the influence of other factors which are not so pronounced in the sulfonamide series. Apparently the SO_2 and PO groups cannot compete equally for the negative charge left by the dissociation of the proton of the amide. In the bacteriostatically ineffective alkyl hydrogen *p*-aminophenylphosphonates the PO_2^- carries a formal negative charge as does the CO_2^- in the *p*-aminobenzoate ion, and the pK_a (3.8 to 4.0) of these esters approaches that of PABA (4.68). The effects of solubility, steric influences, etc., may outweigh those of acid dissociation; alkyl N^1-heterocyclically substituted P-(*p*-aminophenyl)-phosphonamidates are uniformly more soluble in base than the corresponding sulfonamide drugs. The valence requirements of the phosphonamidates place an alkyl group on one of the oxygens whereas this is absent in the sulfonamides. While it is conceivable that the extra group could interfere with the fit at an essential receptor site, the bacteriostatic activity of *p*-aminophenylphosphonamidates containing two relatively bulky substituents in the amidate group[7] cannot readily be reconciled with this view.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE]

Synthesis of Peptides of Arginine Related to Arginine-vasopressin[1]

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The preparation of L-prolyl-L-arginylglycinamide dihydrobromide and S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycin amide dihydrobromide, peptides containing sequences that are found in arginine-vasopressin, is described.

As a result of degradation studies on purified preparations of arginine-vasopressin, the principal pressor and antidiuretic hormone of the beef posterior pituitary gland, the sequence of amino acids in arginine-vasopressin was obtained[3] and a structure was postulated.[4,5] For the purpose of investigating synthetic routes to arginine-vasopressin, the peptides L-prolyl-L-arginylglycinamide dihydrobromide (I) and S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide dihydrobromide (II) were desired. The preparation of these peptides and their intermediates are described in this paper.

N^{α}-*p*-Nitrobenzyloxycarbonyl-L-arginylglycinamide hydrochloride (III) was prepared by the method of Gish and Carpenter[6] and the peptide derivative was isolated as the crystalline picrate IV. The picrate IV was cleaved with acetic acid saturated with hydrogen bromide to give L-arginylglycinamide dihydrobromide (V) as an amorphous solid. The dipeptide amide V was characterized as its crystalline dipicrate VI. The dihydrobromide V was converted to the monohydrobromide and the monohydrobromide was condensed with carbobenzoxy-L-proline by the tetraethyl pyrophosphite method[7] to give carbobenzoxy-L-prolyl-L-arginylglycinamide hydrobromide (VII). The tripeptide amide derivative VII was purified by countercurrent distribution and then treated with acetic acid saturated with hydrogen bromide to give L-prolyl-L-arginylglycinamide dihydrobromide (I). The amorphous product was converted to the crystalline diflavianate for characterization.

S-Benzyl-N-*p*-nitrobenzyloxycarbonyl-L-cysteine was converted to its acid chloride and then condensed with proline benzyl ester to give S-benzyl-N-*p*-nitrobenzyloxycarbonyl-L-cysteinyl-L-proline benzyl ester (VIII) and with proline methyl ester to give the corresponding methyl ester IX. The esters VIII and IX were obtained as oils, but could be converted to the crystalline hydrazide in high yield. Saponification of these esters yielded S-benzyl-N-*p*-nitrobenzyloxycarbonyl-L-cysteinyl-L-proline (X). Yields of 50–85% were obtained on various runs of the saponification of the benzyl ester of the *p*-nitrobenzyloxycarbonyl derivative of this dipeptide, whereas a yield of 52% was obtained in the saponification of the methyl ester. Saponification of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-proline methyl ester (XI), prepared in a manner similar to that used for the preparation of IX, gave consistent yields of about 85%. No difficulties had been encountered previously during the saponification of *p*-nitrobenzyloxycarbonyl derivatives of peptides.[6,8] The dipeptide derivative X was condensed with L-arginylglycinamide monohydrobromide by the tetraethyl pyrophosphite method to give the S-benzyl-N-*p*-nitrobenzyloxycarbonyl-L-cysteinyl-L-prolyl-L-arginylglycinamide hydrobromide (XII). The crude product was purified by countercurrent distribution and the tetrapeptide amide derivative was characterized as its crystalline picrate XIII. Treatment of the purified tetrapeptide amide derivative XII with acetic acid saturated with hydrogen bromide gave S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide dihydrobromide (II) as an amorphous solid. Countercurrent distribution of this material in the system 2-butanol–0.1% acetic acid revealed a single peak and the tetrapeptide amide dihydrobromide

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was generally used for further work directly following the cleavage in acetic acid–hydrogen bromide without further purification except for precipitation from methanol with ether in order to remove acetic acid and excess hydrogen bromide.

Experimental[9,10]

Nα-*p*-Nitrobenzyloxycarbonyl-L-arginylglycinamide Picrate.—Nα-*p*-Nitrobenzyloxycarbonyl-L-arginyl chloride hydrochloride was prepared as previously described[6] from 3.53 g. (0.01 mole) of Nα-*p*-nitrobenzyloxycarbonyl-L-arginine. To the acid chloride was added a cold solution of 1.11 g. (0.01 mole) of glycinamide hydrochloride[11] and 3.0 ml. (0.0215 mole) of triethylamine in 15 ml. of dimethylformamide. The mixture was stirred for one hour in the cold. The precipitate of triethylamine hydrochloride was filtered off, washed with a few ml. of cold dimethylformamide and the washings were added to the filtrate. The product was precipitated with chloroform and the sticky solid was collected on a Buchner funnel and washed with chloroform. The material was dissolved in 25 ml. of warm water and 2.6 g. (1.1 moles) of picric acid dissolved in 25 ml. of warm ethanol was added. The crystalline picrate separated as the solution cooled to room temperature. The mixture was not cooled below room temperature since further cooling caused the separation of an oily product. The picrate was collected, washed with 50% ethanol and dried over P_2O_5 *in vacuo*. The yield was usually 60–65%, m.p. 148–158°. For further work the material was twice recrystallized from 50% ethanol to give an over-all yield of 50%, m.p. 165–168°. For analysis a sample was further recrystallized from 50% ethanol, m.p. 165–168°, and dried over P_2O_5 *in vacuo* at 75°; $[\alpha]^{22}D$ −3.8° (*c* 1, acetone–water (4:1)).

Anal. Calcd. for $C_{16}H_{23}O_8N_7 \cdot C_6H_3O_7N_3$: C, 41.38; H, 4.10; N, 21.94. Found: C, 41.40; H, 4.17; N, 21.82.

Sometimes during crystallization of this material a crystalline product of m.p. 110–120° was obtained. This was presumably a hydrate and could be converted to the material of m.p. 165–168° by seeding a warm solution with crystals of m.p. 165–168°.

L-Arginylglycinamide Dihydrobromide.—Nα-*p*-Nitrobenzyloxycarbonyl-L-arginylglycinamide picrate (10 g.) was suspended in 75 ml. of acetic acid which had been saturated with hydrogen bromide in the cold. The mixture was warmed to 50–55° for 1.25 hours. Ether was then added to the cooled mixture to precipitate the dihydrobromide as a white amorphous solid. The material was collected and washed with ether. To free the product from excess hydrogen bromide and acetic acid it was precipitated several times from methanol with ether. The yield was quantitative. The product was easily soluble in water, alcohol and dimethylformamide and insoluble in the usual organic solvents. The material was hygroscopic. Titration of a sample with standard alkali, using the Model G Beckman *p*H meter, gave the expected titration curve, showing the absence of any free carboxyl group and the presence of an α-amino group with a pK' of 7.3. The electrodes used did not permit titration of the guanido group. For titration of the α-amino group, 0.0620 g. requires 1.58 ml. of 0.0998 *N* NaOH. Found: 1.56 ml.

In order to characterize this hygroscopic compound it was converted to its crystalline dipicrate. The dihydrobromide (0.94 g., 2.4 mmoles) was dissolved in 5 ml. of warm water and 1.32 g. (20% excess) of picric acid dissolved in 10 ml. of hot ethanol was added. The crystalline dipicrate separated from the cooled mixture; yield 1.21 g., m.p. 206–207°. The material was recrystallized from water–ethanol (8:1), m.p. 209–210°; $[\alpha]^{24}D$ +15.9° (*c* 1, acetone–water (1:1)). A sample was dried *in vacuo* over P_2O_5 at 56° for analysis.

Anal. Calcd. for $C_8H_{18}O_2N_6 \cdot 2C_6H_3O_7N_3$: C, 34.89; H, 3.51; N. 24.42. Found: C, 34.95; H, 3.62; N, 24.31.

S-Benzyl-N-*p*-nitrobenzyloxycarbonyl-L-cysteine.—This was prepared from 21.13 g. (0.1 mole) of S-benzyl-L-cysteine[12] according to procedure A previously described.[13] The yield was 34.75 g. (89%), m.p. 127–133°. One recrystallization from 1600 ml. of 50% acetic acid gave 31.5 g., m.p. 134–135°. Further recrystallization did not raise the melting point. For analysis a sample was dried *in vacuo* over P_2O_5 at 75°; $[\alpha]^{22}D$ −47.0° (*c* 1, 95% ethanol).

Anal. Calcd. for $C_{18}H_{18}O_6N_2S$: C, 55.37; H, 4.65; N, 7.18; S, 8.21. Found: C, 55.42; H, 4.88; N, 7.19; S, 8.08.

S-Benzyl-N-*p*-nitrobenzyloxycarbonyl-L-cysteinyl-L-proline Benzyl Ester.—S-Benzyl-N-*p*-nitrobenzyloxycarbonyl-L-cysteine (7.8 g., 0.02 mole) was suspended in 100 ml. of dry absolute ether and the mixture was cooled in an ice-salt bath. To the mixture was added 4.6 g. (10% excess) of phosphorus pentachloride and the mixture was stirred for 1.25 hours. The solution was filtered free of excess phosphorus pentachloride and the ether was evaporated at reduced pressure, with a bath temperature less than 20°. The acid chloride crystallized from the concentrated solution. The product was washed with cold hexane by decantation and then collected on a Buchner funnel and washed on the funnel with cold hexane. The acid chloride was added to 100 ml. of cold ether (almost all dissolved) and a cold solution of proline benzyl ester, prepared from 5.8 g. (0.024 mole) of proline benzyl ester hydrochloride,[14] and 2.8 ml. of triethylamine in ether was added. (The free ester was prepared by dissolving the hydrochloride in 30 ml. of chloroform, adding 3.4 ml. of triethylamine and evaporating the solution to a small volume. Dry ether (75 ml.) was added, triethylamine hydrochloride was filtered off, washed with ether and the washings were added to the filtrate. The ethereal solution of the free ester was kept cold until used and triethylamine equivalent to the acid chloride was added just before use.) The reaction mixture was stirred in the cold for about 15 minutes and then at room temperature for 1.5 hours. The triethylamine hydrochloride was removed by filtration and washed with ethyl acetate, the washings being added to the filtrate. The solution was washed successively with *N* hydrochloric acid, water, *N* sodium bicarbonate and saturated sodium chloride and dried over magnesium sulfate. The solvent was removed *in vacuo* and the oil was washed with hexane and then dried *in vacuo* over concentrated sulfuric acid and paraffin. The yield varied from 89–98%. This compound could not be crystallized.

S-Benzyl-N-*p*-nitrobenzyloxycarbonyl-L-cysteinyl-L-proline.—The ester was saponified by stirring a dioxane solution with a 5% excess of *N* sodium hydroxide for 2–3 hours. The mixture was acidified and the product was extracted into ethyl acetate–ether (1:1). The organic solution was washed with *N* hydrochloric acid and water and the product was then extracted into *N* sodium bicarbonate. The bicarbonate solution was washed with ethyl acetate–ether and acidified and extracted with ethyl acetate. The organic solution was washed with *N* hydrochloric acid, water and saturated sodium chloride and dried over magnesium sulfate. The solvent was removed *in vacuo* and the oil was dried over concentrated sulfuric acid *in vacuo*. The yield generally varied from 50–85% and gave a product with a neutral equivalent within a few per cent. of the calculated value.

S-Benzyl-N-*p*-nitrobenzyloxycarbonyl-L-cysteinyl-L-proline Methyl Ester.—This compound was prepared in the same manner as that described for the benzyl ester. The yield varied from 73 to 80%.

Saponification of the methyl ester, carried out as described for the benzyl ester, gave, in a yield of 52%, a product with a neutral equivalent within 3% of the calculated value.

S-Benzyl-N-*p*-nitrobenzyloxycarbonyl-L-cysteinyl-L-proline Hydrazide.—The conversion of both the methyl and benzyl ester to the hydrazide was slow and required more drastic conditions than usually employed. The methyl ester (5.54 g.) was refluxed for one hour with 15 ml. of ethanol and 1.5 ml. of hydrazine hydrate (99–100%). The solution was then stored at about 37° for three weeks. The solution was filtered and crystallization was then induced. The hydrazide was filtered from the cooled mixture and

(9) Corrected capillary melting points are reported.

(10) The analyses were performed by Mr. Joseph Albert of this Laboratory.

(11) Prepared essentially as described by Yang and Rising (THIS JOURNAL, **53**, 3183 (1931)) using glycine ethyl ester hydrochloride as the starting material; yield 40%, m.p. 195–200° with decomposition.

(12) J. L. Wood and V. du Vigneaud, *J. Biol. Chem.*, **130**, 109 (1939).

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

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washed with cold ethanol. The yield was 5.06 g. (91%), m.p. 149–151°. Recrystallization was from ethanol, m.p. 150–151°; $[\alpha]^{24}D$ −51.6° (*c* 1, dimethylformamide). A sample was dried *in vacuo* over P_2O_5 at 75° for analysis.

Anal. Calcd. for $C_{23}H_{27}O_6N_5S$: C, 55.08; H, 5.43; N, 13.96; S, 6.39. Found: C, 55.16; H, 5.57; N, 13.64; S, 6.30.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-proline Methyl Ester.—This preparation was carried out in the same manner as that used for the preparation of the *p*-nitrobenzyloxycarbonyl derivative. The yield was 69%.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-proline.—The saponification was carried out as described above for the *p*-nitrobenzyloxycarbonyl derivative except that 1.5 hours was required for complete saponification. The yield was 85%. Neutral equivalent: Calcd. for $C_{23}H_{26}O_5N_2S$: 442.5. Found: 450.

S-Benzyl-N-*p*-nitrobenzyloxycarbonyl-L-cysteinyl-L-prolyl-L-arginylglycinamide Hydrobromide.—L-Arginylglycinamide dihydrobromide (1.18 g., 0.003 mole) was dissolved in 5 ml. of dimethylformamide, 0.47 ml. of triethylamine was added, the mixture was stirred for a few minutes and the monohydrobromide was then precipitated by addition of chloroform. The gummy material was washed with chloroform, then ether, and was dried over P_2O_5 *in vacuo*. The material was dissolved in 5 ml. of diethyl phosphite, 1.46 g. (0.003 mole) of S-benzyl-N-*p*-nitrobenzyloxycarbonyl-L-cysteinyl-L-proline and 1.55 g. (100% excess) of tetraethyl pyrophosphite were added and the mixture was heated at 100° with stirring for 30 minutes. The mixture was cooled and the product, along with unreacted L-arginylglycinamide, was precipitated by the addition of ethyl acetate. The amorphous solid was collected on a Buchner funnel, washed with ethyl acetate and ether and dried *in vacuo* over P_2O_5 and sodium hydroxide pellets. The crude material weighed 1.78 g. The material was purified by countercurrent distribution in the system 2-butanol–0.1% acetic acid. One hundred and twenty transfers were sufficient to obtain complete separation of the tetrapeptide (K = 1.1) from unreacted dipeptide (K = 0.2). The distribution curves were obtained by use of the Sakaguchi reaction.[15] The solvent from the tubes containing the tetrapeptide was concentrated on the flash evaporator[16] and lyophilized to yield a white amorphous solid, weight 1.38 g. (59%). The material was easily soluble in water, alcohol and dimethylformamide and insoluble in the usual organic solvents. For the purpose of characterization the amorphous tetrapeptide was converted to its crystalline picrate. The purified tetrapeptide (175 mg.) was dissolved in 5 ml. of warm water and 55 mg. of picric acid in 5 ml. of warm ethanol was added. The picrate crystallized from the cooled solution; yield 184 mg. (88%), m.p. 155–160°. Recrystallization from 50% ethanol gave 153 mg., m.p. 181.5–184.5°; $[\alpha]^{22}D$ −47.7° (*c* 1, acetone–water (4:1)). A sample was dried over P_2O_5 *in vacuo* at 75° for analysis.

Anal. Calcd. for $C_{31}H_{41}O_8N_9S \cdot C_6H_3O_7N_3$: C, 47.84; H, 4.77; N, 18.10. Found: C, 47.78; H, 4.95; N, 17.73.

S-Benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide Dihydrobromide.—The cleavage of the *p*-nitrobenzyloxycarbonyl derivative was carried out in the same manner as for N^{α}-*p*-nitrobenzyloxycarbonyl-L-arginylglycinamide picrate except that warming was at 50° for 45 minutes. The product was freed from excess hydrogen bromide and acetic acid as before. The yield was nearly quantitative. The white amorphous solid was hygroscopic and was easily soluble in water, alcohol and dimethylformamide and insoluble in the usual organic solvents. The tetrapeptide amide dihydrobromide was titrated using the Model G Beckman *p*H meter. No free carboxyl group was detectable. The pK' of the α-amino group was 6.7. For titration of the α-amino group, 0.035 g. requires 0.52 ml. of 0.100 *N* NaOH; found, 0.52 ml. When subjected to countercurrent distribution up to 200 transfers in the system 2-butanol–0.1% acetic acid this material gave a single peak. The distribution constant was about 0.2 but varied with concentration. As a result the distribution curve (the Sakaguchi reaction was used as before) was sharp on the leading edge but skewed to the rear, as expected.[17]

Carbobenzoxy-L-prolyl-L-arginylglycinamide Hydrobromide.—Carbobenzoxy-L-proline[18] (0.5 g., 0.002 mole, m.p. 74.5–76.5°), L-arginylglycinamide monohydrobromide, prepared from 0.78 g. (0.002 mole) of the dihydrobromide as described above, 3 ml. of diethyl phosphite and 1.0 g. (100% excess) of tetraethyl pyrophosphite were heated at 100° for 30 minutes with stirring. Ether was added to the cooled mixture to precipitate the product along with unreacted arginylglycinamide. The amorphous solid was collected, washed with ether and dried over P_2O_5 and sodium hydroxide pellets. The crude material was purified by countercurrent distribution in the system 2-butanol–0.1% acetic acid. One hundred and fifty transfers were sufficient for complete separation. The distribution constant of the tripeptide was 0.6. The Sakaguchi reaction was used for preparation of the distribution curve. From concentration and lyophilization of the solvent containing the tripeptide was obtained 0.80 g. (74%) of a white amorphous solid. Yields from other preparations varied from 65–75%.

L-Prolyl-L-arginylglycinamide Dihydrobromide.—The carbobenzoxy group was removed by warming the carbobenzoxy derivative in acetic acid saturated with hydrogen bromide for one hour at 40–45°. The white amorphous product was freed from excess hydrogen bromide and acetic acid in the manner described for L-arginylglycinamide dihydrobromide. The yield of the hygroscopic dihydrobromide varied from 80–84%. It was easily soluble in water, alcohol and dimethylformamide and insoluble in the usual organic solvents. The pK' of the imino group was 8.5. To characterize this compound it was converted to its crystalline diflavianate. The dihydrobromide (0.36 g., 0.74 mmole) was dissolved in 2 ml. of warm water and 0.51 g. of flavianic acid in 1.5 ml. of warm water was added along with 0.5 ml. of ethanol. The diflavianate crystallized from the cooled mixture; yield 0.58 g. (80%), m.p. 173° (opaque melt). After recrystallization from water–ethanol (3:1) the diflavianate hydrate was allowed to dry in air for analysis, m.p. 180° (forms opaque melt), $[\alpha]^{24}D$ −18.2° (*c* 1, acetone–water (1:1)).

Anal. Calcd. for $C_{13}H_{25}O_3N_7 \cdot 2C_{10}H_6O_8N_2S \cdot 1^1/_2H_2O$: C, 40.32; H, 4.10; N, 15.68; H_2O, 2.75. Found: C, 40.63; H, 4.16; N, 15.38; H_2O, 2.43.

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