TABLE VII

PROTON N.S.R. SHIELDING VALUES, τ, FOR ACIDIC AMINO ACIDS AND PEPTIDES IN TRIFLUOROACETIC ACID

Compound	$CONH_2$	NH_3^+	α-H	CH_2
L-Aspartic acid	..	2.24 m, b; $W = 16$	5.31 d; $J = 5$	6.45 m, b; $W = 16$
L-Asparagine	2.68 m, b; $W \leq 17$	2.15 m, b; $W \leq 18$	5.33 m, b; $W = 17$	6.51 m, b; $W = 12$
Glycyl-L-asparagine	CONH: 1.73 d; $J = 7$ $CONH_2$: 2.48 (in NH_3^+ peak)	2.48 m, b; $W = 21$	a: 4.77 m, b; $W = 19$ g: 5.67 m, b; $W = 19$	6.73 m, b; $W = 17$
L-Glutamic acid	..	2.32 m, b; $W = 15$	5.45 d; $J = 5$	β: 7.18 γ: ~7.45 (poor) both m, b
Glycyl-L-glutamic acid	2.12 d; $J \leq 7$	2.39 m, b; $W = 24$	glut.: 5.12 m, b; $W = ?$ gly.: 5.76 m, b; $W \leq 22$	$(C_\gamma H_2)_2$: 7.42 m, b; $W = 24$
L-Glutamine	1.74	2.20 m, b; $W = 14$	5.47 m, b; $W = 22$	$(CH_2)_2$: 7.26 m, b; $W = 18$

trum of 2-thiolhistidine, in which this peak is missing. (In this compound, the sulfhydryl hydrogens, unlike those of cysteine, evidently exchange rapidly with the solvent and give rise to no distinguishable peak.)

The spectral peak positions of 1-N-methylhistidine and L-histidine methyl ester are in accordance with expectation. The interpretation of the spectrum of L-histidyl-L-histidine (Fig. 5-c) shows the α-ammonium resonance to be at an unusually low τ-value, as compared to other dipeptides. The pK' for the α-amino group of histidylhistidine in water is 7.80,[21] appreciably lower than the value of 8.2 ± 0.1 characteristic of dipeptides having one basic group and one carboxyl.[21] In trifluoroacetic acid this difference may be expected to be considerably exaggerated because the α-amino group is being observed in a medium of low dielectric constant and under conditions where both imidazole groups are probably positively charged. The displacement of the peak due to the α-amino group is too great to be attributed to the direct inductive effect of the nearest charged imidazole group, but may well result from π-electron ring currents.[7,16-19] The peaks of the α-amino groups of histidine and its other one-ring derivatives (Table VI) are also at somewhat lower τ-values than those of most other compounds reported in this study, but the displacement is much less than found for histidylhistidine.

Acidic Amino Acids and Peptides

The peak positions of the acidic amino acids, aspartic and glutamic, and of asparagine and glutamine, together with two glycyl peptides, are given in Table VII; the spectrum of glutamic acid in Fig. 6. The amide proton peak for asparagine is at considerably higher τ-value than that of glutamine; in glycylasparagine, a similar deviation is seen, the peak due to the peptide proton (identifiable by virtue of its splitting) appearing at considerably lower τ-values than that for the $CONH_2$ protons, which is evidently included under the ammonium peak.

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[CONTRIBUTION FROM THE DEPARTMENT OF ORGANIC CHEMISTRY, UNIVERSITY OF ATHENS, AND THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Studies on Arginine Peptides. II. Synthesis of L-Arginyl-L-arginine and other N-Terminal Arginine Dipeptides

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Nα,Nω-Dicarbobenzoxy-L-arginine and tricarbobenzoxy-L-arginine have been utilized for the preparation of a variety of dipeptides containing an N-terminal arginine residue. The former compound, either as its acid chloride–hydrochloride derivative or under the condensing action of dicyclohexylcarbodiimide, was coupled with diethyl L-glutamate and the condensation product subsequently converted to the corresponding free peptide *via* successive saponification and catalytic hydrogenolysis; the formation of Nα,Nω-dicarbobenzoxyanhydro-L-arginine accompanied the coupling reaction, an occurrence presumably attributable to the fact that the basicity of the guanido moiety of Nα,Nω-diacylated arginines is not completely masked. No analogous evidence of intramolecular cyclization was revealed in the case of the more exhaustively protected tricarbobenzoxy-L-arginine upon like condensation, *via* its mixed carbonic–carboxylic acid anhydride derivative, with the benzyl ester derivatives of L-alanine, L-aspartic acid, L-glutamic acid, glycine, L-isoleucine, D-alloisoleucine, L-leucine, L-phenylalanine, L-tyrosine and L-valine. Catalytic hydrogenolysis of the tricarbobenzoxy-L-arginylamino acid benzyl esters so procured led to the corresponding dipeptides, which were isolated in high over-all yield. A comparable condensation between tricarbobenzoxy-L-arginine and benzyl Nω-carbobenzoxy-L-argininate permitted the ultimate synthesis of L-arginyl-L-arginine, which was isolated and characterized as its diflavianate and dipicrolonate derivatives.

Introduction

The successful union of an arginine residue with another amino acid residue in peptide linkage requires that the basicity of the former be adequately masked prior to its implication in the peptide-forming step. For such purpose, the basicity of the

guanido function is customarily suppressed *via* (a) substitution with a nitro function, (b) salt formation with a hydrogen halide or (c) acylation. The first of these techniques was introduced by Bergmann, Zervas and Rinke[1] who, in 1934, exploited the strongly electronegative character of the nitro function to effectively depress the basicity of the guanido group and thereupon facilitate the incorporation of the arginine residue into peptides; subsequent removal of the nitro function could be achieved through catalytic reduction.[2] An analogous technique was applied to the synthesis of a variety of C-terminal arginine peptides[3-7] and, with the introduction of the mixed carboxylic–carbonic acid anhydride[8,9] and the dicyclohexylcarbodiimide[10] methods, was applied to the preparation of N-terminal arginine peptides as well.[11] Although the use of hydrohalides[12-15] to block the basicity of the guanido moiety has been less frequent than the aforementioned method, it has nevertheless found important application in the synthesis of such important biological compounds as arginine-vasopressin.[16] The final method, with which the initial studies[17,18] of this series were concerned, involves the conversion of arginine to its $N^{\alpha},N^{\omega},N^{\omega}$-tricarbobenzoxy derivative, followed by application of the latter to the synthesis of L-arginyl-L-glutamic acid.[19] It is the purpose of the present communication to report the use of N^{α},N^{ω}-dicarbobenzoxy-L-arginine in like connection, as well as to describe the extended application of tricarbobenzoxy-L-arginine to the preparation of a variety of N-terminal arginine peptides, with particular emphasis on the synthesis of L-arginyl-L-arginine.

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(19) A preliminary report from this Laboratory[20] described the use of an analogous procedure for the preparation of L-arginyl-L-arginine, but without experimental detail.

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Results and Discussion

The basic character of the guanido group in such N^{α},N^{ω}-diacylated L-arginines as the dibenzoyl,[21,22] dibenzylsulfonyl,[23] di-*p*-nitrocarbobenzoxy[12] and dibenzenesulfonyl[24] derivatives is not completely masked. This fact was established as early as 1928, both by Felix and Dirr[22] and by Zervas and Bergmann,[21] who described the hydrochloride salt of N^{α},N^{ω}-dibenzoylarginine, and was reaffirmed, in 1953, by Gish and Carpenter[12] who determined the apparent dissociation constants of N^{α},N^{ω}-di-*p*-nitrocarbobenzoxyarginine and reported a fruitless attempt to employ this compound in the peptide-forming step. That N^{α},N^{ω}-dicarbobenzoxy-L-arginine possesses a like basicity was more recently evidenced[18] when its treatment with carbobenzoxy chloride under Schotten–Baumann conditions led to the formation of the tricarbobenzoxy derivative. Nevertheless, the ready accessibility of N^{α},N^{ω}-di-carbobenzoxy-L-arginine (II) *via* the selective saponification of tricarbobenzoxy-L-arginine (I or Ia) afforded an opportunity to reinvestigate the potential utility of the N^{α},N^{ω}-diacylated arginines as intermediates in the synthesis of N-terminal arginine peptides. Initial studies in this direction involved the treatment of N^{α},N^{ω}-dicarbobenzoxy-L-arginine (II) with phosphorus pentachloride in anhydrous chloroform at 0 to −5°; the acid chloride hydrochloride[25] so derived was immediately coupled with diethyl L-glutamate and the condensation product (III, $R = EtO_2CCH_2CH_2\overset{|}{C}HCO_2Et$) converted to L-arginyl-L-glutamic acid, in modest over-all yield, by saponification followed by catalytic hydrogenolysis. Although a somewhat better yield of the desired intermediate III could be attained through the dicyclohexylcarbodiimide-induced condensation of N^{α},N^{ω}-dicarbobenzoxy-L-arginine with the hydrochloride salt of diethyl L-glutamate in the absence of added base, no appreciable coupling reaction was observed when the latter reactant was present as the free base. Such a situation is explicable on the basis of a migration of protons from diethyl glutamate to the dipolar N^{α},N^{ω}-dicarbobenzoxyarginine as a prelude to the actual condensation. In any event, the isolation of pure L-arginyl-L-glutamic acid demonstrates that N^{α},N^{ω}-diacylated arginines may provide an alternative synthetic route to N-terminal arginine peptides.

During the course of the condensation of N^{α},N^{ω}-dicarbobenzoxy-L-arginine with diethyl L-glutamate by either of the above methods, a crystalline by-product precipitated whose elemental analytical values and chemical properties unequivocally identified it as N^{α},N^{ω}-dicarbobenzoxyanhydro-L-arginine (IV). Whilst the latter was found to be

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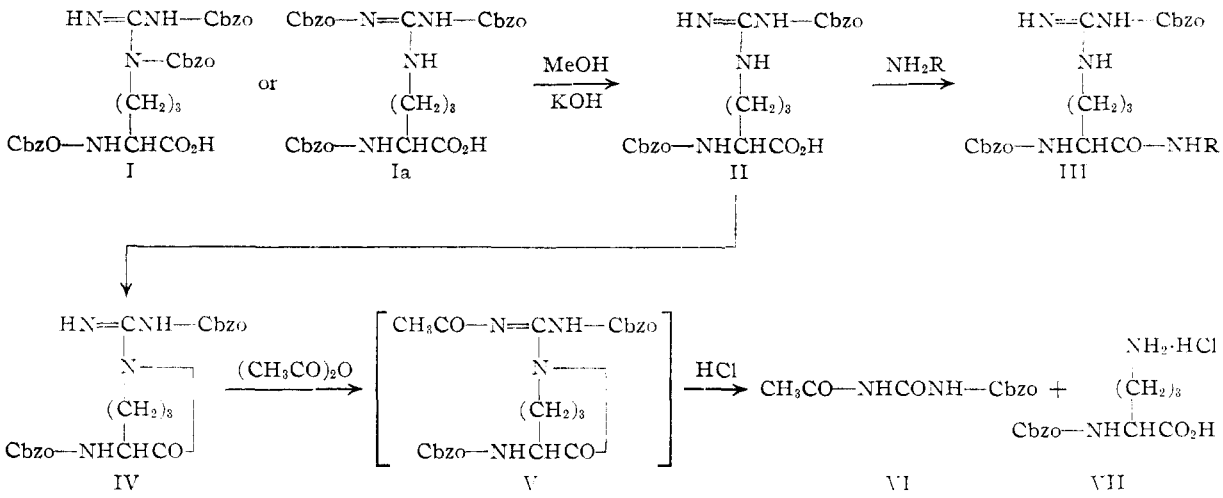
(25) Whilst this intermediate was not characterized, its existence as the hydrochloride salt in solution was indicated by the absence of hydrogen chloride evolution during the phosphorus pentachloride treatment.



stable in neutral aqueous solution, the addition of one equivalent of alkali thereto readily converted it to N^{α},N^{ω}-dicarbobenzoxy-L-arginine in nearly quantitative yield. Treatment of the substance with acetic anhydride at room temperature proceeded with the formation of an intermediate (V) which, when treated with cold water and cold 5 *N* HCl, respectively, decomposed into N-acetyl-N′-carbobenzoxyurea (VI) in addition to α-carbobenzoxyaminopiperidone in the former instance and N^{α}-carbobenzoxy-L-ornithine hydrochloride (VII) in the latter. Substance IV presumably arose as a result of the preliminary activation of the carboxyl group of dicarbobenzoxyarginine by the condensing agent, followed by an intramolecular cyclization. Such formation of an anhydroarginine derivative is reminiscent of the early observation of Bergmann and Köster[26] that L-arginine, when treated with an excess of acetic anhydride at the boiling temperature, is converted to triacetylanhydro-DL-arginine. An analogous situation prevails in the conversion of L-nitroarginine to N^{α}-acetylanhydro-DL-nitroarginine by the action of acetic anhydride in boiling glacial acetic acid.[27] The intramolecular cyclization which accompanies reactions of this type may be ascribed to the fact that the basicity of the guanido moiety of the precursor arginine derivative is not completely masked.

With the above in mind, it becomes clear that by virtue of the completely or almost completely suppressed basicity of the guanido moiety of tricarbobenzoxy-L-arginine, a synthetic route to N-terminal arginine peptides is available wherein no danger of anhydro-compound formation exists. Indeed, the condensation of tricarbobenzoxy-L-arginine, *via* the mixed carbonic–carboxylic acid anhydride[8] and the dicyclohexylcarbodiimide[10] methods, with the benzyl esters of L-alanine, L-aspartic acid, L-glutamic acid, glycine, L-isoleucine, D-alloisoleucine, L-leucine, L-phenylalanine, L-tyrosine and L-valine proceeded smoothly and without complication, and permitted the isolation of the corresponding tricarbobenzoxy-L-arginylamino acid benzyl esters in high yield; in no instance was the formation of a tricarbobenzoxyanhydroarginine side product detected. Subsequent palladium black-catalyzed hydrogenolysis of the condensation products led to the simultaneous removal of the carbobenzoxy and benzyl ester substituents with the formation of the unprotected dipeptides; the latter, with the exception of the aspartic acid and glutamic acid derivatives, were isolated in analytically pure state as their respective acetate salts. L-Arginine amide dihydrochloride was prepared from the precursor tricarbobenzoxy derivative in like manner. Specific rotation values of the peptides so derived were generally in agreement with the literature values of those compounds previously prepared by other routes.

The success attained with tricarbobenzoxy-L-arginine in the aforementioned syntheses suggested that this compound would probably prove of like utility in the synthesis of L-arginyl-L-arginine. For such purpose, tricarbobenzoxy-L-arginine was coupled with N^{ω}-carbobenzoxy-L-arginine benzyl ester[18] *via* both the mixed anhydride[8] and dicyclohexylcarbodiimide[10] methods. This process led to a mixture of products wherefrom tricarbobenzoxy-L-arginyl-N^{ω}-carbobenzoxy-L-arginine benzyl ester[18,20] could be isolated in a satisfactory degree of purity only after repeated recrystallization from various solvents; side product formation in such situation is not unexpected in view of the fact that the benzyl ester of N^{ω}-carbobenzoxy-L-arginine possesses at least two reactive groups capable of partaking in the coupling reaction, namely, one at the α-position and the other at the guanido nucleus. *However, when an identical condensation was effected in the presence of one equivalent of a strong acid, the desired dipeptide derivative was secured directly in both satisfactory yield and a high degree of purity;* such occurrence presumably arose from the proton-mediated neutralization of the basic guanido nucleus prior to the peptide-forming reaction. Catalytic hydrogenolysis of the coupling product in methanol–acetic acid solution and in the presence of freshly prepared palladium black, followed by evaporation of the reaction mixture *in vacuo*, yielded a non-crystallizable sirup which gave only a single ninhydrin positive spot on paper in three different solvent systems. That the sirup

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TABLE I

PHYSICAL CONSTANTS AND ANALYTICAL VALUES OF SOME N-TERMINAL ARGININE DIPEPTIDES AND THEIR CARBOBENZOXYLATED DIPEPTIDE BENZYL ESTER PRECURSORS

Acylated derivatives, benzyl ester	M.p., °C. (cor.)	Empirical formula	Calculated C	Calculated H	Calculated N	Found C	Found H	Found N
TriCbzo-L-arginyl-L-alanine	171–172	$C_{40}H_{43}N_5O_9$	65.1	5.9	9.5	65.1	6.1	9.6
TriCbzo-L-arginyl-N^{ω}-Cbzo-L-arginine	162–164	$C_{61}H_{57}N_8O_{11}$	64.0	6.0	11.7	63.8	6.0	11.6
TriCbzo-L-arginyl-L-aspartic di-	159–160	$C_{48}H_{49}N_5O_{11}$	66.1	5.7	8.0	66.0	5.8	8.0
TriCbzo-L-arginylglycine	118–120	$C_{39}H_{41}N_5O_9$	64.7	5.8	9.7	64.2	5.9	9.7
TriCbzo-L-arginyl-L-isoleucine	147–150	$C_{43}H_{49}N_5O_9$	66.2	6.3	9.0	65.8	6.4	9.2
TriCbzo-L-arginyl-D-alloisoleucine	128–130	$C_{43}H_{49}N_5O_9$	66.2	6.3	9.0	66.4	6.4	9.0
TriCbzo-L-arginyl-L-leucine	158–160	$C_{43}H_{49}N_5O_9$	66.2	6.3	9.0	66.0	6.3	9.0
TriCbzo-L-arginyl-L-phenylalanine	168–170	$C_{46}H_{47}N_5O_9$	67.8	6.1	8.6	67.5	5.9	8.6
TriCbzo-L-arginyl-L-tyrosine	166–168	$C_{46}H_{47}N_5O_{10}$	66.6	5.7	8.4	66.3	5.9	8.6
TriCbzo-L-arginyl-L-valine, monohydrate	129–131.5	$C_{42}H_{47}N_5O_9.H_2O$	64.4	6.3	8.9	64.6	6.2	8.9
Free peptide	$[\alpha]^{24-25}D$							
L-Arginyl-L-alanine monoacetate	+11.9[b]	$C_{11}H_{23}N_5O_5$	43.3	7.6	22.9	43.1	7.6	22.9
L-Arginyl-L-aspartic acid	+32.1[c]	$C_{10}H_{19}N_5O_5$	41.5	6.6	24.2	41.4	6.8	24.1
L-Arginylglycine acetate, acetic acid solvate	+37.6[d]	$C_{12}H_{25}N_5O_7$	41.0	7.2	19.9	40.6	7.3	19.7
L-Arginyl-L-isoleucine monoacetate	+17.4	$C_{14}H_{29}N_5O_5$	48.4	8.4	20.2	48.1	8.6	20.4
L-Arginyl-L-leucine monoacetate	+10.3[e]	$C_{14}H_{29}N_5O_5$	48.4	8.4	20.2	48.5	8.7	19.9
L-Arginyl-L-phenylalanine monoacetate	+29.3[f]	$C_{17}H_{27}N_5O_5$	53.5	7.1	18.4	53.2	7.0	18.0
L-Arginyl-L-tyrosine monoacetate	+32.0[g]	$C_{17}H_{27}N_5O_6$	51.4	6.9	17.6	51.5	6.9	17.8
L-Arginyl-L-valine monoacetate	+15.8	$C_{13}H_{27}N_5O_5$	46.8	8.2	21.0	46.8	8.3	20.7

[a] Rotations were measured in a photoelectric polarimeter as 1% solutions in water. [b] $[\alpha]^{19}D$ +9.3°[11] and $[\alpha]^{32}D$ +9.7°[6] have been reported in the same solvent. [c] $[\alpha]^{21}D$ +22.2° for the dihydrate in water.[11] [d] $[\alpha]^{28}D$ +38.9°[6] and $[\alpha]^{22}D$ +47.1°[9] in water. [e] $[\alpha]^{19}D$ +9.4°,[11] $[\alpha]^{24}D$ +11.6°,[12] $[\alpha]^{28}D$ +9.6°[6] and $[\alpha]^{22}D$ +8.9°[9] have been reported in the same solvent. [f] $[\alpha]^{28}D$ +29.5°[6] and $[\alpha]^{22}D$ +30.1°[9] in water. [g] $[\alpha]^{32}D$ +33.3°, $[\alpha]^{22}D$ +32.0°[9] and $[\alpha]^{19}D$ +33.2°[11] in water.

constituted the desired dipeptide was attested to by the fact that its treatment with flavianic acid and picrolonic acid, respectively, led to the precipitation of crystalline materials whose elemental analyses were in agreement with those calculated for the corresponding diflavianate and dipicrolonate derivatives of L-arginyl-L-arginine. On the basis of this evidence, as well as additional chromatographic and analytical evidence described in the Experimental section, it may be concluded that the compound prepared by the synthetic procedure described above is L-arginyl-L-arginine.

Experimental

I. Preparation of N-Terminal Arginine Peptides from Tricarbobenzoxy-L-arginine. Sodium Tricarbobenzoxy-L-argininate.—The following directions represent a modification of the earlier described procedure.[18] A solution of 35 g. of L-arginine (free base)[28] in 200 ml. of 1 *N* NaOH is cooled to −10° and treated, with vigorous stirring, with 100 ml. of pre-cooled 2 *N* NaOH and 27 ml. of freshly prepared carbobenzoxy chloride. Some 3 to 4 minutes later, a second addition of 100 ml. of pre-cooled 2 *N* NaOH and 27 ml. of carbobenzoxy chloride is effected. Three more identical additions are made at 5-minute intervals and the vigorous stirring and cooling continued for 20–30 minutes beyond the final addition. The white precipitate which forms during the reaction is filtered off in the cold room, pressed sharply to remove the liquid, washed with 100 ml. of ice-cold 5% sodium carbonate solution, and the residue again pressed sharply. The crude sodium salt so secured is dissolved in about 800 ml. of chloroform and, after removal of the slight water layer, washed with two 100-ml. portions of 5% sodium chloride at room temperature. After acidification of the chloroform layer with 2 *N* sulfuric acid, the liberated free acid is reconverted to the sodium salt with sodium acetate trihydrate as described previously.[18]

(28) The free base of L-arginine is readily prepared by treating an aqueous solution of L-arginine hydrochloride with exactly 1 equivalent of standardized lithium hydroxide solution and then adding several volumes of ethanol thereto. The precipitated free base is recrystallized from water–ethanol and then stored *in vacuo* over phosphorus pentoxide until needed.

Tricarbobenzoxy-L-arginine is prepared from the purified sodium tricarbobenzoxy-L-argininate by the earlier described procedure.[18]

Amino Acid Benzyl Ester *p*-Toluenesulfonates.—The benzyl ester *p*-toluenesulfonates of L-alanine, L-aspartic acid (di-ester), L-glutamic acid (di-ester), glycine, L-isoleucine, D-alloisoleucine, L-leucine, L-phenylalanine, L-tyrosine and L-valine here employed were described previously.[18]

Tricarbobenzoxy-L-arginylamino Acid Benzyl Esters. Method A.—A solution of 28.8 g. (0.05 mole) of tricarbobenzoxy-L-arginine and 6.9 ml. (0.05 mole) of triethylamine in 400 ml. of a 1:1 chloroform–toluene mixture is cooled to −5° and treated with 6.5 ml. (0.05 mole) of isobutyl chloroformate. After storage at −5° for 25 minutes, the reaction mixture is treated with a pre-cooled solution of 0.05 mole of the pertinent amino acid benzyl ester *p*-toluenesulfonate and 6.9 ml. of triethylamine in about 100 ml. of chloroform. The reaction mixture is then permitted to stand at room temperature overnight, after which time it is washed twice with a dilute aqueous solution of triethylamine, twice with water, twice with dilute acetic acid and finally twice with water. The organic layer is separated, dried over anhydrous sodium sulfate, filtered and concentrated under a stream of dry air. A residue is obtained which is treated with 1 l. of petroleum ether, cooled, filtered, and the precipitate crystallized from boiling ethanol or ethyl acetate.

The condensation of tricarbobenzoxy-L-arginine with the benzyl esters of L-alanine, L-aspartic acid (di-ester), L-glutamic acid (di-ester), glycine, L-isoleucine, D-alloisoleucine, L-leucine, L-phenylalanine, L-tyrosine and L-valine has been effected by essentially this procedure. With the exception of the L-aspartic acid and D-alloisoleucine derivatives which are purified by crystallization from ethyl acetate, all are crystallized from hot ethanol; in addition, a second crystallization of the L-phenylalanine derivative is effected from chloroform–petroleum ether. Analytical and melting point values for each of these compounds are given in Table I. The over-all yield in most instances ranges from 75–90%.

Method B.—To a solution of 5.8 g. (0.01 mole) of tricarbobenzoxy-L-arginine, 0.01 mole of the pertinent amino acid benzyl ester *p*-toluenesulfonate and 1.38 ml. (0.01 mole) of triethylamine in 30 ml. of dry chloroform is added 2.2 g. of dicyclohexylcarbodiimide. The reaction mixture is maintained at room temperature, with momentary immersion in an ice-bath when necessary. A precipitate appears within a few minutes which is removed by filtration

after storage of the mixture overnight at room temperature. The filtrate is treated with 2 ml. of acetic acid and permitted to stand for an additional 30 minutes (if an insoluble residue forms during this time, it should be removed by filtration). The chloroform solution is then washed twice with dilute aqueous triethylamine, twice with water, twice with dilute acetic acid and finally twice with water. After the chloroform solution has been dried by filtration through a dry filter paper, it is concentrated *in vacuo* at 35°. The residual material is triturated with petroleum ether, collected by filtration and then crystallized from hot ethanol or ethyl acetate.

Use of the above procedure permitted the preparation of tricarbobenzoxy-L-arginyl-L-alanine benzyl ester in some 70% yield after crystallization of the product first from hot ethanol and subsequently from hot ethyl acetate. When dioxane was employed as the reaction solvent under comparable conditions, tricarbobenzoxy-L-arginyl-L-glutamic acid dibenzyl ester could be procured in 75% yield. The pertinent analytical data are given in Table I.

L-Arginylamino Acids.—Hydrogenolysis of the tricarbobenzoxy-L-arginylamino acid benzyl esters listed in Table I is carried out either in 95% acetic acid or in a solution of 10% acetic acid in methanol in the presence of palladium black or 10% palladium-on-charcoal.[2] Upon completion of the reaction, the catalyst is filtered off, washed first with methanol, then with water, and the combined filtrates finally evaporated to dryness. The evaporation is repeated three times after the addition each time of a little ethanol. Crystallization of the residue is effected from acetic acid–ethyl acetate except for the aspartic acid derivative which is crystallized from hot water, the tyrosine derivative which is soldified by repeated precipitation from methanol–acetone and then crystallized from boiling methanol, and the glutamic acid derivative which is crystallized from hot water. All of the dipeptides are isolated as their monoacetate derivatives except for the glutamic and aspartic acid derivatives which are obtained as the corresponding free base. The products are dried at 78–100° over phosphorus pentoxide *in vacuo* for 2–4 hours prior to analysis. Yields of product generally range from about 60–90% of theory. Specific rotation and elemental analytical values are revealed in Table I.

Tricarbobenzoxy-L-arginine Amide.—A solution of 13.0 g. of tricarbobenzoxy-L-arginine and 3.1 ml. of triethylamine in 200 ml. of 1:1 chloroform–toluene is cooled to −5° and treated with 2.9 ml. of isobutyl chloroformate. After storage at −5° for 25 minutes, a cold saturated solution of ammonia gas in chloroform is added thereto. A solid white mass forms immediately which, after storage at room temperature overnight and subsequent admixture with 20 ml. of chloroform and a solution of 2 ml. of triethylamine in 25 ml. of water, is recovered by filtration. The residue is washed successively with water, 0.05 *N* acetic acid and water. Recrystallization is achieved from hot ethyl acetate; yield 10.0 g. (77%), m.p. 182–185° (cor.).

Anal. Calcd. for $C_{30}H_{33}N_5O_7$: C, 62.6; H, 5.8; N, 12.2. Found: C, 62.5; H, 5.9; N, 11.9.

L-Arginine Amide Dihydrochloride Monohydrate.—A suspension of 8.8 g. of tricarbobenzoxy-L-arginine amide in 50–100 ml. of methanol is treated with 3 ml. of concd. HCl and subjected to hydrogenolysis in the presence of palladium black catalyst. Upon termination of the reaction, the catalyst is removed *via* filtration and the filtrate concentrated to dryness under reduced pressure at a temperature not exceeding 40°. The evaporation is repeated twice after the addition each time of a little methanol. The residual material is then dissolved in a small amount of methanol, filtered, and the filtrate treated with ether. On cooling the mixture overnight, a solid material separates which is filtered off, washed with ether and air-dried. The slightly yellowish solid is then taken up in methanol, and the solution decolorized with charcoal and concentrated to dryness. An oil ensues which crystallizes as the monohydrate after washing several times with ether and then rubbing under ether with a glass rod; yield 3.4 g. (84%), $[\alpha]^{24}D$ +13.8 (1.0% in water). The material is dried at 60° for 2 hours prior to analysis.

Anal. Calcd. for $C_6H_{19}N_5O_2Cl_2$: C, 27.3; H, 7.3; N, 26.5. Found: C, 27.4; H, 6.6; N, 26.5.

II. Synthesis and Characterization of L-Arginyl-L-arginine. N^{ω}-Carbobenzoxy-L-arginine Benzyl Ester.—An alternative route to that previously described[18] for this compound is given in what follows: A suspension of 22.0 g. of dry N^{α}, N^{ω}-dicarbobenzoxy-L-arginine (dried at 78° for 2 hours) in 120 ml. of anhydrous chloroform is cooled to −10° and treated with 11.0 g. of phosphorus pentachloride. The reaction mixture is shaken in the cold until all of the latter reagent disappears and is then stored at room temperature for 1 hour, during which time a sirupy precipitate forms. The mixture is evaporated to dryness under reduced pressure at about 55°, the residue treated with a little chloroform and the evaporation repeated. Removal of the benzyl chloride and phosphorus oxychloride from the residual material is effected upon washing the latter several times with petroleum ether by decantation. After removal of the traces of petroleum ether by evaporation *in vacuo*, 100 ml. of benzyl alcohol containing 3.5 g. of hydrogen chloride gas is added to the sirupy residue which thereupon begins to dissolve slowly with the evolution of carbon dioxide. The reaction mixture is stored at room temperature for 3 hours, after which time the benzyl ester hydrochloride is precipitated as a sirup by the addition of ether. Conversion of the hydrochloride salt to the free base, as described previously, yields 11.5 g. (58%) of the desired product, m.p. 129–130° (cor.).

Tricarbobenzoxy-L-arginyl-N^{ω}-carbobenzoxy-L-arginine Benzyl Ester. Method A.—Preparation of this compound was achieved *via* the mixed carboxylic–carbonic acid anhydride method[8] as described earlier,[18] with the exception that isobutyl chloroformate was used *in lieu* of ethyl chloroformate as the anhydride-forming reagent. After recrystallization of the product once from boiling methanol and twice from ethyl acetate, a melting point of 158–160° was obtained; yield 28% theory.

Method B.—A suspension of 8.7 g. of tricarbobenzoxy-L-arginine, 6.0 g of N^{ω}-carbobenzoxy-L-arginine benzyl ester and 2.6 g. of *p*-toluenesulfonic acid in 30 ml. of dioxane is treated with 3.3 g. of N,N′-dicyclohexylcarbodiimide at room temperature and the reaction mixture is shaken thoroughly. As the reaction is mildly exothermic, the mixture is maintained at room temperature by periodic immersion in an ice-bath. After storage at room temperature overnight, the insoluble precipitate of dicyclohexylurea is removed by filtration and washed with a little dioxane. To the combined filtrate and washings is added 1 ml. of acetic acid and a few drops of water and, after storage for 30 minutes at room temperature, the slight turbidity which develops is removed by filtration. The clear filtrate is concentrated to dryness under reduced pressure and the residue taken up in a large volume of ethyl acetate. The resulting solution is washed twice with dilute aqueous triethylamine, once with water, twice with dilute acetic acid and finally three times with water. After removal of water droplets by passage through a dry filter paper, the organic layer is evaporated *in vacuo* and the residue treated with a little methanol which is removed *in vacuo*. A crystalline material ensues which is recrystallized twice from hot ethyl acetate; yield 6.2 g. (43%), m.p. 162–164°.

L-Arginyl-L-arginine.—A suspension of 4.62 g. of tricarbobenzoxy-L-arginyl-N^{ω}-carbobenzoxy-L-arginine benzyl ester in 150 ml. of methanol is treated with 2–3 ml. of glacial acetic acid and a few drops of water and then subjected to catalytic hydrogenolysis in the presence of freshly prepared palladium black catalyst. Upon completion of the reduction, the reaction mixture is filtered, the catalyst washed several times with 5% acetic acid, and the combined filtrate and washings diluted to a volume of 500 ml. with 5% acetic acid. Although various attempts to secure a crystalline reduction product proved unsuccessful, the fact that it was indeed L-arginyl-L-arginine was indicated by its successful conversion to the crystalline dipicrolonate and diflavianate derivatives as well as by the analytical tests given in what follows:

(a) **Chromatography.**—When the above solution of the reduction product is chromatographed on Whatman No. 1 paper in (1) formic acid–water–*t*-butyl alcohol (15:15:70), (2) methanol–water–pyridine (80:20:2) or (3) phenol saturated with 10% disodium citrate, only a single ninhydrin-positive spot ensues which possesses a different R_f than that revealed by a reference standard of arginine under the same conditions. Thus, in the solvents mentioned, the R_f values for arginine and for arginylarginine are, in order, 0.28 and 0.18, 0.26 and 0.15, and 0.65 and 0.83. The room temperature was 25° and the period of observation 18 hours.

(b) **Ninhydrin-CO_2.**—Analysis of 1.7 ml. of the dipeptide solution for the presence of free α-amino acid by the Van Slyke manometric ninhydrin-CO_2 method proceeds with no significant amount of carbon dioxide liberation. However, when a mixture of 4 ml. of the dipeptide solution and 4 ml. of concentrated HCl is heated in a sealed tube at 95–100° for 24 hours and an aliquot of the hydrolysate subsequently subjected to like analysis after removal of the excess hydrochloric acid, some 93% of the theoretical amount of carbon dioxide is obtained if calculations are based upon the hydrolysis of the proposed dipeptide. In addition, chromatographic analysis of the hydrolysate now reveals a very strong ninhydrin-positive spot corresponding to arginine and a faint spot corresponding to the unaltered parent material.

(c) **Specific Rotation before and after Hydrolysis.**—To determine the specific rotation prior to hydrolysis, 30 ml. of the dipeptide solution is evaporated to dryness *in vacuo* and the evaporation repeated twice after the addition each time of a little water and once after the addition of acetone; a solution of the residue in 5 ml. of 5 *N* HCl reveals an $[\alpha]^{25}D$ +15.0° for the free arginylarginine. In order to ascertain the specific rotation of the same amount of starting material after hydrolysis, a mixture of 18.0 ml. of the parent dipeptide solution and 18.0 ml. of concentrated HCl is heated in a sealed tube at 95–99° for 24 hours, after which time the contents are evaporated *in vacuo* and the excess hydrochloric acid removed from the residual material by repeated evaporation in the presence of added water; the sirupy residue reveals an $[\alpha]^{25}D$ +26.0° when dissolved in 3 ml. of 5 *N* HCl; this value, based on free arginine, is that characteristic of the free amino acid.

(d) **Diflavianate Derivative.**—A 120-ml. aliquot of the parent dipeptide solution is evaporated to dryness *in vacuo* and the residue taken up in a mixture of 1.9 ml. of 1 *N* HCl and 90 ml. of water. The latter is treated first with a solution containing 1 g. of flavianic acid in 10 ml. of water and immediately thereafter with 75 ml. of methanol. Upon storage overnight in an open vessel, L-arginyl-L-arginine diflavianate is deposited as beautiful clusters of needles, yield 78%.

Anal. Calcd. for $C_{32}H_{38}N_{12}O_{19}S_2$: C, 40.1; H, 4.0; N, 17.5. Found: C, 39.8; H, 4.0; N, 17.2.

(e) **Dipicrolonate Derivative.**—A suspension of 4.78 g. of tricarbobenzoxy-L-arginine-N$^{\omega}$-carbobenzoxy-L-arginine benzyl ester in about 150 ml. of methanol containing 2–3 ml. of acetic acid and a few drops of water is subjected to catalytic hydrogenolysis as above. The reaction is filtered, the catalyst washed several times with 5% acetic acid, and the combined filtrate and washings again filtered. The filtrate so secured is diluted to 300 ml. with water. A 200-ml. aliquot of this solution is concentrated to a heavy sirup *in vacuo* at a temperature not exceeding 40°. The evaporation process is repeated twice more after the addition each time of a little water. To a solution of the residual sirup in 50 ml. of water is added 500 ml. of a saturated picrolonic acid solution (prepared by stirring 5.5 g. of recrystallized picrolonic acid (from hot ethanol) in 500 ml. of water overnight at room temperature and filtering off the undissolved material). A bright yellow precipitate of the dipicrolonate forms almost immediately which, after some 12 hours storage in the dark, is recovered by filtration, washed with ice-water until a colorless filtrate is obtained, and air-dried in the dark; yield, 2.25 g. (79%), m.p. 289–290°, $[\alpha]^{25}D$ +5.5° (1% in dimethylformamide).

Anal. Calcd. for $C_{32}H_{42}N_{16}O_{13}$: C, 44.8; H, 4.9; N, 26.1. Found: C, 44.6; H, 5.0; N, 25.9.

The arginylarginine dipicrolonate so secured revealed a picrolonic acid content of 60.4% upon analysis,[29] as compared with a calculated value of 61.5%. That the dipicrolonate[30] formulation is valid is reaffirmed by the fact that a sample of the compound, after subjection to acid hydrolysis with 6 *N* HCl in a sealed tube at 100° for 24 hours, shows an arginine content of 40.6% (calcd. for arginine in the dipicrolonate, 40.6) as determined by a manometric ninhydrin-CO_2 measurement of the amino acids in the hydrolysate.

(29) F. Alten, H. Weiland and E. Knippenberg, *Biochem. Z.*, **265**, 85 (1933); *cf.* F. J. Welcher, "Organic Analytical Reagents," Vol. III, D. Van Nostrand Co., Inc., New York, N. Y., 1948, p. 39.

(30) Although the tripicrolonate derivative of L-arginyl-L-arginine was previously described,[19] several subsequent preparations have since yielded elemental analyses as well as picrolonic acid values which were most consistently in agreement with the dipicrolonate derivative.

III. **Preparation of N-Terminal Arginine Peptides from N$^{\alpha}$,N$^{\omega}$-Dicarbobenzoxy-L-arginine. N$^{\alpha}$,N$^{\omega}$-Dicarbobenzoxy-L-arginine.**—The following procedure represents a modification of the previously reported[18] directions for this compound. Thus, the crude solid precipitate of sodium tricarbobenzoxy-L-argininate which results from the percarbobenzoxylation of 35 g. of L-arginine is dissolved in approximately 500 ml. of 95% ethanol containing 15 g. of potassium hydroxide, with simultaneous cooling in an ice-bath and stirring. After the solution has stood at room temperature for 2 hours, about 300 ml. of water is added and the reaction mixture is concentrated to about 300–400 ml. *in vacuo* at 30–35°. About 400 ml. of water and an excess of acetic acid are added, the mixture is cooled, and the sirup which results is washed several times with ice-water by decantation. The sirup is then dissolved in approximately 400–500 ml. of hot methanol and the solution permitted to cool first at room temperature and finally in the cold. A precipitate of the desired compound ensues which is recovered and washed with methanol; yield 52 g., m.p. 147°. Recrystallization as described previously[18] raises the melting point to 150°.

N$^{\alpha}$,N$^{\omega}$-Dicarbobenzoxy-L-arginyl-L-glutamic Acid. Method A.—To a solution of 2.4 g. of L-glutamic acid diethyl ester hydrochloride in 15 ml. of anhydrous dioxane are added 4.4 g. of N$^{\alpha}$,N$^{\omega}$-dicarbobenzoxy-L-arginine (dried over phosphorus pentoxide *in vacuo* at 78°) and 2.2 g. of N,N′-dicyclohexylcarbodiimide. The mixture is shaken for 8 hours at room temperature, during which time the dicarbobenzoxy-L-arginine dissolves and dicyclohexylurea separates out. One milliliter of water is added and, after an additional 3 hours, the urea derivative filtered off and washed with dioxane. The combined filtrate and washings is concentrated to dryness *in vacuo*, the residue dissolved in ethyl acetate, and the resulting solution washed successively with 4% sodium carbonate (twice), dilute acetic acid and water. The organic fraction is evaporated to dryness and the residual material dissolved in approximately 15 ml. of hot ethanol. Upon standing in the cold, 0.8 g. of a crystalline material deposits which, after filtration and subsequent recrystallization from ethanol, melts at 149° and shows an $[\alpha]^{25}D$ −13.6° (2% in chloroform); this compound is N$^{\alpha}$,N$^{\omega}$-dicarbobenzoxyanhydro-L-arginine.

Anal. Calcd. for $C_{22}H_{24}O_5N_4$: C, 62.2; H, 5.7; N, 13.2. Found: C, 62.3; H, 5.9; N, 13.2.

To the parent ethanolic filtrate is added 6 ml. of 5 *N* KOH. After storage at room temperature for 1.5 hours, water is added and the solution acidified by treatment with 4.5 ml. of 5 *N* H_2SO_4 and acetic acid. A sirup deposits which is repeatedly triturated with water and then crystallized by storage under water in the cold; yield 2.6 g. (46%), m.p. 158°. Recrystallization from ethanol–water raises the melting point to 160°.

Anal. Calcd. for $C_{27}H_{33}O_9N_5$: N, 12.2. Found: N, 12.1.

Method B.—A suspension of 4.4 g. of N$^{\alpha}$,N$^{\omega}$-dicarbobenzoxy-L-arginine (dried at 78° *in vacuo* over phosphorus pentoxide) in 30 ml. of anhydrous chloroform is cooled at −5° and 2.1 g. of phosphorus pentachloride added thereto. After shaking the reaction mixture for several minutes at 0 to −5°, a clear solution ensues in the absence of hydrogen chloride evolution. The addition of cold petroleum ether leads to the precipitation of the acid chloride hydrochloride derivative of N$^{\alpha}$,N$^{\omega}$-dicarbobenzoxy-L-arginine which is washed several times with cold petroleum ether and then dissolved in cold anhydrous chloroform. The latter solution is treated with a cold solution of 2.4 g. of L-glutamic acid diethyl ester hydrochloride and 5.2 ml. of triethylamine in chloroform. After storage at room temperature for 2 hours, the reaction mixture is washed successively with water, 4% sodium carbonate (twice), dilute acetic acid and water, dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The residual material is taken up in 15 ml. of ethanol and allowed to stand in the cold, during which time 0.7 g. of N$^{\alpha}$,N$^{\omega}$-dicarbobenzoxyanhydro-L-arginine separates and is recovered by filtration; m.p. 149°, $[\alpha]^{25}D$ −13.4° (2% in chloroform). From the ethanolic mother liquors, N$^{\alpha}$,N$^{\omega}$-dicarbobenzoxy-L-arginyl-L-glutamic acid is obtained by saponification as described under method A above; yield 1.3 g.

L-Arginyl-L-glutamic Acid.—Hydrogenolysis of N$^{\alpha}$,N$^{\omega}$-dicarbobenzoxy-L-arginyl-L-glutamic acid in the presence of

palladium catalyst in the usual manner leads to the ultimate procurement of L-arginyl-L-glutamic acid[18] in nearly quantitative yield.

IV. Properties of Nα,Nω-Dicarbobenzoxyanhydro-L-arginine. Behavior toward Water.—A solution of Nα,Nω-dicarbobenzoxyanhydro-L-arginine in acetone is treated with an amount of water just sufficient to keep the material in solution. After storage at room temperature for 24 hours, the acetone is removed by evaporation under reduced pressure and the precipitate recovered by filtration. Analysis of the precipitate identified it as the unchanged starting material in nearly quantitative yield; $[\alpha]^{25}D$ −13.0° (4% in chloroform).

Action of Alkali.—A solution of Nα,Nω-dicarbobenzoxyanhydro-L-arginine in acetone is treated with 1 equivalent of aqueous alkali and stored at room temperature for 1 hour. After removal of the acetone under reduced pressure at a temperature not exceeding 25°, the residual material is acidified with acetic acid. The sirupy deposit is washed with a small volume of water and then dissolved in hot methanol. Upon cooling, crystalline Nα,Nω-dicarbobenzoxy-L-arginine precipitates in some 85–90% yield, m.p. 150°, $[\alpha]^{25}D$ −5.2° (2% in dimethylformamide) and $[\alpha]^{25}D$ −10.9° (1% in pyridine).

Degradation with Acetic Anhydride.—To 2.6 g. of Nα,Nω-dicarbobenzoxyanhydro-L-arginine is added 50 ml. of warm acetic anhydride. The reaction mixture is heated at 100° for 3 hours[31] and then cooled to room temperature. After the addition of a small volume of ethanol and water, the solution is permitted to stand at room temperature with occasional cooling; by such means most of the acetic anhydride is destroyed. The solution is then evaporated to dryness, the residue is dissolved in about 40 ml. of acetone and the latter solution is treated with 25 ml. of water and permitted to stand at room temperature in an open vessel. After a few hours, precipitation commences due to a slow evaporation of the acetone. Two days later, the crystalline deposit is collected by filtration and recrystallized from hot ethanol; yield 1 g., m.p. 141°. Elemental analysis of the product agree with the values calculated for N-acetyl-N′-carbobenzoxyurea.

Anal. Calcd. for $C_{11}H_{12}N_2O_4$: C, 55.9; H, 5.1; N, 11.9. Found: C, 56.3; H, 5.2; N, 11.9.

The mother liquors from the above reaction are kept at room temperature for one week, during which time a small amount of crystals precipitate. These are filtered off and the filtrate is concentrated to dryness under reduced pressure. A residue remains which is dissolved in approximately 20 ml. of 5 *N* HCl and then stored at room temperature for several days. During this time crystalline needles precipitate which, after storage in the cold, are collected by filtration and washed with a small amount of cold water; yield 0.5 g., m.p. 187–189°, $[\alpha]^{25}D$ −11.5° (1.0% in water). Elemental analysis of the product identified it as Nα-carbobenzoxy-ornithine hydrochloride.

Anal. Calcd. for $C_{13}H_{18}N_2O_4.HCl$: C, 51.6; H, 6.3; N, 9.2; Cl, 11.7. Found: C, 51.7; H, 6.4; N, 9.2; Cl, 11.9.

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(31) Optical rotation measurements of the reaction mixture indicate no difference in reading at the end of 15 minutes, 2 hours and 3 hours of heating.

CONTRIBUTION FROM THE LABORATORY OF ORGANIC CHEMISTRY, UNIVERSITY OF ATHENS, GREECE]

On the Trityl Method for Peptide Synthesis[1]

BY GERASSIMOS C. STELAKATOS,[2] DIMITRIOS M. THEODOROPOULOS[3] AND LEONIDAS ZERVAS[4]

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Trityl derivatives of amino acids can be simply and effectively isolated by chloroform extraction of their diethylammonium salts from the tritylation reaction mixture. N,N′-Ditrityl-L-histidine, N-trityl-L-histidine, N′-trityl-L-histidine methyl ester hydrochloride, O-trityl-L-tyrosine benzyl ester hydrochloride, amongst other tritylamino acids, were prepared. Coupling of tritylamino acids and trityldipeptides with amino acid esters was achieved *via* the carbodiimide method and the phosphorazo method. Trityl dipeptides may alternatively be coupled *via* the mixed anhydride procedure without any influence of the bulky trityl group.

Introduction

As was reported in an earlier communication,[5] the applicability of the trityl function in synthetic peptide techniques was limited not only by the difficulty generally encountered in the preparation of optically active tritylated amino acids (II) but by the failure of these compounds (with the exception of tritylalanine and tritylglycine), as well, to couple with other amino acids *via* the mixed anhydride procedure. The tritylation of amino acid esters proceeds with facility and in satisfactory yield; however, the appreciable steric hindrance afforded by the bulky trityl group makes the saponification of tritylamino acid esters (Ia) under mild conditions difficult and directs the coupling of mixed anhydrides toward carbamate rather than peptide formation.[5] Pure, optically active tritylamino acids (II) may be prepared by tritylation of the benzyl esters of amino acids (Ib) followed by catalytic hydrogenolysis of the

$$\underset{\text{I}}{RCH(NHTr)COOR'} \qquad \underset{\text{II}}{RCH(NHTr)COOH}$$

Ia, R′ = alkyl or $C_6H_5CH_2$
Ib, R′ = $C_6H_5CH_2$
Tr, $(C_6H_5)_3C$

tritylamino acid benzyl ester so derived.[1,2,6] By virtue of the markedly greater rate of hydrogenolysis exhibited by the O-benzyl than the N-trityl substituent, cessation of the hydrogenation after the uptake of approximately 1 mole of hydrogen permits the ultimate isolation of the desired tritylamino acid (II) in satisfactory yield. Prolonged hydrogenolysis, on the other hand, leads to the re-

(1) Previous presentation of this work was given at the XIV International Congress of Pure and Applied Chemistry, Zurich, July 27, 1955; *cf.* Communications du XIV Congrés International de Chimie Pure et Appliqué, Zurich, 1955, p. 224.

(2) This paper is based in part on the doctoral dissertation of G. C. Stelakatos, Division of Natural Sciences (Chemistry Section), University of Athens, Greece, October, 1954. Now at Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda Md.

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