[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

Preparation of Arginyl Peptides

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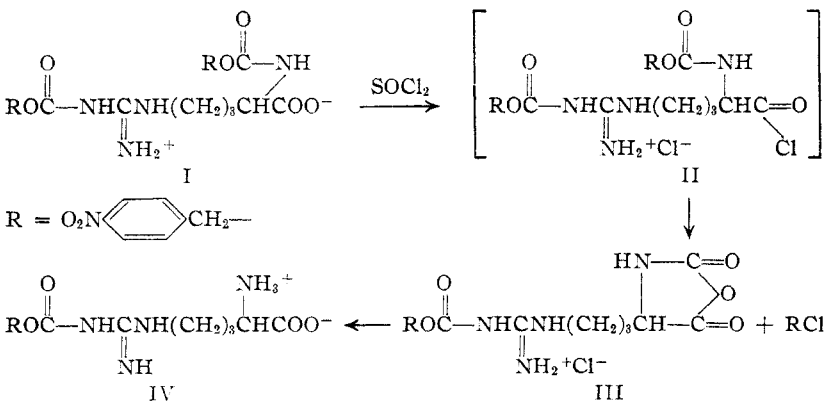
A method of synthesis of arginyl peptides employing the reagent N$^{\alpha}$-*p*-nitrobenzyloxycarbonyl-L-arginyl chloride hydrochloride (VI), prepared by the action of thionyl chloride on N$^{\alpha}$-*p*-nitrobenzyloxycarbonyl-L-arginine (V), is described. Its application in the preparation of N$^{\alpha}$-*p*-nitrobenzyloxycarbonyl-L-argininеamide hydrochloride, the corresponding anilide and L-arginyl-L-leucine and L-arginyl-L-glutamic acid is reported. The preparation of N$^{\omega}$-*p*-nitrobenzyloxycarbonyl-L-arginine-N-carbonic acid anhydride hydrochloride (III) from di-*p*-nitrobenzyloxycarbonyl-L-arginine (I) and unsuccessful attempts to prepare arginyl derivatives through its use are also described. The dissociation constants (pK_D values) of various derivatives of arginine determined in dioxane–water (1:1) are reported and compared to the pK_D values of arginine, histidine and alanine.

Less has been accomplished, perhaps, in the synthesis of peptides of arginine than with any other of the commonly occurring amino acids.[2] Up until this work, no well-defined peptide had been synthesized in which the carboxyl group of arginine was linked to another amino acid; although recently Katchalski and Spitnik[3] have prepared polyarginine, a mixture of polypeptides of arginine with an average chain length of 26 residues. Polyarginine was prepared by the guanidization of polyornithine. Because of the physiological importance of arginine and its derivatives, many attempts have been made to overcome the difficulties involved in the synthesis of peptides of arginine (for a review of past work see Fruton[2]).

As reported in an earlier paper[4] we have been able to prepare N$^{\alpha}$,N$^{\omega}$-di-*p*-nitrobenzyloxycarbonyl-L-arginine (I) by a method similar to that which we have used to prepare other *p*-nitrobenzyloxycarbonyl derivatives of many of the commonly occurring amino acids.

It was thought that the presence of the *p*-nitrobenzyloxycarbonyl group on the guanido group might mask the reactivity of the guanido group and make it possible to use this compound I in the preparation of arginyl peptides. The disubstituted



derivative I was readily converted to an N-carbonic acid anhydride III with the release of *p*-nitrobenzyl chloride by the action of thionyl chloride at 25–40°. However, it was soon recognized that the anhydride was formed as a hydrochloride salt, thus indicating the presence of a basic group in the compound. Furthermore, when the anhydride III was allowed to react with alcoholic ammonia or with a dimethylformamide solution of hydrazine, phenylhydrazine or glycine ethyl ester containing an equivalent of triethylamine, non-crystalline products were obtained which could not be purified and characterized. Since the guanido group in the anhydride III still retained some basic properties, it was considered possible that these results were due to the formation in alkaline solution of a mixture of products which included, in addition to the desired product, a product with a piperidone structure formed by intramolecular cyclization between the highly reactive N-carbonic anhydride group and the substituted guanido group. Intramolecular cyclizations of this type are known to take place readily.[5] When the anhydride III was decomposed in dilute acid, conditions which would keep a positive charge on the substituted guanido group, it was converted to N$^{\omega}$-*p*-nitrobenzyloxycarbonyl-L-arginine (IV). This product was converted to L-arginine acetate by catalytic hydrogenation. No racemization occurred.

In order to confirm the basic character of the substituted guanido group, various derivatives of arginine were titrated in dioxane–water (1:1). This solvent system was chosen because several of the derivatives are insoluble in water at the isoelectric point. The apparent pK_D values were obtained from the titration curves constructed by plotting moles of acid or base combined *versus* the apparent *p*H as determined by the glass electrode. For comparative purposes the titration curves in dioxane–water for arginine, histidine and alanine were obtained in a similar manner. Table I shows the calculated pK_D values obtained from these titrations.[6]

An examination of the titration curve for di-*p*-nitrobenzyloxycarbonyl-L-arginine revealed that when two *p*-nitrobenzyloxycarbonyl groups were

(1) Public Health Service Research Fellow of the National Institutes of Health.

(2) J. Fruton, "Advances in Protein Chemistry," Vol. 5, ed. by M. Anson, J. Edsall and K. Bailey, Academic Press, Inc., New York, N. Y., 1949, p. 1.

(3) E. Katchalski and P. Spitnik, *Nature*, **164**, 1092 (1949).

(4) D. T. Gish and F. H. Carpenter, THIS JOURNAL, **75**, 950 (1953).

(5) (a) P. Hamilton, *J. Biol. Chem.*, **198**, 587 (1952); (b) E. Fischer and G. Zemplén, *Ber.*, **42**, 4878 (1909).

(6) The titration curves and experimental conditions are contained in the doctoral dissertation of Duane T. Gish, University of California, Berkeley, 1953.

TABLE I

APPARENT DISSOCIATION CONSTANTS OF DERIVATIVES OF ARGININE AND OF SEVERAL AMINO ACIDS IN DIOXANE–WATER

Substance	pK_{D1}	pK_{D2}	pK_{D3}	pI_D
Alanine	3.30 (COOH)	10.05 (NH_3^+)		6.7
Histidine	2.65 (COOH)	5.80 (imid.)	9.45 (NH_3^+)	7.6
Arginine	2.95 (COOH)	9.25 (NH_3^+)	? (guan.)	(11)
N^{ω}-*p*-Nitrobenzyloxycarbonyl-L-arginine	2.85 (COOH)	5.80 (subst. guan.)	9.65 (NH_3^+)	7.7
Di-*p*-nitrobenzyloxycarbonyl-L-arginine methyl ester	5.35 (subst. guan.)			
Di-*p*-nitrobenzyloxycarbonyl-L-arginine	4.25 (COOH)	6.45 (subst. guan.)		5.35
N^{α}-*p*-Nitrobenzyloxycarbonyl-L-arginine	4.80 (COOH)	? (guan.)		(8.5)

introduced into arginine, the α-amino group with a pK_D of about 9.5 disappeared and a group of pK_D of about 6.5 appeared. This latter group is assumed to be the guanido group which has been substituted with a *p*-nitrobenzyloxycarbonyl group. Felix and Dirr[7] reported a similar basic group in dibenzoylarginine and were able to obtain a crystalline hydrochloride of that compound. The increase in the pK_D of the carboxyl group from 2.95 for arginine to about 4.25 for di-*p*-nitrobenzyloxycarbonylarginine was also found for N^{α}-*p*-nitrobenzyloxycarbonylarginine with a carboxyl pK_D of 4.8. The increase in the pK_D value of the carboxyl group when the α-amino group was substituted was expected since substitution on the α-amino group is generally accompanied by an increase in the pK value of the carboxyl group.[8] Since the glass electrode used was not sensitive in a *p*H range much above *p*H 11, it was not possible to determine the pK_D of the guanido group of N^{α}-*p*-nitrobenzyloxycarbonylarginine and arginine.

Good evidence that the *p*-nitrobenzyloxycarbonyl group in the compound called "N^{ω}-*p*-nitrobenzyloxycarbonyl-L-arginine" was actually on the guanido group and not the α-amino group was obtained from the titration curve for that compound. The pK_D for the carboxyl group was 2.85 compared to 2.95 for arginine and 4.8 for N^{α}-*p*-nitrobenzyloxycarbonylarginine and a basic group, apparently the α-amino group, with a pK_D of 9.65, compared to a pK_D of 9.25 for the α-amino group of arginine, was present in the molecule. A second basic group, presumably the substituted guanido group, with a pK_D of 5.80 was found as expected.

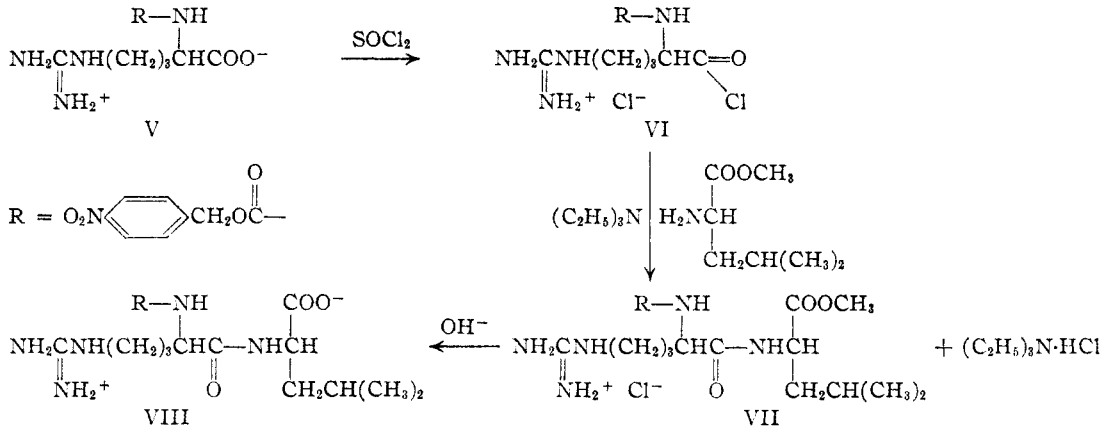
The pK_D of the substituted guanido group of N^{ω}-*p*-nitrobenzyloxycarbonyl-arginine-N-carbonic acid anhydride would be near the pK_D of the same group in a similar type compound such as di-*p*-nitrobenzyloxycarbonylarginine methyl ester. The pK_D of the latter compound was about 5.5. In alkaline medium interaction between this weakly basic group, which possesses a replaceable hydrogen atom, and the N-carbonic acid anhydride group would be expected to occur and to lead to serious side reactions.

Thus the basic problem in the synthesis of arginyl peptides remained: how to mask completely the reactivity of the guanido group. It occurred to us that a simple solution to this problem might rest in the high basicity of the guanido group as compared to that of an α-amino group. Since the pK of the guanido group is about 12.5 as compared to a pK of about 8 for the α-amino group of amino acid esters,[8] a pK of about 9 for ammonia and a pK of about 5 for aniline, the guanido group would remain largely bound to a proton in the presence of equivalent amounts of these or any other bases of comparable strength. Thus one should be able to mask the reactivity of the guanido group by combining it with a proton, and keeping it in a positively charged form, either as the zwitterionic salt or as a hydrochloride or similar salt, while permitting an acid chloride or other reactive group to react selectively with a discharged α-amino group or other group of similar basicity. This was found to be the case. Thus, the acid chloride hydrochloride VI of N^{α}-*p*-nitrobenzyloxycarbonyl-L-arginine (V) was prepared by the action of thionyl chloride on the latter compound. This is analogous to the preparation of an amino acid chloride hydrochloride by the action of phosphorus pentachloride on an acetyl chloride suspension of the amino acid.[9] N^{α}-*p*-Nitrobenzyloxycarbonyl-L-arginyl chloride hydrochloride (VI) was allowed to react with alcoholic ammonia to yield N^{α}-*p*-nitrobenzyloxycarbonyl-L-argininеamide hydrochloride in 78% yield. When the acid chloride VI was allowed to react with a dimethylformamide solution containing one equivalent of aniline and one equivalent of triethylamine the corresponding anilide was isolated in 45% yield. In a similar manner N^{α}-*p*-nitrobenzyloxycarbonyl-L-arginyl-L-leucine methyl ester hydrochloride (VII) was obtained in about 40% yield and N^{α}-*p*-nitrobenzyloxycarbonyl-L-arginyl-L-glutamic acid diethyl ester hydrochloride was obtained in about 50% yield. The esters were saponified by maintaining the *p*H of an aqueous-alcoholic solution between *p*H 10.5 and *p*H 11.5. L-Arginyl-L-leucine acetate and L-arginyl-L-glutamic acid were obtained by catalytic hydrogenation of an acetic acid solution of the N^{α}-*p*-nitrobenzyloxycarbonyl-L-arginyl-L-leucine (VIII) and N^{α}-*p*-nitrobenzyloxycarbonyl-L-arginyl-L-glutamic acid thus obtained. Under the conditions used the guanido group was always masked with a proton. One factor which was found to influence the yield in these reactions was the instability of the acid chloride VI. Usually about 0.2 mole of *p*-nitrobenzyl chloride was isolated from the preparation of the acid chloride. The presence of this *p*-nitrobenzyl chloride indi-

(7) K. Felix and K. Dirr, *Z. physiol. Chem.*, **176**, 29 (1928).

(8) E. Cohn and J. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 99.

(9) E. Fischer, *Ber.*, **38**, 615 (1905).



cated the concomitant formation of some N-carbonic anhydride during the preparation of the acid chloride.

Chromatograms of L-arginyl-L-leucine acetate and L-arginyl-L-glutamic acid and of the hydrolysates of these compounds showed that they contained no ninhydrin-positive impurities, and that the hydrolysates contained ninhydrin-positive substances with R_f's identical to those for arginine and leucine and arginine and glutamic acid, respectively.

The N^{α}-*p*-nitrobenzyloxycarbonyl-L-arginine (V) which was used in this work was prepared in about 90% yield by the addition of a dioxane solution of *p*-nitrobenzyl chloroformate to an aqueous solution of L-arginine buffered at a *p*H of about 10. Sufficient alkali was added along with the *p*-nitrobenzyl chloroformate to neutralize the hydrogen chloride evolved during the reaction. Thus, the *p*-nitrobenzyl chloroformate was allowed to react selectively with the α-amino group while the guanido group was masked with a proton.

Experimental[10]

N$^{\omega}$-*p*-Nitrobenzyloxycarbonyl-L-arginine-N-carbonic Acid Anhydride Hydrochloride.—Di-*p*-nitrobenzyloxycarbonyl-L-arginine[4] (3.4 g., 0.0064 mole) was dissolved in 15 ml. of thionyl chloride, and the solution was warmed at about 40° under a reflux condenser fitted with a calcium chloride tube. After about 30 minutes the solution became cloudy and an oil began to rise to the surface. Crystallization soon began. After an additional 30 minutes the reaction appeared to be complete (the lower thionyl chloride layer became clear) and the reaction flask was transferred to an ice-bath for 1.5 hours for the completion of crystallization. The excess thionyl chloride was then removed with the aid of a filter stick. The product was washed several times with 5-ml. portions of cold thionyl chloride and was then collected on a sintered glass filter. The anhydride was washed rapidly with four portions of absolute ether and dried for two hours over phosphorus pentoxide and sodium hydroxide pellets. The yield was 2.13 g. (80%), m.p. 148.5–151.5°. After recrystallization by precipitation from dimethylformamide with ether (50% recovery), the anhydride melted at 150–151°. It was dried over phosphorus pentoxide *in vacuo* at room temperature for analysis.

Anal. Calcd. for $C_{15}H_{17}O_7N_5 \cdot HCl$: C, 43.33; H, 4.36; N, 16.85; Cl, 8.53. Found: C, 43.61; H, 4.52; N, 16.64; Cl, 8.50.

When a few crystals of the anhydride were treated with aniline a rapid stream of bubbles was evolved. It gave a postive ninhydrin test[11] due to hydrolysis in the color developing solution.

The thionyl chloride mother liquor and washes were combined with the ether washes and the mixture was filtered free of a precipitate which consisted of additional impure product. The solvent was then removed *in vacuo* leaving a yellowish crystalline residue weighing 1.1 g. It was extracted with 20 ml. of hot petroleum solvent (b.p. 75–81°) in two portions. A white crystalline material weighing 0.84 g. was collected from the cooled mixture. It melted at 73.5–74.5° and no depression of the melting point was noted when it was mixed with authentic *p*-nitrobenzyl chloride. A second crop weighing 0.12 g., m.p. 55–71°, was recovered by concentration of the mother liquor. The crystalline *p*-nitrobenzyl chloride recovered amounted to about 90% of the theoretical.

N$^{\omega}$-*p*-Nitrobenzyloxycarbonyl-L-arginine.—N$^{\omega}$-*p*-Nitrobenzyloxycarbonyl-L-arginine-N-carbonic acid anhydride (2.4 g., 0.0058 mole) was dissolved in 15 ml. of dimethylformamide and was added over a period of one hour and 20 minutes to 20 ml. of 0.5 *N* hydrochloric acid. The reaction mixture was warmed at 30° and stirred with a magnetic stirrer. The effluent gas was trapped in saturated barium hydroxide. The evolution of carbon dioxide began soon and ceased after the final addition of the anhydride. The mixture was stirred for an additional 30 minutes. The solvent was removed *in vacuo*, 10 ml. of water was added and the distillation was repeated. The residual oil was then dissolved in 20 ml. of water and the *p*H was adjusted to about 7.2. A crystalline product separated. After the mixture had been allowed to stand in the cold, the crop was collected, washed with water and dried *in vacuo* over phosphorus pentoxide yield 1.87 g. (83%), m.p. 140–141°. It was hygroscopic. It was recrystallized from water (90% recovery), m.p. 140.5–142°. A sample was dried at 100° *in vacuo* over phosphorus pentoxide for analysis, $[\alpha]^{24}D$ +16.8° (*c* 1, 6 *N* hydrochloric acid).

Anal. Calcd. for $C_{14}H_{19}O_6N_5$: C, 47.59; H, 5.42; N, 19.82. Found: C, 47.17; H, 5.61; N, 19.65.

Catalytic Hydrogenation of N$^{\omega}$-*p*-Nitrobenzyloxycarbonyl-L-arginine.—N$^{\omega}$-*p*-Nitrobenzyloxycarbonyl-L-arginine (1.0 g., 0.0028 mole) was dissolved in 20 ml. of glacial acetic acid to which had been added 0.2 g. of palladous oxide.[12] Hydrogen was bubbled through as the mixture was stirred with a magnetic stirrer. The reduction was followed by trapping the carbon dioxide evolved in saturated barium hydroxide. Carbon dioxide evolution ceased after about one hour. The catalyst was filtered off and the solvent was removed *in vacuo*. The material was placed in a desiccator *in vacuo* (mechanical pump) over phosphorus pentoxide and sodium hydroxide pellets overnight in order to remove the slightly volatile *p*-toluidine acetate. The yellowish-brown oil was then dissolved in 95% ethanol and crystallization soon followed. The L-arginine acetate was collected, washed with 95% ethanol and dried, yield 0.51 g. (80%), m.p. 222–

(10) Melting points, unless otherwise noted, were taken on the hot stage. The analyses reported were performed by the Microchemical Laboratory, University of California, Berkeley. In several instances low nitrogen values (Dumas) were obtained. Slow combustion improved the values. Felix and Dirr[7] reported low nitrogen values (Dumas) were often encountered with arginine and its derivatives. In the case of most of the derivatives reported here, the Kjeldahl method would also give low nitrogen values because of the presence of the nitro group. The water analyses were by the method of Karl Fischer as modified by E. Almy, *et al.*, *Anal. Chem.*, **12**, 392 (1940).

(11) S. Moore and W. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

(12) R. Shriner and R. Adams, THIS JOURNAL, **46**, 1683 (1924).

225°, $[\alpha]^{24}D$ +25.4° (*c* 0.75, 6 *N* hydrochoric acid) (corrected for one mole of acetic acid). The rotation of the L-arginine hydrochloride used for the preparation of the di-*p*-nitrobenzyloxycarbonyl-L-arginine was $[\alpha]^{24}D$ +24.8° (*c* 0.8, 6 *N* hydrochloric acid) (corrected for one mole of hydrogen chloride). When a sample of the product obtained above was mixed with a sample of authentic L-arginine acetate (described below) no depression of the melting point was noted. The *Rf* of the above reduction product was identical with that of authentic L-arginine.

L-Arginine Acetate.—L-Arginine free base (2.0 g.) was dissolved in 20 ml. of glacial acetic acid and the excess acetic acid was removed *in vacuo*. The residual oil was dissolved in 95% ethanol and crystallization soon began. After the mixture had been allowed to stand at room temperature and then in the cold, the acetate was collected, washed with 95% ethanol and allowed to dry. The yield was 2.55 g. (95%), m.p. 221–223°, with slow heating. It was noted that with this sample and the sample prepared above that if the material was placed upon the block at about 190° and heated rapidly it melted at about 210–215° and then recrystallized and melted at about 222°. If the sample was placed on the block at about 100° a change in crystalline form took place gradually with the rise in temperature and the sample melted at 221–223°. For analysis the sample was recrystallized from water–ethanol.

Anal. Calcd. for $C_6H_{14}O_2N_4 \cdot CH_3COOH$: C, 41.02; H, 7.74; N, 23.92. Found: C, 41.34; H, 7.70; N, 24.18.

Di-*p*-nitrobenzyloxycarbonyl-L-arginine Methyl Ester.—Di-*p*-nitrobenzyloxycarbonyl-L-arginine[4] (2.0 g.) was suspended in 100 ml. of methanol and hydrogen chloride was passed into the mixture for 20 minutes. The material went into solution rapidly. The clear solution was allowed to stand at room temperature overnight. The excess methanol and hydrogen chloride were removed *in vacuo*. This procedure was repeated twice more. The oil was then dissolved in about 50 ml. of dioxane–water (1:1) and the *p*H was adjusted to about 10 with 4 *N* sodium hydroxide. The mixture was saturated with sodium carbonate and extracted with about 60 ml. of ethyl acetate in 3 portions. The organic phase was dried over magnesium sulfate and the solvent was removed *in vacuo*. The oily residue was dried *in vacuo* over phosphorus pentoxide and sodium hydroxide pellets, weight 1.46 g. (71%). Although the ester was not obtained in crystalline form, it was of sufficient purity for the titration experiments.

N$^{\alpha}$-*p*-Nitrobenzyloxycarbonyl-L-arginine.—L-Arginine monohydrochloride (10.5 g., 0.05 mole) was dissolved in 15 ml. of 4 *N* sodium hydroxide and 40 ml. of *N* sodium bicarbonate. The resulting *p*H was 10.0. In the usual manner[4] were added 10.8 g. (0.05 mole) of *p*-nitrobenzyl chloroformate dissolved in purified dioxane[13] (total volume 30 ml.), and 12.5 ml. of 4 *N* sodium hydroxide in five approximately equal portions. About ten minutes were allowed between additions. After completion of the reaction the *p*H of the mixture was adjusted to about 5.5 with concentrated hydrochloric acid and a small amount of the disubstituted product was removed by filtration. The solution was extracted with three 50-ml. portions of ethyl acetate and the *p*H of the aqueous mixture was then adjusted to about 7.5 with a few drops of alkali. The clear solution was concentrated *in vacuo* to about 65 ml., and crystallization began as soon as most of the dioxane had been removed. The mixture was cooled and transferred to the filter with aid of cold water. The material was washed with water and ethanol and allowed to dry in air, yield 15.5 g. (88%), m.p. 192–193.5°. After the material had been recrystallized from water (85% recovery), it melted at 193–193.5° and analysis showed it to contain one-half mole of water of crystallization. It gave a negative ninhydrin test.[11]

Anal. Calcd. for $C_{14}H_{19}O_6N_5 \cdot {}^1/_2H_2O$: C, 46.40; H, 5.84; N, 19.33; H_2O, 2.49. Found: C, 46.17; H, 5.57; N, 19.59; H_2O, 2.5.

After a sample had been dried at 100° *in vacuo* over phosphorus pentoxide for several hours it lost 2.5% in weight, $[\alpha]^{25}D$ +11.8° (*c* 0.5, dioxane–water (1:1)).

Anal. Calcd. for $C_{14}H_{19}O_6N_5$: C, 47.59; H, 5.42; N, 19.82. Found: C, 47.22; H, 5.38; N, 19.50.

(13) According to E. Eigenberger, see A. Weissberger and E. Proskauer, "Organic Solvents," Oxford University Press, New York, N. Y., 1935, p. 139.

N$^{\alpha}$-*p*-Nitrobenzyloxycarbonyl-L-arginyl Chloride Hydrochloride.—N$^{\alpha}$-*p*-Nitrobenzyloxycarbonyl-L-arginine (1.78 g., 0.005 mole) was layered over with 15 ml. of cold thionyl chloride and the reaction was then allowed to proceed at room temperature for about 5 minutes with vigorous stirring. The crystalline material was rapidly converted to a heavy oil. The flask was transferred to an ice–salt-bath and stirring was continued for another 5–10 minutes. The excess thionyl chloride was decanted off and the product was washed with 5 ml. of cold thionyl chloride and then stirred with cold absolute ether which caused it to solidify. The white solid material was finely pulverized, collected on a sintered glass filter and washed several times with dry ether. This material was used immediately for the preparation of the amide.

N$^{\alpha}$-*p*-Nitrobenzyloxycarbonyl-L-arginineamide Hydrochloride.—The acid chloride prepared above was added portionwise with stirring to 25 ml. of methanol which had been saturated with ammonia and cooled in an ice–salt-bath. The solution was then allowed to stand overnight at room temperature. The mixture was filtered to remove a small amount of insoluble material and the methanol and excess ammonia were removed *in vacuo*. The material crystallized readily when stirred with 8 ml. of hot water. After the mixture had been cooled, the product was collected, washed with cold water and dried, yield 1.51 g. (78%), m.p. 116–129°. It was recrystallized from water for analysis (85% recovery), m.p. 125–140°, $[\alpha]^{25}D$ +4.8° (*c* 1, dioxane–water (1:1)). The rotation is that calculated on the basis of the anhydrous material. When a sample was dried *in vacuo* over phosphorus pentoxide at 100°, it lost 4.5% in weight, calculated for one mole of water, 4.43%.

Anal. Calcd. for $C_{14}H_{20}O_5N_6 \cdot HCl \cdot H_2O$: C, 41.33; H, 5.70; N, 20.66; Cl, 8.96; H_2O, 4.43. Found: C, 41.23; H, 5.80; N, 20.79; Cl, 8.68; H_2O, 4.6.

N$^{\alpha}$-*p*-Nitrobenzyloxycarbonyl-L-arginineanilide Hydrochloride.—The acid chloride prepared from 2.0 g. (0.0057 mole) of N$^{\alpha}$-*p*-nitrobenzyloxycarbonyl-L-arginine was added portionwise with stirring to a dimethylformamide solution of 0.53 g. (0.0057 mole) of aniline and 0.58 g. (0.0057 mole) of triethylamine which was cooled in an ice–salt-bath. The mixture was then allowed to stand overnight at room temperature. The precipitate of triethylamine hydrochloride was removed by filtration. Several volumes of ether were then added to the dimethylformamide solution in order to precipitate the anilide hydrochloride. The mother liquor was decanted from the oil and the product was washed with ether. The oil crystallized rapidly when stirred with a few ml. of water. The crystalline material was collected, washed with cold water and dried *in vacuo* over phosphorus pentoxide, yield 1.2 g. (45%), m.p. 173–181°. It was recrystallized from water (73% recovery), m.p. 180–182°, $[\alpha]^{24}D$ −4.4° (*c* 1, acetic acid–water (1:1)).

Anal. Calcd. for $C_{20}H_{24}O_5N_6 \cdot HCl$: C, 51.67; H, 5.42; N, 18.08; Cl, 7.63. Found: C, 51.54; H, 5.55; N, 18.46; Cl, 7.38.

N$^{\alpha}$-*p*-Nitrobenzyloxycarbonyl-L-arginyl-L-leucine Methyl Ester Hydrochloride.—The acid chloride prepared from 3.53 g. (0.01 mole) of N$^{\alpha}$-*p*-nitrobenzyloxycarbonyl-L-arginine was added portionwise with stirring to a dimethylformamide solution of L-leucine methyl ester (prepared from 1.82 g. or 0.01 mole of the hydrochloride by dissolving the hydrochloride in dimethylformamide, adding one equivalent of triethylamine and removing the triethylamine hydrochloride precipitate by filtration) and 1.01 g. (0.01 mole) of triethylamine which was kept cooled in an ice–salt-bath. After the addition of the acid chloride the mixture was allowed to stand at room temperature for one hour, then in the cold for one hour. The triethylamine hydrochloride was removed by filtration and washed twice with small portions of cold dimethylformamide, and the washings were added to the solution of the product. To the clear filtrate was added 300 ml. of ether and the dimethylformamide–ether mother liquor was decanted from the oil which had separated. After the oil had been washed with ether it was dissolved in 10 ml. of hot water. The product separated as a crystalline solid from the cooled aqueous solution. After the mixture had been allowed to stand in the cold overnight the product was collected and washed twice with a little cold water, yield 2.09 g., m.p. 158–164°. After the mother liquor had been allowed to stand several days in the cold a second crop was collected, weight 0.1 g., m.p. 156–165°.

The total yield was 2.19 g. or 42%. The ester hydrochloride was recrystallized from ethanol–ether (70% recovery), m.p. 167–169°, $[\alpha]^{24}D$ −15.2° (*c* 1, 95% ethanol). It gave a negative ninhydrin test.[11] A sample was allowed to dry in air for analysis.

Anal. Calcd. for $C_{21}H_{32}O_7N_6 \cdot HCl$: C, 48.79; H, 6.43; N, 16.26; Cl, 6.86. Found: C, 48.67; H, 6.05; N, 16.34; Cl, 6.85.

Nα-*p*-Nitrobenzyloxycarbonyl-L-arginyl-L-leucine.—The ester hydrochloride (1.0 g., 0.0019 mole) was dissolved in 20 ml. of 50% ethanol. Sodium hydroxide (0.5 *N*) was then added at a rate necessary to maintain the *p*H between 10.5 and 11.5 as measured on a *p*H meter with a glass electrode. After about an equivalent amount of alkali had been consumed a 10% excess was added and the mixture was allowed to stand overnight. The product separated as a mixture of crystals and oil. The entire crop readily crystallized when stirred. The *p*H was adjusted to 8.0 with a little dilute acid, the mixture cooled, and the product was collected and washed with water. It was dried *in vacuo* over phosphorus pentoxide, weight 0.75 g. (83%), m.p. 160–166°. The peptide derivative, which was recrystallized from ethanol–water (90% recovery), separated with three moles of water of crystallization, m.p. 167.5–169°, $[\alpha]^{24}D$ −6.3° (*c* 0.9, dioxane–water (1:1)). The specific rotation is that calculated for the anhydrous material.

Anal. Calcd. for $C_{20}H_{30}O_7N_6 \cdot 3H_2O$: C, 46.15; H, 6.97; N, 16.15; H_2O, 10.38. Found: C, 46.32; H, 6.95; N, 16.07; H_2O, 10.5.

After a sample had been dried *in vacuo* over phosphorus pentoxide at 75° for 16 hours it still contained one mole of water according to analysis. When the drying period was extended to 72 hours, elementary analysis indicated that the sample still contained a small amount of water.

Anal. Calcd. for $C_{20}H_{30}O_7N_6$: C, 51.49; H, 6.48; N, 18.02. Found: C, 50.91; H, 6.62; N, 17.94.

L-Arginyl-L-leucine Acetate.—Nα-*p*-Nitrobenzyloxycarbonyl-L-arginyl-L-leucine trihydrate (0.7 g., 1.34 mmoles) was dissolved in 20 ml. of glacial acetic acid to which had been added 140 mg. of palladous oxide and the reduction was carried out in the usual manner. The reduction appeared to be complete in about 30 minutes. The catalyst was removed by filtration and the acetic acid was removed *in vacuo*. About 10 ml. of water was added and the distillation was repeated, leaving a crystalline residue. It was dried *in vacuo* (mechanical pump) over phosphorus pentoxide and sodium hydroxide in order to remove the slightly volatile *p*-toluidine acetate, weight 0.47 g. (theoretical), m.p. 175–185° on the hot stage. The peptide acetate was recrystallized from acetic acid–ether (89% recovery), m.p. 181–184° on the hot stage, 207–208° in a sealed capillary, $[\alpha]^{24}D$ +11.6° (*c* 0.4, water). The rotation is that calculated for the free peptide, corrected for one mole of acetic acid. The ninhydrin test was positive. A sample was dried at 75° *in vacuo* over phosphorus pentoxide for analysis.

Anal. Calcd. for $C_{12}H_{25}O_3N_5 \cdot CH_3COOH$: C, 48.40; H, 8.41; N, 20.16. Found: C, 48.09; H, 8.16; N, 20.60.

Nα-*p*-Nitrobenzyloxycarbonyl-L-arginyl-L-glutamic Acid Diethyl Ester Hydrochloride.—The acid chloride prepared from 3.53 g. (0.01 mole) of Nα-*p*-nitrobenzyloxycarbonyl-L-arginine was added in portions to a cold dimethylformamide solution of L-glutamic acid diethyl ester (prepared from 2.40 g. or 0.01 mole of the hydrochloride[14] in the same manner as L-leucine methyl ester) and one equivalent of triethylamine. After the reaction mixture had been allowed to stand at room temperature for two hours the mixture was cooled and the triethylamine hydrochloride was removed by filtration. About 400 ml. of ether was added to the dimethylformamide solution and the mother liquor was decanted from the oil. After the product had been washed with ether several times it was dissolved in 10 ml. of warm water and crystallization was induced by scratching the wall of the flask. After the mixture had been allowed to stand overnight in the refrigerator the product was collected, washed with cold water and dried over phosphorus pentoxide *in vacuo*, weight 2.8 g. (48%), m.p. 51–55°, some material then crystallized and melted at 123–126°. For analysis the ester hydrochloride was recrystallized from water–ethanol–ether (1:5:30) (70% recovery) from which it crystallized with three moles of water (calculated for three moles of water: H_2O, 10.62. Found: H_2O, 10.3), m.p., loses water at 51.5°, melts at 139–140°, $[\alpha]^{25}D$ −9.8° (*c* 0.9, 95% ethanol) (calculated for the anhydrous material). A sample was dried at 56° over phosphorus pentoxide *in vacuo* for analysis.

Anal. Calcd. for $C_{23}H_{34}O_9N_6 \cdot HCl$: C, 48.04; H, 6.14; N, 14.62; Cl, 6.17. Found: C, 47.78; H, 5.99; N, 14.61; Cl, 6.21.

Nα-*p*-Nitrobenzyloxycarbonyl-L-arginyl-L-glutamic Acid. The ester hydrochloride prepared above (1.13 g., 1.96 mmoles) was dissolved in 10 ml. of 50% ethanol and the saponification carried out in the same manner as the saponification of Nα-*p*-nitrobenzyloxycarbonyl-L-arginyl-L-leucine methyl ester hydrochloride. After the completion of the saponification the clear solution was titrated with 0.5 *N* hydrochloric acid to *p*H 4.2 which is approximately the isoelectric point of the compound in this solvent. An oil separated which crystallized after it had been allowed to stand in the refrigerator overnight. The product was dried *in vacuo* over phosphorus pentoxide, weight 0.83 g. (88%), m.p. 138.5–140.5°. For analysis the peptide derivative was crystallized from ethanol–water (84% recovery), crystallizing as a hydrate, m.p. 125–142°. When dried it melted at 141–143°, $[\alpha]D^{26}$ −15.9° (*c* 1, 6 *N* hydrochloric acid). A sample was dried at 56° *in vacuo* over phosphorus pentoxide for analysis.

Anal. Calcd. for $C_{19}H_{26}O_9N_6$: C, 47.30; H, 5.43; N, 17.42. Found: C, 46.92; H, 5.21; N, 17.11.

L-Arginyl-L-glutamic Acid.—Nα-*p*-Nitrobenzyloxycarbonyl-L-arginyl-L-glutamic acid (1.7 g., 3.5 mmoles) was dissolved in 20 ml. of acetic acid and 0.3 g. of palladous oxide was added. The hydrogenation was carried out as before. After the catalyst was removed by filtration and the solvent was removed *in vacuo* the residual oil was dried over phosphorus pentoxide and sodium hydroxide pellets *in vacuo* (mechanical pump) in order to remove the slightly volatile *p*-toluidine acetate. The oil was dissolved in about 20 ml. of water and ethanol was added to opalescence (35 ml. was required). The material was induced to crystallize, and was collected and washed with ethanol and dried over phosphorus pentoxide *in vacuo*. The hygroscopic material weighed 0.89 g. (83%), m.p. 203.5–204.5°. The peptide was recrystallized from water–ethanol (90% recovery), separating as a hydrate, which was observed on the hot stage to undergo a change in crystalline form at about 55°, sinter at 170° and melt at about 205–210°, $[\alpha]^{25}D$ +22.0° (*c* 1, water) (calculated for anhydrous material). A sample was allowed to dry in air for analysis.

Anal. Calcd. for $C_{11}H_{21}O_5N_5 \cdot 2H_2O$: C, 38.93; H, 7.43; N, 20.64; H_2O, 10.62. Found: C, 38.73; H, 7.49; N, 20.45; H_2O, 10.3.

A sample was dried at 100° over phosphorus pentoxide *in vacuo* and submitted for analysis.

Anal. Calcd. for $C_{11}H_{21}O_5N_5$: C, 43.55; H, 6.98; N, 23.09. Found: C, 43.58; H, 6.94; N, 23.01.

Chromatography of L-Arginyl-L-leucine Acetate, L-Arginyl-L-glutamic Acid and their Hydrolysates.—About 10 mg. of each peptide was hydrolyzed by refluxing with 6 *N* hydrochloric acid for 14 hours. The solvent was removed *in vacuo* and the distillation was repeated twice after the addition of water. The residue from each hydrolysate was dissolved in one ml. of water and aqueous solutions of each peptide, L-arginine, L-leucine and L-glutamic acid of about the same concentration were prepared. L-Arginyl-L-leucine, its hydrolysate, arginine and leucine were chromatogrammed on Whatman No. 1 paper using 1-butanol–acetic acid–water (5:1:4) as the solvent. The chromatogram showed that the peptide (R_f = 0.46) was free of any other ninhydrin-positive material. It traveled close to, but was well separated from leucine (R_f = 0.58). The hydrolysate contained two ninhydrin positive substances with R_f's identical to those of arginine and leucine. L-Arginyl-L-glutamic acid, its hydrolysate, arginine and glutamic acid were chromatogrammed on Whatman No. 1 paper buffered with *p*H 9.3 borate buffer using the *m*-cresol–phenol–*p*H 9.3 borate buffer system of Levy and Chung.[15] The peptide (R_f = 0.28) was chromatographically pure and the hy-

(14) We are indebted to Michael Gumbman for this compound which was prepared according to the method of H. M. Chiles and W. A. Noyes, THIS JOURNAL, **44**, 1802 (1922).

(15) A. Levy and D. Chung, *Anal. Chem.*, **25**, 396 (1953).

drolysate contained two ninhydrin-positive substances with R_f's identical to those of arginine (R_f = 0.82) and glutamic acid (R_f = 0.06).

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

A Synthesis of *dl*-Thyronine *via* 4-Chloromethyl-4′-nitrodiphenyl Ether[1]

BY PHILIP L. SOUTHWICK, GEORGE E. FOLTZ AND WILLIAM E. MCINTYRE, JR.[2]

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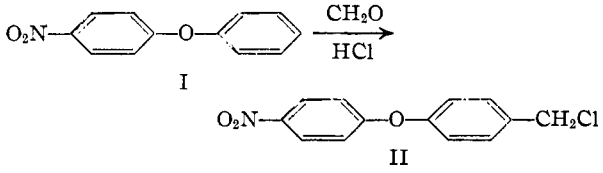
dl-Thyronine has been prepared in 30% yield from 4-nitrodiphenyl ether by means of a sequence of reactions (four separate steps) beginning with chloromethylation to give 4-chloromethyl-4′-nitrodiphenyl ether, and condensation of the latter compound with the sodium derivative of diethyl acetamidomalonate.

In the compound 4-nitrodiphenyl ether (I) the ring holding the nitro group should be relatively inert toward electrophilic reagents for aromatic substitution, whereas the other ring should show a higher reactivity and ortho and para orientation of an entering group. Previous investigations of the reactions of this substance have, in fact, shown that halogenation in the 4′-position is strongly favored.[3] It seemed likely, therefore, that chloromethylation of this compound, if it occurred at all, would lead almost exclusively to the formation of 4-chloromethyl-4′-nitrodiphenyl ether (II), a potentially useful synthetic intermediate. We were particularly interested in the utility of this substance as a starting point for the preparation of compounds related to thyroxine. In the present investigation satisfactory conditions were found for the preparation of compound II by means of the proposed chloromethylation reaction, and a useful synthesis was developed for *dl*-thyronine (V), the desiodo derivative of *dl*-thyroxine. The new thyronine synthesis produced a considerably better over-all yield than methods previously reported.[4]

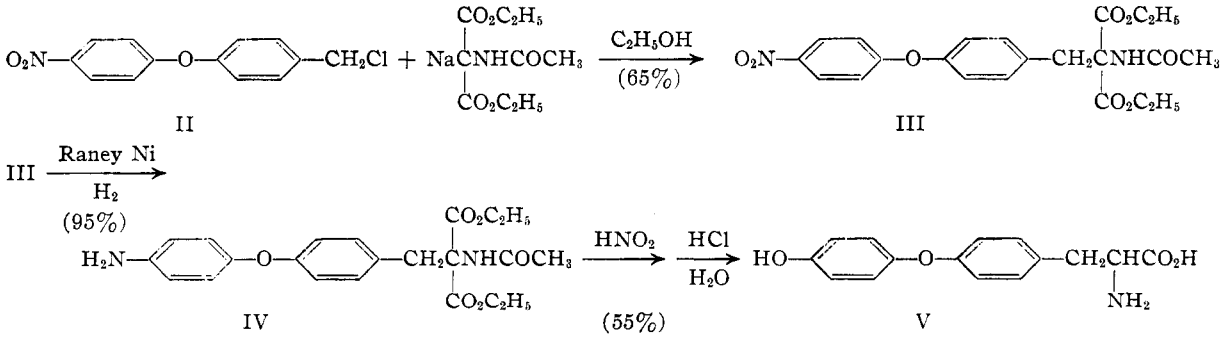


I II

The best conditions found for the chloromethylation of 4-nitrodiphenyl ether (I) involved the use of a glacial acetic acid–phosphoric acid mixture as the reaction medium and a 60-hour heating period on a steam-bath. In this way the crystalline chloromethyl derivative II, m.p. 54–55° when fully purified, was obtained in 90% yield. The use of higher reaction temperatures reduced the time required for complete reaction but resulted in lower yields because of the decomposition of the product. That the chloromethyl group had been introduced into the 4′-position of 4-nitrodiphenyl ether (I) was established by oxidation of the chloromethyl derivative II with potassium permanganate solution to give a sample of 4-(4′-nitrophenoxy)-benzoic acid which was identical with a sample prepared by oxidizing 4-nitro-4′-methyldiphenyl ether.[5]

For the synthesis of *dl*-thyronine (V) from the chloromethyl derivative II a method based on the use of diethyl acetamidomalonate was developed. A 65% yield of the key intermediate, the malonic ester derivative III, was obtained from the reaction of compound II with the sodium derivative of diethyl acetamidomalonate in absolute ethanol solution.

A number of routes for the conversion of the malonic ester derivative III into *dl*-thyronine (V) were investigated. The one which proved most satisfactory was that involving the transformations indicated below.



Analytical results, the melting point and the melting points of derivatives confirmed the identification of the product as *dl*-thyronine. A new

(1) Abstracted from a thesis submitted by William E. McIntyre, Jr., in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Carnegie Institute of Technology, December, 1952.

(2) Institute Fellow in Organic Chemistry, 1951–1952.

(3) (a) R. Q. Brewster and R. Slocombe, THIS JOURNAL, **67**, 562 (1945); (b) H. A. Scarborough, *J. Chem. Soc.*, 2361 (1929).

(4) (a) G. R. Harington, *Biochem. J.*, **20**, 300 (1926); (b) C. R. Harington and W. McCartney, *ibid.*, **21**, 852 (1927); (c) A. Canzanilli, C. R. Harington and S. S. Randall, *ibid.*, **28**, 68 (1934); (d) C. R. Harington and R. V. Pitt Rivers, *J. Chem. Soc.*, 1101 (1940).

(5) Both samples of the acid melted at 236–237° and a mixed melting point showed no depression. C. Haussermann and E. Bauer, *Ber.*, **29**, 2083 (1896), gave the m.p. 236–237°, and C. M. Suter and E. Oberg, THIS JOURNAL, **53**, 1566 (1931), gave the m.p. 235–236°, but H. A. Scarborough and J. L. Sweeten, *J. Chem. Soc.*, 52 (1934), recorded a value of 245°.