II was omitted from the reaction mixture, no hydroxylamine-reactive material was detected by the hydroxamic acid assay.

(b) **With DL-Dihydrolipoamide.**—To a solution containing 4.0 mg. (19.3 μmoles) of DL-dihydrolipoamide,[20] 2 μmoles of sodium acetate in 0.02 ml. of water and 0.98 ml. of dimethoxyethane was added 2.1 μmoles of compound II. After 30 min. at 25° the mixture was evaporated by means of a stream of nitrogen. To the residue was added 1 ml. of water, and the mixture was extracted with a total of 3 ml. of benzene. The combined benzene extracts were washed with 0.5 ml. of water and evaporated with a stream of nitrogen. The residue was dissolved in 0.1 ml. of ethanol and 1 ml. of 2 *M* aqueous hydroxylamine, *p*H 7.0, was added. After 10 min. at 25° 1 ml. of 3 *N* hydrochloric acid was added and the mixture was extracted with two 2-ml. portions of benzene. To the aqueous phase were added 1 ml. of 5% ferric chlorice in 0.1 *N* hydrochloric acid and water to give a total volume of 6 ml., and the absorbance was measured at 540 mμ. The yield of acethydroxamic acid was 0.8 μmole (38%). When either sodium acetate or the 2-acetylthiazolium salt II was omitted from the reaction mixture, no hydroxylamine-reactive material was detected by the hydroxamic acid assay.

In a parallel experiment the benzene extract of the reaction mixture was evaporated *in vacuo* and the residue was dissolved in 2 ml. of methanol. One milliliter of this solution was treated with sodium hydroxide and then acidified as described above. The difference spectrum of this solution *versus* the remaining 1 ml. of methanol solution (acidified) showed a maximum at 236–238 mμ.[21] Assuming the value, $\epsilon = 4{,}400$, for S-acetyldihydrolipoamide, the yield of thiolester calculated from the absorbance was 27%.

Acknowledgments.—We are indebted to Dr. C. G. Skinner and staff of the Clayton Foundation Biochemical Institute for the elemental analyses and to Mrs. Elizabeth Thompson for the acetokinase assays.

(20) L. J. Reed, M. Koike, M. E. Levitch and F. R. Leach, *J. Biol. Chem.*, **232**, 143 (1958).

(21) Crude ethyl 6,8-diacetylthioöctanoate, prepared from ethyl 6,8-dibromoöctanoate and potassium thiolacetate (ref. 22), showed $\lambda_{max}^{CH_3OH}$ 232 mμ (ε 8800). The difference spectrum of a mixture of this thiolester and a 10-fold amount of dihydrolipoamide *versus* dihydrolipoamide showed a maximum at 236–238 mμ.

(22) L. J. Reed and C-I. Niu, *J. Am. Chem. Soc.*, **77**, 416 (1955).

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

Synthesis of Some N-Lipoyl Amino Acids and Peptides[1]

BY KOJI DAIGO,[2] WILLIAM T. BRADY AND LESTER J. REED

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N-Lipoyl amino acids were prepared from DL-lipoic-isobutyl carbonic anhydride. The products were reduced to the dihydrolipoyl derivatives with sodium borohydride. The synthesis of N^{α}-(α-L-aspartyl)-N^{ϵ}-lipoyl-L-lysine[3] (I), N^{α}-(β-L-aspartyl)-N^{ϵ}-lipoyl-L-lysine (II) and N^{α}-(L-asparaginyl)-N^{ϵ}-dihydrolipoyl-L-lysine (III) is described.

During the course of studies on the nature of protein-bound lipoic acid a number of N-lipoyl and N-dihydrolipoyl (6,8-dithioloctanoyl) amino acids and peptides were synthesized. The N-lipoyl amino acids were prepared by the mixed carbonic–carboxylic anhydride method,[4] using DL-lipoic-isobutyl carbonic anhydride.[5] Some of the products were obtained as yellow oils, which polymerized readily. These oils were converted to benzhydrylammonium salts, which were obtained in crystalline form. The N-lipoyl amino acids were reduced with sodium borohydride to obtain the N-dihydrolipoyl amino acids.

Evidence has been presented in previous papers[6,7] which indicates that the sequence Asp.ε-Lipoyl-Lys is present in the *Escherichia coli* pyruvate and α-ketoglutarate dehydrogenation complexes. Three of the peptides isolated from partial hydrolysates of the performic acid-oxidized pyruvate dehydrogenation complex appeared to have the structure Asp. ε-6,8-disulfoöctanoyl-Lys.[7] To confirm the structures assigned to these peptides N^{α}-(α-L-aspartyl)-N^{ϵ}-lipoyl-L-lysine (I), N^{α}-(β-L-aspartyl)-N^{ϵ}-lipoyl-L-lysine (II) and N^{α}-(L-asparaginyl)-N^{ϵ}-dihydrolipoyl-L-lysine (III) were synthesized and then oxidized with performic acid to the corresponding disulfonic acids.[7]

Condensation of N^{ϵ}-(6,8-dibenzylthioöctanoyl)-L-lysine with N-trifluoroacetyl-L-aspartic anhydride, and then treatment with ammonium hydroxide to remove the trifluoroacetyl group,[8] gave a mixture of the α- and β-aspartyl peptides (IV and V), as indicated by paper chromatography. Two ninhydrin-positive spots were observed, one purple and the other blue. According to the findings of Bryant, *et al.*,[9] and other investigators (*cf.* ref. 9), α-aspartyl peptides give a purple color when heated with ninhydrin at 100–120°, whereas β-aspartyl peptides give a blue color. The α-aspartyl peptide IV was found to be more soluble than the β-isomer V in hot methanol, permitting isolation of the β-isomer in pure form. The benzyl groups were removed from the latter isomer by reduction with sodium in liquid ammonia and the dithiol obtained was oxidized to the disulfide II with oxygen in the presence of ferric ion. The α-aspartyl peptide IV could not be separated completely from the β-isomer V. However, after reduction of the mixture with sodium in liquid ammonia, the N^{α}-(α-L-aspartyl)-N^{ϵ}-dihydrolipoyl-L-lysine was separated from the corresponding β-isomer by chromatog-

(1) This investigation was supported in part by a research grant (RG-6590(C1)) from the Division of General Medical Sciences, United States Public Health Service. Abstracted in part from the doctoral dissertation of William T. Brady, The University of Texas, 1960.

(2) Rosalie B. Hite Postdoctoral Fellow, 1959–1961.

(3) In the present study no attempt was made to determine the proportion of the two diastereoisomers in the products prepared from DL-lipoic acid or DL-6,8-dibenzylthioöctanoic acid and L-amino acids.

(4) J. R. Vaughan, Jr., *J. Am. Chem. Soc.*, **74**, 6137 (1952).

(5) L. J. Reed, M. Koike, M. E. Levitch and F. R. Leach, *J. Biol. Chem.*, **232**, 143 (1958).

(6) H. Nawa, W. T. Brady, M. Koike and L. J. Reed, *J. Am. Chem. Soc.*, **82**, 896 (1960).

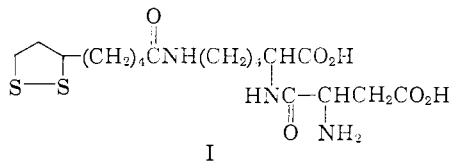
(7) K. Daigo and L. J. Reed, *ibid.*, **84**, 659 (1962).

(8) F. Weygand, P. Klinke and I. Eigen, *Ber.*, **90**, 1192 (1957).

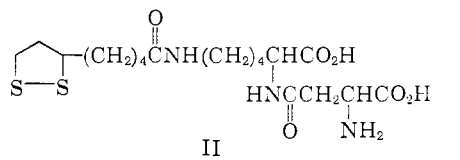
(9) P. M. Bryant, R. H. Moore, P. J. Pimlott and G. T. Young, *J. Chem. Soc.*, 3868 (1959).

raphy on alumina. The dithiol was oxidized to the disulfide I as described above.

In the synthesis of the asparaginyl peptide III, the *t*-butyl ester of N$^{\epsilon}$-(6,8-dibenzylthioöctanoyl)-L-lysine, prepared by the isobutylene method,[10] was acylated with the *p*-nitrophenyl ester of N-benzyloxycarbonyl-L-asparagine.[11] Removal of the *t*-butyl group was achieved by refluxing with *p*-toluenesulfonic acid,[10] and the benzyloxycarbonyl and benzyl groups were removed by reduction with sodium in liquid ammonia.



I



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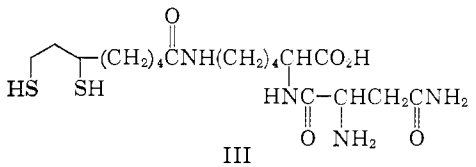
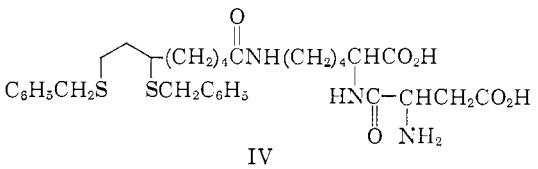
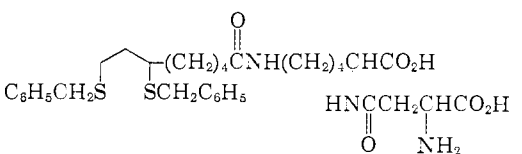


III



IV



V

Experimental[12]

Benzhydrylammonium Salt of DL-Lipoylglycine.—To a stirred solution of 1.03 g. (0.005 mole) of DL-lipoic acid and 0.51 g. (0.005 mole) of triethylamine in 10 ml. of tetrahydrofuran at −5° was added dropwise with stirring a solution of 0.68 g. (0.005 mole) of isobutyl chloroformate in 2 ml. of tetrahydrofuran. The reaction mixture, which contained DL-lipoic-isobutyl carbonic anhydride,[5] was stirred for an additional 10 min., and then a cold solution of 0.38 g. (0.005 mole) of glycine in 5 ml. of 1 *N* sodium hydroxide was added rapidly. The mixture was allowed to warm to room temperature. The clear yellow solution was cooled and 5 ml. of 1 *N* hydrochloric acid was added dropwise with stirring. The mixture was extracted with a total of 10 ml. of ether. The extract was washed with 10 ml. of water and then dried over anhydrous magnesium sulfate. To the ethereal solution was added 0.92 g. (0.005 mole) of benzhydrylamine in 15 ml. of anhydrous ether. An oil separated, which crystallized when allowed to stand in a refrigerator overnight. The product was recrystallized from methanol–ether to yield 0.74 g. (33%) of yellow crystals, m.p. 130–131°.

Anal. Calcd. for $C_{23}H_{30}N_2O_3S_2$: C, 61.85; H, 6.77; N, 6.27. Found: C, 61.91; H, 6.88; N, 6.42.

Benzhydrylammonium Salt of Lipoyl-L-alanine.—This compound was prepared from DL-lipoic acid (1.03 g., 0.005 mole) and L-alanine (0.45 g., 0.005 mole) by the procedure described above. The yield of yellow, crystalline benzhydrylammonium salt was 0.95 g. (42%), m.p. 119–120°.

Anal. Calcd. for $C_{24}H_{32}N_2O_3S_2$: C, 62.58; H, 7.00; N, 6.08. Found: C, 62.45; H, 7.08; N, 5.83.

Benzhydrylammonium Salt of DL-Lipoyl-β-alanine.—The yield of yellow, crystalline benzhydrylammonium salt obtained from 0.45 g. (0.005 mole) of β-alanine was 1.22 g. (53%), m.p. 122–123°.

Anal. Calcd. for $C_{24}H_{32}N_2O_3S_2$: C, 62.58; H, 7.00; N, 6.08. Found: C, 62.38; H, 7.17; N, 6.38.

Benzhydrylammonium Salt of Nα-Acetyl-N$^{\epsilon}$-lipoyl-L-lysine.—The yield of yellow, crystalline benzhydrylammonium salt obtained from 0.51 g. (0.0025 mole) of DL-lipoic acid and 0.47 g. (0.0025 mole) of Nα-acetyl-L-lysine[13] was 0.51 g. (37%), m.p. 136.5–137.5°, $\lambda_{max}^{CH_3OH}$ 330 mμ (ε 136).

Anal. Calcd. for $C_{29}H_{41}N_3O_4S_2$: C, 62.22; H, 7.38; N, 7.50. Found: C, 61.78; H, 7.16; N, 7.46.

DL-Lipoylglycylglycine.—This compound was prepared from DL-lipoic acid (1.03 g., 0.005 mole) and glycylglycine (0.66 g., 0.005 mole) *via* the mixed anhydride procedure described above. The product was extracted from the acidified reaction mixture with chloroform and crystallized from a minimal amount of 95% ethanol; yield 0.76 g. (48%) of yellow crystals, m.p. 138–139°, $\lambda_{max}^{0.1\ N\ NaOH}$ 330 mμ (ε 113).

Anal. Calcd. for $C_{12}H_{20}N_2O_4S_2$: C, 45.31; H, 6.25; N, 8.75. Found: C, 45.40; H, 6.19; N, 9.01.

Nα-Acetyl-N$^{\epsilon}$-lipoyl-L-lysinamide.—To a stirred solution of 8.0 g. (0.025 mole) of Nα-acetyl-N$^{\epsilon}$-carbobenzyloxy-L-lysine[13] and 2.50 g. (0.025 mole) of triethylamine in 60 ml. of chloroform at −5° was added dropwise a solution of 3.38 g. (0.025 mole) of isobutyl chloroformate in 10 ml. of chloroform. The mixture was stirred for an additional 10 min. at this temperature, and then 40 ml. of cold chloroform saturated with anhydrous ammonia was added. A large amount of white material separated. Forty milliliters of chloroform was added and ammonia was bubbled into the reaction mixture for 15 min. The mixture was allowed to stand overnight at room temperature. The solvent was removed *in vacuo* and the white residue was washed with warm water and then dissolved in 350 ml. of warm chloroform. The solution was cooled to obtain 1.78 g. of white solid, *Nα-acetyl-N$^{\epsilon}$-carbobenzyloxy-L-lysinamide*, m.p. 158.5–160°. The filtrate was concentrated *in vacuo* to obtain an additional 2.20 g. of product; total yield 50%. A sample was crystallized from ethanol–water for analysis; m.p. 159–160°.

Anal. Calcd. for $C_{16}H_{23}N_3O_4$: C, 59.80; H, 7.21; N, 13.07. Found: C, 60.45; H, 7.59; N, 13.20.

To a solution of 4.3 g. (0.013 mole) of this compound in 90 ml. of ethanol was added 0.50 g. of palladium black. Hydrogen was bubbled into the mixture, with vigorous stirring, for approximately 8 hr. The mixture was filtered and the filtrate was evaporated *in vacuo*. The residue was extracted with hot ethanol, and the extract was cooled to obtain 0.77 g. of starting material. The mother liquor was evaporated *in vacuo* to obtain 1.60 g. of a viscous oil, *Nα-acetyl-L-lysinamide*.

This product was acylated with DL-lipoic-isobutylcarbonic anhydride in chloroform. The reaction mixture was evaporated *in vacuo* and the residue was washed with water. The yellow solid was extracted with hot ethanol. A considerable amount of white material remained, which presumably was polymer. The ethanol extract was cooled to obtain a yellow solid; yield 26%, m.p. 169–170°, $\lambda_{max}^{CH_3OH}$ 330 mμ (ε 150).

Anal. Calcd. for $C_{16}H_{29}N_3O_3S_2$: C, 51.17; H, 7.78; N, 11.19. Found: C, 51.35; H, 7.46; N, 10.93.

N$^{\epsilon}$-Dihydrolipoyl-L-lysine.—To a stirred solution of 0.05 g. (0.00015 mole) of N$^{\epsilon}$-lipoyl-L-lysine[6] in 1 ml. of 0.25 *N* sodium hydroxide was added 0.05 g. of sodium borohydride. Stirring was continued until the solution became colorless (approximately 2 hr.). The reaction mixture was adjusted

(10) G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, **82**, 3359 (1960).

(11) M. Bodansky and V. du Vigneaud, *ibid.*, **81**, 5688 (1959).

(12) Melting points are uncorrected.

(13) A. Neuberger and F. Sanger, *Biochem. J.*, **37**, 515 (1943).

to *p*H 7 with dilute hydrochloric acid. The white solid which separated was washed successively with cold water, ethanol and ether. The yield was 33 mg. (66%), m.p. 228–230° dec.

Anal. Calcd. for $C_{14}H_{28}N_2O_3S_2$: C, 49.97; H, 8.38; N, 8.32; SH, 19.6. Found: C, 50.27; H, 7.98; N, 8.04; SH, 19.0.[14]

DL-Dihydrolipoylglycine.[15]—To a cold solution of DL-lipoic-isobutylcarbonic anhydride in tetrahydrofuran, prepared from 1.03 g. (0.005 mole) of DL-lipoic acid as described above, was added a cold solution of 0.38 g. (0.005 mole) of glycine in 5 ml. of 1 *N* sodium hydroxide. The mixture was allowed to warm to room temperature. The *p*H was adjusted to 9 with 1 *N* sodium hydroxide, and sodium borohydride was added in portions with stirring until the solution became colorless. The mixture was acidified with 2 *N* hydrochloric acid and extracted with ethyl acetate. The extract was dried over anhydrous sodium sulfate and evaporated *in vacuo*. The residue was crystallized twice from ethyl acetate to obtain 0.61 g. (47%) of white needles, m.p. 63–64°.

Anal. Calcd. for $C_{10}H_{19}NO_3S_2$: C, 45.25; H, 7.21; N, 5.28; SH, 25.3. Found: C, 45.06; H, 7.41; N, 5.52; SH, 25.9.[14]

Benzhydrylammonium Salt of Nα-Acetyl-Nε-dihydrolipoyl-L-lysine.—Nα-Acetyl-L-lysine (0.73 g., 0.0038 mole) was acylated with DL-lipoic-isobutyl carbonic anhydride (0.0038 mole), and the reaction mixture was treated with sodium borohydride as described in the preceding experiment. The benzhydrylammonium salt was prepared by the procedure described previously; yield 1.2 g. (56%), m.p. 123.5–125°.

Anal. Calcd. for $C_{29}H_{43}N_3O_4S_2$: C, 61.99; H, 7.71; N, 7.48. Found: C, 61.63; H, 7.61; N, 7.11.

Nε-(6,8-Dibenzylthioöctanoyl)-L-lysine.—To a solution of 1.94 g. (0.005 mole) of DL-6,8-dibenzylthioöctanoic acid[16] and 0.51 g. (0.005 mole) of triethylamine in 10 ml. of tetrahydrofuran at −3° was added dropwise with stirring a solution of 0.68 g. (0.005 mole) of isobutyl chloroformate in 2 ml. of tetrahydrofuran. After an additional 10 min. at this temperature, the mixture was filtered to obtain a clear solution of DL-6,8-dibenzylthioöctanoic-isobutyl carbonic anhydride.

To a boiling solution of 1.82 g. (0.01 mole) of L-lysine monohydrochloride in 10 ml. of water was added an excess of copper carbonate. The mixture was filtered and the blue filtrate was cooled to −3°. A tetrahydrofuran solution of DL-6,8-dibenzylthioöctanoic-isobutyl carbonic anhydride, prepared as described above, was added in portions with vigorous stirring during a 15-min. period. The *p*H of the mixture was maintained at 9 or above by additions of 1 *N* sodium hydroxide. Stirring was continued for approximately 30 min., while the mixture was allowed to warm to room temperature. The mixture was filtered and the pale blue solid, which contained the copper complex of Nε-(6,8-dibenzylthioöctanoyl)-L-lysine, was washed with water and ethanol and then dried *in vacuo*. The yield was 2.32 g.

A 2.67-g. portion of the copper complex was dissolved in a warm solution of 2.92 g. (0.01 mole) of ethylenediaminetetraacetic acid in 50 ml. of 1 *N* sodium hydroxide. The deep blue solution was adjusted carefully to *p*H 7 with dilute hydrochloric acid. A white solid separated, which was collected by filtration and washed with water and ethanol.[17] This material was dissolved in hot 95% ethanol. When the solution was cooled, 1.25 g. (48%) of a white amorphous solid separated, m.p. 209–212° dec.

Anal. Calcd. for $C_{28}H_{40}N_2O_3S_2$: C, 65.29; H, 7.80; N, 5.42. Found: C, 64.90; H, 7.83; N, 5.52.

Nα-(β-L-Aspartyl)-Nε-(6,8-dibenzylthioöctanoyl)-L-lysine (V).—To a stirred suspension of 7.28 g. (0.014 mole) of Nε-(6,8-dibenzylthioöctanoyl)-L-lysine in 150 ml. of tetrahydrofuran was added 2.98 g. (0.014 mole) of N-trifluoroacetyl-L-aspartic anhydride.[8] The mixture was heated under reflux for approximately 90 min. The clear solution was evaporated *in vacuo* to obtain a viscous oil. Attempts to crystallize the oil were unsuccessful. It was dissolved in 100 ml. of 10% ammonium hydroxide, and the solution was allowed to stand in a refrigerator overnight. The solution was adjusted to *p*H 5 with dilute hydrochloric acid, and the precipitate was collected by filtration and washed with water and ethanol.

A sample of the product was examined by paper chromatography with 2,6-lutidine-collidine (1:1) saturated with water. Two ninhydrin-positive spots, one blue (R_f 0.38) and the other purple (R_f 0.44), were detected under the conditions described by Bryant, *et al.*[9] Hydrolysis of a sample with 6 *N* hydrochloric acid for 23 hr. at 110° yielded lysine and aspartic acid, as indicated by paper chromatography in butanol–acetic acid–water (4:1:5). Amino end group determination by the Sanger method[18] gave N-(2,4-dinitrophenyl)-aspartic acid.

The crude product was extracted with 100 ml. of hot methanol. The insoluble residue (1.2 g., m.p. 187–189° dec.) was reextracted with 25 ml. of hot methanol to obtain 1.0 g. of white solid, m.p. 189–190° dec. The melting point was not changed after crystallization from N,N-dimethylformamide. Paper chromatography with 2,6-lutidine–collidine–water showed a single ninhydrin-positive spot (blue color), R_f 0.35.

Anal. Calcd. for $C_{32}H_{45}N_3O_6S_2$: C, 60.82; H, 7.17; N, 6.65. Found: C, 61.08; H, 7.04; N, 6.96.

Nα-(α-L-Aspartyl)-Nε-(6,8-dibenzylthioöctanoyl)-L-lysine (IV).—The methanol extract obtained in the previous experiment was cooled to obtain 2.25 g. of a white solid, m.p. 155–159° dec. When the mother liquor was concentrated, an additional 1.14 g. of solid was obtained, m.p. 157–161° dec. A 1.0-g. portion of the first crop was recrystallized from 40 ml. of methanol to obtain 0.55 g. of solid, m.p. 159–162° dec. Paper chromatography of this product with 2,6-lutidine–collidine–water showed that it was a mixture of the α- and β-aspartyl peptides, with the former isomer predominating.

Anal. Calcd. for $C_{32}H_{45}N_3O_6S_2$: C, 60.82; H, 7.17; N, 6.65. Found: C, 60.92; H, 7.03; N, 6.44.

Ammonium Salt of Nα-(β-L-Aspartyl)-Nε-lipoyl-L-lysine (II).—The β-aspartyl peptide V (0.125 g.) and sodium (0.05 g.) were added in portions to approximately 30 ml. of liquid ammonia. When the reduction was complete, as evidenced by persistence of a blue color, 0.5 g. of Dowex 50W-X8 (200 to 400 mesh) in the ammonium cycle was added to the mixture, and the ammonia was allowed to evaporate. The residue was extracted with a total of 5 ml. of water. To the aqueous extract (*p*H 9) was added 0.01 ml. of a 1% ferrous acetate solution, and air was bubbled into the solution until the reddish brown color changed to pale yellow (approximately 25 min.). This solution was passed through a column (1 ml.) of Dowex 50 in the ammonium cycle. The effluent was lyophilized and the residue was extracted with a minimal amount of water. Ethanol was added until the solution became turbid. The solution was cooled to obtain a pale yellow, amorphous solid, yield 0.035 g., m.p. 178–180° dec., $\lambda_{max}^{H_2O}$ 330 mμ (ε 106). A sample was subjected to paper electrophoresis in 0.02 *M* phosphate buffer,[19] *p*H 7.0, for 4 hr. at 400 volts. A single ninhydrin-positive spot (greenish gray color) was detected at a distance of 4.5 cm. from the origin (anode end).

Anal. Calcd. for $C_{18}H_{34}N_4O_6S_2$: C, 46.33; H, 7.34; N, 12.01. Found: C, 46.01; H, 6.96; N, 11.76.

Nα-(α-L-Aspartyl)-Nε-lipoyl-L-lysine (I).—The α-aspartyl peptide IV (0.20 g., 0.00032 mole), which contained some of the β-isomer V, was reduced with sodium (0.055 g.) in liquid ammonia. After addition of 1.0 g. of Dowex 50 in the ammonium cycle, the ammonia was allowed to evaporate. The residue was extracted with 5 ml. of water and the solution was lyophilized. The white solid was dissolved in 1 ml. of water, and the solution was passed through a column (5 ml.) of Amberlite IRC-50 in the hydrogen cycle. Water was passed through the column until approximately 20 ml. of effluent was collected. This solution was lyophilized to obtain 0.125 g. of an amorphous

(14) Determined by iodine titration, as described by S. Siggia, "Quantitative Organic Analysis *via* Functional Groups," John Wiley and Sons, Inc., New York, N. Y., 1949, p. 85.

(15) This compound was synthesized by P. K. Martin.

(16) L. J. Reed and C-I. Niu, *J. Am. Chem. Soc.*, **77**, 416 (1955).

(17) Alternatively, the copper complex may be decomposed with a warm aqueous solution of sodium sulfide. The copper sulfide is removed by filtration and the filtrate adjusted to *p*H 6 with dilute hydrochloric acid to obtain the desired product.

(18) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(19) S. P. L. Sørensen, *Ergeb. Physiol.*, **12**, 393 (1912).

solid. A 93-mg. portion of this solid was dissolved in a minimal amount of water, and ethanol was added until the solution became turbid. The solution was cooled to obtain 50 mg. of solid, N^{α}-(α-L-aspartyl)-N^{ϵ}-dihydrolipoyl-L-lysine, m.p. 172–175° dec.

Anal. Calcd. for $C_{18}H_{33}N_3O_6S_2 \cdot H_2O$: C, 46.06; H, 7.51; N, 8.95. Found: C, 46.45; H, 7.30; N, 9.04.

A sample of the product was subjected to paper electrophoresis in Sørensen phosphate buffer, *p*H 7.0, for 4 hr. A major ninhydrin-positive spot (purple) was detected at a distance of 5.3 cm. from the origin. A lesser amount of a greenish gray spot, corresponding to the β-isomer, was detected at a distance of 4.6 cm. from the origin.[20]

Separation of the two isomers was achieved by chromatography on alumina.[21] A solution of 0.95 g. of the mixture in 50 ml. of 50% ethanol in water was applied to a column prepared from a slurry of 70 g. of alumina in 50% ethanol. The column was eluted with 50% ethanol. The first 140 ml. of effluent was ninhydrin-negative and was discarded. The next 330 ml. gave a purple color with ninhydrin. This solution was concentrated *in vacuo* and then passed through a column (10 ml.) of Amberlite IRC-50 in the hydrogen cycle. The effluent was lyophilized to obtain 0.19 g. of a white solid. This solid was triturated with 1 ml. of water. The filtrate was diluted to 5 ml. with water and adjusted to *p*H 9 with ammonium hydroxide. To this solution was added 0.01 ml. of 1% ferrous acetate, and oxygen was bubbled in until the reddish brown color changed to pale yellow (approximately 17 min.). This solution was passed through a column of Amberlite IRC-50 in the hydrogen cycle. The effluent was lyophilized, the residue was dissolved in 0.4 ml. of water, and 2.0 ml. of ethanol was added. A pale yellow, amorphous solid separated, 0.11 g., m.p. 200–202° dec., $\lambda_{max}^{H_2O}$ 330 mμ (ε 111). Paper electrophoresis in 0.02 *M* phosphate buffer, *p*H 7.0, showed a single ninhydrin-positive spot (purple color) at a distance of 5.0 cm. from the origin.

Anal. Calcd. for $C_{18}H_{31}N_3O_6S_2$: C, 48.09; H, 6.95; N, 9.35. Found: C, 48.24; H, 7.50; N, 9.64.

When the alumina column subsequently was eluted with water (250 ml.), the effluent gave a greenish gray color with ninhydrin and showed a single component (β-aspartyl peptide) by paper electrophoresis as described above.

t-Butyl N^{ϵ}-(6,8-dibenzylthioöctanoyl)-L-lysinate.—Concentrated sulfuric acid (1.75 ml.) was added to a suspension of 6.9 g. (0.013 mole) of N^{ϵ}-(6,8-dibenzylthiooctanoyl)-L-lysine in 25 ml. of methylene chloride and 12.5 ml. of isobutylene. The mixture was shaken gently in a stoppered bottle for 48 hr. at 20°. The clear solution was diluted with 100 ml. of methylene chloride and extracted with 15 ml. of 2 *N* sodium hydroxide. The solvent was removed *in vacuo* to obtain 3.87 g. (51%) of a viscous oil. Attempts to crystallize this material were unsuccessful.

t-Butyl N^{α}-(N-Benzyloxycarbonyl-L-asparaginyl)-N^{ϵ}-(6,8-dibenzylthioöctanoyl)-L-lysinate.—A mixture of 2.86 g. (0.005 mole) of the crude product obtained in the previous experiment and 1.94 g. (0.005 mole) of *p*-nitrophenyl N-benzyloxycarbonyl-L-asparaginate[11] in 8 ml. of N,N-dimethylformamide was allowed to stand at room temperature for 48 hr. When 25 ml. of water was added to the solution an oil separated, which gradually solidified. The solid was dissolved in 150 ml. of methylene chloride, and the solution was washed consecutively with 40 ml. of 0.5 *N* sodium hydroxide and water. The organic layer was dried over anhydrous sodium sulfate, and the solvent was removed *in vacuo*. The residue was triturated with ether and then crystallized from N,N-dimethylformamide-water; yield 1.47 g. (36%), m.p. 109–110°.

Anal. Calcd. for $C_{44}H_{60}N_4O_7S_2$: C, 64.36; H, 7.37; N, 6.82. Found: C, 63.80; H, 7.63; N, 6.61.

N^{α}-(N-Benzyloxycarbonyl-L-asparaginyl)-N^{ϵ}-(6,8-dibenzylthioöctanoyl)-L-lysine.—A solution of 1.35 g. (0.0016 mole) of the *t*-butyl ester and 0.3 g. of *p*-toluene sulfonic acid in 7 ml. of benzene was heated under reflux for 45 min. The solvent was removed *in vacuo* and the residue was extracted with 10 ml. of 5% ammonium hydroxide. A trace of insoluble material was removed by filtration and the filtrate was lyophilized. The residue was dissolved in 10 ml. of water and the solution was acidified with dilute hydrochloric acid. The precipitate was washed with water, dried and then triturated with hot ethyl acetate. Crystallization from 75% methanol yielded 0.66 g. (53%) of colorless crystals, m.p. 143–145°.

Anal. Calcd. for $C_{40}H_{52}N_4O_7S_2$: C, 62.80; H, 6.85; N, 7.32. Found: C, 63.45; H, 6.71; N, 7.28.

N^{α}-(L-Asparaginyl)-N^{ϵ}-dihydrolipoyl-L-lysine (III).—To a solution of 0.30 g. (0.0048 mole) of N^{α}-(N-benzyloxycarbonyl-L-asparaginyl)-N^{ϵ}-(6,8-dibenzylthioöctanoyl)-L-lysine in 30 ml. of liquid ammonia was added a total of 0.055 g. of sodium in small portions. Dowex 50 in the ammonium cycle (1.2 g.) was then added and the ammonia was allowed to evaporate. The residue was extracted with a total of 5 ml. of water and the solution was lyophilized. The solid was dissolved in 1 ml. of water and the solution was applied to a column (4 ml.) of Amberlite IRC-50 in the hydrogen cycle. Water was passed through the column until 20 ml. of effluent was collected. The solution was lyophilized to obtain 0.10 g. (58%) of an amorphous solid, m.p. 220° dec.,[22] sintering at 160°. Ninety-five milligrams of this product was dissolved in 0.3 ml. of water and 1.5 ml. of ethanol was added. The precipitate was removed by filtration; 16 mg., m.p. 220° dec. The filtrate was allowed to stand in a refrigerator overnight. The crystalline product was washed with 95% ethanol; 46 mg., m.p. 220° dec.

Anal. Calcd. for $C_{18}H_{34}N_4O_5S_2$: C, 48.00; H, 7.61; N, 12.43. Found: C, 47.80; H, 7.63; N, 12.40.

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(20) A better method of differentiating the α- and β-isomers consists of oxidizing these compounds to the corresponding disulfonic acids, then doing paper electrophoresis in 1 *N* acetic acid (*cf.* ref. 7).

(21) Grade F-20, obtained from the Aluminum Co. of America.

(22) The bath was preheated to 150°.