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Studies on Arginine Peptides. I. Intermediates in the Synthesis of N-Terminal and C-Terminal Arginine Peptides*

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Tricarbobenzoxy-L-arginine, as its sodium salt, was prepared in strongly alkaline medium *via* the carbobenzoxylation of L-arginine. This sodium salt, which was in fact a mixture of at least two different isomeric forms, was converted into a mixture of $N^{\alpha}N,^{\omega}$-dicarbobenzoxy-L-arginine and a single pure isomer of sodium tricarbobenzoxy-L-argininate upon treatment with boiling ethanol. Fractionation of these compounds, followed by acidification of the latter material, yielded pure tricarbobenzoxy-L-arginine wherein the basic properties of the guanido group were completely masked. The utility of this compound in the preparation of *N*-terminal arginine peptides was demonstrated upon its condensation with amino acid benzyl esters, *via* the mixed carbonic–carboxylic acid anhydride procedure, followed by catalytic hydrogenolysis of the condensation product. In addition, the *N*-carboxyanhydride procedure was utilized to effect the transformation of tricarbobenzoxy-L-arginine into N^{ω},N^{ω}-dicarbobenzoxy-L-arginine, and of N^{α},N^{ω}-dicarbobenzoxy-L-arginine into N^{ω}-carbobenzoxy-L-arginine, as well as the benzyl and methyl ester derivatives of the latter. All of these products are of potential value in the synthesis of *C*-terminal arginine peptides. In this connection, the esters of N^{ω}-carbobenzoxy-L-arginine were employed in the synthesis of the benzyl and methyl esters of tricarbobenzoxy-L-arginyl-N^{ω}-carbobenzoxy-L-arginine.

INTRODUCTION

By virtue of the complex nature of the guanido group, the incorporation of the highly basic arginine molecule into synthetic peptides has posed more formidable problems than have been encountered with any of the other protein-derived amino acids. In this connection, the development of an unequivocal synthetic route to arginylarginine affords a particularly tempting challenge, not only in consequence of the natural occurrence of this same residue sequence in polypeptides of biological interest, *e.g.*, adrenocorticotropic hormone, protamines, etc., but also because of the role played by many outstanding investigators in its ill-fated chemical history. Thus, although "arginylarginine," which was characterized as the picrate and nitrate derivatives, was presumed by Fischer and Suzuki[1] in 1905 to have arisen from the autocondensation of L-arginine methyl ester, such presumption was nevertheless qualified by certain reservations. Not only did a Dumas nitrogen analysis of the substance reveal values which were too high, but prolonged treatment of the material with hot concentrated hydrochloric acid resulted in no regeneration of free arginine, a fact which cast some doubt upon its alleged peptide nature. Despite these observations, Edlbacher and Bonem,[2] some twenty years later, reported that the "arginylarginine" of Fischer and Suzuki was completely hydrolyzed upon heating with 25% sulfuric acid for fourteen hours, and that the free amino acid could be recovered from the hydrolyzate in nearly quantitative yield as its picrolonate derivative. In addition, the "arginylarginine" exhibited formol-titratable nitrogen values which were in good agreement with those theoretically expected for this compound, while the action of arginase upon this substance resulted in the liberation of some 20–21% of the total nitrogen as urea. The major arguments barring definite assignment of the arginylarginine structure, tentatively proposed by Fischer and Suzuki, were thereby seemingly removed. However, a disturbing element crept into the picture in 1927 with the announcement by Kossel and Staudt[3] that, in contradistinction to the results of Edlbacher and Bonem, not only did they fail to observe any arginine formation upon prolonged treatment of "arginylarginine" with hot sulfuric acid, but that the picrolonate derivative of arginine described by these latter investigators was, in actuality, the corresponding derivative of unhydrolyzed "arginylarginine." Just one year later, Waldschmidt-Leitz, Schäffner, Schlatter, and Klein[4] reported that an original preparation of Fischer and Suzuki was completely resistant to the hydrolytic action of either trypsin-kinase or intestinal erepsin and thus supported the previous doubt as to the peptide nature of this substance. Such doubt was resolved during the same year with the demonstration by Zervas and Bergmann[5] that the autocondensation of arginine methyl ester to the corresponding dipeptide ester is rapidly succeeded by a disproportionate cleavage of this latter compound into ornithine methyl ester and an anhydride of α,δ-diguanidinovaleric acid. It was the latter product which constituted the controversial "arginylarginine" of Fischer and Suzuki. A somewhat ironical note is presented by the fact

* This paper is a contribution in honor of Lyndon F. Small, former Editor of the Journal.

(1) E. Fischer and U. Suzuki, *Ber.*, **38**, 4173 (1905).

(2) S. Edlbacher and P. Bonem, *Z. physiol. Chem.*, **145**, 69 (1925).

(3) A. Kossel and W. Staudt, *Z. physiol. Chem.*, **170**, 91 (1927).

(4) E. Waldschmidt-Leitz, A. Schäffner, H. Schlatter, and W. Klein, *Ber.*, **61**, 299 (1928).

(5) L. Zervas and M. Bergmann, *Ber.*, **61**, 1195 (1928).

that although reference to this peptide as an arginase-susceptible substrate has persisted to the present day in published investigations and reviews, and although a number of synthetic procedures are currently available for incorporating arginine into the peptide chain,[6-11] no authentic or well-defined sample of arginylarginine[12] has yet been described.

The present series of studies was conceived both with an eye toward clarifying the past confusion which surrounded the synthesis and biological properties of this most intriguing compound, as well as with the goal of developing more facile techniques for "joining" an arginine residue at the *C*-terminal or *N*-terminal end of a peptide chain. The first of these studies, presented herein, is concerned primarily with the preparation of suitably substituted arginine derivatives which may serve as starting materials toward the fulfillment of this aim.

RESULTS AND DISCUSSION

Intermediates in the preparation of N-terminal arginine peptides. An earlier communication[13] has alluded to the successful employment of tricarbobenzoxy-L-arginine in the preparation of peptides which incorporate an *N*-terminal arginine residue. Synthesis of this intermediate was inadvertently achieved during an attempt to synthesize the N^{α}, N^{ω}-dicarbobenzoxy derivative of L-arginine, by treatment of a strongly alkaline solution of L-arginine with 2–4 equivalents of carbobenzoxy chloride, in a manner reminiscent of that previously utilized to prepare the corresponding dibenzoyl derivative.[5,14] During the reaction an insoluble material precipitated, which was filtered in the cold and washed with cold sodium carbonate solution, and the wet cake was taken up in alcohol-free chloroform, dried, and concentrated *in vacuo*; on treatment with ether, the residue solidified. Elemental analyses of the material, so derived, unexpectedly conformed to the composition possessed by sodium tricarbobenzoxy-L-argininate (I). Such preparation of a triacylated arginine derivative brings to mind the early observation of Bergmann and Köster[15] that the action of excess acetic anhydride upon L-arginine proceeds with the formation of triacetyl anhydro-DL-arginine. In any event, the ready accessibility of an N^{α}, N^{ω}, N^{ω}-tricarbobenzoxylated arginine, wherein the reactivity of the strongly basic guanido moiety is completely suppressed by a selectively removable acyl substituent,[16] unveiled a compound which qualified as a practical intermediate in the synthesis of N-terminal arginine peptides.[13]

That the sodium salt of tricarbobenzoxy-L-argininate (I) described above was, in reality, a mixture of at least two isomeric forms was suggested by the fact that a 12% solution of the material in ethanol, upon boiling for several minutes, followed by chilling, led to the deposition and ultimate recovery of some 50% of a crystalline material (II) which, although now only sparingly soluble in ethanol, revealed analyses identical with that of the parent mixture, and which, upon neutralization, was converted to the corresponding crystalline free acid (III); m.p. 138–139°; $[\alpha]_D^{25}$ +15.5° (1% in alcohol-free chloroform). Upon concentration of the ethanolic mother liquors, an exceedingly alcohol-soluble material was secured which, subsequent to acidification with acetic acid and crystallization from methanol, analyzed for the hitherto unreported N^{α},N^{ω}-dicarbobenzoxylated amino acid (IV);[17] m.p. 150°; $[\alpha]_D^{25}$ −10.0° (1% in pyridine). That the precursor of N^{α},N^{ω}-dicarbobenzoxy-L-arginine (IV) was, in fact, a highly *alcohol-susceptible* isomer of II, was indicated, aside from elemental analyses, by the following: (*a*) the *alcohol-stable* sodium salt of tricarbobenzoxy-L-arginine (II), after prolonged treatment with boiling ethanol, could be recovered in nearly quantitative yield without having suffered any detectable change in its constitution; (*b*) conversion of the crystalline sodium salt (II), or its parent isomeric mixture (I), to the N^{α},N^{ω}-dicarbobenzoxylated derivative (IV), could be readily achieved upon reaction with one equivalent of alkali in cold ethanol; and (*c*) carbobenzoxylation of IV, under Schotten-Baumann conditions, proceeded with the remark-

(6) M. Bergmann, L. Zervas, and H. Rinke, *Z. physiol. Chem.*, **224**, 40 (1934).

(7) D. T. Gish and F. H. Carpenter, *J. Am. Chem. Soc.*, **75**, 5872 (1953).

(8) G. W. Anderson, *J. Am. Chem. Soc.*, **75**, 6082 (1953).

(9) K. Hofmann, A. Rheiner, and W. D. Peckham, *J. Am. Chem. Soc.*, **75**, 6084 (1953); K. Hofmann, W. D. Peckham, and A. Rheiner, *J. Am. Chem. Soc.*, **78**, 238 (1956).

(10) H. O. Van Orden and E. L. Smith, *J. Biol. Chem.*, **208**, 751 (1954).

(11) C. Berse and L. Fiche, *J. Org. Chem.*, **21**, 808 (1956).

(12) Although K. Felix and H. Schuberth [*Z. physiol. Chem.*, **273**, 97 (1942)] have reported the isolation of arginylarginine from hydrolysates of clupeine and have analyzed the material as its flavianate derivative, the proximity of the calculated elemental values between the latter compound and arginine flaviate suggests that the assignment of an arginylarginine structure to this isolation product be regarded as tentative until the time that an authentic, well-defined synthetic material becomes available for comparison purposes.

(13) L. Zervas, M. Wintz, and J. P. Greenstein, *Arch. Biochem. and Biophys.*, **65**, 573 (1956).

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

(15) M. Bergmann and H. Köster, *Z. physiol. Chem.*, **159**, 179 (1926).

(16) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

(17) Other N^{α},N^{ω}-diacylated arginines which have been previously described include the dibenzoyl,[14] dibenzenesulfonyl [H. T. Clarke and H. B. Gillespie, *J. Am. Chem. Soc.*, **54**, 1964 (1932)], di-*p*-nitrocarbobenzoxy [D. T. Gish and F. H. Carpenter, *J. Am. Chem. Soc.*, **75**, 5872 (1953)], and dibenzylsulfonyl [H. B. Milne and C-H. Peng, *J. Am. Chem. Soc.*, **79**, 642 (1957)] derivatives.

ably facile production of sodium tricarbobenzoxy-L-argininate which, upon fractionation with ethanol, again yielded II and IV.

Although the basicity of N^{α},N^{ω}-diacyl arginines was established as early as 1928 both by Felix and Dirr[14] and by Zervas and Bergmann,[5] who described the hydrochloric salt of N^{α},N^{ω}-dibenzoylarginine, and was more recently reaffirmed by Gish and Carpenter,[17] who determined the apparent dissociation constants of N^{α},N^{ω}-di-*p*-nitrocarbobenzoxyarginine, the carbobenzoxylation of N^{α},N^{ω}-dicarbobenzoxy-L-arginine described herein represents the first reported instance whereby compounds of this general class have been acylated. In this connection, it is of interest to point out that like attempt to carbobenzoxylate N^{α}-carbobenzoxy-L-arginine, under strongly alkaline Schotten-Baumann conditions, resulted in the recovery only of unchanged starting material. No reason for the apparent resistance of this latter compound to acylation can be offered at the present time. As the N^{α}-carbobenzoxy-L-arginine here employed was prepared by the carbobenzoxylation of L-arginine in sodium bicarbonate solution, which is only slightly alakline, possible acylation of the highly basic guanido group was circumvented.

That tricarbobenzoxy-L-arginine may serve as a valuable intermediate in the synthesis of arginyl peptides was attested to by its ready condensation with amino acid esters *via* the mixed carboxylic–carbonic anhydride procedure.[18–20] This intermediate could be equally well utilized either as the free acid, m.p. 138–139°, or as the sodium salt (II) precursor. Where the latter was employed, best results were obtained by preliminary treatment of its chloroform solution with one equivalent of triethylamine hydrochloride in order to effect its conversion to the corresponding triethylamine salt; concomitant deposition of sodium chloride occurred. Coupling was effected with one equivalent of dibenzyl L-glutamate *p*-toluenesulfonate (or hydrochloride) in a chloroform solution containing one equivalent of triethylamine. The corresponding tricarbobenzoxydipeptide ester was secured in some 75% over-all yield and subsequently converted, *via* catalytic hydrogenolysis,[16] to L-arginyl-L-glutamic acid with an $[\alpha]_D$ value of +21.4° as a 1% solution in water. Chromatographic analysis of the compound in several solvent systems revealed its association with only a single ninhydrin positive spot.

Reported $[\alpha]_D$ values for the aforementioned L-arginyl-L-glutamic acid (as anhydrous material), in water, are as follows: +22°,[7] + 24.8°,[9] + 25.1°,[11] and +27.2°.[10] The differences revealed by these values are too large to be accounted for on the basis of experimental deviations in the measurements alone. In this connection, it should be recalled that the magnitude of the optical rotation of analytically pure peptides or peptide derivatives which contain a single center of asymmetry bears an inverse relationship to the amount of optical contamination by its enantiomorph, *i.e.*, the higher the optical rotation value, the greater the degree of optical purity. Polarimetric measurements thereby provide a means whereby the relative degrees of optical purity of a given mono-asymmetric compound, secured through different synthetic procedures, might be roughly estimated. In sharp contrast, however, the magnitude of the rotation of derivatives with two or more asymmetric centers may be expected to provide practically no information with respect to the degree of contamination by the various stereoisomers. This may be vividly demonstrated with the stereoisomers of alanylalanine, the D-D and L-D diastereomeric forms of which possess specific rotation values in water of +21.3° and +72.2°, respectively.[21] Thus, an increasing degree of contamination by L-alanyl-D-alanine in D-alanyl-D-alanine will lead to an increase in the magnitude of the observed rotation, with a 20% contamination, for example, leading to a specific rotation of +31.4°, a value some 50% higher than that possessed by the pure D-D form. No reliable information with regard to the optical integrity of the aforementioned L-arginyl-L-glutamic acids should therefore be expected from a comparison of the relative magnitudes of their respective optical rotation values alone.

The amino acid benzyl ester *p*-toluenesulfonate employed in the above synthesis was conveniently synthesized following a modification[22] of the method of Cipera and Nicholls.[23] Constants for this, as well as several other amino acid benzyl ester *p*-toluenesulfonates, prepared through this same route, are recorded in Table I.

Intermediates in the preparation of C-terminal arginine peptides. The tricarbobenzoxy- and N^{α},N^{ω}-dicarbobenzoxyarginines, described above, may serve as useful intermediates in the synthesis of *C*-terminal arginine peptides after removal of the N^{α}-carbobenzoxy substituent by an appropriate method.[7,24] Thus, conversion of the former compound to N^{ω},N^{ω}-dicarbobenzoxy-L-arginine *N*-carboxyanhydride by the action of thionyl chloride, followed by hydrolysis, led to the formation of N^{ω},N^{ω}-dicarbobenzoxy-L-arginine. Similar treatment of N^{α},N^{ω}-dicarbobenzoxy-L-arginine with thionyl chloride, followed by the action of aqueous

(18) J. R. Vaughan and R. L. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1952).
(19) R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951).
(20) T. Wieland and H. Bernhard, *Ann.*, **572**, 190 (1951).
(21) B. F. Erlanger and E. Brand, *J. Am. Chem. Soc.*, **73**, 3508 (1951).
(22) K. C. Hooper, H. N. Rydon, J. A. Schofield, and G. S. Heaton, *J. Chem. Soc.*, 3148 (1956).
(23) J. D. Cipera and R. V. V. Nicholls, *Chemistry & Industry*, 16 (1955).
(24) M. Bergmann, L. Zervas, and W. F. Ross, *J. Biol. Chem.*, **111**, 245 (1935).

acetic acid, methanol, or benzyl alcohol upon the N^{ω}-carbobenzoxy-L-arginine *N*-carboxyanhydride, so derived, led to the ultimate isolation, respectively of the free acid, the methyl ester, and the benzyl ester of N^{ω}-carbobenzoxy-L-arginine. Condensation of each of the latter two ester derivatives with tricarbobenzoxy-L-arginine, *via* the mixed carbonic-carboxylic acid anhydride procedures, resulted in the production of the methyl and benzyl esters of tricarbobenzoxy-L-arginyl-N^{ω}-carbobenzoxy-L-arginine. Conversion of these two latter compounds to L-arginyl-L-arginine, as well as exploitation of the aforementioned intermediates for the synthesis of various *C*-terminal and *N*-terminal arginine peptides,[25] will be the subject of a later communication.

EXPERIMENTAL

I. *Preparation of carbobenzoxylated argininines*

Sodium tricarbobenzoxy-L-argininate (I). To a solution of 42.2 g. of L-arginine·HCl[26] in 400 ml. of normal NaOH, chilled to about 2–5°, was added a total of 500 ml. of 2*N* NaOH and 136 ml. of freshly prepared carbobenzoxy chloride, in five equal and alternate portions. The addition, which required some 30 min., was accompanied by vigorous shaking and cooling in an ice bath. During this time, a precipitate of the sodium salt of tricarbobenzoxy-L-arginine formed which, after an additional 30 min. of stirring, was filtered over suction *in the cold* and washed with 200 ml. of *cold*, 5% sodium carbonate solution. The wet precipitate was dissolved in 1 l. of cold, alcohol-free chloroform, the aqueous layer separated, and the chloroform fraction washed with 100 ml. of cold, 5% sodium carbonate solution. Subsequent to filtration, the chloroform layer was dried over anhydrous sodium sulfate and then concentrated to an oily residue under reduced pressure and a bath temperature of 25°. Treatment of the residue with about 1 l. of dry ether, followed by storage for some 12 hr. in the cold, caused it to solidify. This material was filtered, washed with dry ether, and finally dried *in vacuo* over phosphorus pentoxide. Yields of the crude sodium salt ranged from 78 to 85 g.[27]

Anal. Calcd. for $C_{30}H_{31}O_8N_4Na$: N, 9.4; Na, 3.8. Found: N, 9.4; Na, 3.8.

The aforementioned material constitutes a mixture of at least two isomeric forms of sodium tricarbobenzoxy-L-argininate which, upon treatment by the methods given immediately below, may yield one of the component isomers in extremely pure form.

Sodium tricarbobenzoxy-L-argininate (II). *Method A.* Twelve grams of the crude sodium salt (I), described above, was suspended in 100 ml. of absolute ethanol, and solution effected upon boiling for several minutes. The hot solution was filtered and stored at 4° for several hours. A crystalline sodium salt (II) deposited which was filtered over suction and washed first with cold ethanol, then with ether; the combined filtrate and washings were saved for the recovery of α,ω-dicarbobenzoxy-L-arginine (Method A), described below. The yield of crystalline sodium salt, which amounted to 5.8 g., remained unchanged even in those instances where the aforementioned treatment with boiling ethanol was extended to 1 hr. Recrystallization was effected from hot absolute ethanol.

Anal. Calcd. for $C_{30}H_{31}O_8N_4Na$: N, 9.4; Na, 3.8. Found: N, 9.4; Na, 3.8.

Method B. The carbobenzoxylation of 42.2 g. of L-arginine hydrochloride was achieved as described for the preparation of the crude sodium salt of tricarbobenzoxy-L-arginine (I), above. The precipitate was filtered over suction, washed with 100 ml. of cold 5% sodium carbonate, dissolved in about 1 l. of ethanol-free chloroform, and the aqueous layer which formed discarded while the chloroform extract was washed successively with two 100 ml. portions of 5% sodium carbonate, two 100 ml. portions of 2*N* sulfuric acid, and several times with water. All of the above operations were effected at 1–5°C. After drying over anhydrous sodium sulfate, the chloroform fraction was concentrated to dryness under reduced pressure at 35–40°, with complete removal of final traces of chloroform being ensured by the addition of absolute ethanol, followed by reconcentration *in vacuo*. To the residual sirup was added 200–220 ml. of a hot ethanolic solution of 27 g. of sodium acetate trihydrate, and the mixture first boiled for 2 min., with stirring, then permitted to stand at room temperature for some 12–20 hr., during which time the desired crystalline sodium salt (needles) separated. This was filtered off, washed with 30 ml. of absolute ethanol, and filtered with suction, and the wet cake was extracted into 100 ml. of boiling ethyl acetate. Upon chilling the solution at 4°, a solid deposited which was filtered off, washed with 20 ml. of cold ethyl acetate, and then redissolved, while yet moist, in the requisite amount of hot absolute ethanol. A highly pure sodium salt of tricarbobenzoxy-L-arginine precipitated which, after some 24 hr. at 4°, was filtered over suction and washed first with a little cold absolute ethanol, then ether; yield, 21–24 g.

The sodium tricarbobenzoxy-L-argininate, so derived, is extremely pure and yields the free acid, m.p. 138–139°, upon acidification (see procedure below).

Tricarbobenzoxy-L-arginine (III). Two and a half grams of the above-described crystalline sodium salt (II) was suspended in 100 ml. of ethyl acetate and slowly brought into solution by vigorously shaking with 25 ml. of 2% sulfuric acid. The ethyl acetate layer was separated and washed, first with 25 ml. of 2% sulfuric acid, then three times with water, dried over anhydrous sodium sulfate, and then concentrated to dryness *in vacuo*. A crystalline residue was secured which was triturated with petroleum ether and then recovered by filtration over suction; yield, 1.9 g.; m.p. 135°. Two recrystallizations from ethyl acetate raised the melting point to 138–139°; $[\alpha]_D^{25}$ +15.5° (1% in alcohol-free chloroform).

Anal. Calcd. for $C_{30}H_{32}O_8N_4$: C, 62.5; H, 5.6; N, 9.7. Found: C, 62.2; H, 5.7; N, 9.8.

N^{α},N^{ω}-Dicarbobenzoxy-L-arginine (IV). *Method A.* The combined filtrate and washings saved during the preparation of sodium tricarbobenzoxy-L-argininate II (Method A) were concentrated, under reduced pressure, to a residual sirup. This was dissolved in water, the solution acidified with acetic acid, and the tacky precipitate which resulted dissolved in hot methanol. Subsequent cooling proceeded with the deposition of crystalline N^{α},N^{ω}-dicarbobenzoxy-L-arginine; yield, 3.2 g.; m.p. 150°; $[\alpha]_D^{25}$ −10.0° (1% in pyridine).

Anal. Calcd. for $C_{22}H_{26}O_6N_4$: C, 59.7; H, 5.9; N, 12.6. Found: C, 59.3; H, 6.0; N, 12.5.

Method B. To a solution of 8 g. of NaOH in 200 ml. of methanol was added 60 g. of crude sodium tricarbobenzoxy-L-argininate, with cooling in an ice bath. The reaction mixture, after storage at room temperature for 1 hr., was concentrated to approximately 100 ml. at 20–25° under re-

(25) We have recently observed that N^{α},N^{ω}-dicarbobenzoxy-L-arginine will serve as an excellent intermediate in the preparation of *N*-terminal arginine peptides if its condensation with an amino acid ester is effected in anhydrous dioxane according to the dicyclohexyl carbodiimide method [*cf.* J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955) and later papers]. Full details will appear in a subsequent communication.

(26) An equivalent quantity of the free base of L-arginine has also been employed, with somewhat higher yields.

(27) Although the use of 68 ml. (2 equivalents) of carbobenzoxy chloride also led to the separation of sodium tricarbobenzoxy-L-argininate, the yields were necessarily lower than when more of the reagent had been employed.

duced pressure, and 500 ml. of water, then 20 ml. of acetic acid added thereto. A gummy material deposited which was separated from the aqueous layer and repeatedly washed with water by decantation. The residue was finally dissolved in hot methanol; this solution, upon standing first at room temperature for several hours, then at 4°, deposited 38–40 g. of the desired dicarbobenzoxy derivative: m.p. 148°. Purification was effected by solution of the substance in dilute potassium carbonate, acidification, and subsequent crystallization of the precipitated gum from hot methanol. The material exhibited a melting point of 150°, which remained unaltered upon mixture with a sample secured by method A, above. Pure N^{α},N^{ω}-dicarbobenzoxy-L-arginine shows a sparing solubility in methanol and ethanol, and is insoluble in ether, chloroform, ethyl acetate, and benzene.

Carbobenzoxylation of N^{α},N^{ω}-dicarbobenzoxy-L-arginine. To a solution of 22.1 g. of N^{α},N^{ω}-dicarbobenzoxy-L-arginine in 50 ml. of normal NaOH, chilled to about 2–3°, was added a total of 8.5 ml. of carbobenzoxy chloride and 25 ml. of 2*N* NaOH, in three equal and alternate portions. The addition, which required some 10 min., was accompanied by vigorous shaking and cooling in an ice bath. A precipitate appeared during this time which, after an additional 30 min. was filtered over suction. The moist filter cake was dissolved in cold alcohol-free chloroform, the aqueous layer separated, and the chloroform layer washed with 10 ml. of 5% sodium carbonate and dried over anhydrous sodium sulfate. At no time was any of the above operations effected at a temperature greater than 4°. Evaporation of the chloroform solution to dryness at 20–25° *in vacuo*, followed by treatment of the oily residue with absolute ether, resulted in the formation of crystals. These were recovered by filtration, after some 12 hr. at 4°; yield, 20 g. Elemental analyses of the material identified it as sodium tricarbobenzoxy-L-argininate.

Anal. Calcd. for $C_{30}H_{31}O_8N_4Na$: N, 9.4; Na, 3.8. Found: N, 9.4; Na, 3.8.

That the aforementioned substance was a mixture of at least two isomeric forms of sodium tricarbobenzoxy-L-argininate was shown by the fact that 6 g. of the substance, when treated with 50 ml. of boiling absolute ethanol, ultimately yielded 0.7 g. of the crystalline alkali-stable form of sodium tricarbobenzoxy-L-argininate (II) upon standing for 2 days at 4°. The parent material therefore presumably consisted, in the main, of the alcohol-susceptible form.

N^{ω},N^{ω}-Dicarbobenzoxy-L-arginine (V). A solution of 2.9 g. of pure tricarbobenzoxy-L-arginine in 20 ml. of purified thionyl chloride was stored at room temperature for 5 hr. Petroleum ether was then added and the sirup which deposited was washed repeatedly with petroleum ether. The sirup was then dissolved in 20 ml. of acetone and, subsequent to the addition of 0.5 ml. of concentrated hydrochloric acid, was stored at room temperature for 6 hr., after which time it was concentrated to dryness *in vacuo* at 40°. The residue was taken up in water, a small amount of insoluble material was removed by filtration, and the filtrate was adjusted to a faintly alkaline *p*H by the addition of potassium bicarbonate. A precipitate deposited which was filtered over suction; the moist filter cake was crystallized from methanol. Yield, 50% of theory; m.p. 160°.

Anal. Calcd. for $C_{22}H_{26}O_6N_4$: C, 59.7; H, 6.0; N, 12.7. Found: C, 59.6; H, 6.0; N, 12.7.

N^{ω}-Carbobenzoxy-L-arginine (VI). *Method A.* A solution of 4.4 g. of N^{α},N^{ω}-dicarbobenzoxy-L-arginine (IV) in 30 ml. of pure thionyl chloride was stored at room temperature for 1 hr., then concentrated to dryness *in vacuo* at 40–45°. The residual sirupy N^{ω}-carbobenzoxy-L-arginine N-carboxyanhydride hydrochloride was washed repeatedly with petroleum ether in order to remove the benzyl chloride formed during the reaction, and then dissolved in 50 ml. of 10% acetic acid. After 4 hr. at room temperature, the reaction mixture was concentrated to dryness under reduced pressure, the resulting residue dissolved in 50 ml. of water, filtered, and the filtrate, after adjustment to *p*H 9 by the addition of concentrated aqueous ammonia, stored at room temperature in an open vessel in order to permit the excess ammonia to escape. Some 24 hr. later, the precipitate was filtered over suction and washed with cold water; yield 2.6 g. (84%). Recrystallization was effected by the dropwise addition of water to a suspension of the material in boiling ethanol until solution was achieved, followed by the further addition of alcohol and storage at 4°; the crystalline product, recovered by filtration, was ninhydrin positive. M.p. 190°; $[\alpha]_D^{25}$ +9.5° (6.12% in 1 equivalent of aqueous HCl).

Anal. Calcd. for $C_{14}H_{20}N_4O_4$: C, 54.5; H, 6.5; N, 18.2. Found: C, 54.5; H, 6.6; N, 18.2.

Method B. To an ice-cold suspension of 4.4 g. of N^{α},N^{ω}-dicarbobenzoxy-L-arginine, in 30 ml. of dry chloroform, was added 2.1 g. of phosphorus pentachloride. Upon shaking for several minutes at 0°, a homogeneous solution was secured without evident evolution of hydrogen chloride gas (presumably due to the formation of the hydrochloride salt of the acid chloride). The reaction mixture, which became turbid upon storage at room temperature for 1 hr. (formation of the *N*-carboxyanhydride), was concentrated to dryness *in vacuo* at 40–45°, and the residue washed repeatedly with petroleum ether, then treated with 50 ml. of 10% acetic acid. The solution so derived was permitted to stand at room temperature for 4 hr., washed twice with ethyl acetate, and subsequently concentrated to dryness at 40–45° *in vacuo*. An oily residue was secured which was dissolved in 50 ml. of water. Treatment with aqueous ammonia, as described in method A above, led to the desired product in some 65% yield; m.p. 190°.

N^{α}-Carbobenzoxy-L-arginine (VII). To a suspension of 63 g. of sodium bicarbonate in 250 ml. of water, in a 1-l. beaker, was added 42.2 g. of L-arginine·HCl, and the mixture stirred vigorously at room temperature. To the mixture was added 37.4 g. of carbobenzoxy chloride, in about 5 equal portions, over a period of 30 min. The stirring was continued for 1 hr. after the addition was complete, and the reaction mixture then adjusted to *p*H 8.5 with concentrated aqueous ammonia and subsequently stored for 2 hr. at 4°. A crystalline precipitate of the desired product which deposited was filtered off, washed with cold water, and finally recrystallized from boiling water to which a few drops of ammonia had been added; yield, 58.6 g.; m.p. 175°. A melting point of 175° was previously reported[16] for this compound.

II. *Preparation of some amino acid ester derivatives*

N^{ω}-Carbobenzoxy-L-arginine methyl ester (VIII). A solution of 4.4 g. of N^{α},N^{ω}-dicarbobenzoxy-L-arginine in 30 ml. of pure thionyl chloride was stored at room temperature for 1 hr., petroleum ether added thereto, and the resulting sirupy *N*-carboxyanhydride washed repeatedly with petroleum ether. The sirup was then dissolved in 30 ml. of absolute methanol containing about 0.7 g. of hydrogen chloride, and the reaction mixture permitted to stand at room temperature for 3 hr. After this time the solution was evaporated to dryness *in vacuo* at 30–35°, methanol was added, and the evaporation was repeated. The residual sirup was taken up in 7–8 ml. of methanol and 60 ml. of methyl ethyl ketone was added thereto. Upon storage at room temperature in an open vessel, the methanol slowly escaped, and a crystalline substance (prisms) was deposited. After some 24 hr., this was filtered over suction and washed first with methyl ethyl ketone, then with ether; yield, 3.3 g. The substance softened at about 100° and decomposed at 110°; repeated recrystallization of the material from methanol–methyl ethyl ketone did not alter this value. Analytical values indicated the substance to be the dihydrochloride monohydrate.

Anal. Calcd. for $C_{15}H_{22}N_4O_4 \cdot 2HCl \cdot H_2O$: C, 43.6; H, 6.5; N, 13.6; Cl, 17.2. Found: C, 43.5; H, 6.5; N, 13.6; Cl, 17.2.

N^{ω}-Carbobenzoxy-L-arginine benzyl ester (IX). A solution of 4.4 g. of N^{α},N^{ω}-dicarbobenzoxy-L-arginine in 30 ml. of pure thionyl chloride was stored at room temperature for 1 hr. Addition of petroleum ether led to the deposition of a

TABLE I

PHYSICAL CONSTANTS AND ANALYTICAL VALUES OF SOME AMINO ACID BENZYL ESTER *p*-TOLUENESULFONATES

p-Toluenesulfonate Salt	M.P., °C. (Corr.)	$[\alpha]_D^{25a}$	Calculated C	Calculated H	Calculated N	Found C	Found H	Found N
D-Alloisoleucine benzyl ester	162–164	− 0.2°	61.0	6.9	3.56	61.1	7.0	3.56
L-Aspartic acid dibenzyl ester	158–160[b]	+ 1.0°	61.8	5.6	2.89	61.7	5.7	2.86
L-*S*-Benzylcysteine benzyl ester	162–163[c]	−20.9°	60.9	5.8	2.96	61.1	5.8	2.90
L-Glutamic acid dibenzyl ester	144–145	+ 7.6°	62.6	5.9	2.80	62.4	5.9	2.80
Glycine benzyl ester	132–134	—	57.0	5.7	4.15	56.7	5.7	4.18
L-Isoleucine benzyl ester	153–154	− 0.1°	61.0	6.9	3.56	61.1	7.0	3.55
L-Leucine benzyl ester	158.5–160	− 1.7°	61.0	6.9	3.56	61.0	6.8	3.49
L-Phenylalanine benzyl ester	170.5–171.5	− 7.2°	64.6	5.9	3.28	64.6	5.8	3.23
L-Tyrosine benzyl ester	179–180.5	−12.2°	62.3	5.7	3.16	62.4	5.8	3.09
L-Valine benzyl ester	158–160	+ 1.2°	60.1	6.6	3.69	59.5	6.6	3.75

[a] Rotations were measured in a photoelectric polarimeter; solutions were 1–2% in methanol. [b] L. Velluz, G. Amiard, J. Bartos, B. Goffinet, and R. Heymes, *Bull. soc. chim. France*, 1464 (1950), reported a m.p. of 155° and an $[\alpha]_D^{20}$ +6.0° (2% in chloroform). [c] K. C. Hooper, H. N. Rydon, J. A. Schofield, and G. S. Heaton, *J. Chem. Soc.*, 3148 (1956), reported a m.p. of 155°.

sirup, which was washed repeatedly with petroleum ether and subsequently dissolved in 20 ml. of benzyl alcohol containing approximately 0.7 g. of hydrogen chloride. The reaction mixture was permitted to stand at room temperature for 3 hr., after which time treatment with dry ether led to the precipitation of the benzyl ester hydrochloride as a sirup. This was dissolved in a small amount of water, the solution was treated with an excess of anhydrous potassium carbonate, and the liberated free ester was extracted into ethyl acetate. Concentration of the ethyl acetate extracts *in vacuo*, followed by the addition of petroleum ether, resulted in the deposition of N^{ω}-carbobenzoxy-L-arginine benzyl ester in the form of prisms. The product was recrystallized from ethyl acetate; yield, 82% of theory; m.p. 121°.

Anal. Calcd. for $C_{21}H_{26}N_4O_4$: C, 63.3; H, 6.6; N, 14.1. Found: C, 63.4; H, 6.5; N, 14.0.

Preparation of the α-acetyl derivative of the above compound was effected upon treatment of a solution of 2 g. of N^{ω}-carbobenzoxy-L-arginine benzyl ester, in 20 ml. of dry chloroform, with 2 ml. of anhydrous pyridine, and 0.6 ml. of acetic anhydride. After some 2 hr., the reaction mixture was washed copiously, first with 10% acetic acid, then with potassium bicarbonate solution, and subsequently evaporated to dryness. The residue was first triturated with cold water, then crystallized from ethyl acetate. A 70% yield of N^{α}-acetyl-N^{ω}-carbobenzoxy-L-arginine benzyl ester m.p. 82° was thereupon secured. Hydrogenolysis of this compound, in the presence of palladium catalyst, resulted in the formation of N^{α}-acetyl-L-arginine monohydrate in nearly quantitative yield; m.p. 270°; $[\alpha]_D$ +7.8° (in water as the anhydrous material).

Glycine benzyl ester p-toluenesulfonate. Into a 500-ml. round-bottom flask were placed 18.8 g. (0.25 mole) of glycine, 48.5 g. (0.255 mole) of *p*-toluenesulfonic acid monohydrate, 100 ml. of benzyl alcohol, and 50 ml. of benzene. The mixture was then heated under reflux, with the liberated water being removed azeotropically and trapped with the aid of a Stark and Dean distilling receiver. A clear solution was obtained soon after reflux began. When water (about 9 ml.) was no longer distilled off (about 2–5 hr.), the reaction mixture was permitted to cool to room temperature and 250 ml. of benzene plus 400 ml. of dry ether added thereto. After standing for about 2 hr. at 4°, the crystalline benzyl glycinate *p*-toluenesulfonate was filtered, washed with anhydrous ether, and recrystallized from methanol-ether. Yield, 84% of theory. Melting point and analytical values are given in Table I.

Other amino acid benzyl esters. The benzyl ester *p*-toluenesulfonate derivatives of L-isoleucine, L-valine, L-leucine, L-aspartic acid (di-ester), L-glutamic acid (di-ester), L-phenylalanine, L-tyrosine, L-S-benzylcysteine, and D-alloisoleucine were prepared by essentially the same procedure as described above for the corresponding glycine derivative. Analytical and optical rotation values for each of these compound are revealed in Table I. The over-all yields, in most instances, ranged from 80–90% of theory.

III. *Preparation of arginine peptides*

Tricarbobenzoxy-L-arginyl-N^{ω}-carbobenzoxy-L-arginine benzyl ester. A solution of 2.85 g. (0.005 mole) of tricarbobenzoxy-L-arginine (III) and 0.7 ml. of anhydrous triethylamine in 15 ml. of dry chloroform was cooled to 0°, and 0.47 ml. of ethyl chloroformate was added. After 15 minutes at 0°, this solution was added to a chloroform solution of 2.0 g. of ω-carbobenzoxy-L-arginine benzyl ester, and the condensation permitted to proceed at room temperature for 1 hr. The chloroform solution was then washed first with dilute acetic acid, then with water, dried over anhydrous sodium sulfate, and concentrated to dryness *in vacuo*. Complete removal of the final traces of chloroform was ensured by the addition of a little methanol, followed again by evaporation to dryness. The crystalline residue obtained was dissolved in hot methanol, 0.3 ml. of triethylamine[28] was added, the solution was cooled for 12 hr. at 4°, and the precipitate which formed was filtered over suction and washed with a little cold methanol, then with ether; yield, 3.5 g.; m.p. 135°. The material, after two recrystallizations from hot methanol, exhibited a constant melting point at 147–148°.

Anal. Calcd. for $C_{51}H_{57}N_8O_{11}$: C, 64.0; H, 6.0; N, 11.7. Found: C, 63.8; H, 6.0; N, 11.6.

Tricarbobenzoxy-L-arginyl-N^{ω}-carbobenzoxy-L-arginine methyl ester. The condensation of 2.85 g. of tricarbobenzoxy-L-arginine (III) and 1.8 g. of ω-carbobenzoxy-L-arginine methyl ester·2HCl·H_2O was effected in essentially the same manner as described above for the corresponding benzyl ester derivative. A 2.3 g. yield of crude product, which melted at 128°, was obtained. After recrystallization from hot ethyl acetate, the substance revealed a m.p. of 135°.

Anal. Calcd. for $C_{45}H_{52}N_8O_{11}$: C, 61.4; H, 5.8; N, 12.7. Found: C, 61.0; H, 6.2; N, 12.4.

(28) Addition of triethylamine was necessary in order to prevent contamination with an unreacted tricarbobenzoxy-L-arginine. Employment of the conventional procedure, which would involve preliminary washing with carbonate or bicarbonate solution in order to remove unchanged acylamino acid, is useless here by virtue of the high solubility of the sodium and potassium salts of tricarbobenzoxy-L-arginine in chloroform solution, and their relatively low solubility in water.

The melting point of a mixture of the above product and tricarbobenzoxy-L-arginine was depressed to 110–115°.

Tricarbobenzoxy-L-arginyl-L-glutamic acid dibenzyl ester. The condensation of tricarbobenzoxy-L-arginine with the hydrochloride or *p*-toluenesulfonate salt of L-glutamic acid dibenzyl ester was carried out in chloroform solution as above. Isolation of the condensation product was achieved in comparable manner, with the exception that the crystalline residue, obtained upon evaporation of the reaction mixture, was first triturated with *cold* methanol containing triethylamine, then filtered ove" suction and recrystallized twice from ethyl acetate; yiel , 75%; m.p. 120–121°.

Anal. Calcd. for $C_{49}H_{51}O_{11}N_5$: C, 66.4; H, 5.8; N, 7.9. Found: C, 66.1; H, 6.0; N, 7.8.

L-Arginyl-L-glutamic acid. Hydrogenolysis of tricarbobenzoxy-L-arginyl-L-glutamic acid dibenzyl ester in 95% acetic acid was effected in the presence of palladium black catalyst.[16] Upon completion of the reaction, the catalyst was filtered off, washed first with methanol, then with water, and the combined filtrates finally evaporated to dryness. The residue was dissolved in a small amount of hot water and crystallized as plates upon the addition of hot ethanol; yield, 90% of theory. Recrystallization from water yielded the crystalline tetrahydrate with a recovery of 90%.

Anal. Calcd. for $C_{11}H_{21}O_5N_5 \cdot 4H_2O$: C, 35.2; H, 7.8; N, 18.7. Found: C, 35.2; H, 7.9; N, 18.4.

The dipeptide tetrahydrate lost 19.1% of its weight upon drying for 2 hr. *in vacuo* at 78° (calcd. for $4H_2O$, 19.2%) and melted at 210–214° prior to, and after its conversion to the anhydrous material; $[\alpha]_D^{24}$ +21.4° for a 1% solution in water, calculated as the anhydrous material.[29]

Anal. Calcd. for $C_{11}H_{21}O_5N_5$: C, 43.6; H, 7.0; N, 23.1. Found: C, 43.5; H, 7.1; N, 23.2.

A sample of the above dipeptide was hydrolyzed with acid to the corresponding free amino acids with 5*N* HCl and the excess acid was removed *in vacuo*. The hydrolysate was taken up in water and the aqueous solution "spotted," together with a sample of the parent dipeptide and reference standards of glutamic acid and arginine·HCl, on Whatman No. 1 paper. Prior to chromatography, the paper was briefly exposed to ammonia vapors. Four different solvent systems were employed, with the dipeptide revealing only a single ninhydrin positive spot with R_f values as follows: formic acid–water-*tert*-butyl alcohol (3:3:14), 0.26: methanol-water-pyridine (20:5:1), 0.28; 70% aqueous ethanol, 0.29; 88% phenol with 10% sodium acetate, 0.53. The hydrolyzate, on the other hand, revealed only two spots which corresponded to glutamic acid and arginine, respectively.

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(29) These values are in agreement with those reported by Gish and Carpenter[7]; m.p. 205–210° for the dihydrate; $[\alpha]_D$ +22°, calcd. for the anhydrous material in water. Berse and Piche[11] and Hofmann, Peckham, and Rheiner[9] reported melting point values for the anhydrous material as 202–205° and 251–252° dec., respectively, but made no mention of the possibility of hydrate formation. In our experience, the free dipeptide invariably deposits as the tetrahydrate upon crystallization from hot water. However, when precipitation is achieved by the addition of methanol or ethanol to the aqueous solution, a mixture of hydrates is obtained with values found which approach those of either the dihydrate or the tetrahydrate, depending upon the temperature employed. Isolation of the material as the monohydrate has been reported.[10]

[CONTRIBUTION NO. 464 FROM THE RESEARCH LABORATORIES OF HOFFMANN-LA ROCHE INC.]

Piperidine Derivatives. IV. 1,3-Disubstituted-4-aryl-4-acyloxy Piperidines*[1]

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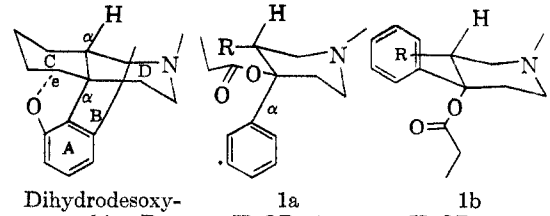
On the basis of the infrared spectra of α-1,3-dimethyl-4-phenyl-4-hydroxypiperidine and β-1,3-dimethyl-4-phenyl-4-hydroxypiperidine, the α-form was tentatively designated *cis* and the β-form *trans* with respect to H and OH. It was also shown that the α and β forms of 1-methyl-3-R-4-phenyl-4-propionoxypiperidine (R = ethyl, allyl, crotyl) can be distinguished by several bands in the infrared. Other esters have been prepared where R is butyl, propyl, hexyl, and benzyl. All of the esters have been examined for their analgesic activity.

In a previous paper,[2] we have described the diastereomeric forms of the low melting (β) [Fig. 1 (1b), R=CH_3] and the high melting (α) [Fig. 1 (1a), R=CH_3] forms of 1,3-dimethyl-4-phenyl-4-propionoxypiperidine hydrochloride.[3] The infrared spectra of the two forms are shown in Fig. 2.

We have now prepared homologous compounds



Dihydrodesoxymorphine-D C:D *trans* — 1a H:OR *cis* — 1b H:OR *trans*

FIGURE 1.

where R is allyl, crotyl, ethyl, propyl, butyl, hexyl, and benzyl. In the case of the first three mentioned, diastereomeric pairs, α and β forms were encoun-

*This paper is a contribution in honor of Lyndon F. Small, former Editor of the Journal.

(1) Presented in part before the Meeting-in-Miniature of the North Jersey Section of the A.C.S., January 28, 1957.

(2) A. Ziering and J. Lee, *J. Org. Chem.*, 12, 911 (1947).

(3) The higher melting isomer is also known as alphaprodine [Nisentil®] and the lower melting form as betaprodine (World Health Organization designations).