*Structure of Roseothricin A***

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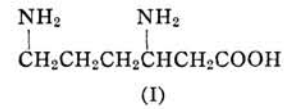
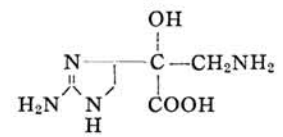
Roseothricin is an antibiotic substance produced by *Streptomyces Roseochromogenus*[1] and has a strong resemblance to streptothricin, streptolin, geomycin etc. Hitherto, two components, β-lysine (I)[2] and roseonine (II)[3], both of which are β-amino acids, have been obtained from the hydrolysate of roseothricin. These two amino acids have also been obtained from the acid hydrolysates of streptothricin[4], streptolin[5], geomycin[6], and racemomycin[7].

Roseothricin is not a simple compound, but consists of three components, roseothricin A, B and C, and these components can be separated by means of paper chromatography, paper electrophoresis or ion exchange chromatography[8]. In order to obtain greater amounts of roseothricin A, the method of ion exchange chromatography was employed: the roseothricin complex was passed through a column packed with Amberlite IRC-50 (Na-form) resin, the column was subsequently eluted with 2% ammonium sulfate solution, and roseothricin A was finally precipitated from the eluate as its phosphotangstate by addition of sodium phosphotangstate solution (cf. experimental part). By this method roseothricin A was obtained in a pure state from the roseothricin complex, and was employed for the following experiments.



(I)



(II)

Components of Roseothricin A.—Acid hydrolysis of roseothricin A with 6 N hydrochloric acid at 100° afforded two ninhydrin positive substances, β-lysine and roseonin, but during the hydrolysis considerable amounts of insoluble black resinous substances were produced[3]. Roseothricin A was therefore hydrolysed with 6 N hydrochloric acid at 50° for 11 days in nitrogen atmosphere, the hydrolysate was passed through a column of Dowex 50 (H-form) and the column was eluted with hydrochloric acid. The results are shown in Fig. 1.

Further hydrolysis of compound B afforded compound A and roseonine, and of compound C afforded β-lysine in addition to the two substances. Thus β-lysine, roseonine, compound A, carbon dioxide and ammonia resulted from the hydrolysis of roseothricin A.

Compound A is a new substance, properties of which are as follows: colorless crystals, dec. p. 145—155°, it shows positive ninhydrin, Fehling, Elson-Morgan, triphenyl tetrazolium chloride, and Tollens reaction and is, in these respects, similar to glucosamine. Paper chromatography: buthanol: acetic acid: water (4 : 1 : 1) R_f. 0.33 (glucosamine [G]. 0.33); collidine 0.44 (G. 0.43); phenol : 1% ammonia (concd.) 0.74 (G. 0.70); ethyl acetate : pyridine : water (2:1:2) 0.86 (G. 0.87); methyl ethyl ketone 0.03 (G.

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** A communication (the part of compound A) has appeared in this Bulletin, **30**, 725 (1957).

1) S. Hosoya, M. Soeda, N. Komatsu, S. Imamura, M. Iwasaki, Y. Sonoda and K. Okada, *Jap. J. Exptl. Med.*, **20**, 121 (1949); *J. Antibiotocs*, **3**, (4), 217 (1950).

2) E. E. van Tamelen and E. E. Smissman, *J. Am. Chem. Soc.*, **75**, 2031 (1953).

3) K. Nakanishi, T. Ito, M. Ohashi, I. Morimoto and Y. Hirata, This Bulletin, **27**, 539 (1954); *J. Am. Chem. Soc.*, **76**, 2843 (1954); This Bulletin **30**, 725 (1957).

4) H. E. Carter, R. K. Clark, Jr., P. Kohn, J. W. Rothrock, W. R. Taylor, C. A. West, G. B. Whitfield and W. G. Jackson, *J. Am. Chem. Soc.*, **76**, 566 (1954).

5) E. E. Smissman, R. W. Sharpe, B. F. Aycock, E. E. van Tamelen and W. H. Peterson, ibid., **75**, 2029 (1953).

6) H. Brockmann and H. Musso, *Chem. Ber.*, **88**, 648 (1955).

7) H. Taniyama and S. Takemura, "Abstracts of Papers", 10th Annual Meeting, Pharm. Soc. Japan, April, 1957.

8) S. Hosoya, M. Soeda, N. Komatsu, N. Hara, Y. Sonoda and R. Arai, *J. Antibiotics*, **4** (5), 314 (1951); *Jap. J. Exptl. Med.*, **20**, 683 (1950); Y. Saburi, *J. Antibiotics*, Ser. B **6** (8), 402 (1953).

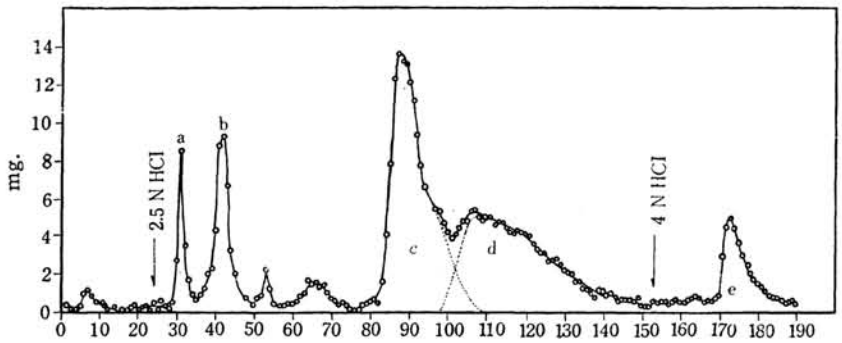


Fig. 1.

Four hundred mg. of roseothricin A HCl salt was hydrolysed to afford 470 mg. of the hydrolysate.

Tube number	Yield	
1—27	9 mg.	
a. 28—34	18 mg.	compound A
b. 35—50	45 mg.	ammonium chloride
51—80	23 mg.	(negative ninhydrin test)
c. 81—100	153 mg.	125 mg. β-lysine. 2HCl 28 mg. roseonine. 2HCl*
d. 101—138	129 mg.	compound B
139—169	21 mg.	
e. 170—190	40 mg.	compound C
Total	438 mg.	(recovery 93%)
	25 mg.	CO_2 (as 111 mg. $BaCO_3$)

* It was calculated from the periodate consumption of fraction C.

0.03). Compound A was scarcely distinguishable from glucosamine by means of paper chromatography using several solvent systems, but there was a small difference between their infrared spectra (Fig. 2). Compound A could not be analysed because of its very minute quantity and of the difficulty obtaining it in a crystalline state. However, according to the analysis and the periodate consumption of compound B, it was shown that compound A was neither a pentosamine nor a branched chain hexosamine, but a straight-chain hexosamine. Recently a hexosamine[9] (m. p. 152—162° dec.) has been isolated from streptothricin and streptolin B, and its structure elucidated as 2-amino-2-deoxy-α-D-gulose (α-D-gulosamine), which is a stereoisomer of glucosamine. Therefore, although no direct comparison between the two has as yet been made, it may be safe to regard compound A as being identical with gulosamine (III).

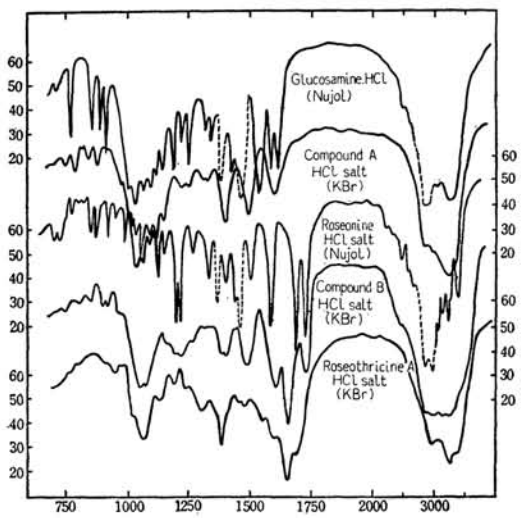
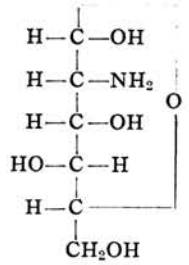


Fig. 2

Structure of Compound B.—Compound B was 2,4-dinitrophenylated incompletely by the method described in the experimental part, and the products were separated by means of counter current distribution. The result is shown in Fig. 3. The two peaks at $K=0.16$ and 9.0 suggest that compound B has two amino

9) E. E. van Tamelen, J. R. Dyer, H. E. Carter, J. V. Pierce and E. E. Daniels, *J. Am. Chem. Soc.*, **78**, 4817 (1956).



(III)

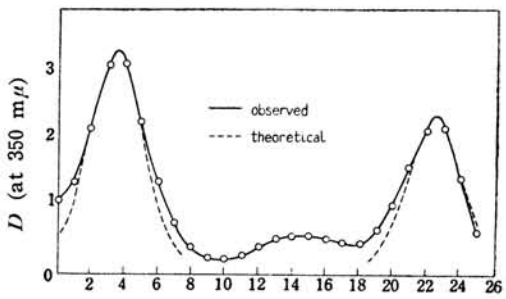


Fig. 3.

groups capable of being 2, 4-dinitrophenylated. The product of complete DNP-ation of compound B has an $E^{1\%}_{1cm}$ value of 306 at 350 mμ, and assuming the presence of two amino groups and taking into account the ε value of 15,900[10] (350 mμ) for a single DNP group, the calculated molecular weight of compound B is ca. 700 for

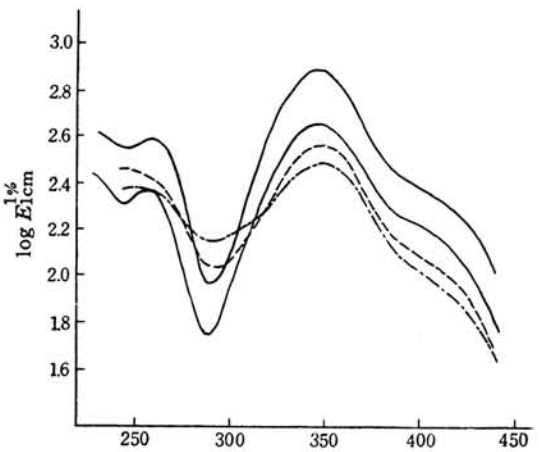


Fig. 4

—— (upper) Di-DNP-β-lysine in tetrahydrofuran $\varepsilon_{350m\mu}=18,000$
—— (lower) DNP-Roseonine in methanol $\varepsilon_{350m\mu}=15,900$
---- DNP-Roseothricin A in T. H. F.
—·— DNP-Compound B in T. H. F.

10) A. R. Batterby and L. C. Craig, ibid., **73**, 1887 (1951). The ε value of 15,900 at 350 mμ is that of DNP-roseonine (roseonine can only be mono DNP-ated).

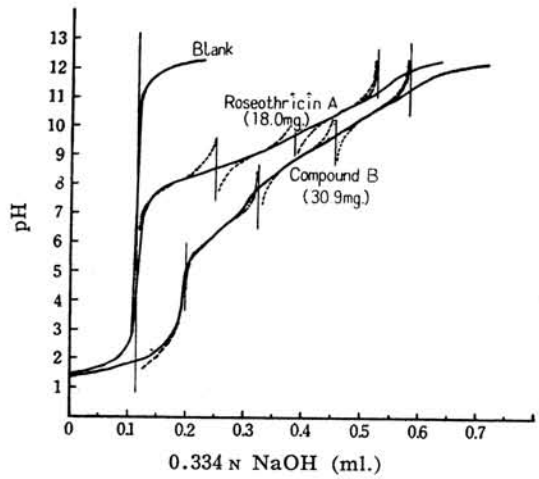


Fig. 5

—— Titration curve
---- Titration curve minus blank
····· Theoretical curve

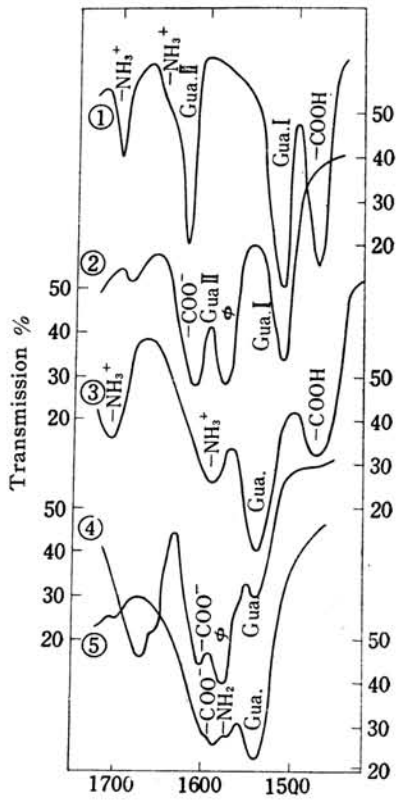


Fig. 6

① Roseonine-2HCl
② DNP- 〃
③ Compound B-HCl
④ DNP-Compound B
⑤ Compound B added NMe_3

the free base (Fig. 4). Though compound B itself could not be hydrolysed without decomposition, roseonine and hexosamine were found in a 1:0.8 ratio in the hydrolysate of roseothricin A (Fig. 1). Coupled with the molecular weight (ca. 700) it is assumed that compound B is composed of 2 moles each of roseonine and hexosamine. Compound B, therefore, should have the molecular formula of $C_{24}H_{44}O_{13}N_{10}$ (2 moles each of roseonine and hexosamine minus $3H_2O$) and a molecular weight of 681 as the free base.

Compound B has eight pK_a's: two at 2.4, two at 6.5, two at 8.9 and two at 10.4 (Fig. 5). Since the first two pK_a's at 2.4 undoubtedly arise from the carboxyl groups evident in the IR spectrum (vide infra), it follows that compound B forms a hexa-hydrochloride with the remaining six groups.

Anal. Found; C, 32.65, H, 6.25, N, 16.12 %. Calcd. for $C_{24}H_{44}O_{13}N_{10}$. 6HCl; C, 32.05, H, 5.60, N, 15.57%.

The IR spectrum of compound B showed a band at 1732 cm^{-1}; when trimethylamine was added and the mixture was immediately evaporated to dryness, this band was displaced to ca. 1600, a position corresponding to the absorption of a carboxylate group. Furthermore, this band is present in DNP-compound B, and this is due to the fact that the guanidine portion cannot be DNP-ated and forms an intramolecular salt with this carboxyl group (Fig. 6). The IR of DNP-roseonine led to similar conclusions. This observation coupled with the two pK_a's at ca. 2.4 suggests that the two carboxyl groups of compound B arising from two molecules of roseonine are free, and neither exist in the form of an ester or an amide group. By comparing the pK_a's of compound B with those of roseonine and hexosamine (Table I), it is apparent that the pK_a's at 10.2 originate from the guanidine portion and those at 6.5 and 8.9 originate from amino groups.

Though the IR spectrum of compound B is very similar to the addition spectrum of roseonine and hexosamine (Fig. 2), the spectra of compound B and roseonine differ in the fact that two bands at 1682 and 1581 in the latter are replaced by a single band at 1658 in the former. These two bands of roseonine originate in the N, N′-disubstituted guanidinium group, and if the guanidinium group is N, N′, N″-trisubstituted, its IR spectrum shows only one band around 1650 cm^{-1} [11]. The guanidinium groups in compound B is, therefore, symmetrically trisubstituted. Thus the data so far mentioned point to the presence of the following groups in compound B: two amino groups which can be DNP-ated, two carboxyl groups, and two symmetrically trisubstituted guanidine groups. Since hydrolysis of compound B

TABLE I

pK_a' VALUES

Compound	−COOH	$-NH_3^+$	Guanidinium$^+$
β-Lysine	3.2	9.5, 10.8	—
Roseonine	2.4	9.3	11.9
Glucosamine	—	7.8	—
Compound B	2.4(2)*	6.5(2)*, 8.9(2)*	10.4(2)*
Roseothricin A	—	8.1(2)*, 9.0(2)*	10.4(2)*

* There are two pK_a's which have the same values; they may not completely coincide but they cannot be distinguished from each other.

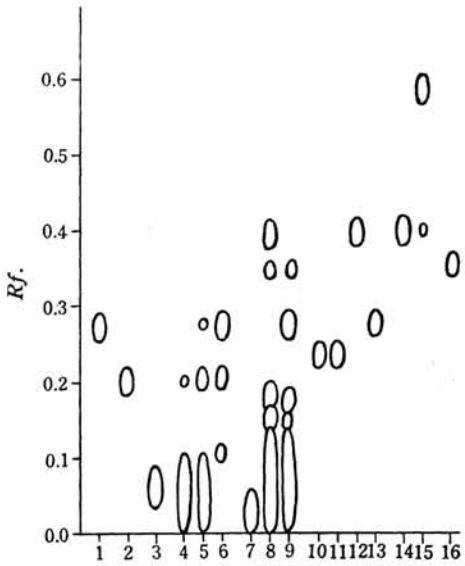
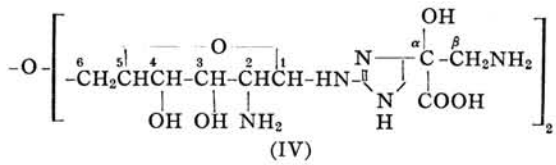


Fig. 7

Solvent system: buthanol : acetic acid : water (4 : 1 : 1). 1. β-lysine. 2. roseonine. hydrolysis of roseothricin A with 0.2 N NaOH at 30°: 3. before hyd. 4. after 1 hour. 5. after 1 day. 6. completely. methanolysis of roseothricin A: 7. with 5% HCl-methanol at 60° for 15 minutes. 8. 20% HCl-methanol, 90°, 3 hrs. 9. hydrolysis of No. 8 with aq. HCl. 10. β-lysine amide 11. treatment of No. 10 with 5% HCl-methanol at 60° for 15 min. 12. treatment of No. 10 with 20% HCl-methanol at 90° for 3 hrs. 13. hydrolysis of No. 12 with aq. HCl. 14. β-lysine methyl ester. 15. treatment of No. 14 with 5% HCl-propanol at 60° for 15 min. 16. compound A.

11) T. Goto, K. Nakanishi and M. Ohashi, This Bulletin, **30**, 723 (1957).

affords roseonine and hexosamine, the only position capable of forming a linkage in the hexosamine moiety is at C_1, which in turn is linked to the guanidine group of roseonine to form a trisubstituted guanidine group (N-glucoside linkage). This is consistent with the fact that compound B gives a positive Elson-Morgan test (glucosamine test) but a negative Fehling test, and the fact that it also gives a positive tetrazolium reaction (semiacetal test) after a longer heating period than that of hexosamine. The strong electron attracting influence of the guanidinium ion probably accounts for the pK_a' (6.5) which is lower than that of hexosamine (ca. 8). The fact that the N-glucoside linkage is more stable under acidic condition than alkaline, is also explicable in terms of this effect. Namely, compound B is extremely resistant towards acid hydrolysis, and no appreciable hydrolysis occurred upon heating for 22 days with concentrated hydrochloric acid at 34°C. It was, however, easily hydrolysed by alkali (1 N sodium hydroxide at 34°C), and gave roseonine (Fig. 7). In this case hexosamine could not be isolated; it was probably decomposed by alkali. As above-menitoned, when compound B was DNP-ated, only two among the four amino groups could be DNP-ated. This partial DNP-ation is probably due to the much slower rate of DNP-ation of the hexosamine residue as compared to the roseonine residue; further DNP-ation of the partial DNP-ated product is thus prevented by precipitation of DNP-compound B. Actually the rate of DNP-ation of glucosamine is less than one fourth of that of roseonine (Fig. 8). Furthermore, it is reported that the amino group of glucosaminide, which has a glucoside linkage, does not react with methyl isothiourea in alkaline condition to form guanidine derivative. This negative evidence may suggest that the glucosidic group affords some protection to the amino group[12]. The positive charge of the guanidinium group in compound B could also retard the reaction between this amino group and the reagent. From these evidences, compound B may be represented by structure (IV).



(IV)

The consumption of periodic acid (6 moles to one mole of compound B, Fig. 9)

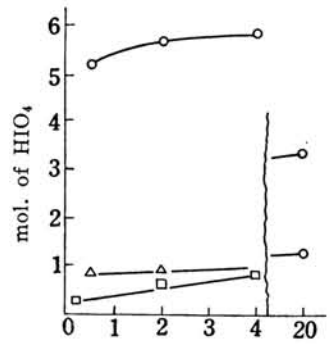


Fig. 9.
○— Compound B
□— Roseonine
△— Roseothricin A

suggests that 2-NH_2, 3- and 4-OH, α-OH, and β-NH_2 are all free. Though there exists no direct evidence concerning the linkage between the two hexosamines, ester or amide bands are not observable in the region 1750–1500 cm^{-1}, and this together with the amount of periodic acid consumption suggests that the primary hydroxyl groups and not the secondary hydroxyl groups participate in an ether linkage.

Structure of Roseothricin A.—Roseothricin A was 2,4-dinitrophenylated incompletely, and the products were separated by means of counter current distribution. The result is shown in Fig. 10.

Since there are four peaks (at K=0.026, 0.15, 2.0 and 6.9) roseothricin A should have four amino groups capable of being 2,4-DNP-ated. The complete DNP-ated

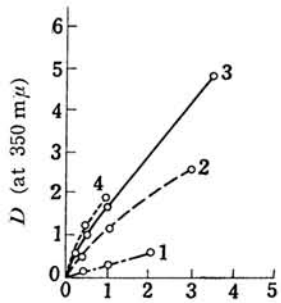


Fig. 8
1 Glucosamine
2 β-Lysine (1/2 eq.)
3 Roseonine
4 Compound B

12) M. Viscontini and J. Meier, *Helv. Chim. Acta*, **35**, 807 (1952).

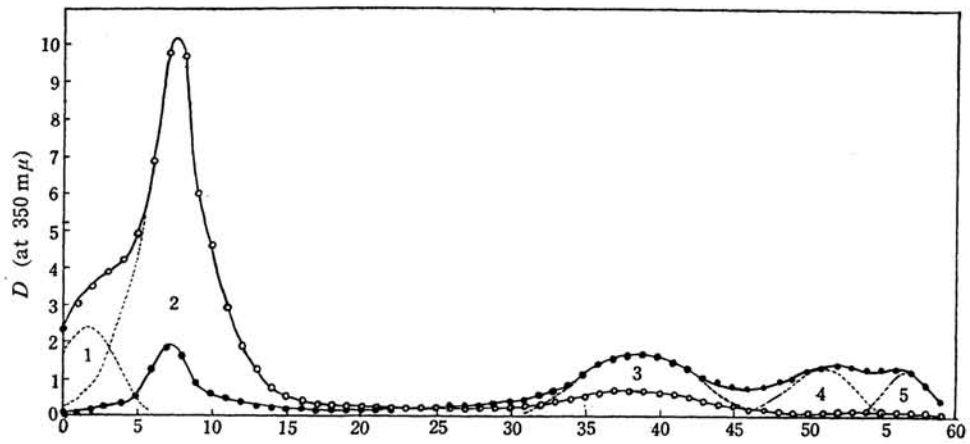


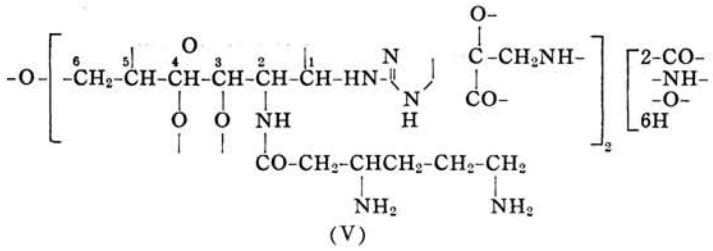
Fig. 10.

—○— upper phase —●— lower phase ····· theoretical

product of roseothricin A has an $E^{1\%}_{1cm}$ value of 357 at 350 mμ (Fig. 4). The E value[10] coupled with the presence of four amino groups leads to a molecular weight of ca. 1100 for roseothricin A free base. Acid hydrolysis of DNP-roseothricin A gave di-DNP-β-lysine as the sole product and no derivatives of roseonine or hexosamine were produced. It is thus inferred that there exists two moles of β-lysine in one mol. of roseothricin A and that the four amino groups are all free. Acid hydrolysis of roseothricin A (Fig. 1) afforded 2 mol. of β-lysine (as standard), 1.6 mol. of roseonine, 1.5 mol. of hexosamine, 1.9 mol. of carbon dioxide and 2.2 mol. of ammonia (compound B was divided between roseonine and hexosamine for convenience of calculation). Roseothricin A is, therefore, composed of two moles each of β-lysine, roseonine, hexosamine, and carbon dioxide and one mole of ammonia (the further mole of ammonia was probably produced from roseonine or hexosamine by decomposition, otherwise analytical data do not agree with calculation), and its molecular weight should be 966 as the free base.

Anal. Found: C, 37.97; H, 6.31; N, 16.75%. (no acetyl group). Calcd. for $C_{38}H_{65}O_{16}N_{15}\cdot 6HCl$: C, 37.83; H, 5.93, N, 17.41%.

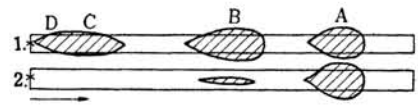
Roseothricin A has six pK_a's: two at 8.1, two at 9.0, two at 10.4 (Fig. 5). This result coupled with the fact that the IR spectrum of roseothricin A is not changed by addition of trimethylamine, suggests that no free carboxyl group is in roseothricin A. Since the amino groups of β-lysine are all free, the four pK_a's at 8.1 and 9.0 must originate from the β- and ε-NH_2 of β-lysine, respectively; the pK_a's at 10.4 are caused by the guanidinium groups. The amino groups of roseonine and hexosamine in roseothricin A are therefore linked to some other groups. When potentiometric titration was carried out in the presence of formaldehyde, the six pK_a's were lowered to two at 3.5, two at 6.1, and two at 8.2. The last pK_a' values (8.2) are higher than that of ordinary amino groups, the pK_a' of which is usually below 7. These data are also consistent with the presence of two guanidinium groups in roseothricin A. Since hydrolysis of roseothricin A with 0.2 N sodium hydroxide at 30°C, afforded first roseonine and subsequently β-lysine, the latter could not be linked to roseonine but should be linked to the hexosamine portion. Heating of roseothricin A with 5% hydrochloric acid-methanol at 60°C afforded neither β-lysine nor its methyl ester, but boiling with 20% hydrochloric acid-methanol afforded β-lysine methyl ester. This behavior was similar to that of synthetic β-lysine amide and not to β-lysine methyl ester (Fig. 7). β-Lysine in roseothricin A, therefore, must be linked to hexosamine through the amino group of the latter. Since roseothricin A did not consume periodate (Fig. 9), at least either the C_3-— or C_4—OH in hexosamine is engaged in a linking (IR spectrum of roseothricin A shows a band at ca. 1717 cm^{-1}, which may be caused by an ester or urethane linkage). We wish to present partial structure (V) for roseothricin A.



(V)

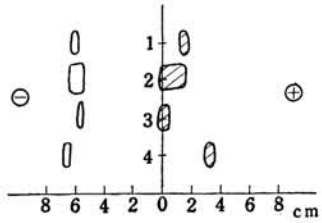
Experimental

Purification of Roseothricin A.—Roseothricin complex (6 g.) was dissolved in 50 ml. of water, and passed through a column of Amberlite IRC-50 (2.5×100 cm tube, 300 c.c. of the resin), after which the column was washed well with water, and eluted with 2% ammonium sulfate solution. The first 10 l. of the eluate did not show any activity on *Bacillus subtilis*, and was discarded. Since the next 20 l. showed strong activity, this fraction was treated with 100 g. of active charcoal. The active charcoal was filtered, washed with water, and added to 250 ml. of 20% acetone-water and an appropriate amount of 1 N hydrochloric acid to adjust the pH to 2, and then filtered. After repetition of this elution procedure for four times, the filtrates were combined, and neutralized to pH 7 by addition of 10% sodium hydroxide solution, and then there was added sodium phosphotangstate solution. The precipitate was centrifuged, washed with water thrice, and dried in a desiccator. This phosphotangstate (ca. 5 g.) was dissolved in 60 ml. acetone and 30 ml. of 2 N hydrochloric acid, filtered from any undissolved substance, and added to 200 ml. of acetone. After being hept overnight in a refrigerator, the precipitate was obtained by decantation, and again dissolved in 10 ml. of water and reprecipitated with 300 ml. of acetone. Finally, the precipitate was dissolved in a small amount of water and freeze dried. Yield ca. 1 g., dec. p. 208–215°. The results of paper chromatography and paper electrophoresis are shown in Fig. 11. The spots were determined by the methods using ninhydrin or *B. subtilis*. The results of electrophoresis suggested that roseothricin had strong basic groups and no acidic groups.



1. Roseothricin complex. 2. Roseothricin A. descending method: solvent; ethanol and 0.5% ammonium sulfate (1 : 1).

Paper electrophoresis



1. Roseonine. 2. Roseothricin complex. 3. Roseothricin A. 4. β-Lysine.
White spot: buffer; 80 ml. of 0.5 M veronal sodium, 2.0 ml. of 2.0N HCl, 72 ml. of 5 M NaCl are diluted to 4 l. (pH 9). 20 mA. 2 hr. Black spot: buffer; 4 ml. of 10% NaOH, 6 ml. of 5 MNaCl are diluted to 500 ml. (pH ca. 12). 20 mA. 4 hr.

Fig. 11.

Hydrolysis of Roseothricin A.—Four hundred mg. of roseothricin A was dissolved in 40 ml. of 6 N hydrochloric acid, and heated at about 50°C under an atmosphere of nitrogen gas which was introduced through two traps containing saturated barium hydroxide solution. After 11 days, the hydrolysate was evaporated to dryness in vacuo, redissolved in a small amount of water and evaporated in a vacuum desiccator containing sulfuric acid and sodium hydroxide. Yield 470mg. The solution of the hydrolysate in 6 ml. water was poured on a column of Dowex 50 (H-form) resin (2×60 cm. tube, ca. 150 c.c. of resin). The column was eluted with water (250 ml.), 2.5 N hydrochloric acid (1300 ml.), and 4 N hydrochloric acid (400 ml.), successively, and the eluates were collected in 10 ml. aliquots by means of a fraction collector. The result is shown in Fig. 1. Each fraction was evaporated to dryness in vacuo below 50°C and weighed. The precipitated barium carbonate in the traps was filtered and weighed.

Partial 2,4-Dinitrophenylations of Compound B and Roseothricin A.—Roseothricin A (83 mg.) and sodium bicarbonate (100 mg.) were dissolved in 10 ml. of 50% ethanol, treated with 5 ml. of 2,4-dinitrofluorobenzene (DNFB 25 mg.) in ethanol (5 ml.), and the solution was allowed to stand overnight at room temperature. A small quantity of yellow precipitate was produced. The solution was acidified with acetic acid, evaporated in vacuo, and then placed in a counter current distributing machine using the solvent system of buthanol: methanol: acetic acid: water (18 : 2: 1 : 19). The result is shown in Fig. 10. The peak No. 5 is 2,4-dinitrophenol. The peak No. 2 was evaporated to dryness, and again 2,4-dinitrophenylated by the same procedure. The result of CCD showed the peaks 2, 3, 4, and 5, except No. 1. The peak No. 2 is much sharper than the theoretical curve; the cause is not elucidated yet.

Partial DNP-ation of compound B was also carried out by the same procedure (9 mg. compound B, 15 mg. sodium bicarbonate 10 mg. DNFB). This result is shown in Fig. 3.

Complete 2,4-Dinitrophenylation of Compound B and Roseothricin A.—Compound B (18 mg.) and sodium bicarbonate (50 mg.) were dissolved in 6 ml. of 50% ethanol, treated with 0.6 ml. of 5% DNFB in ethanol, and incubated at 34°C for two days. The solution was acidified with hydrochloric acid, and the precipitate was

filtered and washed with water, ethanol, and ether, successively. It was dissolved in tetrahydrofuran, and reprecipitated by adding water. Yield 10 mg., m.p. 192–196° (dec.).

In the case of roseothricin A, the same procedure was also employed (117 mg. roseothricin A, 300 mg. sodium bicarbonate, 6 ml. 5% DNFB in ethanol). The precipitate was dissolved in tetrahydrofuran and reprecipitated with ether. Yield 158 mg., m.p. 210°C (dec. sintered at ca. 200°C).

Hydrolysis of DNP-Roseothricin A.—DNP-Roseothricin A (50 mg.) was dissolved in 5 ml. conc. hydrochloric acid, and incubated in a sealed tube at 37° for 14 days. A yellow precipitate separated gradually after two days. Water was added to the mixture and the precipitates were filtered. The filtrate was almost colorless and contained about 10 mg. of roseonine and no β-lysine (by semi-quantitative paper chromatography). The precipitate (23 mg.) was extracted with ethyl acetate. The ethyl acetate soluble portion was 14 mg.; IR spectra and paper chromatography showed this to be identical with di-DNP-β-lysine. The ethyl acetate insoluble portion (9 mg.) was identical with unchanged DNP-roseothricin A (IR spectra).

Synthesis of Di-DNP-β-lysine and DNP-Roseonine.—β-Lysine dihydrochloride (22 mg.) and sodium bicarbonate (60 mg.) were dissolved in 4 ml. of 50% ethanol, treated with 1 ml. of 5% DNFB in ethanol and allowed to stand overnight. The solution was acidified with hydrochloric acid extracted with ethyl acetate, and the ethyl acetate layer was washed with water, and then extracted with 5% potassium carbonate solution. The aqueous layer was separated and acidified with hydrochloric acid. The precipitate was filtered, washed with water, and recrystallized from a mixture of methanol and acetone. Yellow crystals, m.p. 195–196°C.

Roseonine dihydrochloride (39 mg.) and sodium bicarbonate (90 mg.) were dissolved in 5 ml. of 50% ethanol, treated with 1.5 ml. of 5% DNFB in ethanol and allowed to stand overnight. The reaction mixture was added to 10 ml. of water, acidified with hydrochloric acid, and extracted thrice with ethyl acetate. The aqueous layer was evaporated to 3 ml. and the pH was adjusted to ca. 5 with sodium acetate. DNP-roseonine gradually crystallized from the solution as yellow needles, m.p. 226–229°C (dec.).

Measurements of DNP-ation Rates.—*Reagents*: 0.1% aq. sodium bicarbonate solution, 0.4% DNFB ethanol solution (freshly prepared), 1% hydrochloric acid.

Each sample (10 μ mol.) was dissolved in a mixture of 20 ml. of sodium bicarbonate, 2 ml. of DNFB, and 18 ml. of ethanol at 15°C. At suitable intervals, 5 ml. of the solution was poured into 5 ml. of hydrochloric acid, stop the reaction. The absorption intensity of this solution was measured at 320 mμ and 350 mμ. Calculation was based on the density at 350 mμ and the ratio of the density at 320 to that at 350 mμ. The reaction mixture contained the DNP derivative of the sample used, 2,4-dinitrophenol and unchanged DNFB. 2,4-Dinitrophenol and DNFB had about equal values for the density ratio at 320 mμ/350 mμ, i.e., 2.27 and 2.07, respectively, and 2,4-dinitrophenylamino groups also had equal values (0.50). The absorption density originating from 2,4-dinitrophenol and DNFB could thus be subtracted from that of the reaction mixture. The results are shown in Fig. 8.

Measurements of Periodic Acid Consumption.—*Reagents*: 0.01 M sodium periodate, 0.005 M arsenous oxide, 0.01 M iodine, saturated aq. sodium bicarbonate solution, 10% potassium iodide, and starch solution.

One ml. of compound B solution (9.5 mg. in 10 ml. water) and 2 ml. of 0.01 M sodium periodate were mixed and allowed to stand at 30°C. At suitable intervals the solution was treated with 1 ml. saturate sodium bicarbonate, 2 ml. 0.005 M arsenous oxide, and 1 ml. 10% potassium iodide solution in the order mentioned. After 2 minutes the mixture was titrated with 0.01M iodine using starch solution as indicator. From the difference between the volumes of this titration and blank, the amount of periodate consumption was calculated. By the same procedure, periodate consumptions of the following compounds were measured: roseothricin A (24.6 mg. in 10 ml. water); roseonine dihydrochloride (9.0 mg. in 10 ml.). The results are shown in Fig. 9. The reason that the periodate consumption of roseothricin A increases slowly but linearly and exceeds 3 moles after 20 hours, may be that roseothricin A is gradually hydrolysed during the incubation and consumes periodate.

pK_a' Measurements.—The pK_a's were measured by potentiometric titration in water using Beckmann Model G pH meter.

For example, compound B (18 mg. of its hydrochloride) was dissolved in 1 ml. water (carbon dioxide free), treated with 0.10 ml. 0.40 N hydrochloric acid and titrated with 0.334 N sodium hydroxide. The results are shown in Fig. 5 and Table I.

pK_a' *measurements in the presence of formaldehyde*: As is well known in the formol titration of amino acids, addition of formalin to the solution of primary amino acids causes the pK_a' to drop from a value above 7 to that below 7. For example, β-lysine dihydrochloride (12 mg.) was dissolved in 1 ml. water, treated with 0.3ml. of formalin and 1.0 ml. 0.017 N hydrochloric acid and titrated with 0.268 N sodium hydroxide the results are summarised in the following table;

Compound	$pK_a'_1$	$pK_a'_2$	$pK_a'_3$
β-Lysine	3.2*	4.8	6.9
Roseonine	2.4*	4.9	10.6**
Glucosamine	5.8		
Roseothricin A	3.5(2)+	6.1(2)+	8.2(2)+**

* pK_a' of COOH. ** pK_a' of guanidinium.
\+ Cf. footnote of Table I.

Infrared Spectra.—Infrared spectra were measured with a Hilger H 800 spectrophotometer, sodium chloride prism, by the potassium bromide method.

β-Lysine Ester and Amide.—β-Lysine dihydrochloride (3 mg.) was dissolved in 1 ml. of 5 % hydrochloric acid-methanol, heated on a water bath for twenty minutes, and then evaporated to dryness. The residue was shown to be β-lysine methyl ester by means of paper chromatography. This ester was dissolved in conc. aq. ammonia and evaporated to dryness to produce β-lysine amide. When β-lysine methyl ester was heated with 5 % hydrochloric acid-propanol at 60°C for 15 minutes, β-lysine propyl ester was produced. β-Lysine amide, however, did not produce β-lysine methyl ester by heating with 5 % hydrochloric acid-methanol at 60°C for 15 minutes; the ester was first produced by boiling with 20 % hydrochloric acid-methanol. The same procedures were applied to roseorthricin A. Roseothricin A produced β-lysine methyl ester by boiling with 20 % hydrochloric acid - methanol, but did not produce the ester by heating with 5 % hydrochloric acid-methanol at 60°C for 15 minutes. The ester further hydrolysed to β-lysine by boiling in aq. hydrochloric acid. All procedures were protected from moisture.

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