MICROBIAL METABOLITES WITH *tipA* PROMOTER INDUCING ACTIVITY

II. GENINTHIOCIN, A NOVEL THIOPEPTIDE PRODUCED BY *Streptomyces* sp. DD84†

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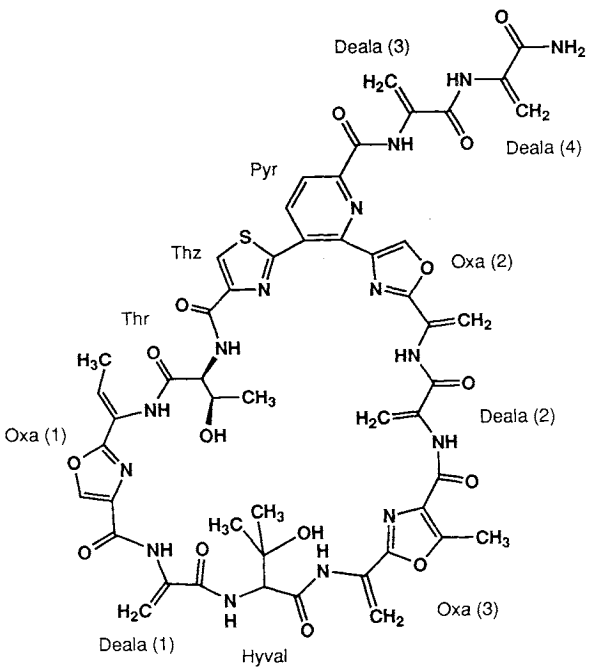
Geninthiocin was isolated from the mycelium of *Streptomyces* sp. DD84 as a *tipA* promoter inducing substance. Based on various NMR studies, its structure was established as a thiopeptide with oxazole and thiazole moieties, and several unusual amino acids.

Thiostrepton is a well known thiopeptide antibiotic which inhibits protein synthesis[1]. Recently, THOMPSON and his colleagues reported that thiostrepton and structurally related substances (several members of the thiopeptide group antibiotics) induced the expression of several genes of unknown function in *Streptomyces lividans*[2,3]. The gene *tipA* which encodes one of these proteins was cloned and sequenced[2]. When the promoter (*ptipA*) of the *tipA* gene was inserted into a promoter probe vector (pIJ486), it also 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 thiostrepton or structurally thiostrepton-related compounds because these substances initiated the transcription of *ptipA*. By utilization of this unique phenomenon, we isolated promothiocins A and B from *Streptomyces* sp. SF2741 which induced *tipA* promoter at ng/ml level[4].

Fig. 1. Structure of geninthiocin.



During the course of our continuing screening for *tipA* promoter inducing

† For part I: see ref. 4.

compounds from microorganisms, a new thiopeptide, geninthiocin, was isolated from the mycelial cake of *Streptomyces* sp. DD84 (Fig. 1). Its physico-chemical properties indicated geninthiocin to be of peptidic nature. The UV, mass, IR and ^{1}H NMR spectral properties of geninthiocin resembled those of neoberninamycin[5] except for its molecular formula established by HR-FAB mass. The structure of neoberninamycin remains still unknown. In this report, we describe the fermentation, isolation, structure elucidation and some biological properties of a new thiopeptide geninthiocin which was produced by *Streptomyces* sp. DD84.

Results

Fermentation

The stock culture of *Streptomyces* sp. DD84, which was isolated from a soil sample collected at Izu-Oshima, Japan, was inoculated into test tubes containing 12 ml of the preculture medium consisting of starch 1.0%, polypepton 1.0%, molasses 1.0% and beef extract 1.0% (pH 7.2 before sterilization). After incubation at 27°C for two days on a reciprocal shaker, an aliquot of the broth (2 ml) was transferred to six 500-ml Erlenmeyer flasks each containing 100 ml of the seed medium consisting of glycerol 2.0%, molasses 1.0%, casein 0.5%, polypepton 0.1% and $CaCO_3$ 0.4% (pH 7.2 before sterilization), and cultivation was carried out at 27°C for two days on a rotary shaker. This seed culture was transferred to a 50-liter jar fermentor 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.

Isolation and Purification

Geninthiocin was isolated from the mycelium as shown in scheme 1 by monitoring the *tipA* promoter inducing activity. The harvested culture was collected with continuous centrifugation at 8,000 rpm 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

Scheme 1. Purification procedure of geninthiocin.

Whole broth (30 liters)
↓ centrifuged
Mycelial cake
↓ extracted with acetone
concentrated *in vacuo*
adjusted to pH 4.0
extracted with EtOAc
Organic layer
↓ concentrated *in vacuo*
Silica gel column
↓ $CHCl_3$-MeOH (20 : 1)
Preparative-HPLC
↓ Capcell-pak C_{18},
eluted with CH_3CN-H_2O
(40 : 60)/20 mM H_3PO_4
Sephadex LH-20 column
↓ $CHCl_3$-MeOH (2 : 1)
Geninthiocin (71 mg)

Table 1. Physico-chemical properties of geninthiocin.

Appearance	White amorphous solid
MP	270~275°C (dec)
Molecular formula	$C_{50}H_{49}N_{15}O_{15}S$
HRFAB-MS (*m/z*)	
Found:	1132.329 $(M+H)^+$
Calcd:	1132.333
$[\alpha]_D^{19}$	+174.3° (*c* 0.1, $CHCl_3$-MeOH, 1 : 1)
UV λ_{max}^{EtOH} nm (ε)	215 (76,000 sh), 240 (85,000), 315 (13,000 sh)
IR ν_{max} (KBr) cm^{-1}	3400 (br), 3000, 2950, 1690~1630, 1590, 1550~1490, 1430, 1200, 1100, 890

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), the active substance was eluted with $CHCl_3$-MeOH (20:1). Further purification was achieved by preparative HPLC with Capcell-pak C_{18} (20×250 mm), eluting with a mixture of CH_3CN-H_2O (40:60)/20 mM H_3PO_4 at a flow rate of 18 ml/minute. The active compound detected by UV absorption at 254 nm produced a peak with a retention time of 18 minutes. The active eluate was concentrated *in vacuo* and the resulting aqueous solution was extracted with EtOAc. After concentration of the organic layer *in vacuo*, the residue was applied to a Sephadex LH-20 column which was developed with $CHCl_3$-MeOH (2:1) to yield a white amorphous

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

position	δ_C	δ_H	position	δ_C	δ_H
Thiazole			Dehydroalanine (2)		
C-2	163.2		NH		9.36
C-4	149.4		αC	133.9	
CH-5	126.9	8.49	βCH_2	105.9	6.36, 5.76
CO	159.9		CO	162.7	
Threonine			Oxazole (2)		
NH		8.00 (d, 9.0)[a]	NH		9.77
αCH	57.8	4.60 (dd, 9.0, 3.0)	αC	129.3	
βCH	67.3	4.29 (m)	βCH_2	111.2	5.70, 5.71
γCH_3	20.5	1.14 (d, 6.2)	C-2	158.1	
OH		4.98 (d, 5.5)	C-4	139.2	
CO	168.8		CH-5	140.5	8.68
Oxazole (1)			Pyridine		
NH		9.60	C-2	149.2	
αC	123.1		C-3	130.2	
βCH	129.6	6.55 (q, 7.3)	CH-4	141.0	8.50 (d, 8.0)
γCH_3	13.8	1.74 (d, 7.3)	CH-5	121.5	8.23 (d, 8.0)
C-2	159.4		C-6	146.8	
C-4	136.1		CO	161.6	
CH-5	142.7	8.70	Dehydroalanine (3)		
CO	158.4		NH		10.53
Dehydroalanine (1)			αC	134.7	
NH		9.39	βCH_2	106.2	6.42, 5.83
αC	133.4		CO	162.0	
βCH_2	103.7	6.46, 5.88	Dehydroalanine (4)		
CO	163.7		NH		9.44
Hydrovaline			αC	135.1	
NH		8.23 (d, 8.5)	βCH_2	106.0	6.03, 5.69
αCH	61.8	4.64 (d, 8.5)	CO	165.1	
βC	71.0		NH_2		7.93, 7.50
γCH_3	27.3	1.22			
γCH_3	26.1	1.20			
OH		5.17			
CO	169.4				
Oxazole (3)					
NH		9.61			
αC	128.5				
βCH_2	105.6	6.13, 5.65			
C-2	155.2				
C-4	129.2				
C-5	154.6				
CH_3-5	11.5	2.62			
CO	159.5				

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

solid (71 mg) of pure geninthiocin.

Structure Elucidation

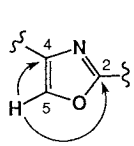
The physico-chemical properties of geninthiocin are summarized in Table 1. Geninthiocin turned brown at 270~275°C. The molecular formula of geninthiocin was determined as $C_{50}H_{49}N_{15}O_{15}S$ by HR-FAB mass spectroscopy using *m*-nitrobenzyl alcohol-glycerol matrix (obsd: 1132.329, calcd: 1132.333 $(M+H)^+$) and by ^1H and ^{13}C NMR spectroscopic analyses. The IR absorption at 3400, 1690~1630 and 1550~1490 cm^{-1} suggested that this compound is a peptide.

The ^1H and ^{13}C NMR spectral data of geninthiocin are summarized in Table 2. The ^{13}C NMR spectrum of geninthiocin revealed the presence of 50 carbons while its ^1H NMR spectrum showed 49 protons including 13 exchangeable ones. DQF-COSY[6], HSQC[7] and FG-HMBC experiments[8] revealed the presence of four partial structures including six terminal methylenes as shown in Fig. 3.

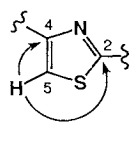
Three sp^2 methine proton signals at 8.70, 8.68 and 8.49 ppm indicated strongly the presence of oxazole and/or thiazole rings. By the HSQC experiment, these protons were correlated to the carbons at 142.7, 140.5 and 126.9 ppm, respectively, suggesting the presence of two oxazole rings and a thiazole one. This assumption was corroborated by HMBC experiments and by good agreement of these NMR chemical shift values with those of known compounds. As shown in Fig. 2, an sp^2 proton signal at 8.70 ppm (Oxa(1), 5-H) showed long range couplings to quaternary carbons at 159.4 ppm (Oxa(1), C-2) and 136.1 ppm (Oxa(1), C-4), and another sp^2 proton signal at 8.68 ppm (Oxa(2), 5-H) to quaternary carbons at 158.1 ppm (Oxa(2), C-2) and 139.2 ppm (Oxa(2), C-4). The chemical shifts of proton and carbon signals showing these long range correlations coincided with those of the corresponding signals of oxazoles in sulfomycin I[9] and A10255G[10] (Fig. 2), and characteristic large coupling constants between 5-H and C-5 of Oxa(1) and Oxa(2) confirmed the presence of two oxazoles ($J_{C-H}=215$ Hz)[11].

A methine signal at 8.49 ppm (Thz, 5-H) showed long range correlations to quaternary carbons at 163.2 ppm (Thz, C-2) and 149.4 ppm (Thz, C-4). These correlations together with comparison of the chemical shifts of proton and carbons with those of known compounds revealed the presence of a thiazole ring (Fig. 2). The characteristic coupling constants between 5-H and C-5 of thiazole supported this conclusion ($J_{C-H}=194.2$ Hz)[11].

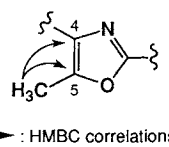
Fig. 2. Chemical shifts of oxazoles, thiazoles and methyloxazoles in geninthiocin and known compounds.



		5-H	C-2	C-4	C-5
Geninthiocin	Oxa (1)	8.70	159.4	136.1	142.7
	Oxa (2)	8.68	158.1	139.2	140.5
Sulfomycin I	Oxa (C)	8.64	158.1	138.8	139.9
A10255G	Oxa (4)	8.60	159.4	136.0	141.9



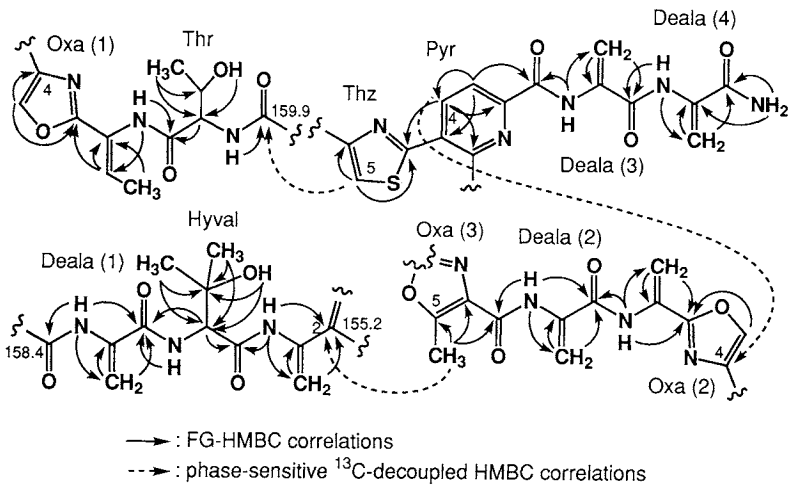
		5-H	C-2	C-4	C-5
Geninthiocin	Thz	8.49	163.2	149.4	126.9
Sulfomycin I	Thz (A)	8.53	162.3	148.9	127.0
A10255G	Thz (3)	8.51	163.6	149.4	127.1



		5-CH_3	C-4	C-5
Geninthiocin	Oxa (3)	2.62	129.2	154.6
Sulfomycin I	Oxa (A)	2.55	128.6	153.2
	Oxa (B)	2.56	129.1	153.9

→ : HMBC correlations

Fig. 3. Partial structures of geninthiocin elucidated by the HMBC experiments.



A methyl signal at 2.62 ppm (Oxa(3), 5-CH_3) showed long range correlations to quaternary carbons at 129.2 ppm (Oxa(3), C-4) and 154.6 ppm (Oxa(3), C-5) indicating the presence of a methyloxazole residue (Fig. 2).

Of the thiopeptide antibiotics, thioxamycin[12], berninamycin[9], sulfomycin I[9] and A10255[10] possess a substructure consisting of thiazole-pyridine-oxazole in the cyclic peptide core, while thiocillin I[13], micrococcin P[14] and GE2270 A[15] possess a thiazole-pyridine-thiazole moiety, which was named as sulfomycinamate in sulfomycin I. Two adjacent aromatic doublet protons at 8.50 ppm and 8.23 ppm in geninthiocin ($J=8$ 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. The ^{13}C chemical shifts of the pyridine moiety in sulfomycin I[9] 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 geninthiocin (Table 2).

The partial structures shown in Fig. 3 were further connected by a new NMR technique, phase-sensitive ^{13}C-decoupled HMBC (D-HMBC)[16]. This enabled the observation of long range ^{13}C-^1H couplings separated by four or five bonds in addition to the very small long range couplings separated by two or three bonds. The results of the D-HMBC experiments are shown in Fig. 3. A D-HMBC experiment optimized for about 4.2 Hz with a delay time of 120 msec revealed a long range coupling of an sp^2 methine proton at 8.49 ppm (Thz, 5-H) to a carbonyl carbon at 159.9 ppm. This indicated the connection of thiazole and threonine residues. In addition, long range correlation from a pyridine proton at 8.50 ppm (Pyr, 4-H) to C-4 of Oxa(2) established the linkage of pyridine and oxazole(2) moieties and this result unambiguously revealed the presence of a thiazole-pyridine-oxazole moiety in the cyclic peptide core as seen in thioxamycin, berninamycin A, sulfomycin I and A10255.

The D-HMBC experiment optimized for 1.0 Hz with a delay time of 500 msec showed a long range coupling from the methyl proton at 2.62 ppm (Oxa(3), 5-CH_3) to the quaternary carbon at 155.2 ppm (Oxa(3), C-2) that was correlated with one of terminal methylenes revealing the presence of the oxazole(3) moiety (Fig. 3). By elimination, C-4 of Oxa(1) was connected to a remaining carbonyl carbon at 158.4 ppm

and thus, the planar structure of geninthiocin was established as shown in Fig. 1. Geninthiocin was structurally very similar to berninamycin[9], the difference being that C-5 of Oxa(1) in geninthiocin was methylated to give the methyloxazole ring in berninamycin.

The partial stereochemistries of geninthiocin were established by NOE experiments and hydrolysis with 6 N HCl at 110°C for 20 hours. The allylic methyl group of Oxa(1) was determined to be *Z* by the NOE effect between the methyl proton and amide proton of Oxa(1). The configuration of L-threonine was established by chiral-TLC plate (Merck, 14285-1M) with MeOH - H_2O - CH_3CN (1 : 1 : 4) as an eluent.

Biological Activity

Minimum induction concentration (MInC) of geninthiocin for *tipA* promoter was 1.2 ng/ml. In addition, we investigated the MInCs of some thiopeptide antibiotics capable of inducing this promoter by the agar dilution method (the previously reported values[4] for promothiocins A and B, 0.2 and 0.1 μg/ml, respectively, were determined by the agar diffusion method) and the values were 19.5, 0.6, 1.2, 2.4 and 78.1 ng/ml for promothiocin A, promothiocin B, berninamycin, thiostrepton and thioxamycin, respectively. Geninthiocin and its methylated derivative, berninamycin showed the same activities. Geninthiocin was 16- and 64-times as active as promothiocin A and thioxamycin, respectively. Among the thiopeptides tested, promothiocin B showed the strongest activity.

Experimental

Spectral Analysis

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 ^1H 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 19 mg of geninthiocin dissolved in 0.8 ml of DMSO-d_6.

The FG-HMBC spectrum with a delay time of 80 msec resulted from a 512×512 ($f_1 \times f_2$) data matrix with 64 scans per t_1 value. The spectral widths were 21,340 Hz for carbon and 5,208 Hz for proton. A pulse repetition time of 1.4 second was used.

The phase-sensitive ^{13}C-decoupled HMBC (D-HMBC) spectra optimized for about 4.2 and 1.0 Hz with delay times of 120 and 500 msec, respectively, were obtained from $512 \times 1{,}024$ ($f_1 \times f_2$) data matrices for both. For each t_1 value, 80 and 64 scans for about 4.2 and 1.0 Hz, respectively, were recorded with a pulse repetition time of 1 second.

Acknowledgments

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