[CONTRIBUTION FROM THE NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Enzymatic Resolution of Some Higher α-Amino Acids and Preparation of Optically Active α-Hydroxy Acids[1]

BY CARL G. BAKER AND ALTON MEISTER

The normal α-amino acids of 7, 8 and 12 carbon atoms were resolved by asymmetric enzymatic hydrolysis of certain derivatives (chloroacetyl of the 7 carbon, amides of the 8 and 12 carbon atom amino acids). A series of D- and L-α-hydroxy acids was prepared from several optically active α-amino acids by the nitrous acid reaction, and the analyses and specific optical rotations are given.

Most of the natural amino acids have been resolved[2-6] by means of asymmetric enzymatic hydrolysis of certain derivatives of the amino acids such that the split, free amino acid derived from one isomer can be separated from the unsplit derivative of the other isomer on the basis of their solubility differences; the latter is subsequently converted into the amino acid by acid hydrolysis. That the method is a general one and applicable to α-amino acids of unnatural origin is shown in the present report, in which is described, with appropriate modifications, the resolution of α-aminoheptylic, α-aminocaprylic and α-aminolauric acids. From a series of optically active α-amino acids including two of the above mentioned ones the corresponding optically active α-hydroxy acids were prepared by means of the nitrous acid reaction. The hydroxy acids were required for biochemical studies which will be reported later.

Experimental

Preparation of Enzymes.—The enzyme preparation for the resolution of DL-α-aminoheptylic acid was that described previously.[2] A similar preparation[6] was used for the resolution of DL-α-aminocaprylic and DL-α-aminolauric acids.

Resolution of α-Aminoheptylic Acid.—DL-α-Aminoheptylic acid, which was prepared by amination of the corresponding α-bromo acid, was converted to chloroacetyl-DL-α-aminoheptylic acid[7] (47% yield, based on the amino acid); m.p. 104–106° (cor.).

Anal. Calcd. for $C_9H_{16}O_3NCl$: N, 6.32; Cl, 16.0. Found: N, 6.31; Cl, 15.6.

A suspension of 142 g. (0.64 mole) of the chloroacetyl derivative in 1500 ml. of water was adjusted to *p*H 8.2 with 6 *N* LiOH, and 90 ml. of enzyme preparation was added. The mixture was incubated at 37° with occasional shaking. After 3 hr., a white, crystalline precipitate settled out, and the *p*H dropped to 6.5. The *p*H was adjusted to 7.6 with 6 *N* LiOH, 60 ml. of fresh enzyme was added, and the incubation continued for 12 hr. The reaction was then stopped by adjusting the *p*H to 5.0 with glacial acetic acid, and the material worked up as described previously[3] with the following modifications because of the greater insolubility of this amino acid. At *p*H 5.0 most of the free L-amino acid precipitated, and it was filtered off with the precipitated protein. This precipitate was exhaustively extracted with hot alcohol to remove adherent chloroacetyl-D-amino acid and then with large volumes of hot water to extract the free amino acid. The combined water extracts were evaporated *in vacuo* at 40°, and the amino acid recrystallized with Norit from water. A second batch of the L-isomer was obtained from the filtrate obtained in the filtration of the protein–L-amino acid mixture.[3] The yield of L-isomer was 58%, based on the acyl derivative. The combined alcoholic mother liquor and washings were adjusted to *p*H 1.0 with concd. HCl, and evaporated to dryness *in vacuo* at 30°. The residual amino acid was removed,[3] and the D-acyl derivative was hydrolyzed by refluxing for 3 hr. with 2 *N* HCl.

The D-amino acid was precipitated at *p*H 5.5 by addition of alcohol and recrystallized with Norit from water (42% yield, based on the acyl derivative).

Anal. Calcd. for $C_7H_{15}O_2N$: C, 57.93; H, 10.34; N, 9.66. Found: L-isomer: $[\alpha]^{27}D$ +23.9°[8]; C, 57.76; H, 10.55; N, 9.77; D-isomer: $[\alpha]^{27}D$ −24.0°[8]; C, 57.90; H, 10.50; N, 9.79.

Resolution of α-Aminocaprylic Acid.—The amide of α-aminocaprylic acid was prepared by treating the methyl ester with methanol saturated with ammonia at 0°[9] (55% yield, based on the ester); m.p. 96.8–97.3° (cor.).

Anal. Calcd. for $C_8H_{18}ON_2$: C, 60.65; H, 11.49; N, 17.70. Found: C, 60.75; H, 11.65; N, 17.78.

A mixture of 28 g. (0.177 mole) of the amide, 450 ml. of water and 225 ml. of enzyme preparation was incubated at 37° for 5 hr. with frequent shaking. Fifty ml. of fresh enzyme preparation was then added and the incubation continued for 3.5 hr. After 1 hr., determinations by the Van Slyke ninhydrin–CO_2 method consistently showed 50% hydrolysis. The protein and L-amino acid were filtered off together and exhaustively extracted with ether. The filtrate was adjusted to *p*H 5.0 with glacial acetic acid and allowed to stand several hours at 5°. The precipitated L-amino acid was filtered off and repeatedly extracted with ether. The mother liquor was evaporated to dryness *in vacuo* at 40°, and this residue was also exhaustively extracted with ether. The combined ether-extracted residues were treated with boiling water and Norit, filtered hot, and allowed to cool. The L-α-aminocaprylic acid was recrystallized twice with Norit from water (36% yield, based on the amide).

The combined ether extracts were filtered and dried over anhydrous sodium sulfate. The ether was evaporated at room temperature, 250 ml. of 3 *N* hydrobromic acid was added, and the solution refluxed for 2.5 hr. On cooling, a small amount of fat appeared which was removed by ether extraction. The D-amino acid was precipitated by addition of 6 *N* LiOH to *p*H 6.0 and was recrystallized twice with Norit from hot water (20% yield, based on the amide).

Anal. Calcd. for $C_8H_{17}O_2N$: C, 60.35; H, 10.80; N, 8.80. Found: L-isomer: $[\alpha]^{22}D$ +23.1°[10]; C, 60.48; H, 10.72; N, 8.83; D-isomer: $[\alpha]^{23}D$ −23.4°[11]; C, 60.09; H, 10.64; N, 8.84.

Resolution of α-Aminolauric Acid.—α-Aminolauric acid was prepared by amination of the α-bromo-acid with liquid ammonia in a bomb (90% yield) and esterified with methanol[12] (88% yield). The methyl ester of the amino acid hydrochloride was aminated with liquid ammonia in a bomb yielding the amide of α-aminolauric acid hydrochloride (45% yield); sublimes with dec. at 228°.

Anal. Calcd. for $C_{12}H_{17}ON_2Cl$: C, 57.45; H, 10.87;

(1) Presented before the Division of Biological Chemistry of the American Chemical Society at Philadelphia, Pa., April, 1950.

(2) P. Fodor, V. E. Price and J. P. Greenstein, *J. Biol. Chem.*, **178**, 503 (1949).

(3) V. E. Price, J. B. Gilbert and J. P. Greenstein, *ibid.*, **179**, 1169 (1949).

(4) J. B. Gilbert, V. E. Price and J. P. Greenstein, *ibid.*, **180**, 473 (1949).

(5) J. P. Greenstein, J. B. Gilbert and P. J. Fodor, *ibid.*, **182**, 451 (1950).

(6) L. Levintow, V. E. Price and J. P. Greenstein, *ibid.*, **184**, 55 (1950).

(7) E. Fischer, *Ber.*, **37**, 2486 (1904).

(8) 4.000% solution in 6 *N* HCl in a 2-dm. tube.

(9) A. Weddige, *J. prakt. Chem.*, **10**, 196 (1874).

(10) 1.000% solution in 6 *N* HCl in 2-dm. tube.

(11) 0.900% solution in 6 *N* HCl in 2-dm. tube.

(12) E. Fischer, *Ber.*, **34**, 433 (1901).

N, 11.17; Cl, 14.14. Found: C, 57.53; H, 11.07; N, 10.90; Cl, 14.19.

A suspension[13] of 22 g. (0.088 mole) of the amide hydrochloride, 10 liters of water and 158 g. of $MnCl_2 \cdot 4H_2O$ (final concentration of Mn^{++} 0.08 *M*) was adjusted to *p*H 7.5 with 6 *N* LiOH. 110 ml. of enzyme preparation was added and the mixture was incubated at 37° for 6.25 hr. with mechanical stirring. Ammonia determinations[14] indicated that 34 and 50% of the amide was hydrolyzed in 4 and 5 hr., respectively; 60 ml. of fresh enzyme preparation was added and the incubation was continued for 4.5 hr. Subsequent ammonia determinations consistently showed 50% hydrolysis. The *p*H of the mixture was adjusted to 4.6 with glacial acetic acid and, after standing overnight at 5°, the precipitate was filtered off, and the clear filtrate taken to near-dryness *in vacuo*. The concentrated filtrate was discarded since it yielded pink, cuboidal crystals, easily soluble in water, which were assumed to be manganese chloride. The precipitate was suspended in sufficient water to form a thick sludge, and the *p*H was adjusted to 11 with 6 *N* LiOH. The suspension was exhaustively extracted with hot alcohol in several portions. The first two alcohol extracts were combined and evaporated *in vacuo*. The D-α-aminolauric acid amide was filtered off, and recrystallized 3 times from absolute alcohol, once from chloroform, and once from ethyl acetate, m.p. 83.2–83.7° (cor.); $[\alpha]^{28}D$ −12.2°.[15,16]

Anal. Calcd. for $C_{12}H_{26}ON_2$: C, 67.22; H, 12.25; N, 13.07. Found: C, 66.75; H, 12.02; N, 12.76.

The D-amide was refluxed for 5 hr. in 3 *N* hydrobromic acid. The *p*H was then adjusted to 6.0 with 6 *N* LiOH and the solution taken to dryness *in vacuo*. The residue was dissolved in hot glacial acetic acid, boiled with Norit, filtered and precipitated by addition of water. After 2 such precipitations, the D-amino acid was crystallized from glacial acetic acid–water, washed thoroughly with water, and dried *in vacuo* over KOH and P_2O_5 (22% yield, based on the amide).

The residue remaining after alcohol extraction was treated with hot glacial acetic acid and Norit and filtered. The L-amino acid was precipitated twice by addition of water. It was crystallized from glacial acetic acid–water, washed thoroughly with water, and dried *in vacuo* over KOH and P_2O_5 (61% yield, based on the amide).

Anal. Calcd. for $C_{12}H_{25}O_2N$: C, 66.94; H, 11.84; N, 6.51. Found: L-isomer: $[\alpha]^{28}D$ +24.3°[17]; C, 66.56; H, 11.36; N, 6.57; D-isomer: $[\alpha]^{26}D$ −23.7°[18]; C, 66.72; H, 11.61; N, 6.34.

Preparation of Optically Active α-Hydroxy Acids.—The α-hydroxy acids were prepared from the corresponding amino acids by the nitrous acid reaction.[19,20] The amino acid was dissolved in 1.5 equivalents of 1 *N* HCl and 1.5 equivalents of silver nitrate was added over a period of 3 days. For the preparation of the α-hydroxycaprylic acids, 3 equivalents of silver nitrate and of HCl were employed. The mixture was allowed to stand at room temperature for an additional 3 to 8 days. The silver chloride was removed by filtration, and the filtrate was treated with H_2S and filtered. The solution was taken to dryness *in vacuo*, the residue extracted with ether and the ether evaporated *in vacuo*. The barium salt of the hydroxy acid was prepared by neutralization with saturated, carbonate-free $Ba(OH)_2$. The barium salts were recrystallized from water–acetone. The specific rotations and analyses of these compounds are given in Table I.

TABLE I

Hydroxy acid	$[\alpha]D^a$	Ba in Ba salt, % Found	Ba in Ba salt, % Calcd.	$[\alpha]D^b$ of corresponding amino acid used[c]
L-Lactic	− 8.2[d]	23.0[d]	23.4[d]	+14.4
D-Lactic	+ 7.9	42.8	43.5	−14.4
L-α-Hydroxybutyric	− 8.6	39.2	39.9	+20.6
D-α-Hydroxybutyric	+ 8.6	39.8		−20.7
L-α-Hydroxyvaleric	− 6.0	36.2	36.9	+24.8
D-α-Hydroxyvaleric	+ 6.4	36.9		−23.9
L-α-Hydroxycaproic	− 9.3	34.7	34.4	+23.3
D-α-Hydroxycaproic	+ 9.5	34.2		−22.8
L-α-Hydroxyheptylic	− 9.5[e]	32.1	32.1	+23.7
D-α-Hydroxyheptylic	+ 9.6[e]	32.0		−24.0
L-α-Hydroxycaprylic	− 9.3[f]	30.5	30.1	+23.1[g]
D-α-Hydroxycaprylic	+ 9.1[f]	30.0		−23.4[h]
L-α-Hydroxyisovaleric[i]	−10.1	37.3	37.0	+27.4
D-α-Hydroxyisovaleric	+10.0	36.7		−26.8
L-α-Hydroxyisocaproic[j]	−19.4[j]	34.7	34.4	+15.6
D-α-Hydroxyisocaproic	+19.4	33.9		−15.6
L-β-Imidazole lactic	−42.3[k]	17.8[l]	18.0[l]	−39.7[m]
D-β-Imidazole lactic	+42.9[k]	17.6[l]		+39.6[m]

[a] 1% Ba salt in water in a 2 dm. tube; temp. range 25–27°. [b] 4% amino acid soln. in 6 *N* HCl in a 2-dm. tube. [c] The amino acids were analytically pure. [d] Zn salt; Zn analyses. E. Fischer and A. Skita (*Z. physiol. Chem.*, **33**, 177 (1901)) reported $[\alpha]^{20}D$ −8.00°; B. Iselin and E. A. Zeller (*Helv. Chim. Acta*, **29**, 1508 (1946)) reported $[\alpha]^{14}D$ −8.4°. [e] 0.500% Ba salt in 1.00 *N* NaOH in a 2-dm. tube. [f] 0.210% Ba salt in 1.00 *N* NaOH in a 2-dm. tube. [g] 1.000% soln. in 6 *N* HCl in a 2-dm. tube. [h] 0.900% soln. in 6 *N* HCl in a 2-dm. tube. [i] Optically active α-hydroxyisovaleric acid has been prepared by E. Fischer and H. Scheibler (*Ber.*, 41, 2894 (1908)). [j] L-α-Hydroxyisocaproic acid, crystallized from ether–pet. ether, as 1% soln. in *N* NaOH in a 2 dm. tube, $[\alpha]^{25}D$ −28.2°, m.p. 80–82° (uncor.). Scheibler and Wheeler[19] reported −27.7°; Iselin and Zeller[d] reported −27.2°, m.p. 80–81° (uncor.). O. Lutz and B. Jorgensons (*Ber.*, **65**, 784 (1932)) reported for 1.3208% water soln. of the barium salt, $[\alpha]^{20}D$ −11.3°. [k] 1% soln. of the free acid in water in a 2-dm. tube; m.p. 204°. Lutz and Jorgensons[j] reported 206°. [l] Nitrogen analyses. [m] 1.5% soln. in water in a 2-dm. tube.

Discussion

α-Aminoheptylic acid was resolved by utilizing the chloroacetyl derivative since the rate of enzymatic hydrolysis was sufficiently rapid. The corresponding caprylic acid derivative, however, was not enzymatically attacked. The relative rates of enzymatic hydrolysis of the chloroacetyl derivatives of straight-chain α-amino acids, expressed as μM of substrate split per hr. per mg. nitrogen of the enzyme preparation, were: alanine, 800; α-aminobutyric acid, 1100; norvaline, 1600; norleucine, 1070; α-aminoheptylic acid, 200; α-aminocaprylic acid, 0. The corresponding value for the amide of α-aminolauric acid in the presence of 0.08 *M* Mn^{++}[21] was 85. Attempts to resolve glycyl-DL-α-aminocaprylic acid[22] failed because of very low enzymatic hydrolytic rates with several enzyme preparations. In 1905 Warburg[23] obtained L-leucine by enzymatic splitting of the methyl

(13) Because of the low solubility of α-aminolauric acid amide (more than 50 liters of water would have been necessary for solution of the 22 g. of amide), a suspension with constant stirring rather than a solution was employed.

(14) The low solubility of α-aminolauric acid precluded use of Van Slyke ninhydrin–CO_2 determinations.

(15) 1.000% solution in absolute alcohol in a 2-dm. tube.

(16) A 0.250% solution of the analytically pure hydrochloride of D-α-aminolauric acid amide, m.p. 251° with dec., in absolute alcohol gave $[\alpha]^{26}D$ −15.8°.

(17) 0.2542% solution in glacial acetic acid in a 4-dm. tube.

(18) 0.2312% solution in glacial acetic acid in a 4-dm. tube.

(19) H. Scheibler and A. S. Wheeler, *Ber.*, **44**, 2684 (1911).

(20) Most of the other amino acids were supplied by Dr. J. P. Greenstein and all were prepared by the methods previously described.[2-5]

(21) The rate with this concentration of Mn^{++} is about ten times faster than with no Mn^{++}. It is also about one-tenth the rate for the hydrolysis of histidine amide under the same conditions.

(22) Prepared[7] by amination of the chloroacetyl derivative in methanolic ammonia (91% yield, based on the acyl derivative); m.p. 220° with dec. *Anal.* Calcd. for $C_{10}H_{20}O_3N_2$: C, 55.55; H, 9.32; N, 12.96. Found: C, 55.65; H, 9.37; N, 12.94.

(23) O. Warburg, *Ber.*, **38**, 187 (1905).

ester with a pancreatic enzyme, and, more recently, Brenner and co-workers obtained both optical isomers of tryptophan[24] and of methionine[25] by the asymmetric action of chymotrypsin on their esters. It was not possible to resolve the methyl ester of α-aminocaprylic acid with crystalline chymotrypsin because of the low rate of enzymatic hydrolysis and because appreciable spontaneous hydrolysis occurred under the conditions employed. The hydrolysis of this ester by chymotrypsin was followed for 21 days during which time fresh enzyme was repeatedly added. The free amino acid formed was filtered off at various intervals and each fraction recrystallized twice. The first fraction of amino acid had a specific rotation of $+18.4°$.[26] The ester remaining after 21 days was hydrolyzed with NaOH and the isolated amino acid had a specific rotation of $-21.7°$.[26] Each isomer was therefore contaminated with its enantiomorph. Spontaneous hydrolysis was also observed in control experiments without enzyme.

The problem of inversion in the nitrous acid reaction has been discussed in detail.[27,28] Inversion appears unlikely in the case of the aliphatic α-amino monocarboxylic acids. The α-hydroxy acids were prepared as the barium salts rather than as the free acids because of the marked hygroscopicity of the latter. Except in one case (the barium salt of L-α-hydroxyisocaproic acid), good agreement with values in the literature was found where comparison could be made with identical compounds (zinc L-lactate, L-α-aminoisocaproic acid and its barium salt, and L-β-imidazole lactic acid).

Acknowledgment.—The authors wish to thank Mr. R. J. Koegel for the analyses herein reported.

(24) M. Brenner, E. Sailer and V. Kocher, *Helv. Chim. Acta*, **31**, 1908 (1948).

(25) M. Brenner and V. Kocher, *ibid.*, **32**, 333 (1949).

(26) 0.900% solution in 6 *N* HCl in a 2-dm. tube.

(27) A. Neuberger, "Advances in Protein Chemistry," Vol. IV, Academic Press, Inc., New York, 1948, pp. 297–383.

(28) P. Brewster, F. Hiron, E. D. Hughes, C. K. Ingold and P. A. D. S. Rao, *Nature*, **166**, 178 (1950).

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2,2-Dialkoxyalkanenitriles

BY JOHN G. ERICKSON

2,2-Dialkoxyalkanenitriles may be prepared in very good yields by reactions of hydrocyanic acid with alkyl esters of aliphatic and aromatic ortho acids, especially in the presence of acidic catalysts.

The only 2,2-dialkoxyalkanenitrile which appears to be described in the literature is diethoxyacetonitrile. It was first prepared by Scheibler, Beiser, Cobler and Schmidt,[1] who obtained it in 30% yield by the dehydration of diethoxyacetamide with quinoline and phosphorus pentoxide. McElvain and Clarke[2] later modified this method.

It has recently been found in these laboratories that compounds of this type may be obtained in excellent yields by reactions of hydrocyanic acid with alkyl esters of ortho acids

$$RC(OR')_3 + HCN \rightleftarrows (R'O)_2CCN + R'OH$$
$$\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad\quad R$$

The ortho esters used may be orthoformic or higher aliphatic ortho esters, such as orthoacetic esters, or may be esters of aromatic ortho acids, such as orthobenzoic acid. In the absence of catalysts, the reactions proceed rather slowly at room temperature but at higher temperatures, such as 150°, the reactions go well. The use of a catalyst is desirable and makes it possible to carry out the reactions in a short time at room temperature. Only acidic materials seem to be effective as catalysts, basic compounds causing polymerization of the hydrocyanic acid. With a catalyst such as zinc chloride, the reaction is rapid and equilibrium, usually well over to the dialkoxynitrile side, is established probably within a few hours at room temperature. Because of this equilibrium, it is desirable to neutralize the catalyst before working up the reaction mixtures; otherwise the equilibrium will be shifted toward the ortho ester side by removal, during distillation, of the hydrocyanic acid.

The structures given the products of these reactions depend upon the mode of formation, analyses and hydrogenation to aminoacetals.[3] The assigned structures are also supported by the close agreement of physical properties of samples of diethoxyacetonitrile prepared in this manner and by McElvain and Clarke in their dehydration of diethoxyacetamide. Further, the infrared absorption spectra of these compounds are fully in agreement with the assigned structures.

These dialkoxynitriles are quite sensitive to moisture, being hydrolyzed to hydrocyanic acid and, presumably, alcohol and the normal ester of the carboxylic acid involved. They are difficult to purify for analytical purposes for this reason and also because compounds of this type, containing alkoxyl groups other than methoxyl, slowly decompose if heated to temperatures of the order of 150° or higher. Even when very carefully purified, the compounds gave some trouble in analysis, the nitrogen and alkoxyl determinations tending to be low.

Acknowledgments.—The high temperature reactions were carried out by the High Pressure Group of these laboratories. Analyses were performed by the Microanalytical Group.

Experimental

All recorded boiling points are corrected.

Reagents.—The hydrocyanic acid was stabilized with a trace of sulfur dioxide. Except in one run, this stabilizer

(1) Scheibler, Beiser, Cobler and Schmidt, *Ber.*, **67**, 1513 (1934).

(2) McElvain and Clarke, THIS JOURNAL, **69**, 2661 (1947).

(3) To be reported elsewhere.