STUDIES ON SIOMYCIN. V

DERIVATIVES OF SIOMYCIN A PREPARED WITH THIOLCARBOXYLIC ACID

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(Received for publication June 9, 1969)

Derivatives of siomycin A were prepared by reaction with thiolcarboxylic acids such as thioglycolic acid or thiomalic acid. Sodium salts of the derivatives were remarkably soluble in water, while the parent siomycin A was quite insoluble. These "soluble derivatives" of the antibiotic were still active against many Gram-positive bacteria, although the activities were approximately one fifth of that of siomycin A.

Siomycin A, a sulfur-containing peptide antibiotic, has been isolated from the cultures of *Streptomyces sioyaensis*[1,2]. This colorless crystalline antibiotic is relatively stable at room temperature. It is highly active against Gram-positive organisms, and shows relatively low toxicity. However, the greatest problem with this antibiotic is its insolubility in water[2].

In the course of the structural study of siomycin A, the authors reported that the antibiotic has dehydroalanine residues in the molecule and that thioglycolic acid reacts with siomycin A. The reaction product contains at least one carboxyl group in their molecule and can form alkali-metal salts.

The present paper deals with the derivatives of siomycin A with thiolcarboxylic acids such as thioglycolic acid or thiomalic acid. Sodium salts of the derivatives were remarkably soluble in water. These "soluble derivatives" of the antibiotic were still active against many Gram-positive bacteria, although the activities were approximately one fifth of that of siomycin A.

Experimental

Siomycin A. Siomycin A was prepared from cultures of *Streptomyces sioyaensis* (unpublished data). Some physicochemical properties and antibacterial activities were described previously[2].

Thiolcarboxylic acids. Thioglycolic acid was purchased from Wako Pure Chem. Ind. Ltd., Osaka. α-Mercaptopropionic acid (thiolactic acid) and its β-isomer were products of Tokyo Kasei Chem. Ind. Ltd. L-Cysteine was purchased from Nakarai Chemicals Ltd., and thiomalic acid was the products of Ishii Pharmaceutical Co., Ltd., and Wako Pure Chem. Ind. Ltd.

Bis-thiolcarboxylic acid-siomycin A. One gram of siomycin A was dissolved in 14 ml of dimethylformamide. Into the solution 1 g of thiolcarboxylic acid was added and dissolved. The mixture was allowed to stand in nitrogen atomosphere for 20 hours at room temperature (22°C). The solvent was distilled under reduced pressure, and a large

amount of ether was added. Precipitates were collected and crystallized from isopropanol. Yield : thioglycolic acid-derivative, 83 % ; α-mercaptopropionic acid-, 81 % ; β-mercaptopropionic acid-, 82 % ; thiomalic acid-, 88 % ; L-cysteine-, 30 % (after purification by a partition column chromatography with Sephadex[3]).

Mono-thiomalic acid-siomycin A*. Procedures were followed as described above except the reaction time. After 6 hours, the reaction was stopped by adding excess amounts of ether. Recrystallized from isopropanol. Yield 70 % ; m. p., darkened at 200°C and not decomposed until 250°C.

Salts of thiomalic acid-siomycin A. Disodium mono-thiomalic acid-derivative: Three grams of mono-thiomalic acid-siomycin A were dissolved in 60 ml of methanol, and into the solution 27 ml of 0.15 M $NaHCO_3$ were added (pH 6.8). After distillation of methanol, a small amount of water was added. The clear solution obtained was lyophilyzed and then further dried in a desiccator over P_2O_5. The quantitative yield was obtained.

Trisodium bis-thiomalic acid-derivative: Bis-thiomalic acid-siomycin A (800 mg) was dissolved in 40 ml of methanol. Eight ml of 0.15 M $NaHCO_3$ added to this solution. The neutralyzed solution was evaporated and lyophilyzed.

Preparations of other salts of bis-thiomalic acid-derivative were obtained by neutralization with LiOH, NH_4OH, $KHCO_3$ and $Ca(OH)_2$.

Analytical methods. Optical rotation was measured in dioxane or water, using a Perkin-Elmer Polarimeter, type 141. Ultraviolet absorption spectra were measured in methanol and water, using a Perkin-Elmer type 202 Spectrophotometer. Infrared spectra were taken by the KBr method with a Nihon Bunko DS-201B Spectrophotometer. Titration experiment was carried out by the Radiometer titrator. Amino acid composition was determined by use of automatic amino acid analyzer, Hitachi KLA modified type 2.

Results

Preparation of Thiolcarboxylic Acid-Siomycins A

Several derivatives of siomycin A were prepared with thiolcarboxylic acids. Thioglycolic acid, α- or β-mercaptopropionic acid, cysteine; and thiomalic acid were used. Bis-thiolcarboxylic acid-siomycins A were obtained when the reactions were proceeded for 20 hours. To prepare mono-thiomalic acid-siomycin A, the reaction time was limited to 6 hours. The properties of the thiolcarboxylic acid-derivatives of the antibiotic are listed in Table 1. Formulas and molecular weights of the derivatives were estimated from the analytical values. The number of added thiolcarboxylic acid was also estimated by alkali titration and elementary analysis.

Salts of Thiomalic Acid-Siomycin A

Bis- or mono-thiomalic acid-siomycins A were neutralized by some cations and solid preparations of the salts-derivatives were made. Values for elementary analysis of these derivatives were listed in Table 2. In the neutralization procedure not only the trisodium salts of bis-thiomalic acid-siomycin A but also mono-, di- or further tetra-sodium salts can be prepared by varying the amount of sodium bicarbonate. Of this group the trisodium salt of the derivative was most stable and soluble in water. The pH values of solutions of the derivative-salts were in the region of pH 6.5~6.8. Disodium mono-thiomalic acid-siomycin A, one of the most simple form of derivative, was also prepared. Specific optical rotations were measured in water as shown in Table 2.

* The finding of this compound is owing to the experiment of Dr. I. KIKKAWA and his staff.

Table 1. Some properties of derivatives of siomycin A reacted with thiolcarboxylic acid

	Thioglycolic acid-siomycin A	α-Mercapto-propionic acid-siomycin A	β-Mercapto-propionic acid-siomycin A	Cysteine-siomycin A	Thiomalic acid-siomycin A	
					Bis-thiomalic acid-	Mono-thiomalic acid-
Appearance	Slightly yellow crystalline			Amorphous	Slightly yellow crystalline	
Elementary analysis (%)	C 47.76 H 5.03 N 13.60 S 11.92	C 47.90 H 5.28 N 12.73 S 11.54	C 48.51 H 5.55 N 13.90 S 11.51	C 55.34 H 6.95 N 15.04 S 11.52	C 47.02 H 4.94 N 13.40 S 10.45	C 49.94 H 5.60 N 13.26 S 10.11
Estimated formula	$C_{78}H_{98}O_{23}N_{19}S_7$	$C_{80}H_{105}O_{28}N_{18}S_7$	$C_{80}H_{109}O_{26}N_{19}S_7$	——	$C_{82}H_{103}O_{27}N_{20}S_7$	$C_{78}H_{105}O_{25}N_{18}S_6$
(mol. wt.)	(1,894)	(1,991)	(1,977)		(2,025)	(1,886)
Number of added thiolcarboxylic acid*	1.65	2.00	1.90	2	1.90	1.22
$[\alpha]_D^{23}$ (*c* 1, dioxane)	−85.9	−64.5	−69.6	−39.7 (methanol)	−69.4	−77.4

* Estimated by alkali titration.

Table 2. Elementary analysis of salts of thiomalic acid-siomycin A

		Bis-thiomalic acid-siomycin A					Mono-thiomalic acid-siomycin A
Type of salts		Na_3	Li_3	K_3	$(NH_4)_3$	$Ca_{3/2}$	Na_2
Elementary analysis (%)	C	46.86	43.42	43.44	46.32	42.72	48.39
	H	5.27	5.70	5.16	5.23	5.45	4.57
	N	13.24	12.55	11.90	13.77	12.40	13.56
	S	—	—	—	10.30	—	—
	Metal	3.02	1.12	8.22	—	3.96	3.71
$[\alpha]_D^{23}$ (*c* 1, water)		−47.6					−39.4

Properties of Derivatives

Some properties of the derivatives of siomycin A with thiolcarboxylic acid were investigated. Fig. 1 shows the ultraviolet absorption spectra of thiomalic acid-derivatives. Molar extinction coefficient (ε) were calculated based on the molecular weights of siomycin A (1,712)[2], mono-thiomalic acid-siomycin A (calc. value, 1,862) and the disodium derivative (calc. value, 1,906). The infrared absorption spectra were shown in Fig. 2. They were quite similar.

The thiolcarboxylic acid-derivatives were relatively soluble in alcohols, for example in methanol, compared to the original antibiotics. The salts of the derivatives were strikingly soluble in water, while it has been shown that siomycin A is insoluble in water. Solubilities of the derivatives and the some of the salts in several solvents were shown in Table 3.

When the derivatives were hydrolyzed by 6 N hydrochloric acid, approximately 1 mole of unusual amino acid was found in the hydrolysate. S-Carboxymethylcysteine, DL- and meso-lanthionine, and S-dicarboxyethylcysteine were found in the hydrolysate of thioglycolic acid-, L-cysteine- and mono-thiomalic acid-siomycin A, respectively. The unusual amino acids were identified by comparing to the synthetic sample of the amino acids. Table 4 shows the amino acid composition of some derivatives of the antibiotic with thiolcarboxylic acid. This results indicate that at least

Fig. 1. Ultraviolet absorption spectra of siomycin A in methanol, monothiomalic acid-siomycin A in methanol, and disodium mono-thiomalic acid-siomycin A in water.

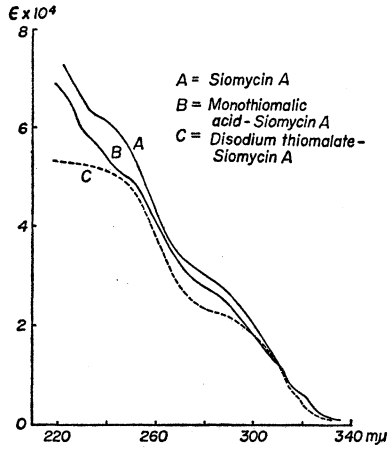


Fig. 2. Infrared absorption spectra of thioglycolic acid-, bis-thiomalic acid- and mono-thiomalic acid-siomycin A by the KBr method.

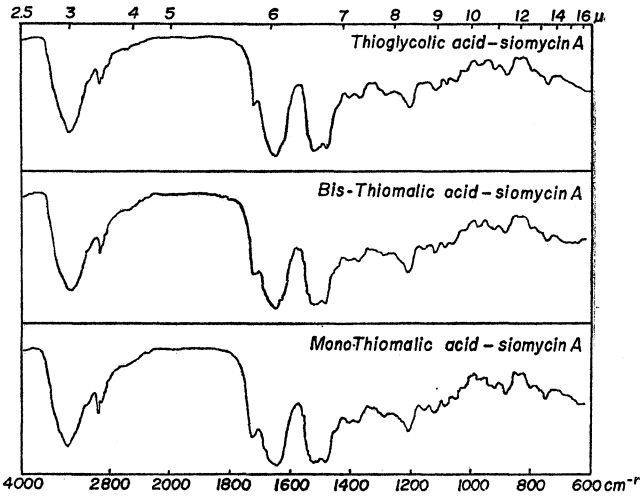


Table 3. Solubility of the thiolcarboxylic acid-siomycins A and their sodium salts (g/100 ml) at 20°C

	Bis-thioglycolic acid-siomycin A	Bis-thiomalic acid-siomycin A	Mono-thiomalic acid-siomycin A	Ma_8, bis-thiomalic acid-siomycin A	Na_2, mono-thiomalic acid-siomycin A	Siomycin A
Methanol	>1	3	1.1	>2	>6	0.004
Acetone	>1	>2	0.2	—	—	0.07
Water	0.009	0.1	0.1	7	6	0.00
dil. NaOH	++	++	++	++	++	—
dil. HCl	±	±	±	±	±	—
Chloroform	±	±	±	—	—	+
Dimethyl-formamide	++	++	++	++	++	++
Dioxane	++	++	3.5	±	—	++
Ether	—	—	—	—	—	—
Petroleum ether	—	—	—	±	—	—

++, very soluble; +, poorly soluble; ±, almost insoluble; —, insoluble.

Table 4. Amino acid composition of some derivatives of siomycin A with thiolcarboxylic acid

	Thoglycolic acid-siomycin A	Cysteine-siomycin A	Mono-thiomalic acid-siomycin A
Threonine	1.01	1.00	1.01
Alanine	1.06	1.05	1.03
Valine	1.00	1.00	1.00
S-Carboxymethyl-cysteine	0.72	—	—
Lanthionine	—	0.49	—
S-(α, β-Dicarboxy-ethyl)-cysteine	—	—	0.68

Data were given as molar ratios when values for valine were assumed to be 1.00.

one residue of the dehydroalanine moiety of siomycin A reacted with sulfhydryl group of the thiolcarboxylic acids.

Antibacterial activities of the derivatives are listed in Table 5. These typical five preparations of the thiolcarboxylic acid-siomycin A and their Na salts are still active against many Gram-positive bacteria and mycobacteria. The potency

Table 5. Antibacterial activity of some derivatives of siomycin A with thiolcarboxylic acid

Test organisms	Minimal inhibitory concentration (mcg/ml)				
	Bis-thioglycolic acid-(free acid)	Bis-thiomalic acid-siomycin A		Mono-thiomalic acid-siomycin A	
		Free acid	Na_3 salt	Free acid	Na_2 salt
Escherichia coli, Umezawa	>20	>20	>20	>50	>50
Pseudomonas aeruginosa	〃	〃	〃	〃	〃
Bacillus anthracis	2.1	1.0	1.0	1.0	5.0
Bacillus subtilis PCI-219	1.0	0.2	0.2	0.2	0.2
Staphylococcus aureus, FDA 209P	0.5	0.2	0.2	0.2	0.2
Sarcina lutea	0.5	0.2	0.2	0.2	0.2
Diplococcus pneumoniae,					
type I	0.2	0.1	0.1	—	—
type I-V	0.2	0.1	0.1	—	—
type II	0.2	0.2	0.1	—	—
type III	0.2	0.1	0.1	—	—
Streptococcus hemolyticus, Denk.	0.2	0.2	0.1	—	—
Corynebacterium diphtheriae, S	0.2	0.1	0.1	—	—
Mycobacterium tuberculosis var. *hominis*, $H_{37}Rv$	>20	20	20	10	10
Potency against siomycin A (μg/mg)	82	270	213	210	215

of the derivatives were approximately one tenth to one fifth compared to that of siomycin A.

A preliminary toxicity test revealed that the intravenous acute LD_{50} for mice was about 150 mg/kg body weight. The intraperitoneal acute LD_{50} was also estimated to be 900 mg/kg.

Of the derivatives which have been prepared from siomycin A and thiolcarboxylic acids, the tri-ammonium bis-thiomalic acid-siomycin A was most unstable as the salt preparation for its storage. This salt gradually reverted to the original acid (bis-thiomalic acid-siomycin A).

Free acids of the derivatives, however, were quite stable and did not show the decrease of the potency as the biological activity for at least 4 months at room temperature.

Aqueous solutions of the derivatives (Na-salts) were relatively unstable at high temperature even in weakly acidic medium. After several days a siomycin A-like substance was found as the precipitates.

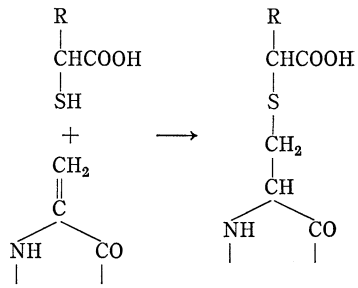
Discussion

Water-soluble derivatives of siomycin A could be prepared by adding thiolcarboxylic acids such as thioglycolic acid into the antibiotic. Among them the thiomalic acid-siomycin A has the most high antibacterial activity. Mono- and bis-thiomalic acid-siomycins A were also prepared independently by selecting the reaction time. Both derivatives were repeatedly compared each other for all properties of them including their purification procedures. It was found consequently, that the mono-thiomalic acid-derivatives of siomycin A was better than the bis-derivative, particularly, in the preparation procedure, no side-products was found.

The mode of addition of thiolcarboxylic acid to the antibiotic molecule was investigated to determine their compositions (Table 4). As far as the amino acid composition of

the derivatives is concerned, it seems that one of the dehydroalanine moieties was displaced by one mole of thiolcarboxylic acid (Fig. 3). However, analytical values revealed that the products of 20-hour reaction would be the bis-derivatives of siomycin A. This discrepancy has not yet been clarified since the full structure of siomycin A has not been elucidated. Addition experiments using hydrogen, amine and thioglycolic acid emphasized that a steric hindrance might be present in the dehydroalanine part near the valine residue[4]. From results described above, it might be expected that two molecules of thiomalic acid would be introduced two parts of the antibiotic, one is dehydroalanine moiety and the other is as yet unknown. As evidence of this, approximately 2 moles of cysteic acid were found, together with approximately one mole of carboxymethyl cysteine in the complete acid hydrolysis of the performic acid oxidized-bis-cysteine-siomycin A.

Fig. 3. The mode of addition of thiolcarboxylic acids into the dehydroalanine part of siomycin A.



Acknowledgement

The authors wish to express their gratitude to Dr. I. KIKKAWA and his staff for their finding of mono-thiomalic acid-siomycin A and helpful discussion. We are indebted to Dr. H. NISHIMURA and Dr. M. MAYAMA for the measurements of antibacterial activities and animal toxicities, and to Mr. Y. TAKAHASHI for his technical assistance. Grateful acknowledgement is also made to Dr. S. MIZUKAMI and his staff for their elementary microanalyses, to Dr. K. KURIYAMA for the optical rotation measurement, and to Dr. Y. MATSUI for the infrared absorption measurements.

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