Notes

MICROBIAL METABOLITES WITH *tipA* PROMOTER INDUCING ACTIVITY

III. THIOXAMYCIN AND ITS NOVEL DERIVATIVE, THIOACTIN, TWO THIOPEPTIDES PRODUCED BY *Streptomyces* sp. DP94†

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Several members of the thiopeptide antibiotics such as thiostrepton, nosiheptide, micrococcin, sulfomycin and berninamycin induce expression of numerous genes of unknown function in *Streptomyces lividans*[3]. Among them, the region containing the *tipA* gene and its promoter region was cloned and sequenced[3]. *TipA* proved to be a regulatory protein which autogenously activates transcription of its own promoter after interacting with thiostrepton or other related thiopeptide antibiotics[4]. 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[1] and geninthiocin[2]. In the course of our continuing screening, thioxamycin and its new derivative, thioactin were isolated from the mycelium of *Streptomyces* sp. DP94 (Fig. 2). The taxonomy of the producing strain, fermentation, purification and physico-chemical properties of thioxamycin were reported[5] but details of its structure determination including NMR data were not presented. This paper presents isolation, fermentation, structure elucidation and *tipA* promoter inducing activities of thioxamycin and its new derivative, thioactin.

Preculture of *Streptomyces* sp. DP94 was inoculated into thirty 500-ml Erlenmeyer flasks each containing 100 ml of the culture medium consisting of starch 2.5%, soybean meal 1.5%, dried yeast 0.2% and $CaCO_3$ 0.4% (pH 6.2 before sterilization), and cultivation was carried out at 27°C for four days on a rotary shaker.

Thioxamycin and thioactin were isolated from the mycelial cake by monitoring the *tipA* promoter inducing activity[1,2]. The mycelium collected by centrifugation 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 Sephadex LH-20 column chromatography developed with $CHCl_3$ - MeOH (2 : 1). Then the active fraction was applied to preparative TLC plates (silica gel TLC, Merck 60 F_{254}) and developed with $CHCl_3$ - MeOH (9 : 1). Two active zones with

Table 1. Physico-chemical properties of thioxamycin and thioactin.

	Thioxamycin	Thioactin
Appearance	White powder	White powder
MP	240~245°C (dec)	250~255°C (dec)
Molecular formula	$C_{52}H_{48}N_{16}O_{15}S_4$	$C_{43}H_{40}N_{14}O_{11}S_4$
HRFAB-MS (*m/z*)		
Found:	1265.250 $(M+H)^+$	1057.190 $(M+H)^+$
Calcd:	1265.245	1057.196
$[\alpha]_D^{19}$	−40.8° (*c* 0.13, $CHCl_3$ - MeOH, 1 : 1)	−42.5° (*c* 0.10, $CHCl_3$ - MeOH, 1 : 1)
UV λ_{max}^{EtOH} nm (ε)	211 (25,000), 237 (23,000, sh)	215 (40,000, sh), 236 (42,000)
IR ν_{max} (KBr) cm^{-1}	3400 (br), 3000, 2930, 1690~1640, 1600, 1550~1490, 1425, 1200, 890, 750	3400 (br), 3000, 2930, 1690~1640, 1600, 1550~1490, 1420, 1200, 900, 750

† For parts I and II: see ref 1 and 2.

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

Position	δ_C		δ_H	
	Thioxamycin	Thioactin	Thioxamycin	Thioactin
Thiazole (1)				
C-2	163.3	163.3		
C-4	149.4	149.6		
CH-5	127.3	127.3	8.46	8.47
CO	160.2	160.2		
Threonine				
NH			8.06 (d, 8.3)[a]	8.06 (d, 8.3)
αCH	59.0	59.1	4.32 (dd, 8.3, 4.2)	4.32 (dd, 8.3, 4.3)
βCH	66.3	66.3	4.13 (m)	4.12 (m)
OH				5.13 (b)
γCH_3	20.3	20.3	1.06 (d, 6.2)	1.05 (d, 6.2)
CO	169.9	169.9		
Oxazole (1)				
NH			8.67 (d, 7.9)	8.69 (d, 8.1)
αCH	46.8	46.8	5.17 (ddd, 7.9, 7.5, 7.2)	5.15 (ddd, 8.1, 7.4, 7.3)
βCH_2	35.3	35.2	3.06 (dd, 13.8, 7.5)	3.06 (dd, 13.7, 7.3)
			2.95 (dd, 13.8, 7.2)	2.95 (dd, 13.7, 7.4)
δCH_3	15.0	15.0	2.04	2.04
C-2	162.6	162.6		
C-4	135.3	135.3		
CH-5	142.4	142.4	8.63	8.64
CO	160.0	160.0		
Thiazole (2)				
NH			8.86 (t, 5.5)	8.89 (t, 5.8)
αCH_2	39.5	39.5	4.72 (d, 5.5)	4.72 (d, 5.8)
C-2	168.7	168.7		
C-4	148.8	148.7		
CH-5	124.7	124.7	8.25	8.25
CO	160.0	160.0		
Thiazole (3)				
NH			8.80 (d, 7.9)	8.84 (d, 7.7)
αCH	46.5	46.5	5.45 (m)	5.44 (m)
βCH_3	20.6	20.6	1.61 (d, 6.8)	1.61 (d, 6.8)
C-2	172.9	172.9		
C-4	147.8	147.8		
CH-5	126.0	126.0	8.38	8.38
CO	158.8	158.8		
Dehydroalanine (1)				
NH			9.78	9.79
αC	133.6	133.6		
βCH_2	104.4	104.5	6.53, 5.81	6.54, 5.81
CO	162.6	162.6		
Oxazole (2)				
NH			9.95	10.01
αC	129.1	129.1		
βCH_2	110.5	111.2	5.74, 5.61	5.73, 5.62
C-2	158.1	158.1		
C-4	139.0	139.0		
CH-5	140.4	140.2	8.64	8.56
Pyridine				
C-2	149.3	149.4		
C-3	130.2	130.2		
CH-4	141.2	141.2	8.47 (d, 8.1)	8.47 (d, 8.1)
CH-5	121.5	121.4	8.26 (d, 8.1)	8.26 (d, 8.1)

Table 2. (Continued)

Position	δ_C		δ_H	
	Thioxamycin	Thioactin	Thioxamycin	Thioactin
C-6	146.7	146.6		
CO	161.4	161.1		
Dehydroalanine (2)				
NH			10.51	10.67
αC	134.0	133.6		
βCH_2	105.9	102.9	6.55, 5.94	6.55, 5.83
CO	162.6	164.8		
NH_2				8.15, 7.65
Dehydroalanine (3)				
NH			9.94	
αC	136.3			
βCH_2	110.5		5.81, 5.76	
CO	162.9			
Dehydroalanine (4)				
NH			9.58	
αC	136.1			
βCH_2	109.2		5.84, 5.68	
CO	162.5			
Dehydroalanine (5)				
NH			9.07	
αC	133.1			
βCH_2	110.0		6.10, 5.77	
CO	164.8			

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

Rf-values of 0.04 and 0.64 for thioxamycin and thioactin, respectively, were scraped off the TLC plates and then extracted with $CHCl_3$ - MeOH (3 : 1). Each crude active compound was further purified by HPLC with Capcell-pak C_{18} eluting with a mixture of CH_3CN - H_2O (42 : 58)/0.1% TFA for thioxamycin and a mixture of CH_3CN - H_2O (38 : 62)/0.1% TFA for thioactin. Thioxamycin and thioactin produced the peaks with retention time of 14.5 and 12.5 minutes, respectively, to give white amorphous solids (thioxamycin 70 mg, thioactin 27 mg).

The physico-chemical properties of thioxamycin and thioactin are summarized in Table 1. Their molecular formulae were determined as $C_{52}H_{48}N_{16}O_{15}S_4$ and $C_{43}H_{40}N_{14}O_{11}S_4$, respectively, by HR-FAB mass spectroscopy using *m*-nitrobenzyl alcohol - glycerol matrix and by ^1H and ^{13}C NMR spectroscopic analyses. The IR absorptions at 1690～1640 and 1550～1490 cm^{-1} suggested that these compounds had a peptidic nature.

The ^1H and ^{13}C NMR spectral data of thioxamycin and thioactin are summarized in Table 2 and the major component thioxamycin was used for further structure analysis. The ^1H NMR spectrum of thioxamycin revealed the presence of 46 protons including 13 exchangeable ones. DQF-COSY[6], HSQC[7] and FG-HMBC[8] experiments revealed the presence of three partial structures as shown in Fig. 1.

Five aromatic methine proton signals at 8.64, 8.63, 8.46, 8.38 and 8.25 ppm indicated the presence of oxazole and/or thiazole rings[1,2,9,10]. The HSQC data correlated these protons to sp^2 methine carbons at 140.4, 142.4, 127.3, 126.0 and 124.7 ppm, respectively, suggesting the presence of two oxazole and three thiazole rings (Fig. 1). This assumption was confirmed by HMBC experimental results and by the good agreement of these NMR chemical shift values with those of sulfomycin I[9] and A10255G[10]. In addition, the characteristic large J_{C-H} coupling constants (192～215 Hz) for the relevant units supported this conclusion[11].

Long range connectivities of two adjacent aromatic doublet protons (J=8.1 Hz) at 8.47 ppm and 8.26 ppm to sp^2 carbons revealed the presence of a 2,3,6-trisubstituted pyridine residue. 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 to C-6, respectively, which were in good agreement with those of the corresponding carbons in thioxamycin (Table 2).

Fig. 1. The partial structures of thioxamycin elucidated by the FG-HMBC experiments and their connectivities.

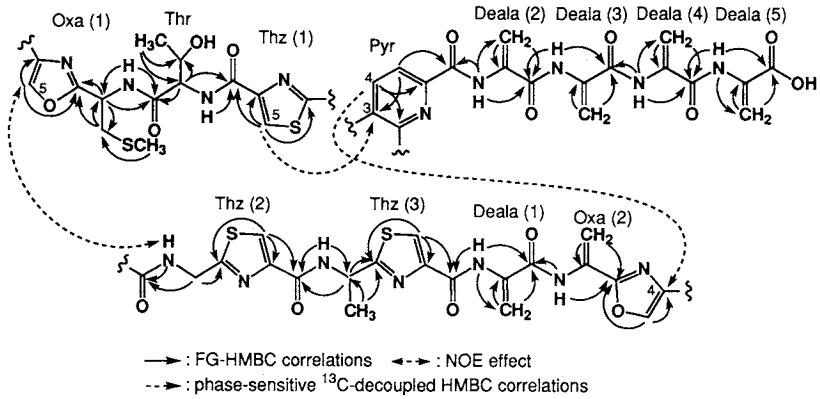
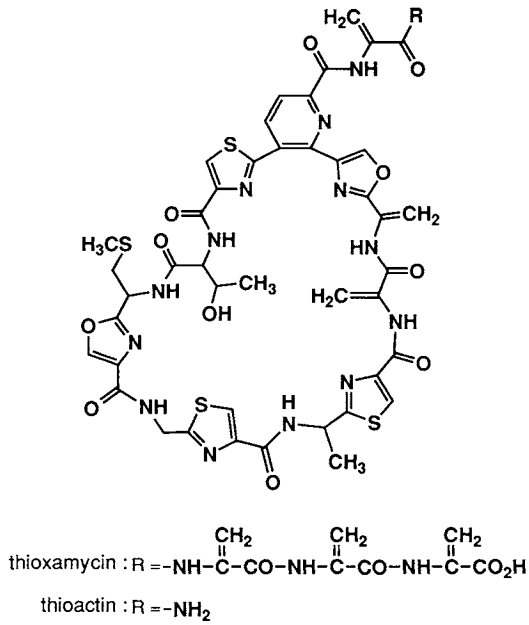


Fig. 2. Structures of thioxamycin and thioactin.



The above partial units and the other remaining structures were connected by the FG-HMBC with delaytime set to 80 msec. This revealed unambiguously the conjugation of the pyridine and dehydroalanine side chain, and the presence of oxazole(1)-threonine-thiazole(1) and thiazole(2)-thiazole(3)-dehydroalanine(1)-oxazole(2) moieties (Fig. 1).

The partial structures shown in Fig. 1 were further connected by the phase-sensitive ^{13}C-decoupled HMBC (D-HMBC)[12] and NOE experiments. A D-HMBC experiment optimized for about 2.1 Hz with a delay time of 240 msec revealed a four-bond long range correlation of an aromatic methine proton at 8.46 ppm (Thz(1), 5-H) to a quaternary carbon at 130.2 ppm (Pyr, C-3). This indicated the connection of thiazole(1) and pyridine rings. In addition, long range correlation from an aromatic proton at 8.47 ppm (Pyr, 4-H) to C-4 of oxazole(2) at 139.0 ppm established the linkage of pyridine and oxazole(2) moiety. These results revealed the presence of a thiazole-pyridine-oxazole moiety in the cyclic peptide core as seen in berninamycin A[9], sulfomycin I[9] and A10255[10]. By elimination, C-4 of oxazole(1) was connected to a remaining carbonyl carbon at 160.0 ppm; this conclusion being sup-

ported by the NOE effect between the methine proton of oxazole(1) at 8.63 ppm and an amide proton of thiazole(2) at 8.86 ppm. The above NMR experiments established the structure of thioxamycin as shown in Fig. 2.

The ^{1}H and ^{13}C NMR spectra of thioactin with the appearance of two new exchangeable protons showed the lack of two dehydroalanine residues in thioxamycin. Based on the ^{1}H, ^{13}C and HR-FAB mass spectra (Table 1 and 2), the structure of thioactin was determined as shown in Fig. 2. The C-terminal carboxylic acid of the dehydroalanine side chain in thioxamycin was replaced with an amide in thioactin.

Minimum induction concentrations of thioxamycin and thioactin for *tipA* promoter were 80 and 40 ng/ml, respectively. Tested so far thioactin showed inhibitory activity against *Streptococcus pyogenes*, *S. pneumoniae* and *Micrococcus luteus* at 6.25 μg/ml (MIC).

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