Synthesis of Tritium Labelled [8-Arginine]-vasopressin[1]

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Ring tritiated phenylalanine has been incorporated into [8-arginine]-vasopressin synthesized by the solid phase method. The peptide produced has levels of biological activities in agreement with those of the highly purified natural hormone.

La phénylalanine tritiée sur le cycle a été incorporée dans l'(Arg-8) vasopressine, synthétisée selon la méthode de la phase solide. Le peptide obtenu possède des degrés d'activité biologique comparables à ceux de l'hormone naturelle hautement purifiée. [Traduit par le journal]

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[8-Arginine]-vasopressin, the antidiuretic hormone of man and most mammals, acts on the kidney. This organ in rats has been shown to bind [8-arginine]-vasopressin (1) and it was suggested that the mechanism of the hormone's action involved an exchange between the peptide disulfide and a membrane thiol group. It is now apparent that this is not an exclusive mechanism of action, since synthetic analogs of [8-arginine]-vasopressin which lack a disulfide group have been shown to retain biological activity (2).

There is considerable evidence that polypeptide hormones bind to sites on the outer surface of their target cell membranes (3), and in order to study the binding properties of [8-arginine]-vasopressin to kidney cell membranes we have found it convenient to synthesize radioactive [8-arginine]-vasopressin by incorporating ring-tritiated phenylalanine in position 3 of the peptide chain (Fig. 1). Tritium, rather than radioactive iodine was chosen as the radioisotope, since it was thought that iodination of [8-arginine]-vasopressin could result in a derivative with significantly altered binding properties. This opinion has recently received support by the demonstration that radio-iodinated oxytocin shows no specific binding to kidney or uterine tissue (4).

The solid phase method of synthesis of [8-arginine]-vasopressin described by Meienhofer and coworkers (5) was used for the synthesis of the required protected nonapeptide precursor to the hormone (Fig. 2). Some modifications were

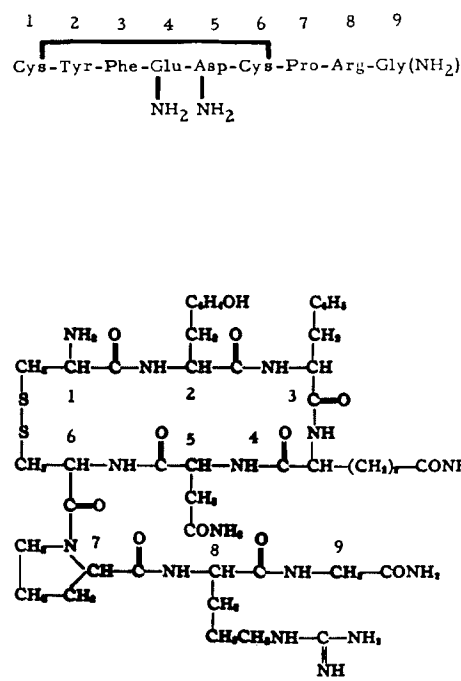

FIG. 1. Structure of [8-arginine]-vasopressin. Numbers indicate amino acid residues.

made in the preparation of the protected amino acids used in the synthesis. The protected nonapeptide derivative was purified by exclusion chromatography on Sephadex LH-20 in *N,N*-dimethylformamide. The protecting groups were removed from the peptide derivative by sodium in liquid ammonia solution and the resultant dithiol compound was oxidatively cyclized using hydrogen peroxide. The [8-arginine]-vasopressin so formed was isolated by sequential exclusion chromatography on Sephadex G-15 in 50% acetic acid, then on Sephadex G-25 in 0.2 *N*

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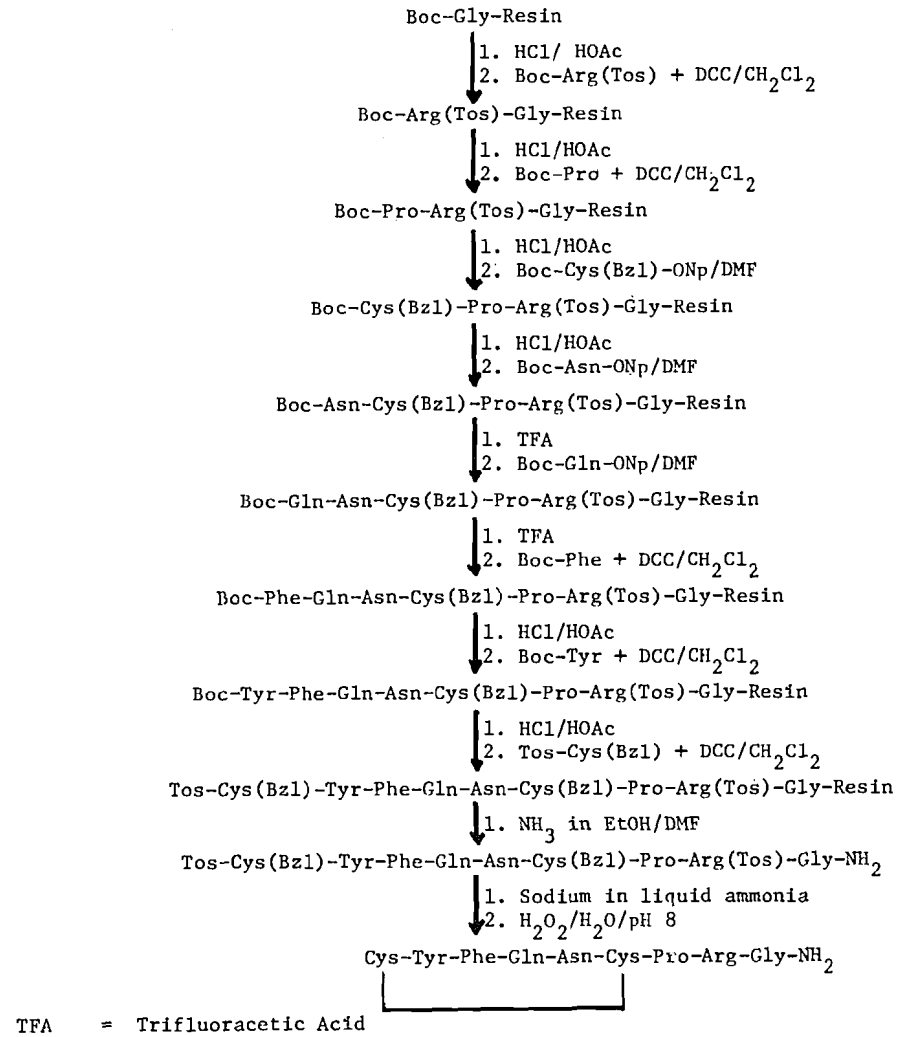

TFA = Trifluoracetic Acid

Boc = tertiary-butyloxycarbonyl protecting group

Resin = Merrifield Resin for solid phase peptide synthesis

HOAc = Acetic Acid

DCC = N,N¹-dicyclohexylcarbodiimide

DMF = N,N-dimethylformamide

EtOH = Ethyl Alcohol

ONp = para-Nitrophenyl ester

FIG. 2. Diagram showing the steps in the solid phase synthesis of [8-arginine]-vasopressin.

acetic acid. This yielded 11% (based on the starting glycine content) of the peptide, which was found to have 470 ± 50 units per mg of rat antidiuretic activity (6), 24 ± 6 units per mg of "milk-ejecting-like" activity measured on an isolated strip of lactating mouse mammary gland (7) and 2.0×10^6 c.p.m. per mg in dioxane-based scintillation fluid. Amino acid analysis was in agreement with the structure of [8-arginine]-vasopressin.

Before the radioactive amino acid was incorporated, a portion of the protected hexapeptidyl-resin was used to complete the synthesis of cold [8-arginine]-vasopressin. This allowed more accurate estimation of the amount of radioactive amino acid needed, and the yield of radioactive hormone which could be expected. Position 3 was chosen as the one to have the radioactive residue since it was the last position to be filled in a protected heptapeptide intermediate to several projected radioactive analogs of [8-arginine]-vasopressin with modifications in the amino acid residues at positions 1 and 2.

The radioactive [8-arginine]-vasopressin prepared is currently being used in studies on its binding to isolated kidney cell membranes.

Experimental

Materials and Methods

The physical properties of the protected amino acids prepared correspond with those reported by Bayer *et al.* (8). All the protected amino acids were homogenous to t.l.c. (Silica Gel G) in the solvent systems, acetone – acetic acid, 98:2, and chloroform–methanol – acetic acid, 85:10:5. The Merrifield resin was obtained from Schwartz/Mann (Orangeburg, New York), and prepared with a substitution level of 1.0 mmol *tert*-butyloxycarbonyl-glycine (Boc–glycine) per gram of resin according to the procedure of Merrifield (9). Ring tritiated phenylalanine was obtained from Schwartz–Mann (Picker Nuclear Canada, Downsview, Ontario).

Exclusion chromatography was carried out in 1.5 × 90 cm columns (Pharmacia Canada Ltd.), monitored by u.v. absorption at 280 nm using a Beckman DB-GT recording spectrophotometer with a Beckman flow cell. A flow rate of 5 ml/h was used and 1 ml fractions were collected. Peptide materials were collected by pooling fractions corresponding to absorption peaks, concentrating *in vacuo*, and lyophilizing. Scintillation counting was carried out in a dioxane-based cocktail (1000 ml dioxane, 100 g naphthalene, 5 g 2,5-diphenyloxazole) in a Beckman LS-233 scintillation counter. All solvents and reagents were the highest purity commercially available and were used without further purification. Amino acid analyses were carried out using a Beckman 120C amino acid analyzer.

All couplings and washings in the synthesis of the peptide were carried out according to the procedure of Meienhofer *et al.* (5).

Assays for antidiuretic activity were performed on anesthetized, hydrated Sprague–Dawley male rats according to the procedure of Sturmer (6). Milk-ejecting like activity was measured using isolated strips of lactating mouse mammary gland according to the procedure of Fielitz *et al.* (7).

tert-Butyloxycarbonyl Amino Acids

The N^{α}-*tert*-butyloxycarbonyl (Boc) derivatives of glycine, proline, glutamine, and phenylalanine were prepared according to the procedure of Schwyzer *et al.* (10) with the modification that reaction times were extended from 20 to 48 h, the first 24 h at 40–50 °C, the remaining 24 h at 25 °C.

The N^{α}-Boc derivatives of cysteine, asparagine, and tyrosine were prepared according to the same procedure with the modifications that a dioxane–water ratio of 1:1 rather than 2:1 was employed and reaction times were extended to a minimum of 48 h at 40–50 °C.

N^{α}-Boc-arginine

Arginine (3.5 g, 20 mmol) was dissolved in dioxane–water, 1:1, (100 ml) and magnesium oxide (1.6 g, 40 mmol) was added. Boc-azide was then added dropwise (4.3 g, 30 mmol) with stirring. The resultant mixture was stirred 24 h at 45 °C. The suspension was filtered, washed twice with water (50 ml), and extracted three times with diethyl ether (50 ml portions). The aqueous phase was cooled to 0° and the p*H* was adjusted to 2 with 2 *N* hydrochloric acid. The solution was warmed to 25° and saturated with sodium chloride. This produced a fine white precipitate which was recovered by decantation (from excess sodium chloride) and filtration. After drying for 24 h over phosphorous pentoxide *in vacuo*, the product (6.16 g, 99% yield based on Boc-arginine·HCl) melted at 113–115 °C, and was homogenous to t.l.c. on silica gel G (R_f 0.70 using butanol – acetic acid – water, 4:1:5). This material was used without further purification.

N^{α}-Boc-N^{G}-tosyl-arginine

N^{α}-Boc-arginine·HCl (3.12 g, 10 mmol) was dissolved in acetone (50 ml) and water (15 ml), the solution was cooled to 0 °C, the p*H* adjusted to 12.5 with 4 *N* sodium hydroxide, and the suspension was stirred until homogenous (1 h). Tosyl chloride (4.75 g, 25 mmol) in acetone (10 ml) was added dropwise with stirring and sodium hydroxide (4 *N*) was added simultaneously to maintain the p*H* in the range 12.0–12.5. Once the addition was complete the solution was stirred at 0 °C for an additional 3 h, maintaining p*H* 12.5. The p*H* was then adjusted to 7.0 with 1 *N* hydrochloric acid at 0 °C, the acetone was removed *in vacuo*, the solution volume was doubled by the addition of water and the solution was extracted three times with diethyl ether (25 ml portions). The aqueous layer was cooled to 0 °C and the p*H* adjusted to 3.0 with 6 *N* hydrochloric acid. The resultant emulsion was saturated with sodium chloride and extracted 3 times with ethyl acetate (50 ml portions). The organic phase was washed three times with 0.1 *N* hydrochloric acid (15 ml portions), then with water (25 ml portions) until neutral, dried (Na_2SO_4), filtered, and concentrated *in vacuo* to yield 2.8 g (60%) of a white glassy material exhibiting a softening point in the range 95–100 °C. T.l.c. (Silica Gel G, acetone – acetic acid, 98:2) showed the

majority of the material to have an R_f of 0.42 with minor components at R_f 0.1 and 0.8. These latter components were removable by extraction with diethyl ether and then 0.1 *N* hydrochloric acid. The optical rotation of the purified material, $[\alpha]_D^{25}$ −3.3° (c = 4, DMF) corresponds to that reported by Ramachandran and Li (11).

Tritiated N^{α}-tert-Butyloxycarbonyl-phenylalanine

A mixture of ring tritiated L-phenylalanine (17 000 mCi/mmol, 2 mCi total) and unlabelled phenylalanine (0.42 mmol) was protected according to the method of Schwyzer *et al.* (10). The resultant ^{3}H-Boc-phenylalanine was found to be greater than 99% pure by radiochromatography on silica gel G using acetone – acetic acid, 98:2.

p-Nitrophenyl Esters

The *p*-nitrophenyl ester of N^{α}-Boc-*S*-Bzl-cysteine was prepared according to the procedure of Beyerman *et al.* (12). N^{α}-Boc-asparagine *p*-nitrophenyl ester was prepared according to the procedure of Schröder and Klieger (13). N^{α}-Boc-glutamine *p*-nitrophenyl ester was prepared according to the procedure of Bodanszky and du Vigneaud (14).

N^{α}-Tosyl-S-Bzl-cysteine

N^{α}-Tosyl-*S*-Bzl-cysteine was prepared from *S*-Bzl-cysteine (15) according to the procedure of du Vigneaud *et al.* (16).

N^{α}-tert-Butyloxycarbonyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N^{G}-tosyl-L-argininyl-glycyl-resin

This Boc-hexapeptide-resin was prepared according to the stepwise procedure of Meienhofer *et al.* (5) from 1.25 g Boc-glycyl-resin (1.25 mmol Boc-glycine) which yielded 2.12 g (88%).

N^{α}-Tosyl-S-benzyl-L-cysteinyl-L-tyrosyl-[^{3}H]-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N^{G}-tosyl-L-arginylglycyl-resin

This material was prepared from the Boc-hexapeptide resin (0.37 g, 0.21 mmol based on starting Boc-glycine content) by the stepwise procedure according to Meienhofer *et al.* (5), with the exception that ^{3}H-Boc-phenylalanine was incorporated using only a two-fold excess (0.42 mmol). The protected nonapeptide-resin was recovered by filtration after thorough washing with ethanol and ether, which yielded 0.43 g (81%) material.

[8-Arginine]-vasopressin

The protected nonapeptide, *S*-benzyl-N^{α}-tosyl-L-cysteinyl-L-tyrosyl-[^{3}H]-phenylalanyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-N^{G}-tosyl-L-arginyl-glycinamide was separated from the resin by ammonolysis of the peptide resin according to the procedure of Meienhofer *et al.* (5). The oil obtained was purified by chromatography on a column of Sephadex LH-20 (0.9 × 55 cm) in dimethylformamide. The absorbance reading at 280 nm was plotted against the elution volume and the peptide material in the fractions corresponding to the main peak of absorbance (elution volume: 32 ml) was recovered by evaporation *in vacuo*. The oil so obtained was converted to a solid by trituration with ethanol and drying *in vacuo*. This yielded 118 mg (32%) pale yellow powder, optical rotation $[\alpha]_D^{22}$ −26.3° (c = 1, DMF).

The solid protected nonapeptide derivative (58 mg) was dissolved in liquid ammonia (100 ml) which had been distilled from sodium. Sodium was added in small quantities to the boiling ammonia solution until a permanent blue color remained for 30 s. The blue color was removed by addition of glacial acetic acid (2 drops) and the ammonia was removed *in vacuo*. The solid product was dissolved in 0.2 *N* acetic acid (150 ml) and the p*H* of the solution adjusted to 8 with concentrated ammonium hydroxide solution, and then hydrogen peroxide solution (30%, 0.5 ml) was added. The solution was stirred for 5 min, then the p*H* was adjusted to 5 with glacial acetic acid, the volume reduced to 50 ml by evaporation *in vacuo*, and evaporation was completed by lyophilization. The resultant solid product was dissolved in 50% acetic acid (4 ml) and purified by chromatography on a column of Sephadex G-15 (1.5 × 90 cm) in 50% acetic acid. The eluent was monitored both by measurement of its u.v. absorption (at 280 nm) and by liquid scintillation counting. A main peak (elution volume: 60 ml) followed by two minor peaks was detected by u.v. absorption measurement while only the main peak was detected by liquid scintillation counting. The fractions corresponding to the leading half and the trailing half of the main peak were pooled and concentrated *in vacuo* to volumes of about 5 ml each, diluted to 50 ml with water and lyophilized to dryness. The material from the trailing half of the main peak was dissolved in 0.2 *N* acetic acid (2.0 ml) and purified by chromatography on a column of Sephadex G-25 superfine (1.5 × 90 cm) in 0.2 *N* acetic acid. The eluent was monitored by u.v. absorption measurement and liquid scintillation counting. The leading and trailing edges of the single peak were discarded and the center portion (elution volume: 123 ml) was concentrated *in vacuo* to a volume of 25 ml and lyophilized to yield 14 mg (11% based on starting glycine) of a solid, white, fluffy material, optical rotation $[\alpha]_D^{22}$ −26.6° (c = 0.20, 1 *N* HOAc). The material possessed 470 ± 50 units/mg rat antidiuretic activity, 24 ± 6 units/mg milk-ejecting like activity and 2.0 × 10^6 c.p.m./mg. On re-chromatography on a column of Bio-Rex 70 (0.9 × 60 cm) in 0.5 *M* ammonium acetate, p*H* 6.4, the material was eluted as a single component (elution volume: 20 ml) when monitored by u.v. absorption, scintillation counting and biological assay (milk-ejecting like activity). No change in biological activity and specific radioactivity of the recovered material was observed.

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