STUDIES ON SIOMYCIN. II

THE COMPOSITION AND DEGRADATION PRODUCTS OF SIOMYCIN A

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From the hydrolysate of siomycin A, an antibiotic from *Streptomyces sioyaensis*, L-threonine, L-alanine, L-valine, D-cysteine and three thiazole-4-carboxylic acid derivatives were isolated. These thiazoles were identified as thiostreptine, 2-aminomethylthiazole-4-carboxylic acid and 2-(1-amino acetonyl) thiazole-4-carboxylic acid. A 4-(α-hydroxyethyl)-8-hydroxyquinaldic acid, and two unidentified C_{10}- and C_{12}-compounds were also isolated in the hydrolysate. The formation of pyruvic acid (2 moles), α-amino-*n*-butyric acid (less than 1 mole), propionic acid (2~3 moles) and acetaldehyde (1 mole) by the action of acid or alkali on siomycin A was demonstrated.

Siomycins A, B and C, sulfur-containing peptide antibiotics, have been isolated from cultures of *Streptomyces sioyaensis* and siomycin preparations[1,2]. Characterization of the three siomycins has been described previously[2]. Among these antibiotics, the main product of the streptomyces was shown to be siomycin A.

The present paper deals with the details of the composition and degradation products of siomycin A, and the purpose of this study was the elucidation of its structure.

Results

Hydrolysis of siomycin A with constant boiling hydrochloric acid yielded four amino acids, threonine, alanine, valine and cysteine, and two derivatives of methylthiazole carboxylic acid. These thiazoles were identified as thiostreptine and 2-aminomethylthiazole-4-carboxylic acid. After oxidation of siomycin A with performic acid, amino acid analysis, by a modification of the STEIN-MOORE procedure[3], gave cysteic acid, α-amino-*n*-butyric acid and a new thiazole in addition to threonine, alanine, valine and the two thiazoles. The new thiazole was identified as 2-(1′-aminoacetonyl)thiazole-4-carboxylic acid.

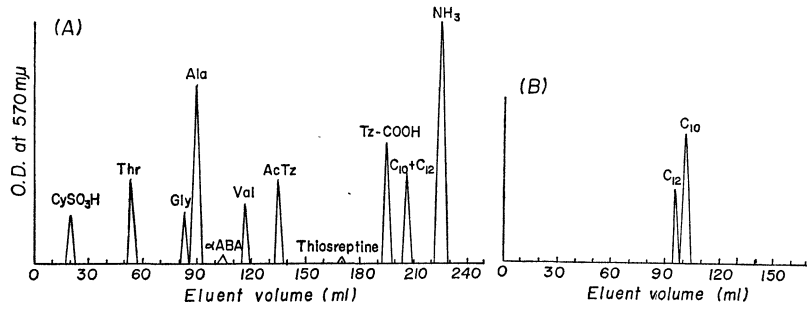
Hydrolysis of reduced siomycin A by sodium borohydride yielded 3 moles of alanine in addition to the ninhydrin-positive substances described above. Furthermore, two unknown C_{10}- and C_{12}-compounds were also found in the hydrolysate of an ammonia-treated (ammonolysis) siomycin A. Molar ratios and positions appear in the analysis chart and are summarized in Table 1 and Fig. 1, respectively.

Amino acids. Quantitative amino acid analysis of 1.72 mg of siomycin A shows

Fig. 1. The position of components and degradation products of siomycin A and modified siomycins A on the 50 cm chromatogram.

Flow rate of the buffer solutions was 30 ml/hr. Temperature was 50°C. (A) 0.25 M citrate, pH 3.25, 170 min; 0.25 M citrate, pH 4 25, 120 min; and 0.7 M citrate, pH 5.28, 240 min. (B) 0.7 M citrate, pH 5.28, 300 min.

Abbreviations: αABA, α-amino-*n*-butyric acid; AcTz, 2-aminoacetonyl thiazole-4-carboxylic acid; Tz-COOH, 2-aminomethylthiazole-4-carboxylic acid; C_{10} and C_{12}, unidentified C_{10} and C_{12} compound.



1 μmole each of threonine, alanine, valine and cysteine. The small amount of glycine found could be a degradation product from threonine or the thiazoles. Approximately 6 moles of ammonia are also liberated from 1 mole of siomycin A. The amino acid components were separated from the acid hydrolysate of siomycin A and the oxidized siomycin A on columns of Amberlite CG 120 resin. Identification of amino acids was accomplished by comparison with authentic samples. Measurements of specific rotations revealed that the threonine, alanine and valine belong to the L-series, while the cysteine has the D-configuration as shown in Table 2.

Table 1. Composition of siomycin A

	Siomycin A	$NaBH_4$-reduced and performic acid-oxidized siomycin A
Threonine	0.88	0.59
Glycine	trace	0.15
Alanine	0.98	2.94
Valine	0.95	0.69
Cysteine	1.18*	1.06
Thiostreptine	0.56	0.68
2-Aminomethylthiazole-4-carboxylic acid	0.15	0.24
α-Amino-*n*-butyric acid	0.00	0.30
Aminoacetonyl-thiazole-4-carboxylic acid	0.00	0.30～0.50
Ammonia	6.1	4.0
4(α-Hydroxyethyl)-8-hydroxyquinaldic acid**	0.79	
$C_{10}H_{20}O_4N_4S$**	0.78	
$C_{12}H_{15}O_5N_4S \cdot HCl$**	0.64	

Hydrolysis was carried out in constantly boiling HCl for 20 hours at 110°C.

* Determined as cysteic acid after oxidation by performic acid.

** By weighing after isolation.

Underlined compounds are considered to be constituents of siomycin A.

Derivatives of quinaldic acid. Intact siomycin A is a ninhydrin-negative substance and has a strong UV absorption in the region of 230～280 mμ. It was considered, therefore, that a UV-sensitive compound such as quinaldic acid might be attached at the nitrogen terminal of siomycin A as in thiostrepton.

When siomycin A was hydrolyzed by 0.1～6 N HCl, patterns of the UV-sensitive

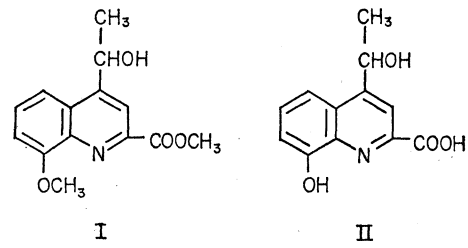
Table 2. Specific optical rotations of components of siomycin A

Component	Derivative	Found $[\alpha]_D^{25}$	Conditions	Form	Reference
4-Hydroxyethyl-8-hydroxyquinaldic acid	Methyl ester methyl ether	−79.0°	*c* 1, EtOH	—	−78°[5]
Alanine	—	+13.1	*c* 1, N HCl	L	+14.7
Threonine	—	−28.5	*c* 1, H_2O	L	−28.2
Valine	—	+26.2	*c* 1, N HCl	L	+27.7
Cysteine	Cysteic acid	−0.32	*c* 1, H_2O	D*	+7.8**
Thiostreptine	—	−2.8	*c* 1, N AcOH	—	−4[5]

* This was also confirmed by an optical rotatory dispersion measurement.
** Values are given as L-form of cysteic acid.

compounds became complicated, and at least two compounds were demonstrated to be present in the hydrolysate. Therefore, methanolysis technique was used to release the UV-sensitive compound from siomycin A. This compound was further methylated by diazomethane and characterization was performed. From the analytical values the formula $C_{14}H_{15}O_4N$ could be calculated for this compound. This was identical with 4-(α-hydroxyethyl)-8-methoxyquinaldic acid methyl ester (Fig. 2, I) which was obtained from thiostrepton[4,5] in melting point, optical rotation, UV absorption, IR absorption and NMR spectra.

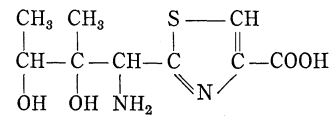
Fig. 2. I, 4-(α-Hydroxyethyl)-8-methoxyquinaldic acid methyl ester; II, 4-hydroxyethyl-8-hydroxyquinaldic acid.



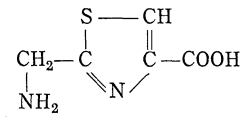
I II

4-Hydroxyethyl-8-hydroxyquinaldic acid (II) was also obtained as semi-hydrate from the methanolysate. This compound seems to be the intact original N-terminal compound of siomycin A. Data for analysis and properties of this compound are shown in the experimental section.

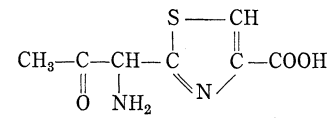
Fig. 3



III : Thiostreptine



IV : 2-Aminomethylthiazole-4-carboxylic acid



V : 2-(1-Aminoacetonyl) thiazole-4-carboxylic acid

Derivatives of thiazole-4-carboxylic acid. Ion exchange column chromatography of the acid hydrolysate of siomycin A and the oxidized siomycin A revealed three peaks of 2-aminomethylthiazole-4-carboxylic acid-derivatives. These thiazole compounds gave yellow colors with ninhydrin but the colors slowly changed to purple. The first isolated thiazole from the hydrolysate of siomycin A was shown to be thiostreptine (Fig. 3, III). Maximum concentrations of this compound in the hydrolysate were under the conditions of 11.4 N HCl-hydrolysis, 105~110°C for 6 hours, and then the concentrations decreased slowly under further hydrolysis. Values from analyses gave a formula, $C_9H_{14}O_4N_2S$. The material is identical with thiostreptine[5,6] by UV absorption, optical rotation, NMR, neutral equivalent determination, Rf-values in paper chromatography and periodate consumption of the DNP-derivative. Thiostreptine converts slowly to

2-aminomethylthiazole-4-carboxylic acid by acid or alkali hydrolysis, and this was also demonstrated on isolated material.

The second isolated thiazole was 2-aminomethylthiazole-4-carboxylic acid ($C_5H_6N_2O_2S$), itself (Fig. 3, IV). Elution with a more concentrated higher pH buffer, 0.4 M, pH 3.4, gave fractions which contained this thiazole. The structure of the compound was confirmed by elementary analysis and other characterization.

From the hydrolysate of the oxidized siomycin A, a new thiazole compound was obtained as the third thiazole derivative. The structure of this compound was also confirmed by elementary analysis, mass spectrum and particularly, NMR spectra. The formula is shown in Fig. 3, V. This compound was stable and shows the typical character of a thiazole. This aminoacetonylthiazole has not been previously derived from thiostreptine.

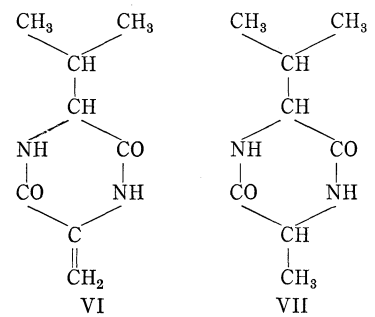
Unidentified compounds. Two unknown S-containing compounds were isolated from the ammonia-treated siomycin A. These compounds were separated as white amorphous or crystalline materials from the acid hydrolysates. Analysis revealed that they each contained one sulfur atom and four nitrogens. Based on the elemental analysis, these compounds were designated as the C_{10}, and C_{12} compounds, respectively. The C_{10} and C_{12} compounds show some characteristics of thiazolidine rather than those of a thiazole or thiazoline. Since the C_{12} compound sulfate was an amorphous material and strongly hygroscopic, the formula ($C_{12}H_{15}O_5N_4S$, mol. wt. 327.3) was deduced from its mono-dinitrophenyl derivative ($C_{18}H_{17}O_9N_6S$, mol. wt. 493.4). The C_{12} compound was ninhydrin-positive and formed a hydrazone with 2,4-dinitrophenyl hydrazine. Analyses and some properties are described in the experimental section.

The C_{10} compound was isolated as a crystalline material ($C_{10}H_{20}O_4N_4S \cdot 2H_2O$, mol. wt. 328.4). This was also ninhydrin-positive and could be derived as the tri-(*p*-bromobenzoyl)-derivative ($C_{31}H_{29}O_7N_4SBr_3$, 841.4). In the partial characterization of the compound, no meaningful UV absorption spectrum was found, but the NMR spectra showed the probable presence of a thiazolidine ring. Properties and derivatives of the C_{10} compound are also mentioned in the experimental section.

Both the C_{10} and C_{12} compounds were, of course, artifacts formed, are independent of each other, from the degradation of siomycin A. Elucidation of the structures of both compounds was very difficult, because no mass spectrum measurements could be obtained as they had no significant volatility.

Diketopiperazine containing valine and dehydroalanine. When siomycin A was heated in a solution of glacial acetic acid, the diketopiperazine, containing valine and dehydroalanine residues (Fig. 4, VI), was liberated. This diketopiperazine should be a fragment of siomycin A rather than a degradation product. Acid hydrolysis of one mole of the compound, gave L-valine (0.93 mole), pyruvic acid and ammonia (0.94 mole). After

Fig. 4



VI
Diketopiperazine of valine and dehydroalanine

VII
Diketopiperazine of valine and alanine

Table 3. Degradation prouducts from siomycin A

Products	Condition	Detected form	Found amounts from 1,712 g of siomycin A
Pyruvic acid	2~6 N HCl, 110°C	2,4-dinitrophenyl-hydrazone	1.81~2.12 moles
α-Amino-*n*-butyric acid	Oxy-Red-6 N HCl, 105°C, 20 hr.	free form and DNP-compound	less than 1 mole
Propionic acid	0.5 N NaOH, RT, 2 hr., H^+ dist.	methyl ester by GLC	2.37, 3.11 moles
Acetaldehyde	0.5 N NaOH, RT, 2 hr., OH^- dist.	2,4-dinitrophenyl-hydrazone	1.06 moles
Ammonia	2 N HCl, 100°C, 3 hr.	by titration	2.2~2.3 moles
Carbon dioxide	(1) 2 N HCl, 105°C, 2 hr. (2) 0.5 N NaOH, RT, 2 hr.	$BaCO_3$	(1) 0.7 mole (2) 0.5 mole

catalytic reduction this compound converts to another diketopiperazine containing L-valine (1.00) and DL-alanine (1.03). The new diketopiperazine was identical with the synthetic one.

The diketopiperazine containing valine and alanine (Fig. 4, VII) was also obtained from reduced siomycin A under the same conditions. This indicates that the dehydroalanine residue might be present in the molecule of siomycin A.

Other degradation products. Treatment of siomycin A with acid or alkali causes the formation of volatile compounds such as pyruvic acid, propionic acid, acetaldehyde, ammonia and carbon dioxide. They are listed in Table 3, together with α-amino-*n*-butyric acid. Pyruvic acid was detected as 2,4-dinitrophenyl hydrazone. It is worth noting that the formation of 2 moles of pyruvic acid accompanied the liberation of 2 moles of ammonia under the conditions of 2 N HCl, 100°C for 3 hours.

α-Amino-*n*-butyric acid was identified by comparison with an authentic sample. Thin-layer chromatography of the synthetic 2,4-dinitrophenylamino-*n*-butyric acid agreed completely with that of the natural one. Two moles of propionic acid and one mole of acetaldehyde were found when the antibiotic was treated with diluted alkali at room temperature. Residues or positions which liberate both volatile compounds are still unknown.

Discussion

When acid hydrolysates of siomycin A and some derivatives of the antibiotic were subjected to amino acid analysis, ten major ninhydrin positive compounds were found (Fig. 1). Five of these substances corresponded to cysteic acid, threonine, alanine, valine and ammonia. These four amino acid residues account for only 22 % of the weight of the siomycin A molecule. The cysteine belongs to the D-series. D-Cysteine has been obtained from thiostrepton[5], malformin[7] and luciferin.[8,9]

The chromophore part of siomycin A has been characterized as a derivative of quinaldic acid. The structure of the derivative was the same as that of thiostrepton[5]. BODANSZKY *et al.* reported that 4-(α-hydroxyethyl)-8-hydroxyquinaldic acid could be an artifact, and its precursor might be an "indol"[5]. However, we have found no evidence so far for this hypothetical "indol". We have attempted to compare siomycin A and thiostrepton, and find that siomycin A is not quite identical with thiostrepton in respect to the chromophore group. In addition, it has been shown that thiostrepton gives a purple color reaction with EHRLICH's reagent[5], while siomycin yields a negative test.

Intact siomycin A is negative with $FeCl_3$, suggesting that the hydroxy group in the 8 position in the quinaldic acid is masked by another group, or that steric hindrance to the reaction with $FeCl_3$ may be present in the area of this OH.

Results obtained for the nature of the quinaldic acid derivative of siomycin C, shows it to resemble strongly 4-ethyl-8-hydroxyquinaldic acid.

Oxidation of siomycin A yielded a new thiazole compound. The acetylthiazole seems to be derived from thiostreptine by oxidation. Isolated thiostreptine, however, has never been converted to acetylthiazole by performic acid oxidation. On the other hand, the acetylthiazole has never been isolated from the acid hydrolysate of intact siomycin A. It should be considered that this thiazole might be an artificial compound and not derived from the compounds under examination.

It has been shown that siomycin A contained 5 atoms of sulfur in the molecule[2]. From the above results, thiostreptine, cysteine and probably a precursor of the new thiazole seem to be constituents of the antibiotic molecule. Two additional S-containing unknown compounds, C_{10} and C_{12}, were found in the hydrolysate of the ammonolyzed siomycin A. Further investigation of both compounds is necessary, since it is quite clear that they are constituents of the antibiotic in their original forms. According to the recently established structure of siomycin A[10], both compounds exist independently in the sequence of the molecule.

The diketopiperazine containing L-valine and dehydroalanine was isolated from siomycin A in a yield approximating 40 %. Another diketopiperazine containing valine and alanine was obtained from the reduced antibiotic. This indicates that the dehydroalanine residue might be present in the molecule of siomycin A. It is interesting to note that a diketopiperazine containing isoleucine and alanine has also been isolated from thiostrepton by heating a solution of the antibiotic in glacial acetic acid[5].

Siomycin B has been also investigated using the acid hydrolysates. While the reduced siomycin A gives 3 moles of alanine, approximately 2 moles of alanine were found in the hydrolysate of the reduced B.

Some volatile compounds formed from siomycin A as degradation products have been characterized. Residues or positions which liberate these compounds are still unknown except for the 2 moles of pyruvic acid. It is clear that the pyruvic acid is liberated from the two dehydroalanine residues although no definite evidence has been found so far on the nature of these residues in siomycin A.

In the present paper we have mentioned more than 10 compounds which were obtained from siomycin A. Among them only six compounds can be considered to occur in the antibiotic. These are: 4-(α-hydroxyethyl)-8-hydroxyquinaldic acid, L-alanine, thiostreptine, L-threonine, D-cysteine and L-valine. Although the acetyl thiazole, the C_{10} and C_{12} compounds might be constituents, they experimentally occur as artificial compounds, and the structures of their original precursors are still obscure. Further, 2-aminomethylthiazole-4-carboxylic acid, pyruvic acid, propionic acid and acetaldehyde should be considered to be degradation products of the antibiotic.

A trial summation of these constituent and the artificial compounds gives an empirical formula of $C_{71}H_{87}O_{21}N_{19}S_5$, while the molecular formula obtained from the elementary analysis is $C_{74}H_{92}O_{19}N_{19}S_5$.

Finally, in the present study on the composition of siomycin A, thiostreptoic acid and the "362 fragment"[5] were not found in any amount.

Experimental

Siomycin A. Some siomycin A preparations were supplied from the pilot plant of our company. Pure siomycin A was isolated from cultures of *Streptomyces sioyaensis* (unpublished data). Siomycin A was recrystallized from a mixture of chloroform and

methanol (1 : 1) before use. Data of elementary microanalysis and molecular weight of siomycin A have been described previously[2].

Analytical method. Melting points were taken on a hot plate and are uncorrected. Paper and thin-layer chromatograms were run with the solvent *n*-butanol - acetic acid - water (4 : 1 : 2) unless otherwise stated.

Ultraviolet absorption spectra were measured with a Perkin-Elmer type 202 Spectrophotometer. Infrared spectra were taken with KBr tablet or Nujol method using a Nihon Bunko DS-201 B Spectrometer. Proton magnetic resonance spectra were taken in trifluoroacetic acid or D_2O by use of Varian Model A-60 Spectrometer.

Analysis for amino acids and ninhydrin-positive substances were perfomed by automatic amino acid analyzer using a Hitachi KLA midified type 2.

Oxidation of siomycin A. One hundred milligrams of siomycin A were dissolved in 10 % performic acid at 0°C and allowed to stand overnight at the same temperature. The solution was evaporated to dryness and the residue was dried over solid alkali *in vacuo*. The oxidized siomycin A was homogeneous by thin-layer chromatography, and soluble in water.

Reduction of siomycin A. One gram of siomycin A was dissolved in 100 ml of 60 % dioxane and stirred. In this solution 0.5 g $NaBH_4$ was added slowly with cooling. After 2 hours the mixture was evaporated and dissolved again in water, and neutralized by adding diluted HCl. Precipitates formed were collected by filtration and recrystallized from dilute acetone. Yield, 0.80 g. $[\alpha]_D^{23}$ −31.2 (*c* 1, 67 % dioxane). Anal. Found: C 50.64 %, H 5.35 %, N 15.19 %, S 9.42 %. Soluble in acetone and scarcely soluble in water. No activity was found against any microorganisms tested.

Ammonolysis of siomycin A. Three hundred milligrams of siomycin A were mixed with 5 ml of concentrated ammonia and 10 mg $(NH_4)_2CO_3$ and poured into a tube. Six tubes were sealed and heated at 60°C for 3 hours and further at 100°C for 3 hours. The mixture was then evaporated to dryness and dissolved in water at 0°C. The solution was lyophilized twice. Yield, 1.82 g. After ammonolysis of siomycin A, considerable decomposition or hydrolysis of the antibiotic was found.

Acid hydrolysis of siomycin A. For the amino acid analysis, exactly weighed samples of about 0.9 mg were hydrolyzed at 110°C for 20~24 hours by constant boiling HCl (5.93 N) in evacuated and sealed tubes. To determine amount of thiostreptine, hydrolysis was carried out by concentrated HCl (11.4 N) at 110°C for 6 hours.

Methanolysis of siomycin A. For the preparation of the quinaldic acid derivatives from siomycin A, methanolysis was a suitable technique rather than the usual acid hydrolysis. Methanolysis procedure was carried out in sealed tubes at room temperature for almost 2 weeks. Detailed procedures are described below.

Amino acids. One gram of the oxidized siomycin A was hydrolyzed by 5.9 N HCl for 24 hours at 110°C. The hydrolysate was evaporated and dried completely over KOH. The residue was applied to a column of Amberlite CG 120 (18×750 mm) which was equilibrated with 0.2 M pyridine-acetic acid, pH 3.10. Fractions (4.8 ml) obtained by eluting with the same buffer system were collected. Cysteic acid (tube Nos. 11~17), threonine (99~113), valine (145~163) and alanine (170~186) were separated from the others. Fractions of the individual amino acid were combined and lyophilized. Semi-crystalline amino acid residues were decolorized with charcoal and lyophilized again.

Derivatives of quinaldic acid. The derivative of quinaldic acid was prepared from the methanolysate of siomycin A.

Experiment 1: One millimole of siomycin A (1.72 g) was dissolved in 12 ml of HCl-saturated methanol and held at room temperature (22°C) for 15 days. This mixture was evaporated to dryness, dissolved in 6 ml of 2 N NaOH and incubated at 24°C for 2 hours. After acidifing with HCl the mixture was extracted repeatedly with ethyl acetate. The extract was washed and evaporated. Yield, 196 mg (79 %).

Experiment 2: Five hundred milligrams of siomycins were dissolved in 5 ml of HCl-saturated methanol and held at room temperature for 15 days. The mixture was evaporated to dryness, dissolved in 2 ml of N NaOH, and held at 4°C for 36 hours to hydrolyze all ester bonds. After neutralization with 2 ml of N HCl, the mixture was lyophilized. The residue was further purified by ion-exchange column chromatography on Amberlite CG 120 (13×85 mm) which had been equilibrated with 0.2 M tri-methylamine-acetic acid, pH 3.18 solution. 4-(α-Hydroxyethyl)-8-hydroxyquinaldic acid was found in the fraction IV by examination of TLC and UV absorption. Yield, 33 mg. m. p. 130°C; λ_{max}^{alc} 257 mμ ($E_{1cm}^{1\%}$, 1476) and 363 mμ ($E_{1cm}^{1\%}$, 93); λ_{max}^{nujol} 1730 cm^{-1} and 3400 cm^{-1}. $FeCl_3$ test, positive; Anal. Calcd. for $C_{12}H_{11}O_4N \cdot \frac{1}{2}H_2O$, C 59.25, H 5.39 %; Found: C 60.28, H 5.72 %. Soluble in 6 N HCl, scarcely soluble in alcohol and insoluble in water.

For further characterization, the compound was methylated by diazomethane and purified by sublimation. The obtained 4-(α-hydroxyethyl)-8-methoxyquinaldic acid methyl ester was compared with that obtained from thiostrepton. M. p., darkened at 161~168°C, melted at 174°C; sublimed at 150°C (1 mm); $[\alpha]_D^{20}$ −79° (*c* 1, EtOH); λ_{max}^{alc} 254 mμ ($E_{1cm}^{1\%}$, 1553), 307 mμ, 317 mμ and 347 mμ ($E_{1cm}^{1\%}$, 128); λ_{max}^{nujol}, 1735 and 3300 cm^{-1}; $FeCl_3$ test, negative; NMR in CD_3COOD, τ 8.40 (3H doublet), 6.00 (OCH_3), 2.84, 2.42 (side chain α H) and 1.63 (aromatic H). Anal. Calcd. for $C_{14}H_{15}O_4N$: C 64.36, H 5.79, N 5.36, O 24.49 %. Found: C 63.72, H 6.00, N 5.48, O 24.05 %.

Thiostreptine. One millimole of siomycin A was hydrolyzed with 11.4 N HCl at 105~110°C for 6 hours. The mixture was evaporated to dryness. Hydrolysis with 6 N HCl or using a longer hydrolysis time caused poorer yields of this compound. The dried residue was applied to a column of Amberlite CG 120 resin. Elution and collection of fractions were performed as described above. After the alanine fraction appeared, a ninhydrin yellow component (thiostreptine) followed. These fractions were combined and lyophilized. Yield of the crude materials, 72 mg (approximately 30 %). The crude compound was passed through a Sephadex G 10 column (10×800 mm) to remove impurities. Recrystallization was performed with dil. acetone. Yield, 49 mg; m. p., unsharp; $[\alpha]_D^{20}$ −2.8° (*c* 1, AcOH); λ_{max}^{alc} 237 mμ ($E_{1cm}^{1\%}$, 195); NMR in CF_3COOH, τ 8.7 (2′ methyl), 6.4 (3′ H), 4.4 (1′ H) and 1.2 (aromatic H). Anal. Calcd. for $C_9H_{14}O_4N_2S \cdot H_2O$ (264.30): C 40.90, H 6.10, N 10.60, S 12.13 %. Found: C 40.29, H 6.11, N 10.29, S 12.02 %. Neutral eq. as acid, 226; Rf-value on PPC for BuOH-AcOH-H_2O (4:1:1), 0.41; and IO_4 consumption of DNP-thiostreptine, 1.77~2.17 moles.

2-Aminomethylthiazole-4-carboxylic acid. Best hydrolysis conditions were by 6 N HCl at 110°C for 20~24 hours. It was purified on an Amberlite CG 120 column by elution with 0.4 M pyridine-acetic acid buffer, pH 4.0, after thiostreptine fraction appeared. M. p. 272~273°C (in a sealed capillary tube); sublimed at 200°C *in vacuo*; $\lambda_{max}^{H_2O}$ 234~235 mμ ($E_{1cm}^{1\%}$, 319); λ_{max}^{nujol} 1280 cm^{-1} and 1320 cm^{-1}; Rf-value on PPC for BuOH - EtOH - H_2O propionic acid (10:10:5:2), 0.22. Anal. Calcd. for $C_5H_6O_2N_2S$: C 37.97, H 3.82, N 17.71, S 20.27 %. Found: C 37.56, H 3.91, N 17.43, S 20.23 %.

2-(1-Aminoacetonyl)thiazole-4-carboxylic acid. One gram of the oxidized siomycin A was hydrolyzed and fractionated as described in the section on amino acids. Fractions (tube Nos. 215~225) were combined, evaporated to dryness, and further purified on a column of Sephadex G 10. Yield, 20 mg as the hydrochloride. M. p. 198~210°C; $\lambda_{max}^{H_2O}$ 236 mμ ($E_{1cm}^{1\%}$, 142); λ_{max}^{KBr} 1375, 1600 and 1715 cm^{-1}; $[\alpha]_{436}^{26}$ −1.9 (*c* 1, H_2O); NMR in D_2O, 8.1 (3H), and 7.2 (NH_2); mass spectrum, found CH_3-C-. Anal. Calcd. for $C_7H_8O_3N_2S \cdot HCl \cdot H_2O$ (254.69): C 33.01, H 4.35, N 11.00, S 12.59, O 25.13, Cl 13.92, H_2O 7.07%. Found: C 33.49, H 3.64, N 11.30, S 12.93, O 24.49, Cl 14.19, H_2O 6.61 %.

The C_{10} and C_{12} compounds. Ammonolysis of siomycin A (6.4 g) was carried out as described above. The residues were hydrolyzed by 5.9 N HCl at 110°C for 18 hours.

Dried hydrolysates were dissolved in water and extracted by water-saturated *n*-butanol (30 ml×5). The water layer was then concentrated and applied to an Amberlite CG 120 column (27×600 mm). Elution by 0.4 M pyridine-acetic acid, pH 3.40 gave fractions of the C_{12} compound and other fractions of C_{10} compound.

The C_{12} compound: Yield of crude material (semi-solid syrup), 557 mg. H_2SO_4 salt: Purified by Sephadex G 10. Very hygroscopic; stable in acidic solutions; unstable in alkaline media; negative to SAKAGUCHI reaction; positive with ninhydrin and the carbonyl test. $\lambda_{max}^{dil.HCl}$ 235 mμ (shoulder) and 286 mμ (shoulder); λ_{max}^{KBr} 1620 (C=O), and 1100 cm^{-1} (-C=N-C-).

2,4-Dinitrophenylhydrazone of N-acetyl C_{12} compound: Recrystallized from methanol. M. p. 228.5~229.5°C; λ_{max}^{KBr} 3420, 1620 and 1090 cm^{-1}; NMR in CF_3COOH, τ 8.26 (CH_3-C), 7.02 (CH_3CO), 5.90 (=N-NH), 1.95, 1.78 and 1.60. Anal. Found: C 45.62, H 6.02, N 17.17, O 23.43, S 4.67, acetyl, 5.13 %.

2,4-Dinitrophenyl C_{12} compound: Recrysctallized from methanol-*n*-hexane; m. p. 170°C; $\lambda_{max}^{dil.alkali}$ 230 mμ, 260 mμ and 360 mμ. Anal. Calcd. for $C_{18}H_{17}O_9N_6S$ (493.43): C 43.82, H 3.47, N 17.03, S 6.50 %. Found: C 43.27, H 3.52, N 16.79, S 5.98 %.

The C_{10} compound: Recrystallized from methanol; m. p. 135°C; $[\alpha]_D^{24}$ +0.9° (*c* 1, AcOH); Titration by alkali, 0.97 COOH; IO_4 consumption, 1.82~2.00 mole; positive, ninhydrin test; negative, SAKAGUCHI reaction; $\lambda_{max}^{CH_3OH}$ no meaningful strong absorption; λ_{max}^{nujol} 1650, 1190 and 1000 cm^{-1}; NMR in CF_3COOH, 7.8 (CH_3), 5.9 doublet (N-CH-CH_2-S) and 5.0 doublet. Anal. Calcd. for $C_{10}H_{20}O_4N_4S \cdot 2H_2O$ (328.38): C 36.58, H 7.37, N 17.06, S 9.76 %. Found: C 36.47, H 7.39, N 17.21, S 9.94 %.

p-Bromobenzoyl C_{10} compound: Recrystallized from 50 % isopropanol; m. p. 207°C. Anal. Calcd. for $C_{31}H_{29}O_7N_4SBr_3$ (841.36): C 44.25, H 3.47, N 6.66, S 3.81, Br 28.49 %. Found: C 43.67, H 3.17, N 6.04, S 3.46, Br 33.63 %.

Pyruvic acid from siomycin A. Pyruvic acid was identified as its 2,4-dinitrophenylhydrazone. An acid hydrolysate of siomycin A (500 mg) was distilled repeatedly, and to the distillate, 80 ml of 0.5 % of 2,4-dinitrophenylhydrazine were added. Hydrazone formed was collected by filtration and recrystallized from 90 % ethanol. Yield, 1.81 mole from one mole of siomycin A (5.9 N HCl, 110°C 18 hours) to 2.12 mole (2 N HCl, 110°C 3 hours). M. p. 216~218°C; IR and TLC, agreed completely with synthetic sample. Anal. Calcd. for $C_9H_8O_4N_6$: C 40.30, H 3.01, N 20.89 %. Found: C 40.20, H 3.15, N 20.59 %.

Ammonia formation from siomycin A. Fourteen exactly weighed 10 mg samples of siomycin A were heated in 2 N HCl at 100°C in sealed tubes. Samples were taken during the time course (15 minutes, 8 hours), the heated samples were dried and ammonia determined by CONWAY's micro-difusion method. Amount of ammonia were plotted as a function of heating time. Linear liberation of ammonia was found in the region of 1 to 4 hours. The extrapolated value for ammonia to time zero shows 2.19~2.27 mole per 1,712 g of siomycin A.

Propionic acid formation from siomycin A. Siomycin A (75 mg) was dissolved in 4.5 ml of 0.5 N NaOH and the solution was stirred for 2 hours at room temperature. After acidifying by addition of 5 ml of 0.5 N HCl, the mixture was distilled at 130°C. The distilled portion was collected in a dilute alkaline solution. Amount of the acid formed was determined by titration (2.37 COOH eq. from 1,712 g of the antibiotic). Detection and characterization were carried out by PPC technique and GLC analysis.

Solvent system in PPC, (1) ethanol - water - ammonia (80 : 16 : 4), (2) 95 % ethanol - ammonia (100 : 1), (3) 1.5 N ammonia-saturated *n*-butanol. 0.1 % BTB indicator solution was used. GLC analysis: detected form, methyl ester; column, 15 % DEGS, diameter, 3 mm; carrier gas, nitrogen; temperature, 24~150°C.

Only propionic acid was found in the acid fraction obtained from the antibiotic.

Acetaldehyde from siomycin A. Four hundred milligrams of siomycin A were dissolved in 25 ml of 0.5 N NaOH and the solution was stirred for 2 hours at room temperature. The mixture was then distilled and 85 ml of the fraction were collected in

an ice bath. The fraction was acidified by adding 2 ml of concentrated HCl and then 25 ml of 0.5 % 2,4-dinitrophenylhydrazine in 2 N HCl were added. Precipitates formed were collected (54.5 mg) and recrystallized from ethanol.

TLC, Rf 0.87 for a solvent of toluene-ethyl acetate (1 : 1); Rf 0.93 for a solvent of *n*-butanol – ethanol – water (7 : 1 : 2). This indicates that this dinitrophenylhydrazone may be a derivative of acetaldehyde. UV absorption in methanol, IR_{nujol} of the sample agreed completely with those of the authentic compound from acetaldehyde. Anal. Calcd. for $C_8H_8O_4N_4$: C 43.32, H 3.91, O 26.95, N 24.87 %. Found: C 42.86, H 3.60, O 28.55, N 24.99 %.

Amounts of the 2,4-dinitrophenylhydrazones of acetaldehyde from siomycin A and the modified siomycins A were determined in the same way. Siomycin A, 1.06 mole; oxidized siomycin A, 0.93 mole, and reduced siomycin A, 0.91 mole from 1,712 g of siomycin or the modified antibiotic.

Diketopiperazine containing valine and dehydroalanine from siomycin A. Procedures for the preparation of the diketopiperazine were performed as reported by BODANSZKY *et al.*[5] Seventy six mg of the diketopiperazine were obtained from 2 g of the antibiotic. M. p. 241°C; sublimed at 175°C *in vacuo*; $[\alpha]_D^{26}$ −95.7° (*c* 0.4, methanol); $\lambda_{max}^{CH_3OH}$ 220 mμ ($E_{1cm}^{1\%}$, 763); Hydrolysis gave 0.93 mole of L-valine, pyruvic acid and 0.94 mole of ammonia. After reduction, L-valine (1.00) and DL-alanine (1.03). Anal. Calcd. for $C_8H_{12}O_2N_2$: C 57.13, H 7.19, N 16.66 %. Found: C 56.85, H 7.72, N 16.39 %.

Synthesis of diketopiperazine, val-ala. N-Benzyloxycarbonyl-L-valyl-L-alanine methyl ester was prepared from Z-L-Val-OH (3.76 g) and L-Ala-OCH_3 (2.29 g) by use of dicyclohexylcarbodiimide as the coupling agent, and recrystallized from ethanol. Yield, almost quantitative; m. p. 164°C; $[\alpha]_D^{26}$ −46.6° (*c* 0.6, methanol). Anal. Calcd. for $C_{17}H_{24}O_5N_2$: C 60.70, H 7.19, N 8.33 %. Found: C, 61.30, H 7.31, N 8.63 %.

Catalytic hydrogenolysis of 2.52 g of Z-Val-Ala-OCH_3 was carried out for 2 hours in a methanol solution containing acetic acid in the presence of 10 % of Pd-charcoal. The mixture was evaporated and dissolved again in methanol, and was heated on a steam bath for 2 hours. The obtained diketopiperazine of valine and alanine was sublimed at 190°C *in vacuo* and recrystallized from ethanol. Yield, 0.70 g; m. p. 274°C; sublimed at 175°C; $[\alpha]_D^{26}$ −48.2° (*c* 1, methanol); Hydrolysis gave valine (1.00) and alanine (1.06). Anal. Calcd. for $C_8H_{14}O_2N_2$; C 56.45, H 8.29, N 16.46 %. Found: C 56.50, H 8.67, N 16.18 %.

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