# STUDIES ON THIOPEPTIN ANTIBIOTICS

## I. CHARACTERISTICS OF THIOPEPTIN B

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(Received for publication January 23, 1970)

The microbiological characteristics of *Streptomyces* strain No. 7906 include formation of white aerial mycelium, light brown growth and thick aerial hyphae forming tufts. Strain No. 7906 was compared with known species and identified as a new species and given the name *Streptomyces tateyamensis*. *Streptomyces tateyamensis* No. 7906 produces new sulfur-containing peptide antibiotics which are named thiopeptins. The major component, thiopeptin B, is obtained as a crystalline powder decomposing at 219~222°C, and exhibiting optical rotation $[\alpha]_D^{23}$ −80° (*c* 1, chloroform). Its elementary analysis suggested the empirical formula $C_{72}H_{90}O_{22}N_{18}S_6$. The ultraviolet absorption maxima (shoulder) were found at 230~250, 295 and 305 mμ. Thiopeptin B showed strong antibacterial activity against Gram-positive bacteria and had no cross resistance with penicillins, aminoglycoside antibiotics, tetracyclines and macrolides. The administration of 500 mg/kg of this antibiotic into mice by intraperitoneal route did not result in any toxic symptom.

In the course of our antibiotic screening program, a *Streptomyces* numbered 7906 was found to produce antibiotics with activity against Gram-positive bacteria. Chemical studies showed that these antibiotics are new sulfur-containing peptide antibiotics and they have been named thiopeptins. The major component of these antibiotics was designated thiopeptin B.

Characteristics of strain No. 7906, antibiotic production and isolation procedures, and chemical and biological properties of thiopeptin B will be described in this paper.

### Characteristics of Strain No. 7906

The organism No. 7906 producing thiopeptins was isolated from a soil sample collected in Tateyama, Toyama Prefecture, Japan. It is thought to be a new species of the genus *Streptomyces* and has been designated *Streptomyces tateyamensis*. A culture has been deposited in the American Type Culture Collection, Maryland, U. S. A., where it is registered as A. T. C. C. 21353.

The morphology of the culture was microscopically observed on BENNETT's agar. Hyphae of this culture are thick and straight and form tufts. The smooth surface conidia are rather large in size and long-elliptical, but some rectangular conidia were observed.

No. 7906 strain has the following cultural characteristics when grown on the indicated media for 10~14 days at 30°C, with the sole exception when grown on gelatin stab culture. The gelatin stab culture was observed after incubation at room temperature for 20 days.

CZAPEK's agar: Growth faint, colorless; no aerial mycelium; no soluble pigment.

Starch ammonium agar: Growth abundant, yellowish brown in periphery, white in center; aerial mycelium, thick, brownish white, powdery; no soluble pigment. Starch vigorously hydrolyzed.

Glucose asparagine agar: Growth flat, dark brown; aerial mycelium, spreading, white, powdery; no soluble pigment.

Calcium malate agar: Growth flat, faint brown; aerial mycelium, white, powdery; no soluble pigment.

Tyrosine agar: Growth faint brown; aerial mycelium, spreading, faintly brownish white, powdery; no soluble pigment.

Bouillon agar: Poor growth, colorless; no aerial mycelium; no soluble pigment.

BENNETT's agar: Growth faint brown; aerial mycelium, faintly grayish white, powdery, spreading, no soluble pigment. Rather poor growth at 37°C.

Gelatin stab: Faint growth; no aerial mycelium; no soluble pigment. Weak liquefaction.

Glucose-CZAPEK's solution: Small white colonies precipitated; no aerial mycelium; no soluble pigment. No nitrite was produced.

Glucose-bouillon: Faint gray colonies grown on surface; no aerial mycelium; no soulble pigment. The pH value changed slightly to acidic range.

Milk: Faint gray colonies grown on surface; no aerial mycelium; no soluble pigment. Peptonization and coagulation negative.

Potato plug: Growth faint brown, wrinckled; no aerial mycelium; no soluble pigment.

Cellulose: No growth.

The carbon utilization of *Streptomyces tateyamensis* was examined according to the method described by PRIDHAM and GOTTLIEB[1] with results as shown in Table 1.

The distinctive characters of strain No. 7906 would be as follows:

1) Non-chromogenic and does not produce soluble pigment on synthetic media.
2) Aerial mycelium is white powdery and the growth is light brown in most cases.
3) Aerial hyphae are thick, straight and form tufts.
4) Diastatic action is strong, whereas proteolytic activities on gelatin or milk media are both weak.

Table 1. Carbon utilization pattern for *Streptomyces tateyamensis* No. 7906

| | |
|---|---|
| Arabinose | (+) |
| Fructose | (−) |
| Glucose | (+) |
| Inositol | (−) |
| Lactose | (+) |
| Mannitol | (−) |
| Mannose | (−) |
| Raffinose | (−) |
| Rhamnose | (−) |
| Salicin | (−) |
| Sucrose | (+) |
| Trehalose | (−) |
| Xylose | (−) |

(+)=probable utilization
(−)=questionable utilization

The taxonomic keys of WAKSMAN's The Actinomycetes Vol. 2[2] were used to compare the culture with recognized species of the genus *Streptomyces*. From the characteristics described above, strain 7906 is considered to belong to the *albus* group, and *St. gougeroti*[3] and *S. sioyaensis*[4] are found as possibly related species. However they differ from No. 7906 in the following characters: *S. gougeroti*'s aerial hyphae are short and gnarled and its proteolytic activities on gelatin and milk are strong. *S. sioyaensis* grows excellently and produces soluble pigment on CZAPEK's agar, gives positive nitrate reduction and

shows significantly different pattern of carbon utilization. From these considerations, it seems to be most reasonable to identify No. 7906 strain as a new species and the name of *Streptomyces tateyamensis* n. sp. is proposed.

## Production and Isolation

During the production and isolation processes, antibacterial activity was assayed by cup plate or paper disc plate method with *Staphylococcus aureus* 209P as a test organism and using the crystalline thiopeptin B as the assay standard.

For production of the antibiotic a 72-hour shake-flask culture was used. This was obtained by submerged cultivation of the strain *Streptomyces tateyamensis* No. 7906 at 30°C in the liquid medium composed of 4 % potato starch, 2 % Pharmamedia (Trader Oil Mill Co., Texas, U.S.A.), 1 % corn steep liquor, 1 % dried yeast, 1 % $CaCO_3$ 2.18 % $KH_2PO_4$, 1.43 % $Na_2HPO_4 \cdot 12H_2O$ (pH 6.2).

In large scale production the antibacterial activity was observed mainly in the mycelium, and the whole broth (pH 6.2; 3,080 liters, 268 mcg/ml) was agitated with filter aid (Radiolite, 180 kg) and filtered with the aid of additional Radiolite (20 kg). The collected filtered cake was extracted twice with each 1,000 liters of acetone. The extract (1,900 liters) was concentrated *in vacuo* and the remaining aqueous solution (430 liters) was extracted twice with 200 liters of chloroform. The extract (350 liters) was concentrated *in vacuo* to about 8 liters. Upon addition of 5 volume of *n*-hexane a brownish precipitate of crude thiopeptin was formed (800 g).

The active crude powder was purified further by chromatography on a column of silica gel (Merck). The column was washed first with mixture of methanol and chloroform (1 : 50, v/v) and eluted with the same solvent system (1 : 9, v/v). The antibacterial activity of the eluates was monitored by thin-layer chromatography and bioautography.

The active eluates were collected and evaporated to dryness. Residual yellowish brown powder was dissolved in chloroform and chromatographed again on a column of silicic acid (Mallinckrodt). This column was eluted with mixture of methanol and chloroform (1 : 50, v/v). Active fractions were concentrated to dryness and recrystallized from acetone. This yellowish crystalline material was named thiopeptin B; 420 g of thiopeptin B was recovered from starting broth and total activity recovery yield is about 51 per cent.

## Chemical Properties

Thiopeptin B, the main component of thiopeptin antibiotics, is a faint yellowish crystalline material having decomposition point between 219~222°C, and exhibiting optical rotation; $[\alpha]_D^{23}$ −80° (*c* 1, chloroform).

The molecular weight of thiopeptin B is 1942 by vapor pressure method and 1782 by amino acid analysis. The elementary analysis showed the following result:

Found. C 49.26, H 5.16, N 14.35, S 10.82, O 19.88
(Tentative formula $C_{72}H_{90}O_{22}N_{18}S_6$, MW 1752)

The ultraviolet absorption spectrum of thiopeptin B in methanol showed shoulders at 230~250 mμ, 295 mμ and 305 mμ as shown in Fig. 1. The infrared absorption spectrum in a Nujol null (Fig. 2) shows absorption bands at following frequencies: 3400, 3300, 3160, 1735, 1685, 1660, 1650, 1640, 1585, 1510, 1485, 1335, 1305, 1270, 1250, 1240, 1205, 1165, 1130, 1120, 1110, 1095, 1070, 1025, 1005, 975, 850, 930, 895, 820, 765, 725, 705 $cm^{-1}$.

Amino acid analysis of acid hydrolysate of thiopeptin B (6 N HCl, 110°C, 24~48 hours) shows about 1 mol of threonine, 2 mols of alanine, 1 mol of valine and 1 mol of cysteine.

Fig. 1. Ultraviolet spectrum of thiopeptin B (in methanol)

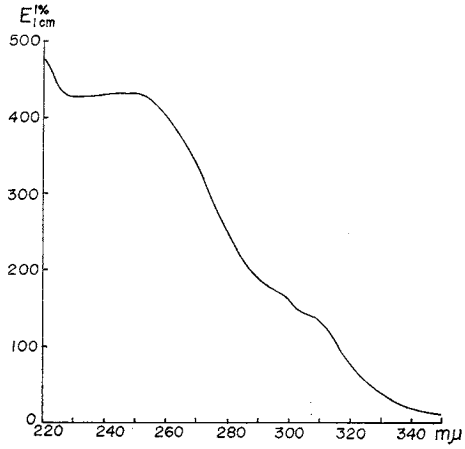
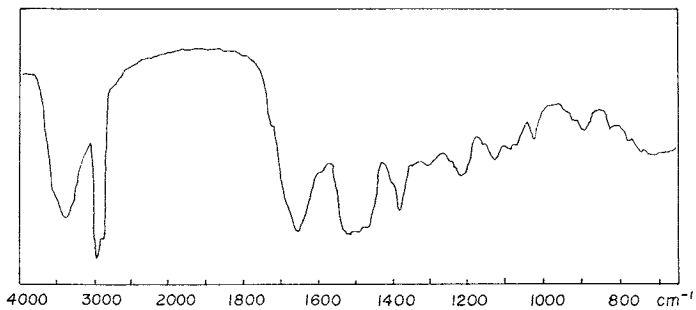



Fig. 2. Infrared absorption spectrum of thiopeptin B in Nujol.




The $R_f$ values for thiopeptin B and other sulfur-containing peptide antibiotics in thin-layer chromatography or paper partition chromatography with various solvent systems are shown in Table 2.

Table 2. Comparison of $R_f$ values of thiopeptin B and other closely related antibiotics

| Antibiotics | $R_f$ values on various experimental conditions* | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| A-59 | 0.50 | 0.30 | 0.27 | 0.43 | 0.14 |
| Siomycin A | 0.50 | 0.30 | 0.29 | 0.43 | 0.14 |
| Sporangiomycin | 0.50 | 0.30 | 0.27 | 0.41 | 0.14 |
| Thiostrepton | 0.50 | 0.30 | 0.38 | 0.43 | 0.38 |
| Thiopeptin B | 0.10 | 0.00 | 0.00 | 0.00 | 0.23 |

*1. Silica gel G TLC; chloroform - methanol (9 : 1)
2. Silica gel G TLC; chloroform - methanol (19 : 1)
3. Spotfilm (Tokyo Kasei Co. silica gel) TLC; chloroform - *n*-butanol (6 : 1)
4. PPC (Toyo Roshi No. 50); ethylacetate - *n*-hexane - 2N $NH_4OH$ (4 : 1 : 1)
5. PPC (Toyo Roshi No. 50); methanol - acetic acid - water (25 : 3 : 72)

Thiopeptin B is soluble in dioxane, dimethylsulfoxide, dimethylformamide, pyridine, chloroform and 3 N hydrochloric acid; slightly soluble in methanol, acetone and ethyl acetate; insoluble in ether, benzene, *n*-hexane and petroleum ether. It exhibits positive reaction in permanganate test, and negative in ninhydrin, biuret, FEHLING, ferric chloride, and DRAGENDORFF tests. Thiopeptin B is stable on treatment at 60°C for one hour at a pH range of 2.0~8.0.

## Biological Characteristics

The antimicrobial spectrum of thiopeptin B obtained by the agar dilution streak method is shown in Table 3. Thiopeptin B is primarily active against Gram-positive bacteria with minimum inhibitory concentrations of 0.01~0.25 mcg/ml. Thiopeptin B showed no cross resistance with penicillin G, aminobenzyl penicillin, streptomycin, chloramphenicol, tetracycline, leucomycin, erythromycin and kanamycin. The intraperitoneal administration of 500 mg/kg of thiopeptin B into mice did not result in any toxic symptom for 2 weeks after injection.

Table 3. Antimicrobial spectrum of thiopeptin B

| Test organism | MIC (mcg/ml) |
|---|---|
| *Bacillus subtilis* ATCC-6633 | 0.25 |
| *Bacillus megatherium* | 0.25 |
| *Staphylococcus aureus* 209P | 0.125 |
| Smith | 0.125 |
| Newman | 0.25 |
| Terashima | 0.25 |
| Pc-G, AB-PC, Sm, Cp-R* | 0.25 |
| Tc, Lm, Em, Pc-G, Sm, Cp-R* | 0.25 |
| *Sarcina lutea* PCI-1001 | 0.03 |
| *Corynebacterium xerosis* | 0.25 |
| *Escherichia coli* | >128 |
| *Proteus vulgaris* | >128 |
| *Pseudomonas aeruginosa* | >128 |
| *Streptococcus hemolyticus* | 0.05 |
| *Diplococcus pneumoniae* | 0.02 |
| *Mycobacterium* 607 | 50 |
| Sm-R* | 50 |
| Km-R* | 50 |
| *Mycobacterium phlei* | 50 |
| *Penicillium chrysogenum* Q176 | >128 |
| *Candida albicans* | >128 |
| *Candida utilis* | >128 |

*; Pc-G, AB-PC, Sm, Cp-R=Penicillln G, ampicillin, streptomycin, chloramphenicol-Resistant
Tc, Lm, Em, Pc-G, Sm, Cp-R=Tetracycline, leucomycin, erythromycin, penicillin G, streptomycin, chloramphenicol-Resistant
Sm-R=Streptomycin-Resistant
Km-R=Kanamycin-Resistant

## Discussion

On the basis of its chemical properties, thiopeptin B is clearly a member of sulfur-containing peptide antibiotics. Comparison with other known antibiotics of this group was listed in Table 4. Among this group, thiopeptin B can readily be differentiated from related antibiotics such as siomycins B and C[8], pepthiomycins[10], thermothiocin[12], and sulfomycins[13] comparing with their analytical

Table 4-1. Comparison of sulfur-containing peptide antibiotics

| | | Thiopeptin B | Thiostrepton | Siomycin A | Siomycin B | Siomycin C |
|---|---|---|---|---|---|---|
| Color of crystals | | Light yellow | | Colorless | Colorless or slight yellow | Colorless |
| m.p. | | >200 (219) | 246~255 | >200 (255~260) | >200 (255~260) | >200 (255~260) |
| $[\alpha]_D$ | | −80 ($CHCl_3$) | −60.2 (Dioxane) | −95.3 (Dioxane) | −102.9 (Dioxane) | −84.6 (Dioxane) |
| Elementary analysis | C | 49.26 | 52.51 | 51.55 | 43.83 | 47.97 |
| | H | 5.16 | 5.08 | 5.36 | 4.71 | 5.11 |
| | O | | | | | |
| | N | 14.35 | 16.08 | 15.81 | 13.11 | 11.17 |
| | S | 10.82 | 9.72 | 9.28 | 8.53 | 8.00 |
| Mol. weight | | 1,942 | 1,628.9 | 1,740 | 1,879 | 1,700 |
| Formula | | $C_{72}H_{90}O_{22}N_{18}S_6$ | $C_{72}H_{83}N_{19}O_{17}S_5$ | $C_{74}H_{92}O_{19}N_{19}S_5$ | $C_{63}H_{80}O_{32}N_{16}S_5$ | $C_{69}H_{87}O_{31}N_{14}S_{4\sim5}$ |
| $UV_{max}$ mμ | | 230~250<br>295<br>305 | 240~250<br>270~285 | 240~250<br>270~300<br>355 | 240~255<br>260~270<br>270~300 | 240~255<br>270~300<br>340, 355 |
| Amino acid present | | Thr, Ala, Cys, Val (Pro)? | Thr, Ala (Cys) Isoleu (Gly) | Thr, Ala, Val, Cys (Gly) | Same as siomycin A | Same as siomycin A |
| Reference | | | [5,6,7] | [8] | [8] | [8] |

Table 4-2. Comparison of sulfur-containing peptide antibiotics

| | A-59 | Peptiomycin A | Peptiomycin B | Sporangiomycin | Thermothiocin |
|---|---|---|---|---|---|
| Color of crystals | White prism | Pale yellow | Pale yellow | Whitish powder | Yellow powder |
| m. p. | 246~255 | 204~210 | 280~290 | 261~262 | 250, 300 (brown) |
| $[\alpha]_D$ | −92 (Dioxane) | +35 (DMF) | −30 (DMF) | −100 ($CHCl_3$) | +29.4 |
| Elementary analysis C | 49.57 | 58.73 | 51.43 | 50.4 | 46.01 |
| H | 5.62 | 6.23 | 5.26 | 5.6 | 7.14 |
| O | | 20.27 | 23.12 | 18.0 | |
| N | 13.94 | 11.23 | 14.81 | 15.5 | 14.5 |
| S | 10.21 | 4.22 | 4.73 | 10.5 | 6.21 |
| Mol. weight | ca. 2,000 | | | 1,800 | 1,500 |
| Formula | | | | $C_{77\sim82}H_{101\sim105}N_{20\sim21}O_{21}S_6$ | $C_{60}H_{110}N_{16}O_{25}S_5$ |
| $UV_{max}$ mμ | 240~250<br>275~285 | 230<br>310 | 246<br>310 | 318 ($H_2SO_4$) | 275 (DMF) |
| Amino acid present | Thr, Ala, Cys, Val, Pro, Lys, Asp, Glu, Try, Gly | Thr, Gly, Ala, Val, Isoleu, 3 unknown | Thr, Gly, Ala, Val, Isoleu, Leu, Phe, 3 unknown | 5 spots | Gly, Cys, Asp, Meth, Lys, Ala, Phe, Pro, γ-ABA |
| Reference | 9) | 10) | 10) | 11) | 12) |

Table 4-3. Comparison of sulfur-containing peptide antibiotics

| | Sulfomycin I | Sulfomycin II | Sulfomycin III |
|---|---|---|---|
| Color of crystals | Colorless powder | | |
| m. p. | >280 | 190 | 183 |
| $[\alpha]_D$ | −16 (MeOH) | −11.8 (MeOH) | +3.2 (MeOH) |
| Elementary analysis C | 49.95 | 50.68 | 50.42 |
| H | 4.50 | 4.25 | 4.29 |
| O | | | |
| N | 16.86 | 16.14 | 16.71 |
| S | 4.80 | 5.81 | 5.14 |
| Mol. weight | 1,218 | 1,138 | 1,233 |
| Formula | $C_{55\sim57}H_{56\sim64}N_{15\sim17}O_{20\sim22}S_2$ | $C_{45\sim47}H_{45\sim49}N_{10\sim14}O_{15\sim17}S_2$ | $C_{50\sim52}H_{50\sim51}N_{13\sim15}O_{16\sim18}S_2$ |
| $UV_{max}$ mμ | 252<br>325 | 252<br>323 | 252<br>324 |
| Amino acid present | Thr, Ala, Gly, 2 unknown | Thr, Ala, Gly, 2 unknown | |
| Reference | 13) | 13) | 13) |

data of sulfur contents. Chromatograhic comparision with related antibiotics of high sulfur-contents including siomycin A[13], A-59[9], thiostrepton[5,6,7] and sporangiomycin[11] showed thiopeptin B to be quite different from these related antibiotics. Thus, it seems reasonable to conclude that thiopeptin B is a new sulfur-containing peptide antibiotic.

Acknowledgement

The authors are grateful to members of Nagoya Pilot Plant of our company for preparation of fermentation product. They are also indebted to Misses E. KAJI and K. KURITA for technical assistance throughout this work.

They would like to express their appreciation for the receipt of antibiotic samples as follows: siomycin A from Shionogi Seiyaku Co., A-59 substance from Meiji Seika Co., thiostrepton from Squibb Laboratories, and sporangiomycin and thermothiocin from Lepetit Laboratories.

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