[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE]

Studies on Polypeptides. VII. The Synthesis of Peptides Containing Arginine[1]

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Methods are described for the preparation of peptides containing the amino acid L-arginine. The applications of these procedures to the preparation of two types of arginine-containing dipeptides are presented. In the first type, the amino group of arginine is involved in peptide bonding; the carboxyl group provides the link with the other amino acid in the second. Nitro-L-arginine, the key intermediate in our scheme, was prepared by a simplified procedure affording the compound in high yield. The degree of homogeneity of the various peptides was determined by paper chromatography in three solvent systems.

In connection with our interest in the synthesis of protein degradation products, we have undertaken a systematic study of approaches to peptides containing arginine.

Nitro-L-arginine, first obtained by Kossel and Kennaway[2] from acid-hydrolyzed nitroclupein and later by direct nitration of L-arginine, served as the key intermediate for our experiments. The introduction of the strongly electronegative nitrofunction depresses markedly the basic nature of the guanido group, thus facilitating the incorporation of arginine into peptides.

In 1934, Bergmann, Zervas and Rinke[3] carried out a systematic study of nitro-L-arginine's usefulness as an intermediate in the synthesis of peptides containing arginine. These authors transformed nitro-L-arginine into the carbobenzoxy derivative and prepared carbobenzoxyglycylnitro-L-arginine from carbobenzoxyglycyl chloride and nitro-L-arginine. Hydrogenolysis in the presence of palladium, in ethanol containing hydrogen chloride, converted carbobenzoxynitro-L-arginine into L-arginine hydrochloride. The arginine retained its optical activity during these transformations. Carbobenzoxyglycylnitro-L-arginine on hydrogenation was converted into the hydrochloride of glycyl-L-arginine which was obtained in the form of an amorphous powder.

Hofmann and Bergmann[4] prepared benzoylglycyl-L-argininamide by hydrogenolysis of benzoylglycylnitro-L-argininamide.

Arginyl peptides, *i.e.*, peptides in which the carboxyl group of arginine is involved in peptide-bond formation, have not been prepared until very recently, when three groups of workers practically simultaneously (but by independent routes) arrived at the solution of this problem. Anderson[5] reported the synthesis of L-arginyl-L-leucine from carbobenzoy-L-arginine hydrobromide by the tetraethyl pyrophosphite route. Hofmann Rheiner and Peckham[6] prepared L-arginyl-L-alanine, L-arginyl-L-phenylalanine and L-arginyl-L-tyrosine (in the form of their crystalline acetate salts) by the use of carbobenzoxynitro-L-arginine, whereas Gish and Carpenter[7] obtained L-arginyl-L-leucine and L-arginyl-L-glutamic acid *via* the acid chloride hydrochloride of *p*-nitrocarbobenzoxy-L-arginine.

Van Orden and Smith,[8] confirming our results, have described the synthesis of four arginyl peptides from nitro-L-arginine, using the mixed anhydride procedure. Only one of these peptides was obtained in crystalline form.

This paper describes the preparation from nitro-L-arginine of two series of dipeptides containing L-arginine. In the first of these, the amino group of arginine is involved in peptide bonding, and the carboxyl group provides the link with the other amino acid in the second. The nitro-L-arginine which was required for this investigation was prepared by a modification of Bergmann's procedure[3] which affords the compound in high yields.[9]

Treatment with methanolic hydrogen chloride converted nitro-L-arginine into its crystalline methyl ester hydrochloride. Attempts to prepare the ethyl ester hydrochloride afforded a gummy material which resisted all crystallization attempts. Methyl nitro-L-arginate (which had been prepared from the hydrochloride with sodium methoxide) reacted smoothly with mixed anhydrides prepared from the carbobenzoxy derivatives of glycine, L-alanine, L-phenylalanine, L-proline and L-lysine to give the respective carbobenzoxylated dipeptide esters. For conversion into the corresponding acylated dipeptides, the esters were saponified with aqueous sodium hydroxide. Hydrogenation over palladium, in methanol containing glacial acetic acid, removed the carbobenzoxy and nitro groups from the acylated dipeptides to give the acetate salts of the respective dipeptides containing L-arginine. The acetates of L-alanyl- and L-propyl-L-arginine crystallized, but the remaining dipeptide acetates could not be induced to crystallize and were obtained in the form of amorphous powders. These were purified by repeated precipitation with ether from methanolic solution. The glycyl-L-arginine was further characterized in the form of its crystalline dipicrate. It is of interest to note that the interaction of L-phenylalanyl-L-arginine with picric acid led to the formation of a crystalline monopicrate whose analysis corresponded with that calculated for the monopicrate of the respective diketopiperazine and not for that of the dipeptide. The material regenerated from the picrate by passage through the ion exchanger, Amberlite IR-4B (acetate form), failed to exhibit a positive ninhydrin test (in contrast to the starting dipeptide which was

(1) The authors wish to express their appreciation to Armour and Company and the U. S. Public Health Service for generous support of this investigation.

(2) A. Kossel and E. L. Kennaway, *Z. physiol. Chem.*, **72**, 487 (1911).

(3) M. Bergmann, L. Zervas and H. Rinke, *ibid.*, **224**, 40 (1934).

(4) K. Hofmann and M. Bergmann, *J. Biol. Chem.*, **138**, 243 (1941).

(5) G. W. Anderson, THIS JOURNAL, **75**, 6081 (1953).

(6) K. Hofmann, A. Rheiner and W. D. Peckham, *ibid.*, **75**, 6083 (1953).

(7) D. T. Gish and F. H. Carpenter, *ibid.*, **75**, 5872 (1953).

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

(9) This method was developed by Dr. Andreas E. Furlenmeier.

TABLE I

PAPER CHROMATOGRAPHIC EVALUATION OF A SERIES OF DIPEPTIDES CONTAINING L-ARGININE

Peptide	R_f Values[a] Part[b]	2-But–NH_3[c]	Phenol[d]	Results of acid hydrolysis[e]	Enzymatic treatment[f]
L-Arg-Gly	0.25	Lys	0.89		
L-Arg-L-Ala	.26	Arg	.91	Only Arg and Ala in theor. amt.	No splitting in 24 hr. by carboxypeptidase
L-Arg-L-Leu	.56	Met	.95		
L-Arg-L-Phe	.50	Val$^+$	.94	Only Arg and Phe in theor. amt.	Split completely into Arg and Phe by carboxypeptidase
L-Arg-L-Tyr	.38	Ser$^+$	.88	Only Arg and Tyr in theor. amt.	Split completely into Arg and Tyr by carboxypeptidase
L-Arg-L-Try[g]	.46	Val$^-$	.93		Split completely into Arg and Try by carboxypeptidase
L-Arg-L-Glu	.19	0.75 × Glu	.67		
Gly-L-Arg	.16	Arg	.88		
L-Ala-L-Arg	.14	Arg$^+$	.91	Only Ala and Arg in theor. amt.	
L-Pro-L-Arg	.25	Ser$^-$	.95	Only Pro and Arg in theor. amt.	No splitting in 24 hr. by carboxypeptidase
L-Phe-L-Arg	.38	Ileu$^-$	.96		
L-Lys-L-Arg[h]	.13	Glu	.95		

[a] Whatman #1 Paper, descending, 20–25°. Except when noted otherwise, the peptides produced one single ninhydrin positive spot in the three solvent systems. [b] Butanol–water–acetic acid (4:5:1). [c] 2-Butanol–3% ammonia (3:1). The R_f values are given in terms of the nearest amino acid. [d] Phenol (72%). [e] Sealed tube, 6 *N* HCl at 105° for 18 hours. [f] Crystalline carboxypeptidase (0.5 mg./ml.) at *p*H 7.7 in 0.1 *N* ammonium acetate buffer for 24 hours at 37°. [g] Some samples contained a ninhydrin-positive impurity of R_f 0.57 in the Partridge System.[17] [h] Slight trace of impurity at position of Arg.

ninhydrin positive). These findings suggest that the picric acid treatment brought about the elimination of water from the dipeptide, to give the respective diketopiperazine. The picrate of the diketopiperazine of D-phenylalanyl-L-arginine was prepared by Bergmann and Köster.[10]

Of particular interest was the preparation of the second series of peptides in which the carboxyl group, instead of the amino group, of arginine is involved in peptide-bonding. Carbobenzoxynitro-L-arginine served as the starting material for these experiments. Previous attempts to convert this compound into an acid chloride or azide were unsuccessful.[11] We have observed[6] that carbobenzoxynitro-L-arginine is readily converted into a mixed anhydride[12–14] when treated with ethyl chloroformate in the presence of tri-*n*-butylamine. This mixed anhydride reacted with aniline to give the anilide of carbobenzoxynitro-L-arginine in crystalline form. Upon reaction with the methyl or ethyl esters of a variety of amino acids, the corresponding carbobenzoxynitro-L-arginyl peptide esters were obtained. Saponification with dilute sodium hydroxide gave the respective carbobenzoxynitro-L-arginyl peptides. Conversion to the L-arginyl dipeptides was again performed by hydrogenation over palladium in methanol–glacial acetic acid. With the exception of L-arginyl-L-glutamic acid, which was obtained in crystalline form as the free dipeptide, the compounds were isolated as the crystalline acetate salts.[15] All the peptides containing arginine exhibited a positive Sakaguchi reaction. Table I summarizes the paper chromatographic evaluation of the homogeneity of the arginine peptides with three solvent systems.[16]

It is apparent from an inspection of Table I that, with the exception of L-lysyl-L-arginine and L-arginyl-L-tryptophan, the peptides produced a single ninhydrin-positive spot in the three systems studied. Five of the peptides were hydrolyzed with hydrochloric acid, and the expected amino acids were obtained in the theoretical proportions. L-Arginyl-L-phenylalanine, L-arginyl-L-tyrosine and L-arginyl-L-tryptophan were readily hydrolyzed by carboxypeptidase; but L-arginyl-L-alanine and L-prolyl-L-arginine resisted the action of this enzyme under our experimental conditions. The identification of L-arginyl-L-tryptophan with a dipeptide fragment resulting from the chymotryptic digestion of corticotropin-A[17] established the sequence Arg·Try in this pituitary hormone.

Experimental[18]

Nitro-L-arginine.—A mixture of fuming nitric acid (40 ml.) and fuming sulfuric acid (25 ml.; containing 30% of sulfur trioxide) was cooled in an ice–salt-bath, and L-arginine (free base; 30 g.) was added slowly with stirring. The last batches of the arginine were washed into the reaction mixture with concentrated sulfuric acid (15 ml.). The mixture was stirred for 1 hour with cooling and was then poured onto cracked ice and the solution adjusted to a *p*H of 8 to 9 with concentrated ammonium hydroxide. The *p*H was then adjusted to 6 with glacial acetic acid, and the solution was kept in a refrigerator for 4 hours. The precipitate was collected, and recrystallized from hot water, washed with 95% ethanol and ether, and dried; yield 30.8 g. (81.5%); m.p. 251–252° (lit.[3] m.p. 262°); $[\alpha]^{25}D$ +24.3° (*c* 4.12, in 2 *N* hydrochloric acid).

Anal. Calcd. for $C_6H_{13}O_4N_5$: C, 32.9; H, 6.0; N, 32.0. Found: C, 33.1; H, 5.7; N, 32.0.

(10) M. Bergmann and H. Köster, *Z. physiol. Chem.*, **173**, 259 (1928).

(11) J. S. Fruton, in "Advances in Protein Chemistry," M. L. Anson and John T. Edsall, Eds., Vol. V, Academic Press Inc., New York, N. Y., 1949, p. 64.

(12) T. Wieland and R. Sehring, *Ann.*, **569**, 122 (1950).

(13) R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951).

(14) J. R. Vaughan, Jr., THIS JOURNAL, **73**, 3547 (1951).

(15) L-Arginyl-L-tryptophan was obtained as an amorphous powder by precipitation from methanol with ether.

(16) We wish to express our appreciation to Dr. W. F. White of the Armour Laboratories in Chicago for these determinations.

(17) W. F. White and W. A. Landmann, THIS JOURNAL, **76**, 4193 (1954).

(18) The melting points are uncorrected. Optical rotations were measured in a Rudolph Precision Polarimeter, Model 70, using a 1-dm. tube.

Nitro-L-arginine Methyl Ester Hydrochloride.—Nitro-L-arginine (10.0 g.) was suspended in methanol (150 ml.) and hydrogen chloride was passed through the solution for 1 hour. The mixture was refluxed for 15 minutes, when the solvent was removed *in vacuo*. The residue was redissolved in absolute methanol (100 ml.) and the procedure was repeated. The solvent was removed *in vacuo*, the resulting foam was redissolved in methanol (40 ml.), and ether was added to the solution until it became slightly turbid. The mixture was kept at room temperature for 4 to 5 hours and was then placed in a refrigerator to complete the crystallization. A sample for analysis was recrystallized from methanol–ether, yield 12.0 g. (97%); m.p. 159–161° (lit.[8] m.p. 154–155°); $[\alpha]^{25}D$ +17.5° (*c* 3.20, in methanol).

Anal. Calcd. for $C_7H_{16}O_4N_5Cl$: C, 31.2; H, 6.0; N, 26.0; Cl, 13.1. Found: C, 31.0; H, 6.3; N, 26.0; Cl, 13.1.

Carbobenzoxyglycylnitro-L-arginine.—Nitro-L-arginine methyl ester hydrochloride (1.1 g.) was dissolved in methanol (10 ml.), and 0.5 *N* methanolic sodium methoxide (8 ml.) was added. The solution was evaporated to dryness *in vacuo* and the residue was triturated with dioxane (10 ml.). To this solution of nitro-L-arginine methyl ester was added a solution of a mixed anhydride prepared from carbobenzoxyglycine (0.84 g.) in dioxane (25 ml.), tri-*n*-butylamine (0.96 ml.) and ethyl chloroformate (0.38 ml.) at 11–12°. The mixture was shaken at room temperature for 45 minutes and was evaporated to dryness *in vacuo*. The residue was dissolved in ethyl acetate and the solution was washed successively with 1 *N* hydrochloric acid, water, 1 *M* sodium bicarbonate and water, dried over sodium sulfate and evaporated to dryness. The residual oily ester (1.9 g.) was saponified in the usual manner, and the resulting carbobenzoxyglycylnitro-L-arginine was recrystallized from dilute acetic acid; yield 887 mg. (54%); m.p. 142–145° (lit.[3] m.p. 145°); $[\alpha]^{23}D$ +3.2° (*c* 1.13, in glacial acetic acid).

Anal. Calcd. for $C_{16}H_{22}O_7N_6$: C, 46.8; H, 5.4; N, 20.5. Found: C, 47.0; H, 5.2; N, 20.6.

Carbobenzoxy-L-alanylnitro-L-arginine.—A solution of a mixed anhydride prepared from carbobenzoxy-L-alanine (2.23 g.), tri-*n*-butylamine (2.4 ml.) and ethyl chloroformate (0.96 ml.) in dioxane (100 ml.) was added to a dioxane solution of nitro-L-arginine methyl ester (prepared from 2.70 g. of the hydrochloride), and the mixture was shaken at room temperature for 45 minutes. The reaction product was isolated in the manner described above and then saponified. The carbobenzoxy-L-alanylnitro-L-arginine was recrystallized from a mixture of ethanol and water, yield 2.8 g. (66%); m.p. 171–172°; $[\alpha]^{25}D$ −9.4° (*c* 1.29, in methanol).

Anal. Calcd. for $C_{17}H_{24}O_7N_6$: C, 48.1; H, 5.7; N, 19.8. Found: C, 47.9; H, 5.4; N, 20.1.

Carbobenzoxy-L-phenylalanylnitro-L-arginine Methyl Ester.—A mixed anhydride was prepared from carbobenzoxy-L-phenylalanine (6.0 g.), tri-*n*-butylamine (4.8 ml.) and ethyl chloroformate (1.92 ml.) in dioxane (200 ml.), and was added to a dioxane solution of nitro-L-arginine methyl ester (prepared from 5.4 g. of the hydrochloride). The mixture was shaken at room temperature for 45 minutes and the ester was isolated in the manner described above. The compound was recrystallized from a mixture of methanol and ether; yield 7.1 g. (70%); m.p. 160–161°; $[\alpha]^{28}D$ −16.2° (*c* 1.12, in methanol).

Anal. Calcd. for $C_{24}H_{30}O_7N_6$: C, 56.0; H, 5.9; N, 16.3. Found: C, 55.9; H, 5.9; N, 16.1.

Carbobenzoxy-L-phenylalanylnitro-L-arginine.—Carbobenzoxy-L-phenylalanylnitro-L-arginine methyl ester (2.0 g.) was saponified by shaking for 1 hour at room temperature with 50% aqueous methanol (20 ml.) containing 240 mg. of sodium hydroxide. The product was isolated in the usual manner and recrystallized from 50% aqueous ethanol; yield 1.7 g. (87%); m.p. 185–186°; $[\alpha]^{27}D$ +1.5° (*c* 1.59, in pyridine).

Anal. Calcd. for $C_{23}H_{28}O_7N_6$: C, 55.2; H, 5.6; N, 16.8. Found: C, 55.4; H, 5.7; N, 16.6.

Carbobenzoxy-L-prolylnitro-L-arginine.—A mixed anhydride was prepared from carbobenzoxy-L-proline (5 g.), (dried by azeotropic distillation with benzene), tri-*n*-butylamine (4.8 ml.) and ethyl chloroformate (1.92 ml.) in dioxane (150 ml.), and was added to a dioxane solution of nitro-L-arginine methyl ester (prepared from 5.4 g. of the hydrochloride). The product was isolated in the usual manner and saponified by shaking for 1 hour at room temperature with 0.5 *N* sodium hydroxide (45 ml.). The material was recrystallized from aqueous acetone; yield 5.4 g. (60%); m.p. 197–198°; $[\alpha]^{30}D$ −32.7° (*c* 3.92, in methanol).

Anal. Calcd. for $C_{19}H_{26}O_7N_6$: C, 50.7; H, 5.8; N, 18.7. Found: C, 51.1; H, 6.2; N, 18.9.

α,ε-Dicarbobenzoxy-L-lysylnitro-L-arginine Methyl Ester.—A mixed anhydride was prepared from α,ε-dicarbobenzoxy-L-lysine (5.9 g.), tri-*n*-butylamine (3.6 ml.) and ethyl chloroformate (1.44 ml.) in dioxane (150 ml.), and was added to a dioxane solution of nitro-L-arginine methyl ester (prepared from 4.1 g. of the hydrochloride). Reaction conditions and the isolation were the same as above. The ester was recrystallized from a mixture of methanol and ether; yield 6.0 g. (64%); m.p. 70–72°; $[\alpha]^{23}D$ −10.6° (*c* 3.58, in methanol).

Anal. Calcd. for $C_{29}H_{39}O_9N_7$: C, 55.3; H, 6.2; N, 15.6. Found: C, 55.4; H, 6.3; N, 15.4.

α,ε-Dicarbobenzoxy-L-lysylnitro-L-arginine.—The above methyl ester (2.0 g.) was suspended in 0.5 *N* sodium hydroxide (10 ml.) and the mixture was shaken for 1.5 hours at room temperature. The material was isolated in the usual manner and was recrystallized from 50% ethanol; yield 1.8 g. (91%); m.p. 142–145°; $[\alpha]^{27}D$ −6.2° (*c* 2.64, in pyridine).

Anal. Calcd. for $C_{28}H_{37}O_9N_7$: C, 54.6; H, 6.1; N, 15.9. Found: C, 54.6; H, 6.1; N, 16.1.

Glycyl-L-arginine Acetate.—Carbobenzoxyglycylnitro-L-arginine (1.0 g.) was dissolved in methanol containing 10% of glacial acetic acid (25 ml.) and was hydrogenated over a palladium catalyst[19] for 12 hours. The catalyst was then removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in methanol (5 ml.) and the product was precipitated with ether. This procedure was repeated twice. An amorphous powder was obtained which was dried for 24 hours at 56° *in vacuo* at 0.03 mm. pressure; yield 0.48 g. (67%); $[\alpha]^{25}D$ +1.6° (*c* 4.31, in water).

Anal. Calcd. for $C_{10}H_{21}O_5N_5$: C, 41.2; H, 7.3; N, 24.1. Found: C, 41.3; H, 7.7; N, 24.4.

Dipicrate.—A sample of the dipeptide acetate (0.35 g.) was dissolved in water (1 ml.), and picric acid (0.7 g.) in hot water (40 ml.) was added. The mixture was kept in a refrigerator for 48 hours, the crystals were collected, dried, washed with ether and recrystallized three times from aqueous acetic acid; yield 0.25 g.

Anal. Calcd. for $C_{20}H_{23}O_{17}N_{11}$: C, 34.8; H, 3.4; N, 22.4. Found: C, 34.3; H, 3.6; N, 22.7.

L-Alanyl-L-arginine Acetate.—Carbobenzoxy-L-alanylnitro-L-arginine (2.8 g.) was hydrogenated in the manner described above. Evaporation of the solvents gave a solid which was recrystallized twice from aqueous ethanol; yield 1.55 g. (77%); m.p. 166–168°; $[\alpha]^{27}D$ +8.7° (*c* 3.32, in water).

Anal. Calcd. for $C_{11}H_{23}O_5N_5$: C, 43.3; H, 7.6; N, 22.9. Found: C, 43.4; H, 7.6; N, 22.9.

L-Phenylalanyl-L-arginine Acetate.—Carbobenzoxy-L-phenylalanylnitro-L-arginine (0.80 g.) in glacial acetic acid (25 ml.) was hydrogenated for 10 hours. The material was isolated in the usual manner and purified by precipitation from methanolic solution with ether. An amorphous powder was obtained which was dried at 56° for 24 hours at 0.03 mm. pressure; yield 0.46 g. (75%); $[\alpha]^{23}D$ +7.0° (*c* 3.16, in water).

Anal. Calcd. for $C_{17}H_{27}O_5N_5$: C, 53.5; H, 7.1; N, 18.4. Found: C, 53.8; H, 6.9; N, 18.7.

Diketopiperazine Monopicrate.—L-Phenylalanyl-L-arginine acetate (0.46 g.) was dissolved in water (2 ml.), and picric acid (0.55 g.) in hot water (40 ml.) was added. The mixture was kept in a refrigerator for 12 hours and the supernatant liquor was decanted from the yellow oil which had precipitated. The residue crystallized on trituration with ethanol. The crystals were collected and recrystallized from aqueous acetic acid; yield 0.16 g. (25%); m.p. 254–255°.

Anal. Calcd. for $C_{21}H_{24}O_9N_8$: C, 47.4; H, 4.5; N, 21.1. Found: C, 47.3; H, 4.6; N, 21.1.

(19) J. Tausz and N. von Pulnoki, *Ber.*, **52**, 1573 (1919).

L-Prolyl-L-arginine Acetate.—Carbobenzoxy-L-prolylnitro-L-arginine (0.67 g.) was hydrogenated in methanol containing 10% of glacial acetic acid (25 ml.), and the hydrogenated product was isolated in the usual manner. The product was obtained in crystalline form and was purified by recrystallization from aqueous ethanol; yield 0.37 g. (76%); m.p. 182–183°; $[\alpha]^{27}D$ −28.6 (*c* 2.59, in water).

Anal. Calcd. for $C_{13}H_{25}O_5N_5$: C, 47.1; H, 7.6; N, 21.1. Found: C, 47.2; H, 7.2; N, 20.7.

L-Lysyl-L-arginine Diacetate.—α,ϵ-Dicarbobenzoxy-L-lysylnitro-L-arginine (0.35 g.) was dissolved in methanol containing 10% by volume of glacial acetic acid (20 ml.), and was hydrogenated in the usual manner for 12 hours. The catalyst was removed by filtration and the solvents were removed *in vacuo*. The product was purified as described for the preparation of glycyl-L-arginine acetate and was obtained in the form of a hygroscopic amorphous powder; yield 0.15 g. (64%); $[\alpha]^{25}D$ +15.5° (*c* 2.13, in water).

Anal. Calcd. for $C_{16}H_{34}O_7N_6$: C, 45.5; H, 8.1; N, 19.9. Found: C, 45.2; H, 8.9; N, 19.7.

Carbobenzoxynitro-L-arginine.—Nitro-L-arginine was carbobenzoxylated essentially as described by Bergmann, *et al.*[3] The crude crystalline material was washed with ice-water, triturated with ether, and dried to constant weight at room temperature over phosphorus pentoxide. The average yield in several preparations was 85–90%; m.p. 122–126°. A sample for analysis was recrystallized from dilute ethanol; m.p. 134–136° (lit.[3] m.p. 132–134°); $[\alpha]^{27}D$ −3.5° (*c* 1.02, in methanol).

Anal. Calcd. for $C_{14}H_{19}O_6N_5$: C, 47.6; H, 5.4; N, 19.8. Found: C, 48.2; H, 5.1; N, 20.1.

Carbobenzoxynitro-L-arginyl Amino Acid Esters.—In general, these compounds were prepared as follows. Carbobenzoxynitro-L-arginine was dissolved in sodium-dried dioxane (10 ml. per mmole), and tri-*n*-butylamine (0.24 ml. per mmole) was added. The solution was cooled at 11–12°, ethyl chloroformate (0.096 ml. per mmole) was added with stirring, and the solution was kept at 11–12° for 15 minutes. This mixture was then added to a dioxane solution of the appropriate amino acid ester (1 mmole per mmole of carbobenzoxynitro-L-arginine). The mixture was shaken for 45 minutes at room temperature and the solvents were evaporated at a bath temperature of 40–50°. The residue was dissolved in ethyl acetate, and the solution was washed successively with 1 *N* hydrochloric acid, water, 1 *N* sodium carbonate and water, dried over sodium sulfate, and evaporated to dryness *in vacuo*. The ensuing sirupy esters were dissolved in a small amount of methanol to induce crystallization. The resulting crystals were purified by recrystallization from an appropriate solvent.

Carbobenzoxynitro-L-arginyl Anilide.—This compound was prepared from carbobenzoxynitro-L-arginine (0.18 g.) and aniline (0.05 ml.) as described above, and recrystallized from methanol–ether; yield 0.12 g. (56%); m.p. 167–168°; $[\alpha]^{25}D$ −3.5° (*c* 1.1, in methanol) (lit.[3] m.p. 166–167°).

Anal. Calcd. for $C_{20}H_{24}O_5N_6$: C, 56.1; H, 5.7; N, 19.6. Found: C, 56.2; H, 5.5; N, 19.6.

Carbobenzoxynitro-L-arginylglycine Methyl Ester.—This compound was prepared from carbobenzoxynitro-L-arginine (5.3 g.) and glycine methyl ester hydrochloride (1.89 g.) as described above, and recrystallized from acetone–ether; yield 2.8 g. (44%); m.p. 70–73°; $[\alpha]^{29}D$ −13.8° (*c* 3.60, in methanol).

Anal. Calcd. for $C_{17}H_{24}O_7N_6$: C, 48.1; H, 5.7; N, 19.8. Found: C, 48.3; H, 5.7; N, 19.6.

Carbobenzoxynitro-L-arginyl-L-alanine Methyl Ester.—This compound was prepared from carbobenzoxynitro-L-arginine (5.3 g.) and L-alanine methyl ester hydrochloride (2.1 g.) as described above, and recrystallized from methanol–ether; yield 3.0 g. (46%); m.p. 157–159°; $[\alpha]^{25}D$ −18.8° (*c* 0.98, in methanol).

Anal. Calcd. for $C_{18}H_{26}O_7N_6$: C, 49.3; H, 6.0; N, 19.2. Found: C, 49.5; H, 5.9; N, 19.3.

Carbobenzoxynitro-L-arginyl-L-leucine Methyl Ester.—This compound was prepared from carbobenzoxynitro-L-arginine (1.77 g.) and L-leucine methyl ester hydrochloride (0.91 g.) was described above, and recrystallized from methanol–ether; yield 1.45 g. (60%); m.p. 170–171° (lit.[3] m.p. 162–163.4°); $[\alpha]^{30}D$ −23.4° (*c* 0.94, in methanol).

Anal. Calcd. for $C_{21}H_{32}O_7N_6$: C, 52.5; H, 6.7; N, 17.5. Found: C, 52.5; H, 6.8; N, 17.9.

Carbobenzoxynitro-L-arginyl-L-phenylalanine Methyl Ester.—This compound was prepared from carbobenzoxynitro-L-arginine (1.06 g.) and L-phenylalanine methyl ester hydrochloride (0.65 g.) as described above, and recrystallized from methanol–ether; yield 1.0 g. (65%); m.p. 132–133°; $[\alpha]^{26}D$ −8.2° (*c* 1.18, in methanol).

Anal. Calcd. for $C_{24}H_{30}O_7N_6$: C, 56.0; H, 5.9; N, 16.3. Found: C, 55.7; H, 5.7; N, 16.1.

Carbobenzoxynitro-L-arginyl-L-tyrosine Methyl Ester.—This compound was prepared from carbobenzoxynitro-L-arginine (1.06 g.) and L-tyrosine methyl ester hydrochloride (0.70 g.) as described above, and recrystallized from methanol–ether; yield, 1.1 g. (66%); m.p. 159–160°; $[\alpha]^{25}D$ −3.6° (*c* 1.22, in methanol).

Anal. Calcd. for $C_{24}H_{30}O_8N_6$: C, 54.3; H, 5.7; N, 15.8. Found: C, 54.2; H, 5.4; N, 15.9.

Carbobenzoxynitro-L-arginyl-L-tryptophan Methyl Ester.—This compound was prepared from carbobenzoxynitro-L-arginine (5.3 g.) and L-tryptophan methyl ester hydrochloride (3.82 g.) as described above, and recrystallized from acetone–ether; yield 3.95 g. (48%); m.p. 83–85°; $[\alpha]^{29}D$ −0.5° (*c* 1.54, in methanol).

Anal. Calcd. for $C_{26}H_{31}O_7N_7$: C, 56.4; H, 5.7; N, 17.7. Found: C, 56.7; H, 5.6; N, 18.0.

Carbobenzoxynitro-L-arginyl-L-glutamic Acid, Diethyl Ester.—This compound was prepared from carbobenzoxynitro-L-arginine (1.77 g.) and L-glutamic acid diethyl ester hydrochloride (1.20 g.) as described above, and recrystallized from methanol–ether; yield 1.39 g. (52%); m.p. 108–110° (lit.[3] m.p. 111–113°); $[\alpha]^{28}D$ −21.3 (*c* 1.50, in methanol).

Anal. Calcd. for $C_{23}H_{34}O_9N_6$: C, 51.3; H, 6.4; N, 15.6. Found: C, 51.2; H, 6.2; N, 15.4.

Carbobenzoxynitro-L-arginylglycine.—Carbobenzoxynitro-L-arginylglycine methyl ester (2 g.) was suspended in 0.5 *N* sodium hydroxide (15 ml.) and the mixture was shaken at room temperature for 45 minutes. The solution was extracted with ethyl acetate, and the aqueous layer was acidified to congo red with concentrated hydrochloric acid. The resulting crystalline product was collected, and was recrystallized from aqueous ethanol; yield 1.5 g. (78%); m.p. 111–113° (lit.[3] m.p. 111–113°); $[\alpha]^{28}D$ −16.8° (*c* 1.05, in methanol).

Anal. Calcd. for $C_{16}H_{22}O_7N_6(^1/_2H_2O)$: C, 45.8; H, 5.5; N, 20.0. Found: C, 46.0; H, 5.6; N, 20.0.

Carbobenzoxynitro-L-arginyl-L-alanine.—Carbobenzoxynitro-L-arginyl-L-alanine methyl ester (1.5 g.) was suspended in 0.5 *N* sodium hydroxide (7 ml.) and the mixture was shaken for 1 hour at room temperature. The product was isolated in the usual manner and recrystallized from 50% aqueous ethanol; yield 1.1 g. (76%); m.p. 207–208°; $[\alpha]^{29}D$ −5.9° (*c* 1.80, in pyridine).

Anal. Calcd. for $C_{17}H_{24}O_7N_6$: C, 48.1; H, 5.7; N, 19.8. Found: C, 48.1; H, 5.7; N, 20.0.

Carbobenzoxynitro-L-arginyl-L-leucine.—Carbobenzoxynitro-L-arginyl-L-leucine methyl ester (1.5 g.) was suspended in 0.5 *N* sodium hydroxide (10 ml.), and methanol (10 ml.) was added. The mixture was shaken at room temperature for one hour; the product was isolated in the usual manner, and recrystallized from 50% aqueous ethanol; yield 1.3 g. (89%); m.p. 165–167° (lit.[3] m.p. 161–162°); $[\alpha]^{27}D$ −5.0° (*c* 1.73, in pyridine).

Anal. Calcd. for $C_{20}H_{30}O_7N_6$: C, 51.5; H, 6.5; N, 18.0. Found: C, 51.4; H, 6.5; N, 18.0.

Carbobenzoxynitro-L-arginyl-L-phenylalanine.—Carbobenzoxynitro-L-arginyl-L-phenylalanine methyl ester (1.03 g.) was suspended in 0.5 *N* sodium hydroxide (6.0 ml.), methanol (6.0 ml.) added, and the mixture was shaken at room temperature until a clear solution resulted. The product was isolated in the usual manner and recrystallized from 50% aqueous ethanol; yield 0.98 g. (98%); m.p. 225–226°; $[\alpha]^{29}D$ +13.1° (*c* 0.63, in pyridine).

Anal. Calcd. for $C_{25}H_{28}O_7N_6$: C, 55.2; H, 5.6; N, 16.8. Found: C, 55.3; H, 5.9; N, 16.9.

Carbobenzoxynitro-L-arginyl-L-tyrosine.—Carbobenzoxynitro-L-arginyl-L-tyrosine methyl ester (1.5 g.) was dissolved in 0.5 *N* sodium hydroxide (16 ml.) and the solution was kept at room temperature for 1 hour. The product was isolated in the usual manner and recrystallized from aqueous

acetone; yield 1.3 g. (90%); m.p. 171–173°; $[\alpha]^{28}D$ +17.9° (*c* 3.46, in pyridine).

Anal. Calcd. for $C_{28}H_{28}O_8N_6$: C, 53.5; H, 5.5; N, 16.3. Found: C, 53.4; H, 5.4; N, 16.2.

Carbobenzoxynitro-L-arginyl-L-tryptophan.—Carbobenzoxynitro-L-arginyl-L-tryptophan methyl ester (1.6 g.) was suspended in 0.5 *N* sodium hydroxide (10 ml.) and the mixture was shaken for 1 hour. The product was isolated in the usual manner and recrystallized from 50% aqueous ethanol; yield 1.36 g. (88%); m.p. 202–203°; $[\alpha]^{30}D$ +20.8° (*c* 1.45, in pyridine).

Anal. Calcd. for $C_{23}H_{29}O_7N_7$: C, 55.7; H, 5.4; N, 18.2. Found: C, 55.6; H, 5.7; N, 18.5.

Carbobenzoxynitro-L-arginyl-L-glutamic Acid.—Carbobenzoxynitro-L-arginyl-L-glutamic acid diethyl ester (1.1 g.) was suspended in 0.5 *N* sodium hydroxide (12 ml.) and the mixture was shaken for 1 hour. The product was isolated in the usual manner and recrystallized from 50% aqueous ethanol; yield 0.84 g. (87%); m.p. 224–225° (lit.[3] m.p. 211–212°); $[\alpha]^{27}D$ 0.0° (*c* 0.95, in pyridine).

Anal. Calcd. for $C_{19}H_{26}O_9N_6$: C, 47.3; H, 5.4; N, 17.4. Found: C, 47.6; H, 5.5; N, 17.5.

L-Arginylglycine Acetate, Acetic Acid Solvate.—Carbobenzoxynitro-L-arginylglycine (1 g.) was dissolved in methanol containing 10% of glacial acetic acid (25 ml.). Palladium catalyst[19] was added and the mixture was shaken in a stream of hydrogen for 12 hours. The catalyst was removed by filtration and the solvent was evaporated *in vacuo*. The ensuing sirup was layered with acetone and kept at room temperature until crystallization was complete. The material was recrystallized from methanol-acetic acid; yield 0.74 g. (88%); m.p. 167–169°; $[\alpha]^{28}D$ +38.9° (*c* 5.75, in water).

Anal. Calcd. for $C_{10}H_{21}O_5N_5(CH_3COOH)$: C, 41.0; H, 7.2; N, 19.9; acetyl, 34.2. Found: C, 41.4; H, 7.0; N, 20.1; acetyl, 32.2.

L-Arginyl-L-alanine Acetate.—Carbobenzoxynitro-L-arginyl-L-alanine (1.0 g.) was hydrogenated in methanol containing 10% of acetic acid (25 ml.) in the manner described above. The sirup resulting from the evaporation of the solvents crystallized on standing. The material was recrystallized twice from aqueous ethanol; yield 0.6 g. (83%); m.p. 173–174°; $[\alpha]^{32}D$ +9.7° (*c* 2.38, in water).

Anal. Calcd. for $C_{11}H_{23}O_5N_5$: C, 43.3; H, 7.6; N, 22.9. Found: C, 42.7; H, 7.1; N, 23.1.

L-Arginyl-L-leucine Acetate.—Carbobenzoxynitro-L-arginyl-L-leucine (1.3 g.) was hydrogenated in methanol containing 10% of acetic acid (25 ml.) in the manner described above. The dipeptide crystallized during the hydrogenation and was redissolved by the addition of glacial acetic acid (15 ml.). The crystals resulting on evaporation of the solvents were recrystallized from methanol–acetic acid; yield 0.85 g. (89%); m.p. 205–206° (lit.[7] m.p. 207–208°); $[\alpha]^{28}D$ +9.6° (*c* 1.35, in water).

Anal. Calcd. for $C_{14}H_{29}O_5N_5$: C, 48.4; H, 8.4; N, 20.2. Found: C, 48.5; H, 8.2; N, 20.0.

L-Arginyl-L-phenylalanine Acetate.—Carbobenzoxynitro-L-arginyl-L-phenylalanine (0.5 g.) was hydrogenated in methanol containing 10% of acetic acid in the manner described. The dipeptide acetate precipitated during the course of the hydrogenation and was redissolved by the addition of small quantities of glacial acetic acid. The material crystallized on removal of the solvent, and was recrystallized from aqueous ethanol; yield 0.24 g. (63%); m.p. 172–173°; $[\alpha]^{28}D$ +29.5° (*c* 1.81, in water).

Anal. Calcd. for $C_{17}H_{27}O_5N_5$: C, 53.5; H, 7.1; N, 18.4. Found: C, 53.6; H, 7.4; N, 18.6.

L-Arginyl-L-tyrosine Acetate.—Carbobenzoxynitro-L-arginyl-L-tyrosine (0.8 g.) was hydrogenated in methanol–acetic acid as described. The sirup obtained on evaporation of the solvents crystallized on standing and the peptide was recrystallized from aqueous ethanol; yield 0.5 g. (81%); m.p. 157–158°; $[\alpha]^{32}D$ +33.3° (*c* 1.53, in water).

Anal. Calcd. for $C_{17}H_{27}O_6N_5$: C, 51.4; H, 6.9; N, 17.6. Found: C, 51.3; H, 7.1; N, 18.0.

L-Arginyl-L-tryptophan Acetate.—Carbobenzoxynitro-L-arginyl-L-tryptophan (0.75 g.) was hydrogenated in methanol–acetic acid for 12 hours. The sirup obtained on evaporation of the solvents failed to crystallize. The dipeptide was obtained in the form of a hygroscopic powder by repeated precipitation from methanol with ether; yield 0.30 g. (52%); $[\alpha]^{28}D$ +5.1° (*c* 4.94, in water).

Anal. Calcd. for $C_{19}H_{28}O_5N_6$: N, 20.0. Found: N, 19.1.

L-Arginyl-L-glutamic Acid.—Carbobenzoxynitro-L-arginyl-L-glutamic acid (0.8 g.) was hydrogenated in methanol–acetic acid for 12 hours. The crystalline residue which remained upon evaporation of the solution, was recrystallized from aqueous ethanol; yield 0.48 g. (96%); m.p. 251–252° dec. (lit.[7] m.p. 205–210°); $[\alpha]^{25}D$ +24.8° (*c* 1.90, in water).

Anal. Calcd. for $C_{11}H_{21}O_5N_5$: C, 43.6; H, 7.0; N, 23.1. Found: C, 43.8; H, 7.0; N, 23.2.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF PENNSYLVANIA]

Metabolite Analogs. V. Preparation of Some Substituted Pyrazines and Imidazo[b]pyrazines

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A number of pyrazines and imidazo[b]pyrazines have been prepared as potential metabolic antagonists of essential pyrimidines and purines.

A number of imidazo[b]pyrazines have been prepared from the corresponding 2,3-diaminopyrazines. The preparation of the imidazo[*b*]pyrazine ring system was first reported in 1952.[1] In general the first step involves the condensation of a suitable 1,2-dicarbonyl compound with aminomalonamide to form the corresponding 2-hydroxy-3-carboxamidopyrazine. Although the conditions for these reactions had been reported previously,[2] it has been found in the course of the present study that reproducible results could not be obtained. Consequently a careful examination of the reaction conditions was made.

It has been found that the procedure of Jones for the condensation of diacetyl with aminomalonamide gave the best yields of 2-hydroxy-3-carboxamido-5,6-dimethylpyrazine (75%) when the sodium hydroxide was omitted and the reaction was carried out at 85°. The condensation of glyoxal with aminomalonamide was also modified considerably. The use of glyoxal bisulfite in place of glyoxal has been reported[2] but neither experimental conditions nor yields were given. In our laboratory the best results were obtained when an aqueous

(1) E. Schipper and A. R. Day, THIS JOURNAL, **74**, 350 (1952).
(2) R. G. Jones, *ibid.*, **71**, 78 (1949).