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

Sir:

Cholecystokinin (CCK) is a hormonal regulator of pancreatic secretion as well as gallbladder contraction and gut motility. It has also been proposed as a neurotransmitter in central nervous system. In our screening program for new binding inhibitors of brain-type CCK (CCK-B) receptors from microorganisms, we discovered a novel CCK-B receptor antagonist, tetronothiodin (**1**) from the culture filtrate of *Streptomyces* sp. NR0489†. In this communication, we describe the fermentation, isolation, structural elucidation and biological properties of **1**.

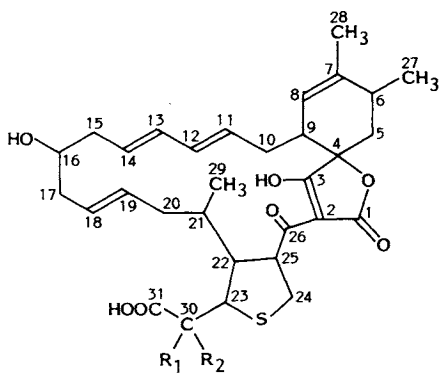
The receptor binding assay was carried out as follows. Broth samples were incubated at 23°C with ^{125}I-labeled CCK8 (*C*-terminal octapeptide of cholecystokinin) and rat cerebral cortex membrane (CCK-B receptors) in a 10 mM 2-(*N*-morpholino)-ethanesulfonate buffer (pH 6.5) containing NaCl 130 mM, $MgCl_2$ 5 mM and bacitracin 0.02%. After 20 minutes, each mixture was filtered by a Durapore HVLP filter and the radioactivity of the filter was counted.

Strain NR0489 was cultured in 50-liter jar fermenters containing each 30 liters of a medium consisting of glucose 1.5%, soluble starch 1%, meat extract 0.75%, Polypepton 0.5%, yeast extract 0.1%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.2% and Nissan Disfoam CA-115 0.05% (pH 7.0). The cultivation was carried out at 27°C for 10 days, agitated at 300 rpm and aerated at 30 liters per minute.

The broth filtrate (191 liters) was adjusted to pH 7 with 6 N HCl and applied to a column of Diaion HP-21. The column was washed with water and 10% aqueous acetone, and the active principle was successively eluted with 50% aqueous acetone. The active eluate was concentrated under reduced pressure and back-extracted with water at pH 7.5. The water layer was applied to a column of QAE Sephadex A-25, which was stepwise developed with NaCl solutions (0~0.5 M). The active eluate (NaCl 0.3~0.5 M) was extracted with ethyl acetate at pH 2 and the organic layer was concentrated under reduced pressure to give an oily residue, which was chromatographed on a Sephadex LH-20 column with MeOH. The active eluate was concentrated under reduced pressure and purified by HPLC over a C-8 reversed-phase silica gel column (YMC-Pack, 30×250 mm; Yamamura Chemical Lab.) 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** (63 mg) as a pale brown powder.

Physico-chemical properties of **1** are summarized in Table 1. Its molecular formula was determined to be $C_{31}H_{38}O_8S$ by HRFAB-MS (negative) and was supported by ^{1}H and ^{13}C NMR spectral data

Fig. 1. Structures of **1** and its derivatives **2a** and **2b**.



1 $R_1, R_2 = =O$
2a, 2b $R_1 = H$ $R_2 = OH$

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

Appearance	Pale brown powder
Molecular formula	$C_{31}H_{38}O_8S$
FAB-MS (positive)	593 $(M+Na)^+$, 609 $(M+K)^+$
HRFAB-MS (negative)	569.2237 $(M-H)^-$, Calcd: 569.2210
UV λ_{max}^{MeOH} nm (ε)	233 (29,900), 273 (12,200)
$\lambda_{max}^{MeOH-NaOH}$ nm (ε)	234 (31,000), 271 (13,500)
$\lambda_{max}^{MeOH-HCl}$ nm (ε)	234 (25,000), 269 (sh, 9,800)
IR ν_{max} (KBr) cm^{-1}	3700~2300 (br), 1760 (sh), 1728, 1638, 1600
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, $CHCl_3$, H_2O

† Taxonomic studies of this organism will be reported elsewhere.

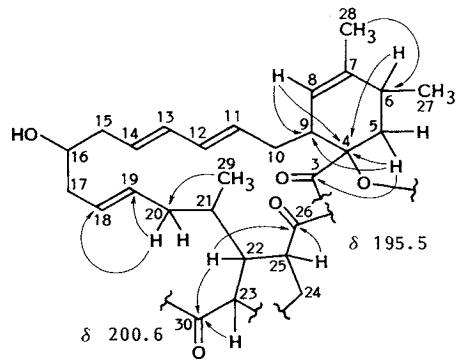
Table 2. NMR spectral data for potassium salt of **1** in D_2O.

No.	δ_C	δ_H
1	177.2	
2	96.6	
3	202.4	
4	85.6	
5	38.6	1.58 (1H, br d, J=13 Hz), 2.10 (1H, dd, J=7, 13 Hz)
6	32.0	2.32 (1H)
7	139.5	
8	123.3	5.28 (1H, br s)
9	39.7	2.73 (1H)
10	34.3	2.23 (2H)
11	131.0	5.50 (1H, m)
12	130.6	5.99 (1H, m)
13	132.5	5.92 (1H, m)
14	128.5	5.45 (1H, m)
15	40.1[a]	2.30 (1H), 2.43 (1H)
16	70.9	3.86 (1H, m)
17	40.05[a]	2.25 (1H), 2.42 (1H)
18	127.5[b]	5.41 (1H)
19	131.5[b]	5.41 (1H)
20	39.3	1.81 (1H), 2.00 (1H, m)
21	33.7	1.99 (1H, m)
22	50.7	2.75 (1H, m)
23	54.1[c]	4.62 (1H, d, J=6 Hz)
24	36.1	3.02 (1H, br d, J=12 Hz), 3.18 (1H, dd, J=7, 12 Hz)
25	54.0[c]	4.50 (1H, m)
26	195.5	
27	19.7	1.19 (3H, d, J=7 Hz)
28	21.1	1.80 (3H, br s)
29	17.7	0.84 (3H, d, J=7 Hz)
30	200.6	
31	169.0	

[a~c] These assignments are interchangeable.

Fig. 2. Partial structure of **1** elucidated by the ^1H-^1H COSY and HMBC experiments on **1**.

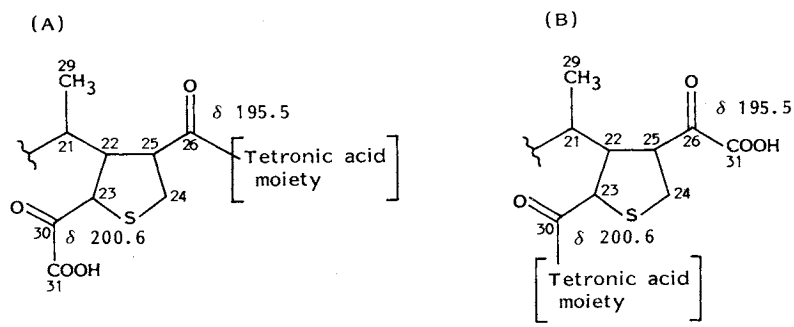
→ ^{13}C-^1H long range coupling.



and qualitative analysis for sulfur. The UV spectrum showed absorption maxima at 233 and 273 nm assignable to α-acyltetronic acid[1]. The IR spectrum showed absorption bands at 3700~2300 (br) and 1728 cm^{-1} assignable to a carboxyl group. Since the free acid of **1** was unstable in solution, the potassium or sodium salt of **1** was used for NMR experiments. The ^1H and ^{13}C NMR spectral data for the potassium salt of **1** in D_2O are shown in Table 2.

The ^1H-^1H COSY experiments on the potassium salt of **1** in D_2O established the following connectivities of the carbons: $-C5H_2-$ C6H–C27H_3, C28H_3–C7=C8H– (by an allylic coupling between 28-H and 8-H), –C9H–(C10H_2···C17H_2)–C18H=, =C19H–C20H_2– and C29H_3–C21H–C22H(C23H)–C25H–C24H_2–. These fragments and 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 a partial structure in Fig. 2 based on the analysis of the ^{13}C-^1H long range couplings obtained by HMBC experiments. The geometries

Fig. 3. Possible partial structures for the tetrahydrothiophene moiety elucidated from the NMR spectral data for **1**.



of the three disubstituted double bonds were determined to be *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 hydroxyl (δ 4.57) and 16-H (δ 3.57) signals in the ^{1}H-^{1}H COSY spectrum of the sodium salt of **1** taken in DMSO-d_6.

UV absorption maxima at 233 and 273 nm[1] and an IR absorption band at 1760 cm^{-1} (sh, γ-lactone) suggested an α-acyltetronic acid structure, which was confirmed by comparison of the ^{13}C NMR spectral data of **1** with those of a carolic acid derivative[2]. 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 revealed to be attached to the cyclohexene ring at C-4 because of the ^{13}C-^{1}H long range coupling between 5-H and C-3.

Although 23-H and 24-H were not coupled each other, a ^{13}C-^{1}H long range coupling was observed between 23-H and C-24 suggesting the linkage of these two carbons through a heteroatom or quaternary carbon. Taking into consideration of as yet remaining units (one sulfur atom and one carboxylic acid), 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. Since the ^{13}C chemical shift of the ketone of concern (δ 200.6) was not compatible with the general trend[3] that the ketones in cyclopentanones were observed at the region lower than 210 ppm, it is most reasonable to locate a sulfur between these two carbons forming a tetrahydrothiophene ring. 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)-3,4-tetrahydrothiophenedicarboxylate[4], and by the structural analysis of a reduced derivative (*vide infra*).

The establishment of the tetrahydrothiophene structure left two possible structures A and B for **1** (Fig. 3). To allocate the only remaining carboxylic function at C-26 or C-30, **1** was reduced by $NaBH_4$ to give epimeric alcohols **2a** and **2b** (Fig. 1). The ^{1}H-^{1}H COSY experiment on potassium salt of **2a** in D_2O revealed a proton sequence; 30-H (δ 3.95), 23-H (δ 3.60), 22-H (δ 3.03), 25-H (δ 4.19) and 24-H (δ 2.90 and 3.36). This result established the structure of this moiety to be represented by A in Fig. 3. The structure of **1** was thus elucidated as shown in Fig. 1.

The inhibitory activities of **1** against the binding of CCK8 to CCK-B receptors prepared from rat cerebral cortex and peripheral-type receptors (CCK-A) prepared from pancreas were measured and compared with those of L-365,260[5] and PD134308[6] which were known as potent CCK-B receptor antagonists (Table 3). **1** inhibited CCK8 binding to rat cerebral cortex membranes (CCK-B) with IC_{50} of 3.2 nM, but did not inhibit the binding to the rat pancreatic membranes (CCK-A). The inhibitory activity of **1** to CCK-B receptors was three or four times more potent than those of the known antagonists. **1** inhibited CCK8-stimulated increase[7] of intracellular calcium concentration in GH3 cells, a rat anterior pituitary tumor cell line exhibiting CCK-B receptors[8]. Detailed biological activities of **1** will be reported elsewhere.

Table 3. Inhibition of CCK8 binding to CCK-A (from rat pancreas) and CCK-B (from rat cerebral cortex) receptors.

Compound	IC_{50} (nM)	
	CCK-A[a]	CCK-B
Tetronothiodin	>100,000	3.2
L-365,260	2,700	9.2
PD134308	Not done	14

[a] The inhibitory activity to CCK-A receptors was observed with rat pancreatic membrane as the receptors by the same method to that for CCK-B receptors with minor modifications.

Acknowledgments

The authors thank Dr. R. IMHOF of F. Hoffmann-La Roche Research Center for kindly providing samples of L-365,260 and PD134308. The authors are grateful for useful discussions with Drs. T. OKUDA, and M. ARISAWA, and special thanks are due Messrs. K. WATANABE, T. SANO and S. OHSHIMA for large scale fermentation at Nippon Roche Research Center.

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(Received September 19, 1991)

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