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Determination of Structure of β,δ-Dihydroxy-β-methylvaleric Acid

BY DONALD E. WOLF, CARL H. HOFFMAN, PAUL E. ALDRICH, HELEN R. SKEGGS, LEMUEL D. WRIGHT AND KARL FOLKERS

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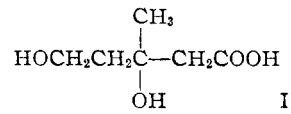
The structure of a new acetate-replacing factor for lactobacilli obtained from distillers' solubles has been established as β,δ-dihydroxy-β-methylvaleric acid. The DL-acid has been synthesized by an unequivocal procedure.

A new acetate-replacing factor for lactobacilli has been discovered[1] and a method for its isolation from distillers' solubles has been published.[2] The investigation of its chemical nature has resulted in the determination of its structure as β,δ-dihydroxy-β-methylvaleric acid (I).[3] The generic name, mevalonic acid, has now been assigned to this substance.[4]



I

The present paper describes the observations from which this structure was deduced, and the confirmation of this structure by synthesis.

The most highly purified preparation of the new factor was a nearly colorless oil, very water soluble, but also soluble in organic solvents, especially polar ones. No ultraviolet absorption was found for the factor, but its infrared absorption behavior was informative. In chloroform solution, strong bands at 2.90 to 2.95 μ (hydroxyl-function) and at 5.78 μ (ester function) were observed. When morpholine was used as a solvent and the infrared absorption followed for 48 hours, the band at 5.78 μ decreased in magnitude and a band at 6.1 μ (carboxyl function) appeared and increased in intensity.

One of the first chemical properties noted for the factor was its acidic character which accounted for its adsorption on anionic exchange resins.[2] The titration curve (Fig. 1) was characteristic of a lactone and indicated an equivalent weight of 128 with a $pH_{1/2}$ value of 4.3. It was a slightly stronger acid than unsubstituted lower aliphatic acids.

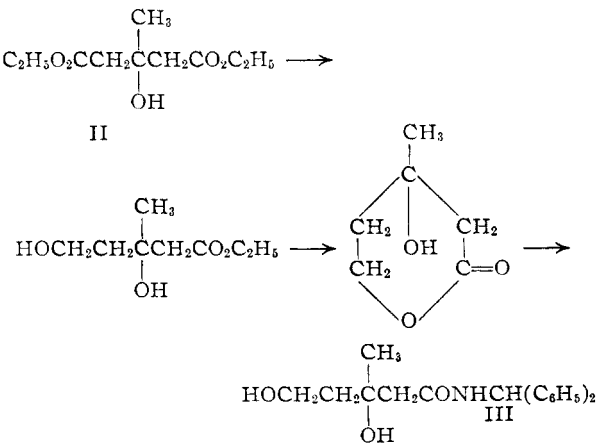
Several attempts to prepare amides with common amines gave neutral but non-crystalline products. The first suitable crystalline derivative was obtained with benzhydrylamine. The benzhydrylamide was found to have one C-methyl group and to form a monoacetate. Both compounds gave analytical data which indicated that the lactone had the formula $C_6H_{10}O_3$.

Treatment with *p*-nitrobenzoyl chloride in pyridine did not give a crystalline derivative, but caused darkening and decomposition. The benzhydrylamide derivative was resistant to periodate oxidation and also to alkaline iodine under conditions for iodoform formation.

These observations led to several restrictive and indicative conclusions concerning the structure.

The infrared absorption data were in better agreement with a δ-lactone than a γ-lactone. For a formula of $C_6H_{10}O_3$, a methyl group and a hydroxyl group remained for assignment of position. It was unlikely that the methyl group is terminal because of the negative iodoform test. The *pK* of the acid was such that an α-hydroxy acid structure is unlikely. A γ-hydroxy structure is impossible because of the lack of reaction with periodate. There remained of the considered structures, the β-methyl-β-hydroxy-δ-lactone as the most likely C_6-structure. The lability of the compound, and the fact that only a monoacetate of the benzhydrylamide was obtained are best explained by the presence of a tertiary hydroxyl group.

The proof of structure by synthesis was achieved by an unequivocal method. β-Hydroxy-β-methylglutaric ester (II) was reduced stepwise by lithium aluminum hydride and by catalytic hydrogenation to give β-hydroxy-β-methyl-δ-valerolactone which was isolated as the benzhydrylamide (III).



The benzhydrylamide of the DL-lactone was hydrolyzed with alkali to give the free acid for microbiological testing. The DL-acid has about one-half the microbial activity of the optically active isomer prepared from distillers' solubles.

Experimental

β,δ-Dihydroxy-β-methylvaleric Acid Benzhydrylamide.—A crystalline derivative was prepared by combining 59 mg. of the active concentrate[2] (70 units/mg. potency) with about 250 mg. of benzhydrylamine and heating the mixture at 100° for one hour in a flask equipped with a drying tube. The reaction mixture was dissolved in 6 ml. of chloroform

(1) H. R. Skeggs, L. D. Wright, E. L. Cresson, G. D. E. MacRae, C. H. Hoffman, D. E. Wolf and K. Folkers, *J. Bact.*, **72**, 519 (1956).

(2) L. D. Wright, E. L. Cresson, H. R. Skeggs, G. D. E. MacRae, C. H. Hoffman, D. E. Wolf and K. Folkers, THIS JOURNAL, **78**, 5273 (1956).

(3) D. E. Wolf, C. H. Hoffman, P. E. Aldrich, H. R. Skeggs, L. D. Wright and K. Folkers, *ibid.*, **78**, 4499 (1956).

(4) The name "divalonic acid" was originally used to designate this new factor. Previous use of this name in the literature was overlooked when a search was made. The name "mevalonic acid" is now being used to designate β,δ-dihydroxy-β-methylvaleric acid.

and the excess base removed by washing with 0.1 *N* hydrochloric acid, then with water until the water extract was nearly neutral. The chloroform solution containing the benzhydrylamide was evaporated at reduced pressure leaving a yellow oil weighing 71 mg. The yellow oil was dissolved in hot benzene and the solution was diluted with petroleum ether (Skellysolve C) until slightly cloudy. On standing, a crystalline precipitate was formed. Recrystallization from the same solvent mixture gave the pure benzhydrylamide, m.p. 92–93°, $[\alpha]^{20}D$ −2.0 (*c* 20 mg./ml. in ethanol).

Anal. Calcd. for $C_{19}H_{23}NO_3$: C, 72.82; H, 7.40; N, 4.47; 1 C-methyl, 4.80. Found: C, 72.70, 72.60; H, 7.17, 7.07; N, 4.74; C-methyl, 5.9.

The benzhydrylamide of the DL-lactone acid was found to have very low activity in the microbiological assay with *Lactobacillus acidophilus* ATCC 4963.[1] To conduct assays with the amide, it was necessary to hydrolyze to liberate the free acid and assay the hydrolyzate. High "recovery" of microbiological activity was obtained by hydrolysis in 0.25 *N* aqueous sodium hydroxide at 120° for one hour. Under these conditions, a sample of the DL-benzhydrylamide showed 75.9 units/mg. activity. This corresponds to 183 units/mg. activity for the β-hydroxy-β-methyl-δ-valerolactone on an equivalence basis. From the average results of numerous assays the activity of this lactone has been established at 180 units/mg.

δ-Acetoxy-β-hydroxy-β-methylvaleric Acid Benzhydrylamide.—Fifty milligrams of the benzhydrylamide of β,δ-dihydroxy-β-methylvaleric acid was dissolved in 0.5 ml. of pyridine and 0.3 ml. of acetic anhydride was added. The reaction mixture was heated at 60° for 30 minutes. The excess reagents were evaporated at reduced pressure leaving the product as an oil which crystallized on standing. It was purified by recrystallization from a mixture of benzene and petroleum ether (Skellysolve C). The pure δ-acetoxy-β-hydroxy-β-methylvaleric acid benzhydrylamide crystallized in colorless needles, m.p. 104–105°, $[\alpha]^{20}D$ +1.6° (*c* 45 mg./ml. in ethanol).

Anal. Calcd. for $C_{21}H_{25}NO_4$: C, 70.97; H, 7.09; acetyl, 12.1. Found: C, 70.70, 70.80; H, 7.09, 6.87; acetyl, 11.4.

DL-β-Hydroxy-β-methyl-δ-valerolactone.—The diethyl β-hydroxy-β-methylglutarate was prepared by the method of Adams and Van Duuren.[5] A solution of 2.18 g. (10 millimoles) of this ester in 15 ml. of anhydrous ether was cooled to −30 to −40° in a flask protected from moisture. Then 5.9 ml. of a solution containing 5 millimoles of lithium aluminum hydride in ether was added slowly, while the mixture was agitated and kept cooled. The mixture was allowed to stand without further cooling for about two hours. At the end of this time, the reaction mixture was agitated with 2 ml. of water, and then treated with anhydrous magnesium sulfate to remove excess moisture.

The ethereal solution was decanted and discarded. It contained only a trace of DL-β,δ-dihydroxy-β-methylvaleric acid as determined microbiologically after saponification.

The residue, including the magnesium sulfate, was treated with 100 ml. of water, cooled and made acidic with dilute hydrochloric acid. It was evaporated at reduced pressure leaving an acidic residue which was extracted with 100 ml. of boiling chloroform. The chloroform was filtered and evaporated at reduced pressure leaving 0.5 g. of an oil which contained some ethyl γ-formyl-β-hydroxy-β-methylbutyrate as indicated by a test with Schiff reagent. It was hydrogenated using 0.1 g. of platinum (oxide) catalyst at 1 to 2 atm. pressure for two hours. The catalyst was removed and the solution evaporated to give the ethyl β,δ-dihydroxy-β-methylvalerate as an oil.

This ester was saponified by autoclaving it with 50 ml. of 0.1 *M* barium hydroxide solution at 120° for one hour. The excess barium ion was precipitated by saturation with carbon dioxide. The barium carbonate was removed and the filtrate evaporated at reduced pressure leaving the barium salt as a solid residue. This was dissolved in methanol, and after filtration, acetone was added to reprecipitate it. The nearly white solid weighed 490 mg. The barium salt

(5) R. A. Adams and B. L. Van Duuren, THIS JOURNAL, **75**, 2377 (1953).

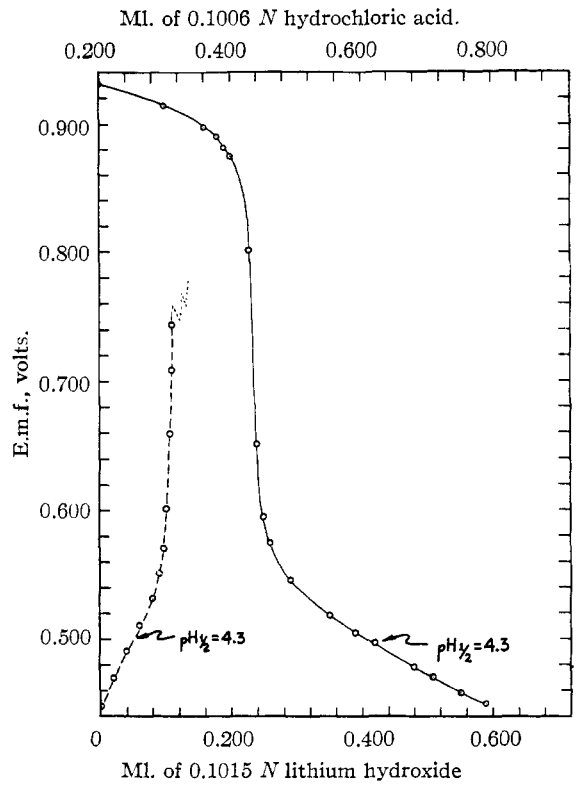


Fig. 1.—Potentiometric titration of mevalonic acid lactone: dashed line, titration with alkali; solid line, subsequent back-titration with acid.

was dissolved in water and dilute sulfuric acid was added until the solution was slightly acid to test paper. The last traces of barium ion were removed by passage of the solution over a resin column of Amberlite IR120(H^+). The column filtrate was evaporated, leaving the DL-β-hydroxy-β-methyl-δ-valerolactone as a clear, slightly brown oil weighing 220 mg.

DL-β-δ-Dihydroxy-β-methylvaleric Acid Benzhydrylamide.—The synthetic lactone was converted to the benzhydrylamide by treatment with 0.4 g. of benzhydrylamine at 100° for 1.5 hours in a flask protected from moisture. The reaction mixture was dissolved in chloroform and extracted successively with dilute hydrochloric acid, water, dilute sodium bicarbonate solution and water. The chloroform solution was dried over anhydrous magnesium sulfate, filtered and evaporated at reduced pressure. The residue was dissolved in benzene and diluted with petroleum ether (Skellysolve C). The crystalline product was twice recrystallized, m.p. 93–95°.

Anal. Calcd. for $C_{19}H_{23}NO_3$: C, 72.82; H, 7.40; N, 4.47. Found: C, 72.22; H, 7.43; N, 4.33.

Under the hydrolysis conditions described above a sample of DL-β,δ-dihydroxy-β-methylvaleric acid benzhydrylamide was first hydrolyzed and the hydrolyzate was then assayed microbiologically using *L. acidophilus* ATCC 4963.[1] It showed an activity of 38 units/mg. after hydrolysis; this corresponds to 91 units/mg. calculated on the basis of the β-hydroxy-β-methyl-δ-valerolactone content.

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RAHWAY, NEW JERSEY