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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[CONTRIBUTION FROM THE CLAYTON FOUNDATION BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS]

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

(1) Rosalie B. Hite pre-doctoral fellow 1957–1959.

(2) R. J. Williams, R. E. Eakin, E. Beerstecher, Jr., and W. Shive, "The Biochemistry of B Vitamins," Reinhold Publishing Corp., New York, N. Y., 1950, pp. 620–651.

(3) W. Drell and M. S. Dunn, THIS JOURNAL, **70**, 2057 (1948).

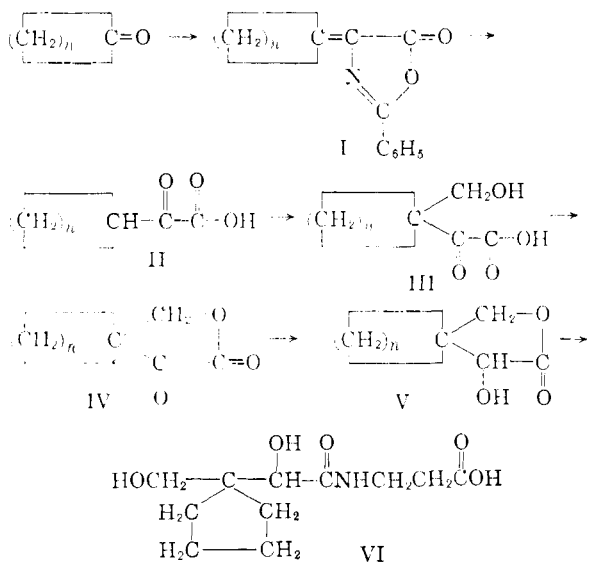
(4) T. Wieland and E. F. Möller, *Ber.*, **81**, 316 (1948).

(5) Bergey's "Manual of Determinative Bacteriology," 7th Ed., The Williams and Wilkins Co., Baltimore, Md., 1957, p. 549.

(6) T. Wieland and W. Maul, *Biochem. Z.*, **326**, 18 (1954).

(7) W. Shive and C. G. Skinner, *Ann. Rev. Biochem.*, **27**, 643 (1958).

(8) J. D. Fissekis, C. G. Skinner and W. Shive, THIS JOURNAL, **81**, 2715 (1959).



acid (II, n = 4) with formaldehyde produced a reaction mixture which contained not only IV, but a large amount of V as well, indication that the formaldehyde was acting as a reducing agent during these condensations. In the case of the cyclohexane analog, the two products could be separated; however, isolation of IV *per se* from the cyclopentane reaction products proved to be difficult, and it was identified in the reaction mixture by conversion to a 2,4-dinitrophenylhydrazone derivative. In later experiments, the formaldehyde reaction product in the cyclopentane series was not isolated, but the reaction mixture was reduced directly to yield exclusively the hydroxy-lactone V (n = 4).

The cyclopentane analog V (n = 4) was condensed with β-alanine, and the combined isomeric reaction products were isolated as a crystalline cinchonine salt by a procedure similar to that described by Wieland and Maul.[6] The diastereoisomeric forms of the cinchonine salt were not resolved by the procedure employed. A paper chromatogram of the analog–cinchonine salt mixture followed by a bioautograph gave a single zone of inhibited growth. The presence of the pantothenic acid analog either free or as a cinchonine salt could also be detected by heating the paper chromatogram at about 150° for 2 hours, and then developing the paper with ninhydrin reagent. The liberated β-alanine gave a typical ninhydrin reaction which was not present in a comparable unheated sample. The R_f values from the bioautograph and the ninhydrin procedure were identical, 0.85 in *sec*-butyl alcohol:98% formic acid:water (15:2:3).

The intermediate cycloalkyl pantolactone analogs IV and V (n = 4 and 5) were examined in several microbial systems for their ability to replace pantothenic acid, and none of the derivatives were found to promote growth, nor were they observed to possess any inhibitory properties. Further, these derivatives also did not promote growth in salicylic acid-inhibited *Escherichia coli*.[9]

N-[α-Hydroxy-1-(hydroxymethyl)-cyclopentaneacetyl]-β-alanine (VI) was inhibitory to the growth of *Streptococcus lactis* 8039 and *Lactobacillns arabinosus* 17–5, and the inhibitions were reversed by appropriate levels of D(+)-pantothenic acid. The racemic mixture of VI has an inhibition index for half-maximal growth of about 2500 and 3300, respectively, for the latter two microörganisms cited above. For *S. lactis*, the cyclopentane analog VI inhibited growth only to a level corresponding to about 20% of the control growth. This results from the analog having a very slight ability to replace partially the pantothenic acid requirement so that the analog alone, at a sufficiently high concentration, can stimulate some growth in the absence of pantothenic acid. The analog inhibits the utilization of pantothenic acid in a manner such that it reduces the growth of *S. lactis* to a level corresponding to the small amount of growth-promoting activity indicated above. For *L. arabinosus* this weak stimulatory growth effect with VI was not observed.

In contrast to the ability of "diethylpantothenic acid" to replace the functions of pantothenic acid in the growth of several microörganisms,[6] the cyclic derivative VI cannot effectively perform the necessary functions of the vitamin, but does possess the ability to compete for interaction at the appropriate enzyme sites of pantothenic acid. Bonding of the two ethyl groups together in a cyclopentane ring apparently introduces a steric hindrance in the resulting analog which prevents subsequent biochemical reactions from taking place. Since a crude reaction mixture containing the corresponding cyclohexyl derivative did not show any inhibitory properties under similar testing conditions the size of the group and degree of planarity may determine the affinity for the site of pantothenic acid utilization. If this is so, a more planar cyclopentene ring (or cyclobutane or cyclopropane) in place of the slightly puckered[10] cyclopentane ring in the appropriate analog might be expected to induce even more effective antagonism of pantothenic acid utilization.

Experimental[11]

Biological Assays.—A previously reported[9] assay medium was used for the studies with *Lactobacillus arabinosus* 17–5 and *Streptococcus lactis* 8039 except that a solution containing 17 g. per liter of commercial casein hydrolysate[12] was used instead of the acid-hydrolyzed casein as indicated in the reference, and the concentration of the vitamin supplement was increased threefold.

The samples to be studied were weighed into sterile tubes, diluted with sterile water, and added aseptically to the sterile assay tubes without being heated. The assay tubes were incubated at 30° for about 17 hours, and the amount of growth was determined turbidimetrically in terms of galvanometer readings so adjusted that in a particular instrument distilled water read 0 and an opaque object 100.

Lactone 1-hydroxymethylcyclohexaneglyoxylic acid (IV, n = 5) was prepared by two different procedures:

A. A sample of 2.5 g. of cyclohexaneglyoxylic acid, prepared by a previously reported procedure through the condensation of cyclohexanone and hippuric acid,[8] was suspended in 65 ml. of water and the *p*H adjusted to 5 with 0.5 *N* potassium hydroxide solution. The reaction mixture

(9) E. M. Lansford, Jr., and W. Shive, *J. Biol. Chem.*, **194**, 329 (1952).

(10) J. E. Kilpatrick, K. S. Pitzer and R. Spitzer, THIS JOURNAL, **69**, 2483 (1947).

(11) All melting points are uncorrected. The R_f values were determined by the ascending technique. The authors are indebted to Miss S. K. Randall for technical assistance with the microbiological assays, and to Mr. A. G. Lane and Miss J. Morehead for the chemical analyses.

(12) Nutritional Biochemicals Corp., Cleveland 28, O.

was stirred vigorously and kept ice-cold throughout the addition of the alkali; after which it was filtered, and reduced to a small volume *in vacuo*. About 200 ml. of acetone was then added to the residue, and the resulting solution was kept at −8° for several hours. The precipitated potassium salt was filtered, washed with cold acetone and then ether, and finally dried *in vacuo* to yield 2.52 g. of product, R_f 0.80 in propionic acid:water:butyl alcohol (5:7:10).[13] To 2.5 g. of this potassium salt dissolved in 3 ml. of water was added 0.8 ml of 37% formaldehyde solution; the reaction mixture was cooled in an ice-bath and 1.3 g. of potassium hydroxide was added in small portions over a 30-minute period with efficient mechanical stirring. After stirring an additional 2 hours, the reaction mixture was allowed to stand at room temperature overnight; subsequently, 6 ml. of water was added, the solution was acidified with 2 *N* hydrochloric acid to a congo red endpoint, and heated over a steam-cone for about 30 minutes. An oil separated at this stage. The combined reaction mixture was adjusted to *p*H 7.2 with dilute potassium hydroxide solution, and continuously extracted with ether to remove the small amount of hydroxy-lactone present.[14] The aqueous residue was then acidified to *p*H 2 with 2 *N* hydrochloric acid, heated for about 15 minutes over a steam-cone, and finally continuously extracted with ether overnight. After drying the ether phase over sodium sulfate, the solvent was removed *in vacuo* to yield a precipitate which was dried *in vacuo* over phosphorus pentoxide. The product was purified by sublimation at 0.01–0.05 mm. and 65° to yield 600 mg. of crystalline material, m.p. 72–74°.

Anal. Calcd. for $C_9H_{12}O_3$: C, 64.27; H, 7.19. Found: C, 64.27; H, 7.15.

B. Using a procedure similar to an analogous process of Wieland and Möller,[4] 1.35 g. of cyclohexaneglyoxylic acid was taken up in 2 ml. of water and neutralized with 0.60 g. of potassium carbonate while the temperature was maintained between 20 and 30°. To this solution was then added 0.67 ml. of 37% formaldehyde solution, followed by, over a period of about 30 minutes, an additional 2.65 g. of potassium carbonate, and the resulting reaction mixture was kept at between 35–40° for about 20 hours. About 5 ml. of water was then added, the solution was acidified to *p*H 3 with 2 *N* hydrochloric acid, and heated over a steam-cone for 30 minutes. The *p*H was maintained at 3 throughout the heating period by dropwise addition of acid when necessary. The resulting solution was treated as described above to yield 310 mg. of product, recrystallized from Skellysolve B–ether, m.p. 72–74°.

Anal. Calcd. for $C_9H_{12}O_3$: C, 64.27; H, 7.19. Found: C, 64.09; H, 7.32.

Spiro{1′-cyclohexane-3-[2,3-dihydrofuro(2,3-b)]quinoxaline}.—The structure of the ketoacid lactone described above was further substantiated by formation of the corresponding quinoxaline derivative. A mixture of 100 mg. of IV (n = 5) and excess *o*-phenylenediamine dissolved in dilute ethyl alcohol containing a few cc. of dilute hydrochloric acid was heated over a steam-cone for 30 minutes, and filtered while still warm. Upon cooling overnight in the refrigerator there was recovered an essentially quantitative yield of product which was twice recrystallized from ethanol to yield white needles which become soft at about 120° and melt at 161°.

Anal. Calcd. for $C_{15}H_{16}N_2O$: C, 75.00; H, 6.71; N, 11.66. Found: C, 75.10; H, 6.74; N, 11.31.

Lactone of α-Hydroxy-1-(hydroxymethyl)-cyclohexaneacetic Acid (V, n = 5).—A sample of 270 mg. of 1-hydroxymethylcyclohexaneglyoxylic acid lactone was taken up in 50 ml. of ethanol and treated with hydrogen gas at atmospheric pressure in the presence of about 50 mg. of platinum oxide for 2 hours. The catalyst was filtered, and the filtrate was reduced to an oily residue *in vacuo*. The residue was then taken up in ether, Skellysolve B (b.p. 60–68°) was added until a slight turbidity persisted, and the mixture was left overnight in a deep freeze. There was obtained 85 mg. of white needles, m.p. 66°.

Anal. Calcd. for $C_9H_{14}O_3$: C, 63.51; H, 8.29. Found: C, 63.22; H, 8.34.

(13) The procedure used for the demonstration of the presence of α-keto acids is given by T. Wieland and E. Fisher, *Naturwiss.*, **36**, 219 (1949).

(14) R. Kuhn and T. Wieland, *Ber.*, **75A**, 121 (1942).

Lactone of α-Hydroxy-1-(hydroxymethyl)-cyclopentaneacetic Acid (V, n = 4).—A sample of cyclopentaneglyoxylic acid prepared as previously reported from 20 g. of α-benzamido-β-cyclopentylideneacetic acid[8] was suspended up in 70 ml. of ice-cold water and taken to *p*H 5 with cold 0.5 *N* potassium hydroxide with efficient stirring. The resulting mixture was filtered, the filtrate was reduced to a small volume *in vacuo*, 200 ml. of acetone–ether (1:1) was added, and the resulting mixture was allowed to stand at −8° overnight. There was recovered 6.5 g. of precipitated potassium salt which was dried *in vacuo* over phosphorus pentoxide. To a cold solution of 3 g. of potassium cyclopentaneglyoxylate in 3 ml. of water was added 2 g. of 37% formaldehyde, followed by the addition of 1.3 g. of potassium hydroxide over a period of about 30 minutes. The reaction mixture was stirred an additional two hours in the cold, and finally left to stand at room temperature overnight. After the addition of 5 ml. of water, the mixture was adjusted to *p*H 2 with 2 *N* hydrochloric acid and heated over a steam-cone for about 1 hour to yield a clear solution. This aqueous solution was extracted continuously with ether for 48 hours, the ether phase was reduced to dryness *in vacuo*, and the resulting residue was dried *in vacuo* over phosphorus pentoxide and potassium hydroxide. The resulting material was sublimed (0.06–0.02 mm. at 60°) to yield 1.49 g. of product, m.p. 78–80°. Recrystallization from ether–Skellysolve B yielded white needles, m.p. 80–82°.

Anal. Calcd. for $C_8H_{12}O_3$: C, 61.52; H, 7.74. Found: C, 61.57; H, 7.58.

In an alternate experiment in which a stoichiometric amount of 37% formaldehyde was added to the potassium salt, the product ultimately isolated after ether extraction was a mixture of the keto- and hydroxy-lactone. The pure keto-lactone could not easily be isolated in a chemically pure form; however, its presence was demonstrated by the formation and isolation of its 2,4-dinitrophenylhydrazone from the crude reaction mixture, m.p. 188° (*Anal.* Calcd. for $C_{14}H_{14}N_4O_6$: C, 50.30; H, 4.22; N, 16.76. Found: C, 50.05; H, 4.48; N, 16.76.) Hydrogenation of this alternate reaction product over platinum black at atmospheric pressure gave the hydroxy-lactone V (n = 4), m.p. 80–82°.

N-[α-Hydroxy-1-(hydroxymethyl)-cyclopentaneacetyl]-β-alanine (VI).—To a reaction mixture containing 25 mg. of sodium in 2.3 ml. of methanol was added 90 mg. of β-alanine; after which, 157 mg. of lactone of α-hydroxy-1-(hydroxymethyl)cyclopentaneacetic acid dissolved in 1.5 ml. of methanol was added, and the mixture was heated to reflux for about 8 hours. The solvent was removed *in vacuo*, and the white glass-like residue was dissolved in water and charged to a 1 × 28 cm. Dowex 50 column. The column was eluted with water in 15-ml. fractions, and those fractions which gave a positive ninhydrin test[15] after heating were combined. These combined fractions were taken to dryness *in vacuo* to yield 130 mg. of a waxy residue which contained a small amount of free β-alanine contaminant as evidenced by paper chromatography. This product was used for the microbiological assays, and its R_f value in *sec*-butyl alcohol:95% formic acid:water (15:2:3) was 0.85. In an effort to obtain a satisfactory elemental analysis on this product, it was taken up in 10 ml. of water, and treated with an equivalent weight of powdered cinchonine.[6] The reaction mixture was heated for about 10 minutes over a steam-cone, evaporated to dryness *in vacuo*, the residue was taken up in 50 ml. of hot 3-pentanone, filtered, and again taken to dryness. The oily residue was repeatedly taken up in a small amount of acetone and the solvent removed *in vacuo* until the residue became a glass-like white solid. This solid was recrystallized from acetone–ether to yield small feather-like crystals of the cinchonine salt of the pantothenic acid analog, m.p. 207–210°.

Anal. Calcd. for $C_{30}H_{41}N_3O_6$: C, 66.77; H, 7.66; N, 7.79. Found: C, 66.92; H, 7.07; N, 7.80.

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(15) A spot of the eluate was placed on a piece of filter paper, and the paper was heated at 150° for two hours before spraying with ninhydrin reagent. The test was negative or very weak before heating; but it became positive after heating for those fractions which contained the pantothenic acid derivative because of its thermal breakdown to β-alanine.