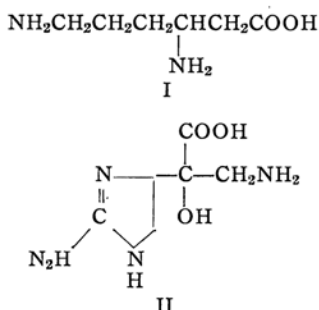


## Two Uncommon Amino Acids obtained from Roseothricin\*

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Roseothricin is a complex antibiotic comprised from several components and produced by *Streptomyces roseochromogenus*. It was formerly designated H-277 and isolated by Hosoya et al.<sup>1)</sup> from the soil of suburban Tokyo during the course of investigations on the production of antibiotics from various molds. Although of rather limited clinical importance due to its somewhat high toxicity, it possesses a high and broad antibacterial spectrum<sup>2)</sup>; furthermore, the acid hydrolysis of either roseothricin complex or of the separated components afforded two amino acids, neither of them belonging to the common natural amino acids. Hence chemical investigations have been undertaken on these hydrolysates, and one has been identified as  $\beta$ ,  $\epsilon$ -diaminocaproic acid (I) ( $\beta$ -lysine)<sup>3)</sup>\*\*\* or isolysine<sup>4)</sup>. The other substance, for which the trivial name of "roseonine" is proposed, has now been shown to be 2-amino-4 (or 5)-(1-carboxy-1-hydroxy-2-amino)-ethyl-2-imidazoline (II), a totally new  $\beta$ -amino acid.\*\*\*\*



The separation of the components of roseothricin complex was accomplished by submitting the substance to paper chromatography,

utilizing butanol-ammonia-*p*-toluenesulfonic acid mixture as solvent and *B. subtilis* test plate for location of the active agents.<sup>5)</sup> Three substances were obtained, which were designated roseothricin A, B and C, in order of their decreasing flow distance; a fourth component was obtained occasionally. Filter paper cataphoresis (100 v., 4 hrs.) at pH 4.6<sup>5)</sup> separated the complex into two fractions, A and B-C. Separation of the complex by means of paper chromatography and filter paper cataphoresis under slightly different conditions have also been reported.<sup>6)</sup> Ion exchange chromatography was found to be effective<sup>5)</sup> for the separation of A and B-C in fairly large quantities; the complex was absorbed on a column of Amb. IRC-50 (Na-form) column at pH 7, and when eluted with citric acid-HCl buffer at pH 4.6 and gradually lowering the pH by 0.2 units, the following three fractions were obtained: pure A, A-B-C mixture and finally B-C mixture. The separation of B and C has as yet not been accomplished. The toxicity against mice (ID<sub>50</sub>) weighing 14.4-15.6 g. (intravenous injection) was as follows: A, 155 mg./kg.; B-C, 1 mg./kg.; complex, 14 mg./kg. The respective fractions were active against gram-positive, -negative and acid-fast bacteria, the activity against *Mycobacterium tuberculosis* 607 being of the same order as that of streptomycin; the activity of A was 1/2-1/100 that of B-C and the complex, depending on the species of the strain<sup>1,2,5)</sup>.

Roseothricin gave a crystalline helianthate (decomp 215-8°, N, 15.51%); reineckate (decomp. 185-190°, N, 22.18%) and picrolonate (decomp. 225-230°, C, 48.23; H, 5.63; N, 20.34%). The ninhydrin, biuret and Fehling reactions were positive, whereas the Pauly, Tollens, maltol, Sakaguchi, Trommer, Nylander, Molisch, Folins, Hopkins-Cole, Millon and Bial reactions were negative<sup>1)</sup>. The acid hydrolysis of roseothricin complex and of roseothricin A both afforded two ninhydrin positive products, I and II, which were separated by the solubility differences of the

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

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4) E. E. van Tamelen and E. E. Smitsman, *ibid.*, 3714.

5) Y. Saburi, *J. Antibiotics*, **6** (8), 402 (1953).

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picrates. Compound I,  $C_5H_{14}N_2O_2$ ,  $[\alpha]_D^{25} +19.0^\circ$  (dihydrochloride in water) was ninhydrin positive, but did not respond normally to the action of the reagent (*vide infra*, Experimental) and therefore it is probably not an  $\alpha$ -amino acid. The Van Slyke amino nitrogen determination gave values of one mole of nitrogen after five minutes and another mole after thirty minutes (Table I). The Kuhn-Roth C-CH<sub>3</sub> determination and periodate test were negative. No absorption maximum was seen in the ultraviolet spectrum, and the infrared spectrum of the hydrochloride in nujol also suggested a simple amino acid structure, i.e., 3.6–4.2  $\mu$  (broad), 5.81  $\mu$  (sharp and strong), 6.25  $\mu$  (medium), 6.7  $\mu$  (weak). The  $pK'_a$  values, 3.20, 9.47 and 10.82 (10°C), indicated that one carboxyl

and two amino groups were present, and that probably a  $\beta$ -amino acid grouping was involved, the other amino group occupying the terminal position. The data allowed only two possible structures, viz.,  $\beta$ ,  $\epsilon$ -diaminocaproic acid and  $\alpha$ -aminomethyl- $\delta$ -amino valeric acid. Two papers by American workers<sup>3,4)</sup> on the elucidation of the structure of an amino acid which had been obtained from streptothricin<sup>7)</sup>, streptolin<sup>7,8)</sup> and viomycin<sup>7,9)</sup> appeared at this point, and it was found that compound I was identical with the new amino acid, i.e.,  $\beta$ ,  $\epsilon$ -diaminocaproic acid ( $\beta$ -lysine or isolysine).

The infrared spectrum of roseonine (II),  $C_5H_{12}O_3N_4$ ,  $[\alpha]_D^{25} +51.0^\circ$  (dihydrochloride in water) (Fig. 1) showed besides bands expected for regular amino acid hydrochlorides, sharp

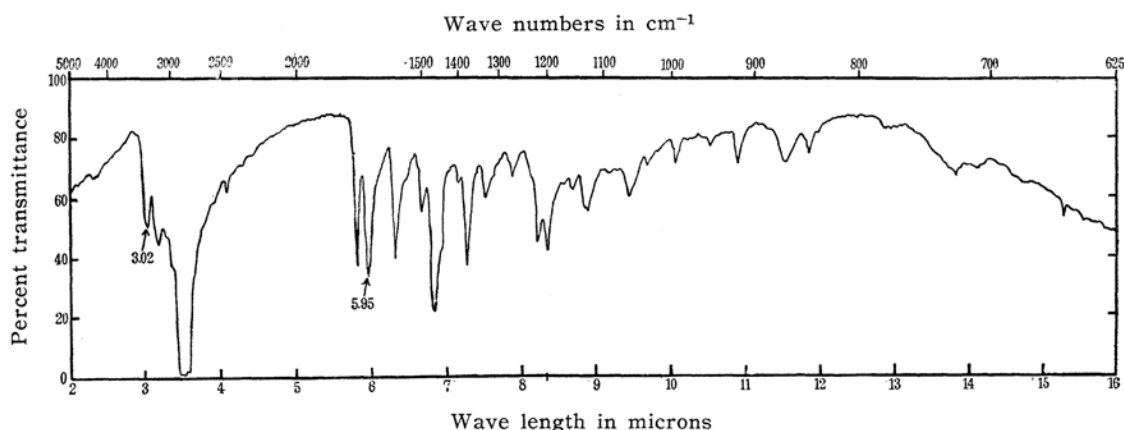
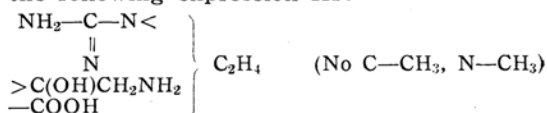


Fig. 1. Roseonine dihydrochloride (Nujol).

absorptions at 3.02  $\mu$  and 5.95  $\mu$ ; the ultraviolet spectrum had an end absorption and a low broad maximum at 262–3  $m\mu$  ( $\log \epsilon$ : 1.22, dihydrochloride in water). Titration of the hydrochloride in aqueous solution gave  $pK'_a$  values of 2.4, 9.3 and 11.9 (17°C), the first constant apparently arising from the carboxyl group, and the second and third from nitrogen functions. The 11.9 acidic constant and the 5.95  $\mu$  infrared band suggested the possible existence of a guanido group and the low ultraviolet maximum was also comparable with that of guanidine hydrochloride, 265  $m\mu$  ( $\log \epsilon$ : 1.26, in water).

Permanganate oxidation of roseonine at room temperature gave guanidine and a

small amount of glycine. When treated with periodate, it consumed one mol. of oxygen in 15 minutes with the formation of one mole each of ammonia and formaldehyde, which meant that one of the two adjacent carbon atoms bearing the hydroxyl and amino groups is at the end of a chain. Coupling this with the formation of glycine and the absence of a strong band in the region 9–10  $\mu$  (infrared spectrum) usually associated with primary alcoholic groups, the presence of the grouping  $>C(OH)CH_2NH_2$  was clearly indicated. The Van Slyke amino nitrogen determination revealed the existence of two amino groups, one of them being liberated somewhat slowly (*vide infra*, Table I-II). The results may be summarized in the following expression III:



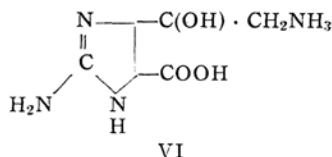
III

7) H. E. Carter, W. R. Hearn and W. R. Taylor, "Abstracts of Papers" 120th Meeting, Am. Chem. Soc., New York, N. Y. September, 1951, p. 3L.

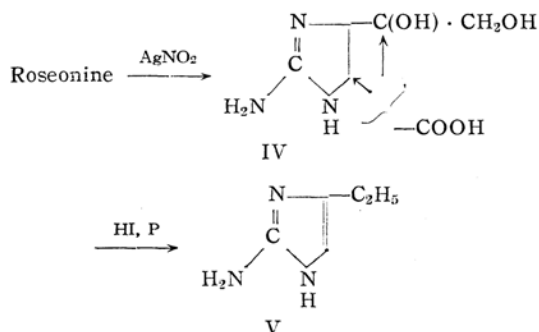
8) E. E. Smismann, R. W. Sharpe and E. E. van Tamelen, "Abstracts of Papers" 121st Meeting, Am. Chem. Soc., Milwaukee, Wisconsin, April 1952, p. 80.

9) T. H. Haskell, S. A. Fusari, R. P. Frohardt and Q. R. Bartz, *J. Am. Chem. Soc.*, **74**, 599 (1952).

It was found at this point that the action of silver nitrite and subsequently hydroiodic acid—red phosphorous brought forth the remarkable conversion of roseonine into an imidazole derivative. Thus silver nitrite reacted normally to convert one amino group into a hydroxyl; the product IV consumed one mole of periodate with the formation of one mole of formaldehyde and it was natural to adopt the view (which received further confirmation, Table I-IV) that the non-guanido amino group had been replaced to give a vic-diol. Heating IV with hydroiodic acid and red phosphorous in a sealed tube gave a substance analyzing as  $C_6H_9N_3$  (V); this coupled with *p*-diazobenzenesulfonic acid to give an orange-red color, a reaction which is typical for imidazoles possessing a free imino group and a hydrogen atom (or carboxyl) in positions 2,4 or 5 (Pauly reaction). The bromine decoloration test which is negative for 2-imidazoles but positive for imidazoles containing a free nuclear methine group was also positive. The Kuhn-Roth determination corresponded to one  $C-CH_3$  whereas the  $N-CH_3$  (or  $N-C_2H_5$ ) determination was negative. Hence V must be 2-amino-4 (or 5)-ethylimidazole. Amongst the conceivable structures for a compound possessing the partial structure III, those represented by II or VI, i.e., 2-



amino-2-imidazoline derivatives, were the only two which could afford V. The possibility of an open chain structure, and hence the formula  $C_6H_{14}O_3N_4$  instead of  $C_6H_{12}O_3N_4$  was also considered, but all were excluded on grounds of apparent inconsistencies with data hitherto mentioned. The reaction sequences then may be formulated as follows:



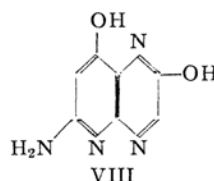
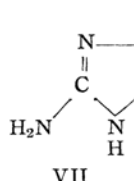
The conversion of IV into V could take place

by dehydration of the side chain hydroxyl group, and rearrangement of the double bond to the more stable aromatic system and decarboxylation.

Interesting supporting evidences for the presence of a ring system in roseonine and V were obtained from comparative studies on the Van Slyke amino nitrogen determination (Table I). Comparing 7 with 6 and 8

TABLE I  
VAN SLYKE AMINO NITROGEN

Compound	Moles of N liberated in	
	7 min.	30 min.
1. $\alpha, \epsilon$ -Diaminocaproic acid (I) dipicrate	1.06	2.13
2. Roseonine (II) dihydrochloride	1.02	1.84
3. Roseonine (II) methyl ester dipicrate	1.04	2.00
4. <i>vic</i> -Diol (IV) from roseonine	0.09	0.71
5. Compound V monopicrate		0.23
6. 2-Amino-2-imidazoline (VII) monopicrate	0.14	0.86
7. Arginine monopicrate	1.01	1.04
8. 2-Amino-4,6-dihydroxypteridine (VIII)	0	0.29



respectively, it may be noted that incorporation of the guanido group into either a non-aromatic or aromatic ring exerts a labilizing effect on the guanido amino group. Thus the presence of a 2-imidazoline ring in roseonine was strengthened by the fact that the response of the guanido amino group of roseonine and products (2, 3 and 4) was similar to that of 2-amino-2-imidazoline, namely, about 0.8 mol. in 30 minutes; furthermore, comparison of 5 (0.23 mol.) and 8 (0.29 mol.) also reveal a striking similarity between the two compounds and hence the probable incorporation of the guanido group of V into an aromatic nuclei.

Of the remaining alternative structures II and VI, the latter was excluded on the following grounds: (i) Roseonine is remarkably stable towards dilute and concentrated hydrochloric acid and thus the functional groups are probably not disposed in space so as to permit easy dehydration leading to formation of  $\gamma$ -lactone, -lactam or hydantoin-imide-(2) rings. These relations are satisfied in struc-

ture II: it may also be noted that normal acid dehydration involving the tertiary hydroxyl group would be hindered due to the presence of the carboxyl and an adjacent positive ammonium ( $\text{NH}_3^+$ ) group. (ii) In the periodate treatment, a second mole of the reagent was consumed after 20 hours, a behavior conforming to that of serine, and hence apparently to that of isoserine.

Structure II is also consonant with the fact that the Sakaguchi and biacetyl reactions were negative since it has been reported that  $\text{N}, \text{N}'$ -disubstituted guanidines do not respond to these color tests<sup>10</sup>.

### Experimental\*\*

**I. Hydrolysis of Roseothricin.**—A solution of 5 g. of roseothricin (complex or A) in 50 cc. of 20% HCl was heated in a sealed tube for 48 hours at 100°C. and the reaction mixture was evaporated to dryness in vacuo. After addition of 20–30 cc. of water and filtration to remove some tarry product, two spoonfuls of Amberlite IR-4B (OH-form) was added and left overnight when the pH was 7–8. The resin was removed and to this there was added 6 g. of picric acid under slight heating upon which a mixture of two picrates and a small amount of resinous product was deposited. The mixture was warmed to bring the picrates into solution, and the resinous material was removed by filtration through cotton. The cooled filtrate containing yellow and orange-yellow picrates was gently rewarmed, when the former dissolved more rapidly. The supernatant was decanted and the separation of the two picrates was effected by repeating the process of dissolution, cooling, warming and separation in the conventional triangular scheme of fractional crystallization. The yellow and orange-yellow picrates corresponded to that of  $\beta, \epsilon$ -diaminocaproic acid and roseonine, respectively. Upon recrystallization from water, each picrate weighed about 2 g. When submitted to paper chromatography using butanol-acetic Acid-water (4:1:5) as solvent, the picrates dissociated and each gave a single spot with ninhydrin,  $R_f$  0.15 ( $\beta, \epsilon$ -diaminocaproic acid) and  $R_f$  0.08 (roseonine).

**II.  $\beta, \epsilon$ -Diaminocaproic Acid (I).** *Dipicrate*: m.p. 200–1° (water). Found: C, 35.55; H, 3.27; N, 18.19; M.W.<sup>11</sup> 596. Calcd. for  $\text{C}_{18}\text{H}_{20}\text{O}_{15}\text{N}_8$  (604.40): C, 35.76; H, 3.31; N, 18.54. *Dihydrochloride*: The picrate was dissolved in a small quantity of hot water and the picric acid which precipitated upon acidification with hydrochloric acid was extracted thrice with ether and the solution was concentrated in vacuo. The addition of ethanol gave the hydrochloride which was recrystallized by adding alcohol to the hot aqueous solution, hygroscopic needles, m.p. 146–7° (water and alcohol). Haskell et al. report 153–5° for this derivative.<sup>9</sup> Found:

C, 33.60; H, 7.66; N, 12.96; Cl, 31.61. Calcd. for  $\text{C}_8\text{H}_{16}\text{O}_2\text{N}_2\text{Cl}_2$ : C, 32.88; H, 7.36; N, 12.79; Cl, 32.36. *N, N*-Dibenzoate (neutral derivative)<sup>12</sup>: The dihydrochloride was benzoated through the Schotten-Baumann procedure, and the product recrystallized from ethanol, m.p. 159–60°. *p*-Hydroxyazobenzene-*p*'-sulfonate<sup>3,4</sup> m.p. 243–5° (water).

$\alpha$ -Amino acid test: (*vide infra*).

**III. Roseonine (II).** *Dipicrate*: decomp. 237° (water). Found: C, 33.48; H, 3.17; N, 22.09; M.W. 654<sup>11</sup>. Calcd. for  $\text{C}_{18}\text{H}_{18}\text{O}_{17}\text{N}_{10}$  (646.40): C, 33.45; H, 2.81; N, 22.09. *Dihydrochloride*: From the dipicrate by conventional methods, m.p. 215° (water and alcohol). Found: C, 27.51; H, 5.40; N, 21.55; Cl, 27.97. Calcd. for  $\text{C}_8\text{H}_{14}\text{O}_3\text{N}_4\text{Cl}_2$ : C, 27.65; H, 5.41; N, 21.46; Cl, 27.16. *Methyl ester dipicrate*: To a suspension of 20 mg. of the dihydrochloride in 3 cc. of methanol, there was added 0.12 cc. of  $\text{BF}_3$  etherate (4 mol. equiv.) and the mixture was refluxed gently for 5 hours. After the addition of 3 cc. of water and picric acid, methanol was removed by boiling and the solution was left at room temperature; the formation of the picrate was slow but the yield was good (35 mg.), m.p. 202° (water). However, there were occasions when the preparation of the ester failed; the cause for this uncertainty was not fully investigated. The ester gave a positive ninhydrin coloration. It was very susceptible to acid hydrolysis. Found: C, 34.66; H, 3.40; N, 21.39. Calcd. for  $\text{C}_{19}\text{H}_{20}\text{O}_{17}\text{N}_{10}$  (660.43): C, 34.56; H, 3.05; N, 21.22.  $\alpha$ -Amino acid test—Reaction with ninhydrin: A solution of 33 mg. ninhydrin (2 mol. equiv.) in 5 cc. of water was added to a solution of 26 mg. of the dihydrochloride in 2 cc. of water and heated at 100°. Contrary to the behavior of usual  $\alpha$ -amino acids (formation of purple precipitate), no reaction occurred. Hence the mixture was heated in a sealed tube for 3 hours at 150°, upon which ca. 10 mg. of purple brown precipitate was obtained; when 2,4-dinitrophenylhydrazine was added to the colorless solution a small amount of hydrazone, decomp. 110° resulted. However, the same two substances were also produced when ninhydrin alone was treated in a similar manner. A small amount of roseonine picrate was recovered after addition of picric acid to the filtrate of the hydrazone. The behavior of  $\beta, \epsilon$ -diaminocaproic acid was also exactly similar.

*Action of periodate*: Sodium metaperiodate oxidized II-dihydrochloride at room temperature to give one mole each of formaldehyde (dime-done) and ammonia (titration) after 15 minutes. The consumption of the reagent was 1.01 mol. (15 min.), 1.05 mol. (1 hr.), 2.32 mol. (20 hrs.) for roseonine dihydrochloride, and 1.15 mol. (10 min.), 1.13 mol. (1 hr.), 1.94 mol. (30 hrs.) for serine. Standard techniques were employed for these analyses. Similar results were obtained with periodic acid.

*Potassium permanganate oxidation*: 100 mg. of II-dihydrochloride and 250 mg. of KOH were dissolved in 5 cc. of water, and 120 mg. of  $\text{KMnO}_4$  in 5 cc. of water

\*\* All melting points were determined on a micro hot stage, and are uncorrected.

<sup>10</sup> J. D. Mold, J. M. Lladino and E. J. Schantz, *J. Am. Chem. Soc.*, **75**, 6321 (1953).

<sup>11</sup> Obtained from the extinction coefficient by the method of K. G. Cunningham, W. Dawson and F. S. Spring, *J. Chem. Soc.*, 2305 (1951).

was added dropwise with stirring over a period of 2 hours at 30°. After leaving for 2 hours, the mixture was filtered and the brown precipitate washed thoroughly with boiling water. The filtrate and wash solution were combined, and after acidification, taken up to dryness in vacuo. The residue was extracted repeatedly with boiling ether, and the ether extract was left overnight to evaporate when a minute amount of colorless crystals, m.p. 126–31° (with partial sublimation; m.p. depression with malonic acid) was obtained. Though the amount was insufficient to permit further purification, paper chromatography gave only a single spot corresponding to oxalic acid; a simultaneous run showed that neither malonic, succinic nor malic acid was present. The residue from ether extraction was extracted with boiling ethanol, leaving a white inorganic salt. Removal of alcohol gave a hygroscopic crystalline residue. An aqueous solution of picric acid was added; the crystals were first washed with ether and then recrystallized several times from water with addition of active charcoal; 3 mg. of yellow plates, m.p. 315°. No depression observed when mixed with authentic guanidine picrate. Found: C, 29.65; H, 3.13; N, 28.81. Calcd. for  $C_7H_8O_7N_6$  (288.18): C, 29.17; H, 2.80; N, 29.17.

The filtrate from the guanidine picrate was acidified and, after extraction of excess reagent with ether, was concentrated in vacuo to give a syrupy mass. This was paper-chromatographed with the following solvents and in each case gave only a single ninhydrin positive spot undistinguishable from glycine. BuOH: AcOH:  $H_2O$  4:1:1 (Rf 0.12). BuOH: AcOH:  $H_2O$  = 4:1:5 (Rf 0.21). Lutidine: collidine:  $H_2O$  = 1:1:1 (Rf 0.15). 40% aq. acetone (Rf 0.45). Phenol: 1% ammonia = 85:15 (Rf 0.38).

Oxidation with  $Ba(MnO_4)_2$  also afforded similar results.

*vic-Diol (IV)*: A solution of 130 mg. of II-dihydrochloride was treated with 154 mg. of  $AgNO_3$  (2 mol. equiv.) at room temperature for 15 hours, and after filtration of  $AgCl$  and concentration in vacuo, a syrupy mass was obtained. White crystals were isolated by the addition of alcohol and filtration; m.p. 190° (water and alcohol), yield 50 mg. Periodate oxidation for 20 minutes produced 0.62 mol. of formaldehyde. Found: C, 37.77; H, 5.62; N, 21.96. Calcd. for  $C_6H_{11}O_4N_3$  (189.17): C, 38.09; H, 5.86; N, 22.21. When only one mole of  $AgNO_3$  was added the yield of IV was poor, and upon addition of picric acid, unchanged starting material was obtained in good yield. Thus removal of both molecules of hydrochloric acid as  $AgCl$  seemed to be necessary for the satisfactory action of  $AgNO_3$ . Incidentally,  $AgNO_3$  is presumably a weaker reagent than  $HNO_3$  as could be seen from the fact that the  $\beta$ -amino group of histamine was converted into a hydroxyl by the latter but not by the former.

*Compound V*: II-Dihydrochloride (130 mg) was treated with 2 mol. of  $AgNO_3$  and the product was worked up to give the syrupy mass as mentioned above. To this there was added 0.48 cc. of HI (b.p. 126°) and 24 mg. of red phosphorus,

and the mixture was heated in a sealed tube for 8 hours at 150°. The clear colorless solution was separated from some tarry precipitate and after evaporating to dryness, water was added and the solution was concentrated again; the processes of concentration were carried out under nitrogen. A syrupy residue containing white crystals resulted. The crystals were separated by filtration and analyzed but were not further investigated, m.p. 168°, yield 30 mg. Found: C, 1.24; H, 5.26; N, 9.52; ash, 30.72. The acidic syrup was neutralized with Amberlite IR-4B when addition of picric acid afforded 30 mg. of a yellow picrate, m.p. 182° (water). The syrup as well as the free compound obtained from the picrate gave an orange-red Pauly test. The same picrate was obtained from the reaction product of one mole of  $AgNO_3$ , but the yield was even lower. Found: C, 39.05; H, 3.72; N, 23.91; M.W. 356<sup>11</sup>) Calcd. for monopicrate,  $C_{11}H_{12}O_7N_6$  (340.25); C, 38.83; H, 3.56; N, 24.70.

*Acid treatment*: Roseonine is remarkably stable towards acid, and was recovered unchanged after heating 50 mg. of the dihydrochloride with 0.6 cc. HCl in a sealed tube for 8 hours at 150°.

### Summary

Roseothricin has been hydrolysed to give two uncommon amino acids, *viz.*,  $\beta,\epsilon$ -diaminocaproic acid (I,  $\beta$ -lysine), and 2-amino-4(or 5)-(1-carboxy-1-hydroxy-2-amino)-ethyl-2-imidazole (II, roseonine) as the sole ninhydrin positive products.

### Acknowledgement

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\*\*\* We are very grateful to Professor Carter for information give us on  $\beta$ -lysine.

\*\*\*\* It has been shown by direct comparison that roseonine is identical with streptolidine, a hydrolysis product of streptolisin (Communication from Professor E. E. van Jamelen, September 10 (1954).