SYNTHESIS OF S-BENZYL-DL-[1-^{13}C]CYSTEINE AND ITS INCORPORATION INTO OXYTOCIN AND [8-ARGININE]VASOPRESSIN AND RELATED COMPOUNDS BY TOTAL SYNTHESIS. SEPARATION OF DIASTEREOISOMERS BY PARTITION CHROMATOGRAPHY AND HPLC*

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Summary

S-Benzyl-DL-[1-^{13}C]cysteine was prepared from Na^{13}CN by a three step synthesis and converted to the t-butyloxycarbonyl derivative which was suitable for use in peptide synthesis. This compound was incorporated into the 1 and 6 positions of a variety of oxytocin and [8-arginine]vasopressin derivatives and analogues via total synthesis using the solid phase method. The compounds were separated and purified by partition chromatography on Sephadex and their purity was checked by high pressure liquid chromatography. The compounds synthesized include [1-hemi-[1-^{13}C]cystine]oxytocin, [1-hemi-D-[1-^{13}C]-cystine]oxytocin, [1-hemi-[1-^{13}C]cystine, 8-arginine]vasopressin, [1-hemi-D-[1-^{13}C]cystine, 8-arginine]vasopressin, [6-hemi-[1-^{13}C]cystine]oxytocin, [6-hemi-D-[1-^{13}C]cystine]oxytocin, [1-hemi-D-[1-^{13}C]cystine, 3-D-leucine]-oxytocin, and [1-hemi-[1-^{13}C]cystine, 3-D-leucine]oxytocin.

Key Words: Carbon-13, labeled peptide hormones, oxytocin, solid phase synthesis

*All amino acids except glycine are of the L-configuration unless otherwise noted. Standard abbreviations for amino acids, protecting groups, and peptides as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature are used. Other abbreviations include: HPLC, high pressure (performance) liquid chromatography; NMR, nuclear magnetic resonance; DMB, 3,4-dimethylbenzyl; HOAc,acetic acid; TFA, trifluoroacetic acid; AVP, arginine vasopressin; TLC, thin layer chromatography.

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INTRODUCTION

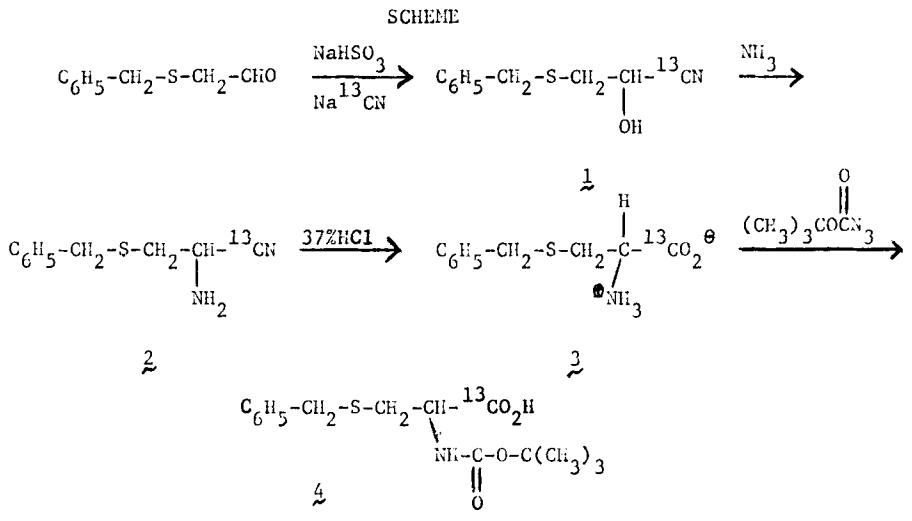
Carbon-13 nuclear magnetic resonance has been used extensively to study the conformational and dynamic properties of peptides in solution and the interactions and binding of these compounds to macromolecules (1-6). In many cases, to obtain unambiguous results(5,6) it is necessary to selectively enrich a carbon atom in the peptide, and this requires the synthesis of specifically labeled amino acids and their incorporation into the peptide chain. For reasons discussed previously (7,8), the most efficient use of the precious labeled amino acid is to incorporate the synthetic racemic amino acid into the peptide and then separate the diastereoisomers by a suitable method such as partition chromatography or high pressure liquid chromatography. We have successfully used this approach to prepare several specifically labeled oxytocin and arginine vasopressin derivatives (5-9). In this paper we report the synthesis of S-benzyl-DL-[1-^{13}C]cysteine and its incorporation by total synthesis into a variety of oxytocin (H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2) and arginine vasopressin (H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2) derivatives and analogues.

DISCUSSION

S-Benzyl-DL-[1-^{13}C]cysteine was prepared using $Na^{13}CN$ as the source of label following the procedure of Gawron and Glaid (10). The bisulfite adduct of benzylthioacetal 1 was allowed to react with $Na^{13}CN$, and the product, ß-benzylthio-α-amino-[1-^{13}C]propionitrile (2) was hydrolyzed in 37% aqueous HCL to give S-benzyl-DL-[1-^{13}C]cysteine (3) as outlined in the scheme. This was converted to N—Boc-S-benzyl-DL-[1-^{13}C]cysteine (4) using t-butyloxycarbonyl-azide, and this protected amino acid derivative was incorporated into either the one or the six position of the hormone derivatives.

The solid phase synthesis (11) of all the specifically labeled hormone derivatives and analogues as well as the subsequent removal of protecting groups, work-up and purifications were done as described elsewhere for similar oxytocin and arginine vasopressin derivatives (7-9) to give the diastereomeric peptide mixture. The diastereoisomer products were separated and purified using

SCHEME



partition chromatography on Sephadex (7,9,12) followed by gel filtration. The diastereoisomeric peptide which were prepared, the solvent systems used to separate and purify these mixtures, the partition chromatography R_f values, and the yield of each isomeric peptide are given in Table 1.

TABLE 1

Compound	Solvent System[a]	R_f[b]	Yield[c]
[1-Hemi-DL-[1-^{13}C]-cystine]oxytocin (5)	A	D-0.33 L-0.23	38.2% 49.3%
[6-Hemi-DL-[1-^{13}C]-cystine]oxytocin (6)	A	D 0.32 L 0.23	32.8% 30.8%
[1-Hemi-DL-[1-^{13}C]-cystine, 3-D Leucine]-oxytocin (7)	A	D-0.39 L-0.20*	30.7% 25.6%
[3-D-Leucine]oxytocin (8)	A	D 0.19	66.0%
[1-Hemi-DL-[1-^{13}C]cystine, 8-arginine]vasopressin (9)	B	D 0.19 L 0.12	50.2% 47.2%

[a]Solvent system used for partition chromatography separation and purification: A. 1-butanol-3.5% aqueous HOAc in 1.5% pyridine (1:1): B. 1-butanol-ethanol-3.5% aqueous HOAc containing 1.5% pyridine (4:1:5); [b]partition chromatography R_f values are given; * [1-hemi-[1-^{13}C]cystine, 3-D-leucine]oxytocin; [c]Yields based on purified product obtained from protected nonapeptide.

The purity of the compounds was checked by direct comparison with authentic unlabeled derivatives previously prepared using thin layer chromatography, high pressure liquid chromatography, bioassay in the milk ejecting assay, and amino acid analysis (Table 2). In the case of the [1-hemi-[1-^{13}C]cystine, 3-D-leucine]-oxytocin diastereoisomers, they were identified by comparison with authentic unlabeled [3-D-leucine]oxytocin synthesized in the usual manner (see Experimental Section for details).

EXPERIMENTAL SECTION

General

Capillary melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Thin-layer chromatography was performed on silica gel G plates using the following solvent systems: (a) 1-butanol-acetic acid-water (4:1:5, upper phase only); (b) 1-butanol-acetic acid-pyridine-water (15:3:10:12); (c) 1-pentanol-pyridine-water (7:7:6); and (d) ethyl acetate-pyridine-acetic acid-water (5:5:1:3). The load size was 40-80 μg, and the chromatogram lengths were 130-160 mm. Detection was made by ninhydrin, iodine vapor, and fluorescamine. Nuclear magnetic resonance spectra were obtained using a Varian Model T-60 spectrometer or a Bruker Model WH-90 FT spectrometer. Amino acid analyses were obtained by the method of Spackman, Stein, and Moore (13) on a Beckman Model 120C amino acid analyzer after hydrolysis in 6 N HCl for 22-24 hr. Solvents used for partition chromatography were purified as previously reported (14). All organic solutions were dried over anhydrous Na_2SO_4 before removal of solvents.

N-Boc protected amino acids were purchased from Vega Biochemicals or from Biosynthetika, or were prepared by published procedures except as discussed below. Before use in synthesis, purity was checked by melting point determination, by thin layer chromatography in solvents systems a, b, and c, and by the ninhydrin teste to detect free amino groups.

For HPLC work the following system (Waters Associates, Milford, Massachusetts)

was used: two Model 6000-A pumps and Model U6K injector, a Model 660 solvent programmer, and a Model 440 dual channel UV detector set for monitoring 254 and 280 nm absorbance simultaneously. Two μ-Bondapac C_{18} reverse phase columns (0.39 cm x 30 cm), connected in series using a 3 cm stainless steel connector, were used for the analytical analyses. In previous studies (8, 15, 16) we have shown how utilizing this equipment and a variety of solvent systems the diastereoisomers of oxytocin and some of its analogues could be separated. In this work a solvent system composed of acetonitrile (Burdick and Jackson, glass distilled, Muskegon, Michigan) and 0.10 M aqueous ammonium acetate, pH 4.0, generally was used. Both solvents were filtered (Millipore, Bedford, Massachusetts), the former through a FHLP-0.5 μM filter, the latter through a HAWP-0.45 μM filter, and then degassed in vacuo just prior to use.

β-Benzylthio-α-hydroxy-[1-^{13}C]propionitrile (1)

To a stirred solution of 6.5 g of sodium bisulfite in 20 ml of water was added 11.1 g of benzylthioacetaldehyde to give a pasty mass. A solution of 20 g $Na^{13}CN$ (90% ^{13}C-enriched) in 7 ml of water was added portionwise. After 30 minutes the reaction mixture was extracted with benzene (4 x 25 ml). The benzene solution was washed with water. Removal of benzene gave 6.3 g (88%) of 1 as an oil; NMR: δ ($CDCl_3$), δ 2.80 (d, 2H), δ 3.65 (broad, 1H), δ 3.85 (s, 2H), δ 4.43 (t, 1H), δ 7.30 (s, 5H).

β-Benzylthio-α-amino-[1-^{13}C]propionitrile (2)

The above hydroxynitrile (6.3 g) was allowed to react with anhydrous ammonia at 100° for 30 minutes. After cooling, the reaction mixture was dissolved in 30 ml of benzene and extracted with 10% aqueous HCl (4 x 20 ml). The combined aqueous extracts were made alkaline to pH 9 with concentrated ammonium hydroxide and extracted with benzene (4 x 25 ml). The benzene solution was evaporated to dryness to give 3.25 g (40.0%) of 2 as an oil; NMR: δ ($CDCl_3$), δ 1.95 (broad, 2H), δ 2.80 (d, 2H), δ 3.75 (t, 1H), δ 3.90 (s, 2H), δ 7.30 (s,5H).

S-Benzyl-DL-[1-^{13}C]Cysteine (3)

The above aminonitrile (3.25 g) was refluxed with 37% HCl (38 ml) for 4 h.

It was then cooled and the pH was brought to 6 with concentrated ammonium hydroxide. The precipitate was filtered and it was further purified by reprecipitating from 29% ammonium hydroxide followed by addition of acetic acid to pH 6 to give 2.2 g (50%) of (3) m.p. 212-213°C (lit. (10) m.p. 213-214°C)TLC in solvent system (a) and (b) showed it to be a single compound identical in behavior to authentic unlabeled S-benzyl-L-cysteine.

N-Boc-S-Benzyl-DL-[1-^{13}C]Cysteine (4).

The title compound was prepared from 3 using a pH stat titration at pH 10.2 in the normal manner (17). Starting from 736 mg of S-benzyl-DL-[1-^{13}C]cysteine and 1.0 g of t-butyloxycarbonylazide there was obtained 900 mg (83%) of the title compound 4 m.p. 109-110°C (lit. (6a) m.p. 108-109°C - [2-^{13}C]-labeled derivative). NMR: δ($CDCl_3$), δ 1.45 (s, 9H), δ 2.90 (d, 2H), δ 3.72 (s, 2H), δ 4.50 (broad, 1H), δ 5.35 (broad, 1H), δ 7.30 (s, 5H), δ 8.35 (s, 1H).

General Procedures for Peptide Syntheses.

All oxytocin and arginine vasopressin peptides and their derivatives were prepared by solid phase method using N-Boc-Gly-O-resin which had a substitution level of 0.40-0.51 mmol/g of resin. Removal of the N-Boc protecting groups by TFA neutralization of the peptide resin salts with diisopropylethylamine, and addition of the next amino acid residue followed methods similar to those previously reported. Coupling steps generally were single 90-minute couplings with a three fold excess of protected amino acid and DCC. The labeled Boc amino acids were coupled at a much reduced excess (1.2-1.5 equivalents) and for longer periods. If the coupling was incomplete (ninhydrin test), as was the case for the synthesis of [6-hemi-DL-[1-^{13}C]cystine]oxytocin and 1-hemi-DL-[1-^{13}C]cystine, 3-D-leucine]oxytocin, a second coupling with 0.6 equivalents of the labeled Boc-amino acid was performed. Uncoupled labeled amino acid was not recovered. For the active ester coupling of asparagine and glutamine an equimolar amount of 1-hydroxybenzotriazole was added to the reaction mixture as a catalyst. N-t-Butyloxycarbonyl protection was used throughout. Benzyl and 3, 4 dimethylbenzyl groups were used to protect the sulfhydryl groups of cysteine; the tyrosine hydroxyl group was unprotected; the guanidyl group of arginine was tosyl protected.

After each amino acid residue was coupled, the synthesis was monitored for completion of coupling by use of the ninhydrin test (18). A negative test (99.4% reaction) was indicated at each step. At the completion of the synthesis the N-Boc protecting group was removed by TFA, the peptide resin neutralized, and the peptide-resin dried in vacuo.

The protected peptide was cleaved from the resin by stirring for 3 days in freshly prepared anhydrous methanol saturated with anhydrous ammonia (7-9). The solvent was removed by rotary evaporation in vacuo and the peptide extracted with two 50 ml portions of DMF at 40°C. The DMF solution was concentrated and diluted with H_2O. The product was filtered and reprecipitated from acetic acid-ethanol and water.

The protected nonapeptide was dissolved in 100 ml of freshly distilled anhydrous ammonia and treated with a sodium stick until a blue color persisted for 30-45 s. The ammonia was removed by evaporation under a slow stream of nitrogen and the last 10 ml by lyophilization. The white powder was dissolved in 300-500 ml of deaerated 0.1% aqueous HOAc under nitrogen. The pH was adjusted to 8.5 with 3 N NH_4OH, and the deprotected peptide was oxidized with 50 ml of 0.01 N $K_3Fe(CN)_6$ for 30 min. The pH was readjusted to 4 with 10% aqueous HOAc and the ferro and excess of ferricyanide ions were removed by addition of Rexyn 203 (Cl^-form). After 20 minutes, the resin was removed by filtration and washed with three 25 ml portions of 20% aqueous HOAc. About 25 ml of 1-butanol was added to the combined aqueous solutions and the solution was concentrated to about 100 ml by rotary evaporation. The solution was lyophilized and the powder was dissolved in 4 ml of upper phase and 2 ml of lower phase of a solvent system and subjected to partition chromatography on a 2.8 x 60 cm column of Sephadex G-25 (block polymerizate, 100-200 mesh)which had been equilibrated with the upper and lower phases. The fractions corresponding to the each peak were pooled and isolated and the required product was futher purified by gel filtration on Sephadex G-25 using 0.2 N HOAc as the eluent solvent. The solvent systems used and the R_f values of each product are given in Table 1. The purity of each compound was checked by TLC in three solvent systems and by HPLC.

Solid Phase Synthesis of [1-Hemi-DL-[1-^{13}C]cystine]oxytocin(5) and Separation and Purification of the Diastereoisomers [1-Hemi-[1-^{13}C]cystine]- (5a) and 1-Hemi-D-[1-^{13}C]cystine]oxytocin (5b).

The solid phase synthesis of H-DL-[1-^{13}C]Cys-(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-O-Resin was accomplished using 2.0 g of N-Boc-Gly-O-Resin which had a substitution level of 0.51 mmol/g resin. Following its synthesis, the nonapeptide was removed from the resin by ammonolysis to give 750 mg of H-DL-[1-^{13}C]-Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2. A 340 mg portion of the peptide gave 67 mg of the all L isomer 5a and 52 mg of the D diastereoisomer 5b. The amino acid analyses of the two diastereoisomers are given in Table 2. Each peptide gave single uniform spots on TLC using solvent systems (a), (b), and (c) identical with authentic samples (9b). Milk ejecting activities (19) were identical with authentic samples. HPLC analysis was done as previously reported (15, 16), and showed the compounds to be pure (>99%) diastereoisomers.

Table 2

Amino Acid Analysis of Oxytocin and Vasopressin Derivatives and Analogues

Compound	Quantitative Amino Acid Ratios								
	Arg	Asp	Glu	Pro	Gly	Half-Cys	Ile	Leu	Phe	Tyr
[1-Hemi-[1-^{13}C]cystine]-oxytocin (5a)		1.03	1.01	1.03	1.00	1.83	1.03	1.00		0.88
[1-Hemi-D-[1-^{13}C]cystine]-oxytocin (5b)		1.00	0.98	1.07	1.00	1.91	0.98	1.02		0.90
[6-Hemi-[1-^{13}C]cystine-oxytocin (6a)		1.01	1.04	1.05	0.98	1.92	0.97	1.03		0.86
[6-Hemi-D-[1-^{13}C]cystine]-oxytocin (6b)		1.04	1.04	0.95	0.95	1.89	0.90	1.04		0.87
[1-Hemi-[1-^{13}C]cystine, 3-D-Leucine]oxytocin (7a)		0.98	1.00	0.95	0.94	2.06		2.06		0.90
1-Hemi-D-[1-^{13}C]cystine, 3-D-Leucine]oxytocin (7b)		0.99	1.00	0.96	0.96	2.10		2.06		0.90
[3-D-Leucine]oxytocin (8)		0.97	1.01	0.97	0.94	2.04		2.06		0.88
[1-Hemi-[1-^{13}C]cystine, 8-Arginine]vasopressin(9a)	0.98	1.00	1.05	1.10	1.00	1.88			0.96	0.96
[1-Hemi-D-[1-^{13}C]cystine, 8-Arginine]vasopressin(9b)	0.96	1.02	1.03	1.07	1.00	1.79			0.98	0.92

Solid Phase Synthesis [6-Hemi-DL-[1-^{13}C]cystine]oxytocin (6) and Separation and Purification of the Diastereoisomers [6-Hemi-[1-^{13}C]cystine]- (6a) and [6-Hemi-D-[1-^{13}C]cystine]oxytocin(6b).

The solid phase synthesis of H-Cys(DMB)-Tyr-Ile-Gln-Asn-DL-[1-^{13}C]Cys(Bzl)-Pro-Leu-Gly-O-Resin was accomplished using 2.5 g of N-Boc-Gly-O-Resin which had a substitution level of 0.40 mmol/g resin. Following the synthesis the peptide resin was subjected to ammonolysis to give 600 mg of H-Cys(DMB)-Tyr-Ile-Gln-Asn-DL-[1-^{13}C]Cys(Bzl)-Pro-Leu-Gly-NH_2. A 320 mg portion gave 41 mg of [6-Hemi-[1-^{13}C]cystine]oxytocin (6a) and 38.5 mg of [6-hemi-D-[1-^{13}C]cystine]oxytocin (6b). The amino acid analyses of the two diastereoisomers are given in Table 2. Each peptide gave single uniform spots on TLC using solvent systems (a), (b), and (c). Milk ejecting activities (19) were identical with authentic samples. HPLC analysis was done as previously reported (15, 16) and showed the compounds to be pure (>99%) diastereoisomers.

Solid Phase Synthesis of [1-Hemi-DL-[1-^{13}C]cystine, 3-D-Leucine]oxytocin (7) and Separation and Purification of Diastereoisomers [1-Hemi-[1-^{13}C]cystine, 3-D-leucine]oxytocin (7a), and [1-Hemi-D-[1-^{13}]cystine, 3-D-leucine]oxytocin(7b).

H-DL-[1-^{13}C]Cys(Bzl)-Tyr-D-Leu-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-O-Resin was prepared using 1.0 g of N-Boc-Gly-O-Resin which had a substitution level of 0.40 mmol/g resin. Following the synthesis the peptide resin was subjected to ammonolysis to give 350 mg of the nonapeptide H-DL-[1-^{13}C]-Cys(Bzl)-Tyr-D-Leu-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-NH_2. From 250 mg there was obtained 25 mg of 7a and 30 mg of 7b. The amino acid analyses of the two diastereoisomers are given in Table 2. Each peptide gave single uniform spots on TLC using solvent systems (a) and (b). HPLC analysis was done using the same solvent system as previously reported for [3-D-Leucine]oxytocin (8) and showed the compounds to be pure (>99%) diastereoisomers.

Solid Phase Synthesis of [3-D-Leucine]oxytocin (8).

The synthesis of H-Cys(DMB)-Tyr-D-Leu-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-O-Resin was carried out using 1.0 g of N-Boc-Gly-O-Resin (substitution level of 0.40 mmol/g resin). The peptide was cleaved from the resin by ammonolysis to give

250 mg of H-Cys(DMB)-Tyr-D-Leu-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-NH_2. From 250 mg of the protected nonapeptide there was obtained 66.5 mg of 8. The amino acid analysis is given in Table 2. TLC in solvent system (a) and (b) gave single uniform spots. HPLC using methods previously reported (8) showed the compound to be the pure isomer identical with the sample previously prepared (8). The compound had 0.31 units/mg of milk ejecting activity (19).

Solid Phase Synthesis of [1-Hemi-DL-[1-^{13}C]cystine, 8-arginine]vasopressin (9) and Separation and Purification of Diastereoisomers [1-Hemi-[1-^{13}C]cystine, 8-arginine]vasopressin (9a) and [1-Hemi-D-[1-^{13}C]cystine, 8-arginine]vasopressin (9b).

H-DL-[1-^{13}C]Cys(Bzl)-Tyr-Phe-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-O-Resin was synthesized using 2.4 g of Boc-Gly-O-Resin with a substitution level of 0.42 mmol/g resin as starting material. The peptide was cleaved from the resin by ammonolysis to give 720 mg of H-DL-[1-^{13}C]-Cys(Bzl)-Tyr-Phe-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2. A 360 mg portion gave 69 mg of [1-hemi-[1-^{13}C]cystine, 8-arginine]vasopressin (9a) and 65 mg of [1-hemi-D-[1-^{13}C]cystine, 8-arginine]vasopressin (9b). The amino acid analyses are given in Table 2. TLC in solvent system (a), (b), and (d) showed the compounds to give single uniform spots identical to diastereoisomers previously prepared (7). HPLC in a solvent system similar to that used for oxytocin diastereoisomers (15, 16) showed the compound to be pure and uncontaminated by each other. The milk-ejecting activity of 9a was the same as that of authentic AVP (18).

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