solve for δ. From the observed δ for pyridine betaine in pure water we calculate its dipole moment μ to be 16.9×10^{-18} e. s. u. In equation (11a) ϵ_1 is replaced by the square of the refractive index of pyridine betaine which is determined from a calculated molar refractivity R. The molar volume of pyridine betaine in water is $v = 99.6$ cc./mole.[6] The dielectric constant of water, ϵ_2, has the value 78.54.

The theoretical and experimental[3b] results are illustrated in the figure.

The deviations of the theoretical results from the observed suggest that the sorting of the water molecules by the dipolar ions is not complete and therefore the actual shielding effect is not as great as would be expected for a shell made up entirely of water molecules.

The writer wishes to thank Professor J. G. Kirkwood for his helpful suggestions.

Summary

A theory of the decrease in dielectric increment of dipolar ions with decrease in dielectric constant of the solvent mixture is presented. On the basis of electrostatic theory dipolar ions would be expected to sort out the solvent constituent of higher dielectric constant. The shell of material of higher dielectric constant thereby produced serves to act as an electrostatic shield thus decreasing the effective dipole moment (and therefore the dielectric increment) of the dipolar ion. The shielding effect becomes more prominent as the difference in dielectric of the shell surrounding the dipolar ion and that of the liquid in the bulk of the solution becomes greater.

This continuum treatment leads to results which reproduce the general features of the observed results.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF HARVARD UNIVERSITY AND RADCLIFFE COLLEGE]

Syntheses of Tyrosyltyrosyltyrosine and Tyrosyltyrosyltyrosyltyrosine

BY ARCHIE E. BARKDOLL[1] AND WILLIAM F. ROSS[2]

In relation to certain protein studies in progress in this Laboratory, there were desired polypeptides containing three, four and additional residues of the amino acid *l*-tyrosine[3] bound one to another with none but tyrosyl residues within the chain. For this reason the syntheses of tyrosyltyrosyltyrosine and tyrosyltyrosyltyrosyltyrosine have been carried out according to the procedures to be described in this paper.

The starting material was the compound O-acetyl-N-carbobenzoxytyrosyltyrosine ethyl ester, I, prepared according to the method of Bergmann, *et al.*,[4] which involves the coupling of O-acetyl-N-carbobenzoxytyrosyl chloride with the ethyl ester of tyrosine. This product may be applied in several ways toward the synthesis of the desired polypeptides. Its hydrogenation yields tyrosyltyrosine ethyl ester, II, to the free amino group of which additional tyrosine residues may be coupled through the action of the chloride or azide of a suitable tyrosine derivative.

The preparation of O-acetyl-N-carbobenzoxytyrosyl-O-acetyltyrosyl chloride, which after reaction with II would lead directly to a derived tetrapeptide, was first investigated. O-Acetyl-N-carbobenzoxytyrosyl-O-acetyltyrosine was obtained in good yield by the saponification and subsequent acetylation of I, but attempts to convert it satisfactorily into the chloride failed. The reaction of the free acid with phosphorus pentachloride yielded an oil, which in exploratory experiments with tyrosine ethyl ester gave no useful products. It appears probable that the failure of this approach was due to an attack on the peptide linkage by the phosphorus pentachloride. Similar results were obtained by Pacsu and Wilson,[5] who studied the action of phosphorus pentachloride and of thionyl chloride on carbobenzoxyglycylglycine.

The azide of N-carbobenzoxytyrosyltyrosine coupled with II, would also yield a derivative of the tetrapeptide. This line of approach was next investigated. N-Carbenzoxytyrosyltyrosine hydrazide was obtained readily by the action of hydrazine hydrate on I. The corresponding azide was prepared and, without isolation, added in ethyl acetate solution to a similar solution of the ester, II. The failure to isolate any definite product and the inaccessibility of the two reagents employed led finally to the simple, direct approach of adding single tyrosine residues stepwise to peptide esters of increasing length.

This procedure, although laborious and time-consuming, led finally to the desired products. For its consummation O-acetyl-N-carbobenzoxytyrosyl chloride was coupled with tyrosyltyrosine ethyl ester, to give O-acetyl-N-carbobenzoxytyrosyltyrosyltyrosine ethyl ester, III. The latter was converted by hydrolysis to the carbobenzoxytripeptide, IV, which was catalytically reduced to the desired tripeptide, V. Repeating this process, addition of another

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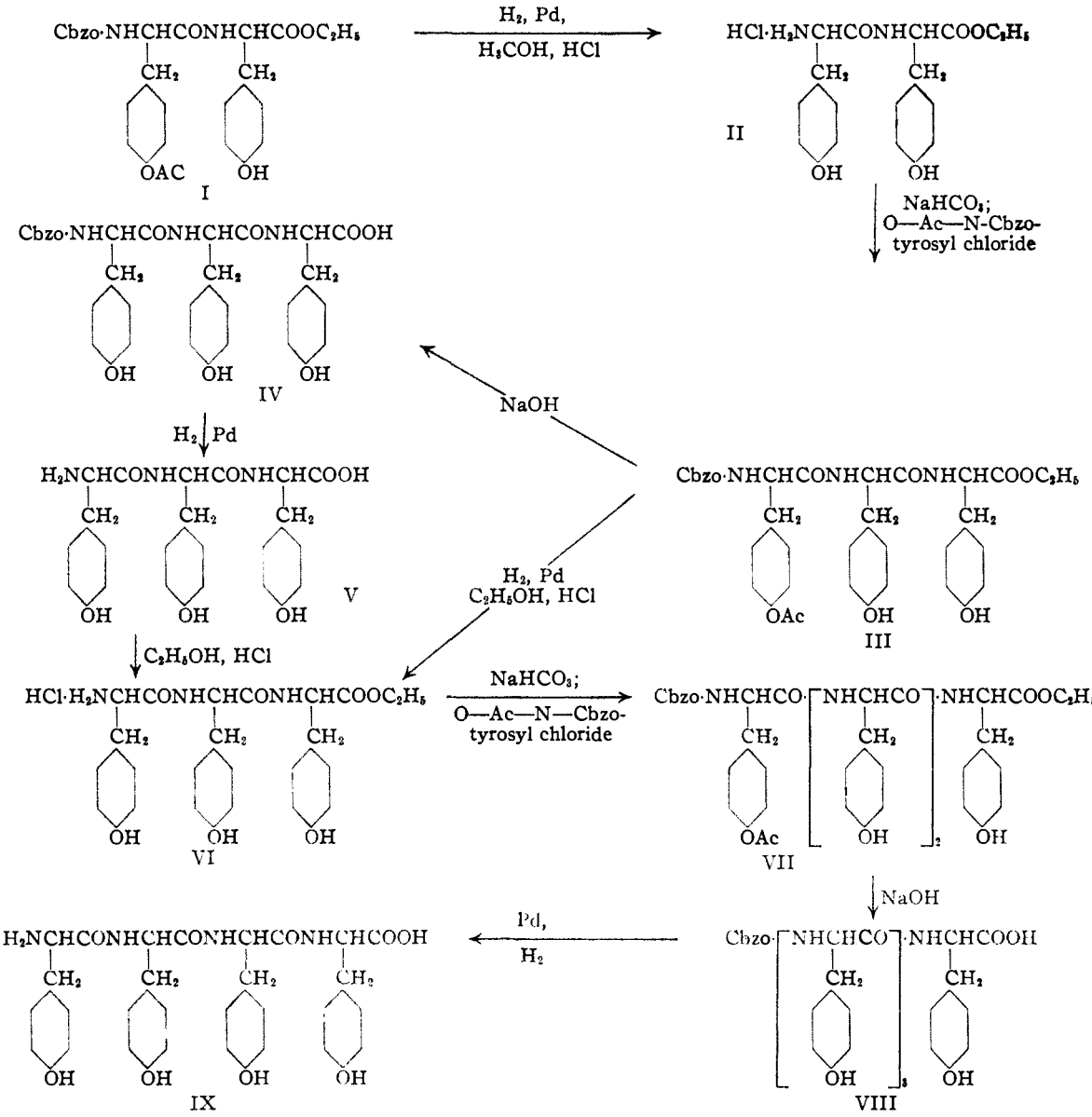
(3) *l*-Tyrosine was used throughout; consequently all of the compounds described in the text contain tyrosine of this configuration.

(4) Bergmann, Zervas, Salzmann and Schleich, *Z. physiol. Chem.*, **224**, 17 (1934).

(5) Pacsu and Wilson, *J. Org. Chem.*, **7**, 117 (1942).

TABLE I

SYNTHESES OF TYROSYLTYROSYLTYROSINE AND TYROSYLTYROSYLTYROSYLTYROSINE



tyrosine residue to the tripeptide ester, VI, yielded the tetrapeptide, tyrosyltyrosyltyrosyltyrosine, IX. An outline of the entire procedure is given in Table I.

It will be noted that catalytic reduction of O-acetyl-N-carbobenzoxytyrosyltyrosine ethyl ester, III, in absolute ethanol containing hydrogen chloride leads directly to an unacetylated product. A parallel case was observed by Bergmann and Fruton[6] in the reduction of O-acetyl-N-carbobenzoxytyrosylglycine ethyl ester, and analogous behavior of derivatives of oestrone has been observed by Miescher and Scholz.[7] The removal of the O-acetyl group during reduction has been verified by the synthesis of tyrosyltyrosine ethyl ester hydrochloride by an unambiguous method. O-Acetyl-N-carbobenzoxytyrosyltyrosine ethyl ester, I, was saponified to N-carbobenzoxytyrosyltyrosine and this was esterified with diazoethane. The resulting ester was reduced to tyrosyltyrosine ester hydrochloride, II, identical with that obtained by the reduction of the acetylated derivative, I. In addition, the dipeptide ester hydrochloride was prepared by the esterification of tyrosyltyrosine.

The O-acetyl group was lost also during the reduction of O-acetyl-N-carbobenzoxytyrosyltyrosyltyrosine ethyl ester, III, to tyrosyltyrosyl-

(6) Bergmann and Fruton, *J. Biol. Chem.*, **118**, 405 (1937).

(7) Miescher and Scholz, *Helv. Chim. Acta*, **20**, 263 (1937).

tyrosine ethyl ester hydrochloride, VI. The same product was formed by the direct esterification of tyrosyltyrosyltyrosine with absolute ethanol and dry hydrogen chloride. This esterification is of interest in view of the report of Pacsu and Wilson[8] that attempts to prepare the ester hydrochlorides of several tripeptides were unsuccessful because of cleavage of the peptide chain. For example, alanylglycylglycine on esterification was converted to a mixture of the ester hydrochlorides of glycine and alanylglycine.

The O-acetyl group, however, is not removed when glacial acetic acid is substituted for methanolic hydrogen chloride, the carbobenzoxy derivative reduced, and gaseous hydrogen chloride then added. In this way, O-acetyl-N-carbobenzoxytyrosine methyl ester gave O-acetyltyrosine methyl ester hydrochloride, and O-acetyl-N-carbobenzoxytyrosine gave O-acetyltyrosine hydrochloride.

The availability of the series consisting of tyrosine, tyrosyltyrosine, tyrosyltyrosyltyrosine and tyrosyltyrosyltyrosyltyrosine makes possible a comparison of the physical and chemical properties of the various members. Most striking is the change in solubility with the number of tyrosine residues within the molecule. Tyrosine itself is almost insoluble in water, tyrosyltyrosine and the tripeptide are freely soluble, but the tetrapeptide only slightly soluble in the cold. There is an increasing solubility in either ethyl or methyl alcohol as the number of residues is increased. Both the tri- and tetrapeptides give positive tests with Millon reagent.

The absorption spectra of the first three members of the series have also been determined.[9] All three compounds contain the same chromophoric group, the phenolic ring, so that little qualitative difference would be anticipated. This expectation is confirmed by the experimental curves of Fig. 1 and the data of Table II. There is only a slight quantitative shift toward the red with increasing molecular weight; this may be associated with the increasing size of the molecule to which the chromophores are bound in each case. The proportionality between the extinction coefficient and the number of tyrosine residues per molecule is also evident from the last column of the table.

TABLE II

For these experiments buffered solutions of *p*H 4.5 were employed. The value of 2750 Å. for λ max of tyrosine agrees with that of Enselme and Pozzi[10] for this amino acid in a solution of *p*H 5.95.

Substance	Mol. wt.	$E_{molar.}$ max.	λ max, Å.	$E_{molar.}$ max./1350
l-Tyrosine	181.2	1350	2750	1.00
Tyrosyltyrosine	344.4	2850	2760	2.11
Tyrosyltyrosyltyrosine dihydrate	543.6	4160	2765	3.08

(8) Pacsu and Wilson, *J. Org. Chem.*, **7**, 126 (1942).

(9) We are especially grateful to Dr. R. Norman Jones for his cooperation in making these determinations.

(10) Enselme and Pozzi, *Bull. soc. chim. biol.*, **17**, 283 (1935).

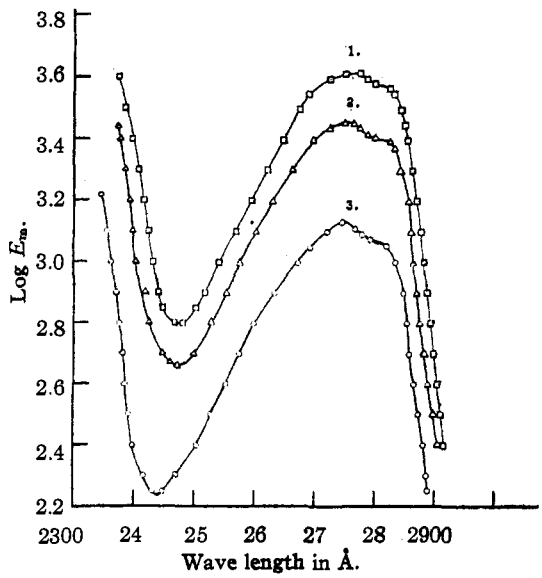


Fig. 1.—Ultraviolet absorption spectra of *l*-tyrosyl-*l*-tyrosyl-*l*-tyrosine dihydrate, □, (1); *l*-tyrosyl-*l*-tyrosine, Δ, (2); and *l*-tyrosine, O, (3); all in a buffer solution of *p*H 4.5.

The Removal of the Carbobenzoxy Group by Alcoholic Hydrogen Chloride.—In addition to the usual catalytic hydrogenation method for the removal of the carbobenzoxy group from amino acids and polypeptides, several others have been found valuable for special cases such as the reduction of sulfur containing carbobenzoxy derivatives. Thus sodium in liquid ammonia has been employed by Sifferd and du Vigneaud,[11] and phosphonium iodide in glacial acetic acid by Harington and Mead.[12] Also, White[13] removed the carbobenzoxy group by treatment with aqueous hydrogen chloride at 60°. We have observed that the action of an absolute ethanol solution of hydrogen chloride also serves to liberate the carbobenzoxy group, with the simultaneous formation of the corresponding ester hydrochloride.

In an attempt to prepare N-carbobenzoxytyrosyltyrosine ethyl ester by the action of absolute ethanol and dry hydrogen chloride at 0° on N-carbobenzoxytyrosyltyrosine, it was found that a major portion of the acid had been converted by esterification of the peptide carboxyl and fission of the carbobenzoxy group to tyrosyltyrosine ethyl ester hydrochloride, while only a small amount of the desired carbobenzoxy ester was obtained. A similar experiment with O-acetyl-N-carbobenzoxytyrosyltyrosine ethyl ester gave the same products.

The action of absolute ethanolic hydrogen chloride on carbobenzoxyglycine at 0° likewise yielded a mixture of glycine ester hydrochloride and carbobenzoxyglycine ethyl ester. But, if

(11) Sifferd and du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).

(12) Harington and Mead, *Biochem. J.*, **29**, 1602 (1935).

(13) White, *J. Biol. Chem.*, **106**, 143 (1934).

the absolute alcohol solution of the acid was saturated at room temperature with dry hydrogen chloride and refluxed for an hour, glycine ester hydrochloride was obtained to the exclusion of carbobenzoxyglycine ester. The simplicity of this procedure for removing the carbobenzoxy group and the concomitant ester formation suggest its further application in synthetic work.

Experimental

The tyrosine employed in this work was natural *l*-tyrosine of $(\alpha)^{20}D$ −9.0° (in 5.8 *N* hydrochloric acid, *C* = 3.613). Consequently all the compounds to be described are of the *l* configuration, although the prefix *l* is omitted throughout for the sake of economy.

The palladium black catalyst was prepared according to the method of Tausz and von Putnoky.[14] It was kept either in the dry state or as a suspension in distilled water.

The analyses have been variously performed by Mr. M. Brown, Dr. Carl Tiedcke, Miss E. Werble, and one of the authors (A. E. B.).

N-Carbobenzoxytyrosyltyrosine Ethyl Ester.—The ethyl ester was prepared by the addition of somewhat less than one equivalent of diazoethane–ether solution to a solution of N-carbobenzoxytyrosyltyrosine hydrate (0.97 g.) prepared by the method of Bergmann,[4] in 15 cc. of methanol. The sirupy residue remaining after concentration of the solution *in vacuo* was dissolved in ethyl acetate and unreacted acid extracted with dilute sodium bicarbonate. The ethyl acetate solution was dried over anhydrous sodium sulfate, the solvent removed *in vacuo*, and the residue recrystallized several times from ethyl acetate–petroleum ether to yield 260 mg. of the pure ester in the form of colorless, silky needles melting at 159–160.5°.

Anal. Calcd. for $C_{28}H_{30}O_7N_2$: C, 66.38; H, 5.97; N, 5.53. Found: C, 65.96, 65.88; H, 6.11, 6.17; N, 5.62.

All melting points are uncorrected.

Tyrosyltyrosine Ethyl Ester Hydrochloride.—Five grams of O-acetyl-N-carbobenzoxytyrosyltyrosine ethyl ester, prepared by the method of Bergmann,[4] was dissolved in 110 cc. of 0.2 *N* absolute ethanolic hydrogen chloride and reduced at atmospheric pressure in the presence of 1.4 g. of palladium black catalyst. The product, recovered after concentration by the addition of ether, represented an almost quantitative yield. For analysis it was recrystallized twice from absolute alcohol–absolute ether in the form of microscopic clusters of platelets; it melted at 216° (dec.).

Anal. Calcd. for $C_{20}H_{25}O_5N_2Cl$: C, 58.50; H, 6.16; Cl, 8.68. Found: C, 58.25, 58.41; H, 6.05, 6.23; Cl, 8.55.

An identical product was obtained by dissolving 100 mg. of tyrosyltyrosine[4] in 10 cc. of absolute ethanol saturated with dry hydrogen chloride at 0°. After two days at 7° the crystalline ester hydrochloride was obtained by concentration and addition of ether. It melted at 217° and showed no depression of the decomposition point when mixed with the product described above.

The same product, melting at 216°, was obtained by the catalytic reduction of N-carbobenzoxytyrosyltyrosine ethyl ester in absolute ethanolic hydrogen chloride.

N-Carbobenzoxytyrosyltyrosine Methyl Ester.—Methylation of N-carbobenzoxytyrosyltyrosine hydrate with diazomethane in methyl alcohol solution gave the ester, which, after several recrystallizations from ethyl acetate–ligroin, formed small needles and rosets melting at 174–175°.

Anal. Calcd. for $C_{27}H_{28}O_7N_2$: C, 65.84; H, 5.73; N, 5.68. Found: C, 65.62; H, 5.57; N, 6.27.

Tyrosyltyrosine Methyl Ester Hydrochloride.—Obtained by the catalytic reduction of N-carbobenzoxytyrosyltyrosine methyl ester, this product melted at 210° after three recrystallizations from absolute methanol–absolute ether.

Anal. Calcd. for $C_{19}H_{23}O_5N_2Cl$: C, 57.79; H, 5.87; Cl, 8.98. Found: C, 57.84, 57.49; H, 6.09, 6.03; Cl, 9.17.

O-Acetyl-N-carbobenzoxytyrosyl-O-acetyltyrosine.—Three grams of finely powdered O-acetyl-N-carbobenzoxytyrosyltyrosine ethyl ester was added to 21 cc. of 1 *N* sodium hydroxide. After shaking for an hour at room temperature, the solution was filtered, and 1.11 cc. of acetic anhydride in 12 cc. of dioxane added. After fifteen minutes at room temperature, the solution was acidified to congo red with 1 *N* hydrochloric acid. The solid thus formed was collected on the filter, washed with water and recrystallized three times from hot acetone–water, to give white tufts of needles melting at 209–210°. The acid was insoluble in cold acetone, and in either hot or cold water, but was moderately soluble in boiling acetone.

Anal. Calcd. for $C_{30}H_{30}O_9N_2$: C, 64.05; H, 5.37; N, 4.98. Found: C, 63.94, 63.82; H, 5.20, 5.35; N, 5.30.

O-Acetyl-N-carbobenzoxytyrosyltyrosyltyrosine Ethyl Ester.—Tyrosyltyrosine ethyl ester hydrochloride (4.24 g.) was dissolved in 35 cc. of water, 25 cc. of pure ethyl acetate was added and the mixture cooled in ice. The free ester was liberated by the addition of 1.70 g. of sodium bicarbonate. It settled out as a thick paste after several minutes of shaking. This was filtered off, pressed as dry as possible on the filter, and then dissolved in 75 cc. of purified dioxane. To this solution 25 cc. of ethyl acetate was added and the resulting solution dried for an hour at 0° over anhydrous potassium carbonate. After filtration the solution was stored at 0° for about an hour, during the preparation of O-acetyl-N-carbobenzoxytyrosyl chloride.

The latter was prepared essentially according to the procedure of Bergmann[4]: 7.20 g. of the finely powdered free acid was suspended in 72 cc. of anhydrous ether, and cooled in an ice–salt-bath; to the suspension was then added 4.20 g. of finely powdered phosphorus pentachloride. The flask was shaken in ice until the white crystalline acid chloride had completely formed. This was filtered off, washed with a 1:2 mixture of anhydrous ether–petroleum ether and dried in an evacuated desiccator at 0° over phosphorus pentoxide. It was used for coupling with the ester as soon as possible.

For this purpose, 2.28 g. of O-acetyl-N-carbobenzoxytyrosyl chloride dissolved in 20 cc. of purified, anhydrous ethyl acetate was added to the tyrosyltyrosine ethyl ester solution as prepared above. Immediate formation of a precipitate of tyrosyltyrosine ester hydrochloride occurred. After standing at room temperature for nine hours, the latter was removed, and the solution concentrated *in vacuo* to a thick yellow sirup which was crystallized by the slow addition of ethyl acetate. After two recrystallizations from hot acetone–ether the product was obtained as a white microcrystalline powder, m. p. 211°. The yield of the crude product, calculated from the weight of amino acid ester hydrochloride recovered, was 78%.

Anal. Calcd. for $C_{39}H_{41}O_{10}N_3$: C, 65.81; H, 5.81; N, 5.90. Found: C, 65.94, 65.80; H, 6.35, 6.15; N, 5.92.

Tyrosyltyrosyltyrosine Ethyl Ester Hydrochloride.—A sample of 2.06 g. of O-acetyl-N-carbobenzoxytyrosyltyrosyltyrosine ethyl ester was dissolved in 40 cc. of anhydrous dioxane by warming, and 160 cc. of absolute ethanol, 7 cc. of 2 *N* absolute ethanolic hydrogen chloride and 2 g. of palladium black catalyst were added. After completion of the hydrogenation, which followed, the filtered solution was concentrated *in vacuo* to a thick sirup which was taken up in 15 cc. of water and treated with 10 cc. of ethyl acetate and 0.8 g. of sodium bicarbonate in order to transfer the ester to the organic phase. After washing the aqueous phase with several additional 10-cc. portions of ethyl acetate, the combined ethyl acetate extracts were dried thirty minutes at 0° over anhydrous potassium carbonate and filtered. When dry hydrogen chloride gas was passed over the surface of the filtrate, the hydrochloride formed as a light yellow sirup which slowly

(14) Tausz and von Putnoky, *Ber.*, **52**, 1573 (1919).

crystallized. The yield of product was 66%. In order to purify a sample for analysis it was necessary to repeat the procedure of transferring the liberated ester to ethyl acetate and adding hydrogen chloride. Hemispherical nodules of crystals ranging up to 2 mm. in diameter were obtained; m. p. 231–231.5° (dec.) with previous coloring and sintering. Attempts to crystallize the compound directly from alcohol–ether gave an oil which crystallized but slowly and retained any impurities present.

Anal. Calcd. for $C_{29}H_{34}O_7N_3Cl$: C, 60.88; H, 5.99; Cl, 6.11. Found: (dried at 100° *in vacuo*) C, 60.67, 60.20; H, 6.82, 6.25; Cl, 6.12.

The same product was obtained by esterification of 150 mg. of tyrosyltyrosyltyrosine in absolute ethanol solution with hydrogen chloride. The procedure was similar to that followed for tyrosyltyrosine ethyl ester, except that the viscous sirup obtained on concentration of the solution was crystallized by allowing it to stand under ethyl acetate for several days; yield, 158 mg. The m. p. was 230–231° (dec.) and a mixture of the product with the tripeptide ester hydrochloride, of m. p. 231–231.5° (dec.), prepared by the reduction of O-acetyl-N-carbobenzoxytyrosyltyrosyltyrosine ethyl ester, gave no depression.

N-Carbobenzoxytyrosyltyrosyltyrosine.—This derivative was obtained by saponification of 1.5 g. of O-acetyl-N-carbobenzoxytyrosyltyrosyltyrosine ethyl ester in 8.5 cc. of 1.1 *N* sodium hydroxide. It formed as a gel when the solution was acidified to congo red, and was finally crystallized as sheaves and rosets of needles from warm methanol; m. p. 182–183°, yield, 1 g.

Anal. Calcd. for $C_{35}H_{35}O_9N_3$: C, 65.51; H, 5.49; N, 6.55. Found: C, 65.45, 65.49; H, 5.56, 5.61; N, 6.49.

Tyrosyltyrosyltyrosine Dihydrate.—To a solution of 400 mg. of N-carbobenzoxytyrosyltyrosyltyrosine in 20 cc. of methanol were added 30 cc. of water, 0.4 g. of palladium black catalyst and a small drop of glacial acetic acid. Removal of the carbobenzoxy group was complete after hydrogenation for thirty minutes. The filtered solution was concentrated *in vacuo* at a bath temperature not exceeding 35°. The peptide crystallized in the form of microscopic white prisms after the removal of approximately half of the solvent. After further concentration, the solid was removed and recrystallized twice from hot water to give fine prisms of m. p. 181–182° in a yield of 150–200 mg.

Anal. Calcd. for $C_{27}H_{29}O_7N_3 \cdot 2H_2O$: C, 59.65; H, 6.11; N, 7.73; H_2O, 6.65. Found: C, 59.54, 59.53; H, 5.92, 6.14; N, 7.90; H_2O, 6.63.

The tripeptide was slightly soluble in cold alcohol and water, somewhat soluble in hot alcohol and quite soluble in hot water. The anhydrous material, formed by drying at 100° over phosphorus pentoxide *in vacuo*, was hygroscopic, tending to revert to the dihydrate on exposure to the air.

N-Carbobenzoxytyrosyltyrosine Hydrazide Hydrate.—The hydrazide was obtained from O-acetyl-N-carbobenzoxytyrosyltyrosine ethyl ester by reaction with hydrazine hydrate in ethanol solution. It melted at 246° (dec.) after recrystallization from pyridine, and lost one molecule of water on drying over phosphorus pentoxide for one hour at 100° (1 mm.).

Anal. Calcd. for $C_{26}H_{28}O_6N_4 \cdot H_2O$: C, 61.16; H, 5.94; N, 10.97; H_2O, 3.53. Found: C, 61.13; H, 5.96; N, 11.09; H_2O, 3.42.

It has already been pointed out that attempts to use the corresponding azide in coupling experiments with tyrosyltyrosine ethyl ester were without success.

O-Acetyl-N-carbobenzoxytyrosyltyrosyltyrosyltyrosine Ethyl Ester.—O-Acetyl-N-carbobenzoxytyrosyl chloride was prepared as described in the preparation of the O-acetyl-N-carbobenzoxy tripeptide, except that in this case it was desirable to have extremely pure chloride on account of the difficulty of purifying the tetrapeptide. Therefore, just as the crystals of chloride began to form, the solution was filtered, the precipitate discarded and the chloride to be used in the coupling obtained from further crystallization in the filtrate.

374 mg. of O-acetyl-N-carbobenzoxytyrosyl chloride was dissolved in 10 cc. of anhydrous ethyl acetate and the solution added to that containing the ester from 1.14 g. of tyrosyltyrosyltyrosine ethyl ester hydrochloride. The latter had been prepared in the same manner as the tyrosyltyrosine ester in the synthesis of the tripeptide. The solution became turbid, and an oil deposited. After standing for two days, this oil had solidified and was found to be a mixture of the tripeptide ester hydrochloride and the desired O-acetyl-N-carbobenzoxy tetrapeptide ester. The two were separated by extraction of the former from the solid with 0.5 *N* hydrochloric acid and crystallization of the tetrapeptide ester from hot acetone–ether. The yields were 233 mg. and 624 mg., respectively. The m. p. of the latter was 235.5–236.5° (dec.).

Anal. Calcd. for $C_{48}H_{50}O_{12}N_4$: C, 65.89; H, 5.76; N, 6.40. Found: C, 65.53; H, 5.80; N, 6.43.

N-Carbobenzoxytyrosyltyrosyltyrosyltyrosine.—A sample of 590 mg. of finely powdered O-acetyl-N-carbobenzoxytyrosyltyrosyltyrosyltyrosine ester was saponified by treatment with 6 equivalents of 1 *N* sodium hydroxide for thirty minutes at room temperature. The free acid was precipitated as an amorphous jell on acidification to congo red. After filtering, washing, and drying, it weighed 530 mg. It was finally obtained as a white solid, appearing under the microscope as ill-formed rosets of crystals, by the slow concentration of a methanol solution in a partially evacuated desiccator over phosphorus pentoxide. The final yield was 201 mg. and the product melted at 224–225° (dec.) with previous darkening at 220°. The acid contained a molecule of methanol which was lost on drying over phosphorus pentoxide at 100° (1 mm.).

Anal. Calcd. for $C_{44}H_{44}O_{11}N_4 \cdot CH_3OH$: CH_3OH, 3.83. Found: (dried *in vacuo* at 100°) 3.70. Calcd. for $C_{44}H_{44}O_{11}N_4$: C, 65.65; H, 5.51; N, 6.96. Found: C, 65.35; H, 5.62; N, 6.38.

On standing in the air, the dried acid rapidly absorbed 1% of its weight of moisture. The acid was insoluble in water and 5% sodium bicarbonate, but soluble in 1 *M* sodium hydroxide and hot methanol.

Tyrosyltyrosyltyrosyltyrosine.—A sample of 118 mg. of N-carbobenzoxy tetrapeptide methyl alcoholate was catalytically hydrogenated in methyl alcohol solution containing a small drop of acetic acid. Concentration of the filtered solution gave a viscous sirup which formed a noncrystalline glassy solid on standing. The latter we were unable to obtain in a clearly crystalline form in spite of many efforts. The analytical sample was obtained as a glass-like solid by treating the tetrapeptide in methanol solution with activated carbon, followed by concentration *in vacuo* and drying over phosphorus pentoxide one hour at 65° (1 mm.).

Anal. Calcd. for $C_{36}H_{38}O_9N_4$: C, 64.46; H, 5.71; N, 8.35. Found: C, 63.45; H, 5.72; N, 7.15.

The tetrapeptide was insoluble in cold water, hot and cold dioxane, ethyl acetate, and acetone, but was soluble in cold 5% sodium carbonate, 0.5 *N* hydrochloric acid, absolute methanol and ethanol, and glacial acetic acid. It could be precipitated from absolute ethanol solution in an amorphous condition by the addition of ether.

Purification of Materials for Determination of Absorption Spectra.—**A. Tyrosine:** Six grams of tyrosine, obtained from the University of Illinois, was recrystallized five times from boiling distilled water, carbon being employed during the first two recrystallizations: yield 2.42 g. The material was dried for two hours over phosphorus pentoxide at 100° (1 mm.).

B. Tyrosyltyrosine: The dipeptide was prepared by the method of Bergmann, *et al.*[4] After two recrystallizations from hot ethanol–water, the peptide was obtained in the form of small, colorless prisms melting at 285–288° (dec.), with preliminary sintering. The material was dried over phosphorus pentoxide for two hours at 100° (1 mm.).

C. Tyrosyltyrosyltyrosine Dihydrate: An analytical sample of tripeptide dihydrate (300 mg.) which gave correct analyses but which had a very faint yellow tint, was

recrystallized three times from hot distilled water, 25 mg. of activated carbon being used in the first recrystallization. There was obtained 220 mg. of white clusters of prisms, m. p. 180–181°. As the anhydrous tripeptide was quite hygroscopic, the stable hydrate was air-dried to constant weight and employed for the spectral analysis.

O-Acetyl-N-carbobenzoxytyrosine Methyl Ester.—A 9.91-g. sample of recrystallized O-acetyl-N-carbobenzoxytyrosine was esterified in methyl alcohol with diazomethane. The product after two recrystallizations from ether melted at 73–74.5°.

Anal. Calcd. for $C_{20}H_{21}O_6N$: C, 64.66; H, 5.70; N, 3.77. Found: C, 64.68, 64.77; H, 6.00, 5.81; N, 4.13.

Tyrosine Methyl Ester Hydrochloride from the Reduction of O-Acetyl-N-carbobenzoxytyrosine Methyl Ester.—Three grams of O-acetyl-N-carbobenzoxytyrosine methyl ester, dissolved in 30 cc. of 0.27 *N* absolute methanolic hydrogen chloride, was reduced in the presence of palladium black catalyst. From the reaction solution was obtained 1.75 g. of fine needles, which after two recrystallizations from absolute methanol–absolute ether melted at 187° (dec.). A mixed melting point with authentic tyrosine methyl ester hydrochloride showed no depression.

Anal. Calcd. for $C_{10}H_{14}O_3NCl$: C, 51.83; H, 6.09. Found: C, 51.63, 51.55; H, 6.15, 6.31.

O-Acetyltyrosine Methyl Ester Hydrochloride.—One gram of O-acetyl-N-carbobenzoxytyrosine methyl ester was dissolved in 40 cc. of glacial acetic acid and hydrogenated in the presence of palladium black catalyst. The filtered solution was saturated with dry hydrogen chloride and concentrated *in vacuo* at a bath temperature of 25° until the hydrochloride crystallized. There was obtained 0.68 g. of O-acetyltyrosine methyl ester hydrochloride, which melted at 201° (dec.), after two recrystallizations from hot glacial acetic acid–ether.

Anal. Calcd. for $C_{12}H_{16}O_4NCl$: C, 52.65; H, 5.89; Cl, 12.95. Found: C, 52.47, 52.50; H, 5.77, 5.97; Cl, 12.8.

O-Acetyltyrosine Hydrochloride.—O-Acetyl-N-carbobenzoxytyrosine dissolved in glacial acetic acid was reduced with palladium black catalyst in the usual manner. The reduction product separated as a white, flocculent precipitate which was dissolved by saturating the solution with dry hydrogen chloride and heating on the steam-bath. The solution was filtered and the hydrochloride obtained as small white needles by cooling the filtrate and resaturating it with hydrogen chloride. Three recrystallizations from glacial acetic acid–hydrogen chloride gave a product melting at 223° (dec.).

Anal. Calcd. for $C_{11}H_{14}O_4NCl$: C, 50.87; H, 5.43; Cl, 13.65. Found: C, 50.81, 50.66; H, 5.53, 5.40; Cl, 13.21.

Action of Absolute Alcoholic Hydrogen Chloride on N-Carbobenzoxytyrosyltyrosine.—Anhydrous N-carbobenzoxytyrosyltyrosine (1.16 g.) was dissolved in 15 cc. of absolute ethanol, and the solution saturated at 0° with dry hydrogen chloride and stored in ice for thirty-one hours. By concentrating the alcohol solution and diluting it with ether, altogether 590 mg. of tyrosyltyrosine ethyl ester hydrochloride, m. p. 214°, was obtained. By allowing the mother liquor and washings to evaporate spontaneously overnight white needles of N-carbobenzoxytyrosyltyrosine ethyl ester were formed; weight 189 mg. After recrystallization from ethyl acetate–petroleum ether the latter melted at 159°, and gave no depression on admixture with an authentic sample.

Action of Absolute Alcoholic Hydrogen Chloride on O-Acetyl-N-carbobenzoxytyrosyltyrosine Ethyl Ester.—Five grams of O-acetyl-N-carbobenzoxytyrosyltyrosine ethyl ester was treated with 90 cc. of ethanolic hydrogen chloride as described in the preceding section. The product consisted of 2.0 g. of tyrosyltyrosine ethyl ester hydrochloride, m. p. 216° (dec.) and 1.2 g. of an impure product melting at 132–160°. The latter, which appeared to be composed of the starting material and N-carbobenzoxytyrosyltyrosine ethyl ester, we were unable to purify further.

N-Carbobenzoxyglycine Ethyl Ester.—The ester from 14 g. of glycine ethyl ester hydrochloride was coupled in ethyl acetate solution with 20.2 g. of carbobenzoxy chloride, employing the customary technique. The product was purified by vacuum distillation, b. p. 147–151° (0.5–1.0 mm.), and 17.8 g. was obtained. It formed beautiful long needles which were readily recrystallized from aqueous ethanol, m. p. 35.5–36.5°.

Anal. Calcd. for $C_{12}H_{15}O_4N$: C, 60.74; H, 6.37; N, 5.90. Found: C, 60.45, 60.72; H, 6.26, 6.46; N, 5.96.

Action of Absolute Alcoholic Hydrogen Chloride on Carbobenzoxyglycine.—A solution of 3 g. of carbobenzoxyglycine[15] in 15 cc. of absolute ethanol was saturated with dry hydrogen chloride at 0° and stored at this temperature for 42 hours. By cooling and seeding the solution there was obtained altogether 1.25 g. of glycine ester hydrochloride, which melted at 144.5–145° alone and at 143.5–144.5° when mixed with an authentic sample. The concentrated mother liquor was dissolved in ether, and the resulting solution dried over sodium sulfate and concentrated on the steam-bath. The light yellow residual oil was crystallized from alcohol–water to give 0.86 g. of carbobenzoxyglycine ester, m. p. 35.5°. This gave no depression in a mixed m. p. determination with an authentic sample.

An ethanolic hydrogen chloride solution of 10 g. of carbobenzoxyglycine was refluxed for one hour while a slow stream of dry hydrogen chloride was passed through the solution. 5.80 g. of glycine ester hydrochloride melting at 143–144° was obtained, and no carbobenzoxyglycine ester could be recovered from the mother liquor. This represents an 87% yield.

Summary

1. Tyrosyltyrosyltyrosine and tyrosyltyrosyltyrosyltyrosine have been synthesized.

2. The ultraviolet absorption specta of tyrosine, tyrosyltyrosine and tyrosyltyrosyltyrosine are compared.

3. The use of alcoholic hydrogen chloride for the removal of the carbobenzoxy group is described.

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