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Thiopeptin, a New Feed Additive Antibiotic: Microbiological and Chemical Studies

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The thiopeptins are a new group of sulfur-containing peptide antibiotics produced by *Streptomyces tateyamensis*. The antibiotic consists of a major component (designated as thiopeptin B) and four minor ones (thiopeptins A_1 to A_4). These components were isolated by solvent extraction from mycelium followed by chromatography on silica gel with various ratios of chloroform and methanol as elution solvents. Acid hydrolysis of each of the thiopeptin components yielded 1 mole of valine, 1 of threonine, 1 of cysteine, and 2 of alanine as amino acids. Each component of the thiopeptin A group has chemical and biological properties closely similar to those of thiopeptin B, but detailed characterization has established that thiopeptins A_1, A_3, and A_4 are new antibiotics. We could not obtain accurate data for determination of the uniqueness of A_2 because of insufficient sample. Thiopeptin has strong antibacterial activity against gram-positive bacteria and *Mycoplasma*, and exhibits no cross-resistance to major human-use antibiotics.

During screening for new antibiotics, a previously undescribed antibiotic was isolated from culture broth of a new *Streptomyces* species which was isolated from soil samples collected at Tateyama, Toyama Prefecture, Japan. The antibiotic was characterized as a sulfur-containing peptide complex consisting of very closely related compounds and was designated thiopeptin. Thiopeptin inhibits primarily gram-positive bacteria, and is valuable as a feed additive for animals, because of its marked growth-promoting action.

In a previous paper (5), we reported the chemical and biological characteristics of thiopeptin B, the main component of the antibiotic complex. We continued our studies on components of thiopeptin and found out that four components were produced in thiopeptin fermentation broth, which were designated A_1, A_2, A_3, and A_4. Each component was isolated in pure form except for A_2. Thiopeptins A_1, A_3, and A_4 were established as new antibiotics on the basis of their chemical and biological properties. Definite data for component A_2 could not be obtained because of its poor production in broth.

In this paper, fermentation characteristics, isolation procedures, and the chemical and biological properties of the thiopeptin A antibiotics are compared with those of thiopeptin B.

MATERIALS AND METHODS

Fermentation and extraction. A medium consisting of 6.0% potato starch, 3.0% Pharmamedia (Trader Oil Mill Co.), 1.5% corn steep liquor 1.5% dried yeast, 0.3% $FeSO_4 \cdot 7H_2O$, 0.2% *N*-acetyl methionine, 1.09% KH_2PO_4, and 0.71% Na_2HPO_4 was prepared with 650 liters of tap water in a 1,000-liter stainless-steel fermentor and was sterilized with steam under high pressure.

Streptomyces tateyamensis ATCC 21389 was precultured in 30 liters of the same medium described above for 48 hr at 30 C. The seed culture was inoculated into the main fermentor, where it was cultured for 120 hr at 30 C with aeration at 680 liters/min and agitation at 183 rev/min.

Antibacterial activity was assayed by cup plate or paper-disc plate method with *Staphylococcus aureus* 209P as a test organism and with crystalline thiopeptin B as the assay standard.

The harvested broth (480 liters) was filtered after addition of 25 kg of filter acid (Radiolite). Thiopeptin was extracted by adding 120 liters of acetone to the filter cake, and extraction was performed by repeating the process twice. The extract (230 liters) was concentrated in vacuo, and the residual aqueous solution (55 liters) was extracted twice with 25 liters of chloroform each time. The chloroform extracts were combined (40 liters) and concentrated in vacuo. The residue (1 liter) was added to 5 volumes of *n*-hexane and allowed to settle at 5 C overnight. Crude crystalline thiopeptin was precipitated, and the precipitates were collected, washed with a small amount of ace-

TABLE 1. *Characteristics of the thiopeptin A group and thiopeptin B*[a]

Thiopeptin	Melting point, decomposition (C)	α_D^{23} $CHCl_3$ ($c = 1$)	Molecular wt[b]	Elementary analysis (%)			
				C	H	N	S
A_1	>200, 223–226	−71.0°	1,637	49.68	4.96	14.32	11.00
A_2	>200						
A_3	>200, 232–236	−10.8°	1,972	48.45	5.11	14.46	12.09
A_4	>200, 236–245	−81.5°	1,854	50.13	5.30	15.22	12.02
B	>200, 219–222	−80.0°	1,942	49.26	5.16	14.53	10.82

[a] All of the thiopeptins were pale yellow crystals.
[b] Measured by the vapor pressure method.

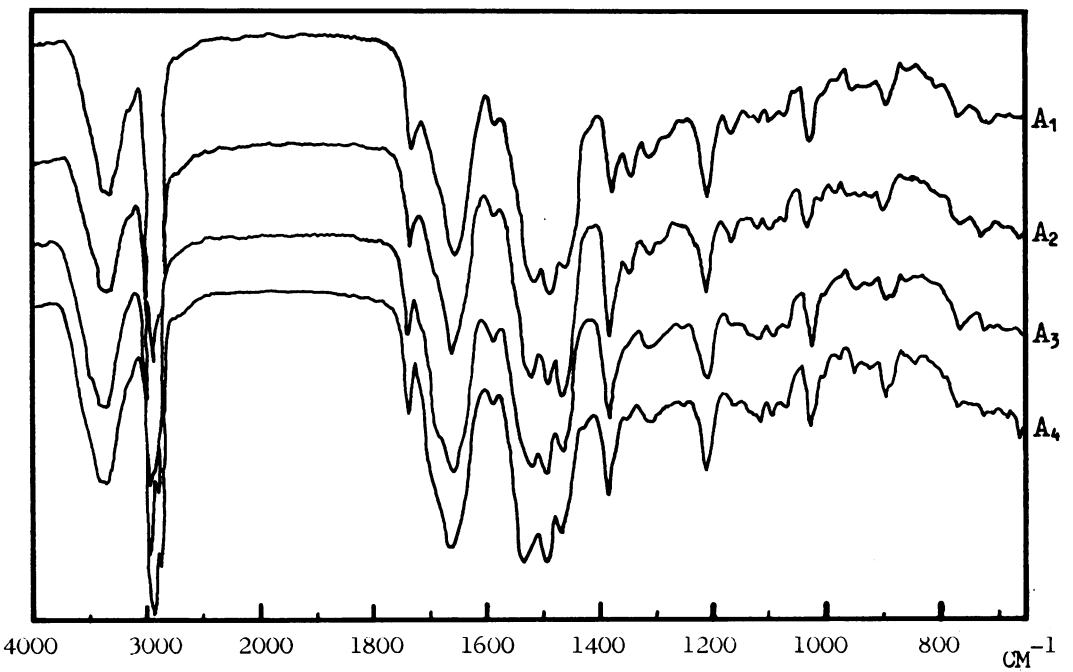

FIG. 1. *Infrared absorption spectra of thiopeptins A_1, A_2, A_3, and A_4 (in Nujol).*

tone, and dried. About 100 g of crude crystalline powder was obtained from 480 liters of broth (yield was about 70% of total biological activity).

Purification. A 10-g amount of crude crystalline thiopeptin was dissolved in 20 ml of chloroform and subjected to chromatography by use of a column (5 by 40 cm) packed with silica gel (Merck). After washing the column thoroughly with chloroform, elution was carried out with a mixture of chloroform-methanol (19:1, v/v). The fraction of thiopeptin A_1 was eluted out first, and a mixture of thiopeptins A_1, A_2, A_3, and A_4 followed. After these processes, thiopeptin B, the major component, was eluted by using a mixture of chloroform-methanol (9:1, v/v) as the developing solvent.

The 1-g mixture of $A_1 \sim A_4$ was dissolved in 5 ml of chloroform and chromatographed on a column (3 by 100 cm) containing silica gel (Merck); a mixture of chloroform-methanol (50:1, v/v) was used for elution. By this process, A_1, the A_1 A_2 A_3 mixture, A_3, the A_3 A_4 mixture, and A_4 could be separated. The A_1 A_2 A_3 mixture was further separated by the same process on a silica gel (Merck) column (2 by 80 cm).

The remaining mixture of A_1 A_2 A_3 (30 mg) was dissolved in 1 ml of chloroform. The chloroform solution was spotted on five thin-layer plates (20 by 20 cm by 0.5 mm) of Silica gel GF 254 (Merck) and was developed with a chloroform-methanol mixture (20:1, v/v). The spot of A_2 fraction (R_F 0.42) was scratched and collected under an ultraviolet lamp in a dark room.

The collected silica gel powder was extracted with

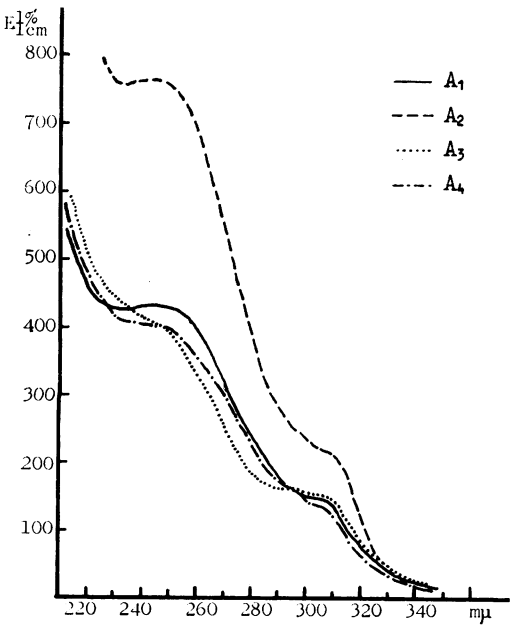

FIG. 2. *Ultraviolet absorption spectra of thiopeptins A_1, A_2, A_3, and A_4 (in methanol).*

chloroform-methanol (4:1, v/v), after which the extract was concentrated in vacuo to yield A_2 crystal.

Each component of thiopeptin thus prepared was dissolved in warm acetone and crystallized to obtain the pure crystalline form.

RESULTS

Physical and chemical properties. Thiopeptins A_1, A_2, A_3, and A_4 had similar properties. They were pale yellowish crystals which showed indefinite melting points, and turned to brown at about 200 C. The characteristics of thioptin B and the A group are shown in Table 1. However, definite data could not be obtained for thiopeptin A_2 because of insufficient sample. Optical rotations were determined in chloroform (c = 1), and molecular weights were estimated by the vapor pressure method.

The infrared spectra (in Nujol) and ultraviolet spectra (in methanol) of thiopeptin B and the A group are shown in Fig. 1 and 2, respectively.

A comparison of the thin-layer chromatography characteristics of thiopeptins $A_1 \sim A_4$ with thiopeptin B and other related antibiotics on Silica gel G is shown in Table 2.

The solubilities of thiopeptins A_1, A_3, A_4, and B were similar and were found to be as follows: easily soluble in dioxane, dimethylsulfoxide, dimethylformamide, pyridine, and chloroform; fairly soluble in methanol, acetone, and ethyl acetate; insoluble in ether, benzene, *n*-hexane, petroleum ether, and water. The color reactions of the thiopeptin A group were as follows: they decolorized permangantate, but gave negative reactions with ninhydrin, biuret, ferric chloride, and Dragendorff's reagent. They were stable to heat treatment of 1-hr duration at 60 C in the *p*H range of 2 to 8.

TABLE 2. *Comparison of R_F values of thiopeptins and other closely related antibiotics*

Antibiotic	R_F values under various experimental conditions[a]				
	1	2	3	4	5
Thiostrepton....	0.50	0.30	0.38	0.43	0.38
Siomycin A.....	0.50	0.30	0.29	0.43	0.14
Sporangiomycin	0.50	0.30	0.27	0.41	0.14
A-59 substance..	0.50	0.30	0.27	0.43	0.14
Thiopeptin					
A_1.....	0.83	0.48	0.73	0.95	0.18
A_2.....	0.70	0.42	0.62	0.85	0.25
A_3.....	0.60	0.37	0.48	0.57	0.52
A_4.....	0.50	0.30	0.38	0.57	0.38
B.....	0.10	0.00	0.00	0.00	0.23

[a] Conditions: (1) Silica gel G thin-layer chromatography, chloroform-methanol (9:1); (2) Silica gel G thin-layer chromatography, chloroform methanol (19:1); (3) Spotfilm (Tokyo Kasei Co. silica gel) thin-layer chromatography, chloroform-*n*-butanol (6:1); (4) PPC (Toyo Roshi No. 50), ethylacetate-*n*-hexane-2 N NH_4OH (4:1:1); (5) PPC (Toyo Roshi No. 50), methanol-acetic acid-water (23:3:72). PPC = Paper chromatograpy.

Hydrolysis of thiopeptin A_1, A_3, A_4, and B with 6 N hydrochloric acid yields a number of ninhydrin-positive components. Amino acid analysis indicated the presence of 2 moles of alanine, 1 mole of valine, 1 mole of threonine, and 1 mole of cysteine.

Biological properties. In Table 3, the antimicrobial spectra of thiopeptins A_1, A_3, and A_4 are compared with that of thiopeptin B. The minimal inhibitory concentrations were determined by an agar-dilution method. Thiopeptins A_1, A_3, and A_4 had strong activity against gram-positive bacteria, in accordance with thiopeptin B, and did not show any cross-resistance with chloramphenicol, penicillin G, erythromycin, or streptomycin. The thiopeptin antibiotics also proved to be highly active against some species of *Mycoplasma* (Table 4).

The intraperitoneal administration to mice of 500 mg of thiopeptins A_1, A_3, A_4, and B per kg did not result in any toxic symptoms.

TABLE 3. *Antimicrobial spectra of thiopeptins A_1, A_3, A_4, and B*

Test organism	Minimal inhibitory concn (μg/ml)			
	A_1	A_3	A_4	B
Staphylococcus aureus 209P	0.125	0.25	0.06	0.125
S. aureus Smith	0.125	0.25	0.06	0.125
S. aureus Terashima	0.125	0.25	0.06	0.25
S. aureus Cp-R[a]	0.125	0.25	0.06	0.25
S. aureus Pc-R[a]	0.125	0.25	0.06	0.25
S. aureus Em, Sm, Pc-R[a]	0.125	0.25	0.125	0.25
Sarcina lutea PCI-1001	0.125	0.06	0.015	0.03
Bacillus subtilis ATCC-6633	0.125	0.25	0.25	0.25
B. megaterium BB-105	0.125	0.25	0.25	0.25
Corynebacterium xerosis BO-404	0.125	0.5	0.25	0.25
Escherichia coli NIHJ	>128	>128	>128	>128
Pseudomonas aeruginosa BP-145	>128	>128	>128	>128
Proteus vulgaris BO-701	>128	>128	>128	>128
Mycobacterium 607	>128	32	128	64
M. phlei	32	8	8	32
Penicillium chrysogenum Q 176	>128	>128	>128	>128
Candida albicans YC-109	>128	>128	>128	>128

[a] CP-R = chloramphenicol-resistant; Pc-R = penicillin-resistant; Em, Sm, Pc-R = erythromycin-, streptomycin-, penicillin-resistant.

TABLE 4. *Minimal inhibitory concentrations of thiopeptins against Mycoplasma*

Method[a]	Organism	Minimal inhibitory concn (μg/ml)				
		A_1	A_4	B	TC[b]	EM[b]
PDM	*M. gallisepticum*	0.15	0.7	3.0		
	M. hominis type I	15.0	15.0	30.0		
	M. laidlawii A	0.07	0.07	0.07		
	M. salivarium	3.0	3.0	12.0	5.0	>50
	M. pneumoniae Mac	0.15	0.07	0.07	1.25	0.1
	M. fermentans	0.7	0.3	1.5	10	>100
	M. pulmonis	0.03	0.03	0.3	1.0	0.1
TDM	*M. gallisepticum*	0.015	0.00075	0.15	0.1	0.1
	M. hominis type I	5.0	10.0	>10	1.0	>10
	M. laidlawii A	0.0015	0.0015	0.003	0.1	0.1

[a] PDM = paper-disc method; TDM = tube dilution method.
[b] TC = tetracycline; EM = erythromycin.

DISCUSSION

The novelty of thiopeptin B, the main component of the thiopeptin complex, was established in a previous paper (5). On the basis of their chemical properties, thiopeptins A_1, A_3, and A_4 apparently should also be classified in the sulfur-containing peptide antibiotic group. Thiostrepton (1, 8, 11), siomycin A, B, and C (2, 7), A-59 substance (4), pepthiomycin A and B (6), sporangiomycin (10), thermothiocin (R. Craveri, British Patent 1,106,148, 1968), sulfomycin I, II, and III (3), thiopeptin B (5), and multhiomycin (9) can be mentioned as known antibiotics in this group.

Thiopeptins A_1, A_3, and A_4 can be readily distinguished from members of the group that have a lower content of sulfur, namely, siomycin B and C, pepthiomycin A and B, thermothiocin, and sulfomycin I, II, and III. Multhiomycin, on the other hand, has a distinctively higher sulfur content than the thiopeptin A group.

The remaining antibiotics, thiostrepton, siomycin A, A-59 substance, sporangiomycin, and

TABLE 5. *Amino acid composition of thiopeptins, siomycin, and thiostrepton*[a]

Antibiotic	Alanine	Threonine	Valine	Cysteine	Isoleucine
Thiopeptin A_1..	2.00	0.93	1.01	0.91	0
Thiopeptin A_3..	2.00	0.94	1.01	0.98	0
Thiopeptin A_4..	2.00	0.91	1.06	1.00	0
Thiopeptin B...	2.00	0.99	1.03	0.97	0
Siomycin A....	1	1	1	1	0
Thiostrepton...	2	1	0	1	1

[a] Data for thiopeptins are also expressed as the molar ratio, but assuming alanine = 2.00.

thiopeptin B, were compared with thiopeptins A_1, A_2, A_3, and A_4 by use of thin-layer chromatography. The results (Table 2) indicated that the thiopeptin A group is clearly different from the other antibiotics tested.

A comparison of the amino acid composition of thiopeptins, siomycin, and thiostrepton is made in Table 5. These results also clearly distinguish the thiopeptin A group from siomycin and thiostrepton.

On the basis of the above discussion, it is reasonable to conclude that thiopeptins A_1, A_3, and A_4 are new sulfur-containing peptide antibiotics. As for thiopeptin A_2, any conclusions as to its uniqueness must be suspended at present because of the lack of available data on its chemical and biological characteristics.

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