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Thiotipin, A Novel Thiopeptide with a *tipA* Promoter Inducing Activity Produced by *Streptomyces* sp. DT31

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Abstract: Thiotipin was isolated from the mycelium of *Streptomyces* sp. DT31 as a *tipA* promoter inducing substance. Based on various NMR studies including ^{1}H-^{1}H COSY, HSQC, FG-HMBC, phase-sensitive ^{13}C-decoupled HMBC (D-HMBC) and NOE, its structure was established as a thiopeptide with oxazoles, thiazoles and several modified amino acids. Minimum induction concentration of thiotipin for *tipA* promoter was 80 ng/ml.

The thiopeptide antibiotics are characterized by structural features containing thiazole(s) and/or oxazole(s) and unusual amino acids in mono-, di- or tri-cyclic peptide ring(s). This class antibiotics give rise to great concern in biologists because of their new unique biological activities. Several members of the thiopeptide antibiotics such as thiostrepton, nosiheptide, berninamycin, micrococcin, thiopeptin and sulfomycin induced the expression of many genes of unknown function in *Streptomyces lividans*[1]. Of them, the *tipA* gene including its promoter region was cloned and sequenced[1]. *TipA* proved to be a regulatory protein which autogenously activates transcription of its own promoter after interacting with thiostrepton or other related thiopeptide antibiotics[2]. This powerful inducible promoter (*ptipA*) has been incorporated into a series of vectors to allow regulated expression of genes in *Streptomyces*. In addition, *ptipA* has been employed in a very sensitive and specific microbiological disc assay to screen for compounds inducing its transcription resulting in isolation of promothiocins A and B[3]. In the course of our continuing screening, thiotipin, a novel thiopeptide, was isolated from the mycelium of *Streptomyces* sp. DT31 (Fig. 1). This paper presents its isolation, structure elucidation and biological properties.

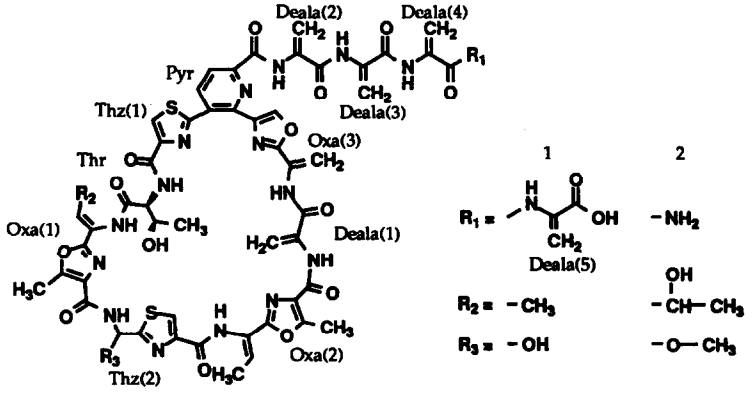


Figure 1. Structures of thiotipin (1) and sulfomycin I (2)

The structures of the thiopeptide antibiotics have been elucidated by X-ray crystallography[4-6] and by chemical degradation studies[6-8]. In recent years, the mass/mass technique provided us with great convenience in their structure determination, especially in settlement of hetero atoms for thiazole and oxazole units[9]. We elucidated the structure of thiotipin by the new NMR technique, phase-sensitive ^{13}C-decoupled HMBC[10], which enabled to observe ^{13}C-^{1}H long range couplings separated by four or five bonds in addition to the very small long range couplings separated by two or three bonds that were not observable under the normal HMBC conditions.

Table 1. ^{13}C and ^{1}H NMR data for thiotipin in DMSO-d_6.

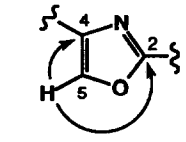
position	δ_C	δ_H	position	δ_C	δ_H
Thiazole (1)			Dehydroalanine (1)		
C-2	162.6		NH		9.25
C-4	149.0		αC	133.7	
CH-5	127.3	8.47	βCH_2	105.8	6.35, 5.76
CO	160.3		CO	162.6	
Threonine			Oxazole (3)		
NH		8.12 (d, 7.7)[a]	NH		9.79
αCH	58.9	4.39 (dd, 7.7, 3.9)	αC	129.2	
βCH	66.5	4.18 (m)	βCH_2	111.4	5.74, 5.73
γCH_3	20.1	1.09 (d, 6.4)	C-2	158.1	
OH		5.18 (b)	C-4	138.9	
CO	169.0		CH-5	140.4	8.67
Oxazole (1)			Pyridine		
NH		9.39	C-2	149.1	
αC	123.3		C-3	130.5	
βCH	127.8	6.43 (q, 7.3)	CH-4	140.6	8.65 (d, 8.1)
γCH_3	13.5	1.73 (d, 7.3)	CH-5	121.7	8.27 (d, 8.1)
C-2	156.6		C-6	146.7	
C-4	128.9		CO	161.4	
C-5	153.3		Dehydroalanine(2)		
CH_3-5	11.5	2.58	NH		10.48
CO	160.7		αC	134.0	
Thiazole (2)			βCH_2	106.0	6.54, 5.94
NH		8.48 (d, 9.0)	CO	162.7	
αCH	70.2	6.63 (dd, 9.0, 3.9)	Dehydroalanine (3)		
OH		7.33 (d, 3.9)	NH		9.97
C-2	171.6		αC	136.3	
C-4	148.9		βCH_2	110.9	5.80, 5.76
CH-5	126.4	8.42	CO	162.9	
CO	159.3		Dehydroalanine (4)		
Oxazole (2)			NH		9.60
NH		9.92	αC	136.2	
αC	123.5		βCH_2	109.2	5.84, 5.68
βCH	129.4	6.45 (m)	CO	162.5	
γCH_3	13.7	1.76 (d)	Dehydroalanine (5)		
C-2	156.9		NH		9.11
C-4	129.1		αC	133.3	
C-5	153.4		βCH_2	109.8	6.10, 5.76
CH_3-5	11.5	2.56	CO	164.8	
CO	159.9				

[a] Proton signal multiplicity and coupling constant (J = Hz) in parentheses.

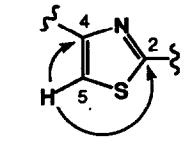
The culture conditions of the producing strain and bioassay-directed (*tipA* promoter inducing activity)[3] isolation procedures of thiotipin are outlined in the Experimental section.

The molecular formula of thiotipin was established as $C_{55}H_{50}N_{16}O_{17}S_2$ by HR-FAB mass spectroscopy (*m/z* 1271.3052(M+H)$^+$ -0.8mmu) in combination with ^{1}H and ^{13}C NMR data. The IR absorptions at

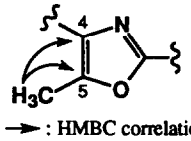
1690~1620 and 1540~1490 cm^{-1} suggested that this compound had a peptidic nature. The ^1H and ^{13}C NMR spectral data of thiotipin are summarized in Table 1. The ^1H NMR spectrum of thiotipin revealed the presence of 49 protons including 12 exchangeable ones. The ^1H-^1H COSY data indicated the presence of ten partial structures including some allylic and homoallylic relations; -CH=CH-, CH_3-CH(OH)-CH-NH-, 2 × CH_3-CH=C-NH-, OH-CH-NH- and 5 × CH_2=C-NH-. The HSQC experiments[11] established all one-bond ^1H-^{13}C connectivities and assigned 12 olefinic or aromatic protons at 6.54, 6.35, 6.10, 5.94, 5.84, 5.80, 3 × 5.76, 5.74, 5.73 and 5.68 ppm as six terminal methylenes.



		H-5	C-2	C-4	C-5
Thiotipin	Oxa (3)	8.67	158.1	138.9	140.4
Sulfomycin I	Oxa (C)	8.64	158.1	138.8	139.9
A10255G	Oxa (4)	8.60	159.4	136.0	141.9



		H-5	C-2	C-4	C-5
Thiotipin	Thz (1)	8.47	162.6	149.0	127.3
	Thz (2)	8.42	171.6	148.9	126.4
Sulfomycin I	Thz (A)	8.53	162.3	148.9	127.0
A10255G	Thz (2)	8.39	172.8	148.0	125.8
	Thz (3)	8.51	163.6	149.4	127.1



		CH_3-5	C-4	C-5
Thiotipin	Oxa (1)	2.58	128.9	153.3
	Oxa (2)	2.56	129.1	153.4
Sulfomycin I	Oxa (A)	2.55	128.6	153.2
	Oxa (B)	2.56	129.1	153.9

→ : HMBC correlations

Figure 2. Chemical shifts of oxazoles, thiazoles and methyloxazoles in thiotipin and known compounds

Three methine protons at 8.67, 8.47 and 8.42 ppm indicated strongly the presence of oxazole and /or thiazole rings. By the HSQC data, these protons were correlated to the carbons at 140.4, 127.3 and 126.4 ppm, respectively, suggesting the presence of one oxazole and two thiazole rings. This assumption was corroborated by HMBC experiments[12] and by good agreement of these NMR chemical shift values with those of sulfomycin I[6] and A10255G[9]. As shown in Fig. 2, a methine proton signal at 8.67 ppm (Oxa(3), H-5) was long range coupled to two quaternary carbons at 158.1 ppm (Oxa(3), C-2) and 138.9 ppm (Oxa(3), C-4). The chemical shifts of the proton and carbon signals showing these long range correlations coincided with those of the corresponding signals of oxazoles in sulfomycin I and A10255G, and the characteristic large coupling constant between H-5 and C-5 of oxazole(3) confirmed the presence of an oxazole ring ($J_{C\text{-}H}$ = 215 Hz)[13]. A methine proton signal at 8.47 ppm (Thz(1), H-5) showed long range couplings to sp^2 quaternary carbons at 162.6 ppm (Thz(1), C-2) and 149.0 ppm (Thz(2), C-4), and another methine proton signal at 8.42 ppm (Thz(2), H-5) to sp^2 quaternary carbons at 171.6 ppm (Thz(2), C-2) and 148.9 ppm (Thz(2), C-4). These correlations together with good agreement of the chemical shifts of protons and carbons with those of known compounds revealed the presence of two thiazole rings (Fig. 2). The characteristic coupling constants between H-5 and C-5 of these thiazoles supported this conclusion ($J_{C\text{-}H}$ = 192 Hz for both)[13]. ^1H-^{13}C long range correlations were observed from a methyl proton at 2.58 ppm (Oxa(1), CH_3-5) to quaternary carbons at 128.9 ppm (Oxa(1), C-4) and 153.3 ppm (Oxa(1), C-5), and from another methyl proton at 2.56 ppm (Oxa(2), CH_3-5) to quaternary carbons at 129.1 ppm (Oxa(2), C-4) and 153.4 ppm (Oxa(2), C-5); these results together

with the good agreement of the NMR spectral data between thiotipin and sulfomycin I[6] suggested the presence of two methyloxazole rings in thiotipin.

Two adjacent aromatic doublet protons at 8.65 and 8.27 ppm in thiotipin (J = 8.1 Hz), their long range connectivities with sp^2 carbons and comparison of the chemical shifts with those of the corresponding pyridine moiety in sulfomycin I revealed the presence of a 2,3,6-trisubstituted pyridine moiety. The ^{13}C chemical shifts of the pyridine moiety in sulfomycin I were 149.0, 130.5, 140.0, 121.5 and 146.6 ppm for C-2, C-3, C-4, C-5 and C-6, respectively, which were in good agreement with those of the corresponding carbons in thiotipin (Table 1).

The connectivities of the above partial units and the other remaining fragments were established by FG-HMBC experiment[12] with delay time set to 80 msec. As shown in Fig. 3, long range couplings from H-5 of pyridine and amide proton of dehydroalanine (2) to an sp^2 carbon at 161.4 ppm indicated that the pyridine ring and dehydroalanine side chain was bridged by an amide carbonyl carbon. Long range correlations from the amide proton of threonine and H-5 of thiazole (1) to a carbonyl carbon at 160.3 ppm, and from H-4 of pyridine to C-2 of thiazole (1) revealed the presence of a threonine-thiazole (1)-pyridine moiety. In addition, CH_3-5 of oxazole (1) showed four-bond long range correlation to a carbonyl carbon at 160.7 ppm indicating the linkage of oxazole (1) and thiazole (2) rings (under this experimental condition, four-bond long range couplings could be observed only with methyl proton signals, which appeared as sharp strong peaks). The FG-HMBC data unambiguously established the four partial structures shown in Fig. 3.

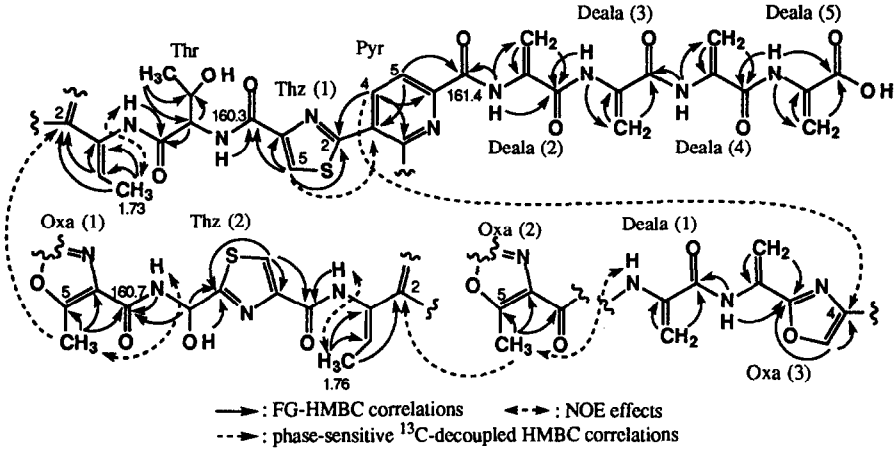


Figure 3. Partial structures of thiotipin and their connectivities

These partial structures were connected by phase-sensitive ^{13}C-decoupled HMBC (D-HMBC)[10]. A D-HMBC experiment optimized for 4.2 Hz with a delay time of 120 msec revealed a long range coupling of an aromatic methine proton at 8.65 ppm (Pyr, H-4) to an sp^2 quaternary carbon at 138.9 ppm (Oxa (3), C-4), which proved the presence of a thiazole-pyridine-oxazole moiety in the cyclic peptide core as seen in sulfomycin[6], berninamycin[7] and A10255[9]. The D-HMBC experiment optimized for 1.25 Hz with a delay time of 400 msec showed long range couplings from the methyl proton signal at 2.58 ppm (Oxa(1), CH_3-5) to an sp^2 quaternary carbon at 156.6 ppm (Oxa(1), C-2) that was correlated with a methyl proton signal at 1.73 ppm, and from another methyl proton signal at 2.56 ppm (Oxa(2), CH_3-5) to an sp^2 quaternary carbon at

156.9 ppm (Oxa(2), C-2) that was correlated with a methyl signal at 1.76 ppm. These results revealed the presence of the two dehydrobutyrines masked by methyloxazoles (1) and (2) at their carboxyl group (Fig. 3). In addition, a four-bond long range coupling from H-5 of Thz(1) to C-3 of Pyr confirmed the linkage of these two moieties. By elimination, the carbonyl carbon of oxazole(2) was suggested to be connected to a remaining amide proton of dehydroalanine(1). This conclusion was proved by NOE effect between the methyl protons of oxazole (2) and amide proton of Deala(1). The above NMR experiments established the structure of thiotipin as shown in Fig. 1. The stereochemistries of the propenyl side chain in Oxa(1) and Oxa(2) were determined to be *Z* by the NOE effects between the methyl protons and amide protons of Oxa(1) and Oxa(2) (Fig. 3). The configuration of L-threonine was established by chiral-TLC analysis (Merck, 14285-1M) of acid hydrolysate. Since the α-position of Thz(2) racemized during the acid hydrolysis[14], its stereochemistry could not be determined. Thiotipin is closely related to sulfomycin I; the structural difference between them which may affect the biological activities is that the terminal carboxylic acid in thiotipin is protected by an amide group in sulfomycin I.

Minimum induction concentration of thiotipin for *tipA* promoter was 80 ng/ml. From the preliminary experiments for structure-activity relationship of the thiopeptides capable of inducing the *tipA* promoter, we found that the dehydroalanine (Deala) side chain of the thiopeptides was closely related to their activities but not essential. Thiopeptides with a longer Deala side chain were more active than those with a shorter one, and Deala chain with a C-terminal amide (thiostrepton, berninamycin and promothiocins) showed higher activity than that with a C-terminal carboxylic acid (thioxamycin and thiotipin). Thiotipin exhibited antibacterial activity against some Gram-positive bacteria including *Streptococcus pneumoniae*, *S. pyogenes* and *Micrococcus luteus* at the level of 3~6 μg/ml.

Experimental

General Methods

Specific rotation was determined on a Jasco DIP-371 digital polarimeter. Mass spectra were measured on a JEOL HX-110 spectrometer in the FAB mode using *m*-nitrobenzyl alcohol-glycerol matrix with polyethylene glycol as internal standard. UV and IR spectra were recorded on a Shimadzu UV-300 and a Jasco A-102 spectrophotometer, respectively. NMR spectra were obtained on a JEOL JNM-A500 spectrometer with ^{1}H NMR at 500 MHz and with ^{13}C NMR at 125 MHz. Chemical shifts are given in ppm using TMS as internal standard. All NMR experiments were performed on 25 mg of thiotipin dissolved in 0.8 ml of DMSO-d_6. The FG-HMBC spectrum set delay time to 80 msec resulted from a 512 × 1,024 ($t_1 \times t_2$) data matrix with 96 scans per t_1 value. The spectral widths were 23,878 Hz for carbon and 5,249 Hz for proton. A pulse repetition time of 1.0 sec was used. The phase-sensitive ^{13}C-decoupled HMBC (D-HMBC) spectrum optimized for about 4.2 Hz with a delay time of 120 msec were obtained from 256 × 2,048 ($t_1 \times t_2$) data matrix. For each t_1 value, 64 scans were recorded with a pulse repetition time of 1.2 sec. The D-HMBC spectrum set delay time to 400 msec for about 1.25 Hz were obtained from 256 × 1,024 ($t_1 \times t_2$) data matrix. 160 scans were recorded with a pulse repetition time of 1.7 sec for each t_1 value.

Fermentation, Extraction and Isolation Procedures

A preculture broth (starch 1.0 %, polypepton 1.0 %, molasses 1.0 % and beef extract 1.0 %; pH 7.2 before sterilization, 12 ml in a test-tube) was inoculated with the stock culture of *Streptomyces* sp. DT31. After incubation for 48 h at 27 °C on a reciprocal shaker, an aliquot of the broth was transferred to six 500-ml

Erlenmeyer flasks each containing 100 ml of the seed medium consisting of glycerol 3.0 %, fish meal 2.0 % and $CaCO_3$ 0.2 % (pH 7.4 before sterilization), and cultivation was carried out for 48 h at 27 °C on a rotary shaker. This seed culture was transferred to a 50-liter jar fermenter containing 30-liters of the same medium as for the seed culture. The fermentation was carried out at 27 °C with aeration of 30 liters per minute and agitation at 400 rpm for 72 hours. The mycelium was collected by centrifugation from harvested culture and extracted with acetone. After concentration *in vacuo*, the resulting aqueous solution was adjusted to pH 4.0 with HCl and then extracted with EtOAc. The solvent layer was dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and the residue was washed with diethyl ether. The precipitate was subjected to stepwise silica gel column chromatography. The active fraction eluted with $CHCl_3$- MeOH (5 : 1) was concentrated, applied to preparative TLC plates and developed with $CHCl_3$: Me_2CO : MeOH (8:8:7). After scraped off the TLC plates and then extracted with $CHCl_3$-MeOH (7:1), the crude active compound was further purified by HPLC with Capcell-pak C_{18} (20 × 250 mm), eluting with a mixture of CH_3CN-H_2O (42:58) / 20 mM H_3PO_4 at a flow rate of 18 ml/min. The active eluate with a retention time of 11 minutes was concentrated *in vacuo*, and the resulting aqueous solution was extracted with EtOAc. The solvent layer was concentrated *in vacuo* to give a white amorphous solid (40 mg) of pure thiotipin; White powder. mp 265~270°C (dec.). $[\alpha]_D$ = -9.5° (*c* 0.15, $CHCl_3$:MeOH (1:1)). UV (EtOH) λ_{max} (ε): 215 (53000, sh), 247 (67000), 315 (9000, sh). IR (KBr): 3400, 1690~1620, 1540~1490, 1430, 1200, 1110, 1085 and 890 cm^{-1}. HRFAB-MS: *m/z* = 1271.3052 (MH^+), $C_{55}H_{50}N_{16}O_{17}S_2$ requires 1271.3060. For NMR data, see Table 1.

Acknowledgments

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