TABLE IV

TOTAL ACTIVITY AND SPECIFIC ACTIVITY OF FRACTIONS OBTAINED AT VARIOUS STAGES OF THE PURIFICATION OF D-AMINO ACID OXIDASE

Stage of purification	Total activity, μl. O_2/10 min.	Specific activity, μl. O_2/10 min./mg. protein
Crude extracts	764,000[a]	6.4
Ppt. I First ammonium sulfate ppt.	1,200,000	640
Ppt. II Second ammonium sulfate ppt.	1,010,000	1390

[a] This figure must be doubled due to the presence of catalase in the crude extract. Catalase is not present in subsequent fractions.

monium sulfate to 0.4 and the *p*H to 7.0. The white precipitate which formed was collected by filtration after first adding 10 g. of Hyflo Super-Cel filter aid. The "Cel"-enzyme filter cake was extracted four times with 130-ml. portions of *M*/25 pyrophosphate buffer, *p*H 8.3, to give a final volume of 530 ml. of a clear yellow solution. This solution was brought to 0.3 saturation with respect to ammonium sulfate and the yellow precipitate which formed was collected by centrifugation at 317 × gravity for 20 min. The yellow precipitate was resuspended in distilled water to yield 100 ml. of dark amber solution. Data on the activity of the various fractions obtained in the course of this procedure are presented in Table IV.

Specific Property Test.—The procedure of Falconer and Taylor[6] was used. A graphic representation of the results is presented in Fig. 4.

Column Chromatography.—Hyflo Super-Cel was treated according to the procedure of Clauser.[12] Five grams of this treated material was added, as a slurry with the buffer (pyrophosphate *p*H 6.0) in which absorption was to take place, to a 7 × 350 mm. column. The column was washed with more of the same buffer and then 3.0 ml. of the enzyme containing 10 mg. of protein in buffer *p*H 6.0, added. The enzyme was eluted from the column by slowly raising the *p*H of the column.

A continuous *p*H gradient was maintained along the column by allowing a more alkaline pyrophosphate buffer, *p*H 8.3, to siphon into a well stirred acidic pyrophosphate buffer, *p*H 6.0, which in turn siphoned into the column. The rate of elution was adjusted to 5 ml./hr. An automatic fraction collector was used to collect the fractions. The whole procedure was conducted in a cold room at 0°. All of the protein (10 mg.) was recovered in 100 ml. of eluate.

Filter Paper Electrophoresis.—The apparatus was of the hang-strip modification built after the design of Kunkel.[13] The purified enzyme solution was added from a 15 *lambda* micropipet to inch wide strips of Whatman No. II paper. The application was made at the apex of the strip after dialyzing the enzyme against *M*/20 alanine buffer, *p*H 8.3 for four hours. A small drop of dextran was also placed at the apex in order to account for electroösmosis. Applied potentials ranged from 200 to 500 volts and times from 2–12 hours. At the completion of an experiment the position of the protein band was determined by staining with brom phenol blue in the manner described by Durrum.[14] In addition several of these strips were segmented immediately after electrophoresis and activity determinations made on each segment. The position of maximum activity always corresponded to the position of the protein band within the limits of experimental error. The results of such an experiment are presented in Fig. 3.

Conventional Electrophoresis.—Purified enzyme solutions were first dialyzed overnight against the buffer in which the electrophoresis was to take place. Protein determinations made before and after dialysis were the same within experimental error. The positions of the boundaries were recorded photographically at appropriate intervals. Fractions were withdrawn from the cell at the end of the experiment by means of a syringe and needle. The activity and protein content of these fractions were determined in the usual manner. The results of these experiments are presented in Fig. 4 and Table III.

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COLLEGE PARK, MARYLAND

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

Peptide Derivatives Containing Hydroxyamino Acids

BY JOHN C. SHEEHAN, MURRAY GOODMAN[1] AND GEORGE P. HESS[2]

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With the use of N,N′-dicyclohexylcarbodiimide as the condensing agent, several peptide derivatives containing L-serine, L-threonine and L-hydroxyproline have been synthesized.

In view of the outstanding importance of proteins and peptides containing hydroxyamino acids[3–8] and the complex problems associated with their synthesis, we have extended our recently devised method of peptide formation[9] to include derivatives of serine, threonine and hydroxyproline. Difficulties previously encountered have involved mainly interference by the hydroxyl group. Successful syntheses of these compounds have been accomplished only *via* the azide method, a multistep procedure.[10–14] With our technique, N,N′-dicyclohexylcarbodiimide[15] was used to form the peptide bond. Although the first aliphatic carbodiimide, diethyl carbodiimide, was prepared as early as 1893,[16] the reactions of these compounds with

(1) Aided by a contract from the Office of Naval Research.

(2) Aided by a fellowship from the National Foundation for Infantile Paralysis.

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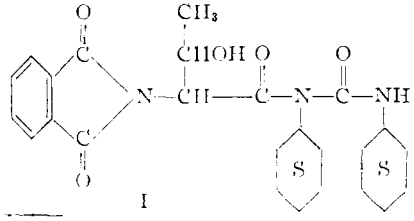
carboxylic acids and phosphoric esters have been investigated only in recent years.[17-23]

In the preparation of the peptide derivatives the phthaloyl group[24] was employed. The synthesis of phthaloylhydroxyamino acids could not be accomplished readily under the conditions of fusion[25] previously reported for amino acids with no additional functional groups. We have found that optically active phthaloyl-L-threonine can be obtained by allowing phthalic anhydride to react directly with L-threonine in dioxane suspension. In the case of serine it is advantageous to block the hydroxyl function of L-serine by preparing O-acetyl-L-serine before treatment with phthalic anhydride. This can be done by a reverse Fischer esterification of L-serine by interaction of the amino acid with an excess of glacial acetic acid and hydrogen chloride. The resulting O-acetyl-L-serine was then condensed with phthalic anhydride in dioxane solution.

Phthaloyl-L-threonine, phthaloyl-O-acetyl-L-serine and carbobenzoxy-L-hydroxyproline were coupled with amino acid esters in a one-step, room temperature reaction using N,N'-dicyclohexylcarbodiimide as the condensing agent.[26] Similarly, a tripeptide derivative of phthaloyl-L-threonyl-L-phenylalanine was prepared. When phthaloyl-L-threonine was coupled with amino acid esters in dioxane or tetrahydrofuran, there was obtained, in addition to the desired peptide derivative, a side product, phthaloyl-L-threonyl-N,N'-dicyclohexylurea (I).[27] In purified methylene chloride or acetonitrile, no evidence of this type of side reaction could be detected in the formation of peptide derivatives of hydroxyamino acids. The desired peptide derivatives were formed rapidly and conveniently in high yields, illustrating that reactions can be carried out in the presence of unprotected primary[9] and secondary hydroxyl groups. Since the reagent and its reaction products are both neutral, the synthesis can take place in the presence of acid- and base-sensitive groups.



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(27) Isolated in 20% yield, m.p. 210-211°; $[\alpha]^{26}D$ −74.0° (0.0350 g. in 1.5 ml. of absolute ethanol). *Anal.* Calcd. for $C_{25}H_{33}N_3O_3$: C, 66.04; H, 7.25; N, 9.25. Found: C, 65.93; H, 7.59; N, 9.40.

Experimental[28]

Phthaloyl-L-threonine.—To a solution of 4.54 g. (0.0306 mole) of phthalic anhydride in 25 ml. of dry dioxane, there was added 3.04 g. (0.0255 mole) of L-threonine. The heterogeneous mixture was submerged in an oil-bath at 105° and stirred vigorously for five hours. After evaporation of the solvent under reduced pressure, the phthaloyl-L-threonine crystallized from acetone–water, yield, 6.0 g. (94%), m.p. 135–140°. A portion of the product was recrystallized twice from ethanol–water, m.p. 143–144°, $[\alpha]^{26.0}D$ −36.7° (0.0480 g. in 1.5 ml. of absolute ethanol).

Anal. Calcd. for $C_{12}H_{11}NO_5$: C, 57.80; H, 4.44; N, 5.61. Found: C, 58.06; H, 4.75; N, 5.54.

O-Acetyl-L-serine.—A solution of 3.5 g. (0.0286 mole) of L-serine in 150 ml. of glacial acetic acid was saturated with hydrogen chloride at 0°. The mixture was stored for 15 hours at room temperature, and the solvent removed under reduced pressure. After repetition of this process, the Oa-cetyl-L-serine hydrochloride was crystallized from ethanol-ether, yielding 5.20 g. (99%), d. 160°, $[\alpha]^{27}D$ −7.4° (0.0334 g. in 1.5 ml. of absolute ethanol). To a solution of 3.0 g. (0.0163 mole) of this product in ethanol there was added 1.62 g. (0.0160 mole) of triethylamine. The resulting O-acetyl-L-serine was recrystallized from water–ethanol, 2.42 g. [98%], d. 167–168°, $[\alpha]^{27}D$ +9.15° (0.0268 g. in 1.5 ml. of 0.1 *N* hydrochloric acid).

Anal. Calcd. for $C_5H_9NO_4$: C, 40.82; H, 6.12; N, 9.51. Found: C, 40.92; H, 6.41; N, 9.39.

Phthaloyl-O-acetyl-L-serine.—To a solution of phthalic anhydride, 2.0 g. (0.0135 mole) in 20 ml. of dry dioxane, there was added 1.65 g. (0.0112 mole) of O-acetyl-L-serine. The heterogeneous mixture was submerged in an oil-bath at 95° and stirred vigorously for 12 hours. The dioxane solution was lyophilized. The resulting oil was taken up in an ether–chloroform mixture (3:1) and filtered. The solution was chromatographed on a 100-g. silica gel column (Davison silica gel, lot no. 923-08-08-226, 100–200 mesh), previously washed with ether, then with ether–chloroform (3:1). The crystalline product, 1.93 g. (63%), emerged with the solvent front, m.p. 140–147°. A portion of this material was recrystallized from ethanol–water for analysis, m.p. 151–153°, $[\alpha]^{27}D$ −63.1° (0.0363 g. in 1.5 ml. of absolute ethanol).

Anal. Calcd. for $C_{13}H_{11}NO_6$: C, 56.32; H, 4.00; N, 5.05. Found: C, 56.33; H, 3.85; N, 5.04.

Phthaloyl-L-threonyl-L-phenylalanine Methyl Ester.—To a solution of 0.350 g. (0.0014 mole) of phthaloyl-L-threonine in 6 ml. of purified methylene chloride,[27] there was added freshly prepared L-phenylalanine methyl ester[29] (0.500 g., 0.0028 mole) and N,N'-dicyclohexylcarbodiimide[15] (0.290 g., 0.0014 mole). A precipitate of N,N'-dicyclohexylurea formed immediately, but the reaction was allowed to proceed for 5 hours at room temperature.

The urea was removed by filtration and the methylene chloride solution was extracted successively with *N* hydrochloric acid, *N* potassium bicarbonate and water. After drying and subsequent removal of the solvent, the residue was crystallized from acetone–ether, yielding a total of 0.520 g. (91%) of product, m.p. 149–152°. An analytical sample was obtained after two recrystallizations from acetone–ether, m.p. 153–154°, $[\alpha]^{27}D$ +1.93° (0.0986 g. in 1.5 ml. of dimethylformamide).

Anal. Calcd. for $C_{22}H_{22}N_2O_6$: C, 64.38; H, 5.40; N, 6.83. Found: C, 64.63; H, 5.65; N, 7.11.

Phthaloyl-L-threonyl-L-leucine Benzyl Ester.—To a solution of 0.600 g. (0.00241 mole) of phthaloyl-L-threonine in 10 ml. of distilled acetonitrile, there was added L-leucine benzyl ester[30] (0.810 g., 0.0036 mole) and N,N'-dicyclohexylcarbodiimide[15] (0.495 g., 0.00241 mole).

(28) All melting points are corrected. We are indebted to Dr. S. M. Nagy and his associates for the microanalytical data.

(29) Prepared by neutralizing an aqueous solution of L-phenylalanine methyl ester hydrochloride to *p*H 9.0 with potassium carbonate and subsequent extraction into ether. The hydrochloride was prepared by Fischer esterification.

(30) Prepared by neutralizing an aqueous solution of L-leucine benzyl ester hydrochloride to *p*H 9.0 with potassium carbonate and subsequent extraction into ether. The hydrochloride was synthesized according to the procedure of H. K. Miller and H. Waelsch, THIS JOURNAL, **74**, 1092 (1952).

The reaction was carried out as described for phthaloyl-L-threonyl-L-phenylalanine methyl ester. After the acetonitrile was removed by distillation under reduced pressure, the residue was taken up in ethyl acetate and extracted with acid, bicarbonate and water. After drying and removal of the solvent the residue was crystallized from acetonitrile–water, yielding 0.980 g. (89%), m.p. 103–106°. Two recrystallizations from acetone–ether–hexane yielded an analytical sample, m.p. 109–110°, $[\alpha]^{26}D$ −16.7° (0.0770 g. in 1.5 ml. of absolute ethanol).

Anal. Calcd. for $C_{25}H_{28}N_2O_6$: C, 66.36; H, 6.24; N, 6.19. Found: C, 66.14; H, 6.25; N, 6.32.

Phthaloyl-L-threonyl-L-phenylalanine.—To a solution of 0.840 g. (0.00205 mole) of phthaloyl-L-threonyl-L-phenylalanine methyl ester in 30 ml. of acetone, there was added 10 ml. of water and 5 ml. of concentrated hydrochloric acid. The solution was refluxed for 2.5 hours and the acetone removed by distillation. Ethyl acetate was added and the product was subsequently extracted into bicarbonate solution. After acidification and re-extraction into ethyl acetate, drying and removal of the solvent under reduced pressure, the product was obtained as a crystalline mass. Recrystallization from ethanol–water yielded 0.510 g. (64%) of analytically pure material, m.p. 207–208°, $[\alpha]^{27}D$ +23.2° (0.025 g. in 1.5 ml. of absolute ethanol).

Anal. Calcd. for $C_{21}H_{20}N_2O_6$: C, 63.60; H, 5.09; N, 7.07. Found: C, 63.30; H, 5.16; N, 7.13.

Phthaloyl-L-threonyl-L-phenylalanyl-L-phenylalanine Methyl Ester.—A mixture of 0.200 g. (0.00051 mole) of phthaloyl-L-threonyl-L-phenylalanine, L-phenylalanine methyl ester[29] (0.110 g., 0.00062 mole) and N,N′-dicyclohexylcarbodiimide[15] (0.105 g., 0.00051 mole) in methylene chloride, was allowed to react as described for phthaloyl-L-threonyl-L-phenylalanine methyl ester.

The recrystallized product (ethanol–water) amounted to 0.245 g. (92%), m.p. 147.2–148°, $[\alpha]^{27}D$ −25.6° (0.0271 g. in 1.5 ml. of absolute ethanol).

Anal. Calcd. for $C_{31}H_{31}N_3O_7$: C, 66.77; H, 5.60; N, 7.54. Found: C, 66.71; H, 5.86; N, 7.48.

Phthaloyl-O-acetyl-L-seryl-L-phenylalanine Methyl Ester.—A methylene chloride solution of 0.400 g. (0.00145 mole) of phthaloyl-O-acetyl-L-serine, L-phenylalanine methyl ester[29] (0.310 g., 0.00165 mole) and N,N′-dicyclohexylcarbodiimide[15] (0.300 g., 0.0014 mole) was allowed to react as described for phthaloyl-L-threonyl-L-phenylalanine methyl ester.

Recrystallization from ethanol–water afforded a total of 0.570 g. (89%), m.p. 131–132°, $[\alpha]^{27}D$ +14.7° (0.0395 g. in 1.5 ml. of absolute ethanol).

Anal. Calcd. for $C_{23}H_{22}N_2O_7$: C, 63.01; H, 5.06; N, 6.39. Found: C, 63.21; H, 5.34; N, 6.44.

Carbobenzyloxy-L-hydroxypropyl-L-phenylalanine Methyl Ester.—A solution of carbobenzyloxy-L-hydroxyproline[10] (0.8 g., 0.0032 mole), L-phenylalanine methyl ester[29] (0.54 g., 0.0032 mole) and N,N′-dicyclohexylcarbodiimide[15] (0.625 g., 0.0033 mole) in methylene chloride was treated as described for phthaloyl-L-threonyl-L-phenylalanine methyl ester. Crystallization occurred from an ethyl acetate solution, yield 1.1 g. (86%), m.p. 108–112°. A portion of the material was recrystallized from acetone–ether–hexane, m.p. 114–115°, $[\alpha]^{27}D$ −29.2° (0.0416 g. in 1.5 ml. of absolute ethanol).

Anal. Calcd. for $C_{23}H_{26}N_2O_6$: C, 64.78; H, 6.10; N, 6.57. Found: C, 64.76; H, 6.36; N, 6.60.

CAMBRIDGE 39, MASSACHUSETTS

[CONTRIBUTION NO. 2046 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY OF THE CALIFORNIA INSTITUTE OF TECHNOLOGY]

Selective Acetylation of the Hydroxyl Groups in Gelatin

BY J. BELLO AND J. R. VINOGRAD

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The hydroxyl groups of gelatin have been selectively acetylated by two methods: treatment with acetic acid, acetic anhydride and perchloric acid, and treatment with acetic anhydride and trifluoroacetic acid. The latter method gives products that are less degraded and more reproducibly acetylated. Acetylation of the hydroxyl groups was shown by infrared spectra and selectivity by O-acetyl and amino nitrogen analyses and isoionic *p*H data.

As part of a study of the effect of chemical modification on the physical properties of gelatin it was decided to block the hydroxyl groups with non-ionizing substituents. Acetylation appeared to be a desirable reaction, but no methods were found in the literature for selective acylation of hydroxyl groups of proteins with carboxylic acids.

We have developed two methods for the selective acetylation of the hydroxyl groups of gelatin. One is an adaptation of the method of Sakami and Toennies[1] for the selective acetylation of the hydroxyl groups of amino acids with acetic acid, acetic anhydride and perchloric acid. The second utilizes the recently reported[2] solubility of proteins in trifluoroacetic acid and the trifluoroacetic acid catalysis of acylations of hydroxyl groups by acid anhydrides.[3]

Experimental

The gelatin used was Wilson Laboratories U-COP-CO, Special Non-Pyrogenic Gelatin, an acid extracted pigskin gelatin of isoionic point and isoelectric point of *p*H 9.2 (turbidity maximum, viscosity minimum and mixed-bed, ion-exchange resin).[4]

Acetylation with Acetic Acid, Acetic Anhydride and Perchloric Acid.—The gelatin was prepared for the reaction by freeze-drying and subsequent vacuum-drying of the porous solid for one day at room temperature. An ice-cold mixture of 150 ml. of glacial acetic acid, 8 g. of 60% perchloric acid and 35 ml. of acetic anhydride (all must be added together or the gelatin may become gummy) was added to 5 g. of gelatin in a standard Waring Blendor with stainless steel blades. The mixture was stirred for 5 minutes and then stood 20 minutes. After excess liquid was removed with a filter stick, the gelatin was washed with 50 ml. of acetic acid and five 100-ml. portions of acetone. During the last two washings the gelatin was shredded in the blendor. It was then transferred to a sintered glass funnel and washed with 200 ml. of ether. The ether was pumped off *in vacuo* and 150 ml. of water was added to the dry white powder. The remaining acidity was neutralized with a small amount of 2 *N* sodium hydroxide added dropwise. During neutralization most of the gelatin dissolved, and the remainder was dissolved by brief warming at 35°. To remove salts the solution was passed through a column 20 mm. in diameter, containing 40 ml. of Amberlite MB-3, mixed-bed, ion exchange resin. The flat portion of the *p*H *vs.* eluate volume curve indicated an isoionic point of 8.2.[4] In one run the product was freed of salts by dialysis against 2% sodium bicarbonate followed by distilled water. The salt free solutions were

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