

AMINO ACIDS AND DERIVATIVES OF THIAZOLE-4-CARBOXYLIC  
ACID AS CONSTITUENTS OF THIOPEPTIN B

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L-Alanine, L-threonine, L-valine, D-cystine and three derivatives of thiazole-4-carboxylic acid (thiostreptin, 2-aminomethylthiazole-4-carboxylic acid and thiostreptoic acid) were isolated from the acid-hydrolysate of thiopeptin B. In addition, the presence of dehydrobutyrine and dehydroalanine residues in the antibiotic was determined. Other components remain unidentified.

Thiopeptins are sulfur-containing peptide antibiotics produced by *Streptomyces tateyamensis*<sup>1,2)</sup>. Quinaldic acid derivatives, were isolated from hydrolysates of thiopeptin B<sup>3)</sup>, the major components of the antibiotics. Consequently, thiopeptin B seems to resemble other sulfur-containing peptide antibiotics, thiostrepton<sup>4)</sup> and siomycins<sup>5)</sup>. However, their amino acid constituents differ from one to the other. In this paper, we wish to report on the amino acids and thiazole compounds isolated from the acid-hydrolysate of thiopeptin B.

On amino acid analysis of the hydrolysate, threonine, valine and alanine were found in the molar ratio of 1:1:2 (Table 1).

On isolation by ion-exchange chromatography, the amino acids were found to belong to the L series. Similarly, the antibiotic treated with sodium borohydride gave one equivalent of 2-amino-

Table 1. Amino acid analysis of acid hydrolysates of untreated, oxidized and reduced thiopeptin B\*

Pretreatment	Untreated**			HCO <sub>2</sub> H-*** oxidation	NaBH <sub>4</sub> -*** reduction
	5	15	30	15	15
Hydrolysis time (hour)					
Cysteic acid	—	—	—	0.82	—
Threonine	0.98	0.97	0.96	1.00	1.00
Alanine	1.37	1.69	1.77	1.81	3.81
Butyrine	—	—	—	—	0.84
Valine	0.74	0.93	0.93	0.74	0.91
Cystine	trace	0.04	0.10	—	trace
TST	0.71	0.41	0.19	0.26	0.39
ATC	0.06	0.19	0.28	0.22	0.25
TSA	0.08	0.11	0.13	—	0.17

\* Peptide samples were hydrolyzed with constant-boiling HCl in evacuated-sealed tubes at 110°C.

\*\* Values are given in  $\mu$  mole of products resulting from 1.94 mg of thiopeptin B.

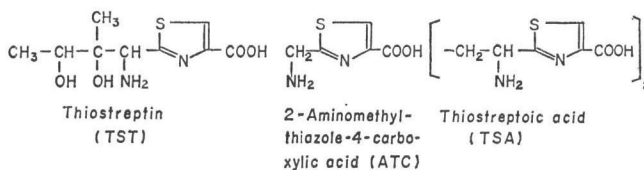
\*\*\* The sodium borohydride-reduction was carried out according to the Reference 7, and the performic acid-oxidation in a similar way to that described in the experimental section. Values represent molar ratios to threonine content.

butyric acid (butyric) and two of alanine in addition to the amino acids described above. This observation is similar to that found for thiostrepton<sup>7,8)</sup>, and suggests that the thiopeptin B molecule also contains one residue of 2-aminocrotonic acid (dehydrobutyric) and two residues of 2-aminoacrylic acid (dehydroalanine). This was substantiated further by formation of the 2,4-dinitrophenylhydrazones of 2-ketobutyric and pyruvic acids after acid-hydrolysis of thiopeptin B.

Although cystine could neither be detected by amino acid analysis of the hydrolysate of thiopeptin B nor be isolated from the hydrolysate of either the intact or the NaBH<sub>4</sub>-reduced antibiotics, cysteic acid was recovered in equimolar quantity by hydrolyzing the performic acid-oxidized antibiotic. Since cysteic acid was isolated as a racemic mixture, assignment of the configuration of its precursor was unsuccessful. Similarly, performic acid-treated glutathione also gave optically inactive cysteic acid. Determination of the configuration of a cysteine or cystine residue in a peptide by isolating cysteic acid after performic acid-oxidation followed with hydrolysis is difficult. Even if cysteic acid partially retains its configuration after this treatment, it is not easy to determine whether the acid is D or L because of its low rotatory activity.

The acid hydrolysate of thiopeptin B gave a small cysteine peak on amino acid analysis. After air-oxidation of the hydrolysate in a neutral solution, this peak disappeared and the amount of cystine increased. Consequently, isolation of cystine was undertaken as follows: hydrolysis with distilled HCl under steaming nitrogen, air-oxidation of the hydrolysis product in neutral solution, and isolation by ion-exchange chromatography on a Dowex-50W column. Cystine thus obtained was considered almost pure based on amino acid analysis and it was of D-configuration by optical rotation. The cystine, however, appeared partially racemized as its rotation was less than the literature value. The result indicates that the cysteine precursor in thiopeptin B belongs to the D-series. Thiopeptin B itself was negative in the sodium nitroferricyanide test, but it yielded cysteine after acid hydrolysis. Suggesting that the cysteine residue in thiopeptin B, like thiostrepton, might be present as a thiazoline derivative.

From the acid-hydrolysate of thiopeptin B, derivatives of thiazole carboxylic acid were obtained; including thiostreptin (TST), 2-aminomethylthiazole-4-carboxylic acid (ATC) and thiostreptoic acid (TSA). The amounts measured by amino acid analysis are shown in Table 1. The derivatives were



isolated by ion-exchange chromatography, and identified by comparing their spectra (UV, IR, NMR), optical rotations and elemental analyses with similar derivatives obtained from thiostrepton. With prolonged hydrolysis, the amount of TST decreased with an accompanying increase in the amount of ATC (Table 1). This correlation was also observed for thiostrepton<sup>4)</sup>. It is presumed that ATC resulted from further degradation of TST.

Comparison of molar ratios of hydrolysis products from performic acid-treated thiopeptin B with hydrolysis products from the non-treated antibiotic, showed that about 20% of the valine and almost all of the TSA were decomposed by the performic acid-oxidation. Similar results were obtained with

thiostrepton.

In the case of thiostrepton<sup>9)</sup>, thiostreptonic acid was obtained from the water-insoluble portion of the residue obtained after concentrating the HCl-hydrolysate. In contrast, the residue from thiopeptin B was almost completely soluble in water, and no detectable thiostreptonic acid was present. Based on the structure of thiostrepton proposed by X-ray crystallographic analysis<sup>10)</sup>, thiostreptoic and thiostreptonic acids are assumed to arise from the same portion of the parent molecule. Hence it is proposed that thiopeptin B and thiostrepton differ in this portion of the molecules.

A comparison of the molecular formula of thiopeptin B<sup>1)</sup> with the sum of identified constituents suggests that some components might still be undetected. Studies on the structure of thiopeptin B continued.

### Experimental

Melting points are uncorrected. UV spectra were obtained by measurement with a Hitachi Spectrometer, Model 323; IR spectra with a Hitachi Grating Infrared Spectrophotometer, Model 215; and NMR spectra with a Varian Associates Spectrometer HA-100 or a Hitachi-Perkin Elmer R-20A Spectrometer.

#### Analysis of amino acids and thiazole carboxylic acid derivatives

These were analyzed with a Jeol Automatic Amino Acid Analyzer, Model JLC-6AS, using a LC-R-2 resin column (0.8 × 14.0 cm). The conditions were as follows: flow rate, 0.96 ml/min; column temperature, 40°C (55 min), 52°C. Eluting buffer (sodium citrate); pH 2.97 (0.2 N), 45 min; pH 3.25 (0.2 N), 15 min; pH 3.82 (0.2 N), 22 min; pH 3.94 (0.38 N), 23 min; pH 4.97 (0.2 N). The peaks appeared in the following order with retention times as indicated: cysteic acid, 17 min; threonine, 31 min; cysteine, 42 min; alanine, 50 min; butyric acid, 55.5 min; valine, 61 min; cystine, 73 min; TSA, 93 min; ammonia, 108 min; ATC, 118 min; TSA, 134 min. For the quantitative analysis of thiazole-4-carboxylic acid derivatives, products isolated from the hydrolysate of thiopeptin B were used as reference standards.

#### Isolation of hydrolysis products from thiopeptin B

##### Procedure A.

Thiopeptin B (5 g) was hydrolyzed by refluxing with 250 ml of 6 N HCl for 24 hours. After concentration *in vacuo*, the residue was dissolved in water. The solution was extracted with ether and the aqueous layer was again concentrated. The residue was dissolved in water and applied onto a Dowex 50W × 12 (H-form) column (2.5 × 40 cm). The column was eluted with water (1.6 liters) and then with hydrochloric acid; 0.24 N (1.6 liters), 0.30 N (1.6 liters), 0.36 N (3.8 liters), 0.48 N (3.5 liters), 0.60 N (3.6 liters), 1.0 N (8.2 liters), 2.0 N (3.6 liters), 6.0 N (4 liters). Fractions of 400 ml each were collected.

L-Threonine (227 mg) was isolated from fractions 7 and 8:  $[\alpha]_D^{21} -13.0^\circ$  (*c* 2, 5 N HCl); in Lit.,  $[\alpha]_D^{25} -15.0^\circ$  (*c* 1~2, 5 N HCl). L-Alanine (110 mg) was isolated from fractions 11 and 12:  $[\alpha]_D^{25} +14.5^\circ$  (*c* 2, 5 N HCl); in Lit.,  $[\alpha]_D^{25} +14.6^\circ$  (*c* 2, 5 N HCl). The residue from fractions 13 to 19 was further purified by repeating a similar ion-exchange chromatography (elute, 0.2 N HCl; fraction, each 200 ml), and then L-valine (130 mg) was obtained from fractions 30 to 37:  $[\alpha]_D^{21} +28.8^\circ$  (*c* 1, 5 N HCl); in Lit.,  $[\alpha]_D^{25} +28.3^\circ$  (*c* 1~2, 5 N HCl). The IR spectra of these amino acids were identical with those of the respective authentic samples.

ATC was obtained from fractions 25 to 33 in the first ion-exchange chromatography, and further purified by crystallization from water-ethanol-ether as hydrochloride. Yield, 87 mg. M.p. 258~260°C (in Lit.<sup>11)</sup>, 263~265°C); UV,  $\lambda_{\max}^{H_2O}$  234 nm,  $E_{1\%}^{1\text{cm}}$  286 (in Lit.<sup>11)</sup>,  $E_{1\%}^{1\text{cm}}$  270); NMR (in D<sub>2</sub>O),  $\delta$  4.15 (s, 2H), 8.04 (s, 1H).

Anal. Calcd. for C<sub>5</sub>H<sub>6</sub>O<sub>2</sub>N<sub>2</sub>S·HCl: C 30.85, H 3.63, N 14.39, S 16.47, Cl 18.21%.

Found: C 30.39, H 3.46, N 13.58, S 15.90, Cl 18.18%.

TSA was isolated from fractions 70 to 72 and crystallized as dihydrate from 0.1 N NH<sub>4</sub>OH - 0.1 N AcOH. Yield, 95 mg. M.p. 236~237°C (in Lit.<sup>12)</sup>, 235~237°C); UV,  $\lambda_{\max}^{NH_4Cl}$  236 nm,  $E_{1\%}^{1\text{cm}}$  445 (in Lit.<sup>12)</sup>,  $E_{1\%}^{1\text{cm}}$  396); NMR (in TFA),  $\delta$  2.57 (m, 4H), 5.25 (m, 2H), 8.68 (s, 2H) (in Lit.<sup>4)</sup>,  $\delta$  2.62, 5.36, 8.75).

Anal. Calcd. for  $C_{12}H_{14}O_4N_4S_2 \cdot 2H_2O$ : C 38.09, H 4.79, N 14.81, S 16.95%.  
 Found: C 38.10, H 4.76, N 14.51, S 17.10%.

#### Procedure B.

In order to isolate TST, 2 g of thiopeptin B was refluxed with 100 ml of 10 N HCl for 6 hours. The solution was treated in a similar way to that in procedure A, and the water-soluble and EtOAc-insoluble product was applied to an ion-exchange chromatograph (Dowex 50W $\times$ 12, 3 $\times$ 11 cm). Pyridine-acetic acid buffer (0.2 M, pH 3.1) was used as eluent. Fractions containing a ninhydrin-positive substance (yellow to purple, on Avicel-cellulose tlc plate) were collected and concentrated. The residue was further purified by repeating the chromatography.

TST was isolated as yellow crystals (monoacetate) after recrystallization from water-acetone. Yield, 90 mg. M.p. 165~168°C;  $[\alpha]_D^{25} -4.4^\circ$  (c 1, 1 N AcOH) (in Lit.<sup>41</sup>),  $[\alpha]_D^{20} -4^\circ$  (c 1, 1 N AcOH); UV,  $\lambda_{max}^{H_2O}$  237 nm,  $E_{1cm}^{1\%}$  206 (in Lit.<sup>41</sup>),  $\lambda_{max}^{alc}$  of TST 237 nm,  $E_{1cm}^{1\%}$  275).

Anal. Calcd. for  $C_9H_{14}O_4N_2S \cdot CH_3CO_2H$ : C 43.13, H 5.92, N 9.14, S 10.47%.  
 Found: C 43.21, H 6.02, N 9.64, S 11.01%.

An aliquot of the acetate was changed to the dihydrochloride monohydrate by evaporation after dissolving in 0.1 N HCl. NMR of the dihydrochloride monohydrate (in TFA),  $\delta$  1.28 (s, 3H), 1.56 (d, 3H), 4.39 (m, 1H), 5.56 (s, 1H), 8.69 (s, 1H).

Anal. Calcd. for  $C_9H_{14}O_4N_2S \cdot 2HCl \cdot H_2O$ : C 32.06, H 5.38, N 8.31, S 9.51%.  
 Found: C 32.00, H 5.54, N 8.37, S 10.13%.

#### Isolation of cysteic acid from thiopeptin B and glutathione oxidized with performic acid

From thiopeptin B. Thiopeptin B (2.01 g) was treated at 0°C overnight with performic acid prepared from 27 ml of 88% formic acid and 3 ml of 30% hydrogen peroxide. After evaporation, the product was refluxed with 100 ml of 6 N HCl for 20 hours. After the solution was concentrated to dryness *in vacuo*, the residue was dissolved in 150 ml of water, and the solution was filtered. The filtrate was extracted with ethyl acetate and the aqueous layer was evaporated. From the residue, cysteic acid was isolated by repeating ion-exchange chromatographies; 1st, Dowex 50W $\times$ 12 column (3.5 $\times$ 21 cm) with 0.2 M pyridine-acetic acid buffer (pH 3.3); 2nd, Dowex 1 $\times$ 2 column (1.6 $\times$ 9 cm) with 1.0 N HCl; 3rd, Dowex 1 $\times$ 2 column (1.7 $\times$ 30 cm) with 0.5 M acetic acid (130 ml) followed by 1.0 N HCl. Finally, cysteic acid was isolated in the yield of 155 mg and it was optically inactive. Its IR spectrum was identical to that of authentic DL-cysteic acid.

From glutathione. Glutathione (reduced form, 303 mg) was treated with performic acid in a similar fashion. After concentration *in vacuo*, the residue was dissolved in 50 ml of 6 N HCl and refluxed for 20 hours. After evaporation, the residue was purified with ion-exchange chromatography (Dowex 1 $\times$ 2 column, 1.6 $\times$ 38.5 cm, with 200 ml of 0.5 N acetic acid followed by 1.0 N HCl). Pure cysteic acid was isolated in the yield of 147 mg and it was optically inactive. Its IR spectrum agreed with that of the authentic sample. Nevertheless, glutamic acid concurrently isolated in this procedure did not racemize.

#### Isolation of D-cystine from thiopeptin B

Thiopeptin B (2.58 g) was hydrolyzed with 130 ml of 6 N HCl under reflux in an atmosphere of nitrogen for 12 hours. After concentration *in vacuo*, the residue was dissolved in water and the solution was extracted with ether and ethyl acetate, and then the aqueous layer was again concentrated. The aqueous solution of the residue was adjusted to pH 7.0 with 1 N NaOH. The final volume of the solution was 55 ml. Air was bubbled into the solution for two days in order to oxidize cysteine to cystine. After removing the solvent, the residue was dissolved into 40 ml of 0.1 M pyridine-acetic acid buffer (pH 2.75) and applied onto a Dowex 50W $\times$ 12 (pyridine form) column (2.7 $\times$ 65 cm). The column was eluted with the same buffer, and fractions of 10 g each were collected: eluting buffer; 0.1 M pyridine-acetic acid buffer (pH 2.75), 60 ml; 0.1 M (pH 3.15), 240 ml; linear gradient, 0.1 M (pH 3.15), 600 ml~0.2 M (pH 3.15), 600 ml; 0.2 M (pH 3.15), 200 ml; linear gradient, 0.2 M (pH 3.15), 650 ml~0.2 M (pH 4.40), 650 ml; 0.2 M (pH 4.40). Fraction Nos. 266~317 were combined and concentrated. The residue was triturated with 5 ml of water and filtered. From the result of amino acid analysis, the insoluble solid (50 mg) was almost pure cystine. A part of the solid was further purified by recrystallization from water.  $[\alpha]_D^{27} +134^\circ$  (c 0.1, 1 N HCl); Lit. for L-cystine,  $[\alpha]_D^{20} -223.4^\circ$  (c 1, 1 N HCl). For elemental analysis, the sample was further purified by repetition of ion-exchange chromatography.

Anal. Calcd. for  $C_8H_{12}N_2O_4S_2$ : C 29.99, H 5.03, N 11.66, S 26.69%.

Found: C 29.63, H 4.96, N 11.29, S 26.41%.

Hydrazone formations<sup>6)</sup> from pyruvic and 2-ketobutyric acids in the hydrolysate of thiopeptin B  
Thiopeptin B (322 mg) was hydrolyzed with 6 N HCl (16 ml) in evacuated-sealed tubes at 110°C for 10 hours. Excess of 2,4-dinitrophenylhydrazine in 3 N HCl was added into the hydrolysate solution diluted twofold with water. After a few hours, a precipitate was collected and dissolved into 2 ml of 5% NaHCO<sub>3</sub> solution. After removing an insoluble part, the filtrate was acidified with 6 N HCl. 2,4-Dinitrophenylhydrazones were collected by filtration. They exhibited four spots on tlc (Merck silicagel-precoated plate, CHCl<sub>3</sub> - MeOH - AcOH, 95: 10: 3); R<sub>f</sub>, 0.37, 0.47, 0.62, 0.74. These spots corresponded to those of the authentic hydrazone from 2-ketobutyric acid (R<sub>f</sub> 0.47, 0.74) and that from pyruvic acid (R<sub>f</sub> 0.37, 0.62).

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