ammonium hydroxide to effect solution followed by acidification. Thus, 5.9 g. of *dl*-biotin was dissolved in 50 cc. of dilute ammonium hydroxide. The solution was filtered and acidified to congo red with hydrochloric acid. The *dl*-biotin was removed by filtration, washed with water and dried; m. p. 232–233°; yield, 5.6 g. (95%). When the solution was acidified while hot, the *dl*-biotin crystallized in long needles.

(c) *dl-epi*-Allobiotin.—One and one-half grams of *dl-epi*-allodiamido acid, m. p. 189–190°, was hydrolyzed with barium hydroxide and treated with phosgene as described for *dl*-biotin. After one recrystallization from water, the yield of *dl-epi*allobiotin was only 0.2 g. (22%); this compound decomposed gradually above 195° without melting.

Anal. Calcd. for $C_{10}H_{16}N_2O_3S$: C, 49.16; H, 6.60; N, 11.46. Found: C, 49.23; H, 6.75; N, 11.21.

Preparation of Samples of the Diamido Acids or Esters for Microbiological Assay.—3,4-Dibenzamidotetrahydro-2-thiophenevaleric acid,[6] prepared from biotin, was used as a standard with which to compare the unknown diamido derivatives under the various hydrolysis conditions. In a typical experiment, 10 mg. of a crude fraction of *dl*-diamido ester was placed in a small Pyrex test-tube and to it was added 200 mg. of finely ground barium hydroxide octahydrate and 1 cc. of water. The tube was sealed and heated at 135–140° for sixteen hours. After opening the tube, the turbid solution and the two 0.5-cc. portions of wash water were transferred to a centrifuge tube. This solution was warmed in a hot water-bath and acidified to congo red with 1 *N* sulfuric acid. The barium sulfate was removed by centrifugation and washed with two 1-cc. portions of hot water. The combined solutions were made neutral with saturated aqueous sodium carbonate and about 1 cc. more saturated sodium carbonate solution was added. The solution was cooled in an ice-bath and treated with phosgene until it gave an acid reaction to congo red. It was then diluted to 10 cc. and suitable dilutions made for assay. The microbiological assay[11] showed 20% yield of biotin. Pure *dl*-diamido ester showed slightly better than 50% biotin activity compared to the standard by this procedure. The *dl*-allodiamido ester and the *dl-epi*-allodiamido ester showed no biotin activity.

Sodium hydroxide was tried also as the hydrolytic agent. About 5 mg. of the *d*-3,4-dibenzamido acid was placed in each of three small Pyrex test-tubes. To the samples was added respectively, 0.5–0.6 cc. of 1, 1.5 and 2 *N* aqueous sodium hydroxide. The sealed tubes were heated at about 140° for fifteen hours. The resulting solution from each tube was washed into a six-inch test tube. After cooling in ice, each sample was treated with phosgene until the solution was acid to congo red when silicic acid precipitated as a gel. Each sample was diluted to 10 cc. with water and an aliquot of 3 cc. was withdrawn, diluted to 10 cc. and filtered for assay. The assay showed the following per cent. recovery of biotin from the various hydrolyses: 22% from 1 *N* sodium hydroxide solution; 78% from both 1.5 and 2 *N* sodium hydroxide solution; 89% from the barium hydroxide hydrolysis described above.

Acknowledgment.—The authors wish to thank Messrs. D. F. Hayman, R. N. Boos, W. R. Reiss and H. S. Clark, and Mrs. Edith Meiss for the microanalyses reported in this paper.

Summary

dl-Biotin and two stereochemically related racemates which are called *dl*-allobiotin and *dl-epi*-allobiotin have been synthesized from 4-benzamido-3-ketotetrahydrothiophene and methyl γ-formylbutyrate.

These two intermediates were condensed to yield 4-benzamido-3-keto-$\Delta^{2,5}$-tetrahydro-2-thiophenevaleric acid methyl ester. This unsaturated keto derivative was converted into two isomeric oximes which were reductively acetylated to yield two products: 3-acetamido-4-benzamido-4,5-dihydro-2-thiophenevaleric acid methyl ester and 3-acetamido-4-benzamido-$\Delta^{2,5}$-tetrahydro-2-thiophenevaleric acid methyl ester. These two products were hydrogenated over a palladium catalyst to yield three racemic forms of 3-acetamido-4-benzamido-tetrahydro-2-thiophenevaleric acid methyl ester. These three diamido esters were first hydrolyzed and then treated with phosgene to yield the three racemates stereochemically related to biotin.

RAHWAY, NEW JERSEY RECEIVED AUGUST 17, 1945

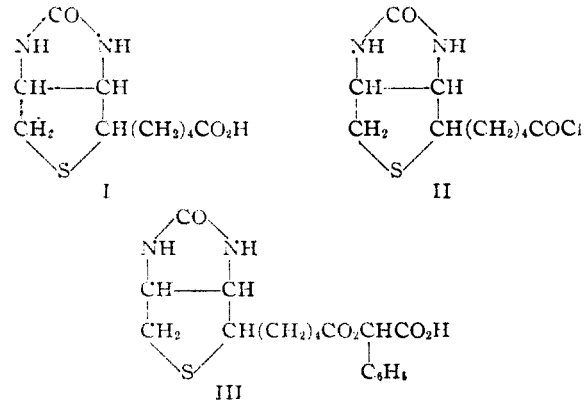
[CONTRIBUTION FROM THE RESEARCH LABORATORIES, MERCK AND CO., INC.]

Biotin. VI. Resolution of *dl*-Biotin

BY DONALD E. WOLF, RALPH MOZINGO, STANTON A. HARRIS, R. CHRISTIAN ANDERSON AND KARL FOLKERS

Biotin was first obtained synthetically[1] by resolution and hydrolysis of the *d*(−)-mandelic acid esters of *dl*-biotin.[2,3] Later the resolution of the *l*(+)-arginine salts of *dl*-biotin gave biotin satisfactorily.

dl-Biotin (I) was converted to its acid chloride (II) with thionyl chloride and the acid chloride was allowed to react with *d*(−)-mandelic acid in chloroform solution to give the corresponding esters (III). The crude esters were then subjected to a series of fractional crystallizations



(1) Harris, Wolf, Mozingo and Folkers, *Science*, **97**, 447 (1943).

(2) Harris, Wolf, Mozingo, Anderson, Arth, Easton, Heyl, Wilson and Folkers, THIS JOURNAL, **66**, 1756 (1944).

(3) Harris, Wolf, Mozingo, Arth, Anderson, Easton and Folkers, *ibid.*, **67**, 2096 (1945).

which yielded the *d*(−)-mandelic acid ester of biotin. The yield was very low because of the similarity in solubility properties of the two diastereoisomeric esters. Hydrolysis of this ester gave biotin. It was found to be identical with the natural product[1] and verified the structure assigned to biotin.

l-Biotin was obtained by fractional crystallization of the esters prepared similarly from *l*(+)-mandelic acid followed by hydrolysis of the *l*(+)-mandelic acid ester of *l*-biotin.

A study was made of a series of optically active bases to find one which would separate biotin from its enantiomorph as a crystalline salt. None of the alkaloids commonly employed for the resolution of racemic acids gave a salt of biotin with the desired properties. A possible explanation for this difficulty is the weak acidity of biotin. From data previously published,[4] it is possible to calculate the acidic ionization constant of biotin which is $K_A = 6.3 \times 10^{-6}$. The relative acidity may be judged by a comparison with acetic acid which has the ionization constant $K_A = 1.75 \times 10^{-5}$. A weak acid such as biotin might easily fail to form stable salts with bases unless the latter were of strong basicity. Therefore, the possibilities for the resolution of *dl*-biotin by way of the salts was correspondingly limited.

A search for a resolving agent of stronger basic properties was then initiated. Previous work in these Laboratories[5] has demonstrated the usefulness of quaternary alkaloid salts for the resolution of weak acids, apparently due to the enhanced basicity of the quaternary ammonium bases. Accordingly, several such bases were studied and it was found that from *dl*-biotin and quinidine methohydroxide a crystalline salt was formed. It was readily purified by recrystallization from a mixture of methanol and acetone and, when decomposed in hydrochloric acid solution, gave nearly pure *l*-biotin. The quinidine metho salt of biotin was isolated from the mother liquors as an oil from which biotin was obtained in a much lower yield and less pure state than its enantiomorph.

Further search for a better method of obtaining biotin from *dl*-biotin led to the study of some of the less commonly employed bases. It was found that *l*(+)-arginine with *dl*-biotin gave a crystalline salt of biotin as the less soluble salt, which separated in 92% yield. Recrystallization of the *l*(+)-arginine salt followed by decomposition yielded biotin in 80% yield without recovery of additional material from mother liquors. This method of resolution of *dl*-biotin to obtain biotin is the most satisfactory one. The use of *l*(+)-arginine as an optically active base for the resolution of racemic acids of this type seems to be new. Its surprising utility for the resolution of *dl*-biotin has encouraged the thought that it may be of more general usefulness, particularly, where weak acids are concerned.

(4) du Vigneaud, Hofmann, Melville and Rachele, *J. Biol. Chem.*, **140**, 763 (1941).

(5) Stiller and Wiley, THIS JOURNAL, **63**, 1237 (1941); Major and Finkelstein, *ibid.*, **63**, 1368 (1941).

Experimental

Resolution of *d*(−)-Mandelic Acid Esters of *dl*-Biotin.—One and two-tenths grams of *dl*-biotin was treated with excess thionyl chloride at slightly below room temperature until all of it was in solution. The excess reagent was removed *in vacuo*. To the residual *dl*-biotin acid chloride, was added a hot solution of 0.9 g. of *d*(−)-mandelic acid in 16 ml. of chloroform. The mixture was agitated until all the residue had dissolved. After standing overnight, the solvent was removed from the ester, leaving it as a gum.

The crude *d*(−)-mandelic acid esters of *dl*-biotin were extracted five or six times with hot ethyl acetate. The ethyl acetate solution was concentrated to dryness *in vacuo* leaving a tan gum. This gum was extracted twice with hot water. The aqueous solution on standing deposited a partially crystalline precipitate. This was recrystallized by dissolving it in hot methanol and diluting the solution with water until it was slightly cloudy. Crystallization occurred giving an ester melting at 100–124°. Two further recrystallizations gave a product melting at 181–189° (micro-block). From 110 mg. of this crude ester, 67 mg. of crude biotin, m. p. 225–229° (micro-block), was obtained by hydrolysis with 5% aqueous sodium hydroxide at 70° for thirty minutes. After four recrystallizations from water, the biotin was pure and melted at 230–231° (micro-block); $[\alpha]^{25}D$ +90.7° (*c*, 2.04 in 0.1 *N* sodium hydroxide); yield 54 mg.

Anal. Calcd. for $C_{10}H_{16}N_2O_3S$: C, 49.16; H, 6.60; N, 11.46. Found: C, 49.12; H, 6.47; N, 11.23.

The *d*(−)-mandelic acid ester of biotin obtained from biotin of natural origin melted at 188–189° (micro-block).

Anal. Calcd. for $C_{18}H_{22}N_2O_5S$: N, 7.40. Found: N, 7.55.

The combined residual material from the resolution was hydrolyzed to the free acid and this in turn was converted to the *l*(+)-mandelic acid ester as described above. Fractional crystallization yielded an ester melting at 184–186° (micro-block). Hydrolysis of this ester gave *l*-biotin which after two recrystallizations from water melted at 229–230° (micro-block); $[\alpha]^{25}D$ −90.6° (*c*, 0.5 in 0.1 *N* sodium hydroxide).

Anal. Calcd. for $C_{10}H_{16}N_2O_3S$: C, 49.16; H, 6.60. Found: C, 49.52; H, 6.87.

Resolution of Quinidine Metho Salts of *dl*-Biotin.—One and seventeen-hundredths grams of *dl*-biotin was dissolved in 31.7 ml. of water containing one equivalent of quinidine methohydroxide. The solution was evaporated *in vacuo* and concentrated with methanol to remove residual water. The residue of quinidine metho salts was dissolved in dry methanol and diluted with dry acetone. A crystalline product, the quinidine metho salt of *l*-biotin, was obtained which was recrystallized from dry methanol–acetone; $[\alpha]^{25}D$ +122.7° (*c*, 1.94 in water); yield 0.92 g. The salt was dissolved in water and decomposed with 3 *N* hydrochloric acid to give *l*-biotin, m. p. 228–230° (micro-block); $[\alpha]^{25}D$ −90.5° (*c*, 1.78 in 0.1 *N* sodium hydroxide); yield 332 mg.

A second crop of crystalline quinidine metho salt of *l*-biotin was obtained which was recrystallized and decomposed as described above to yield 135 mg. of *l*-biotin; m. p. 228–231° (micro-block); $[\alpha]^{25}D$ −86° (*c*, 1.43 in 0.1 *N* sodium hydroxide).

The mother liquor from the second crop of quinidine metho salt when further diluted with acetone gave the quinidine metho salt of biotin as an oil. Decomposition of this product with aqueous hydrochloric acid gave crude biotin. Recrystallization from water purified the product, m. p. 229–231° (micro-block); $[\alpha]^{25}D$ +89.2° (*c*, 1.29 in 0.1 *N* sodium hydroxide); yield 112 mg.

The *l*-biotin was recrystallized from water and dried at 100° for analysis; m. p. 228–231°.

Anal. Calcd. for $C_{10}H_{18}N_2O_3S$: C, 49.16; H, 6.60; N, 11.46. Found: C, 49.21; H, 6.81; N, 11.25.

Resolution of *l*(+)-Arginine Salts of *dl*-Biotin.—A mixture of 1.44 g. of *dl*-biotin and 1.15 g. (10% excess) of *l*(+)-arginine was dissolved in 20 ml. of water and the solution was diluted with isopropyl alcohol. Crystallization was allowed to proceed in the refrigerator overnight. The crystals were collected on a filter and washed with acetone; m. p. 214–218° (micro-block); $[\alpha]^{25}D$ +49.09° (*c*, 1.039 in water); yield 1.13 g. (92%). The *l*(+)-arginine salt of biotin when pure had the following properties: m. p. 228–230° (micro-block); $[\alpha]^{25}D$ +59.9° (*c*, 1.37 in water).

Anal. Calcd. for $C_{16}H_{30}N_6O_5S$: C, 45.91; H, 7.23. Found: C, 46.25; H, 7.51.

One and nine-hundredths grams of the crude *l*(+)-arginine salt was recrystallized twice from aqueous isopropyl alcohol; $[\alpha]^{25}D$ +57.2° (*c*, 1.747 in water). The purified salt was dissolved in 10 ml. of water and acidified with dilute hydrochloric acid. The crystalline biotin was collected on a filter, washed with water and dried; m. p. 229–231° (micro-block); a mixture of this sample with biotin of natural origin melted without depression; $[\alpha]^{25}D$ +88.8° (*c*, 1.025 in 0.1 *N* sodium hydroxide); yield 0.51 g. (80%). Further purification was accomplished by suspending the biotin in 20 ml. of hot water, adding just enough dilute sodium hydroxide solution to dissolve the solid, then acidifying with hydrochloric acid. The pure crystalline biotin melted at 229–231°; $[\alpha]^{25}D$ +90.4° (*c*, 1.87 in 0.1 *N* sodium hydroxide); yield 0.44 g. (64% overall).

Anal. Calcd. for $C_{10}H_{18}N_2O_3S$: C, 49.16; H, 6.60; N, 11.46. Found: C, 49.07, 49.35; H, 6.46, 6.47; N, 11.42.

Acknowledgment.—The authors wish to express their thanks to Messrs. D. F. Hayman, R. N. Boos, Leonard Rosalsky and Edward Thornton for carrying out the microanalyses.

Summary

dl-Biotin has been resolved by three independent methods.

The fractional crystallization of the *d*(−)-mandelic acid esters of *dl*-biotin led to the isolation of biotin. From the *l*(+)-mandelic acid esters, *l*-biotin was obtained.

Quinidine methohydroxide was found to be a satisfactory reagent for the separation of *l*-biotin from its enantiomorph. Biotin was obtained from the mother liquor salts.

The *l*(+)-arginine salt of biotin crystallized as the less soluble salt from the mixed *dl*-biotin salts of *l*(+)-arginine. Decomposition of the salt gave biotin. This resolution of *dl*-biotin is the most satisfactory one.

RAHWAY, NEW JERSEY RECEIVED AUGUST 17, 1945

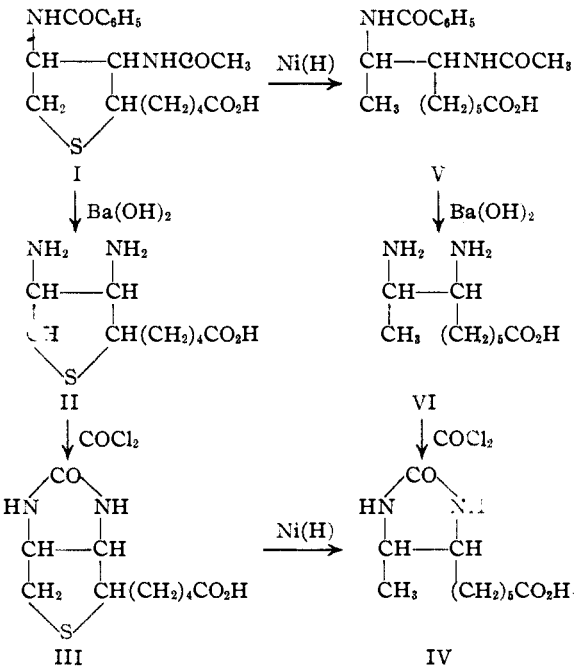
[CONTRIBUTION FROM THE RESEARCH LABORATORIES, MERCK AND CO., INC.]

Biotin. VII. A Stereochemical Correlation of *dl*-Biotin, *dl*-Allobiotin and *dl-epi*-Allobiotin

BY STANTON A. HARRIS, RALPH MOZINGO, DONALD E. WOLF, ANDREW N. WILSON AND KARL FOLKERS

A stereochemical correlation of *dl*-biotin, *dl*-allobiotin and *dl-epi*-allobiotin[1] and of the three intermediate racemic methyl esters of 3-acetamido - 4 - benzamidotetrahydro - 2 - thiophenevaleric acid[2] has been accomplished. This was effected by the use of the sulfur hydrogenolysis reaction[3,4] with Raney nickel catalyst. The application of this reaction is illustrated by reactions III → IV and I → V.

A tetrahydrothiophene which is substituted in the 2, 3 and 4 positions could exist in eight stereoisomeric forms. Four of these forms are represented by formulas VIIId, f, g and h in Diagram I; the other four forms are enantiomorphic to the ones shown and do not need to be illustrated for the purpose of this discussion. If the sulfur atom were replaced by hydrogen atoms, the asymmetry about carbon atom 2 would be lost. Two of these forms are represented by formulas IXj and k; the other two enantiomorphic forms are not illustrated. The racemates as well as the individual isomers may be used to show these



relationships. Thus, the four possible racemates corresponding to formulas VIIId, f, g and h should yield two desthio racemates corresponding to

(1) Harris, Mozingo, Wolf, Wilson, Arth and Folkers, THIS JOURNAL, **66**, 1800 (1944).

(2) Harris, Wolf, Mozingo, Arth, Anderson, Easton and Folkers, *ibid.*, **67**, 2096 (1945).

(3) Mozingo, Wolf, Harris and Folkers, *ibid.*, **65**, 1013 (1943).

(4) du Vigneaud, Melville, Folkers, Wolf, Mozingo, Keresztesy and Harris, *J. Biol. Chem.*, **146**, 475 (1942); Melville, Dittmer, Brown and du Vigneaud, *Science*, **98**, 497 (1943).