STUDIES ON SIOMYCIN. III

STRUCTURAL FEATURES OF SIOMYCIN A

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The sequence of siomycin A was determined from the seventeen fragments of the modified antibiotic, although a complete structural formula is still not available. Siomycin contains eleven constituents, two of which are still unidentified. In the determination of the sequence, the reduced-, oxidized-, ammonia treated-siomycin A, partial acidic hydrolysis was used to provide water soluble fragments. SANGER's (DNFB) method was applied to the fragments to obtain sequence information. The structure surrounding the dehydroalanine moiety, the properties of the sulfhydryl group and the lactone ring were also investigated.

Siomycin, a new S-containing peptide antibiotic was isolated in 1959 by NISHIMURA *et al.* from cultures of *Streptomyces sioyaensis*[1]. It is active against Gram-positive microorganisms and mycobacteria. The high activity against most Gram-positive bacteria and the relatively low toxicity, together with the fact that the antibiotic is closely related to thiostrepton[2], encouraged a more detailed study.

In the present paper, a possible sequential structure of the antibiotic is described, constructed from information derived from peptide fragments. In our postulated sequence, siomycin contains eleven constituents, two of which are still unidentified. Further, the structure surrounding dehydroalanine, the properties of sulfhydryl group and the lactone structure are also described.

Experimental

Siomycin A. Siomycin A was obtained as described previously[3,4]. Data for elementary analysis, molecular weight and composition of the antibiotic were reported previously[3,4].

Derivatives of siomycin A. Derivatives of siomycin A such as oxidized-, reduced- and ammonolyzed-siomycin A, and double-treated siomycin A with combinations of these reactions were as described previously[4].

Alkali treated-siomycin A: Siomycin A (0.50 g) was dissolved in a mixture of 2 ml of dioxane and 4 ml of 5 % $Ba(OH)_2$, and the mixture was stirred for 20 minutes. The clear solution was then neutralized with N H_2SO_4 to pH 5.6. $BaSO_4$ was removed by filtration and the supernatant lyophilyzed to yield a light pink powder. This preparation is homogeneous by paper electrophoresis and thin-layer chromatography: paper electrophoresis (pH 9.6, 200 V, 7.5 hr), siomycin A did not move, the alkali treated-antibiotic 4.1 cm toward the cathode; thin-layer chromatography on silica gel G (butane-1-ol-AcOH-H_2O, 4 : 1 : 2), siomycin A, Rf 0.7, the derivative, Rf 0.40, $[\alpha]_D^{23}$ $-29.6°$ (*c* 1, 50 % dioxane). The ultraviolet spectrum of the derivative is the same as that of siomycin A. The 1730

cm^{-1} band which is present in the infrared spectrum of siomycin A is missing in this alkali-treated siomycin A. Alkali titration of the derivative disclosed two (1.85) carboxylic acid functions (pKa 4.65).

Enzymes. Crystalline papain and chymopapain were prepared by the method of EBATA *et al.*[5]. Pepsin and carboxypeptidase were purchased from Worthington Biochemical Co., New Jersey, nagarse and pronase were products of Nagase & Co., Osaka and Kaken Chem. Co., Ltd., respectively. Thermolysin was a gift from Dr. K. MORIHARA.

Dinitrophenyl derivatives of constituents. The dinitrophenylation of each component or constituent was achieved by the usual DNFB-method in 67 % ethanol solution[6,7].

DNP-derivative of 2-(1'-amino-1'-acetyl)methylthiazole-4-carboxylic acid, 2-aminomethylthiazole-4-carboxylic acid and the unidentified C_{10} compound: Five to ten milligrams of individual compound were converted to corresponding 2,4-dinitrophenyl derivatives by the usual method. Obtained DNP-derivatives of these compounds were used after removal of 2, 4-dinitrophenol by sublimation.

DNP-C_{12} compound: This compound was already described previously[4].

Partial hydrolysis. Ten different procedures were tried to determine the optimal procedure for the partial hydrolysis of the antibiotic, and the following three methods were selected to provide the appropriate fragments of siomycin A.

Experiment 1: Seven grams of derivatives of siomycin A were dissolved in 70 ml of concentrated hydrochloric acid and incubated for 4 days at 37°C. The mixture was then dried *in vacuo* by lyophilization.

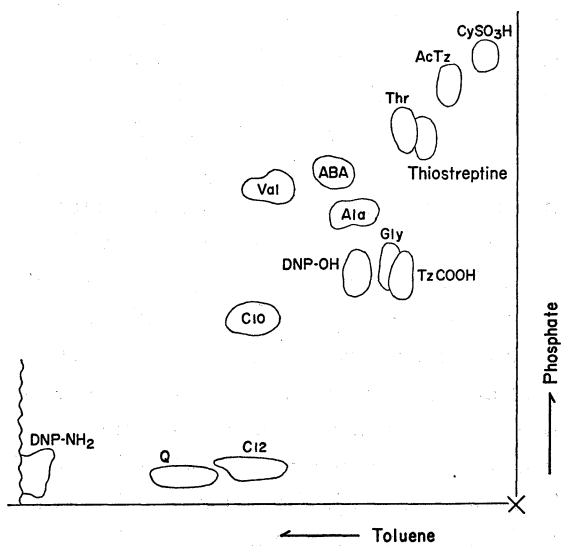
Experiment 2: One gram of siomycin A was mixed with 20 ml of a suspension of Dowex 50×4 (1 g of H^+ form), and was heated at 100°C for 20 hours. After cooling, the mixture was extracted with ether. The washed resin was treated with 3 % ammonia solution to remove the fragments. The eluate was evaporated and lyophilized.

Experiment 3: The antibiotic was dissolved in a mixture of acetic acid and conc. hydrochloric acid (6 : 1). Then the solution was heated at 100°C for 1 hour. The solution was evaporated and lyophilized.

Isolation of fragments. All partial hydrolysates containing amino acids and fragments were subjected to automatic amino acid analysis before separation of the fragments. Preparations which contain water-soluble and ninhydrin-positive fragments were fractionated by ion-exchange column chromatography or partition column chromatography, as described previously[4]: Resin Amberlite CG 120 type III; column size, 22×800 mm; buffer system, pyridine-acetic acid, 0.2~0.4 M, pH 3.10~3.40. Partition chromatography on Sephadex G 50: Thirty grams of Sephadex G 50 were added in 500 ml of 3 % ammonia-saturated butane-1-ol. The mixture was stirred for 3 hours and the slurry was

Fig. 1. Two-dimentional partition paper chromatography of dinitrophenyl components.

Toluene, the upper layer of the mixture of toluene, pyridine, 2-chloroethanol and 0.8 N ammonia (30 : 9 : 18 : 18); Phosphate, 1.5 M phosphate buffer, pH 6.0. Abbreviations: AcTz, 2-(1'-amino-1'-acetyl)methylthiazole-4-carboxylic acid; ABA, α-amino-n-butyric acid; DNP-OH, 2, 4-dinitrophenol; DNP-NH_2, 2, 4-dinitroaniline; C_{10}, unidentified C_{10} compound; C_{12}, unidentified C_{12} compound; Q, quinaldic acid derivatives.



poured into a column (size 12×400 mm). The hydrolysate was dissolved in the solvent and put onto the column equilibrated with the same solvent. The column was eluted and ninhydrin color-yield and UV absorption were measured on each fraction. Fractions with single symmetric peak were evaporated and dried.

Two-dimensional paper chromatography. To characterize DNP-compounds, the system described by LEVY[8] was used. A chromatogram of the DNP-compounds from fragments of siomycin A is shown in Fig. 1.

Estimation of components. All constituents or components were determined by use of automatic amino acid analyzer. Conditions and positions on the analytical chart were described previously[4].

Results

Action of Enzymes on the Derivatives of Siomycin A

Since siomycin A is completely insoluble in water, oxidized-, alkali-treated and KCN-treated siomycin A were used as substrates. Oxidized siomycin A was not hydrolyzed by the action of papain, pepsin, nagase and thermolysin. The alkali-treated antibiotic was also not susceptible to thermolysin, chymopapain and carboxypeptidase A. KCN-treated siomycin A was also resistant to the action of pronase.

Functional Groups of Siomycin A

Analyses of the functional groups of siomycin A revealed that the antibiotic has one SH-like group and four hydroxyl groups. This SH group, however, was masked but could readily be converted to cysteine by reduction. No amino- or carboxyl-group was found in the fresh siomycin A. Results were listed in the Table 1. Treatment with alkali converts the antibiotic to an acidic substance which contains approximately two carboxyl groups. This product was homogeneous and its properties were investigated as described in the experimental section. This indicates that siomycin A contains two lactones in its molecule.

Table 1. Functional groups of siomycin A

Group	Number (corrected)	Detection
Sulfhydryl (-SH)	0.65~0.79 (1)	PCMB-Titration
Hydroxyl (-OH)	4.36 (4)	Acetylation
Amino ($-NH_2$)	0	Acetylation and alkylation
Carboxyl (-COOH)		
Intact siomycin A*	0	Titration
Alkali-treated siomycin A	1.85 (2)	Titration

* Siomycin A did not bind with DEAE-cellulose under any conditions.

Fragments from Partial Hydrolysate of Siomycin A

Seventeen fragments obtained from partial hydrolysates of siomycin A are listed in Table 2. For the isolation of fragments, 12 N HCl hydrolysis (37°C, 4 days) and fractionation by ion-exchange column chromatography were most successful as shown in the Table. Composition of each fragment was estimated by the amino acid analysis after complete hydrolysis. Nitrogen terminals were estimated by the DNFB-method. C-terminals of some fragments were determined quantitatively by use of carboxipeptidase A. In the Table 2, parentheses show the unknown sequence in the fragment. Components outside of the parentheses are the nitrogen terminal ones. Sometimes unknown compounds were found in fragments as shown in the Table. They were distinguished from the C_{10} and C_{12} compounds but are still closely related to them. The amino terminal 2-aminomethylthiazole-4-carboxylic acid in the fragment

Table 2. Fragments obtained from siomycin A

Source	Hydrolysis	Separation	Sequence and molar ratio of constituent
Siomycin A	AcOH : conc. HCl	Cellulose column chromatography	Q—Ala (1.0) (1.0)
	Dowex 50	〃	Q—Ala—Thio (1.0) (0.9) (0.9)
	N HCl	CH_3OH-fractionation	Ala—Thio(- -Thr—Cys) (1.0) (0.3) (1.0) (0.3)
	Dowex 50	Partition in Sephadex G 50	Thio(- -Thr—Cys—Tz) (1.0) (1.0) (1.4)
	〃	〃	Thio(- -Thr—Cys) (0.9) (1.0)
Oxidized siomycin A	12 N HCl	Dowex 50	S_2-Compound—Thr—$CySO_3H$ (1.0) (1.0)
Oxidized-reduced-siomycin A	12 N HCl	Amberlite CG 120	Ala—AcTz—Ala (1.0) (0.7) (1.0)
	AcOH	Sublimation	-Val—Dehydroala- (1.0) (1.0)
Ammonolyzed siomycin A	12 N HCl	Amberlite CG 120	AcTz(- -Val—C_{10}) (1) (1) (1)
	〃	〃	Thr(—$CySO_3H$—C_{10}) (1) (1) (1)
	〃	〃	C_{10}-(Thr-$CySO_3H$-C_{12}-Ala) (1) (1) (0.5) (1) (1)
	〃	〃	Thr-($CySO_3H$-C_{12}-Ala-AcTz-unknown) (1) (1) (1) (0.6)
	〃	〃	Thr-(unknown-Ala) (1) (1) (2)
Ammonolyzed-reduced and oxidized siomycin A	〃	〃	NH_2 | TzCOOH (1) | NH_2-unknown (1)
	〃	〃	NH_2 | TzCOOH-C_{10} (1) (1)
	〃	〃	Ala (1) | TzCOOH (1) | Val(-C_{10}-Thr) (1) (1) (1)
	〃	〃	Val—C_{10} (1) (1)

Abbreviations : Q, derivative of quinaldic acid; Thio, thiostreptine; Tz, thiazole compound; AcTz, 2-(1′-amino-1′-acetyl)methylthiazole-4-carboxylic acid; Dehydroala, dehydroalanine; TzCOOH, 2-aminomethylthiazole-4-carboxylic acid, C_{10}, unidentified C_{10} compound; C_{12}, unidentified C_{12} compound.

of TzCOOH-unknown or TzCOOH-C_{10} seems to be derived from thiostreptine by the action of ammonia.

Sequence of Siomycin A

The seventeen fragments described above can be combined, here, as the eleven-membered sequence as shown in Fig. 2. This figure shows only an order of the binding, but is not the final structural formula.

Latent SH Group of Siomycin A

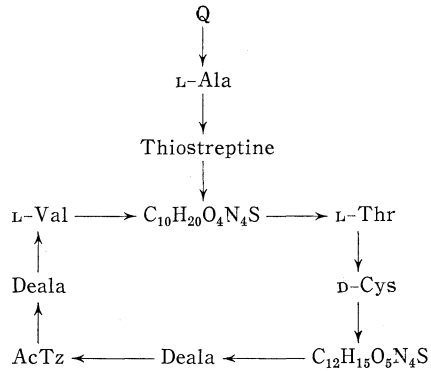
One mole of D-cysteic acid has been isolated from the hydrolysate of the performic acid oxidized-siomycin A[4]. The phosphotungstic acid test on the antibiotic was apparently positive indicating the occurrence of a free thiol group. It seems that *p*-

chloromercuribenzoate (PCMB) acts on the SH group from the results of the UV absorption ($\Delta\varepsilon$ at 245 mμ, see Table 1). Analyses of the mixed crystals show that they contained PCMB and heavy metals (Table 3).

PCMB could be removed readily from the mixed crystals by recrystallization. Sulfhydryl reagents, such as monoiodoacetic acid, ethylenimine and 1-fluoro-2, 4-dinitrobenzene did not react with SH groups of siomycin A as shown in Table 3. The preparation of desthiosiomycin A from the antibiotic by treatment with RANEY nickel in dioxane at 100°C was relatively difficult. Further, siomycin A gave a negative nitroprusside reaction.

Fig. 2. The sequential feature of siomycin A.

Abbreviations : Q, 4-(α-hydroxyethyl)-8-hydroxyquinaldic acid; Deala, dehydroalanine; AcTz, 2-(1'-amino-1'-acetyl)methylthiazole-4-carboxylic acid.



After reduction of siomycin A by $NaBH_4$, Zn-acetic acid and sodium cyanide, positive nitroprusside tests were obtained with these reduced antibiotics. This indicated that the sulfur of siomycin A was masked but could be readily converted to cysteine by reductions. This latent SH group was determined by phosphotungstate as follows: siomycin A, 0.25 SH/mole of siomycin A ; CN-treated, 1.43 ; $NaBH_4$-reduced, 1.16 and Zn-acetic acid-reduced, 0.79. From these results, it was concluded that one latent SH group is contained in one mole of siomycin A.

Table 3. Reactivity of the D-cysteine residue in siomycin A

Reagent	Altered to	Molar ratio	Biological activity*
Performic acid	D-$CySO_3H$	1.00	0%
Monoiodo acetic acid	CM-Cysteine	0.10	±
Ethylenimine	S-Aminoethyl cysteine	0.21	0
L-Cysteine	*meso*-Cysteine**	0.20	50
KCN	SCN-Cysteine	<0.10	±
1-Fluoro-2, 4-dinitrobenzene	S-DNP-Cysteine	0	
p-Chloromercuribenzoate	Mixed crystals***	0.68	
$AgNO_3$	Mixed crystals***	<0.5	
$HgCl_2$	Mixed crystals***	0.68	86

* Compared to siomycin A.
** Another one mole of cysteine was introduced into the one of the dehydroalanine moieties.
*** These compounds or heavy metals could be removed by recrystallization.

Dehydroalanine Moiety

Two moles of pyruvic acid have been found in the acid hydrolysate of siomycin A[4]. In another experiment, a diketopiperazine containing valine and dehydroalanine was isolated when the antibiotic was heated in glacial acetic acid[4]. From these results it was considered that a dehydroalanyl- or α-hydroxy-α-aminopropionyl, or, α, α-diaminopropionyl residue might be present in siomycin A molecule. Among the three residues, only dehydroalanyl residue has an unsaturated character. If the dehydroalanyl moiety was in fact, siomycin A should reveal several types of addition reactions. Addition of hydrogen, amine and thiolcarboxylic acid was tried and corresponding diketopiperazines were obtained with a variety of yields as shown in Table 4.

Table 4. Addition reactions into the dehydroalanine parts

Added reagent	Products in dehydroalanine parts	mole (corrected)	Yielded deketopiperazine	Yield % (ratio)
None	Pyruvic acid ammonia	1.81(2) 2.2 (2)	Val-Dehydroala	43(100)
H ($NaBH_4$)	DL-Alanine	1.96(2)	Val-Ala	39 (90)
CH_3NH_2	α-Amino-β-methylamino propionic acid	2.00(2)	Val-α-amino-β-methyl amino propionyl	22 (50)
Thioglycolic acid	S-Carboxymethyl-cysteine	0.72(1)	Val-CM-Cys	1 (3)

Siomycin A readily reacts with 2 moles of methylamine, and 2 moles of α-amino-β-methylaminopropionic acid could be found in the acid hydrolysate of the reacted antibiotic. Thioglycolic acid was incorporated into the antibiotic, and approximately one mole (0.73) of S-carboxymethyl cysteine could be found after complete acid hydrolysis.

Based on the evidence described above, it is concluded that siomycin A contains two dehydroalanine residues.

Lactone Structure

Evidence that siomycin A contains a lactone structure was obtained as follows. The infrared spectrum of siomycin A possessed a peak at 1730 cm^{-1}, characteristic of an ester or lactone function. On treatment with 0.1 N sodium hydroxide or barium hydroxide at room temperature, the band at 1730 cm^{-1} disappeared, accompanied by a change in the optical rotation from $[\alpha]_D^{23}$ −95.3 to −29.6. The alkali-treated siomycin A contains all the original constituents and has the same ultraviolet spectrum as siomycin A. Potentiometric titration of this compound revealed two carboxylic acid functions (Table 1). On electrophoresis, the alkali-treated siomycin moves toward cathode.

Acyl Groups in Siomycin A

A volatile acid has been found from the preparation of the alkali-treated siomycin A, and has been characterized as propionic acid[4]. The acyl content in the antibiotic and its derivatives are shown in Table 5. Siomycin A liberates approximately 3 moles of propionic acid, and the modified antibiotic also liberates 2~3 moles of the acid. This indicates that the acid did not form the labile lactone, because even the alkali-treated antibiotic could liberate 2.7 moles of the acid. Since the reduced siomycin A still contains 7.27 % acyl group, propionic acid from the derivatives seems to be produced from something other than the dehydroalanine moiety.

Table 5. Content of volatile acyl group

	Alanine content	Formation of pyruvic acid	Content of acyl group
	mole	mole	
Siomycin A	0.98(1)	1.81~2.12(2)	10.33% (3.1 mole)
Reduced siomycin A	2.94(3)	0.14 (0)	7.27 (2.2)
Alkali-treated siomycin A	0.88(1)	—	8.80 (2.7)
Thioglycolic acid-siomycin A	1.05(1)	—	10.87 (3.3)

* Values for % was estimated as acetyl; mole, as propionyl.

Discussion

The sequence of siomycin A was constructed from fragments obtained from the chemically modified siomycins A (Fig. 2). This figure shows only bindings from component to component, and it is not the structural formula. To elucidate the structure of siomycin A, further detail remain for investigation. For the purpose of this study we have even applied the crystalline monoiodoacetyl-siomycin A[9] to X-ray analysis.

Some of derivatives of siomycin A could not be hydrolized at any rate by the action of many different enzymes, including that of the antibiotic different from usual peptides, in another words, siomycin A contains only 22 % amino acids by weight in the molecule.

As functional groups of the antibiotic, only approximately four hydroxyl groups were observed. No free amino-, carboxyl- or SH-groups were found in the molecule. This finding also supports the fact that siomycin A is resistant to, and is not an appropriate substrate for the enzymes.

The cysteine residue which has been converted to cysteic acid by performic acid oxidation, was found to be masked on the SH group. The nature of the masked group was unknown and it was very hard to characterize it. The sulfur, however, was readily converted to cysteine by reduction. This property resembles that of thiazoline. Since siomycin contains four more sulfur atoms as well as UV-sensitive components in its molecule, it was very difficult to determine the exact nature of the masking group.

In a mild alkali hydrolysis experiment, it was demonstrated that siomycin has two lactone structure. This was confirmed from the infrared spectrum data, optical rotation, electrophoresis and potentiometric titration. A preliminary determination of the carboxyl terminals of the lactone revealed the C_{10} or C_{12} compounds, while the O-terminals of those were still unknown. Final determinaton of the O-terminals was difficult since the antibiotic contained approximately four hydroxyl groups.

A diketopiperazine containing valine and dehydroalanine was isolated when siomycin A was heated in acetic acid. This finding indicates that L-valine has to be present with a dehydroalanine residue. However, there is no evidence of a linear structure such as -dehydroalanyl-L-valyl-. In 1964, BODANSZKY *et al.*[2] proposed a structure of thiostrepton such that the diketopiperazine ring structure must already be present in the molecule of the antibiotic. With siomycin A, there is no other information about a cyclic structure of a diketopiperazine. Occurrence of dehydroalanine residue in natural products is worth noting because this particular amino acid residue has also been found in antibiotic nisin[10].

The information so far accumulated is sufficient only to describe the "building stones" of siomycin A. Proposed sequential structure consonant with our results have been presented, but the true structure of siomycin A, it is felt will be a unique one.

Acknowledgement

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References

1) NISHIMURA, H.; S. OKAMOTO, M. MAYAMA, H. OTSUKA, K. NAKAJIMA, L. TAWARA, M. SIMOHIRA & N. SHIMAOKA : Siomycin, a new thiostrepton-like antibiotic. J. Antibiotics, Ser. A 15 : 255～263, 1959

2) BODANSZKY, M.; J. FRIED, J. T. SHEEHAN, N. J. WILLIAMS, J. ALICINO, A. I. COHEN, B. T. KEELER & C. A. BIAKHIMER : Thiostrepton. Degradation products and structural features. J. Am. Chem. Soc. 86 : 2478～2490, 1964

3) EBATA, M.; K. MIYAZAKI & H. OTSUKA : Studies on siomycin. I. Physicochemical properties of siomycins A, B and C. J. Antibiotics 22 : 364～368, 1969

4) EBATA, M.; K. MIYAZAKI & H. OTSUKA : Studies on siomycin. II. The composition and degradation products of siomycin A. J. Antibiotics 22 : 423～433, 1969
5) EBATA, M. & T. YASUNOBU : Chymopapain. I. Isolation, crystallization and preliminary characterization. J. Biol. Chem. 237 : 1086～1094, 1962
6) SANGER, F. : The free amino groups in insulin. Biochem. J. 39 : 507～515, 1945
7) SANGER, F. : The terminal peptides of insulin. Biochem. J. 45 : 563～574, 1949
8) LEVY, A. : A paper chromatographic method for the quantitative estimation of amino-acids. Nature 174 : 126～127, 1954
9) EBATA, M.; K. MIYAZAKI & H. OTSUKA : Studies on siomycin. IV. Acyl derivatives of siomycin A. J. Antibiotics (in press)
10) GROSS, E. & J. L. MORELL : The presence of dehydroalanine in the antibiotic nisin and its relationship to activity. J. Am. Chem. Soc. 89 : 2791～2792, 1967