water and treatment of the residue with ethanol precipitated a white solid which was removed by filtration. The ethanol was evaporated from the filtrate and the residue was dissolved in acetone with heating. Upon standing, a white crystalline compound precipitated m. 138–139°, (0.83 g.; 38%). The analytical sample was recrystallized from acetone, m.p. 139–140°, $[\alpha]^{28}D$ −109.9° (*c* 1.18; H_2O).

Anal. Calcd. for $C_8H_{16}O_6S$: C, 40.00; H, 6.67; S, 13.33. Found: C, 40.68; H, 6.69; S, 13.39.

3-Deoxy-3-methylthio-D-gulose (VI). Methyl 3-deoxy-3-methylthio-β-D-gulopyranoside (II) which had been purified through its benzylidene compound (1.0 g.) was hydrolyzed by heating with 0.2*N* sulfuric acid for 4 hr. on the steam bath. After neutralization with barium carbonate, the filtrate was taken to dryness. The residue was dissolved in ethanol, filtered through a mat of Celite No. 535, and evaporated to constant weight to give a theoretical yield of VI as an oil with $[\alpha]^{25}D$ −25.2° (*c* 2.0; water), showing no mutarotation. A sample purified by chromatography on Whatman No. 3MM filter paper (*n*-butanol–acetic acid–water; 4:1:1) had $[\alpha]^{25}D$ −27.1° (*c*, 1.5; water). A chromatogram on Whatman No. 1MM paper with the same solvent system (16 hr., aniline hydrogen phthalate as developer) gave an R_{xylose} value of 1.72.

GAINESVILLE, FLA.

[CONTRIBUTION FROM THE ROCKEFELLER INSTITUTE]

On Bacitracin F

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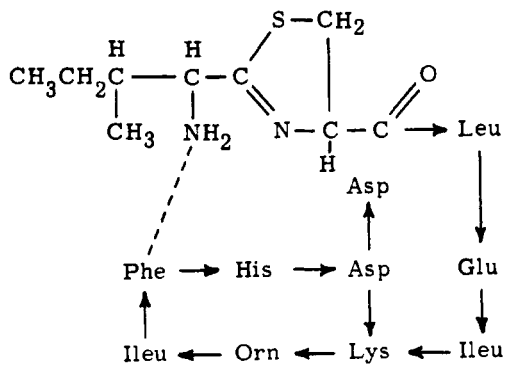
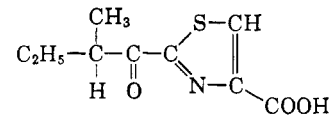
Bacitracin F has been further purified and more precisely characterized by the improved separation and analytical procedures now available. One of the possible structures earlier considered has been shown to be the correct one. Bacitracin F is derived from A by oxidation of the *N*-terminal amino group of the isoleucine residue to form a ketone and by oxidation of the thiazoline ring to a thiazole. Contrary to the earlier view, the amide nitrogen of the aspartic acid residue is retained.

One of the most characteristic features of the bacitracin polypeptides is the limited *p*H range within which the antibiotics retain their activity. In a previous publication,[1] we have described the processes leading to inactivation at low *p*H's. In this paper we will be dealing with the transformation that occurs at *p*H 7 or slightly higher to give the inactive product called bacitracin F.[2,3]



Formula 1

The slow inactivation of bacitracin A (Formula 1) at or above neutral *p*H was first observed rather early in the study.[2,3] Since that time several proposals have been made for the structure of bacitracin F and for the steps involved in its formation from bacitracin A.[4] The structure proposed was based primarily on two lines of evidence. Degradation studies led to the isolation of a ketothiazole acid[5] in 50% yield from an acid hydrolysate of purified bacitracin F. This ketothiazole acid was shown to have Formula 2 by degradation[5] and synthesis.[6] Secondly, the empirical formula for bacitracin F derived from analytical data seemed to require the loss of two nitrogens assuming the Dumas analysis to be reliable. The intermediate was thought to be an amino thiazole.[5]



Formula 2

A more recent proposal postulated the partial structure for bacitracin F[4] shown in Formula 3. Neither this nor the earlier proposal can reasonably account for all the evidence now available. One problem in the earlier work was the liberation of a fractional mole of ammonia during the Conway amide determination. Another problem concerned the electrophoretic behavior of bacitracin F. A third problem centered around the low yields of the

(1) William Konigsberg and L. C. Craig, *J. Org. Chem.*, **26**, 3867 (1961).

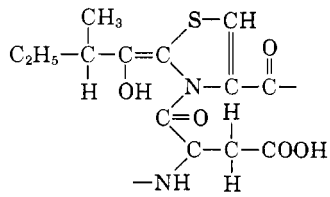
(2) L. C. Craig, J. R. Weisiger, W. Hausmann, and E. Harfenist, *J. Biol. Chem.*, **199**, 259 (1952).

(3) G. G. F. Newton and E. P. Abraham, *Biochem. J.*, **47**, 257 (1950).

(4) L. C. Craig, William Konigsberg, and R. J. Hill, in Ciba Foundation Symposium on Amino Acids and Peptides with Antimetabolic Activity, 1958, p. 226.

(5) J. R. Weisiger, W. Hausmann, and L. C. Craig, *J. Am. Chem. Soc.*, **77**, 3123 (1955).

(6) J. R. Weisiger, unpublished results. This derivative was synthesized by a method similar to that employed by Brookes, Fuller, and Walker [*J. Chem. Soc.*, **137**, 689 (1957)] for the synthesis of 2-isobutyrylthiazole.



Formula 3

ketothiazole acid obtained from bacitracin F. A structure able to account for this result was required since the ketothiazole acid itself was found to be perfectly stable under the same hydrolytic conditions even in the presence of bacitracin A and F. Finally, the extinction coefficient at 290 mμ of bacitracin F was significantly lower than that of the model compound and the minimum did not occur at the position expected, 255 mμ in the case of the ketothiazole amide but 270 in the case of bacitracin F.

Most of the data available regarding bacitracin F were obtained before the present-day refinements of separation and analytical methods. It, therefore, seemed desirable to reinvestigate this product from the standpoint of purity, composition, physical properties, and certain chemical properties.

EXPERIMENTAL

Commercial bacitracin was supplied through the courtesy of the Commercial Solvents Corp. Lot B-55-10 was used throughout the work.

The distributions were made at 25° in an automatic C.C.D. apparatus.[7] All solvents were distilled before use. Amino acid analyses were performed by the method of Moore, Spackman, and Stein.[8]

Purification of the "F" fraction from commercial bacitracin. During the course of the purification of bacitracin A from commercial bacitracin, a considerable amount of the crude F fraction had accumulated. It was free of the A component since its partition coefficient was well removed from that of A in the system initially used to purify A.

This material (a 1-g. sample) was dissolved in 25 ml. of methanol. Chloroform (25 ml.) and water (12.5 ml.) were added. The two phases were placed in the first tubes of a 220 tube 10/10 automatic train already charged with the system methanol–chloroform–water (2:2:1 volume proportions).

After 241 transfers analysis by optical density and by weight gave the pattern shown in Fig. 1. Cuts were taken as indicated and the solute recovered by initial removal of the organic solvents in a rotatory evaporator with final removal of the water by lyophilization. The cut from the major component (BH_1) gave a residue of 350 mg.

Preparation of bacitracin F from bacitracin A. A 600-mg. sample of bacitracin A was allowed to stand for 2 weeks at 37° in 100 ml. of 0.05 *M* phosphate buffer *p*H 7.2. Optical density readings were made at 290 mμ and 255 mμ during this period. At the end of 1.5 weeks, the optical density at 290 mμ had reached a maximum and the 290/255 ratio remained constant. The reaction mixture was then lyophilized and distributed in the system 2-butanol–3% acetic acid (1:1) to 137 transfers using an automatic 200-tube machine, 10 ml. each phase. The pattern was similar to that previously

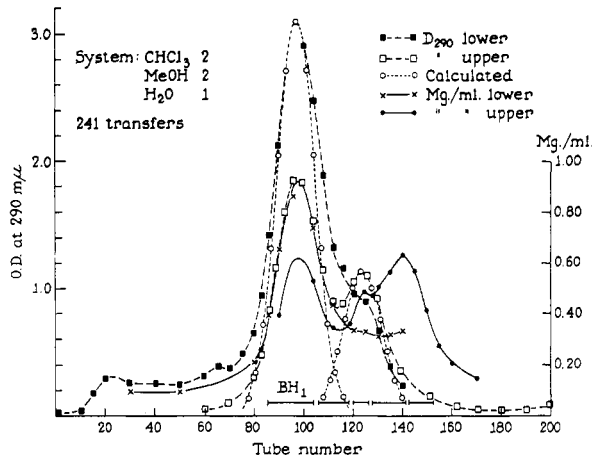


Fig. 1. Countercurrent distribution of crude bacitracin F from commercial bacitracin

published[5]; it showed two components. The material with the higher partition coefficient (K = 1.0) was the only one having a maximum absorption at 290 mμ. The material in the band with the lower partition ratio (K = 0.3) had the same partition coefficient as bacitracin A itself. It was recovered and redistributed in the more selective system *n*-butyl alcohol–0.5 *M* phosphate buffer *p*H 5.45.[9] This gave a pattern which showed that it did indeed consist of unchanged bacitracin A. The material (180 mg.) from the band of K = 1.0, recovered by evaporation and lyophilization, will be referred to as FA_2. A portion was redistributed in the system chloroform–methanol–water (2:2:1). The pattern was similar to that shown for the preparative run (Fig. 1) except for the relative peak heights.

Carboxymethylcellulose chromatography of bacitracin F. Carboxymethylcellulose (0.47 meq./g.) was obtained from the Brown Co. It was washed with 0.2 *M* sodium hydroxide in 1 *M* sodium chloride, then equilibrated with 0.05 *M* pyridine acetate buffer, *p*H 3.97. Columns 34 × 0.9 cm. were used. Two-milliliter fractions were collected using a flow rate of 4 ml./hr. and analyzed by the ninhydrin procedure of Moore and Stein.[10] The pattern obtained from the crude product obtained by transformation of bacitracin A is shown in Fig. 2, pattern A.

Analytical data. Elementary analyses were made on cut 1 (BH_1) from Fig. 1 after drying to constant weight at 100°.

Anal. Calcd. for $C_{66}H_{99}O_{17}N_{16}S$: C, 56.0; H, 7.00; N, 15.8. Found: C, 55.7; H, 7.40; N, 15.6 (Kjeldahl); N. 14.7 (Dumas).

Amide nitrogen determinations were carried out by the micro Conway technique with the results shown in Table I. The amino acid analysis results are shown in Table II.

TABLE I

CONWAY AMMONIA DETERMINATIONS

	Residues of NH_3 per Mole (Average of 3 Determinations)
"Crude" F	0.67
F made from A (FA_2)	0.94
F from cut 1, Fig. 1	1.01
2-Isobutyrylketothiazoleamide	0.98
Bacitracin A	1.06
2-Isobutyrylketothiazole	0.00
"Desamido A"	0.05

(7) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *J. Am. Chem. Soc.*, **23**, 1236 (1951).

(8) S. Moore, D. H. Spackman, and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958).

(9) L. C. Craig and William Konigsberg, *J. Org. Chem.*, **22**, 1345 (1957).

(10) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

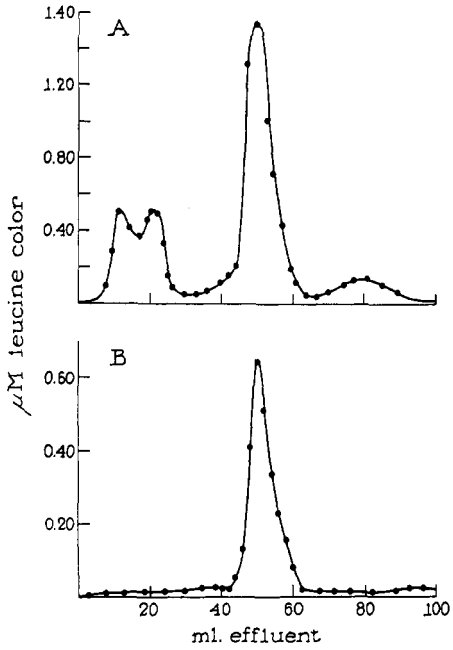


Fig. 2. Effluent patterns from carboxymethyl cellulose chromatography of bacitracin F. Pattern A is from the crude transformation product from bacitracin A. Pattern B is from the cut BH_1 shown in Fig. 1

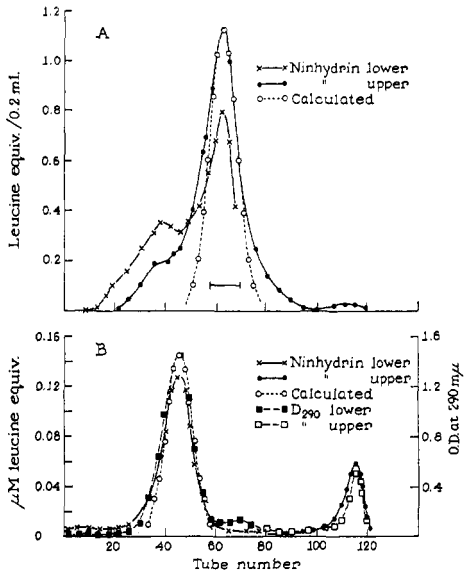


Fig. 3. Countercurrent distribution patterns at 120 transfers of reduced (pattern A) bacitracin F and the product reoxidized (pattern B) after reduction. System = 2-butanol–3% aqueous acetic acid (1:1 volume proportions)

TABLE II

AMINO ACID ANALYSES OF BACITRACIN F AND DERIVATIVES

Amino Acid	F	Residues per Mole: Reduced F	Residues per Mole: Reoxidized product
Asp	1.72	1.89	1.80
Glu	1.01	0.97	0.98
Cys	0.03	0.05	0.05
Val	0.06	0.08	0.07
Allo-ileu	0.08	0.12	0.10
Ileu	1.82	1.71	1.78
Leu	1.00	1.01	1.01
Phe	0.98	0.96	0.96
Lys + orn	1.69	1.77	1.77
His	1.01	1.03	0.99
NH_3	1.21	1.19	1.16
% N accounted for	96	94	94

Reactions of bacitracin F with hydroxylamine and ferric chloride.[11] When a sample from cut 1, Fig. 1, was allowed to react in an aqueous methanolic solution of hydroxyl amine at *p*H 6.5, room temperature, followed by the addition of a drop of ferric chloride solution, a red color was produced.

Reduction of bacitracin F. Attempts were made to reduce bacitracin F with hydrogen gas and Raney nickel as well as palladium- and platinum-on-charcoal using hydrogen at atmospheric pressure. None of these methods worked as shown by an unchanged absorption spectrum of the material after the reaction. When a neutral aqueous methanolic solution of sodium borohydride was used, there was a rapid decrease in the absorption of the material at 290 mμ.

The reaction was repeated using the purified bacitracin F (cut 1, Fig. 1). A 30% methanol aqueous solution was made up and buffered to 0.5 *M* with phosphate, *p*H 7.2. The reaction was carried out at 0° with stepwise addition of sodium borohydride until the absorption at 290 mμ reached a low constant value. The mixture was then extracted four times with *n*-butyl alcohol, the alcohol solution was concentrated, water was added, then concentrated and finally lyophilized. The residue was distributed in the system 2-butanol 3% acetic acid to 120 transfers with the result shown in Fig. 3, pattern A; the yield was 85%. The elemental analysis of the reduced compound is in accord with that expected.

Anal. Calcd. for $C_{66}H_{101}O_{17}N_{16}S$: C, 56.0; H, 7.0; N, 15.8. Found: C, 55.6; H, 7.40; N, 15.6.

Reoxidation of reduced bacitracin F. A few crystals of chromic anhydride and one drop of concd. sulfuric acid were dissolved in acetone and added to an acetone solution of reduced bacitracin F at −40°. The solution was allowed to warm to 4° and to remain at this temperature for 3 hr. during which time the yellow-orange color of the chromic anhydride complex turned greenish indicating the reduction of the chromium(VI). Isopropyl alcohol was added to destroy the excess oxidizing agent. The *p*H was adjusted to *p*H 7 by the addition of 20 ml. of 0.5 *M* phosphate buffer. After extracting three times with *n*-butyl alcohol, washing back once with water to remove chromous salts, concentrating, and lyophilizing, a relatively salt-free product was obtained. This material was distributed in the chloroform system to 120 transfers to give pattern B shown in Fig. 3. The yield based on the absorption of the main peak at 290 was about 80%. The material in the main peak had an absorption spectrum corresponding almost exactly to that obtained with pure bacitracin F. The molar extinction at the maximum (290 mμ) was 4600 in methanol.

Conversion of bacitracin F to desamido bacitracin F. A sample of F (50 mg.) was dissolved in 15 ml. of 0.1 *N* hydrochloric acid and kept for 30 min. at 100°. The solution was cooled and lyophilized. This material had the same molar extinction as the starting material but carried one more negative charge than bacitracin F as shown by electrophoresis at *p*H 7.9 and 5.6. A Conway amide determination on this product showed no release of ammonia. The distribution is shown in Fig. 4A.

Preparation of desamido bacitracin A. A sample of bacitracin A (100 mg.) was allowed to stand at 37° in 0.1 *N* sodium hydroxide for 2 hr. The products were separated by C.C.D. in the phosphate system[9] and carboxymethylcellulose chro-

(11) We are indebted to S. Peter Marfey for this experiment.

TABLE III

SPECTRA OF BACITRACIN F AND RELATED DERIVATIVES

Material	Max. (mμ)	Solvent	ε	Min. (mμ)	O.D. Max./Min.
2-Ketoisovalyryl-4-carboxamidothiazole	287	CH_3OH	4700	255	3.02
2-Ketoisovalyryl-4-carboxamidothiazole	290	0.5 *N* HCl	5600	259	3.02
2-Ketoisovalyryl-4-carboxamidothiazole	290	0.1 *N* NaOH	5250	259	3.04
F	289	CH_3OH	4830	268	1.35
F	290	0.5 *N* HCl	5400	270	1.41
F	290	0.1 *N* NaOH	5100	270	1.60
F prepared from A	287	CH_3OH	2800	270	1.15
"Crude" F	290	CH_3OH	2700	270	1.25
Reduced F	238	CH_3OH			
2-α-Hydroxyisovalyryl-4-carboxythiazole	238	CH_3OH			
Reoxidized F	287	CH_3OH	4750	269	1.42

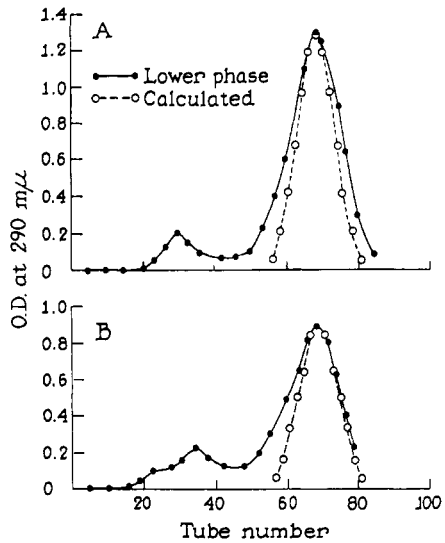


Fig. 4. Countercurrent distribution patterns at 120 transfers of desamido bacitracin F (pattern B) prepared from desamido bacitracin A. System = chloroform–methanol–water (2:2:1 volume proportions)

matography.[12] The resulting desamido A had the same molar extinction coefficient at 255 mμ as bacitracin A but had an additional negative charge as shown by paper electrophoresis at *p*H 5.6 and 7.6.

Conversion of desamido A to desamido F. A sample of desamido A (60 mg.) was allowed to stand at *p*H 7.2 for 1 week. The characteristic spectrum of the bacitracin F type reached a maximum after 3 days and remained constant. The material was distributed as shown in Fig. 4B.

Isolation of the chromophore from bacitracin F. A sample of F (10 mg.) was hydrolyzed for 24 hr. at 110° with 6 *N* hydrochloric acid. The hydrolysate was cooled and diluted with 0.1 *N* citrate buffer *p*H 3.1. It was then distributed for 20 transfers in a system of ethyl ether, 0.1 *M* citrate buffer *p*H 3.1; 1:1. Analysis by absorption at 290 mμ showed that essentially all of the optical density was located in the upper phase of the last three tubes. Final isolation of this material was accomplished by evaporation and subsequent desalting on Sephadex G-25.[13] The chromophore was retained past the volume required to elute the salt. Synthetic isobutyrylketothiazole acid emerged at the same peak position as the chromophore when chromatographed on Sephadex G-25 using 0.2 *M* acetic acid as an eluant. The chromophore had the same molar extinction as F assuming a molecular weight of 213 which is that of the synthetic isobutyrylketothiazole acid. The yield was 90% based on absorption at 290 mμ.

(12) William Konigsberg and L. C. Craig, *J. Am. Chem. Soc.*, **81**, 3452 (1959).

DISCUSSION

The bacitracin F which had been used in the earlier studies was obtained mostly from commercial bacitracin by extensive C.C.D. fractionation. The major component in the F group followed a theoretical curve even after 1200 transfers in one system. Amino acid analysis was consistent with the loss from A of the amino terminal isoleucine and of the cysteine.

Nonetheless, further fractionation seemed desirable and C.C.D. offered the most promise. Several systems with differing selectivities were studied, but one made from chloroform, methanol, and water was finally chosen. The pattern in Fig. 1 showed the bacitracin used in the earlier work to be impure.

Material recovered from the cut shown in the main band of Fig. 1 was used in this study. Its purity was checked by chromatography on carboxymethyl cellulose. A single peak was obtained at a retention of two column volumes.

Paper electrophoresis at *p*H 5.6 and 7.9 gave single sharp spots consistent with the loss of the terminal amino group. This was shown to be absent also by dinitrophenylation experiments.

Amino acid analyses, Table II, made by the most recent procedure of Moore, Spackman, and Stein[8] were consistent with the composition earlier proposed for bacitracin F and showed a low valine content. This indicated that most of the F type derived from bacitracin B had been removed.

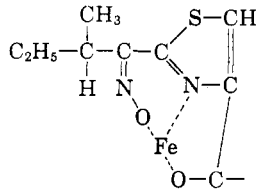
(13) Obtained from the Pharmacia Co., 501 Fifth Ave., New York 17, N. Y. See J. Porath and P. Flodin, *Nature*, **183**, 1657 (1959).

Ultimate analysis was consistent with the formula $C_{66}H_{99}O_{17}N_{16}S$ except for the Dumas nitrogen determination. This repeatedly gave a figure lower than the Kjeldahl. The reason for this is not known. The empirical formula is that derived from the formula of bacitracin A by the loss of one mole of ammonia, two hydrogens and the gain of one oxygen atom. Amide determination by the Conway procedure, Table I, indicated the amide group of bacitracin A still to be present.

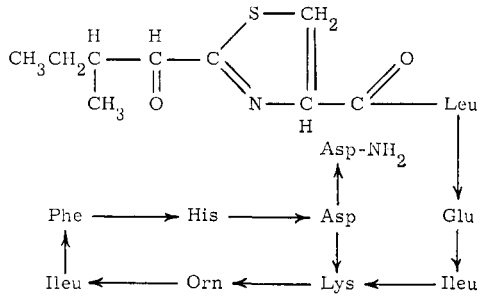
The ultraviolet spectra were compared with various model compounds containing the suspected chromophore and with other samples of F used in the earlier studies.

The results summarized in Table III show that the present preparation, in contrast to other samples of bacitracin F, has the same molar extinction value as the synthetic 2-isobutyrylketothiazole-4-carboxamide but that the peptide bonds contribute end absorption so that there is a difference in the position of the minimum and in the 290/255 ratio. There is an increase in ϵ_m for both the synthetic keto thiazole and F in acid and base. In both cases, the chromophores retain full absorption even after boiling in 1 *N* hydrochloric acid or 1 *N* sodium hydroxide for 3 hr. It can be seen also that "crude" F and bacitracin F made from A have about half the molar extinction at 290 mμ as that of the purified bacitracin F and the synthetic keto thiazole. These data enable us to reconcile the earlier results with impure bacitracin F. A comparison of the infrared spectra of the two compounds supported their identity with respect to the chromophore. There were peaks from the amide, carboxylic, ketone and C—H groups in F and in addition several in the fingerprint region that corresponded with those from the model compound.

Although the analytical data for F are now consistent with the idea that the chromophore is indeed a 2-ketothiazole amide, it remains to be shown that the chromophore is present as such in F and not formed during hydrolysis from a related unstable precursor. The presence of the keto group can be shown by reaction with hydroxylamine and subsequent formation of a chelate with iron-(III) as shown below in Formula 4.



Formula 4



Formula 5

All the evidence available is now consistent with Formula 5 for the bacitracin F derived from bacitracin A.

The synthetic ketothiazole amide behaves in an identical fashion when subjected to this reaction. Neither the synthetic ketothiazole amide nor bacitracin F itself gives any reaction with ferric chloride alone, indicating that very little of an enol form can be present.

Mild reduction of F with sodium borohydride removes the 290-mμ absorption. Most of the reduced material behaves as a single component in C.C.D. (Fig. 3) and has a spectrum very close to that of the corresponding alcohol.

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