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

II. ISOLATION, CHARACTERIZATION AND BIOLOGICAL ACTIVITIES

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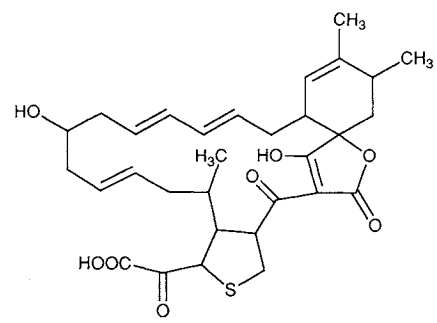
A novel cholecystokinin type-B receptor antagonist named tetronothiodin has been isolated by column chromatography and preparative HPLC from the fermentation broth of *Streptomyces* sp. NR0489. Tetronothiodin inhibited the binding of CCK_8 (*C*-terminal octapeptide of cholecystokinin) to rat cerebral cortex membranes (CCK type-B receptors) with an IC_{50} of 3.6 nM, whereas it did not inhibit CCK_8 binding to rat pancreatic membranes (CCK type-A receptors). It also inhibited CCK_8 induced Ca^{2+} mobilization in GH3 cells, a rat anterior pituitary cell line, but was without effect on the basal cytosolic Ca^{2+} concentration. This finding indicated tetronothiodin was an antagonist of CCK type-B receptors.

Cholecystokinin (CCK) is a hormonal regulator of pancreatic secretion[1] as well as gallbladder contraction[2] and gut motility[3]. It has also been proposed to act as a neurotransmitter in the central nervous system[4]. CCK type-B (CCK-B) receptors are suggested to be related to appetite[5], pain[6,7] and anxiety[8,9]. Some CCK-B receptor antagonists increased food intake[5], enhanced morphine analgesia[6,7] and reduced anxiety[8,9] in rats. However, physiological and pharmacological roles of CCK-B receptors are not yet fully understood in part because of the shortage of potent and specific CCK-B receptor antagonists. To obtain structurally unique and specific CCK-B receptor antagonists, we screened microbial metabolites by employing a binding assay method in which rat cerebral cortex membranes and ^{125}I labeled Bolton-Hunter CCK_8 ([^{125}I]-CCK_8) were used as the receptors and the radioligand, respectively. In this screening program, we discovered a novel CCK-B receptor antagonist named tetronothiodin (**1**) from the culture broth of *Streptomyces* sp. NR0489, and determined the structure to be a macrocyclic compound containing an α-acyltetronic acid and a tetrahydrothiophene ring (Fig. 1). A preliminary communication of this work

Fig. 1. Structure of tetronothiodin (**1**).



has been reported[10]. Details of the taxonomy and fermentation of **1** are reported in the preceding paper[11]. The structural elucidation study of **1** is reported in the succeeding paper[12] in detail. In the present paper, we describe the isolation, physico-chemical characterization and biological activities of **1**.

Isolation

Isolation of **1** was carried out by monitoring the inhibitory activity against ^{125}I labeled Bolton-Hunter CCK_8 ([^{125}I]-CCK_8) binding to rat cerebral cortex membranes. The isolation procedure of **1** is outlined in Fig. 2. After cultivation of the producing organism for ten days in 50-liter jar fermenters by the procedure described in the preceding paper[11], the mycelium was removed by centrifugation. The broth supernatant (181 liters) was adjusted to pH 7 with 6 N HCl and applied to a column (12 × 100 cm) of Diaion HP-21 (Mitsubishi Chemical Industries). The column was washed with water (25 liters) and 10% aqueous acetone (50 liters), and the active principle was eluted with 50% aqueous acetone (60 liters). The active eluate was concentrated to about 15 liters under reduced pressure and extracted with ethyl acetate (25 liters × 2) at pH 2. The organic layer was dried over anhydrous sodium sulfate and concentrated to 3.5 liters under reduced pressure. This solution was back-extracted with water (1.5 liters × 2) at pH 7.5. The water layer was concentrated to 1.5 liters under reduced pressure. The concentrate was applied to a column (5 × 16 cm) of QAE Sephadex A-25 (Pharmacia Fine Chemicals) which was developed stepwise with water (1 liter) and NaCl solutions (0.2, 0.3 and 0.5 M; 3.5 liters each). The active eluate (0.3 and 0.5 M NaCl fractions) was extracted with ethyl acetate (4 liters × 2) at pH 2. The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure to give an oily residue, which was chromatographed on a Sephadex LH-20 column (3.2 × 120 cm) developed with MeOH. The active eluate was concentrated under reduced pressure and purified by preparative HPLC over a C_8 reversed-phase silica gel column (YMC-Pack,

Fig. 2. Isolation procedure of tetronothiodin.

Broth supernatant (181 liters)
- adjusted to pH 7
- Diaion HP-21 column chromatoraphy
  - washed with water and 10% aqueous acetone
  - eluted with 50% aqueous acetone

EtOAc extract (pH 2)
- concd under reduced pressure

Back-extract (pH 7.5)
- concd under reduced pressure
- QAE Sephadex A-25
  - washed with water
  - eluted with NaCl (0.2~0.5 M)

EtOAc extract (pH 2)
- concd under reduced pressure
- Sephadex LH-20 column chromatography
  - eluted with MeOH
- concd under reduced pressure
- Preparative HPLC
  - (C_8 reversed phase silica gel column)
  - eluted with MeOH-phosphate buffer (pH 2.2) (6:4)
- concd under reduced pressure

EtOAc extract (pH 2.5)
- concd under reduced pressure

Tetronothiodin (240 mg)

Table 1. Physico-chemical properties of **1**.

Appearance	Pale brown powder
Molecular formula	$C_{31}H_{38}O_8S$
FAB-MS (positive ion)	593 $(M+Na)^+$, 609 $(M+K)^+$
HRFAB-MS (negative ion)	569.2237 $(M-H)^-$ Calcd: 569.2210
UV λ_{max}^{MeOH} nm (ε)	233 (29,900), 273 (12,200)
$\lambda_{max}^{MeOH-NaOH}$	234 (31,000), 271 (13,500)
$\lambda_{max}^{MeOH-HCl}$	234 (25,000), 269 (sh, 9,800)
IR ν_{max} (KBr) cm^{-1}	3700~2300 (br), 1760 (sh), 1728, 1638, 1600
$[\alpha]_D^{20}$	−56.9° (*c* 1.1, MeOH)
Rf (Silica gel 60 F_{254})	0.69 ($CHCl_3$-MeOH-28% aqueous ammonia, 4:4:1)
Solubility	Soluble in DMSO, MeOH, THF Insoluble in hexane, ether, $CHCl_3$, H_2O

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. The active fraction (retention time, 13 minutes) was concentrated and extracted with ethyl acetate at pH 2.5. The organic layer was concentrated to dryness under reduced pressure to give **1** (240 mg) as a pale brown powder.

Physico-chemical Characteristics

The physico-chemical properties of **1** are summarized in Table 1. The isolation procedure of **1**, extraction with ethyl acetate at pH 2 and back-extraction with water at pH 7.5, indicated its acidic nature. **1** was soluble in MeOH, THF, DMSO and alkaline water but insoluble in ether, chloroform, hexane and water. **1** was positive to $FeCl_3$, vanillin-H_2SO_4 and iodine reactions. The free form of **1** was unstable in solution; it gradually decomposed during NMR experiments for two weeks in DMSO-d_6 or CD_3OD. Its alkaline metal salts were stable for at least five months under the same experimental conditions. The IR (Fig. 3) absorption bands at 3000~2300 and 1728 cm^{-1} suggested the presence of a carboxylic acid. A

Fig. 3. IR spectrum (KBr) of tetronothiodin.

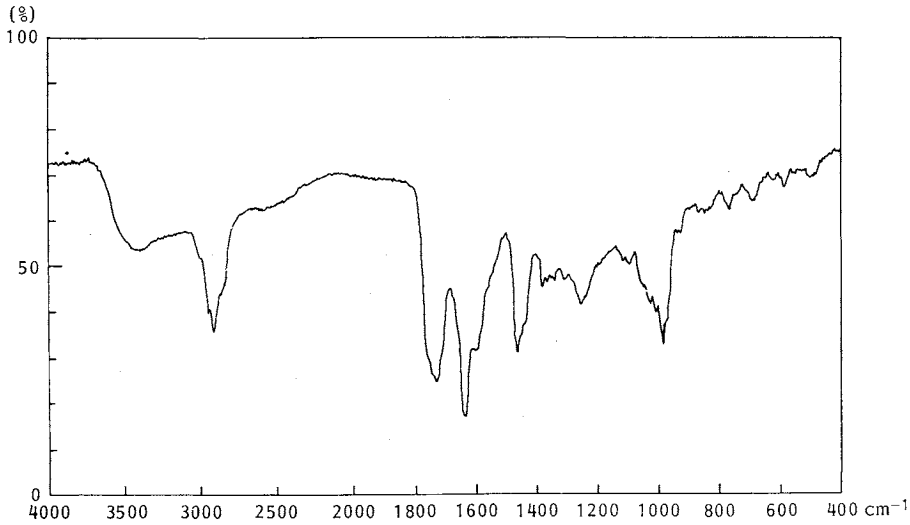
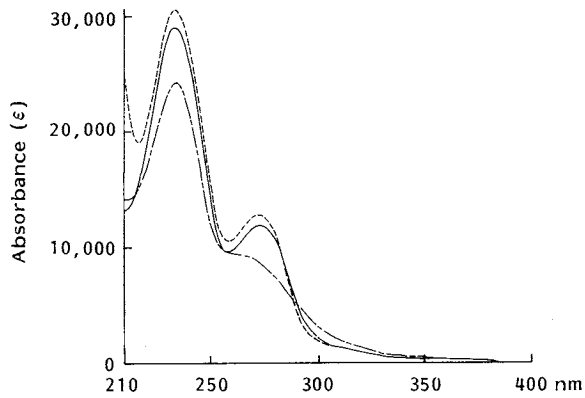


Fig. 4. UV spectrum of tetronothiodin.

—— MeOH, – – – MeOH-NaOH, —·— MeOH-HCl.



γ-lactone function was also suggested by the shoulder band at 1760 cm^{-1} (KBr), which clearly separated from a large carbonyl band (1728 cm^{-1}) in THF. The UV spectrum (Fig. 4) in MeOH showed absorption maxima at 233 and 273 nm and hypochromic effect was observed in acidic methanol. These absorption maxima were attributable to an α-acyltetronic acid chromophore[13,14] with the former absorption maximum being partly due to a diene chromophore. The molecular formula ($C_{31}H_{38}O_8S$) determination was based on positive ion FAB-MS and negative ion HRFAB-MS data [569.2237, calcd for $(M-H, C_{31}H_{37}O_8S)^-$ 569.2210]. The molecular formula was supported by the analyses of the ^1H NMR spectrum (Fig. 5) and the ^{13}C NMR spectrum (Fig. 6) showing 31 carbon signals, and by qualitative analysis for sulfur[15]. These physico-chemical properties indicated that the structure of **1** was different from known CCK receptor antagonists of microbial origin such as virginiamycin M_1 analogues[16], anthramycin[17] and asperlicin[18]. The chromophore, α-acyltetronic acid, is commonly contained in some antibiotics such as kijanimicin[13], tetrocarcins[14] and MM 46115[19]. However **1** was different from these antibiotics in terms of containing a sulfur atom in the molecule.

Biological Activities

The inhibitory activities against the binding of [^{125}I]-CCK_8 to CCK-A and CCK-B receptors were observed by the following procedures. Test samples were incubated at 23°C with [^{125}I]-CCK_8 and rat pancreatic membranes (CCK-A receptors) or rat cerebral cortex membranes (CCK-B receptors) in a

Fig. 5. 400 MHz ^1H NMR spectrum of the potassium salt of tetronothiodin in D_2O.

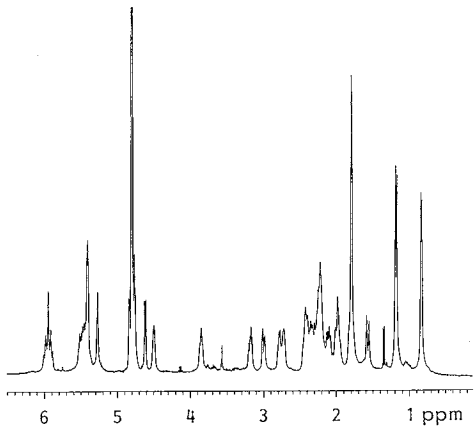
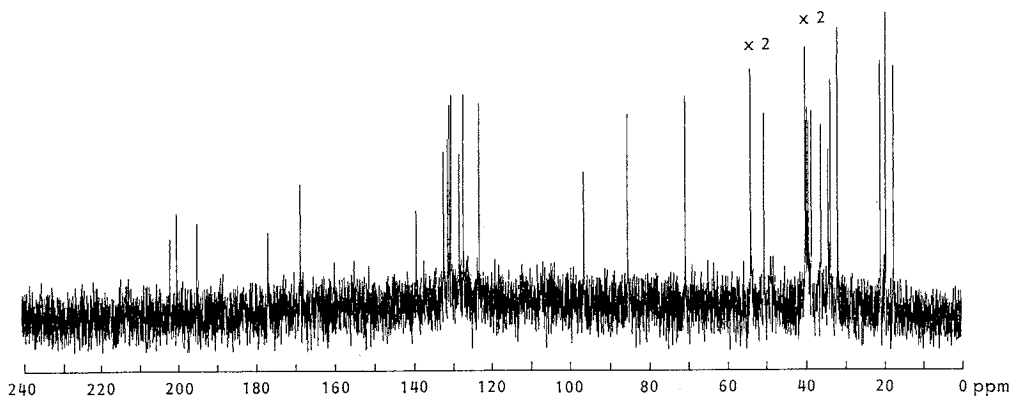


Table 2. Inhibition of [^{125}I]-CCK_8 binding to CCK-A (from rat pancreas) and CCK-B (from rat cerebral cortex) receptors.

Compound	IC_{50} (nM)	
	CCK-A	CCK-B
Tetronothiodin (**1**)	>100,000	3.6
L-365,260	2,700	9.2
CI-988	Not done	14
CCK_8	0.27	1.2

Fig. 6. 100 MHz ^{13}C NMR spectrum of the potassium salt of tetronothiodin in D_2O.



10 mM 2-(*N*-morpholino)ethanesulfonate buffer (pH 6.5) containing NaCl 130 mM, $MgCl_2$ 5 mM, bacitracin 0.02% and bovine serum albumin (0.2% and 0% for CCK-A and CCK-B receptors, respectively). After equilibrium was reached (120 minutes for CCK-A receptors or 20 minutes for CCK-B receptors), each mixture was filtered by a Durapore HVLP filter and the radioactivity of the filter was counted by an autogamma counter.

1 inhibited the binding of [^{125}I]-CCK_8 to CCK-B receptors on rat cerebral cortex membranes in a concentration dependent manner with an IC_{50} of 3.6 nM (Table 2). The affinity to CCK-B receptors of **1** was three or four times more potent than those of L-365,260[20] or CI-988[9] known as potent and selective CCK-B receptor antagonists, and only three times less potent than the natural ligand CCK_8 (IC_{50} = 1.2 nM). However **1** did not inhibit the binding of [^{125}I]-CCK_8 to rat pancreatic membranes (CCK-A receptors). The ratio of the affinity for CCK-A to CCK-B receptors of **1** was more than 27,000, which was 90-fold greater than the -A and -B affinity ratio of L-365,260 (300). **1** was thus revealed to be a highly selective binding inhibitor of CCK-B receptors.

GH3 cells were reported to express CCK-B receptors[21]. This fact was corroborated by our results that **1** inhibited CCK_8 binding to GH3 cells with an IC_{50} of 4.2 nM, which was of the same order as that for brain CCK-B receptors. It had also been demonstrated that the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in GH3 cells was increased by CCK_8 in a concentration dependent manner at 1 to 1,000 nM[22] using the Fura-2 method[23]. CCK_8 (100 nM) transiently increased $[Ca^{2+}]_i$ from 448 nM (basal level) to 739 nM. This stimulation caused by 100 nM CCK_8 was 97% of the maximum stimulation obtained by the treatment with 1 μM CCK_8. The effect of **1** to this Ca^{2+} mobilization was investigated by measuring $[Ca^{2+}]_i$ in GH3 cells. When GH3 cells were treated with **1** (1 μM) one minute prior to the treatment of 100 nM CCK_8 which induced a submaximum increase of $[Ca^{2+}]_i$, **1** inhibited this increase completely without affecting the basal level. This inhibitory activity was concentration dependent. At the concentration of 50 nM of CCK_8 which causes about 80% stimulation of the maximum $[Ca^{2+}]_i$ increase, pretreatments of GH3 cells with 1, 10, 100, and 1,000 nM of **1** inhibited the $[Ca^{2+}]_i$ increase by 12, 55, 70 and 93%, respectively. The IC_{50} against the increase of $[Ca^{2+}]_i$ induced by 50 nM CCK_8 was 26 nM. These results indicate that **1** acted as an antagonist of CCK-B receptors on GH3 cells.

1, at concentrations up to 9 μM, did not show cell growth inhibitory activity against HeLa cells. **1** was inactive against bacteria (*Bacillus subtilis*, *Micrococcus luteus* and *Escherichia coli*) and fungi (*Candida albicans*, *Aspergillus fumigatus*, *Trichophyton mentagrophytes* and *Pyricularia oryzae*) at concentrations up to 450 μM.

Discussion

The structure of **1** is completely different from natural CCK-B receptor antagonists (virginiamycin M_1 analogues[16] and anthramycin[17] produced by *Streptomyces* sp.) and a CCK type-A receptor antagonist (asperlicin produced by *Aspergillus alliaceus*[18]) of microbial origin. It is also different from the other CCK antagonists[24]: (1) cyclic nucleotides (dibutyryl cyclic GMP), (2) amino acids (proglumide, lorglumide and loxiglumide), (3) partial sequences and derivatives of the *C*-terminal heptapeptides of CCK (CCK-JMV-180), (4) benzodiazepines (devazepide and L-365,260) and (5) nonpeptide "peptoids" derived from fragments in the CCK molecule (CI-988). **1** is structurally related to some antibiotics such as kijanimicin[13], tetrocarcins[14] and MM 46115[19] in terms of the macrocyclic molecule containing an α-acyltetronic acid chromophore. In contrast to these antibiotics, **1** is inactive against *Bacillus subtilis* and *Micrococcus luteus*.

A CCK-B receptor antagonist, L-365,260, increased food intake in rats[5]. An anxiolytic activity[8,9] and

enhancement of morphine analgesia[6,7] by CCK-B receptor antagonists in rats were also demonstrated by L-365,260 and CI-988. The possibility of clinical application of CCK-B receptor antagonists was suggested by these studies. However, physiological and pharmacological roles of CCK-B receptors are not yet fully understood partly because of the shortage of potent and specific CCK-B receptor antagonists. **1** is a novel, potent and highly selective CCK-B receptor antagonist. It will be a useful tool for the investigation of the physiological and pharmacological roles of CCK-B receptors. Full details of the biological activities will be reported elsewhere[25].

Acknowledgments

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