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Thiostrepton. Degradation Products and Structural Features

BY MIKLOS BODANSZKY, JOSEF FRIED, JOHN T. SHEEHAN, NINA J. WILLIAMS, JOSEPH ALICINO, ALLEN I. COHEN, BARBARA T. KEELER, AND CAROLYN A. BIRKHIMER

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Thiostrepton, an antibiotic from cultures of *Streptomyces azureus*, has a molecular weight of about 1650. From its acid hydrolysates, L-threonine, L-alanine (2 moles), L-isoleucine, D-cystine, and two thiazole-4-carboxylic acid derivatives, designated as thiostreptoic acid and thiostreptine, were isolated. Derivatives of 8-hydroxyquinaldic acid also found in the hydrolysates of the antibiotic were shown, however, not to be present as such in the parent molecule, but to be products of rearrangement reactions during hydrolysis. The formation of pyruvic acid by the action of trifluoroacetic acid on the antibiotic has also been demonstrated.

The antibiotic substance thiostrepton,[1-4] produced as a metabolic product of the microorganism *Streptomyces azureus*, has been the object of structural investigation in these laboratories[5] for several years. Preliminary reports dealing with the structures of some of its degradation products have been published.[8-10] The present paper reports the details of that work and discusses its significance in terms of the over-all thiostrepton molecule. In addition, the characterization of more recently found hydrolysis products is also presented.

Amino Acids.—Hydrolysis of thiostrepton with constant boiling hydrochloric acid yields a number of ninhydrin-positive components. Of these, cystine, threonine, alanine, and isoleucine, and a trace of glycine are readily recognized on paper chromatograms. Quantitative amino acid analysis by a modification of the Stein–Moore procedure shows that 1 μmole of threonine, 2 μmoles of alanine, 1 μmole of isoleucine, but considerably less than 1 μmole of cystine and glycine are present in 1.8 mg. of the antibiotic. A trace of valine found indicates that the thiostrepton sample might be contaminated by siomycin.[4] About 4 moles of ammonia are also liberated from 1 mole of thiostrepton. The amino acid components were isolated from the acid hydrolysate by countercurrent distribution in a system of 1-butanol–5% hydrochloric acid followed by partition chromatography on a cellulose column. The identification of the above amino acids was completed by comparing their infrared spectra with those of authentic samples. Measurements of the specific rotations revealed that threonine, alanine, and isoleucine belong to the L series, while cystine from thiostrepton has the D configuration. Although D-amino acids occur in antibiotics with a certain regularity, D-cystine, to our best knowledge, had not been obtained from a natural product prior to its isolation from thiostrepton (ref. 9, footnote 5). It is worth noting, however, that very soon after this first isolation was reported, D-cysteine was found as a component in the thiazoline moiety of luciferin.[11] In a number of antibiotics, such as the actinomycins,[12] ostereogrycin B,[13] etc., the D-amino acid is preceded in the sequence of amino acids by L-threonine, the hydroxyl group of which, in turn, participates in a lactone bond. It is not unlikely that the amino group of the D-cysteine moiety of thiostrepton is acylated by the threonine residue.

Although the compound isolated from the hydrolysates of thiostrepton is D-cystine, there is good reason to believe that this moiety does not occur as such in the antibiotic. Treatment of thiostrepton with potassium cyanide does not expose an SH group, but the hydrolysates give a positive reaction with sodium nitroprusside. Furthermore, in countercurrent distribution or on partition chromatograms, cystine appears not only in fractions where it can be expected, but also in some other fractions where it can be present only if it traveled as cysteine, and was oxidized to the disulfide afterwards. The negative sulfhydryl test in the parent antibiotic and the low value found for cystine in the quantitative amino acid analysis show that this amino acid must be present in some unusual form in the parent molecule.

Derivatives of Thiazole-4-carboxylic Acid.—It is interesting to note that apart from bryamycin,[14] which is identical with thiostrepton,[4] and from siomycin,[15-17] which is very closely related to it, only the antibiotic micrococcin[18-21] contains derivatives of thiazole-4-carboxylic acid similar to those found in thiostrepton.[22]

(1) J. F. Pagano, M. J. Weinstein, H. A. Stout, and R. Donovick, *Antibiotics Ann.*, **1955–1956**, 554 (1956).

(2) J. Vandeputte and J. D. Dutcher, *ibid.*, **1955–1956**, 560 (1956).

(3) B. A. Steinberg, W. P. Jambor, and L. O. Suydam, *ibid.*, p. **1955–1956**, 562 (1956).

(4) M. Bodanszky, J. D. Dutcher, and N. J. Williams, *J. Antibiotics* (Tokyo), **16**, 76 (1963).

(5) This antibiotic was studied (in collaboration with the authors) also at the University of Liverpool, England, by the group led by Professor G. W. Kenner (*cf.* ref. 6 and 7).

(6) G. W. Kenner, R. C. Sheppard, and C. E. Stehr, *Tetrahedron Letters*, 23 (1960).

(7) D. F. W. Cross, G. W. Kenner, R. C. Sheppard, and C. E. Stehr, *J. Chem. Soc.*, 2143 (1963).

(8) M. Bodanszky, J. T. Sheehan, J. Fried, N. J. Williams, and C. A. Birkhimer, *J. Am. Chem. Soc.*, **82**, 4747 (1960).

(9) C. N. C. Drey, G. W. Kenner, H. D. Law, R. C. Sheppard, M. Bodanszky, J. Fried, N. J. Williams, and J. T. Sheehan, *ibid.*, **83**, 3906 (1961).

(10) M. Bodanszky, J. Alicino, C. A. Birkhimer, and N. J. Williams, *ibid.*, **84**, 2003 (1962).

(11) E. H. White, F. McCapra, G. F. Field, and W. D. McElroy, *ibid.*, **83**, 2402 (1961); E. H. White, F. McCapra, and G. F. Field, *ibid.*, **85**, 337 (1963).

(12) H. Brockmann, "Progress in the Chemistry of Organic Natural Products," Vol. 18, Springer Verlag, Vienna, 1960, p. 1.

(13) F. W. Eastwood, B. K. Snell, and A. Todd, *J. Chem. Soc.*, 2286 (1960).

(14) R. J. Reedy and C. H. Schaffer, Jr., *Antibiot. Ann.*, 483 (1957).

(15) H. Nishimura, S. Okamoto, M. Mayama, H. Ohtsuka, K. Nakajima, K. Tawakara, M. Shimohira, and N. Shimaoka, *J. Antibiotics* (Tokyo), **14A**, 255 (1961).

(16) H. Nishimura and S. Okamoto, *Ann. Rept. Shionogi Res. Lab.*, **11**, 137 (1961).

(17) H. Nishimura, K. Nakajima, and N. Shimaoka, *ibid.*, **11**, 141 (1961).

(18) P. Brookes, A. T. Fuller, and J. Walker, *J. Chem. Soc.*, 689 (1957).

(19) M. P. V. Mijovic and J. Walker, *ibid.*, 909 (1960).

(20) P. Brookes, R. J. Clark, A. T. Fuller, M. P. V. Mijovic, and J. Walker, *ibid.*, 916 (1960).

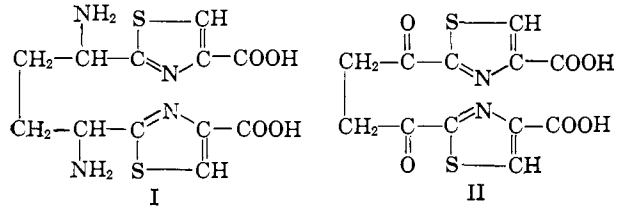
(21) (a) P. Brookes, R. J. Clark, B. Majhofer, M. P. V. Mijovic, and J. Walker, *ibid.*, 925 (1960); (b) M. P. V. Mijovic and J. Walker, *ibid.*, 3381 (1961); (c) B. M. Dean, M. P. V. Mijovic, and J. Walker, *ibid.*, 3394 (1961).

(22) Not only have the reports on the chemistry of Micrococcin P been very helpful in the studies on thiostrepton but our work was also greatly assisted by personal communications and samples of synthetic thiazole-4-carboxylic acid derivatives from Dr. James Walker of the National Institute for Medical Research, London.

Thiazole-4-carboxylic acid itself was shown to be a constituent of the antibiotic althiomycin.[23]

Thiostreptoic Acid.[8]—Thiostrepton was hydrolyzed with a mixture of formic acid and hydrochloric acid.[24] After evaporation of the acid mixture and extraction of the aqueous solution of the residue with 1-butanol, on neutralization of the aqueous layer, a very insoluble compound, which was named thiostreptoic acid, separated in crystalline form. Elemental analysis suggested the formula $C_6H_{6-8}N_2O_2S\cdot H_2O$ and the ultraviolet spectrum, with a maximum at 236 mμ, pointed towards a thiazole derivative. Because of the slow mobility of thiostreptoic acid on paper chromatograms and its marked insolubility in water, it was felt that the correct formula for thiostreptoic acid could be $C_{12}H_{14}N_4O_4S_2\cdot H_2O$. To test this assumption, thiostreptoic acid was treated with somewhat less than 1 equiv. of dinitrofluorobenzene[25] and from the resulting reaction mixture, in addition to some starting material, a mono and a bis-(dinitrophenyl) derivative were isolated by partition chromatography on a cellulose column. The molecular weight of these derivatives was calculated from the intensities of their ultraviolet absorption and from their titration with acid and with alkali. These measurements supported the C_{12} formula. The mass spectrum[26] of diacetylthiostreptoic acid dimethyl ester showed the molecular ion 454 (calcd. mol. wt., 454.5), confirming the C_{12} formula.

Since no C-methyl groups were found on Kuhn–Roth oxidation, and oxidative deamination[18] with permanganate in dilute alkali gave a diketone with no loss in the number of carbon atoms in the molecule, structure I was considered the most likely for thiostreptoic acid, and II for the diketo acid derived from it. The n.m.r.



I II

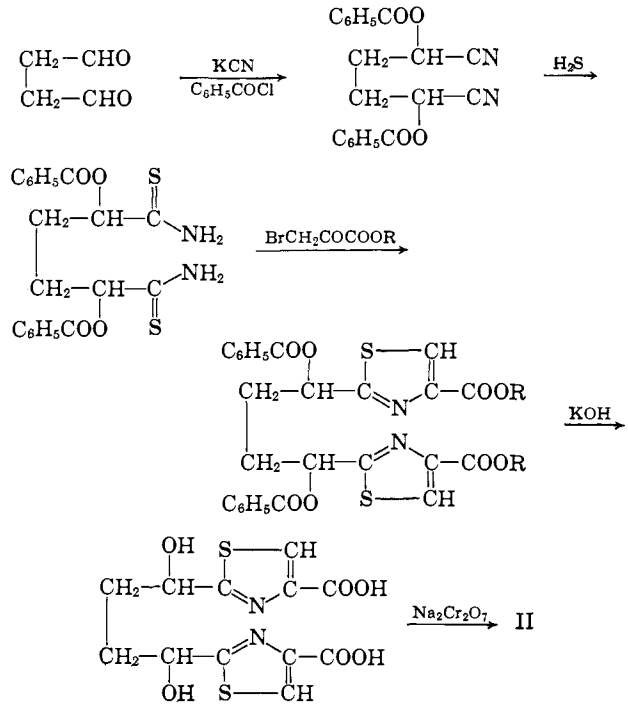
spectrum of thiostreptoic acid in trifluoroacetic acid is in full agreement with structure I; it shows a four-proton peak at τ 7.38, a two-proton peak at τ 4.66, and a sharp two-proton singlet at τ 1.25. The latter corresponds to the aromatic protons of the thiazole rings.

Thiostreptoic acid has no measurable optical activity. This is not too surprising because the asymmetric centers are next to thiazole rings with electron attracting substituents. Therefore, the protons should be easily removed from the asymmetric carbon atoms, leading to racemization. In a symmetrical molecule like thiostreptoic acid, racemization gives a *meso* and a DL species. To avoid complications of this kind, synthesis of the diketo acid II was chosen for the confirmation of structure I.

The diketo acid II was synthesized as shown in Chart I. The synthetic acid was found to be identical in all respects with the diketo acid obtained from thiostrepton.

CHART I

SYNTHESIS OF COMPOUND II[18,27]



Thiostreptoic acid was found to occur in two forms, one decomposing at about 225°, and a second form decomposing at about 308°. The two forms are indistinguishable in behavior on paper chromatograms and by ultraviolet spectra, but show some minor differences in their respective infrared spectra. The question of whether a DL–*meso* relationship is involved here remains open. The yield of this compound is considerably less than would be expected from its stability under the conditions of acid hydrolysis.[28] The question as to the fate of the unaccounted portion of thiostreptoic acid cannot be answered at this time with any great degree of certainty; however, a degradation product was found which probably has its origin in the thiostreptoic acid moiety of the antibiotic. This product separates as a brown amorphous material from acid hydrolysates which are made weakly acidic. It is insoluble in weak but soluble in strong acids and also in alkali. Attempts to crystallize this material failed, and its purification by countercurrent distribution was incomplete even after several thousand transfers. Analysis of the amorphous and probably still impure substance revealed that it contains two sulfur atoms and three nitrogens. One weak basic center and two acidic groups were shown to be present by titration. In the ultraviolet region, this substance exhibits a very characteristic absorption spectrum (Fig. 1) with a maximum at 362 mμ ($E^{1cm}_{1\%}$ *ca.* 500) and shoulders at about 350 and 375 mμ. This compound was designated as the "362 fragment."

(23) D. J. Cram, O. Theander, H. Jager, and M. K. Stanfield, *J. Am. Chem. Soc.*, **85**, 1430 (1963).

(24) C. L. Miller and V. du Vigneaud, *J. Biol. Chem.*, **118**, 101 (1937).

(25) A. R. Battersby and L. C. Craig, *J. Am. Chem. Soc.*, **73**, 1887 (1951); *ibid.*, **74**, 4023 (1952).

(26) We are grateful to Professor Carl Djerassi, Stanford University, for this spectrum.

(27) J. F. Olin and T. B. Johnson, *Rec. trav. chim.*, **50**, 72 (1931).

(28) In constant boiling HCl at 110° thiostreptoic acid remains unchanged except that its dihydrochloride separates in crystalline form. Thiostreptoic acid also forms a salt with sulfuric acid. After hydrolysis of thiostrepton with 60% H_2SO_4 at 110°, an acid sulfate with 4 moles of sulfuric acid per mole of thiostreptoic acid was obtained in crystalline form. The basic character of the thiazole nucleus (or of its nitrogen atom) is best characterized by the isolation of thiazole-4-carboxylic acid oxalate from the oxidation reaction mixture of thiostrepton with concentrated nitric acid. The oxalate not only crystallizes as such, but can be recovered unchanged on sublimation.

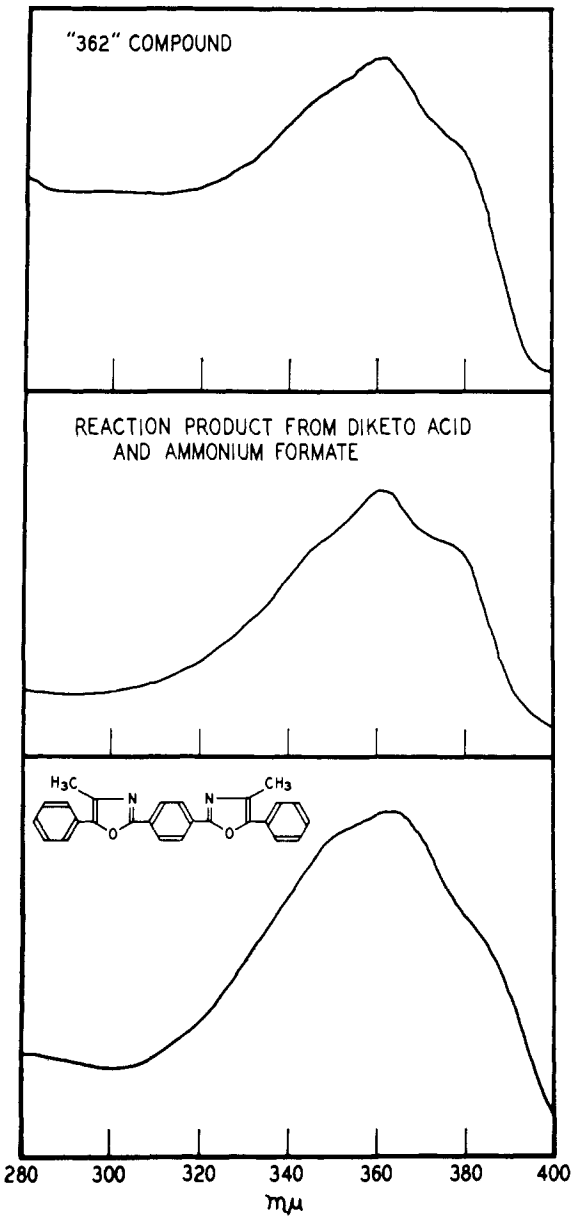
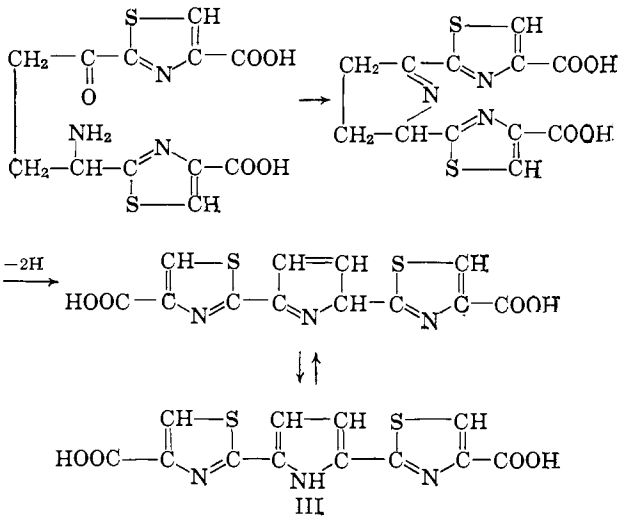


Figure 1.

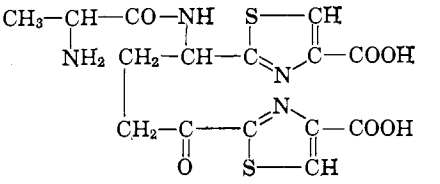
The formation of the diketo acid II from thiostreptoic acid by oxidative deamination suggests that, if the two amino groups are not simultaneously removed, the newly formed keto group and the unchanged amino group could easily form a cyclic Schiff base. The substituted pyrroline, in turn, could lose two hydrogens to form the pyrrole derivative III. That the hypothetical pyrrole derivative could have the ultraviolet absorption spectrum of the "362 fragment" is strongly supported by the ultraviolet spectrum of 1,4-bis[2-(4-methyl-5-phenyloxazoloyl)]benzene, which shows an ultraviolet spectrum (λ_{max} 364 mμ) quite similar to that of the "362 fragment." The latter substance also gives a color test for pyrrole derivatives. The pyrolysis products of a sample turn a strip of newspaper-paper wetted with hydrochloric acid to purple-red. Thiostreptoic acid itself also gives the same color reaction.

Although there is no clear cut evidence that the "362 fragment" has structure III, there can be little doubt about its origin, namely, that it is derived from thiostreptoic acid. Oxidation of the latter with potassium permanganate under mildly alkaline conditions gives,



in addition to the keto acid II, also some by-products with the characteristic spectrum of the "362 fragment". Formation of material with the same ultraviolet absorption pattern was also observed when synthesis of thiostreptoic acid from compound II by the Leuckart–Wallach procedure was attempted (*cf.* Fig. 1).

Additional evidence for the formation of the "362 fragment" from thiostreptoic acid was found in the study of a ninhydrin-positive fragment from a partial acid hydrolysate of the antibiotic. This fragment had an absorption maximum at 290 mμ. (The diketo acid II absorbs at 293 mμ and 2-propionylthiazole-4-carboxylic acid, which will be discussed later, absorbs at 283 mμ.) On completion of the hydrolysis with constant boiling hydrochloric acid at 110°, the "290 fragment" yielded alanine, and the mixture showed the characteristic "362" absorption pattern. This can be interpreted by the assignment of the following structure to the "290 fragment." Removal of the alanyl moiety from



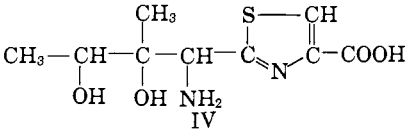
the amino group would allow the ring closure to occur leading to the formation of the "362 fragment."

Thiostreptine.—On hydrolysis with constant boiling hydrochloric acid at 110° for 16 hr., in addition to the amino acids discussed earlier, two more components with positive ninhydrin reaction are liberated from thiostrepton. In a system of 1-butanol–acetic acid–water (4:1:5) one of these travels somewhat faster, the other somewhat slower, than alanine. Both give a yellow color with ninhydrin at room temperature, but the color slowly changes to purple. The slower-moving component is 2-aminomethylthiazole-4-carboxylic acid, which has been already described as a degradation product of thiostrepton by Kenner and his associates.[6] The observation was made that, under the conditions of acid hydrolysis mentioned above, the faster ninhydrin yellow component (from here on, thiostreptine) appears first, and its concentration then slowly decreases. The 2-aminomethylthiazole-4-carboxylic acid appears later, and it reaches maximum concentration at a

time, in about 24 hr., when most of the thiostreptine has already disappeared, indicating that the C_5 acid arises from the degradation of thiostreptine. Subsequently, thiostreptine was isolated from an acid hydrolysate of thiostrepton by countercurrent distribution in a system of 1-butanol–ethanol–0.1% acetic acid (4 : 1 :5). After 1200 transfers, a band with $K = 0.2$ was removed, and the crude thiostreptine was further purified by partition chromatography on a cellulose column in a system of 1-butanol–acetic acid–water (4:1:1). The chromatographically homogeneous amorphous material was still not pure, but any additional purification was hampered by its lack of stability. A salt of thiostreptine with *p*-hydroxyazobenzene-*p*-sulfonic acid has been obtained in crystalline form,[8] but on standing at room temperature after a few weeks, it exhibited on paper chromatograms not the spot corresponding to thiostreptine but that of the C_5 acid. After longer storage, thiostreptine itself shows several spots on paper chromatograms. This lack of stability could explain why some analytical values for thiostreptine itself have been unsatisfactory. These values, nevertheless, pointed towards $C_9H_{14}N_2O_4S$ as the best-fitting formula.[29]

The conversion of thiostreptine to 2-aminomethylthiazole-4-carboxylic acid by hydrolysis, both with acids and alkali, was demonstrated also on isolated material. The ultraviolet spectrum remains practically unchanged during this conversion and, therefore, one has to assume that the reaction occurs outside of the chromophoric system, that is, in the side chain. By subtracting from the formula of thiostreptine ($C_9H_{14}N_2O_4S$) that of 2-aminomethylthiazole-4-carboxylic acid ($C_5H_6N_2O_2S$), the difference, $C_4H_8O_2$, corresponds to acetoin, a compound which was found to be present in the hydrolysis mixture, from which, with the aid of dinitrophenylhydrazine, it was isolated as the osazone. In addition to acetoin, acetaldehyde was also found to be present in the hydrolysis mixture. The antibiotic itself, when treated with dilute alkali, liberated acetaldehyde and acetoin. The former was isolated as the dinitrophenylhydrazone. The acetoin was further identified by oxidation with ferric chloride to diacetyl and conversion of the diacetyl to nickel dimethylglyoxime.

The cleavage of thiostreptine by acid or by base with the formation of acetoin and of 2-aminomethylthiazole-4-carboxylic acid suggests the following structure for thiostreptine.



IV

The validity of structure IV for thiostreptine is supported also by periodate oxidation. Thiostreptine consumes readily 2 moles of the reagent. From the reaction mixture acetaldehyde was removed by distillation and isolated as the dinitrophenylhydrazone. Acetic acid was present in the distillate collected after acidification of the reaction mixture, and extraction of the latter with ether gave crystals of 2-formylthiazole-4-carboxylic acid. The ultraviolet spectrum of this acid (λ_{max} 234 mμ) corresponds to that of the alkyl derivatives of thiazole-4-carboxylic acid, but it is not similar to that of its 2-acyl derivatives. Presumably, a hemiacetal or acetal is formed with the alcohol used as solvent. The stabilization of a hemiacetal by the strong negative group next to the aldehyde carbonyl is a not unreasonable assumption. Moreover, the structure of this compound was confirmed by elemental analysis, and by its mass spectrum,[30] which shows the molecular weight of 157, and finally by its n.m.r. spectrum in acetonitrile [only an aldehydic proton (τ 0.83) and an aromatic proton (τ 2.15) were demonstrable].

The n.m.r. spectrum of thiostreptine in trifluoroacetic acid shows a three-proton singlet at τ 8.7 corresponding to the 2′-methyl group, and a three-proton doublet centered at τ 8.4 ($J = 6$ c.p.s.) of the ω-methyl group of the side chain. Single protons were found at τ 6.4 (3′-H) and 4.4 (1′-H). The aromatic proton appears at τ 1.2. This spectrum is in excellent harmony with the proposed structure (IV) of thiostreptine.

2-Propionylthiazole-4-carboxylic Acid.—This compound was isolated from acid hydrolysates of the antibiotic (*cf.* ref. 8, footnote 15) and was identified by a comparison with an authentic sample obtained from Dr. Walker.[18] However, while this acid has been isolated from micrococcin P in good yield, only a small amount of it was obtained from thiostrepton. This suggests that in the latter it is not an original building component but rather a secondary degradation product.[31]

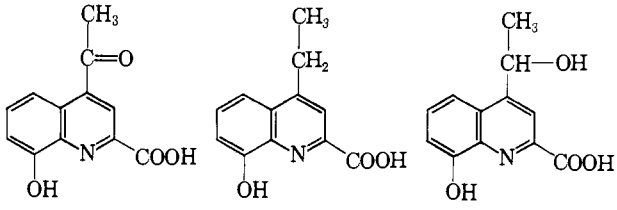
Derivatives of 8-Hydroxyquinaldic Acid.—Thiostrepton was hydrolyzed with boiling *N* hydrochloric acid for 24 hr. Extraction of the solution with ether gave a yellow, crystalline acid (m.p. 190–200°). Its absorption spectrum in the ultraviolet with maxima at 265 and 375 mμ suggested a derivative of 5- or 8-hydroxyquinoline. The analytical values agreed with those calculated for $C_{12}H_9NO_4$. The presence of a phenolic hydroxyl was indicated by the intense green color with ferric chloride and the formation of a chloroform-soluble copper complex pointed towards an 8-hydroxyquinoline derivative. The acid was easily decarboxylated on heating and the decarboxylation product was shown by superimposable infrared spectra to be identical with one of the phenolic products obtained by Kenner and his associates[9] from the pyrolysis of thiostrepton. The low melting point of the decarboxylation product, its solubility in hexane, and its ability to form a chloroform-soluble copper complex can all be reconciled with a quinoline having an hydroxyl group in the 8-position. In view of the ready decarboxylation of the acid, position 2 is the most likely for the carboxyl group. The presence of a carbonyl group (in addition to the carbonyl of the carboxyl) was shown by the infrared spectrum (λ_{max}^{Nujol} 1700 cm.$^{-1}$), and this was confirmed by the formation of a crystalline dinitrophenylhydrazone. Finally, a positive iodoform test and the n.m.r. spectrum of the methyl ether-methyl ester of the acid taken in deuteriochloroform, disclosing no aldehydic proton but a three-proton singlet at τ 7.45, clearly established the presence of a CH_3CO grouping. That this side

(29) Since its original isolation, thiostreptine was secured again, this time as the analytically pure monohydrochloride, and a sample of the latter crystallized from ethanol in the form of thin platelets.

(30) We express our gratitude to Professor Klaus Biemann, Massachusetts Institute of Technology, for this mass spectrum and its interpretation.

(31) Formation of a 2-acylthiazole-4-carboxylic acid from a 2-(α-aminoalkyl)thiazoline-4-carboxylic acid occurs during degradation of bacitracin A [J. R. Weisiger, W. Hausmann, and L. C. Craig, *J. Am. Chem. Soc.*, **77**, 3123 (1955)].

chain is located at position 4 of the quinoline nucleus was shown by oxidative degradation with hot concentrated nitric acid. The oxidation product gave a color reaction with ferrous sulfate which is characteristic for 2,3,4,6-pyridinetetracarboxylic acid, and this acid on decarboxylation formed a mixture of nicotinic, isonicotinic, and cinchomeronic acids. An identical mixture was obtained from cinchomeronic acid but from no other isomeric pyridinedicarboxylic acid. When in place of the quinoline keto acids its decarboxylation product was oxidized with nitric acid, the color reaction of the product with ferrous sulfate corresponded to that of 2,3,4-pyridinetricarboxylic acid; decarboxylation of this acid gave the same mixture of pyridinecarboxylic acids as mentioned above. The only structure compatible with these findings is V.



The mother liquors from the crystallization of V yielded a second quinaldic acid which lacked the ketonic carbonyl group of V, but otherwise was found to be very much like V, yielding the same products upon oxidation. This second acid on decarboxylation yields an 8-hydroxyquinoline derivative, identical with a second pyrolysis product from thiostrepton.[9] From the analytical values the formula $C_{12}H_{11}NO_3$ could be calculated for the second acid; hence, the structure of 4-ethyl-8-hydroxyquinaldic acid (VI) was assigned to this acid.

At first, it was felt that in V and VI two genuine building stones of thiostrepton had been recognized. However, when instead of *N* hydrochloric acid, a mixture of hydrochloric acid and formic acid was used for hydrolysis of the antibiotic, a third quinaldic acid, an optically active compound VII was obtained in fair yield. Compound VII differs from V by having two more hydrogen atoms. It has the molecular formula $C_{12}H_{11}NO_4$, it lacks the ketone function, but has an alcoholic OH as shown by the infrared spectrum. Jones reagent[32] oxidizes the methyl ester-methyl ether of compound VII to the corresponding derivative of keto acid V; therefore, VII is 4-(α-hydroxyethyl)-8-hydroxyquinaldic acid.

That at least the side-chain portion of VII occurs as such in the antibiotic is shown by the optical activity of VII. The question whether, in addition to the acid VII, the acids V and VI are present in thiostrepton could be resolved when it was shown that VII undergoes disproportionation to V and VI on heating in 1 *N* hydrochloric acid. Similar disproportionations are not unknown.[33] The disproportionation proceeds also in boiling acetic acid; it is retarded, however, by strong acids, and this could be the reason for the improved yield of the carbinol acid VII when concentrated acids rather than dilute ones were used in the hydrolysis.

The disproportionation reaction itself does not exclude the possibility that, in addition to VII, also V and VI, or one of them, occurs as such in the antibiotic, but the intensity of the ultraviolet absorption spectrum of thiostrepton in absolute ethanol cannot accommodate more than one of the quinaldic acids. This still would permit the occurrence of more than one mole of quinaldic acid in hydrolysates if the quinaldic acids do not occur as such, but are formed by rearrangement reactions during hydrolysis. Evidence for the absence of V in the molecule was obtained from the n.m.r. spectrum of thiostrepton in deuteriochloroform. This spectrum showed no signal at τ 7.45, where the characteristic CH_3 protons of V appear.

The n.m.r. spectrum of the methyl ester-methyl ether of VII is in full agreement with the proposed structure. Compounds V and VI have been synthesized by Professor Kenner's group[9]; compound VI was also prepared in our laboratory, albeit in low yield, from 4-ethyl-8-methoxyquinoline by the Reissert reaction.[34]

While both V and VI could be excluded as structural units of thiostrepton, compound VII was considered to be one of the moieties in the antibiotic. Yet even this view finally had to be abandoned, for the following reasons.

Thiostrepton gives an intense purple color reaction with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent). This color reaction is not due to tryptophan. The color develops much slower, has a different shade, and persists much longer than in the case of tryptophan. After hydrolysis with constant boiling hydrochloric acid at 110° for 16 hr., none of the liberated fragments gave a similar color reaction. Using milder conditions for hydrolysis, such as concentrated hydrochloric acid at room temperature, a slow fragmentation of thiostrepton can be observed by paper chromatography and, initially, several of these fragments give a positive Ehrlich reaction. Incidentally, both the parent molecule and the Ehrlich-positive fragments which are formed under the last-mentioned conditions also give a positive reaction with diazotized sulfanilic acid, and an even better reaction with *p*-bromobenzenediazonium chloride. This Pauly color reaction, an intense cherry-red color, appears as soon as the chromatograms are sprayed with the solution of diazonium salt and before spraying with sodium carbonate. This coupling in acid solution suggests an aromatic amine. After standing for about 1 week at room temperature, the reaction mixture contains neither Ehrlich- nor Pauly-positive fragments, except that a component can now be detected with positive Pauly reaction after spraying with sodium carbonate. This component can be shown to be VII. Even though the color reaction which indicates the presence of an indole derivative becomes negative after about 1 week in concentrated hydrochloric acid, an absorption maximum in the ultraviolet at around 285 mμ remains unchanged for several months. After standing for 3 months, a portion of this reaction mixture was diluted with water and extracted with ether, with ethyl acetate, and finally

(32) R. G. Curtis, I. Heilbron, E. R. H. Jones, and G. F. Woods, *J. Chem. Soc.*, 457 (1953).

(33) *E.g.*, H. Burton and G. W. H. Cheeseman, *J. Chem. Soc.*, 986 (1953); *cf.* P. D. Bartlett and J. D. McCollum, *J. Am. Chem. Soc.*, **78**, 1441 (1956).

(34) A. Reissert, *Ber.*, **38**, 1603 (1905); R. B. Woodward, *J. Am. Chem. Soc.*, **62**, 1626 (1940).

with 1-butanol. The first two solvents removed the quinaldic acid present and the "362 fragment"; the butanol extracts contained a substance with characteristic maxima at 283, 290, and 303 mμ. From the optical density of subsequent butanol extracts, measured at 283 mμ, a distribution coefficient $K = 0.86$ was calculated for this fragment in the system 1-butanol–1 *N* HCl. This K value was found to be remarkably reproducible; therefore, it was surprising to find that on an attempted isolation of this chromophore by countercurrent distribution in the similar solvent system 1-butanol–0.1 *N* HCl, only a fraction of the material with maxima around 283 mμ was found, as expected, in the center part of the train; the major portion of it traveled near the front with a K value of about 10. Even in the slower band, with K around 1, lack of equilibrium was evident, since the upper phase was much richer in the absorbing material than the lower phase. Evaporation *in vacuo* below room temperature of the butanol layers, both from the band with $K = ca.$ 1 and from the band with $K = ca.$ 10, gave residues which showed very little indole-type absorption but exhibited clearly the very characteristic quinaldic acid spectrum. These transformations can be described in the following terms: the original "indole" in thiostrepton, which gives an unusual Ehrlich reaction and a positive Pauly reaction even in acid media, is slowly converted by concentrated hydrochloric acid at room temperature to "indole A" which does not give these two color reactions and which has a K value of 0.86 in the system of butanol–dilute hydrochloric acid. On further standing in dilute acid, "indole A" is transformed into "indole B" with a K value of about 10. Evaporation, or treatment with hot constant boiling hydrochloric acid, converts "indole B" into the quinaldic acid VII.

Although no definite evidence has been found so far for the nature of the hypothetical "indole" in thiostrepton, it is quite clear that it is the precursor of VII in the degradation procedure.[35]

Composition and Molecular Weight.—Thiostrepton has no free amino or carboxyl groups. As a peptide, it is apparently cyclic and, therefore, the determination of its molecular weight was not possible by the titration of an ionizable group. Under anhydrous conditions in glacial acetic acid the antibiotic can be titrated with perchloric acid, but the equivalent weight thus determined, 512 or 370 in the presence of mercuric chloride, does not permit a simple interpretation. Accurate elemental analysis of the antibiotic is greatly hampered by the capacity of the molecule not only to retain solvents tenaciously, but, following careful drying, to absorb moisture from the air. This causes variations in analytical values; *e.g.*, the sulfur content can vary from 9.2–10.2%. The average value, 9.7%, permits molecular weights which are multiples of 330. Of the sulfur-containing components described in the present paper, only three, thiostreptoic acid, thiostreptine, and cysteine, are considered to be present as structural units, while the other sulfur-containing products are probably formed from these three by secondary reactions. As thiostreptoic acid contains two sulfur atoms, thiostreptine and cysteine one each, a total of four sulfur atoms could be assumed for the molecule. On the other hand, the molecular weight as calculated from the data of quantitative amino acid analysis indicates that an S_5 formula is the correct one, and this view is further supported by X-ray measurements on crystals of thiostrepton.[36] The formula $C_{72}H_{83}N_{19}O_{17}S_5$ comes closest to the values of elemental analysis. A final formula for thiostrepton can be expected only at the completion of the structural elucidation.

The Sequence of Structural Units.—Several attempts were made to isolate products of partial hydrolysis in the hope of obtaining information on the sequence of the building stones of thiostrepton. Rearrangements, secondary degradations which occur concomitantly with the fragmentation of the molecule, seriously hamper such experiments; consequently, only a limited amount of information has become available from partial hydrolysis.

Evidence for the alanylthiostreptoic acid sequence has been mentioned earlier in connection with thiostreptoic acid. It has to be noted that in the same partial hydrolysate not one, but two, ninhydrin-positive fragments were found, chromatographically different, one with a maximum at 287, the other with a maximum at 290 mμ. Both gave on further hydrolysis the ultraviolet absorption pattern of the "362 fragment" and both had alanine as the N-terminal acid as shown by dinitrophenylation followed by hydrolysis. No explanation has been found so far for the difference in the two fragments.

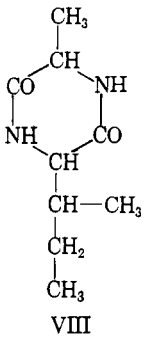
Another degradation product, absorbing in the ultraviolet but ninhydrin-negative, became ninhydrin-positive upon completion of the hydrolysis. The only amino acid liberated was alanine. The ultraviolet spectrum is that of the 8-hydroxyquinaldic acids. This finding points toward a quinaldyl–alanine (or rather "indole"–alanine) sequence in thiostrepton, and the two partial sequences can be combined as "indole"–alanine–thiostreptoic acid partial structure. The fact that there is also a second alanine in thiostrepton has not been overlooked, but as the second alanine can be found in a different part of the molecule the above combination of two "dipeptide" sequences into a "tripeptide" sequence seems to be justified.

A fragment containing isoleucine, alanine, threonine, and cysteine, and another fragment with alanine, threonine, cysteine, and thiostreptine were isolated from partial hydrolysates by preparative paper chromatography. A third fragment was isolated in crystalline form and was also shown by comparison with a synthetic sample to be isoleucylalanine. These three peptides indicate the sequence isoleucylalanylthreonylcysteinylthiostreptine. Since only a fraction of a mole of cysteine is found, on quantitative amino acid analysis, in thiostrepton, and disulfide and mercapto groups are absent, the cysteine must be present in some nonconventional form, perhaps as a thiazoline of threonylcysteine. The isoleucylalanine sequence, too, is not quite free of complications. In addition to this dipeptide, the diketopiperazine VIII, containing isoleucine and alanine residues, was isolated not only after

(35) A ring enlargement of an indole yielding a quinoline has been elegantly demonstrated by E. E. van Tamelen and V. B. Haarstad [*Tetrahedron Letters*, 390 (1960)] in the conversion of α-methyltryptophan into 4-acetylquinaldic acid. It is difficult to visualize a similar rearrangement in thiostrepton because VII is optically active.

(36) Unpublished report from Dr. Kenneth Watson, Chemical Crystallography Laboratory, University of Oxford.

pyrolysis of the antibiotic (ref. 8, footnote 6) but also on heating a solution of thiostrepton in glacial acetic



VIII

acid. The same diketopiperazine also forms, under similar conditions, *e.g.*, hot acetic acid, from synthetic L-isoleucyl-L-alanine, but the ring closure could be prevented by the addition of a proton donor (*p*-toluenesulfonic acid). With thiostrepton, even an excess of *p*-toluenesulfonic acid did not prevent the formation of VIII in hot acetic acid. Therefore, the diketopiperazine ring structure must already be present as such in the molecule of the antibiotic. That this is not the only point of similarity between thiostrepton and the ergot alkaloids will be seen below.[37] Since the carboxyl group of the alanyl moiety has to be linked, not only to the isoleucyl residue, but also to the threonine-containing part of the sequence, a cyclol structure can also be considered.

Pyruvic Acid, Ammonia, and N.m.r. Spectra.—Although thiostrepton is quite soluble in $CDCl_3$ and n.m.r. spectra were taken also in this solvent, several of the degradation products, especially thiostreptoic acid and thiostreptine, are insoluble in $CDCl_3$ and trifluoroacetic acid was used as solvent for these highly polar compounds. In order to correlate the spectra of the thiazole derivatives with that of the parent antibiotic, the n.m.r. spectrum of thiostrepton in trifluoroacetic acid was required. The observation was made that, in this solvent, a peak at τ 7.39, originally absent immediately after the solution of thiostrepton was prepared, gradually emerges. This newly formed singlet reaches its maximum in about a day, 6 hr. being necessary for half-conversion at room temperature. An examination of the n.m.r. spectra in trifluoroacetic acid of a number of methyl ketones revealed that pyruvic acid is the most likely compound to have been formed by the action of trifluoroacetic acid on thiostrepton. The same emergence of a strong signal corresponding to the methyl group of a pyruvyl moiety was observed also in a solution of thiostrepton in concentrated hydrochloric acid, and subsequently pyruvic acid was isolated in the form of its dinitrophenylhydrazone from acid hydrolysates of the antibiotic. The amount of the isolated dinitrophenylhydrazone, and the area under the peak at τ 7.39, suggest that at least 2 moles of pyruvic acid are liberated from 1 mole of thiostrepton.

(37) Siomycin (ref. 15–17) contains no isoleucine but has a valine residue. An additional difference is that only one mole of alanine is present in its molecule. It is interesting to speculate that the two antibiotics may be in a similar relation to each other as are ergosecalin [M. Abe, T. Yamano, S. Yamatodani, Y. Kozo, M. Kusumoto, H. Komatsu, and S. Yamada, *Bull. Agr. Chem. Soc. Japan*, **23**, 246 (1959)]; *cf.* J. H. Bi.kinshaw and C. E. Stickings, "Progress in the Chemistry of Organic Natural Products," Vol. 20, Springer Verlag, Vienna, 1962, p. 18, and, *e.g.*, ergotamine. The diketopiperazine partial structure of the latter is replaced by valine in the former.

It would be convenient to assume that acylated α,α-diaminopropionic acids participate in the structure of the antibiotic, since such an assumption would help to explain the liberation of 4 to 5 moles of ammonia during hydrolysis of thiostrepton. However, a study of the stability of α,α-diacetylaminopropionic acid, of α-acetylaminoacrylic acid, and of α-hydroxyl-α-acetylaminopropionic acid (a complex with 2 moles of acetamide) in trifluoroacetic acid showed that only the last-mentioned compound forms pyruvic acid at room temperature.

Even though the formation of pyruvic acid from thiostrepton is still not well understood, this fact suggests a certain similarity between the structure of this antibiotic and those of the ergot alkaloids.

Beyond the formation of pyruvic acid, not too much information was gained from the n.m.r. spectrum of thiostrepton taken in trifluoroacetic acid. On the other hand, the use of fully deuterated acetic acid, CD_3COOD, as a solvent, leads also to simplified n.m.r. spectra, since all the easily exchangeable protons of the antibiotic are replaced by deuterium in this solvent. The observed chemical shifts are about the same in CD_3COOD as in $CDCl_3$, while CF_3COOH leads to substantial changes in chemical shifts. An additional advantage of CD_3COOD is that the polar components, like threonine, alanine, isoleucine, etc., are also sufficiently soluble in it to allow the recording of n.m.r. spectra, and in the large complex peak of the aliphatic region (τ 7.9 to 9.3) of thiostrepton it is possible to recognize the contributions from the methyl groups of the amino acids just mentioned. The presence of three sharp one-proton singlets at τ 1.70, 1.76, and 1.80 is even more interesting. The aromatic proton of the 2-substituted thiazole-4-carboxylic acids appears at such low τ-values, but the proton on C-3 of the quinaldic acids also appears there. As only three such protons are shown in the n.m.r. spectrum, and thiostreptoic acid has two and thiostreptine has one aromatic proton, the spectrum can be considered in agreement with evidence presented earlier that the quinaldic acid VII must be a secondary degradation product.

Conclusion.—The information so far accumulated is sufficient only to describe the building stones of thiostrepton, and even one of these is known at the present only in a rearranged form. Tentative proposals for some partial sequences have been made, but the structure of this antibiotic as yet cannot be drawn.

Experimental

Melting points were taken in capillary tubes and are uncorrected. Paper chromatograms, except when stated otherwise, were run in 1-butanol–acetic acid–water (4:1:5). For ultraviolet spectra, 95% ethanol was used as solvent.

Thiostrepton.—For analysis thiostrepton was dissolved in hot chloroform, the solution was concentrated to a small volume, and 95% ethanol was added until crystallization started. After several such recrystallizations, the material travels as a single component in countercurrent distribution in a system of toluene–chloroform–methanol–water (5:5:8:2). Before analysis, samples were dried at 110° *in vacuo* for several hours and were kept protected from moist air before combustion. *Anal.* Found: C, 52.15, 52.67; H, 5.2, 5.2; N, 16.1; S, 9.9, 9.5. $C_{72}H_{83}N_{18}O_{17}S_6$ requires C, 52.51; H, 5.08; N, 16.08; S, 9.72. For the quantitative amino acid analysis, exactly weighed samples of about 2 mg. were hydrolyzed at 110° for 16 hr with constant boiling hydrochloric acid (about 0.5 ml.) in evacuated, sealed ampoules. No significant difference was found when the time of hydrolysis was extended to 24 or 48 hr. The average of six determinations

shows that from 1 mg. of thiostrepton the following amounts of amino acids (in μmoles) are liberated: threonine, 0.51; alanine, 1.14; cystine, 0.12; isoleucine, 0.555; 2-aminomethylthiazole-4-carboxylic acid, 0.27; and ammonia, 2.44. The recordings show also the presence of thiostreptine, thiostreptoic acid, and a trace of glycine. Assuming quantitative recoveries, from the isoleucine content a molecular weight of 1800 could be calculated; as recoveries of amino acids have to be less than 100%, the molecular weight is lower than 1800.

L-Isoleucine, L-Alanine, L-Threonine, Ammonia, and Thiostreptoic Acid.—To a solution of thiostrepton (1.0 g.) in formic acid (20 ml.) concentrated hydrochloric acid (20 ml.) was added, and the solution was heated on a steam bath for 22 hr. The mixture was evaporated to dryness, water (10 ml.) was added and re-evaporated, and this was repeated once more. The solution of the residue in the lower phase (50 ml.) of a 1-butanol–5% hydrochloric acid system was placed into the first five tubes of a countercurrent distribution apparatus and distributed, using 10-ml. phases, for 100 transfers. The contents of tubes with similar patterns on paper chromatograms were pooled, and the solvents were removed on a steam bath. A 75% aliquot from the band of tubes no. 33–59 was chromatographed on a Whatman No. 1 cellulose powder column, 29 cm. high and 4 cm. in diameter, using 70% ethanol as eluent. After 190 ml. of eluent was collected, isoleucine was detected in the eluate up to an effluent volume of 330 ml. Evaporation of this fraction left a yellow semicrystalline residue (150 mg.) which was dissolved in a few milliliters of water, filtered, and reevaporated. The still impure, colored residue (98 mg.) has $[\alpha]^{23}D$ +17° (*c* 1, 6 *N* HCl). Recrystallization from dilute ethanol by the addition of ether gave crystals (22 mg.) having $[\alpha]^{24}D$ +32°, and an infrared spectrum (Nujol) indistinguishable from that of authentic isoleucine.

The contents of tubes no. 0–24 of the above distribution were pooled and evaporated, and a 75% aliquot of the residue was chromatographed on a cellulose column as described for isoleucine. From a fraction of elution volume of 175 to 225 ml., a residue (363 mg.) was obtained, which when triturated with ethanol–ether gave an insoluble material (76 mg.). This material on reprecipitation from dilute ethanol with ether afforded a white crystalline solid (71 mg.) which was shown to be ammonium chloride. The soluble portion was fractionated on paper chromatograms using Whatman No. 3MM sheets. Two ninhydrin-positive bands were eluted and further purified by curtain electrophoresis in normal acetic acid. Three fractions were separated, one containing crude alanine, a second containing a mixture of alanine and threonine, and a third with impure threonine. From the alanine fraction on evaporation, a small amount of a solid was left with $[\alpha]^{24}D$ +11° (*c* 1, 1 *N* HCl) and an infrared spectrum which was found to be identical with that of authentic alanine. The third fraction left a residue too impure for direct identification; however, the second fraction when rechromatographed on Whatman No. 3 MM sheets gave, after elution of the two ninhydrin-positive bands, alanine (6 mg.) and threonine (11 mg.), $[\alpha]^{24}D$ −10° (*c* 1, H_2O). The infrared spectrum was used here again to confirm the identity of the latter. The slowest moving fraction (tubes no. 0–14) of the 100-transfer distribution left, on evaporation, a residue which was dissolved in water (8 ml.), and the solution was adjusted first to pH 8 with triethylamine and finally to pH 5.5 with acetic acid. On standing in the refrigerator, the solution deposited needles. The crystals were filtered, washed successively with water and with ethanol, and were then air-dried (wt. 18 mg.). By dissolving the material in dilute ammonium hydroxide and adjusting the pH to 6 with acetic acid and repeating the above procedure, a purified material (5 mg.) was obtained, which was optically inactive (in 1 *N* hydrochloric acid) and gave a single spot with ninhydrin, R_f 0.1. This material was named thiostreptoic acid (I).

Preparation of Thiostreptoic Acid (I). Isolation of D-Cystine. A solution of thiostrepton (2 g.) in constant boiling hydrochloric acid (40 ml.) was kept at 105–110° for 24 hr. After cooling to room temperature, the solution was diluted with water (160 ml.) and extracted with ten 100-ml. portions of water-saturated 1-butanol. The aqueous layer was evaporated on a steam bath. The residue was dissolved in a few milliliters of water and evaporated; when redissolved in water (4 ml.), the solution was made slightly alkaline by the addition of 1 *N* ammonium hydroxide. On readjusting the pH to about 6 with acetic acid and seeding with thiostreptoic acid, crystallization of beautiful needles started. After a few hours, the crystals were collected, washed with water and with ethanol, and finally dried over phosphorus pentoxide *in vacuo* at room temperature. Chromatographically pure I (30 mg.) was obtained. On drying *in vacuo* at 110° a weight loss of 10.7% was observed (2 moles of water require 9.5%).

Anal. Found: C, 42.48; H, 4.21; N, 16.39; S, 18.86. $C_{12}H_{14}N_4O_4S_2$ requires C, 42.10; H, 4.11; N, 16.36; S, 18.73.

Thiostreptoic acid when first isolated melted with decomposition at 235–237°; a second preparation obtained in a similar way decomposed at 225° (12% loss of weight on drying at 110°). A second crop of thiostreptoic acid isolated from the mother liquor of this preparation melted with decomposition at 308°. Finally, in some subsequent preparations, the main crop had the higher (308°) decomposition point.

When ethanol was added, the mother liquors of thiostreptoic acid gave a second precipitate. After standing at room temperature for 2 weeks, this material was collected by centrifugation, and was purified by precipitation from a solution of dilute ammonium hydroxide with acetic acid at pH 6. On repetition of this purification step, the slow formation of crystals (not needles) was observed. The crystals were washed with water and ethanol, and dried. This material (7.4 mg. from 2 g. of the antibiotic) was identified as cystine by a comparison with an authentic sample using both paper chromatograms and infrared spectra; its $[\alpha]^{22}D$ +180° (*c* 0.33, 1 *N* HCl) shows that it is of the D configuration. The paper chromatograms of thiostrepton hydrolysates reveal that both cystine and cysteine are present in the mixture.

Thiostreptoic Acid Hydrogen Sulfate. A.—A solution of thiostrepton (2 g.) in a mixture of concentrated sulfuric acid (37 ml.) and water (33 ml.) was heated to 110° for 20 hr., while nitrogen was passed through the solution and then into a trap of barium hydroxide solution. Titration of this solution showed that about 1.5 moles of carbon dioxide was formed from 2 g. (about 1.7 g., dry wt.) of thiostrepton. The reaction mixture was cooled to room temperature, extracted with ether, and set aside for several weeks. The crystals which deposited were collected by centrifugation, washed with acetone, and dried over phosphorus pentoxide *in vacuo* at room temperature. The practically pure material weighed 0.20 g. A part (113 mg.) of this product was dissolved in water (1 ml.), concentrated ammonium hydroxide solution (0.2 ml.) was added, and a few drops of acetic acid brought the pH to 6. The crystals which formed were collected, washed with water and ethanol, and dried. The product (50 mg.) darkened at 280° and melted with decomposition at 305–308°.

Anal. Found: C, 42.23; H, 4.80; N, 16.65; S, 18.69; loss of weight on drying *in vacuo* at 130°, 10.65%.

B.—To a suspension of thiostreptoic acid (21 mg.) in water (0.5 ml.) an equal volume of concentrated sulfuric acid was added. Thiostreptoic acid dissolved on the addition of the first drop of sulfuric acid, but crystals appeared soon after the addition of all the acid. The crystals were collected, washed with acetone, and dried over phosphorus pentoxide *in vacuo* at room temperature overnight, giving 39 mg. (calculated for a salt of thiostreptoic acid with 4 moles of sulfuric acid, 41 mg.) of material which darkened at 250° and melted with decomposition at 270°.

Anal. Calcd. for $C_{12}H_{22}N_4O_{20}S_6$: C, 19.61; H, 3.02; N, 7.63; S, 26.19. Found: C, 19.23; H, 3.34; N, 7.53; S, 25.85.

Dinitrophenyl Derivatives of Thiostreptoic Acid.—To a solution of I (1.9 mg.) in water (0.5 ml.) containing 2% triethylamine, an ethanolic solution of dinitrofluorobenzene (5 mg. in 0.5 ml.) was added. After 2 hr. at room temperature, additional 2% triethylamine solution (0.25 ml.) and water (2 ml.) were added, and the mixture was extracted with three 3-ml. portions of ether. The residue from the evaporation of the aqueous solution was chromatographed on Whatman No. 1 paper in a system of 1-butanol–acetic acid–water (4:1:1). Two yellow spots appeared, one with R_f 0.8, a second with R_f 0.4, the latter being also ninhydrin-positive; a third spot became visible only after spraying with ninhydrin, R_f 0.3. Presumably the fastest spot, yellow, ninhydrin-negative, is that of the bisdinitrophenyl derivative; the slower yellow spot which is stained purple by ninhydrin corresponds to the monodinitrophenyl derivative, while the slowest, colorless, but ninhydrin-positive, spot is unchanged starting material (I). The experiment was repeated using a larger amount (40 mg.) of I in 2% triethylamine solution (5 ml.) and dinitrofluorobenzene (20 mg.) in ethanol (5 ml.). The products were chromatographed on a Whatman No. 1 cellulose powder column (200 ml.) in the system used for the paper chromatogram just mentioned; 10-ml. fractions were taken. Yellow eluates were found in fractions no. 14–21 with a clear minimum of color

at 16–17. Evaporation of fractions 14–16 gave the bisdinitrophenyl derivative, while from fractions 17–21 the monosubstituted derivative of I was isolated. This latter, in 95% ethanol containing 10% acetic acid, had λ_{max} 337 mμ ($E^{1\%}_{1\,cm}$ 241); by comparison with the corresponding value of dinitrophenylglycine under the same conditions ($E^{1\%}_{1\,cm}$ 475), the molecular weight of 475 (calcd. 508) was found for the dinitrophenyl I. The neutralization equivalent, when titrated with $HClO_4$, was found to be 257; when titrated with $KOCH_3$, the neut. equiv. was found to be 539.

Diacetylthiostreptoic Acid.—To a suspension of I (lower melting form, 20 mg.) in H_2O (2 ml.), triethylamine (0.125 ml.) was added and the solution was treated with acetic anhydride (0.125 ml.). The same amounts of triethylamine and acetic anhydride were added again, and after short standing the solution was acidified with 5 *N* hydrochloric acid (0.5 ml.). Diacetyl-I slowly separated in nice crystals, which were collected after 2 days, washed with water, and dried. The product (20 mg.) melted at 275–277° dec. On drying at 110° it lost 10.4% of its weight (calcd. for $3H_2O$, 11.2%).

Anal. Calcd. for $C_{16}H_{18}N_4O_6S_2$: C, 45.06; H, 4.25; S, 15.04. Found: C, 45.33; H, 4.38; S, 15.1.

Diacetylthiostreptoic Acid Dimethyl Ester.—To a solution of diacetyl-I (50 mg.) in methanol (25 ml.), an ethereal solution of diazomethane was added to permanent yellow color; soon, crystals appeared. The solvents were removed and the residue was washed with methanol. The crystals, rhombic platelets (36 mg.), darkened at 265° and decomposed at 295–298°. A sample of this material, after being sublimed at 0.15 mm. and 290°, gave in its mass spectrum a peak at mass 454 (mol. wt. 454.5). In the n.m.r. spectrum in CD_3COOD, the aromatic protons of the diester appear as a sharp singlet at τ 1.80.

Oxidation of I to the Diketo Acid II.—Compound I (38 mg.) was dissolved in water by the addition of normal sodium hydroxide (0.5 ml.), and a 2% solution of potassium permanganate in water was added in small portions until a pink color persisted. A total of 3.3 ml. was necessary for this. During the oxidation, the liberation of ammonia could be detected. On addition of 2 *N* sulfuric acid (0.5 ml.) and a small amount of sodium bisulfite, a clear solution was obtained from which rectangular plates deposited slowly. The crystals were collected, washed with water, and dried at 70°. The acid (14 mg.) decomposed on the hot stage at 280–290°, $\lambda^{95\%\ alc.}_{max}$ 293 mμ ($E^{1\%}_{1\,cm}$ 690). Solutions of II give an intense orange color with sodium hydroxide.

Anal. Calcd. for $C_{12}H_8N_2O_6S_2$: C, 42.35; H, 2.37; S, 18.84. Found: C, 42.45; H, 2.19; S, 18.83.

Ether extraction of the mother liquors of II gave a material (14.5 mg.) which in 95% ethanol showed a maximum at 360 mμ with shoulders at 345 and 375 mμ.

Synthesis of Compound II. A. α,δ-Dibenzoyloxyadipic Acid Dinitrile.—A mixture of succindialdehyde (30 g.), crushed ice (750 g.), potassium cyanide (44 g.), and benzoyl chloride (78 ml.) was stirred for 1 hr. The mixture was filtered and the solid was washed with water. Extraction of this product (15 g.) with ether and recrystallization of the ether-insoluble material from hot dimethylformamide gave a crystalline product (3.5 g.), m.p. 214–216°.

Anal. Calcd. for $C_{20}H_{16}N_2O_4$: C, 68.96; H, 4.63; N, 8.04. Found: C, 68.96; H, 4.81; N, 8.10.

Filtrates and washings from the above mixture were combined and extracted with chloroform; the organic layer was washed with dilute potassium bicarbonate then with water, dried, and evaporated. Trituration of the residue with hexane left a second product (19 g.) which was extracted with ether, and the ether-insoluble portion was recrystallized from hot toluene to give the lower melting (134–136°) form (5.0 g.) of the dinitrile.

Anal. Found: C, 68.89; H, 4.60; N, 7.93.

B. α,δ-Dibenzoyloxyadipic Acid Dithioamide.—Into a solution of the dinitrile (4.6 g., m.p. 134–136°) in absolute ethanol (25 ml. and dimethylformamide (25 ml.) containing triethanolamine (0.3 ml.), H_2S was passed for 6 hr. The flask was stoppered and left to stand at room temperature for 3 days. Hexane was then added to the mixture, followed by enough ethanol to give a homogeneous system. On standing, a yellow solid (4.0 g.) separated, and was recrystallized from ethanol to give a purified product (2.55 g.), m.p. 203–205°.

Anal. Calcd. for $C_{20}H_{20}N_2O_4S_2$: N, 6.73; S, 15.40. Found: N, 6.94; S, 15.37.

C. Condensation of the Thioamide with Ethyl Bromopyruvate.—A solution of the thioamide (2.5 g.) described above and of ethyl bromopyruvate (2.5 g.) in ethanol (100 ml.) was heated under reflux for 1.5 hr. On cooling, some of the condensation product separated; an additional amount was obtained on concentration of the mother liquor. The crude product (2.9 g.) was recrystallized from boiling ethanol to give a purified product (2.3 g.) of m.p. 144–145°.

Anal. Calcd. for $C_{30}H_{28}N_2O_8S_2$: N, 4.60; S, 10.53. Found: N, 4.68; S, 10.61.

D. Dihydroxy Acid.—To a suspension of the condensation product (2.1 g.) (described in the last paragraph) in absolute ethanol (50 ml.), a solution of potassium hydroxide (0.76 g.) in ethanol (5 ml.) was added. On stirring at room temperature solution occurred, and the mixture was left to stand at room temperature overnight. The solid which separated was filtered, washed with ethanol, then dissolved in water (10 ml.), and the solution was acidified with *N* hydrochloric acid. A precipitate slowly formed and was collected on a filter, washed with water, and dried (1.0 g., m.p. 235–240°). An additional crop (0.2 g.) was obtained from the filtrate of the reaction mixture. Recrystallization from boiling water raised the melting point to 240–242°; $\lambda^{alc.}_{max}$ 237 mμ ($E^{1\%}_{1\,cm}$ 400).

Anal. Calcd. for $C_{12}H_{12}N_2O_6S$: C, 41.85; H, 3.51; S, 18.62. Found: C, 41.87; H, 3.95; S, 18.83.

E. Oxidation to Diketo Acid II.—To a suspension of the dihydroxy acid (0.60 g.) in glacial acetic acid (15 ml.), sodium bichromate (0.50 g.) and water (0.5 ml.) were added, and the mixture was heated on the steam bath for 2 hr. On cooling, the diketo acid II separated as an insoluble product; it was washed with liberal quantities of acetic acid. After drying, the crude acid weighed 0.40 g. The acid was recrystallized from dimethylformamide or water. The melting point of the purified material was 284–286° dec. (hot stage) or 275–280° dec. (capillary tube). The infrared spectrum is identical with that of II obtained by the oxidation of thiostreptoic acid.

Anal. Found: C, 42.32; H, 2.61; S, 18.54.

"362 Fragment".—Thiostrepton (10 g.) was hydrolyzed with boiling 1 *N* hydrochloric acid for 24 hr. The mixture was cooled, filtered, and extracted several times with ether; the aqueous layer was evaporated to dryness; and the residue was dried over sodium hydroxide in a desiccator. On redissolving this material in water (30 ml.), adjustment of the pH to about 3 caused the formation of a precipitate which was collected, washed with water, and dried (0.80 g.).

Anal. Found: N, 11.8; S, 15.2.

A similar material was obtained when a mixture of concentrated hydrochloric acid and formic acid was used for hydrolysis. Reprecipitation from a methanolic solution by the addition of ethyl acetate gave an amorphous precipitate. The ultraviolet spectrum of the "362 fragment" is shown in Fig. 1.

Anal. Found: C, 49.08; H, 4.31; N, 11.9; S, 17.1; neut. equiv. as base, 561; as acid, 270.

Thiazole-4-carboxylic Acid Oxalate.—Thiostrepton (2.0 g.) was treated with boiling nitric acid (20 ml.). After the vigorous reaction subsided, the reaction mixture was evaporated on a steam bath, and the residue was dissolved in water, re-evaporated, and stored *in vacuo* over sodium hydroxide pellets. The orange-yellow solid (1.8 g.) was distributed between ethyl acetate and water, using 50-ml. layers and the complete withdrawal technique for a total of five fractions of upper and five fractions of lower layers. The contents of the fundamental fractions except no. 0 and the last three withdrawn fractions were pooled, the solvents were removed *in vacuo*, and the residue was sublimed *in vacuo* between 160 and 200° (0.2 mm.). The sublimate (0.30 g.) was dissolved in chloroform containing a few per cent ethanol, and hexane was added to the solution until crystals appeared. These crystals (0.16 g.) were resublimed, and the fraction which was collected up to 155° was sublimed again, this time at 125°. The white product shrinks at 182° and melts at 188–190°, $\lambda^{alc.}_{max}$ 232 mμ ($E^{1\%}_{1\,cm}$ 395).

Anal. Calcd. for $2C_4H_3NO_2S \cdot (COOH)_2$: C, 34.49; H, 2.32; N, 8.04; S, 18.41; neut. equiv., 87. Found: C, 34.94; H, 2.75; N, 8.39; S, 18.33; neut. equiv., 86.

To a hot solution of the oxalate (60 mg.) in water (5 ml.), a hot aqueous calcium chloride solution (52 mg. in 1 ml.) was added, and the precipitate which formed was removed by centrifugation and identified as calcium oxalate by its infrared spectrum. The supernatant solution, on continuous extraction with ethyl acetate, gave thiazole-4-carboxylic acid (41 mg.) which was purified by sublimation and showed $\lambda^{alc.}_{max}$ 232 mμ ($E^{1\%}_{1\,cm}$ 502).

Anal. Calcd. for $C_4H_3NO_2S$: C, 37.21; H, 2.34; N, 10.85; S, 24.82. Found: C, 37.35; H, 2.47; N, 10.86; S, 23.76.

A sample of thiazole-4-carboxylic acid oxalate (71 mg.) was dissolved in methanol (7 ml.) and treated with an ethereal solution of diazomethane until a yellow color persisted. After removal of the solvents *in vacuo*, the residue was dissolved in chloroform. This solution after washing with a solution of potassium bicarbonate and with water was dried, the chloroform was evaporated, and the residue was sublimed *in vacuo* at 90–100°. The methyl ester (24.5 mg.) (*Anal.* Calcd. for OCH_3: 21.6. Found: 21.05.) showed in its n.m.r. spectrum, which was taken in $CDCl_3$, a singlet at τ 6.03 (OCH_3) and doublets (J = 2 c.p.s.) at τ 1.6 (2-H) and 1.76 (5-H).

Thiostreptine (IV).—A solution of the antibiotic (7 g.) in a mixture of concentrated hydrochloric acid (35 ml.) and formic acid (35 ml.) was heated at 110° for 5 hr. The mixture was evaporated to dryness by passing a stream of nitrogen over the gently heated solution. The residue was stored over sodium hydroxide pellets *in vacuo* for 2 days, then dissolved in water (100 ml.), filtered, and extracted with six 100-ml. portions of ether. The aqueous layer was concentrated to a small volume and then diluted with the lower layer of the system 1-butanol–ethanol–0.1% acetic acid (4:1:5) and placed into the first 20 tubes of the countercurrent distribution apparatus (10-ml. phases). After 1200 transfers, the component with a fast-moving, ninhydrin-positive, yellow spot was found in a band with K = 0.2. The contents of tubes 200 to 264 were pooled, and the solution was concentrated *in vacuo* to about 20 ml. and then neutralized with 1 N sodium hydroxide. After filtration from a small amount of insoluble material, the filtrate was evaporated and the residue was extracted with ethanol. Addition of ethyl acetate to the ethanol extract precipitated crude thiostreptine which was collected by centrifugation, washed with ethyl acetate, and dried (0.59 g.). A portion (0.19 g.) of this material was chromatographed on Whatman No. 1 cellulose powder with 1-butanol–acetic acid–water (4:1:1), collecting 10-ml. fractions. Following the elution of a small amount of a component which gives a purple spot with ninhydrin (R_f 0.48), thiostreptine (R_f 0.41) was found in tubes 54–64. Evaporation of the content of these tubes to a small volume followed by lyophylization gave a white solid (125 mg.), $[\alpha]^{20}D$ −4° (*c* 1, 1 N acetic acid), λ_{max}^{alc} 237 mμ ($E_{1\,cm}^{1\%}$ 275). The n.m.r. spectrum in trifluoroacetic acid shows a singlet (3 protons, 2′-methyl) at ~8.7, a doublet (3 protons, ω-methyl) centered at τ 8.4 (J = 6 c.p.s.), and single protons at τ 6.4 (3′-H) and 4.4 (1′-H). The aromatic proton appears at τ 1.20.

Anal. Calcd. for $C_9H_{14}N_2O_4S$: C, 43.89; H, 5.73; N, 11.38; S, 13.02. Found: C, 45.6; H, 6.7; N, 11.4; S, 13.7; neut. equiv., 230 (as acid), 233 (as base).

In a similar isolation, but without neutralization after countercurrent distribution, thiostreptine was obtained as the hydrochloride.

Anal. Calcd. for $C_9H_{15}ClN_2O_4S \cdot H_2O$: C, 35.94; H, 5.70; Cl, 11.79; N, 9.31; S, 10.66; neutr. equiv., 300.8. Found: C, 35.40; H, 5.97; Cl, 12.63; N, 9.29; S, 10.53; neutr. equiv., 157 (acid); 299 (base).

Acid Hydrolysis of Thiostreptine.—A solution of IV (37 mg.) in constant boiling hydrochloric acid (1 ml.) was heated in an evacuated, sealed ampoule at 110° for 30 hr. The mixture was diluted with water (25 ml.) and distilled until about 20 ml. of distillate was collected. A solution of dinitrophenylhydrazine in hydrochloric acid (15 ml., 3 mg./ml.) was added; an oily precipitate soon appeared which crystallized in the form of clusters of needles. The dinitrophenylhydrazone (17 mg.) was extracted with hot ethanol. This extraction left some undissolved material which does not melt up to 295° and which was identified as biacetyl 2,4-dinitrophenylhydrazone by a comparison of its infrared spectrum with that of an authentic sample (m.p. *ca.* 325° dec.[38]) Dilution of the ethanol extracts with water gave crystals (10 mg.) of rectangular plates, m.p. 120–121°. After sublimation *in vacuo* at about 240°, the infrared spectrum of this material is indistinguishable from that of acetaldehyde dinitrophenylhydrazone. This identity was confirmed also by mixture melting point with an authentic sample and by chromatography on a thin layer of acid-washed alumina with chloroform.

(38) The melting point of the dinitrophenylhydrazone of biacetyl has been reported erroneously (ref. 10, footnote 11) to be 235° dec. instead of 325° dec. It is identical with the product (osazone) prepared from acetoin and dinitrophenylhydrazine. *Anal.* Calcd. for $C_{16}H_{14}N_8O_8$: C, 43.06; H, 3.16; N, 25.10. Found: C, 43.09; H, 3.23; N, 25.20.

The distillation residue was evaporated to dryness to a dark crystalline material which was purified by crystallization from methanol–ethyl acetate. The crystals (12 mg.) which shrink at 215°, m.p. 275° dec., were found to be identical with 2-aminomethylthiazole-4-carboxylic acid as they were indistinguishable from an authentic sample[6,7] by a comparison of paper chromatograms and also by a comparison of their infrared spectra.

Periodate Oxidation of Thiostreptine. A.—One mole of IV consumes 2.07 moles of periodate. Distillation of the reaction mixture into sodium bisulfite and titration of the excess of the latter shows the formation of 19% acetaldehyde (calcd. 19%).

B.—To a solution of IV (46 mg.) in water (15 ml.), a saturated aqueous solution of sodium periodate (10 ml.) as added. A slow current of air was passed through a solution of 2,4-dinitrophenylhydrazine in 1.5 N hydrochloric acid (*ca.* 3 mg./ml.), then through the reaction mixture, and finally through a series of three traps each containing 50 ml. of the 2,4-dinitrophenylhydrazine solution described above. After 20 hr. the air current was stopped, and the precipitate which formed in the first two traps was collected, washed with 1 N hydrochloric acid and with water, and dried over sodium hydroxide *in vacuo* at 60° (wt. 19 mg., m.p. 140–150°). This material was shown to be (impure) acetaldehyde 2,4-dinitrophenylhydrazone by comparison with an authentic sample *via* mixture melting point, infrared spectrum, and chromatography on a thin layer of acid-washed alumina (chloroform). Ether extraction of the reaction mixture gave a white solid (22 mg.) which was sublimed at about 0.01 mm. and 90°. The purified product (12 mg.) has no well-defined melting point; it melts at about 165–184°, λ_{max}^{alc} 235 mμ. It gives a 2,4-dinitrophenylhydrazone. Molecular weight of the carbonyl compound by mass spectrum was found to be 157. The mass spectrum reveals also the presence of one sulfur, one nitrogen, and a COOH group. The n.m.r. spectrum in CH_3CN shows one aldehydic proton (τ 0.83) and an aromatic proton (τ 2.15).

Anal. Calcd. for $C_8H_3NO_3S$: C, 38.21; H, 1.92; S, 20.39. Found: C, 38.96; H, 2.17; S, 19.95.

C.—To an aqueous solution of III (40 mg. in 10 ml.), an aqueous solution of H_5IO_6 (75 mg. in 0.75 ml.) was added and the pH of the mixture adjusted to 8 with 1 N sodium hydroxide (0.5 ml.). After 40 min. at room temperature, 2 N sulfuric acid (2 ml.) and some water were added, and the reaction mixture was distilled until 20 ml. of distillate was collected. After the addition of more water (20 ml.), the distillation was continued until a second 20 ml. of distillate was collected. Neutralization of the first distillate required 1.5 ml. of 0.1 N sodium hydroxide; the second needed 0.5 ml. The two neutralized solutions were pooled and evaporated to dryness. The residue was shown to consist of sodium acetate by comparison with an authentic sample on paper chromatograms in the system 1-butanol–ethanol–3 N ammonium hydroxide (4:1:5)[39] and this identity was confirmed with the aid of the infrared spectrum. No formate or propionate was found in the residue.

Acetaldehyde and Acetoin from Thiostrepton. A.—A suspension of the antibiotic (0.82 g.) in 0.5 N sodium hydroxide (50 ml.) was stirred at room temperature. The white material turned pink, and in about 2 hr. an orange solution was obtained. After dilution with water (50 ml.) the mixture was distilled until about 50 ml. of distillate was collected, then more water (100 ml.) was added, and a second 50 ml. of distillate was collected. Both distillates were acidified with concentrated hydrochloric acid (10 ml.), and a 0.5% solution of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid (50 ml.) then was added. The precipitates which soon formed were collected, washed with 2 N hydrochloric acid followed by water, and dried. From the first distillate, 91 mg. of material was obtained, and from the second, 8.5 mg. of material was obtained. They were combined and extracted with hot ethanol. The alcohol-insoluble residue (19 mg.) shrank at 280° and decomposed (charred) at 325°; its infrared spectrum is identical with that of the osazone from acetoin.

Anal. Calcd. for $C_{16}H_{14}N_8O_8$: C, 43.06; H, 3.16; N, 25.10. Found: C, 43.12; H, 4.39; N, 24.70.

Addition of water to the alcohol extracts gave the 2,4-dinitrophenylhydrazone of acetaldehyde (66 mg.), m.p. (shrank at 146°) 160.5–164°, which was identified by methods already described in this paper.

B.—In a similar experiment, a stream of air was passed through the combined distillates and then through the 2,4-dinitrophenylhydrazine solution. In this way 110 mg. of acetaldehyde 2,4-

(39) F. Brown, *Biochem. J.*, **47**, 598 (1950).

dinitrophenylhydrazone was obtained from 0.80 g. of thiostrepton.

C.—The antibiotic (0.80 g.) was treated with dilute alkali and the solution was distilled as described under A and **B**. Aeration of the pooled distillates using two traps containing 0.4% aqueous dimedone solution (50 ml.) gave the dimedone derivative of acetaldehyde (106 mg.), m.p. 141–143°. The distillate from which the acetaldehyde had been removed by the aeration was treated with ferric chloride (2 g.), acidified with a few drops of acetic acid, and redistilled. The first 125 ml. of the new distillate was neutralized with dilute potassium bicarbonate solution and treated with hydroxylamine hydrochloride (0.2 g. in 1 ml. of water) and sodium acetate (0.4 g. in 2 ml. of water). After briefly warming the solution on the steam bath, a 10% solution of nickel chloride (1 ml.) was added. While warming continued, beautiful red crystals of nickel dimethylglyoxime separated. The crystals were collected, washed with water and with ethanol, and dried (wt. 17 mg.).

2-Propionylthiazole-4-carboxylic Acid and Methyl Ester.—Thiostrepton (1 g.) was hydrolyzed with strong sulfuric acid as described for thiostreptoic acid hydrogen sulfate. Ether extracts of the hydrolysate were concentrated to an oil which was dissolved in chloroform; the solution was dried over magnesium sulfate and evaporated. Sublimation of the residue at 100° and 0.15 mm. gave crystals (15 mg.), m.p. 164–166°. These were treated with an ethereal solution of diazomethane until the yellow color persisted. Removal of the solvents left a crystalline residue, m.p. 89–91°, which was identified by a comparison (infrared and n.m.r. spectra) with a synthetic sample[18] from Dr. J. Walker. The n.m.r. spectrum of the acid in $CDCl_3$ showed a triplet (CH_3) at τ 8.72 (J = 7 c.p.s.), a quadruplet (CH_2) at 6.72 (J = 7 c.p.s.), and singlets (aromatic) at 1.38 and (COOH) 0.66. The n.m.r. spectrum of the ester in $CDCl_3$ showed peaks at τ 8.75, 6.72, 6.00 (OCH_3), and 1.55; in CD_3COOD the ester showed peaks at τ 8.81, 6.83, 6.09, and 1.50.

4-Acetyl-8-hydroxyquinaldic Acid (V).—A suspension of thiostrepton (5 g.) in 1 N hydrochloric acid (150 ml.) was heated to 110° for 24 hr. Some insoluble material was removed by filtration, and the solution was extracted with seven 100-ml. portions of ether. The ether extracts were dried over magnesium sulfate and evaporated to dryness, and the residue (1.13 g.) was dissolved in absolute ethanol (7 ml.). The solution was kept in the cold, and needles separated. The crystals were collected by centrifugation, washed with 95% ethanol, and dried (wt. 110 mg.). Recrystallization from hot 95% ethanol gave purified material (50 mg.), m.p. 190–200° dec., λ_{max}^{Nujol} 1730 and 1700 cm.$^{-1}$, λ_{max}^{alc} 261 and 360 mμ.

Anal. Calcd. for $C_{12}H_9NO_4$: C, 62.34; H, 3.92; N, 6.06; neut. equiv. 231. Found: C, 62.61; H, 4.03; N, 6.10; neut. equiv. 225.

The compound can be titrated with alkali but not with anhydrous perchloric acid; with ferric chloride in methanol it gives an intense green color; with $Cu(OAc)_2$ an orange precipitate forms which is soluble in chloroform.

4-Acetyl-8-methoxyquinaldic Acid Methyl Ester.—A solution of V (231 mg.) in methanol (50 ml.) and ether (50 ml.) was treated with an ethereal solution of diazomethane until a sample of the mixture no longer gave a green color with ferric chloride. After 45 min. at room temperature, acetic acid (0.5 ml.) was added, and the solvents were removed *in vacuo*. A few milliliters of ethanol was added to the residue and evaporated; this operation was repeated twice more. The methyl ester-methyl ether was washed on a filter with ethanol and dried, weighing 150 mg., m.p. 165–167°, distilling unchanged at about 300°, λ_{max}^{alc} 263 mμ ($E_{1\,cm}^{1\%}$ 1140), 366 mμ ($E_{1\,cm}^{1\%}$104). In the n.m.r. spectrum in $CDCl_3$ a 3-proton singlet at τ 7.45 corresponds to the CH_3CO-protons.

Anal. Calcd. for $C_{14}H_{13}O_4N$: C, 64.86; H, 5.05; N, 5.40; OCH_3, 23.95; mol. wt. 259. Found: C, 65.09; H, 5.43; N, 5.58; OCH_3, 24.16; mol. wt. (Rast), 270.

4-Acetyl-8-methoxyquinaldic Acid.—To a suspension of the methyl ester-methyl ether (78 mg.), described in the preceding paragraph, in ethanol (3 ml.), 1 N sodium hydroxide (0.40 ml.) was added. Almost immediately a precipitate (sodium salt) formed. After 20 min. at room temperature, water (4 ml.) was added. The resulting clear yellow solution was acidified with hydrochloric acid (0.60 ml.); silky needles separated and were collected on a filter, washed with water, and dried over sodium hydroxide (wt. 45.6 mg., m.p. 128–130°).

Anal. Calcd. for $C_{13}H_{11}NO_4$: C, 63.67; H, 4.52; N, 5.71. Found; C, 63.86; H, 4.68; N, 5.70.

Decarboxylation of V to 4-Acetyl-8-hydroxyquinoline.—A sample (15 mg.) of the keto acid V in a small test tube was immersed in a metal bath, the temperature of which was raised from 220° to 245° within a few minutes and kept at this temperature until the evolution of gas ceased. The contents of the tube solidified on cooling to a substance with m.p. *ca.* 100°, λ_{max}^{Nujol} 1700 cm.$^{-1}$. *Anal.* Calcd. for $C_{11}H_9NO_2$: neut. equiv., 187.2. Found (titration with $HClO_4$): 190.

4-Ethyl-8-hydroxyquinaldic Acid (VI).—From mother liquors of the crystallization of V, a crop of needles was obtained, m.p. 190–200° dec.; λ_{max}^{Nujol} 1700 cm.$^{-1}$; λ_{max}^{alc} 255, 357 mμ.

Anal. Calcd. for $C_{12}H_{11}NO_3$: C, 66.35; H, 5.10; N, 6.45. Found: C, 65.82; H, 4.67; N, 6.47.

Decarboxylation of VI to 4-Ethyl-8-hydroxyquinoline.—A sample (51 mg.) of the acid VI was heated in a small test tube from 200 to 270° until the evolution of gas ceased. After cooling, the residue was extracted with warm hexane (5 ml.) from which, on cooling, a small amount of solid separated and was removed. The filtrate on evaporation gave a light yellow solid (18 mg.). The product from several such experiments, using 300 mg. of crude acid as starting material, was pooled, and was distilled *in vacuo* at *ca.* 200° and 1 mm. The purified material shows no CO bands in the infrared spectrum, m.p. *ca.* 75°.

Anal. Calcd. for $C_{11}H_{11}NO$: C, 76.27; H, 6.40; N, 8.09; neut. equiv., 173.2. Found: C, 76.36; H, 6.39; N, 8.36; neut. equiv. 171.

Nitric Acid Oxidation of V, VI, and Their Decarboxylation Products.—Compound V (43 mg.) was heated with concentrated nitric acid (1 ml.) in a test tube on the steam bath for 90 min. The mixture was evaporated to dryness, and the residue was twice evaporated with water. The product (58 mg.) gave a violet-purple color with ferrous sulfate. A sample on heating darkened from 120°, and gas evolution was observed at 150–160°. A second period of gas evolution took place at 175–185°, and at 210–220° sublimation of some white solid was observed. Finally, strong gas evolution with copious sublimation of white material occurred at 240°. The temperature of the bath was held at 280° for 40 min. The sublimate was compared on paper chromatograms using the system 1-butanol–acetic acid–water (4:1:2) with products similarly obtained from cinchomeronic, isocinchomeronic, and quinolinic acid. The spots were revealed with bromphenol blue, but were also visible under ultraviolet light. The oxidation–decarboxylation product from V gives 3 spots: R_f 0.71, nicotinic acid; R_f 0.61, isonicotinic acid; and R_f 0.42, cinchomeronic acid. The same pattern was obtained from cinchomeronic acid but not from the other pyridinedicarboxylic acids. A comparison of infrared spectra of the sublimates confirmed this observation. The oxidation product of VI behaved exactly as that of V, while the oxidation products from the two quinolines prepared by decarboxylation of V and VI gave a rusty red color with ferrous sulfate. On decarboxylation again, the formation of nicotinic, isonicotinic, and cinchomeronic acids was observed.

Synthesis of 4-Ethyl-8-hydroxyquinaldic Acid (VI). A. 4-Ethyl-8-methoxyquinoline.—A mixture of *o*-anisidine hydrochloride (25 g.), ferric chloride (68 g.), and zinc chloride (3 g.) in 95% ethanol (125 ml.) was heated with stirring in a bath at 60–65° while ethyl vinyl ketone (13 ml.) was added dropwise in about 1.5 hr. After refluxing for 2 hr. and standing overnight at room temperature, 40% sodium hydroxide (100 ml.) was added. The mixture was evaporated to dryness *in vacuo* and the residue was dried by evaporation with absolute ethanol. The solid was extracted with five 500-ml. portions of benzene and the benzene was evaporated. The residue was dissolved in ether, was filtered, and the solvent was removed giving a product which distilled at 150–160° (2 mm.). The distillate (12.6 g.) was crystallized from hexane to give a product (6.5 g.) with m.p. 49–51°. Redistillation *in vacuo* raised the m.p. to 52–53°.

Anal. Calcd. for $C_{12}H_{13}NO$: C, 76.97; H, 7.00; N, 7.48; neut. equiv. 187.2. Found: C, 76.66; H, 7.00; N, 7.23; neut. equiv. 187.

B. Reissert Reaction and Demethylation.—To a mixture of 4-ethyl-8-methoxyquinoline (2.8 g.), benzoyl chloride (4 ml.), and potassium cyanide (3 g.) in a glass pressure bottle, liquid sulfur dioxide (35 ml.) was added while cooling with an acetone–Dry Ice bath. The vessel was closed and left to stand overnight at room temperature, then it was opened to allow the evaporation of sulfur dioxide. The residue was washed successively with water, 5% sodium bicarbonate solution, 5% hydrochloric acid, and water. The crude product (1.8 g.) solidified under ether. It was dissolved in ethanol (15 ml.) and the solution was filtered

and diluted with water. The solid which separated was extracted with hot hexane to give a crude product (1.5 g.), m.p. 161° dec. A sample (100 mg.) of this material was heated under reflux with 48% hydrobromic acid (5 ml.) for 5 hr. After dilution with water (50 ml.) and extraction with five 20-ml. portion of ether, the ether solution was washed with water and the ether was evaporated. Sublimation of the residue *in vacuo* gave a small amount of a product the ultraviolet and infrared spectra of which were identical with those of VI.

4-(α-Hydroxyethyl)-8-methoxyquinaldic Acid Methyl Ester.—From the final mother liquors of the crystallization of V and VI, 4-(α-hydroxyethyl)-8-hydroxyquinaldic acid (VII) was isolated. Better yields of VII were obtained, however, if a mixture of formic acid (35 ml.) and concentrated hydrochloric acid (35 ml.) were used for the hydrolysis of thiostrepton (7.35 g.). After 16 hr. at 100°, the mixture was evaporated to dryness on the steam bath with a stream of nitrogen; water was added to the residue and re-evaporated. The material was taken up in water (60 ml.), the insoluble material was filtered off, and both the solution and the solid were extracted repeatedly with ether. Evaporation of the ether extracts left an orange-yellow residue (1.53 g.) which was dissolved in methanol (50 ml.) and methylated with an ethereal solution of diazomethane. The solvents were removed *in vacuo*, and the product was dissolved in hot benzene. On cooling a part of the ester separated in crystalline form and was filtered off (0.14 g.). The filtrate was chromatographed on a column of acid-washed alumina using first benzene as eluent, followed by a gradient elution with increasing concentration of chloroform, and finally chloroform itself. Only this last solvent removed the methyl ester-methyl ether of VII from the column. The chromatographic procedure was monitored by an examination of the column with an ultraviolet lamp, since the ester-ether of VII shows intense white fluorescence. Chromatography of effluent samples on a thin layer of alumina developed with chloroform also was helpful for orientation. The ester-ether of V and VI are much faster on these plates than that of VII, and they show a yellow fluorescence under the ultraviolet lamp. Evaporation of the fractions with the ester-ether of VII gave a residue (0.90 g.) which was crystallized from benzene (wt. 0.20 g.). More material could be secured from the mother liquor. The crystalline product was further purified by sublimation *in vacuo* at about 1 mm. and 150°. This raised the melting point from about 160–168° to 175–177°; $[\alpha]^{20}D$ −78° (*c* 1.6, absolute EtOH); λ_{max}^{Nujol} 1750, 3400 cm.$^{-1}$; λ_{max}^{alc} 254 mμ ($E_{1\,cm}^{1\%}$ 1500), 347 mμ ($E_{1\,cm}^{1\%}$115). The n.m.r. spectrum in CD_3COOD shows a three-proton doublet centered at τ 8.41 (*J* = 6.5 c.p.s.), a six-proton singlet (OCH_3) at 6.04, a one-proton doublet at 4.37 (side-chain α-carbon proton), one proton multiplet at 2.83, a two-proton doublet at 2.50, and, finally, the sharp singlet of the aromatic proton of the pyridine ring at 1.76.

Anal. Calcd. for $C_{14}H_{15}NO_4$: C, 64.36; H, 5.79; N, 5.36. Found: C, 64.50; H, 6.09; N, 5.58.

Disproportionation of VII to V and VI. A.—The crude acid VII, obtained as described earlier, was crystallized from a mixture of ether and benzene. The material, while still not pure, showed no ketone carbonyl band in its infrared spectrum; $[\alpha]^{22}D$ −68° (*c* 1, EtOH). A sample (33 mg.) of this acid was heated with hydrochloric acid (1 ml.) in an evacuated sealed ampoule at 110° for 24 hr. On cooling, orange-brown prisms deposited from the clear solution. The crystals were collected by centrifugation, washed with hydrochloric acid and water, and dried (wt. 15 mg.). The product was identical with V (infrared spectrum).

Anal. Found: C, 62.31; H, 4.01; N, 5.99.

Extraction of the supernatant with ethyl acetate gave crude VI (13 mg.), identified *via* infrared and ultraviolet spectra.

B. A sample of the methyl ester-methyl ether of VII (53 mg.) in acetic acid (0.8 ml.) was heated in an evacuated, sealed ampoule at 100° for about 40 hr. The solvent was removed, and the residue was compared with the starting material by chromatography on a thin layer of alumina. Only a small part of the starting material was left unchanged, and considerable amounts of the ether-esters of V and VI were formed. The infrared spectrum confirms the formation of the keto derivative (λ_{max}^{Nujol} 1700 cm.$^{-1}$).

Formation of Quinaldic Acid Derivatives from "Indole".—Thiostrepton (1.0 g.) was dissolved in concentrated hydrochloric acid (10 ml.) and was left to stand at room temperature. After 3 months, water (110 ml.) was added and the solution was extracted with two 100-ml. portions of ether, with two 100-ml. portions of ethyl acetate, and with five 100-ml. portions of water-saturated 1-butanol. The butanol solutions showed maxima at 283, 290, and 303 mμ. From the optical densities at 283 mμ of the individual butanol extracts, a distribution coefficient of 0.87 was calculated for the "indole". On evaporation of the pooled butanol extracts to about 30 ml., an increase in the absorption at 256 mμ was observed. The concentrated solution was placed into the first four tubes of a countercurrent distribution apparatus in the system 1-butanol–0.1 *N* hydrochloric acid for 96 transfers. Examination of the contents of each fifth tube by ultraviolet absorption at 283 mμ revealed that contrary to expectations only a small peak was present at tube no. 42 (*K* = 0.8), while the bulk of the material was found in a peak around tube no. 88 (*K* = 10). Evaporation of the solution from tubes 80 to 99 gave a residue with λ_{max}^{alc} at 258 and 357 mμ, the characteristic spectrum of VII.

Pyruvic Acid from Thiostrepton.—A solution of the antibiotic (2.0 g.) in constant boiling hydrochloric acid (20 ml.) was kept in a bath of 110° under an air condenser for 16 hr. Water (30 ml.) was added to the clear brown solution, and the latter was distilled with a free flame until 30 ml. of distillate were collected. Addition of water and distillation was repeated twice more; finally, the mixture was distilled until only a very small volume was left. The first three distillates were pooled and treated with a 0.5% solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid (300 ml.) to give a precipitate (0.20 g.). The last distillate also gave a dinitrophenylhydrazone (0.20 g.). The dinitrophenylhydrazones were purified with about 88% recovery by dissolution in dilute potassium bicarbonate solution and acidification of the filtered solutions, m.p. 208–212°. A sample of 2,4-dinitrophenylhydrazone of authentic pyruvic acid melted at 217–218°, the mixture at 214–216°. Their identities were confirmed by infrared spectra and by chromatography on a thin layer of acid-washed alumina using chloroform–methanol (10:1) for development; identical patterns of three spots were demonstrated. For analysis the 2,4-dinitrophenylhydrazone originating from thiostrepton was sublimed at 210° and 0.1 mm.

Anal. Calcd. for $C_9H_8N_4O_8$: C, 40.30; H, 3.01; N, 20.89; neut. equiv., 268.2°. Found: C, 40.60; H, 3.50; N, 21.14; neut. equiv., 264.

In a second similar experiment, except that distillation was repeated eight times, 0.54 g. (2 mmoles) of purified 2,4-dinitrophenylhydrazone of pyruvic acid was obtained from 2.0 g. of thiostrepton.

Diketopiperazine, $\overline{\text{L-Ileu-Ala,-}}$ from Thiostrepton.—A solution of the antibiotic (2.0 g.) in acetic acid (20 ml.) was heated to 100° for 20 hr. The solvent was removed by drying from the frozen state, and the fluffy residue was extracted with three 300-ml. portions of ethyl acetate. Evaporation of the solvent and crystallization of the residue from ethyl acetate–ether gave 0.15 g. of an off-white crystalline material, which sublimes unchanged at 200–210° (0.5 mm.), m.p. 236–239°, $[\alpha]^{22}D$ −25° (*c* 1, ethanol). On hydrolysis, two ninhydrin-positive components are liberated: isoleucine and alanine. The compound is identical in all respects (melting point, mixture melting point, infrared spectra) with synthetic $\overline{\text{L-isoleu-L-ala-}}$.

Anal. Calcd. for $C_9H_{16}N_2O_2$: C, 58.67; H, 8.75; N, 15.21. Found: C, 58.29; H, 8.91; N, 15.13.

A solution of thiostrepton (100 mg.) and of *p*-toluenesulfonic acid monohydrate (50 mg.) in acetic acid (2 ml.) was heated as described above. Removal of the acetic acid followed by extraction with ethyl acetate and re-extraction of the ethyl acetate soluble part with ether gave an impure diketopiperazine, which was sublimed and then identified by its infrared spectrum. No diketopiperazine was obtained from a mixture of isoleucylalanine (100 mg.) and *p*-toluenesulfonic acid monohydrate (100 mg.) in acetic acid (2 ml.), when treated as described above.

Synthesis of L-Isoleucyl-L-alanine and of the Corresponding Diketopiperazine. A. Methyl Benzyloxycarbonyl-L-isoleucyl-L-alaninate.—To a solution of *p*-nitrophenyl benzyloxycarbonyl-L-isoleucinate[40] (37 g.) in chloroform (100 ml.) methyl L-alaninate hydrochloride[41] (13.7 g.) and triethylamine (14 ml.) were added. After standing overnight at room temperature, the chloroform was evaporated *in vacuo;* the residue was dissolved in ethyl acetate (350 ml.) and water (300 ml.), and the organic layer was washed with 1 *N* hydrochloric acid, water, 0.5 *N* ammonium hy-

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droxide, and water. After drying, the ethyl acetate was evaporated to a small volume; hexane (200 ml.) was added, and the crystals which formed were collected (11.63 g., m.p. 167–169°). A second crop (7.3 g.), m.p. 157–165°, on recrystallization from hot 95% ethanol gave a product (1.4 g.), m.p. 171–173.5°. This was combined with the main crop and recrystallized from 95% ethanol yielding 8.4 g., m.p. 172.5–175°, $[\alpha]^{24}D$ −44° (*c* 4, MeOH). Additional crystallization did not raise this melting point.

Anal. Calcd. for $C_{18}H_{26}N_2O_5$: C, 61.70; H, 7.48; N, 8.00; OCH_3, 8.80. Found: C, 61.85; H, 7.56; N, 7.75; OCH_3, 9.20.

B. Benzyloxycarbonyl-L-isoleucyl-L-alanine.—A sample of the protected dipeptide ester was saponified in aqueous methanol with the calculated amount of sodium hydroxide. On acidification, the protected dipeptide acid, m.p. 155–160°, was obtained in 90% yield. After recrystallization from dilute ethanol, it melted at 169–171°, $[\alpha]^{22}D$ −27° (*c* 4.95, ethanol).

Anal. Calcd. for $C_{17}H_{24}N_2O_5$: C, 60.70; H, 7.19; N, 8.33; neut. equiv., 336.4. Found: C, 60.94; H, 7.27; N, 8.53; neut. equiv., 340.

The same protected dipeptide acid (m.p. 167–168°) was obtained by acylating L-alanine in dilute pyridine with *p*-nitrophenyl benzyloxycarbonyl-L-isoleucinate at pH 9.5, kept constant by the addition of dilute sodium hydroxide.

C. L-Isoleucyl-L-alanine.—The protected dipeptide acid (1.58 g.) in a mixture of methanol (26 ml.), acetic acid (4 ml.), and water (20 ml.) was hydrogenated in the presence of a 10% palladium-on-charcoal catalyst (0.35 g.). After removal of the catalyst and evaporation of the solvents *in vacuo*, the residue was triturated with absolute ethanol. The precipitate which formed was collected and washed on the filter with ethanol (0.79 g., m.p. 234–237°). Recrystallization from water–ethanol raised the m.p. to 236–238°, while a small second crop melted at 240–242°, $[\alpha]^{23}D$ +6.5° (*c* 4, 1 *N* acetic acid).

Anal. Calcd. for $C_9H_{18}N_2O_3$: C, 53.44; H, 8.97; N, 13.85. Found: C, 53.30; H, 8.87; N, 13.77.

D. L-Ileu-L-ala.—The protected dipeptide methyl ester (3.5 g.) was hydrogenated in methanol (90 ml.) containing acetic acid (10 ml.) in the presence of a 10% palladium-on-charcoal catalyst (0.70 g.). After the evolution of carbon dioxide ceased, the catalyst was filtered off and the solvents were removed. A 50% aliquot of the residue was heated on a steam bath to give a solid (0.90 g.), m.p. 232–239°, $[\alpha]^{22}D$ −22° (*c* 1, 95% ethanol). Sublimation *in vacuo* raised the melting point to 243–246°, $[\alpha]^{20}D$ −30°.

Anal. Calcd. for $C_9H_{16}N_2O_3$: C, 58.67; H, 8.75; N, 15.21. Found: C, 58.95; H, 8.98; N, 14.90.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY 4, CALIF.]

Photochemical Transformations. XVI.[1] The Structure of Photolevopimaric Acid[2]

BY WILLIAM G. DAUBEN AND ROBERT M. COATES[3]

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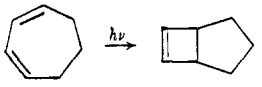
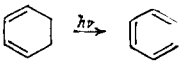
Levopimaric acid (**9**) upon ultraviolet irradiation was transformed into its valence isomer **10**. The presence of a bicyclo[2.2.0]hexene structure was established by conversion of **10** to the triester **25** by a series of specific degradations.

Conjugated cyclic dienes upon ultraviolet irradiation have been found to undergo two modes of reactions, bond formation and bond cleavage. The pathway to be followed depends upon the particular system at hand. When the diene moiety is enclosed in a seven-membered ring, irradiation effects valence isomerization to a bicyclo[3.2.0]heptene[4] and the numerous cycloheptadienes which have been transformed in this fashion attest to the generality of the process.[5,6] The photochemistry of cycloheptatrienes,[4,7] tropolones,[1,8]



and functionalized cycloheptadienes[9] usually follows the same course. The consistent behavior in this series may be attributed, in part, to the fact that simple ring cleavage is not possible in an odd-membered ring system.[10]

With six-membered ring analogs the photochemistry generally takes the alternative course of ring opening. Having a carbon–carbon bond allylic to each terminus of the diene, a cyclohexadiene may undergo the cleavage reaction. That ring opening to trienes is most frequently observed is not to be unexpected since valence isomerization demands the formation of a highly strained bicyclo[2.2.0]hexene system. Thus, cyclohexadiene,[11–13] α-phellandrene,[12] α-terpinene,[14] allo-ocimene,[15] and 5,6-dimethyl-1,3-cyclohexadiene[15] all furnish the corresponding trienes by radiative scission of the doubly allylic bond. In a similar fashion, *o*-cyclohexadienones[11,16,17] are converted to ketene derivatives.



(1) For previous paper in this series, see W. G. Dauben, K. Koch, S. L. Smith, and O. L. Chapman, *J. Am. Chem. Soc.*, **85**, 2616 (1963).

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