[CONTRIBUTION FROM ALLERGEN RESEARCH DIVISION, BUREAU OF AGRICULTURAL AND INDUSTRIAL CHEMISTRY, AGRICULTURAL RESEARCH ADMINISTRATION, U. S. DEPARTMENT OF AGRICULTURE]

Some Derivatives of L-Tryptophan[1]

BY JOSEPH R. SPIES

Several derivatives of L-tryptophan were required for an investigation on the quantitative determination of tryptophan in proteins. This paper describes the preparation and properties of seven derivatives of which five—N-phenoxyacetyl-L-tryptophan, *p*-phenylphenacyl-N-acetyl-L-tryptophan, *p*-phenylphenacyl-N-phenoxyacetyl-L-tryptophan, ethyl-N-acetyl-L-tryptophan and N-acetyl-L-tryptophan amide—have not been previously reported. N-Benzoyl-L-tryptophan and ethyl-L-tryptophan hydrochloride, first prepared by Berg, Rose and Marvel,[2] are included because of differences in details of preparation and because additional properties are recorded. The melting point, optical rotation and nitrogen content of the compounds prepared are shown in Table I.

TABLE I

DERIVATIVES OF L-TRYPTOPHAN

Derivative	Formula	Nitrogen content[a] Calcd.	Found av.	Melting point,[b] °C.	Optical rotation[c] Solvent	$[\alpha]^{20}D$
N-Benzoyl-L-tryptophan	$C_{18}H_{16}O_3N_2$	9.09	9.14[d]	[f]	Acetone	−37°
N-Phenoxyacetyl-L-tryptophan	$C_{19}H_{18}O_4N_2$	8.28	8.33	196–197	Acetone	+ 1.5°
p-Phenylphenacyl-N-acetyl L-tryptophan	$C_{27}H_{24}O_4N_2$	6.36	6.38[e]	[f]	Chloroform	−79°
p-Phenylphenacyl-N-phenoxyacetyl L-tryptophan	$C_{33}H_{28}O_5N_2$	5.26	5.39	163–164	Chloroform	−58°
Ethyl-N-acetyl-L-tryptophan	$C_{15}H_{18}O_3N_2$	10.22	10.21	109–109.5	Chloroform	+50°
Ethyl-L-tryptophan hydrochloride	$C_{13}H_{17}O_2N_2Cl$	10.43	10.43	225–226 dec.	Methanol	+12°
N-Acetyl-L-tryptophan amide	$C_{13}H_{15}O_2N_3$	17.13	16.92	193–194	Methanol	+19°

[a] Determined by the Kjeldahl micromethod. The author is indebted to Dorris C. Chambers for these determinations. [b] Melting points were determined with an apparatus described by Markley, *Ind. Eng. Chem., Anal. Ed.*, 6, 475 (1934). National Bureau of Standards calibrated Anschütz thermometers were used. Different rates of heating cause slight variations in the melting points. [c] Solvents were freshly distilled from reagent quality chemicals. Blank readings were determined for each solvent. Rotations were determined with a 2-decimeter, semimicro tube of approximately 3 ml. capacity. The concentrations were all 10 mg. per ml. except for ethyl N-acetyl L-tryptophan and acetyl L-tryptophan amide which were 5 mg. per ml. Specific rotations were calculated by the formula $[\alpha]^{20}D = \frac{\alpha v \text{ (ml.)}}{1 \text{ (decimeters)} w \text{(grams)}}$. [d] Dry basis. A dry sample which was exposed to air for three days lost 2.74% of its weight when heated in a vacuum Abderhalden drier for three hours at 61°. [e] Dry basis. [f] See text.

Experimental

The L-tryptophan and organic reagents used in this study were obtained from Eastman Kodak Company. The L-tryptophan samples contained the theoretical nitrogen content and those lots tested were microbiologically twice as potent in producing growth with *S. faecalis R.* as was an analytically pure sample of DL-tryptophan. The microbiological comparison is a sensitive method for showing that the L-tryptophan is the naturally occurring form.

N-Benzoyl-L-tryptophan.—One and five-tenths grams (7.3 millimoles) of L-tryptophan in a 50-ml. glass-stoppered Erlenmeyer flask was dissolved in 16.0 ml. of *N* sodium hydroxide. The solution was cooled in ice, and 0.84 g. (6.0 millimoles) of benzoyl chloride was added. After five minutes of shaking the benzoyl chloride was all dissolved, and the flask was allowed to stand at room temperature for twenty minutes. The flask was again cooled in ice, and 8 ml. of 2 *N* sulfuric acid was added slowly with stirring. The crystalline precipitate was washed successively on the filter once with 25 ml. of 0.2 *N* hydrochloric acid and then with 30-ml. portions of water until the filtrate was free from chloride ions. The white solid was recrystallized from 1400 ml. of boiling water. The product was dried in a vacuum desiccator over calcium chloride. A yield of 1.116 g. (63% based on the benzoyl chloride) of pinkish platelets was obtained. This substance became hydrated on exposure to air and the melting point varied with its moisture content. The melting point of a sample that had been dried in the melting point tube in a vacuum Abderhalden drier at 61° for seven and one-half hours was: softened at 95°, gas evolved and sample appeared to be melted at 104–105° but the melt was full of bubbles and did not run down the sides of the tube until temperature was about 150°. Berg, *et al.*,[2] gave 104–105° as the melting point but they did not mention the hygroscopic property of this substance:

N-Phenoxyacetyl-L-tryptophan.—One gram (4.9 millimoles) of L-tryptophan in a 50-ml. glass-stoppered Erlenmeyer flask was dissolved in 12.0 ml. of *N* sodium hydroxide. The solution was cooled in ice and 0.877 g. (5.1 millimoles) of phenoxyacetyl chloride was added. The solution set to a solid, so 10 ml. of water was added and the suspension was shaken at room temperature for 20 minutes whereupon solution was complete. To the solution was slowly added with stirring 6.0 ml. of 2.0 *N* sulfuric acid. The precipitate was washed on the filter with 30 ml. of 0.2 *N* hydrochloric acid and then with 30-ml. portions of water until the filtrate was free from chloride ions. The product was dried in a vacuum desiccator over calcium chloride. A yield of 1.110 g. (67% based on tryptophan) of crystalline solid was obtained which melted at 195 to 196° after softening at 194°. This solid after two recrystallizations from 200 ml. of 25% ethanol had a sharp constant melting point. This compound crystallized as white silky needles.

***p*-Phenylphenacyl-N-acetyl-L-tryptophan.**—This derivative was prepared by the general method of Drake and

(1) Paper III in a series entitled, "Chemical Determination of Tryptophan." For the previous paper in this series, see THIS JOURNAL, 70, 1682 (1948). Not subject to copyright.

(2) Berg, Rose and Marvel, *J. Biol. Chem.*, 85, 207 (1929–1930).

Bronitsky.[3] Six tenths of a gram (2.4 millimoles) of N-acetyl-L-tryptophan[4] and 129 mg. (2.4 milliequivalents) of sodium carbonate were dissolved in 2.5 ml. of water in a small flask. Five ml. of ethanol was added, followed by 670 mg. (2.5 millimoles) of *p*-phenylphenacyl bromide. The suspension was refluxed for one and one-half hours. The clear solution was filtered hot. An oil-like substance precipitated and crystallized on cooling. The crystals were filtered and washed with cold 66% ethanol, after which they were dried in a vacuum desiccator over calcium chloride. A yield of 0.89 g. (76% based on tryptophan) was obtained. The solid was recrystallized once from 75 ml. of 50% ethanol and once from 25 ml. of 60% ethanol. This compound crystallized as well-defined short rods or needles. The product after drying in a vacuum desiccator over calcium chloride contained 6.15% nitrogen. On further drying in a vacuum Abderhalden drier at 80° for four hours the compound lost 3.63% in weight. The nitrogen content on a dry basis was 6.38%; the theoretical nitrogen content is 6.36%. A typical melting point of the hydrate was: softened at 96°, became semi-solid at 105°, gas evolved at 110 to 115°, the substance then solidified and remelted sharply at 150 to 152°.

***p*-Phenylphenacyl-N-phenoxyacetyl-L-tryptophan.**—Four hundred and forty-six milligrams (1.3 millimoles) of N-phenoxyacetyl-L-tryptophan and 70 mg. (1.3 milliequivalents) of sodium carbonate were suspended in 5.0 ml. of water in a small flask. Then 10 ml. of ethanol and 362 mg. (1.3 millimoles) of *p*-phenylphenacyl bromide were added, and the mixture was refluxed for one and three-quarters hours. The solution became clear at first, but became turbid on refluxing; so 45 ml. of 89% ethanol was added to keep practically all of the solid in solution during refluxing. The hot solution was filtered and crystallization occurred on cooling. A yield of 504 mg. (83%) was obtained. This compound, after two recrystallizations from 75 ml. of 80% ethanol, melted sharply and constantly. The solid crystallized as small colorless rods.

Ethyl-N-acetyl-L-tryptophan.—One-half gram (2.0 millimoles) of N-acetyl-L-tryptophan[4] was dissolved in 15 ml. of absolute ethanol in a 50-ml. flask. The contents of the flask were protected from atmospheric moisture and saturated with dry hydrogen chloride while being cooled in ice. The solution, which became pink, was allowed to stand at room temperature overnight. Most of the ethanol and hydrogen chloride were removed at room temperature in a current of air and finally in a vacuum desiccator over calcium chloride and sodium hydroxide solution. The sirupy residue was dissolved in 5 ml. of ethanol, and the solution was poured into 50 ml. of water. Crystallization occurred after the suspension had stood at −7° for a few hours. A yield of 253 mg. (45%) of crystals, which melted at 106 to 107° after softening at 104°, was obtained. The product was recrystallized by dissolving in 10 ml. of boiling ethanol to which was added 20 ml. of water. Crystallization was induced by cooling to −7° and scratching the side of the beaker. The 151 mg. of solid obtained was recrystallized similarly from solution in 5 ml. of boiling ethanol to which was added 10 ml. of water. The crystals were filtered off and washed once with a little 25% ethanol. After drying a yield of 87 mg. was obtained. The compound crystallized as colorless short rods which had a constant melting point.

N-Acetyl-L-tryptophan Amide.—To 653 mg. (2.7 millimoles) of crude crystalline ethyl N-acetyl-L-tryptophan in a 25-ml. glass-stoppered Erlenmeyer flask was added 20 ml. of concentrated ammonium hydroxide solution. The slightly turbid solution obtained by shaking for one hour was allowed to stand overnight. The ammonia and part of the water were removed in a partially evacuated desiccator over calcium chloride. When the volume was 10 ml., some crystallization occurred. The suspension was cooled at 5° overnight and the solid was filtered off with suction. The precipitate was washed with a little water and dried. A yield of 310 mg. (48%) of a granular product was obtained which melted at 194 to 195°. The solid was dissolved in 5 ml. of absolute ethanol and filtered. Crystallization occurred after cooling at −7° for four hours. The product was filtered, washed twice with 1 to 2 ml. of ethanol and dried in a vacuum desiccator over calcium chloride. A yield of 160 mg. of crystalline product which melted at 193 to 194° was obtained. The 1° higher melting point obtained with the first product is attributed to the effect of rate of heating.

Ethyl L-Tryptophan Hydrochloride.—This compound was prepared by the method used by Abderhalden and Kempe[5] to prepare the methyl ester of tryptophan. Berg, *et al.*,[2] prepared the ethyl ester hydrochloride by this method. Three-fourths of a gram (3.7 millimoles) of L-tryptophan was suspended in 10 ml. of absolute ethanol in a 50-ml. flask. The suspension was cooled in ice, saturated with dry hydrogen chloride and allowed to stand overnight at room temperature in the dark. A precipitate weighing 149 mg. was obtained which was discarded because the nitrogen content was higher than the theoretical value. The mother liquor after standing twenty-four hours more in the dark at room temperature deposited crystals which were filtered off, washed once with a few ml. of cold ethanol and dried in a vacuum desiccator over calcium chloride and Ascarite. The yield was 215 mg. (22%) of crystals which contained the theoretical nitrogen content. The compound softened at 223° and decomposed at 225 to 226°. Berg, *et al.*, reported that their ethyl-L-tryptophan hydrochloride softened at 219° and decomposed at 221° (uncor.).

Discussion

One of the main uses of the derivatives described herein was for the determination of the extent of racemization during hydrolysis with 5 *N* sodium hydroxide at 100°.[6] Conditions of synthesis were chosen, therefore, which minimized the possibility of partial racemization. That partial racemization did not occur in the preparation of N-acetyl-L-tryptophan was conclusively shown by du Vigneaud and Sealock[4] who resolved N-acetyl-DL-tryptophan. The N-acetyl-D-tryptophan so obtained melted at 190°, like the L form, and had an optical rotation of −30.2°. When equal quantities of the two forms were mixed and recrystallized together the product was optically inactive. Prevention of racemization of amino acids during acetylation has been shown by du Vigneaud and Sealock,[4] du Vigneaud and Meyer[7] and Sealock[8] to require the presence of a slight excess of alkali. Therefore, in the preparation of derivatives, by the Schotten–Baumann reaction, a slight excess of alkali was used. The other derivatives were prepared under conditions not likely to cause racemization. All of the derivatives were optically active as shown in Table I. The value of +1.5° for the rotation of N-phenoxyacetyl-L-tryptophan appears to indicate a nearly racemic compound but when N-phenoxyacetyl-L-tryptophan was hydrolyzed with sodium hydroxide at 100°, 87% of the tryptophan was in the L form as shown by microbiological analysis.[6] The effect of hydrolysis with sodium hydroxide on the extent of

(3) Drake and Bronitsky, THIS JOURNAL, **52**, 3715 (1930).

(4) Prepared as described by du Vigneaud and Sealock, *J. Biol. Chem.*, **96**, 511 (1932). The preparation contained 11.44% nitrogen (the theoretical nitrogen content is 11.38%), melted at 189 to 190°, and $[\alpha]^{20}D$ +29°.

(5) Abderhalden and Kempe, *Z. physiol. Chem.*, **52**, 207 (1907).

(6) Unpublished work.

(7) du Vigneaud and Meyer, *J. Biol. Chem.*, **98**, 295 (1932).

(8) Sealock, *ibid.*, **166**, 1 (1946)

racemization of free tryptophan and of these derivatives will be described in detail elsewhere.[6]

Summary

The preparation, nitrogen content, melting point and optical rotation of seven derivatives of L-tryptophan are described. Five of these derivatives—N-phenoxyacetyl-L-tryptophan, *p*-phenylphenacyl-N-acetyl-L-tryptophan, *p*-phenylphenacyl-N-phenoxyacetyl-L-tryptophan, ethyl-N-acetyl-L-tryptophan and N-acetyl-L-tryptophan amide—have not been previously described.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF OKLAHOMA]

The Use of Amberlite Resin in a Separation of Xanthine and Guanine[1]

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Hitchings[2] has described a method for the estimation of xanthine and guanine by making use of the color forming reaction of these purines with phenol reagent. In this method only 0.0007–0.0028 millimole of xanthine or guanine is required. These two purines can be separated from other interfering substances, but heretofore there has been no satisfactory method for separating such small amounts of xanthine and guanine from each other in a mixture of the two.

A description of a method for separating small amounts of xanthine from guanine from a mixture containing only these two purines by the use of amberlite resin and the analysis for each purine comprises the subject matter of the present article. Essentially, the method consists of the adsorption of xanthine and guanine from *p*H 1 hydrochloric acid by the sodium derivative of Amberlite IR-100 resin with subsequent elution of the xanthine by means of a suitable solution. (It is not known definitely that the phenomena reported are true examples of ion exchange. Hence, the terms "adsorption" and "elution" are used throughout this article.)

The amounts of xanthine and guanine are estimated by means of the reaction with the phenol reagent by the method of Hitchings, but modified for use with a Fisher Electrophotometer. The modified analysis enables one readily to obtain correct values on standards with the phenol reagent for the purine concentrations described in this article, although these concentrations of purines are even less than those of Hitchings. The amount of guanine is determined by difference between the amount of purine nitrogen in the original solution and the xanthine nitrogen eluted.

Experimental

Amberlite Resin.—The Amberlite IR-100-H cation exchanger used was manufactured by the Resinous Products and Chemical Company of Philadelphia. This resin, of analytical quality and in the form of the hydrogen derivative, was purchased from Fisher and Eimer and Amend Company of St. Louis. The resin was processed by a method based on the successful findings of Cleaver and others[3] in their studies of amino acids on exchange resins.

Amberlite IR-100 resin was ground in a ball mill and then sieved. The portion of resin which passed through a 60-mesh but not an 80-mesh screen was selected. The resin was then washed with several portions of distilled water to remove all small particles. The washing was continued till the supernatant liquid was practically clear. Next, 5% hydrochloric acid was added to the beaker containing the resin and allowed to stand, with intermittent stirring, for about twenty minutes. The acid was then decanted off, and the resin was washed with distilled water until the wash water gave no test for chloride ion with silver nitrate. A 5% solution of sodium carbonate was then added to the resin, and this was allowed to stand, with intermittent stirring, for about twenty minutes. The resin was again washed with distilled water until the wash water was neutral to litmus paper. The treated resin was then stored in a stoppered container in distilled water till ready for use.

The treated resin was introduced in a slurry into a well-cleaned 11 × 130 mm. chromatographic adsorption column to a height of 3 cm. Several small portions of distilled water were then put through the column until the effluate was absolutely clear. The level of liquid was at all times maintained above the top surface of the resin to prevent the formation of air pockets which would prevent the solution from contacting part of the resin.

Xanthine.—The xanthine used in this investigation was purchased from Schwarz Laboratories, and was further purified by the method of Levene and Bass.[4]

Guanine Hydrochloride.—Guanine hydrochloride purchased from Eastman was further purified according to the method of Hunter and Hlynka.[5]

Standard Purine Nitrogen Solution.—For constructing a calibration graph to be used in conjunction with the electrophotometer readings in determining the purine nitrogen content of solutions, a solution was made up containing 31.9 mg. of the previously purified guanine hydrochloride dihydrate in 100 cc. of 0.1 *N* hydrochloric acid. One cc. of this solution contained 0.1 mg. of nitrogen. In constructing the graph various different size samples of this solution were treated with phenol reagent, and the electrophotometer readings obtained, using filter number 650A, were plotted against mg. of purine nitrogen.

Eluting Solution.—For elution of xanthine from the adsorption column a solution of the following composition was used: Each 1000 cc. of solution contained 950 cc. of sodium hydroxide adjusted to *p*H 9, uncorrected, with a MacBeth *p*H meter, Model A, 25 cc. of sodium hydroxide–boric acid buffer, and 25 cc. of 0.1 *N* citric acid. The buffer was prepared by adding 21.3 cc. of 0.1 *N* sodium hydroxide to 50 cc. of 0.1 *M* boric acid and diluting to 100 cc.

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(1) Taken from part of a thesis submitted by Sam C. Smith to the Graduate Faculty of the University of Oklahoma in partial fulfillment of the requirements for the degree of Master of Science. Present address: Department of Agricultural Biochemistry, University of Wisconsin, Madison, Wisconsin.

(2) Hitchings, *J. Biol. Chem.*, **139**, 847 (1941)

(3) Cleaver, Hardy and Cassidy, THIS JOURNAL, **67**, 1343 (1945).

(4) Levene and Bass, "Nucleic Acids," The Chemical Catalog Co., Inc., New York, N. Y., 1931, p. 124.

(5) Hunter and Hlynka, *Biochem. J.*, **31**, 486 (1937).