5-Ha and 5-Hb for the latter. Their half value widths were 3 and 4 Hz, respectively. Since the coupling constant between an axial proton (or pseudoaxial proton) and an axial carbon was expected to be rather large (6~9 Hz) due to the *trans* relation, these small coupling constants indicated the *cis* relation between C-3 and 5-Hb (or 9-H). The relation between C-3 and 5-Hb (or 9-H) was thus determined to be *cis*.

The *cis* relation between 23-H and 25-H was established by the NOE between these protons (Fig. 8). Treatment of the free acid of **1** in CD_3OD for 5 hours resulted in the complete disappearance of the signal of the active hydrogen 25-H. This sample was then treated with CH_3OH to exchange the deuterium for hydrogen. After these treatments, **1** was recovered without epimerization at C-25, which was revealed by ^{1}H NMR experiments on the sodium salt of the treated sample in DMSO-d_6. This finding meant there existed only one stable configuration at C-25 of **1** in solution. Thus, a *trans* relation was reasonably assigned to the substituents on C-22 and C-25 because the sterically less hindered *trans* isomer was believed to be thermodynamically more stable than the *cis* isomer if the Dreiding model is considered.

Discussion

The structure of **1** is completely different from known CCK receptor antaogonists including those of microbial origin (anthramycin[10], asperlicin[11] and virginiamycin M_1 analogues[12]). **1** can be structurally related to some antibiotics such as kijanimicin[5], tetrocarcins[6], M139603[13] and MM 46115[14] in terms of containing an α-acyltetronic acid chromophore in their structures. However, none of them contain a tetrahydrothiophene ring linked to an α-acyltetronic acid.

The relative stereochemistry for the cyclohexene ring in **1** is the same as those of kijanimicin[5] and chlorothricin[15~17] which have been determined to be macrocyclic compounds containing a cyclohexene ring bearing a spirotetronic acid like **1** by X-ray crystallographic studies on their derivatives. Although

the chemical shifts (5-Ha and 5-Hb) and the coupling constants ($J_{5\text{-Ha-6-H}}=1.8$ Hz and $J_{5\text{-Hb-6-H}}=7$ Hz) for **1** are incompatible with the corresponding originally reported values[18] (δ 2.37 for 5-Ha and δ 1.84 for 5-Hb, and $J_{5\text{-Ha-6H}}=7.5$ Hz and $J_{5\text{-Hb-6-H}}=0$ Hz) for kijanimicin, the latter chemical shifts and coupling constants have been reversed based on NOE experiments on the aglycone of kijanimicin by MALLAMS[19]. Our conclusion on the stereochemistry at C-6 is thus confirmed. It will be interesting to investigate biosynthetic pathway of **1** in relation to that of kijanimicin or chlorothricin[16].

Experimental

General Procedures

UV and IR spectra were recorded on a Kontron Uvicon 860 spectrometer, and on a Hitachi 270-30 infrared spectrometer, respectively. Fast atom bombardment mass spectra (FAB-MS) were obtained with a JEOL DX-300 spectrometer using thioglycerol-glycerol-DMSO (positive ion) and glycerol-pyridine (nagative ion) as matrices. ^{1}H and ^{13}C NMR spectra were recorded on JEOL-GSX-400, -GSX-500, or -A-500 spectrometers.

Reduction of Tetronothiodin (1) with $NaBH_4$

1 (6.5 mg) was dissolved in MeOH (1.5 ml) and cooled to 0°C. To this solution was added $NaBH_4$ (5 mg) with stirring. After stirring the solution for 2.5 hours at 0°C, the solvent was evaporated under reduced pressure. The residue was suspended in water, adjusted to pH 2 with 1 N HCl and extracted with EtOAc. The organic layer was washed with water, dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in MeOH - 0.1 M phosphate buffer (pH 2.2) (6:4) and purified by preparative HPLC over a C_8 reversed-phase silica gel column (YMC-Pack, 30 × 250 mm; YMC Co., Ltd.) with MeOH - 0.1 M phosphate buffer (pH 2.2) (6:4) at a flow rate of 43 ml/minute to give 1.8 mg of **2a** (retention time, 14.5 minutes) and 2.2 mg of **2b** (retention time, 17.0 minutes) as powders. **2a**: Negative-ion HRFAB-MS *m/z* 571.2341 $(M-H)^-$ (Calcd for $C_{31}H_{39}O_8S$ 571.2366); IR ν_{max} (THF) 1736, 1642 cm^{-1}; UV λ_{max} (MeOH) 230 nm (ε 23,000), 271 (8,800), λ_{max} (MeOH - NaOH) 231 nm (ε 24,000), 273 (9,700), λ_{max} (MeOH - HCl) 231 nm (ε 19,000), 269 (sh; 7,800); ^{1}H NMR (400 MHz, D_2O) of the potassium salt of **2a**; 1.0 (3H, d, $J=7$ Hz, H-29), 1.17 (3H, d, $J=7$ Hz, H-27), 1.50 (1H, m, H-21), 1.57 (1H, br d, $J=13$ Hz, H-5a), 1.72 (1H, m, H-20a), 1.76 (3H, s, H-28), 1.90 (1H, m, H-20b), 2.00 (1H, m, H-17a), 2.06 (1H, dd, $J=7.5$, 13 Hz, H-5b), 2.1 ~ 2.45 (6H, H-6, -10, -15, -17b), 2.61 (1H, m, H-9), 2.90 (1H, dd, $J=7$, 11 Hz, H-24a), 3.03 (1H, m, H-22), 3.36 (1H, dt, $J=11$, 7 Hz, H-24b), 3.60 (1H, dd, $J=1 \sim 2$, 5 Hz, H-23), 3.85 (1H, m, H-16), 3.95 (1H, d, $J=5$ Hz, H-30), 4.19 (1H, dt, $J=11$, 7 Hz, H-25), 5.22 (1H, s, H-8), 5.31 (2H, m, H-18, -19), 5.37 (1H, ddd, $J=5$, 9, 15 Hz, H-14), 5.45 (1H, dt, $J=15$, 7 Hz, H-11), 5.80 (1H, dd, $J=11$, 15 Hz, H-13), 6.00 (1H, dd, $J=11$, 15 Hz, H-12). **2b**: Negative-ion HRFAB-MS *m/z* 571.2339 $(M-H)^-$ (Calcd for $C_{31}H_{39}O_8S$ 571.2366); IR ν_{max} (THF) 1736, 1642 cm^{-1}; UV λ_{max} (MeOH) 230 nm (ε 25,000), 274 (9,500), λ_{max} (MeOH - NaOH) 231 (ε 26,000), 271 (13,000), λ_{max} (MeOH - HCl) 233 (ε 21,000), 269 (sh; 7,500); ^{1}H NMR (400 MHz, D_2O) of the potassium salt of **2b**; 1.0 (3H, d, $J=6$ Hz, H-29), 1.14 (3H, d, $J=7$ Hz, H-27), 1.54 (1H, br d, $J=13$ Hz, H-5a), 1.74 (3H, s, H-28), 1.80 (1H, m, H-20a), 1.81 (1H, m, H-21), 1.99 (1H, m, H-20b), 2.00 (1H, m, H-17a), *ca.* 2.03 (1H, H-5b), 2.1 ~ 2.4 (6H, H-6, -10, -15, -17b), 2.64 (1H, m, H-9), 2.70 (1H, m, H-22), 2.97 (1H, H-24a), 3.03 (1H, H-24b), 3.84 (1H, m, H-16), 3.99 (1H, br, H-23), 4.15 (1H, br, H-30), 4.31 (1H, m, H-25), 5.22 (1H, s, H-8), 5.3 ~ 5.4 (4H, m, H-11, -14, -18, -19), 5.83 (1H, dd, $J=11$, 15 Hz, H-13), 5.99 (1H, dd, $J=11$, 15 Hz, H-12).

Acknowledgments

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