Solid-Phase Synthesis of [2-Isoleucine,4-leucine]oxytocin and [2-Phenylalanine,4-leucine]oxytocin and Some of Their Pharmacological Properties†

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[Ile^2,Leu^4]oxytocin and [Phe^2,Leu^4]oxytocin have been synthesized using the solid-phase method. In the synthesis of [Phe^2,Leu^4]oxytocin a benzhydrylamine resin support was used, and a diphenylmethyl carboxamide protecting group was used for the asparagine residue. The protected asparagine residue was coupled to the peptide resin with dicyclohexylcarbodiimide. The peptide was cleaved from the resin and the diphenylmethyl carboxamide and *S*-*p*-methoxybenzyl protecting groups were removed by HF(*l*). The disulfhydryl peptide was oxidized and purified to give the desired cyclic peptide. In the synthesis of [Ile^2,-Leu^4]oxytocin, *tert*-butyloxycarbonylasparagine was coupled to the peptide resin with dicyclohexylcarbodiimide and 1-hydroxybenzotriazole. No cyano formation was detected. [Ile^2,Leu^4]oxytocin and [Phe^2,-Leu^4]oxytocin were found to possess weak pressor activities and no antidiuretic activities. [Ile^2,Leu^4]-oxytocin had a mild natriuretic–diuretic activity whereas [Phe^2,Leu^4]oxytocin had only a weak natriuretic activity. Neither compound had a detectable oxytocic activity when assayed in the isolated uterus in a Mg^{2+}-free van Dyke–Hastings solution. On the contrary, they inhibited the oxytocic response of oxytocin in this *in vitro* system. Their oxytocic inhibitory activity was found to be dependent on Ca^{2+} concentration. When the Ca^{2+} concentration in the bathing medium was increased from 0.5 to 1.0 m*M*/l., the inhibitory activity of [Phe^2,Leu^4]oxytocin was markedly reduced while [Ile^2,Leu^4]oxytocin became a weak agonist.

Structure–activity relationship studies of the neurohypophysial hormone, oxytocin (Figure 1), have shown that replacement of the tyrosine residue at position 2 of the hormone by a more lipophilic amino acid (*e.g.*, [2-*O*-ethyltyrosine]oxytocin) can afford an analog with antagonist activities to the natural hormone (for a recent review, see ref 1). Recently it has also been found that replacement of the Gln residue at position 4 by a Leu residue afforded an analog, [Leu^4]oxytocin, that possessed anti-ADH activity (inhibitory to the antidiuretic effect of vasopressins).[2-4] The [Leu^4]oxytocin was also found to inhibit the toad bladder response to the hydroosmotic activity of arginine-vasopressin[5] and was found to possess considerable diuretic and natriuretic effects during both water diuresis and vasopressin-suppressed water diuresis in rats.[3,4] It has also been reported that [Asp^4]oxytocin and [Ser^4]oxytocin can block the antidiuretic effect of ADH.[6,7] Other analogs of oxytocin containing aliphatic amino acids at position 4 possess small or negligible antidiuretic and pressor activities,‡‡ but none have been reported to possess anti-ADH activity.

More recently it was reported[8] that [Leu^2,Leu^4]oxytocin, an analog of oxytocin in which the tyrosine residue at position 2 and the glutamine residue at position 4 are replaced by leucine residues, is an inhibitor to oxytocin in the oxytocic *in vitro* assay system and possesses natriuretic and diuretic activities. [Ile^2,Ile^4]oxytocin,[9] [Leu^3,Leu^4]oxytocin,[10] and [Leu^4,Ile^8]oxytocin[11] were also found to have natriuretic–diuretic effects, but none of these had inhibitory activity to oxytocin in the oxytocic assay. All of these compounds possess little or none of the activities usually associated with the hormone oxytocin (*e.g.*, oxytocic and avian vasodepressor activities).

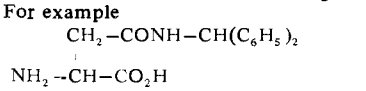
To further assess the effect of substitution of highly lipophilic amino acids at positions 2 and 4 of oxytocin on the various agonist and especially antagonist activities previously mentioned, we have prepared [Ile^2,Leu^4]oxytocin and [Phe^2,Leu^4]oxytocin. In the former compound the tyrosine residue at position 2 has been replaced by an isoleucine residue and the glutamine residue at position 4 by a leucine residue, and in the latter compound the tyrosine was replaced by phenylalanine and the glutamine by leucine (Figure 1).

For the synthesis of [Phe^2,Leu^4]oxytocin we have utilized a polystyrene resin cross linked with 1% divinylbenzene substituted with a phenylmethylamine group (the benzhydryl amine resin first suggested by Pietta and Marshall[12]) to prepare the precursor peptide by the solid-phase method of peptide synthesis.[13] (For a recent review of solid-phase methodology, see ref 14.) We have also utilized a diphenylmethyl protecting group for the carboxamide group of asparagine and during the synthesis coupled this residue to the peptide resin with dicyclohexylcarbodiimide (DCC). [Ile^2,Leu^4]oxytocin was also synthesized by the solid-phase method. *tert*-Butyloxycarbonylasparagine was coupled to the growing peptide chain by DCC with added *N*-hydroxybenzotriazole.

Experimental Procedure

Thin-layer chromatography (tlc) was done on silica gel G plates in the following solvent systems: A, benzene–ethanol–petroleum ether (bp 30–60°) (25:70:5); B, chloroform–methanol–acetic acid

†All optically active amino acids are of the L variety. N^{CA} is used to indicate substitution on the carboxamide nitrogen of asparagine and glutamine. For example



would be N^{CA}-diphenylmethylasparagine. PMB = *p*-methoxybenzyl and DPM = diphenylmethyl. Other abbrevations follow the rules of the IUPAC-IUB Commission on Biochemical Nomenclature.

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‡‡See Table I, ref 3, for a summary.

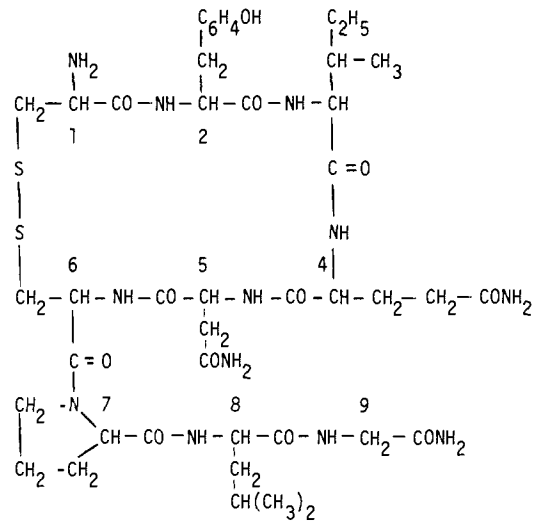


Figure 1. Structure of oxytocin, with numbers indicating the position of the individual amino acid residues. In [2-phenylalanine,4-leucine]oxytocin the Tyr residue in position 2 of oxytocin is replaced by Phe, and the Gln residue in position 4 is replaced by a Leu residue. In [2-isoleucine,4-leucine]oxytocin the Tyr residue in position 2 is replaced by an Ile residue, and the Gln residue in position 4 is replaced by a Leu residue.

(95:5:3); C, 1-butanol-acetic acid-pyridine-water (15:3:10:12). Capillary melting points were determined and are corrected. Amino acid analyses were carried out by the method of Spackman, Stein, and Moore[15] on a Beckman 120C amino acid analyzer after hydrolysis in 6 *N* HCl. A Parr apparatus was used for hydrogenations.

α-Benzyl *tert*-Butyloxycarbonylaspartate (2). A stirred solution of 23.5 g (0.10 mol) of N^{α}-*tert*-butyloxycarbonylaspartic acid (prepared by the pH stat method of Schnabel[16]) in 60 ml of anhydrous tetrahydrofuran (THF, freshly distilled from $LiAlH_4$) was cooled to −5° and a cooled (−5°) solution of 22.76 g (0.11 mol) of DCC in 60 ml of anhydrous THF was added. The solution was stirred for 6 hr at 0 to −5°. The dicyclohexylurea was filtered off and washed with three 40-ml portions of chloroform. The solution was evaporated to dryness *in vacuo* at 25–30°. The powder was dissolved in 60 ml of freshly distilled anhydrous THF and 10.8 g (0.10 mol) of anhydrous benzyl alcohol (distilled from Linde 4A molecular sieves) was added. The solution was stirred about 1 day at room temperature, 0.5 ml of acetic acid was added, and the dicyclohexylurea was filtered off and washed with two 20-ml portions of chloroform. The solvents were removed on a rotary evaporator *in vacuo*. The crude oil was dissolved in 120 ml of anhydrous ether and 27.3 g (0.15 mol) of dicyclohexylamine was added dropwise. A precipitate slowly formed (20 hr). The precipitate was filtered off and washed with three 40-ml portions of ether and dried *in vacuo*: wt, 39.9 g; mp 141–142°. The powder was added to a rapidly stirred two-phase system containing 250 ml of ethyl acetate and 170 ml of 20% aqueous citric acid. After stirring for 20 min, the organic layer was removed and the aqueous layer was extracted with two 150-ml portions of ethyl acetate. The combined organic layers were dried over sodium sulfate and the solvents were removed *in vacuo* on a rotary evaporator. The oil was dissolved in 50 ml of ethanol and 50 ml of deionized water was added. A small amount of dicyclohexylurea precipitated out overnight at room temperature and was filtered off. The product was crystallized from ethanol-water at 2° and dried *in vacuo*: wt, 21.54 g (66.7%); mp 99.5–101°; tlc, single spot in the solvent systems A and B. *Anal.* ($C_{16}H_{21}NO_6$) C, H, N.

α-Benzyl *tert*-Butyloxycarbonyl-N^{CA}-diphenylmethylasparaginate (3). A mixture of 4.85 g (15 mmol) of α-benzyl *tert*-butyloxycarbonylaspartate and 2.7 g (22.5 mmol) of *N*-hydroxysuccinimide in 30 ml of methylene chloride was cooled to −5° with stirring. A solution of 3.58 g (17.4 mmol) of DCC in 14 ml of methylene chloride was added, and the mixture was stirred at −5° for 50 min. A solution of 3.02 g (16.5 mmol) of diphenylmethylamine was added and the mixture was stirred at −5° for an additional 50 min and at room temperature for 24 hr. Acetic acid (1 ml) was added, the mixture was stirred for 15 min, and the dicyclohexylurea was filtered off off and washed with three 30-ml portions of methylene chloride. The solvents were removed *in vacuo* on a rotary evaporator and the residue was dissolved in 20 ml of methylene chloride. The insoluble material was filtered off and washed with two 5-ml portions of methylene chloride. A further 80 ml of methylene chloride was added, and the solution was washed with three 90-ml portions of 5% aqueous citric acid, two 120-ml portions of 5% aqueous sodium bicarbonate, and three 180-ml portions of deionized water. The organic layer was dried over anhydrous sodium sulfate, and the solvents were removed *in vacuo* on a rotary evaporator. The white solid was dissolved in 90 ml of hot ethyl acetate. The solution was cooled and then filtered: wt, 2.2 g; mp 156.5–158°. A second crop was obtained by addition of petroleum ether (bp 30–60°): wt, 4.6 g; mp 156–158°; total wt, 6.8 g (95%); $[\alpha]^{25}D$ −5.8° (*c* 0.5, DMF); tlc, the compound gave a single spot on tlc in the solvent systems A and B. *Anal.* ($C_{29}H_{32}N_2O_5$) C, H, N.

***tert*-Butyloxycarbonyl-N^{CA}-diphenylmethylasparagine (4).** A solution of 1.5 g of α-benzyl N^{α}-*tert*-butyloxycarbonyl-N^{CA}-diphenylmethylasparaginate (3) in 50 ml of *tert*-butyl alchol, 25 ml of deionized water, and 10 ml of acetic acid was prepared and 1.5 g of 5% Pd on C was added. The mixture was shaken for 24 hr at 55 psi of hydrogen in a Parr apparatus. The catalyst was filtered off over Celite and the Celite was washed with three 20-ml portions of ethyl acetate. The aqueous layer was extracted with two 15-ml portions of ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4, and then the solvent was removed on a rotary evaporator *in vacuo*. The product was recrystallized from ethyl acetate–petroleum ether (bp 30–60°) at 2°: wt, 0.95 g (77%); mp 147–148°; $[\alpha]^{25}D$ +1.2° (*c* 0.5, DMF); tlc, the compound showed a single spot on tlc in solvent systems A and B. *Anal.* ($C_{22}H_{26}N_2O_5$) C, H, N.

The cleavage of the diphenylmethyl carboxamide protecting group from *tert*-butyloxycarbonylasparagine in liquid HF containing anisole (9:1) was studied under a variety of conditions (the Boc group was found to be completely removed under all of these conditions). The amount of asparagine and N^{CA}-diphenylmethylasparagine was quantitatively determined on an amino acid analyzer. The following results were obtained (temperature, time, per cent of diphenylmethyl group removed): (1) 0°, 30 min, 100%; (2) 0°, 60 min, 100%; (3) 20°, 30 min, 100%; (4) 20°, 60 min, 100%; (5) 20°, 90 min, 100%.

Copolystyrene–1% Divinylbenzene Phenyl Ketone Resin. To a mixture of 30 g of polystyrene resin cross linked with 1% divinylbenzene (Bio-Beads, X-1, 200–400 mesh, Bio-Rad Corp., Richmond, Calif.) in 225 ml of methylene chloride was added, under nitrogen, 7.26 g (51.8 mmol) of benzoyl chloride. The mixture was cooled to 0° in ice and 6.92 g (51.8 mmol) of aluminum chloride was added in three approximately equal portions. The brown reaction mixture was stirred at 0° for 2 hr, at 25° for 1.5 hr, and finally at reflux for 2.5 hr. The mixture was then cooled, poured into 1 l. of ice-water, and treated with 200 ml of concentrated HCl with stirring. The resin was filtered off, suspended in 200 ml of water, treated with 50 ml of concentrated HCl, and again filtered off. It was then suspended in 200 ml of 0.5 *N* NaOH, stirred for 5 min, filtered, and washed three times each with water–dioxane (3:1), methanol, and methylene chloride. The cream-colored resin was dried overnight *in vacuo*: wt, 33.6 g; ir (KBr) 3100 (aromatic), 1655 (C=O), and 1600 cm^{-1} (aromatic). Based on weight gain, the carbonyl content corresponds to about 1.0 mmol/g of resin.

Benzhydrylamine Resin. A mixture of 167 ml (3.96 mol) of 88% formic acid and 200 ml (2.96 mol) of concentrated ammonium hydroxide was added to a three-necked flask equipped with a thermometer, an overhead mechanical stirrer, and a distillation apparatus. The water was removed by distillation as the inner temperature gradually increased to 150–160°. To the hot mixture was added 5 g of the ketone resin. The mixture was stirred and maintained at 150–160° (outer bath temperature 180°) for 48 hr during which time it was necessary to occasionally wash the resin which had accumulated on the sides of the flask down into the reaction mixture with the reaction solution. The mixture was cooled and filtered through a sintered glasss funnel. The resin was washed three times each with water–dioxane (3:1), methanol, and methylene chloride. It was then suspended in 80 ml of concentrated hydrochloric acid–propionic acid (1:1) and refluxed for 5 hr. After filtration, the resin was washed three times each with 10% aqueous sodium carbonate, water–dioxane (3:1), methanol, and methylene chloride to give a cream-colored resin: wt, 4.8 g; ir (KBr) 3400 (weak, NH_2), 3075 (aromatic), 1655 (unreacted ketone), 1600 (aromatic), 1485 and 1435 cm^{-1}. Elemental analysis indicated an

amino content of 0.49–0.56 mmol/g of resin.§§

Glycinamide Resin. In a 60-ml solid-phase reaction vessel,[17] 5 g of the benzhydryl resin was suspended in 35 ml of methylene chloride and shaken for 10 min. After removal of the solvent, the resin was treated with 1.82 g (10.4 mmol) of *tert*-butyloxycarbonylglycine in 8 ml of methylene chloride and 2.14 g (10.4 mmol) of DCC in 18 ml of the same solvent for 20 min at room temperature. After several washes with methylene chloride and ethanol, the coupling procedure was repeated twice more for 60 min each with half the quantities of *tert*-butyloxycarbonylglycine and DCC in the same volume of methylene chloride. Then the resin was washed with three 35-ml portions each of methylene chloride, ethanol, and methylene chloride, and the unreacted amino groups were blocked by treatment with 8 g (72 mmol) of *N*-acetylimidazole in 60 ml of methylene chloride for 2 hr at 23°. The ninhydrin test[18] for the presence of free amino groups indicated that there were no exposed amino groups. The Boc protecting group was removed by treatment of 2 g of the resin with 18 ml of trifluoroacetic acid–methylene chloride–anisole (50:48:2) for 25 min at 23°. After neutralization with two 18-ml portions of diisopropylethylamine–chloroform (7:93) for 10 min each and washing with three 18-ml portions each of chloroform and methylene chloride, the modified[19] quantitative aldimine test[20] for primary amino groups (~2-mg portions of resin were used) showed a glycine substitution of 0.48 mmol/g of resin. In the modified test, the peptide resin (2–5 mg) was dried *in vacuo* over KOH and carefully weighed in a test vessel [a 2.5-ml vessel with fritted (coarse frit) glass filter fitted with 1-A glass stopcock outlet and 10/18 ST ground glass mouth with stopper]. A solution of 1.0 *N* 2-hydroxy-1-naphthaldehyde in methylene chloride (~2 ml) was added to the test vessel, and the mixture was stirred vigorously for 30–40 min such that resin did not stick to the wall of the vessel. The solution was drained from the vessel by dry nitrogen pressure and the resin and vessel were thoroughly washed with about ten portions of methylene chloride. Then ~1 ml of 1.0 *N* benzylamine in methylene chloride was added to the test vessel and the vessel was again vigorously shaken for 20 min. The solution was carefully drained into a volumetric flask (5 or 10 ml), and washes (with the benzylamine solution) of the resin and vessel sufficient to fill the volumetric flask were used. The absorbance at 420 nm was determined using the benzylamine solution as a blank. Calculations of amount of Schiff base (using a standard curve) were made, and the millimoles of amino acid (or primary amino group) were then obtained. In model studies amino acid analysis of amino acid–resin hydrolysates gave the same results. The resin was used directly for the preparation of the peptide resin 4 starting at step 7 in the cycle *(vide infra)*.

***tert*-Butyloxycarbonyl-*S*-*p*-methoxybenzylcysteinylphenylalanylisoleucylleucyl-N^{CA}-diphenylmethylasparaginyl-*S*-*p*-methoxybenzylcysteinylprolylleucylglycinamide Resin (1).** The glycinamide resin from the previous step *(vide supra)* (2 g) was utilized for the preparation of the title compound. The following cycles of deprotection, neutralization, and coupling were carried out for the introduction of each new residue in the peptide:[17,21] (1) three washings with 18-ml portions of methylene chloride; (2) cleavage of the Boc group by treating with 18 ml of trifluoroacetic acid–methylene chloride–anisole (48:50:2) for 25 min at room temperature; (3) five washings with 18-ml portions of methylene chloride; (4) four washings with 18-ml portions of chloroform; (5) neutralization with two 18-ml portions of diisopropylethylamine in chloroform (7:93) for 6 min at room temperature; (6) three washings with 18-ml portions of chloroform; (7) four washings with 18-ml portions of methylene chloride; (8) addition of 2 mmol of the appropriate *tert*-butyloxycarbonylamino acid in 9 ml of methylene chloride and 5 min of mixing; (9) addition of 2 mmol of DCC in 9 ml of methylene chloride followed by a reaction period of 90 min at room temperature; (10) three washings with 18-ml portions of methylene chloride; (11) three washings with 18-ml portions of dimethylformamide; (12) three washings with 18-ml portions of methylene chloride; (13) repetition of steps 8 and 9 but using 1 mmol of the same *tert*-butyloxycarbonylamino acid and 1 mmol of DCC, respectively; (14) three washings with 18-ml portions of methylene chloride; (15) three washings with 18-ml portions of dimethylformamide. Unless otherwise specified, each washing and mixing step lasted for 2 min. The ninhydrin test was run to monitor the coupling steps (sample was run just before step 2). In each step the coupling appeared to be quantitative. *tert*-Butyloxycarbonylamino acids were used, and the cysteine sulfhydryl groups were protected by *p*-methoxybenzyl groups and the carboxamide group of asparagine was protected by a diphenylmethyl group.

After the synthesis of 1 was completed, the polypeptide resin was washed with three 18-ml portions of acetic acid, three 18-ml portions of absolute ethanol, and three 18-ml portions of methylene chloride and then dried *in vacuo*, wt 3.18 g.

[Phe^2,Leu^4]oxytocin. Boc-*S*-PMB-Cys-Phe-Ile-Leu-N^{CA}-DPM-Asn-*S*-PMB-Cys-Pro-Leu-Gly-resin (1) (0.75 g) was placed in a Telfon cleavage vessel and 1.5 ml of anisole was added. The vessel was attached to the HF cleavage apparatus and 30 ml of anhydrous HF (distilled from CoF_3) was collected into the vessel. The mixture was stirred for 75 min at 20°. The HF was removed under reduced pressure and the resin dried *in vacuo* to remove the anisole. The solid was washed with four 30-ml portions of ethyl acetate and dried *in vacuo* over KOH for 35 min. The peptide material was extracted into four separate 30-ml portions of 1 *N* acetic acid. The resin was then washed with 200 ml of deionized water, and the total volume was brought to 400 ml with deionized water. The solution was adjusted to a pH of 8 with 3 *N* ammonium hydroxide, and the compound was oxidized with 50 ml of 0.01 *N* potassium ferricyanide. After stirring for 30 min, the pH was adjusted to 5 with dilute acetic acid and Rexyn 203 (Cl^- form) resin was added to remove ferro- and excess ferricyanide. The mixture was stirred for 15 min, the resin was filtered off, and the solution was lyophilized.

The residue was dissolved in 5 ml of 50% acetic acid and applied to a 120 × 1.8 cm column of Sephadex G-15 that had been equilibrated with 50% acetic acid solution. The column was eluted with 50% acetic acid and 100 fractions of 4.2 ml each were collected. The fractions corresponding to the peptide material (tubes 34–51) as determined by the Folin–Lowry method[22] were pooled. Deionized water (150 ml) was added, and the mixture was lyophilized, wt 150 mg. The powder was dissolved in 4 ml of the lower phase and 4 ml of the upper phase of the solvent system 1-butanol–benzene–3.5% aqueous acetic acid containing 1.5% pyridine (1:1:2) and placed on a 64 × 2.8-cm column of Sephadex G-25 (100–200 mesh) that had been equilibrated with both the lower and upper phases according to the method of Yamashiro, *et al.*[23,24] One hundred fractions were collected, and the fractions corresponding to the major peak (R_f 0.45) as determined by the Folin–Lowry method were pooled. The tubes from the major fraction were washed with 250 ml of deionized water, and the combined solvents were concentrated to 50 ml *in vacuo* and lyophilized to give a white powder, wt 65 mg. The powder was dissolved in 4 ml of 0.2 *N* aqueous acetic acid and placed on a 2.8 × 65 cm column of Sephadex G-25 (200–270 mesh) that had been equilibrated with 0.2 *N* aqueous acetic acid for gel filtration.[25] One hundred ten fractions of 4.4 ml each were collected, and the fractions corresponding to the major peak (tubes 61–72) as determined by the Folin–Lowry method were pooled. The solution was lyophilized: wt, 52 mg (22.8% yield based on the glycinamide substitution on the resin); tlc using solvent system C showed the material as a single spot; $[\alpha]^{22}D$ −52° (*c* 0.5, 1 *N* acetic acid). *Anal.* ($C_{44}H_{69}N_{11}O_{10}S_2 \cdot H_2O$) C, H, N.

Amino acid analysis after 44 hr of hydrolysis gave the following molar ratios: aspartic acid, 1.0; proline, 1.0; glycine, 1.0; cystine, 0.91; isoleucine, 1.1; leucine, 1.8; phenylalanine, 1.0; and ammonia, 2.1. The long hydrolysis time was necessitated by the difficulty in the hydrolysis of the isoleucylleucine peptide bond.[8,26]

Synthesis of [Phe^2,Leu^4]oxytocin by Typical Methods of Solid-Phase Peptide Synthesis. A sample of 2.0 g of *tert*-butyloxycarbonylglycyl resin prepared by standard methods[27,##] and found to contain 0.46 mmol of glycine/g of resin was placed in a 35-ml solid-phase reaction vessel. The cycles of deprotection, neutralization, and coupling were essentially identical with those used in the synthesis on the benzhydrylamine resin except that a single coupling with a 2.5 *M* excess of the appropriate *tert*-butyloxycarbonylamino acid (step 7) and DCC (step 8) was used. The sulfhydryl group of cysteine was protected with the *p*-methoxybenzyl group. The coupling reaction involving *tert*-butyloxycarbonylasparagine was carried out *via* its nitrophenyl ester. The following steps of the procedure were modified: (7) four washings with 18-ml portions of dimethylformamide; (8) addition of Boc-asparagine-ONp in 15 ml of dimethylformamide followed by 12 hr of reaction time; (9–13) those steps were skipped; (14) three washings with 18-ml portions

§§ A referee has pointed out that benzhydrylamine resins which still contain carbonyl groups tend to lose "functional" amine on standing and therefore recommends that the resins be stored as salts. We have stored our benzhydrylamine resins as free base for periods of 6–12 months over P_2O_5 with no apparent loss of "functional" amine.

##See ref 14, Table II, p 236.

of dimethylformamide; (15) three washings with 18-ml portions of ethanol.

After the synthesis of the peptide resin Boc-*S*-PMB-Cys-Phe-Ile-Leu-Asn-*S*-PMB-Cys-Pro-Leu-Gly-resin was completed, the Boc group was removed by going through steps 1-7; the peptide resin was then washed with three 18-ml portions each of glacial acetic acid, absolute ethanol, and methylene chloride for 2 min each. The peptide resin was dried *in vacuo*, wt 2.51 g. This resin was stirred with 100 ml of freshy prepared anhydrous methanol at 0° and dry ammonia was bubbled into the mixture to saturation. The mixture was stirred for 60 hr at 0°. The methanol and ammonia were removed under aspirator vacuum, and the residue was suspended in 125 ml of dimethylformamide. The mixture was stirred for 2 hr, and the resin was filtered and washed with two 20-ml portions of dimethylformamide. The solvents were removed on a rotary evaporator at 30–40° *in vacuo*, and the residue was reprecipitated from dimethylformamide–water. The product was filtered off and washed with two 15-ml portions of absolute ethanol. The powder was dried *in vacuo*, wt 0.36 g.

A solution of the powder (175 mg) in 125 ml of anhydrous ammonia (freshly distilled from sodium) was treated with a sodium stick until a blue color persisted for 45 sec. The ammonia was removed by evaporation and lyophilization. The salt was dissolved in 400 ml of deaerated deionized water containing 0.40 ml of acetic acid. The pH of the solution was adjusted to 8.5 with 2 *N* ammonium hydroxide, and the compound was oxidized with 30 ml of 0.01 *N* potassium ferricyanide. After stirring for 30 min, the pH was adjusted to 5 with dilute acetic acid and the ferro- and excess ferricyanide ions were removed by treatment with Rexyn 203 (Cl^- cycle). The resin was filtered off and the solution was lyophilized. The residue was subjected first to partition chromatography and then to gel filtration on Sephadex G-25 using the same solvent systems as used in the previous synthesis of [Phe^2,Leu^4]oxytocin. The final lyophilized powder weighed 24 mg (this is a 6% yield based on the glycyl substitution on the resin). The compound was identical with that prepared by the previous synthesis.

***S*-PMB-Cys-Ile-Ile-Leu-Asn-*S*-PMB-Cys-Pro-Leu-Gly-NH_2 (5).** A sample of 2.50 g of *tert*-butyloxycarbonylglycine resin which contained 0.40 mmol of glycine/g of resin as determined by the modified aldimine test was placed in a 50-ml vessel for solid-phase peptide synthesis and swollen in methylene chloride (25 ml). The cycles of deprotection, neutralization, and coupling were similar to those used in the other syntheses except that 25-ml portions of solvent were used, the Boc group was removed by trifluoroacetic acid–methylene chloride–anisole (40:60:1) (step 2), and only a single coupling with a twofold excess of the appropriate protected amino acid and DCC was used. Steps 11-13 were skipped and in step 15 the solvent was ethanol. For the coupling of *tert*-butyloxycarbonylasparagine, the following changes were made: (7) three washings with 25-ml portions of dimethylformamide; (8) addition of 3 mmol of *tert*-butyloxycarbonylasparagine and 6 mmol of 1-hydroxybenzotriazole (Aldrich Chemical Co., Milwaukee, Wis.) in 15 ml of dimethylformamide and 5 min of mixing; (9) addition of 3 mmol of dicyclohexylcarbodiimide in 10 ml of dimethylformamide followed by an overnight reaction period at room temperature; (10) three washings with 25-ml portions of dimethylformamide.

The coupling steps were monitored by the ninhydrin test. All residues except *tert*-butyloxycarbonylasparagine, the second *tert*-butyloxycarbonylisoleucine, and the last *tert*-butyloxycarbonyl-*S*-*p*-methoxybenzylcysteine residues coupled quantitatively. Though the incomplete coupling reactions occurred to an extent of ≥98%, the coupling reactions were repeated for each of these residues. After the second coupling, the couplings were quantitative as determined by the ninhydrin test.

The incorporation of the last protected cysteine residue was followed by deprotection and neutralization (steps 1-7). The resin was dried *in vacuo*, wt 4.1 g. The peptide was cleaved from the resin with anhydrous ammonia (distilled from Na) in anhydrous methanol at 0° and isolated by the same methods as used above: wt, 0.60 g (51% based on the initial glycine substitution); mp 242–243°; $[\alpha]^{22}D$ −46.0° (*c* 0.5, DMF); ir (KBr and Nujol) no absorption in the 2500–2000-cm^{-1} region. (The cyano absorption of β-cyanoalanine in peptides is found at ~2250 cm^{-1} as a medium to weak peak. To observe its presence at low concentrations it is necessary to take the infrared spectrum (KBr pellet) under conditions where the N-H and C-H regions (~2900–3500 cm^{-1}) and the carbonyl region (~1500–1750 cm^{-1}) are opaque (0% transmittance). Under these conditions we estimate that ≥5% contamination of peptide **5** with the β-cyanoalanine containing peptide could be detected. For example, treatment of *S*-Bzl-Cys-Pro-Leu-Gly-NH_2 with *tert*-butyloxycarbonylasparagine and DCC under conditions identical with those used in the synthesis of **5** gave a mixture of peptides in which the cyano group was readily detected (KBr or film). On dilution of the peptide mixture with authentic Boc-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2 (tenfold) the cyano absorption was still visible (KBr).) For elemental analysis, a small portion of the compound was reprecipitated from dimethylformamide–95% ethanol. *Anal.* ($C_{57}H_{89}N_{11}O_{12}S_2 \cdot 2H_2O$) C, H, N.

[Ile^2,Leu^4]oxytocin. The nonapeptide **5** (180 mg) was dissolved in 150 ml of anhydrous NH_3 (freshly distilled from sodium) and treated with a sodium stick until a blue color persisted for 45 sec. The ammonia was removed by evaporation and lyophilization. The white powder was dissolved in 350 ml of deaerated 0.1% aqueous acetic acid and oxidized as in synthesis of [Phe^2,Leu^4]oxytocin by typical methods. The solution was lyophilized, and the powder was dissolved in 5 ml of the upper phase and 2 ml of the lower phase of the solvent system 1-butanol–benzene–3.5% aqueous acetic acid containing 1.5% pyridine (1:1:2). The solution was placed on a 63 × 2.85 cm column of Sephadex G-25 (100–200 mesh) for partition chromatography. The fractions corresponding to the major peak (R_f 0.25) as determined by the Folin–Lowry method[22] were pooled, 250 ml of washes with deionized water were added, the combined mixture was concentrated to 50 ml, and the solution was lyophilized, wt 43.2 mg. The powder was dissolved in 3 ml of 0.2 *N* acetic acid and placed on a 65 × 2.85 cm column of Sephadex G-25 (200–270 mesh) for gel filtration using 0.2 *N* acetic acid as eluent solvent. Eighty fractions of 5.0 ml each were colllected and the product was eluted as a single peak. The fractions corresponding to this peak were collected and lyophilized to give a white powder: wt, 33.3 mg; $[\alpha]^{22}D$ −32.6° (*c* 0.5, 1 *N* HOAc). *Anal.* ($C_{41}H_{71}N_{11}O_{10}S_2 \cdot H_2O$) C, H, N.

A sample was hydrolyzed for 60 hr at 110°. Amino acid analysis gave the following molar ratios: aspartic acid, 1.0; proline, 1.0; glycine, 1.0; cystine, 0.91; isoleucine, 1.7; leucine, 2.1. When a sample was hydrolyzed for 120 hr at 110°, amino acid analysis gave the following molar ratios: aspartic acid, 1.0; proline, 1.0; glycine, 1.0; cystine, 0.91; isoleucine, 1.9; leucine, 2.1. The long hydrolysis time was necessitated by the difficulty in the hydrolysis of isoleucylisoleucine[3,9,28,29] and isoleucylleucine.[8,26]

Bioassay Methods. The oxytocic activity was measured according to the method of Holton[30] as modifed by Munsick[31] on isolated uteri from rats in natural estrus with the use of Mg^{2+}-free van Dyke–Hastings solution. Pressor assays were performed on anesthetized rats.[32] Antidiuretic and diuretic assays were performed on anesthetized rats by methods of Jeffers, Livezy, and Austin[33] as modified by Sawyer.[34] Urinary Na^+ concentrations were determined by a Baird-Atomic flame photometer, with Li_2SO_4 as the internal standard. The USP Posterior Pituitary Reference Standard served as a reference for all activities measured.

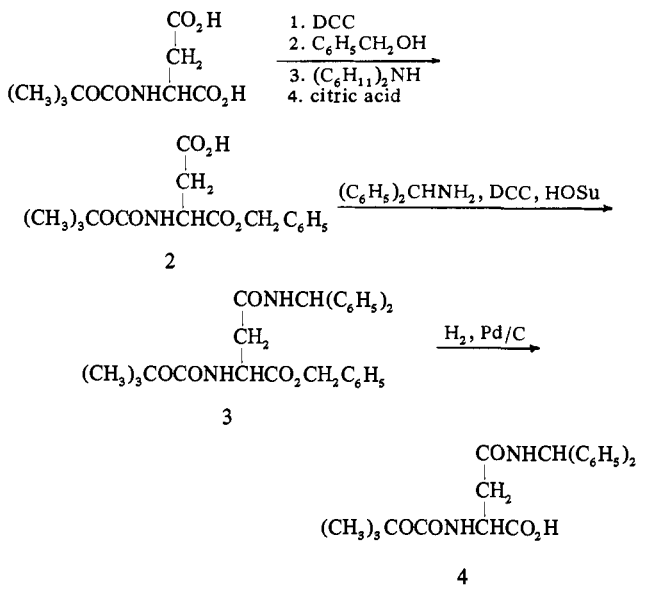
Results and Discussion

The solid-phase synthesis of the desired protected nonapeptide resin intermediate Boc-*S*-PMB-Cys-Phe-Ile-Leu-N^{CA}-DPM-Asn-*S*-PMB-Cys-Pro-Leu-Gly-NH_2 (**1**) was carried out on a benzhydrylamine resin. The carboxamide terminal amino acid, *tert*-butyloxycarbonylglycine, was attached to the resin using dicyclohexylcarbodiimide coupling (substitution values of 0.30–0.50 mmol of amino acid/g of resin could be obtained). Unreacted resin amino groups were then blocked by acetylation with *N*-acetylimidazole.[35] After acetylation, no free amino group could be detected on the resin by the ninhydrin method.[18] The synthesis of the desired nonapeptide resin intermediate **1** was then continued using N^{α}-Boc-protected amino acids by the general procedures of solid-phase peptide synthesis. The exception was for attachment of the asparagine residue.

In solid-phase peptide synthesis most amino acids are coupled to the growing peptide chain by dicyclohexylcarbodiimide (DCC).[13,14] However, *tert*-butyloxycarbonylglutamine and *tert*-butyloxycarbonylasparagine cannot be coupled by this method due to dehydration of the carboxamide groups to cyano groups by this reagent[36-38] and subsequent incorporation of the cyano amino acids into the growing peptide chain. The usual coupling of these amino

acids has therefore been by the active ester method, usually the *p*-nitrophenyl ester.[14,27,39] This coupling procedure generally requires long reaction periods, especially for extended syntheses,[40] and coupling is often incomplete. Furthermore, side reactions such as pyroglutaminyl formation for glutamine[41,42] and hydrolysis of the carboxamide group[43] can occur under certain conditions of peptide synthesis. For these and other reasons we have been studying potential carboxamide protecting groups. Previous work by Weygand and coworkers[44,45] has suggested the use of the rather labile and difficulty prepared bis(2,4-dimethoxybenzyl)carboxamide protecting group. While this report was being prepared, the use of the 2,4-dimethoxybenzyl group was reported[46] as a potential carboxamide protecting group for the above and other side reactions. We have been investigating potential carboxamide protecting groups which would be stable to the conditions for removal of Boc groups (TFA, etc.) but could be readily removed by liquid HF.[47,48] The diphenylmethyl group appeared to be a quite promising group. Hence, we have utilized *tert*-butyloxycarbonyl-N^{CA}-diphenylmethylasparagine (4) in the solid-phase synthesis of **1** and have coupled this residue to the growing peptide chain at the appropriate place using DCC as the coupling agent. For this purpose, protected amino acid **4** was prepared by the method outlined in Scheme I starting with *tert*-butyloxycarbonylaspartic acid.

Scheme I. Preparation of *tert*-Butyloxycarbonyl-N^{CA}-diphenylmethylasparagine



The N^{CA}-diphenylmethyl and *S*-*p*-methoxybenzyl protecting groups were removed and the carboxamide terminal sulfhydryl peptide precursor to [Phe^2,Leu^4]oxytocin was obtained by treating the peptide-resin **1** with anhydrous HF at 20°. The disulfhydryl peptide which was obtained was oxidized with potassium ferricyanide[49] to crude [Phe^2,Leu^4]-oxytocin. The peptide material was desalted on a Sephadex-G-25 column purified by partition chromatography[23,24] on Sephadex G-25 followed by gel filtration[25] on Sephadex G-25.

The [Phe^2,Leu^4]oxytocin was also prepared by standard solid-phase methods similar to those used in the solid-phase synthesis of oxytocin[50] and deaminooxytocin.[39]

The synthesis of carboxamide terminal peptides such as the neurohypophysial hormones on benzhydrylamine resin offers an attractive alternative to the previously used chloromethylated resin. In this one example, a greater overall yield of the desired product was obtained than when using the standard resin. Furthermore, the diphenylmethyl carboxamide protecting group was readily cleaved in liquid HF under mild conditions and the N^{α}-*tert*-butyloxycarbonyl-N^{CA}-diphenylmethylasparagine could be coupled to the growing peptide chain in solid-phase peptide synthesis with dicyclohexylcarbodiimide in quantitative yield.

For the synthesis of [Ile^2,Leu^4]oxytocin, preparation of the requisite nonapeptide intermediate *S*-PMB-Cys-Ile-Ile-Leu-Asn-*S*-PMB-Cys-Pro-Leu-Gly-NH_2 (**5**) was accomplished by the usual solid-phase method. The exception was the asparagine residue.

Recently it has been shown by König and Geiger[51] that in solution syntheses, *N*-hydroxybenzotriazole and dicyclohexylcarbodiimide could be used with N^{α}-protected asparagine and glutamine for peptide bond formation without formation of the cyano derivatives. We have applied this technique to coupling the asparagine residue in the solid-phase synthesis of the nonapeptide resin, using a mixture of *tert*-butyloxycarbonylasparagine, 1-hydroxybenzotriazole (2 equiv), and dicyclohexylcarbodiimide (1 equiv). No dehydration of the carboxamide group to the cyano group could be detected by infrared spectroscopy.

The partially protected nonapeptide was cleaved from the resin by ammonolysis by methods similar to those used in the solid-phase synthesis of deaminooxytocin[39] and converted to [Ile^2,Leu^4]oxytocin.

The [Ile^2,Leu^4]oxytocin and [Phe^2,Leu^4]oxytocin were assayed for oxytocic, pressor, antidiuretic, and diuretic activities, and urinary Na^+ concentrations were measured.

[Ile^2,Leu^4]oxytocin and [Phe^2,Leu^4]oxytocin were found to possess weak pressor activites of approximately 0.5 unit/mg. The compounds had no antidiuretic activities. However, [Ile^2,Leu^4]oxytocin was found to have a mild natriuretic–diuretic activity very much like that found for [Ile^2,Ile^4]-oxytocin.[9] The slight increase in urine output could be accounted for by the natriuretic activity. [Phe^2,Leu^4]oxytocin had only a very weak natriuretic effect. No anti-ADH activity could be found for either compound.

[Ile^2,Leu^4]oxytocin had no detectable oxytocic activity in Mg^{2+}-free van Dyke–Hastings solution. In this solution, the peptide was found to be a weak inhibitor to the oxytocic response of oxytocin. To produce a 50% inhibition of the the response of oxytocin (10 mU in a 10-ml bath), it required a 700:1 ratio of inhibitor to oxytocin (w/w). [Phe^2,-Leu^4]oxytocin also had no detectable oxytocic activity. It, too, inhibited the oxytocic response of oxytocin in this *in vitro* system. Its inhibitory activity was about twice as strong as that of [Ile^2,Leu^4]oxytocin. The inhibitory activity of both [Ile^2,Leu^4]oxytocin and [Phe^2,Leu^4]oxytocin was dependent on the Ca^{2+} concentration of the bathing medium. When the Ca^{2+} concentration in the van Dyke–Hastings solution was increased from 0.5 to 1.0 m*M*/l., the inhibitory activity of both peptides was markedly reduced. In fact, in the 1.0 mmol Ca^{2+} van Dyke–Hastings solution, [Ile^2,Leu^4]oxytocin became a weak agonist, possessing approximately 0.2 U/mg of oxytocic activity. This indicates that the two peptides are partial agonists. In the low Ca^{2+} medium, when the sensitivity of the uterus to oxytocin is low, they function as antagonists. When the sensitivity of the uterus is enhanced by Ca^{2+}, the low intrinsic activity of the peptide becomes demonstrable.

[Ile^2,Leu^4]oxytocin and [Phe^2,Leu^4]oxytocin have oxytocic and pressor activities similar to those found for [Leu^2,-Leu^4]oxytocin.[8] All three peptides are natriuretic like [Leu^4]oxytocin.[2-4] Their natriuretic activities, however,

are weaker than that of [Leu^4]oxytocin and in the case of [Phe^2,Leu^4]oxytocin its natriuretic activity is almost negligible. Furthermore, neither of these two peptides had the anti-ADH activity found for [Leu^4]oxytocin. Recently, however, it has been shown[52] that [Ile^2,Leu^4]oxytocin inhibited the arginine-vasopressin (AVP) stimulated cyclic AMP production in subcellular preparations of toad bladder epithelium[53] and in this case its inhibitory activity is very similar to that of [Leu^4]oxytocin. [Phe^2,Leu^4]oxytocin was also shown to have some inhibitory activity in this system, but the effect was much less than that of [Ile^2,-Leu^4]oxytocin. This suggests that there may be a correlation between the natriuretic activity of these peptides and their inhibitory effect on the AVP stimulated cyclic AMP production.

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