Communication to the Editor

PROMOTHIOCINS A AND B, NOVEL THIOPEPTIDES WITH A *tipA* PROMOTER INDUCING ACTIVITY PRODUCED BY *Streptomyces* sp. SF2741

Sir:

Thiostrepton is well known as an antibiotic which inhibits protein synthesis[1]. Recently, THOMPSON and his colleagues discovered that this compound induced the expression of many genes of unknown function in *Streptomyces lividans*[2,3]. The *tipA* gene which encodes one of these proteins was cloned and sequenced[2]. The *tipA* promoter (*ptipA*) was inserted in a promoter probe vector (pIJ486) so that it controlled the expression of a kanamycin resistance gene (pAK114). Although *S. lividans* (pAK114) was sensitive to kanamycin in the absence of thiostrepton, it became highly resistant to the antibiotic in the presence of very small amounts of thiostrepton ($<10^{-9}$ M). Antibiotics structurally related to thiostrepton such as nosiheptide also induced *ptipA*, however, unrelated antibiotics did not. Here we report the use of this specific and sensitive assay to screen for thiostrepton-like compounds.

Paper disks saturated with screening broth were placed on a kanamycin containing (5 μg/ml) nutrient agar plate seeded with a spore suspension of *S. lividans* (pAK114). After incubation for 24 hours at 30°C, a zone of *S. lividans* (pAK114) growth around the disk was presumed to indicate the presence of a thiopeptide antibiotic. As a result of this screening, novel thiopeptides, promothiocins A and B were isolated from *Streptomyces* sp. SF2741. In this communication, we report the fermentation, isolation and structure elucidation of these two compounds.

An inoculum was prepared by cultivating the producing organism for 48 hours at 28°C in a 30-liter jar fermentor containing 20 liters of the medium consisting of starch 2.0%, glucose 1.0%, polypeptone 0.5%, oatmeal 0.6%, yeast extract 0.3%, soybean meal 0.2% and $CaCO_3$ 0.2%. The fermentation was carried out in a 2,000-liter jar fermentor for 96 hours at 28°C containing 1,000 liters of the medium consisting of starch 1.0%, glucose 2.0%, soybean meal 1.5%, oatmeal 0.8%, polypeptone 0.1%, NaCl 0.1%, $ZnSO_4$ 0.001% and $CaCO_3$ 0.2%. The pH of the medium was adjusted to 7.0 before sterilization.

The harvested culture was filtered with a filter press and the mycelial cake was extracted with acetone. After concentration *in vacuo*, the resulting aqueous solution was adjusted to pH 4.0 and extracted with EtOAc. The solvent layer was dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and the residue was subjected to silica gel column chromatography. After washing with $CHCl_3$-MeOH (50:1), active substances were eluted with $CHCl_3$-MeOH (20:1). The active eluate was concentrated *in vacuo*, and applied to a Sephadex LH-20 column which was developed with $CHCl_3$-MeOH (1:2). After concentration *in vacuo*, the active fraction was purified by preparative TLC with $CHCl_3$-acetone (1:1.3) to yield promothiocins A and B as pure non-crystalline solids (30 mg and

Table 1. Physico-chemical properties of promothiocins A and B.

	Promothiocin A	Promothiocin B
Appearance	White powder	White powder
MP	268~272 (dec)	250~255 (dec)
Molecular formula	$C_{36}H_{37}N_{11}O_8S_2$	$C_{42}H_{43}N_{13}O_{10}S_2$
HRFAB-MS (*m/z*)		
Found:	816.2405 $(M+H)^+$	954.2809 $(M+H)^+$
Calcd:	816.2347	954.2780
$[\alpha]_D^{21}$	+79.2° (*c* 0.69, $CHCl_3$-MeOH, 1:1)	+62.5° (*c* 0.30, $CHCl_3$-MeOH, 1:1)
UV λ_{max}^{MeOH} nm (ε)	224 (34,000), 313 (9,000, sh)	225 (42,000), 313 (10,000, sh)
IR ν_{max} (KBr) cm^{-1}	3420, 3350, 2940, 1690~1640, 1550~1500, 1190, 1040	3420, 3360, 2950, 1695~1645, 1550~1490, 1195, 1030
Amino acid analysis	Alanine, glycine, valine	Alanine, glycine, valine

Table 2. ^{13}C NMR and ^{1}H NMR data for promothiocins A and B in DMSO-d_6.

Position	δ_C		δ_H	
	A	B	A	B
Thiazole (1)				
C-2	163.7	163.5		
C-4	149.0	149.0		
C-5	126.9	127.0	8.38 (s)	8.37 (s)
CO	159.7	159.7		
Valine				
NH			8.03 (d, 9.0)	8.03 (d, 9.0)
α-CH	57.4	57.5	4.40 (dd, 9.0, 6.4)	4.39 (dd, 9.0, 6.4)
β-CH	31.2	31.2	2.20 (m)	2.19 (m)
γ-CH_3	18.3	18.3	0.97 (d, 6.6)	0.97 (d, 6.6)
γ-CH_3	19.4	19.4	0.98 (d, 6.9)	0.98 (d, 6.9)
CO	170.6	170.7		
Methyloxazole (1)				
NH			8.84 (dd, 7.4, 4.2)	8.84 (dd, 7.2, 4.2)
CH_2	35.2	35.2	4.57 (dd, 16.0, 7.4), 4.21 (dd, 16.0, 4.2)	4.57 (dd, 16.0, 7.2), 4.20 (dd, 16.0, 4.2)
C-2	158.5	158.5		
C-4	128.6	128.6		
C-5	152.6	152.7		
CH_3-5	11.2	11.3	2.54 (s)	2.54 (s)
CO	160.1	160.2		
Thiazole (2)				
NH			8.11 (d, 8.5)	8.12 (d, 8.6)
α-CH	45.2	45.2	5.44 (m)	5.44 (m)
β-CH_3	19.8	19.7	1.62 (d, 6.9)	1.62 (d, 7.0)
C-2	171.8	171.7		
C-4	148.5	148.5		
C-5	125.0	125.1	8.25 (s)	8.26 (s)
CO	159.8	159.9		
Methyloxazole (2)				
NH			8.21 (d)	8.22 (d)
α-CH	43.1	43.2	5.04 (m)	5.05 (m)
β-CH_3	18.0	18.1	1.50 (d, 7.0)	1.50 (d, 7.0)
C-2	161.8	162.0		
C-4	132.3	132.3		
C-5	149.2	149.1		
CH_3-5	11.5	11.4	2.67 (s)	2.60 (s)
Pyridine				
C-2	148.0	148.0		
C-3	130.3	130.5		
C-4	141.2	141.1	8.54 (d, 8.1)	8.57 (d, 8.1)
C-5	120.6	120.8	8.19 (d, 8.1)	8.21 (d, 8.1)
C-6	149.0	148.8		
CO	161.2	161.5		
Dehydroalanine (1)				
NH			10.68 (s)	10.49 (s)
α-C	133.6	133.5		
β-CH_2	102.4	104.6	6.58 (s), 5.81 (s)	6.68 (s), 5.97 (s)
CO	164.9	162.9		
Dehydroalanine (2)				
NH				10.09 (s)
α-C		136.9		
β-CH_2		112.2		5.76 (s), 5.73 (s)
CO		162.2		
Dehydroalanine (3)				
NH				9.10 (s)
α-C		134.6		
β-CH_2		104.3		6.14 (s), 5.68 (s)
CO		165.1		
NH_2			8.13 (s), 7.64 (s)	7.91 (s), 7.49 (s)

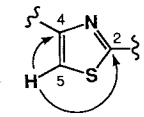
225 mg, respectively).

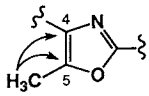
Their physico-chemical properties are shown in Table 1. The molecular formulae of promothiocins A and B were determined as $C_{36}H_{37}N_{11}O_8S_2$ and $C_{42}H_{43}N_{13}O_{10}S_2$, respectively, by HRFAB-MS using *m*-nitrobenzylalcohol and glycerol as a matrix. The IR absorptions at 3420~3350, 1695~1640 and 1550~1490 cm^{-1} suggested that these compounds were of peptidic nature. Amino acid analysis revealed one mole of glycine, alanine and valine in promothiocins A and B.

The ^1H and ^{13}C NMR spectral data of promothiocins A and B are summarized in Table 2. The major component B was used for further structure elucidation. The ^{13}C NMR spectrum of promothiocin B revealed the presence of 42 carbons while its ^1H NMR spectrum showed 43 protons including 9 which were exchangeable. The DQF-COSY experiments[4] revealed the presence of partial structures; $(CH_3)_2$-CH-CH-NH-, CH_3-CH-NH- × 2, -CH=CH- and $-CH_2$-NH-. The presence of thiazole and methyloxazole units were deduced by comparison of the corresponding ^1H and ^{13}C chemical shifts with those of known compounds[5,6]. As shown in Fig. 1, an aromatic proton signal at 8.37 ppm (Thz(1), 5-H) showed long range couplings to carbons at 163.5 ppm (Thz(1), C-2) and 149.0 ppm (Thz(1), C-4), and another aromatic proton signal at 8.26 ppm (Thz(2), 5-H) to 171.7 ppm (Thz(2), C-2) and 148.5 ppm (Thz(2), C-4). These correlations suggested the presence of two thiazoles, which was confirmed by the characteristic coupling constants between 5-H and C-5 of Thz(1) and Thz(2)[7] (J_{C-H} = 192.4 and 194.2 Hz, respectively).

^1H-^{13}C long range correlations were observed from a methyl signal at 2.54 ppm (Oxa(1), CH_3-5) to 128.6 ppm (Oxa(1), C-4) and 152.7 ppm (Oxa(1), C-5), and from a methyl signal at 2.60 ppm (Oxa(2), CH_3-5) to 132.3 ppm (Oxa(2), C-4) and 149.1 ppm

Fig. 1. Chemical shifts of thiazoles and methyloxazoles in promothiocin B and known compounds.





→ : HMBC correlation

		5-H	C-2	C-4	C-5
Promothiocin B	Thz (1)	8.37	163.5	149.0	127.0
	Thz (2)	8.26	171.7	148.5	125.1
Sulfomycin I	Thz (1)	8.53	162.3	148.9	127.0
	Thz (2)	8.45	167.3	148.8	126.5
A10255G	Thz (1)	8.51	163.6	149.4	127.1
	Thz (2)	8.24	168.7	148.7	124.9

		CH_3-5	C-4	C-5
Promothiocin B	Oxa (1)	2.54	128.6	152.7
	Oxa (2)	2.60	132.3	149.1
Sulfomycin I	Oxa (1)	2.55	128.6	153.2
	Oxa (2)	2.56	129.1	153.9

Fig. 2. ^1H-^{13}C long range couplings observed by the HMBC experiments.

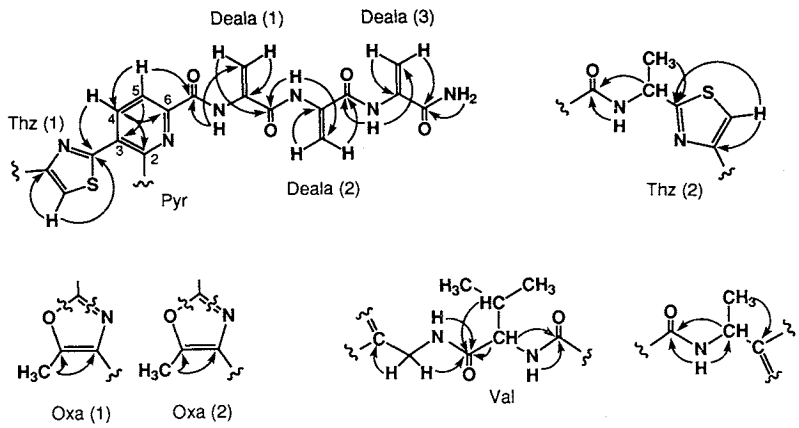
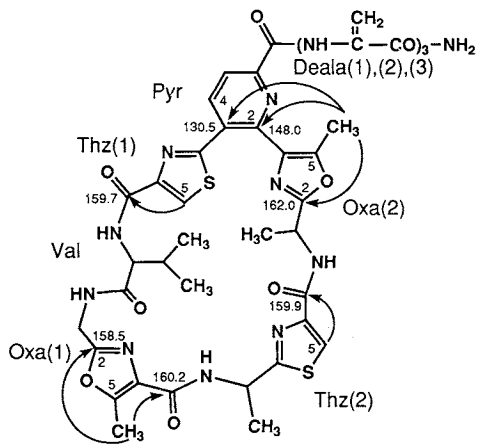


Fig. 3. Structure of promothiocin B elucidated by D-HMBC.



→ : D-HMBC correlations

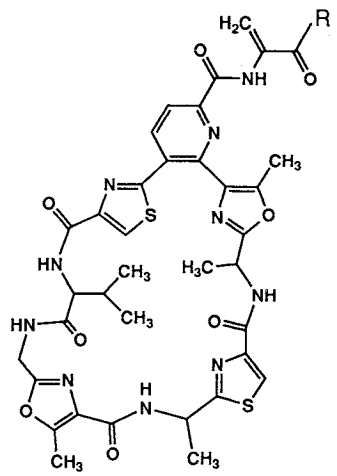
(Oxa(2), C-5), which suggested the presence of two methyloxazoles by comparison of the chemical shifts of these protons and carbons with those of sulfomycin I as shown in Fig. 1.

Six olefinic protons at 6.68, 6.14, 5.97, 5.76, 5.73 and 5.68 ppm were assigned as three terminal methylenes by HSQC experiments[8] in the dehydroalanine residues attached to a carbonyl carbon on the pyridine ring (Fig. 2). The dehydroalanine side chain has often been observed in thiopeptide antibiotics. The large vicinal-coupling constants between two aromatic doublet protons at 8.57 ppm and 8.21 ppm ($J_{4-5}=8.1$ Hz), and their ^{1}H-^{13}C long range connectivities revealed the presence of a 2,3,6-trisubstituted pyridine residue (Fig. 2).

The connectivities of the above partial structures and the other remaining fragments were established by HMBC experiments[9]. The partial structures shown in Fig. 2 were further connected by a new technique, phase-sensitive ^{13}C-decoupled HMBC (D-HMBC)[10] which enabled observation of long range ^{13}C-^{1}H couplings separated by four or five bonds in addition to the very small long range couplings separated by two or three bonds. This technique revealed the correlation from 5-H of Thz(1) to 159.7 ppm (Thz(1), CO), from 5-H of Thz(2) to 159.9 ppm (Thz(2), CO), from CH_3-5 of Oxa(1) to 160.2 ppm (Oxa(1), CO) and 158.5 ppm (Oxa(1), C-2) and from CH_3-5 of Oxa(2) to 162.0 ppm (Oxa(2), C-2) and to 148.0 ppm (Pyr, C-2) and 130.5 ppm (Pyr, C-3). Thus, the planar structure of B was established as shown in Fig. 3.

^{1}H and ^{13}C NMR spectra of promothiocin

Fig. 4. Structures of promothiocins A and B.



A: R = $-NH_2$

B: R = $-NH-\overset{CH_2}{\overset{\|}{C}}-CO-NH-\overset{CH_2}{\overset{\|}{C}}-CO-NH_2$

A lacking the signals due to two dehydroalanine residues implied its structure as shown in Fig. 4. Details of the structure determination for promothiocins A and B will be reported elsewhere. Minimum induction concentrations of promothiocins A and B for *tipA* promoter were 0.2 and 0.1 μg/ml, respectively. The biological activities of promothiocins are now under investigation.

Acknowledgments

We are grateful to Meiji Seika Kaisha for providing us with *Streptomyces* sp. SF2741 and for large scale fermentation.

BONG-SIK YUN
TOMOMI HIDAKA
KAZUO FURIHATA†
HARUO SETO*

Institute of Molecular and Cellular Bioscience,
The University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan
†Department of Agricultural Chemistry,
The University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan

(Recieved December 13, 1993)

References

1) CUNDLIFFE, E.: The mode of action of thiostrepton *in vivo*. Biochem. Biophys. Res. Commun. 44:

912～917, 1971
2) MURAKAMI, T.; T. G. HOLT & C. T. THOMPSON: Thiostrepton-induced gene expression in *Streptomyces lividans*. J. Bacteriol. 171: 1459～1466, 1989
3) HOLMES, D. J.; J. L. CASO & C. J. THOMPSON: Autogenous transcriptional regulation of a thiostrepton-induced gene in *Streptomyces lividans*. EMBO J. 8: 3183～3191, 1993
4) PIANTINI, U.; O. W. SORENSEN & R. R. ERNST: Multiple quantum filters for elucidating NMR coupling networks. J. Am. Chem. Soc. 104: 6800～6801, 1982
5) ABE, H.; K. KUSHIDA, Y. SHIOBARA & M. KODAMA: The structures of sulfomycin I and berninamycin A. Tetrahedron Lett. 29: 1401～1404, 1988
6) DEBONO, M.; R. M. MOLLOY, J. L. OCCOLOWITZ, J. W. PASCHAL, A. H. HUNT, K. H. MICHEL & J. W. MARTIN: The structure of A10255B, -G and -J: New thiopeptide antibiotic produced by *Streptomyces gardneri*. J. Org. Chem. 57: 5200～5208, 1992
7) KALINOWSKI, H.; S. BERGER & S. BRAUN: Carbon-13 NMR Spectroscopy, pp. 495～512, John Wiley & Sons Ltd., 1988
8) BODENHAUSEN, G. & D. J. RUBEN: Natural abundance nitrogen-15 NMR by enhanced heteronuclear spectroscopy. Chem. Phys. Lett. 69: 185～189, 1980
9) BAX, A.& M. F. SUMMERS: ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. J. Am. Chem. Soc. 108: 2093～2094, 1986
10) FURIHATA, K. & H. SETO: HMBC-COSY and HMBC-HOHAHA, new application of HMBC method. Abstract papers of 15th NMR symposium, pp. 5～8, Himeji, Nov. 17～19, 1992