[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY AND CHEMISTRY, UNIVERSITY OF WISCONSIN]

The Chemistry of Antimycin A. VIII.[1] Degradation of Antimycin A_1[2]

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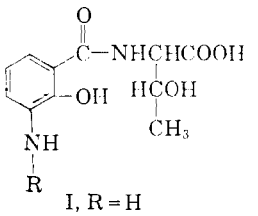
Mild alkaline hydrolysis of the antibiotic antimycin A_1 yields two primary products, N-formylantimycic acid (IV) and a "neutral fragment" (II). Further alkaline treatment of the latter gave isovaleric acid and an hydroxylactone, $C_{11}H_{20}O_3$, which was identified as α-*n*-hexyl-β-hydroxy-γ-methyl-γ-butyrolactone by degradation to acetaldehyde and *n*-hexylmalonic acid. These results establish the structure of II as α-*n*-hexyl-β-isovaleryloxy-γ-methyl-γ-butyrolactone, and thus complete the structural elucidation of the component parts of antimycin A_1.

Previous chemical studies of the antimycin A complex have shown that mild alkaline treatment results in cleavage to antimycic acid (I), formic acid and a neutral, nitrogen-free liquid designated as the "neutral fragment" (II).[4-6] The structure



I, R = H
IV, R = CHO

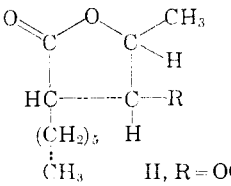
of antimycic acid was established by degradation[5] and synthesis.[7] On the basis of: (i) further hydrolysis of the crude "neutral fragment" by means of hot aqueous alkali to L-(+)-methylethylacetic acid and an unidentified keto acid, $C_{11}H_{20}O_3$ (III); and (ii) study of model compounds, II was regarded as conforming to the β-acyloxy-γ-lactone type.[4,6,8]

Much of the difficulty encountered in the earlier studies of the "neutral fragment" was occasioned by the fact that the antimycin preparations available at that time consisted of mixtures of at least four individual components. Degradation of this complex led to difficultly-crystallizable mixtures, and interpretation of the results was tentative. This situation was greatly improved when a procedure for separating the individual components was developed,[9] and the present reinvestigation of the degradation of the antimycins was therefore undertaken.

In the meantime Yonehara, *et al.*, isolated blastmycin,[10] which proved to consist essentially of antimycin A_3,[11] and carried out an extensive investigation of its chemistry,[11] similar to that reported earlier from this Laboratory, as well as in the present account.

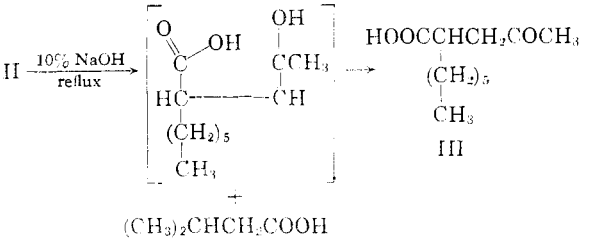
Mild alkaline hydrolysis of antimycin A_1 with 5% aqueous sodium hydroxide at room temperature yielded not only antimycic acid and the "neutral fragment," but also N-formylantimycic acid (blastmycic acid, IV). In contrast to earlier procedures, saponification of the purer neutral product II was carried out by stirring with 10% aqueous sodium hydroxide at room temperature until the lactone had completely dissolved. From this reaction there were obtained two products after acidification, one of which was identified as isovaleric acid. The other product, $C_{11}H_{20}O_3$ (V), isomeric with the keto acid III previously obtained, was formulated as an hydroxy lactone, formed by simple saponification of the β-acyloxy group.

Further degradation of compound V was accomplished by alkaline periodate oxidation. This treatment gave rise to a volatile carbonyl compound which was identified as acetaldehyde. The residual solution after removal of the acetaldehyde was treated with silver oxide and from the mixture, *n*-hexylmalonic acid (VI) was isolated. These results, apart from substantiating the earlier generalized structural assignment to the "neutral fragment,"[4,8] establish the structure of compound V as α-*n*-hexyl-β-hydroxy-γ-methyl-γ-butyrolactone, and that of the neutral fragment II from antimycin A_1 as the isovaleryl ester of V



II, R = $OOCCH_2CH(CH_3)_2$
V, R = OH

These findings also suggest that the keto acid III be formulated as α-*n*-hexyllevulinic acid. As suggested earlier,[8] hydrolytic cleavage of the "neutral fragment" under more drastic alkaline condi-



$(CH_3)_2CHCH_2COOH$

(1) Paper VII, F. S. Okumura, M. Masumura and T. Horie, THIS JOURNAL, **81**, 5215 (1959).

(2) Presented in part at the 136th Meeting of the American Chemical Society, Atlantic City, N. J., September, 1959, Abstracts p. 16-C. Supported in part by grant G-3527 from the National Science Foundation.

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(10) K. Watanabe, T. Tanaka, K. Fukuhara, N. Miyairi, H. Yonehara and H. Umezawa, *J. Antibiotics (Japan), Ser. A*, **10**, 39 (1957).

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tions involves to a greater extent β-elimination of the β-acyloxy substituent followed by conversion of the resulting unsaturated product to the observed γ-keto acid.

Antimycin A_1 and A_3 have thus been shown to be isovaleryl esters,[12] and differ only in the nature of the alkyl side chain appearing in the lactone degradation product (II): *n*-hexyl in A_1 and *n*-butyl in A_3. The present results complete the elucidation of the structures of the primary cleavage products of antimycin A_1.

Experimental

Separation of Subcomponents.—Five grams of antimycin complex m.p. 138–139° was subjected to countercurrent distribution as previously described.[9] A distribution curve was prepared, using the results of dry weight measurements of every tenth tube of a 675 transfer distribution. On the basis of this curve the tubes containing the A_1 and A_3 peaks were combined and the crystalline antibiotics recovered in the manner previously described. A yield of 1.12 g. of A_1 (m.p. 149–150°) and 0.72 g. of A_3 (m.p. 171–173°) was obtained.

Alkaline Hydrolysis of Antimycin A_1.—A sample of 2.2 g. of A_1 was mixed with 10 ml. of 5% aqueous sodium hydroxide solution at room temperature, and the mixture stirred for 7 minutes. At the end of this time the solid material had dissolved, and a layer of oily liquid floated on the top of the solution. The oil was extracted 3 times with 20-ml. portions of petroleum ether (b.p. 28–38°). After drying the extract over anhydrous sodium sulfate and removing the solvent, 1.12 g. of oily liquid was obtained. This degradation product is referred to as the "neutral fragment," compound II.

The aqueous solution was acidified to *p*H 2 with dilute hydrochloric acid, and a tan-colored precipitate formed. This product was filtered off and, after drying, weighed 280 mg. After crystallization from absolute methanol, it melted at 193–195°[13] and showed no depression with authentic antimycic acid (m.p. 220°).

The filtrate remaining after removal of the tan precipitate was extracted 3 times with ether using 20-ml. portions each time. Removal of the solvent left 500 mg. of a crude crystalline product which after crystallization from ethanol yielded needle-like crystals, m.p. 141.5–142.5°. *Anal.* Calcd. for $C_{12}H_{14}N_2O_6$: C, 51.06; H, 5.00; N, 9.93. Found: C, 51.84; H, 5.56; N, 9.34. The mixed melting point with authentic blastmycic acid,[11,14] m.p. 140°, was 140–142°. Both ultraviolet and infrared spectra of this product were identical with those of blastmycic acid. The same acid was also obtained by similar degradation of antimycin A_3.

Saponification of "Neutral Fragment" II.—The 1.12. g. of oily neutral product obtained as described above was vigorously stirred for 23 hours at room temperature with 10 ml. of 10% aqueous sodium hydroxide solution. At the end of this time the oil had completely dissolved. The mixture was then acidified to *p*H 2 with dilute hydrochloric acid and extracted 4 times with 15-ml. portions of ether. After drying the ether extract over anhydrous sodium sulfate and removing the solvent, a colorless oil remained.

The above oily product was fractionally sublimed at a pressure of approximately 0.01 mm. to yield two products which were collected on a cold trap cooled with acetone–Dry Ice freezing mixture. The first product was collected at a bath temperature below 40° and amounted to 370 mg. of colorless oily acidic material. The second sublimation product was obtained at a bath temparature of 40–65° and comprised 320 mg. of a white solid, compound V.

Identification of Isovaleric Acid.—The above oily sublimate was twice resublimed at 0.01 mm. and 30–40°. On paper chromatograms in a solvent system of 1-butanol–1.5 N ammonia (1:1 by volume) it showed the same R_f value as both isovaleric acid and methylethylacetic acid. After methylation with diazomethane the resulting ester showed a retention time of 13.25 minutes on a 2-meter gas chromatogram column composed of diisodecyl phthalate distributed on Celite. The column was operated at 146° and helium was used as the carrier gas. Under the same conditions the retention time for methyl isovalerate was 13.50 minutes and that for the methyl ester of methylethylacetic acid was 13.00 minutes. Thus both the paper and gas chromatographic results were indecisive. The infrared spectrum of the resublimed acid, however, was identical with that of isovaleric acid but differed from that of DL-methylethylacetic acid.

The *p*-bromophenacyl ester of the unknown acid was prepared in the usual way and crystallized without difficulty. This derivative melted at 64–65° and when mixed with the corresponding ester of authentic isovaleric acid, m.p. 65–66°, melted at 65–66°. The *p*-bromophenacyl ester of DL-methylethylacetic acid melted at 52.5-53.5° and the mixed melting point with the derivative of the unknown acid was 40–45°. The corresponding values for the L-acid were 53.6–54° and 41–47°. The infrared spectrum of the *p*-bromophenacyl ester of the unknown acid was identical with that of isovaleric acid but different from that of methylethylacetic acid.

Oxidative Degradation of Compound V.—The 320 mg. of white solid sublimate was recrystallized from an ether–petroleum ether mixture to a constant melting point of 62–62.5°. The yield of the purified product was 205 mg. The infrared spectrum of this compound showed major bands at 2.95, 3.40, 5.70, 6.85, 8.50 and 9.42 μ. The product was soluble in ether, ethanol, chloroform, and warm 5% aqueous sodium bicarbonate solution but insoluble in petroleum ether and in water.

Anal. Calcd. for $C_{11}H_{20}O_3$: C, 65.96; H, 10.06; sapon. equiv., 200.27; mol. wt., 200.27. Found: C, 65.48; H, 9.75; sapon. equiv., 209; mol. wt. (Rast), 181; C-methyl, 1.8 moles.

A quantity of 150 mg. of purified compound V was dissolved in 4 ml. of 1 *N* sodium hydroxide solution in a 50-ml. distillation flask. The mixture was saturated with carbon dioxide and 4 ml. of 0.54 *M* periodic acid solution added. The flask was then wrapped in aluminum foil, connected to a condenser for downward distillation, and a trap containing a solution of 2,4-dinitrophenylhydrazine sulfate attached to the condenser outlet. After the mixture was allowed to stand at room temperature for 1 hour, distillation was carried out at 15 mm. pressure. A precipitate which formed in the 2,4-dinitrophenylhydrazine solution was filtered off and recrystallized from methanol to a constant melting point of 153–155°.

Anal. Calcd. for $C_8H_8N_4O_4$: C, 42.86; H, 3.59; N, 24.99. Found: C, 43.23; H, 3.45; N, 24.0.

This product showed the same R_f value (0.26) as the 2,4-dinitrophenylhydrazone of acetaldehyde when subjected to paper chromatography in a solvent system of 1-heptane–methanol (2:1 by volume). The infrared spectra of the two hydrazones were identical and the mixed melting point showed no depression.

The residual solution remaining after distillation was diluted with 2 ml. of 10% aqueous sodium hydroxide solution, and 1 g. of powdered silver oxide was added. The mixture was refluxed for 8 hours, filtered, and the residue washed twice with small portions of water. The combined filtrate and washings were acidified with sulfuric acid, and the turbid solution extracted continuously with ether for 40 hours. Removal of the solvent left a solid residue which was crystallized from a mixture of chloroform and petroleum ether to a constant melting point of 102–104°.

Anal. Calcd. for $C_9H_{16}O_4$: C, 57.43; H, 8.58. Found: C, 56.47; H, 8.43.

Synthetic *n*-hexylmalonic acid[15] melted at 103–105°. The mixture of the synthetic and isolated acids melted at 102–105°. Likewise the infrared spectra (taken in chloroform) of the two samples were identical.

Preparation of Keto Acid III.—A small sample of the neutral oil II was refluxed with 10% aqueous sodium hydroxide solution for 1.5 hr., after which time the mixture was acidified to *p*H 3 with sulfuric acid and extracted with ether.

(12) The L-(+)-methylethylacetic acid previously obtained[4] presumably must have been derived from one of the other components of the antimycin complex.

(13) In another experiment antimycic acid, m.p. 213–215°, mixed m.p. 216–217°, resulted when antimycin A_1 was warmed for 10 minutes with 5% aqueous sodium hydroxide solution. The low m.p. observed here may have been due to contamination with blastmycic acid, but this point was not investigated.

(14) We wish to thank Dr. H. Yonehara for samples of blastmycic acid.

(15) We wish to thank J. P. Dickie for synthesizing this acid.

Removal of the solvent left a liquid residue which readily formed a crystalline derivative with 2,4-dinitrophenylhydrazine. The product was recrystallized to a constant m.p. 124–125°. *Anal.* Calcd. for $C_{17}H_{24}N_4O_8$: C, 53.70; H, 6.36; N, 14.72; mol. wt. 380.4. Found: C, 53.76; H, 6.26; N, 14.80; mol. wt. (Rast) 366. These values correspond to the formula $C_{11}H_{20}O_3$ for the ketoacid III. An acid of the same formula was previously isolated from antimycin hydrolysates as the 2,4-dinitrophenylhydrazone of the ethyl ester.[4]

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Cycloalkyl Analogs of Pantothenic Acid

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Cyclopentane- and cyclohexaneglyoxylic acids were condensed with formaldehyde and the hydroxymethyl analogs formed were then converted to the corresponding α-ketolactones. Reduction of the α-keto grouping to an alcohol produced cycloalkyl analogs of pantolactone. The lactone of the cyclopentane derivative, α-hydroxy-1-(hydroxymethyl)-cyclopentaneacetic acid, was then condensed with β-alanine to form the pantothenic acid analog. The latter compound, N-[α-hydroxy-1-(hydroxymethyl)-cyclopentaneacetyl]-β-alanine, was isolated as a crystalline derivative through formation of its cinchonine salt. The cyclopentane analog of pantothenic acid inhibited the growth of *Streptococcus lactis* 8039 and *Lactobacillus arabinosus* 17-5, and the inhibitions were competitively reversed by pantothenic acid. The inhibition indices for half-maximal growth of these two organisms were about 2500 and 3300, respectively.

Although modifications of the β-alanine moiety of pantothenic acid have frequently resulted in inhibitory analogs,[2] only substitution on the γ-carbon of the pantoyl (α,γ-dihydroxy-β,β-dimethylbutyryl-) portion has resulted in effective antagonists of pantothenic acid, *i.e.*, ω-methylpantothenic acid.[3] Substitution of an ethyl group in place of one of the β-methyl groups of the pantoyl moiety resulted in analogs which possessed the ability to replace pantothenic acid[4]; the D-forms of the two possible diastereoisomers with configurations resembling isoleucine and alloisoleucine are 62 and 27%, respectively, as effective as pantothenic acid in promoting the growth of *Streptobacterium plantarum* 10 S (*Lactobacillus plantarum*[5]). More recently, a corresponding analog with both β-methyl groups of the pantoyl grouping replaced by ethyl groups has been prepared, and found to be about one-thousandth as active as pantothenic acid in promoting the growth of *S. plantarum* and *Saccharomyces carlsbergensis*.[6] Thus, it appears that the introduction of an additional methyl group on the β-methyl groups of the pantoyl moiety produces analogs which possess the growth-promoting activity of pantothenic acid rather than inhibitory properties.

In the case of the β,β-diethyl analog of pantothenic acid described above, the effect of joining the ends of the ethyl groups to form a cyclopentane ring would be of interest since this ring system has been demonstrated to be structurally similar to the *sec*-butyl grouping in isoleucine. In the latter instance, substitution of the cycloalkyl group for the *sec*-butyl grouping results in the formation of a competitive metabolite antagonist.[7] Although it might be anticipated that the planar cyclopentane derivative would be less sterically hindered than the diethyl analog in performing the functions of pantothenic acid, the ring system might ultimately prevent the analog from performing the normal functions of pantothenic acid, without affecting its ability to compete with pantothenic acid for enzymatic sites involved in its utilization. Accordingly, the structural modification containing a cyclopentane ring, and several related compounds containing a cyclohexane ring, were prepared and their biological properties were determined. In contrast to the slight growth-promoting effects of "diethylpantothenic acid,"[6] the cyclopentane analog inhibited the utilization of pantothenic acid by certain lactobacilli.

The pantoic acid derivatives were prepared as indicated in the accompanying equations by condensing the appropriate cycloalkanone with hippuric acid to form the corresponding 2-oxazoline-5-one derivatives (I) which were subsequently hydrolyzed to the α-keto acid analogs[8] II. The α-keto acids were then treated with formaldehyde to yield a reaction mixture containing the hydroxymethyl derivatives III which were subsequently lactonized to the α-keto-γ-lactones IV. Catalytic reduction of the α-keto grouping produced the desired cycloalkyl analogs of pantolactone (V). The cyclopentane analog (V), (n = 4) was finally condensed with β-alanine to produce the corresponding pantothenic acid analog (VI). The hydroxymethyl derivatives III were not isolated, but were converted directly to the γ-lactone by acidification of the reaction mixture followed by gentle warming over a steam-cone. An ether extraction of the latter reaction mixture under controlled *p*H conditions, followed by a vacuum distillation using a cold-finger, yielded the cyclohexane analog of IV (n = 5). The structure of this derivative was confirmed by elemental analysis and by conversion to the quinoxaline derivative using *o*-phenylenediamine. The interaction of cyclopentaneglyoxylic

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