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SYNTHESIS OF DL-[2-^{13}C]LEUCINE AND IT USE IN THE PREPARATION OF [3-DL-[2-^{13}C]LEUCINE]OXYTOCIN AND [8-DL-[2-^{13}C]LEUCINE]OXYTOCIN

PREPARATIVE SEPARATION OF DIASTEREOISOMERIC PEPTIDES BY PARTITION CHROMATOGRAPHY AND HIGH PRESSURE LIQUID CHROMATOGRAPHY[1]

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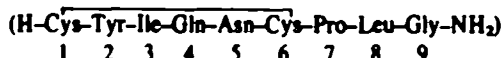
Abstract—DL-[2-^{13}C]Leucine was prepared by condensing the sodium salt of ethyl acetamido-[2-^{13}C]cyanoacetate with isobutylbromide in hexamethylphosphoroustriamide followed by acid hydrolysis. N-Boc-DL-[2-^{13}C]Leucine was prepared and incorporated into [8-DL-[2-^{13}C]leucine]oxytocin by total synthesis. The ^{13}C-labeled hormone derivative [8-[2-^{13}C]leucine]oxytocin was separated from its 8-position diastereoisomer by partition chromatography. The specifically ^{13}C-labeled peptide hormone diastereoisomeric analog [3-DL-[2-^{13}C]leucine]oxytocin also was prepared by solid phase peptide synthesis. No suitable solvent system for partition chromatography separation of the latter diastereoisomeric peptide mixture could be found. However an excellent preparative separation of the diastereoisomers could be obtained by reverse phase high pressure liquid chromatography on a partisil 10 M9 ODS column using the solvent system 0.05 M ammonium acetate (pH 4.0), acetonitrile (81:19, v/v) to give pure [3-[2-^{13}C]leucine]oxytocin and [3-D-[2-^{13}C]leucine]oxytocin. An excellent separation of [8-[2-^{13}C]leucine]oxytocin and the corresponding 8-D-leucine diastereoisomer derivative could also be accomplished by high pressure liquid chromatography.

The potential use of specific ^{13}C-enriched amino acids, peptides, and proteins for a variety of biological and chemical-physical studies[2-8] of their structure, dynamics, metabolism, etc. has been recognized recently. However, at present only a limited number of studies have been done owing to the limited availability and high cost of these compounds. Some of the common amino acids can be obtained from hydrolysis of proteins obtained from micro-organisms which are grown on ^{13}C-enriched carbonate or other ^{13}C-enriched sources.[9,10] However, a number of common amino acids are not obtained or are obtained only in small quantities by these methods. In addition they usually are uniformly labeled, often at low levels (< 70%) of ^{13}C enrichment. While this is acceptable for some applications, it is often necessary or desirable to have available specifically labeled amino acids. One of the major methods for identifying the labeled compound and for using the ^{13}C labeled amino acids and peptides for physical-chemical studies is ^{13}C NMR spectroscopy.[2-8,11-13] Uniformly labeled amino acids give complicated ^{13}C NMR spectra with each resonance line of greatly reduced intensity relative to the single line of an isolated ^{13}C atom in a specifically labeled compound. For these and other reasons, including the need for a wide variety of natural and non-natural ^{13}C labeled amino acids and peptides, it is desirable to have available specific ^{13}C-labeled derivatives of these compounds at a high level of ^{13}C-enrichment. For these purposes, simple synthetic methods in which the highly enriched ^{13}C label is introduced only where desired, are needed. In addition, it is desirable to introduce the labeled amino acid derivatives into peptides in the most efficient manner possible so that a maximum of useful physical, chemical, and biological data can be obtained. We needed diastereoisomers of the peptide hormone oxytocin



(H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH$_2$)
1 2 3 4 5 6 7 8 9

with [2-^{13}C]leucine at positions 3 and 8 of the hormone to study the interactions of the hormone at these positions with its natural carrier proteins, the neurophysins by NMR.§ In this paper we report a simple, high yield synthesis of DL-[2-^{13}C]leucine (90% ^{13}C-enriched), its incorporation by total synthesis into the peptide hormone, and the separation and purification of these diastereoisomeric peptides.

Most syntheses of amino acids give at least partially racemic products, and require resolution before incorporation into the peptide. The alternative approach is to incorporate the racemic amino acid into the peptide and then separate the diastereoisomeric peptides. In view of the fact that D-amino acid-containing diastereoisomers often have interesting biological,[16-18] physical and chemical properties, the latter approach seemed to be more attractive because it not only affords the diastereoisomers simultaneously, but also eliminates the necessity of going through the extra steps involved in the resolution of the racemic amino acids. Moreover, the resolution of some amino acids (particularly radiolabeled) can be difficult and expensive. We have been successfully utilizing this approach[19,20,24] in separating various diastereoisomeric hormone peptides by partition chromatography.

The synthesis of DL-[(2-^{13}C)]leucine (3) with 90% ^{13}C

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§The NMR results will be discussed elsewhere.

enrichment was accomplished in about 56% overall yield according to the literature procedure,[21] except that the condensation of the sodium salt of ethyl acetamido [2-^{13}C]cyanoacetate (1) with *isobutylbromide* was done *in* anhydrous hexamethylphosphoroustriamide (HMPT) to give the product 2 in 70% yield. The condensation in anhydrous ethanol[21] gave 2 in only 50% yield in our *hands. The* product was readily converted to 3 in refluxing 6 N HCl (Experimental). Several other syntheses of leucine were examined, but none were superior. A synthesis similar to that outlined above, but utilizing diethyl acetamidomalonate, gave a slightly higher overall yield (about 64%), but the cost of preparing or buying 2-^{13}C-labeled diethyl acetamidomalonate is considerably greater than that of 1 per mmole (~30%).

DL-[2-^{13}C]Leucine was readily converted to the t-butyloxycarbonyl (Boc) derivative by pH stat titration[22] to give Boc-DL-[2-^{13}C]leucine 4 in 87% isolated yield.

The total syntheses of the partially ^{13}C-labeled oxytocin derivatives were accomplished using the solid phase method,[23] as utilized in our laboratory.[19,24] The major exception to these procedures was in the coupling of Boc-DL-[2-^{13}C]leucine (4) to the growing peptide chain. To insure maximal utilization of the valuable labeled amino acid 4, 0.8 equivalents of 4 was coupled to 1.0 equivalent of glycylbenzhydrylamine resin[19] using dicyclohexylcarbodiimide (DCC, 0.8 equivalent) as coupling reagent. After 3 hr, the coupling was nearly quantitative. The unreacted resin amino groups were then acylated with N-acetylimidazole and the synthesis was continued to give the protected peptide resin precursor to [8-DL-[2-^{13}C]leucine]oxytocin (7) (Experimental). In the preparation of the nonapeptide resin precursor to [3-DL-[2-^{13}C]leucine]oxytocin (8), Boc-DL-[2-^{13}C]leucine (4) was incorporated into the growing peptide chain using about 1.2 equivalents of 4 and of DCC, and two additional couplings using about 0.2 equivalents of 4 and DCC (Experimental).

At the conclusion of the synthesis the N-terminal t-butyloxycarbonyl group was removed. The disulfhydryl nonapeptide was cleaved from the resin in its C-terminal carboxamide form by treatment with anhydrous HF containing 10% anisole at 0°,[19] and was then oxidized in aqueous solution under nitrogen[25] with 0.01 N $K_3Fe(CN)_6$.[26] [8-L-[2-^{13}C]Leucine]oxytocin (7a) and [8-D-[2-^{13}C]leucine]oxytocin (7b) were readily separated from one another and from synthetic impurities by partition chromatography on Sephadex G-25[27] using the solvent system 1-butanol-3.5% aqueous acetic acid in 1.5% pyridine (1:1) (Fig. 1). The peptide hormone oxytocin contains a leucine *residue* at position 8, and hence 7a is a ^{13}C-labeled oxytocin, while 7b is the 8-position diastereoisomer. The former compound was indistinguishable from authentic oxytocin in all respects except its

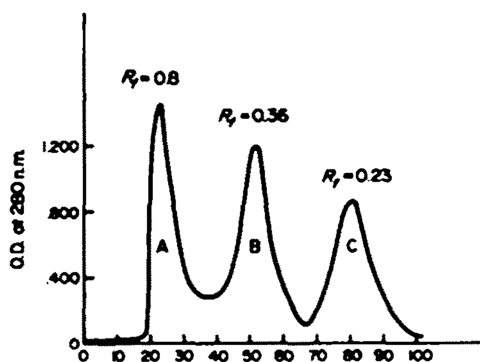

Fig. 1. Partition chromatography separation of [8-[2-^{13}C]-leucine]oxytocin (C) from [8-D-[2-^{13}C]leucine]oxytocin (B) and side products (A) on Sephadex G-25 using the solvent system 1-butanol-3.5% aqueous acetic acid containing 1.5% pyridine (1:1).

^{13}C NMR spectrum in which the α-carbon in Leu-8 is much more intense (>80 times) than the natural abundance nuclei. The chemical shift corresponds to that previously reported for the Leu-8 Cα in dimethylsulfoxide (DMSO)[28] and aqueous[29] solutions. The purity of the diastereoisomers was checked by tlc, by quantitative amino acid analysis, and by reverse phase high pressure liquid chromatography (Fig. 2). The ^{13}C-labeled hormone 7a had identical milk-ejecting activities[30] as the unlabeled natural hormone, while the D-diastereoisomer had only slightly reduced activity (Experimental).

Separation of the diastereoisomeric mixture [3-DL-[2-^{13}C]-leucine]oxytocin (8) could not be accomplished by partition chromatography on Sephadex G-25 using the solvent system 1-butanol-3.5% aqueous acetic acid containing 1.5% pyridine (1:1) (Fig. 3). Evaluation of several other solvent systems[20] provided no system which gave a distinguishable separation of the two diastereoisomers. In addition, separation of the diastereoisomers was not obtained on tlc on silica gel in several different solvent systems. It therefore was decided to examine the potential of preparative reverse phase high pressure liquid chromatography for this separation.

High pressure liquid chromatography has been shown to have considerable potential in the analysis of small peptides,[31-36] and we have recently shown that it can be used to analytically separate diastereoisomeric peptides.[36] Since very few methods are available for preparative separation of diastereoisomeric peptides we decided to examine the potential of hplc for this purpose. A successful development of this procedure would also be useful in numerous other physical, chemical, and biological applications.

For the purpose of obtaining a preparative separation

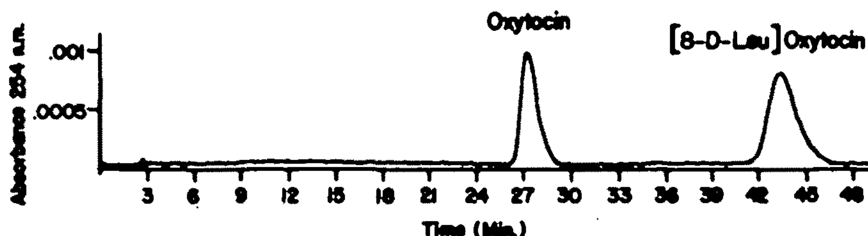

Fig. 2. Composite reverse phase high pressure liquid chromatography of purified [8-D-[2-^{13}C]leucine]oxytocin (right), [8-[2-^{13}C]leucine]oxytocin (left) (ca. 4 nmol ea) on 2μ Bondapak C_{18} columns 0.39 × 30 cm, 0.05 M NH_4OH pH 4.0/CH_3CN (82:18 v/v), 2.0 ml/min, 0.005 AFS at 254 nm.

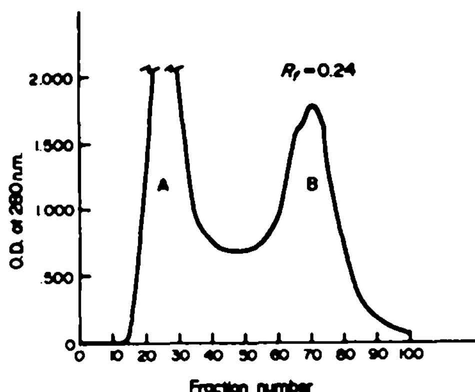

Fig. 3. Partition chromatography of [3-[2-^{13}C]leucine]oxytocin and [3-D-[2-^{13}C]leucine]oxytocin (B) and side products (A) on Sephadex G-25 using the solvent system 1-butanol-3.5% aqueous acetic acid containing 1.5% pyridine (1:1).

of the diastereoisomers, we first evaluated several parameters which effect hplc separations on the analytical scale. A baseline separation of the diastereoisomers [3[2-^{13}C]leucine]oxytocin (8a) and [3-D-[2-^{13}C]leucine]oxytocin (8b) was achieved using reverse phase high pressure liquid chromatography (hplc) employing 2 μ Bondapac C_{18} columns (0.39 × 30 cm), and a solvent system consisting of 82% 0.05 M ammonium acetate (pH 4.0) and 18% acetonitrile. With this information, we then undertook a preparative separation of the diastereoisomers 8a and 8b, using a Whatman Partisil 10 M9 ODS column (0.94 × 50.0 cm). Due to the inherent differences of the preparative and analytical columns, slight adjustments in chromatographic conditions were necessary. Figure 4 shows the chromatogram for a 15 mg single injection separation of the DL diastereoisomers 8a and 8b, using 19% acetonitrile, 81% 0.05 M NH_4OAc, pH 4.0 as eluent solvent. By cutting out a small intermediate fraction (Fig. 4), a complete separation of the DL diastereosiomers was obtained. This is illustrated in Fig. 5

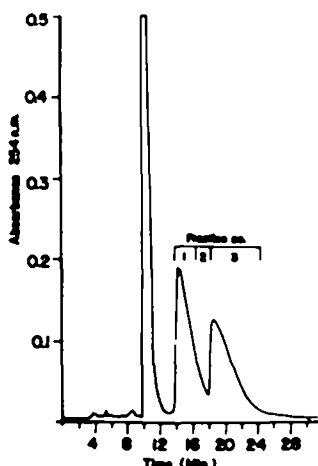

Fig. 4. Preparative reverse phase high pressure liquid chromatography separation of [3-DL-[2-^{13}C]leucine]oxytocin (8) (ca. 15 mg) on a Partisil 10 M9 ODS column, 0.94 × 50 cm, 0.05 M NH_4OAc, pH 4.0/CH_3CN (81:19, v/v), 6.0 ml/min, 0.50 AFS. Fraction 1 is [3-D-[2-^{13}C]leucine]oxytocin (8b); fraction 3 is [3-L-[2-^{13}C]leucine]oxytocin (8a); fraction 2 is the diastereoisomeric mixture; the peak before fraction 1 is non-peptide material, probably pyridinium acetate.

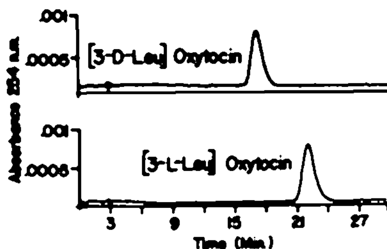

Fig. 5. Reverse phase high pressure liquid chromatography of [3-D-[2-^{13}C]leucine]oxytocin (top) and [3-L-[2-^{13}C]leucine]oxytocin (bottom) (ca. 3 nmol ea.) on 2μ Bondapak C_{18} columns 0.39 × 30 cm, 0.01 M NH_4OAc pH 4.0/CH_3CN (82:18, v/v), 2.0 ml/min, 0.005 AFS at 254 nm, following preparative separation illustrated in Fig. 4.

which shows analytical hplc of the isolated hplc-purified diastereoisomers 8a and 8b. That 8a is actually the [3-L-[2-^{13}C]leucine]oxytocin was shown by an independent synthesis of the unlabeled all L oxytocin analogue [3-leucine]oxytocin by standard procedures (Experimental), and comparison of its hplc with the labeled compound.

Thus, using reverse phase hplc, we have been able to develop the synthesis, separation, and purification of specifically ^{13}C-labeled hormone diastereoisomeric analogs. The general procedure, with appropriate solvent system, column packing, or other modifications should prove very useful in preparing various labeled derivatives (^{14}C, ^{3}H, ^{2}H, ^{125}I, etc.) of peptide hormone without the need to resolve precious labeled enanteomeric amino acid derivatives before incorporating them into the growing chain. We are currently examining the application of these preparative procedures to other biologically active peptides and to other peptide analogs and derivatives in our laboratory.

EXPERIMENTAL

Capillary m.ps were determined on a Thomas–Hoover melting point apparatus and are uncorrected. Tlc was performed on silica gel G plates using the following solvents systems: (A) 1-BuOH–AcOH–water (4:1:5, upper phase only); (B) 1-BuOH–AcOH–pyridine–water (15:3:10:12); (C) 1-pentanol–pyridine–water (7:7:6). The load size was 40–80 μg, and the chromatogram lengths 130–160 mm. Detection was made by ninhydrin, iodine vapor and fluorescamine. Optical rotations were measured at the mercury green line (547 nm) using a Zeiss Old 4 polarimeter. NMR spectra were obtained using a Varian T-60 spectrometer or a Bruker WH-90 FT spectrometer. Amino acid analyses were obtained by the method of Spackman *et al.*[37] on a Beckman 120 C amino acid analyzer after hydrolysis in 6 N HCl for 22–24 hr. Elemental analyses were performed by Chemalytics, Inc. and Spang Microanalytical Laboratory. Hplc was performed using the following Waters Associates (Milford, Mass.) equipment: Two model 6000-A pumps, a model U6K injector, a model 660 solvent programmer and a model 440 dual channel UV detector which was set to monitor 254 nm and 280 nm absorbance simultaneously. Analytical separations reported here were accomplished using 2μ Bondapak C_{18} columns (0.39 × 60 cm) (Waters Assoc.) connected in series. The solvent system consisted of 0.05 M ammonium acetate adjusted to pH 4.0 with AcOH and 18% acetonitrile (Burdick and Jackson, glass distilled, Muskegon, Mich.) (v/v), and the flow rate used was 2 ml/min. Preparative separations employed a Partisil 10 M9 ODS column (0.94 × 50 cm) (Whatman Inc.), the eluent solvent 81% 0.05 M NH_4OAc, pH 4.0, 19% acetonitrile (v/v), and a flow rate of 6 ml/min. Immediately prior to use, both solvents were filtered, the aqueous solution through a Millipore (Bedford, Mass.) HAWP-0.45 μm filter, the acetonitrile through a millipore FHLP-0.50 μm

filter, and then degassed in vacuo. Solvents used for partition chromatography were purified as previously reported.[38]

N-Boc protected amino acids were purchased from Vega-Fox Biochemicals and from Biosynthetica or were prepared by published procedures except as discussed below. Before use in synthesis, purity was checked by m.p. determination, by tlc in solvent systems A, B, C, and by the ninhydrin test[39] to detect free amino groups.

Solid phase peptide synthesis procedures. The chloromethylated resin used in the syntheses (polystyrene crosslinked with 1% divinylbenzene, LS 601 Merrifield Resin, Lab Systems, Inc., San Mateo, Ca.) was substituted with Boc-glycine at the level of 0.40 mmol/g resin by the method of Gisin,[40] and stored at 4° in the protected form. The benzhydrylamine resin was prepared as previously described[19,41] and then converted to the Boc-glycinamido-resin at a glycine substitution level of 0.40 mmol/g resin. Syntheses were performed on semi-automated instruments designed and built in our laboratory.[41] The synthetic procedures were similar to those previously reported except for the coupling of the ^{13}C labeled amino acids.[19,20,34] Syntheses were carried out using 8–10 ml of washing solvent or reagent solution per gram of starting resin. Coupling steps were monitored for completion by the ninhydrin procedure.[39] Cleavage of the peptide from the resins was accomplished as outlined before. Examination of potential solvent systems suitable for partition chromatography separation of diastereoisomeric peptides was accomplished as previously reported.[20,38]

Ethyl acetamidocyano-[2-^{13}C]-isocaproate (2). A mixture of sodium hydride (from 2.50 g of 57% sodium hydride dispersed in oil) and 1.7 g of ethyl acetamidocyano-[2-^{13}C]acetate (1, KOR Isotopes, Cambridge, Massachusetts) in 13 ml hexamethylphosphorotriamide (HMPT) was stirred for 1 hr. To the clear solution 1.4 ml of isobutylbromide was added dropwise and the soln was stirred at room temp. for 3 hr and then at 85–90° for 2 hr. The reaction was run under a N_2 atmosphere. The soln was cooled and diluted with 75 ml of ice-water. The ppt was filtered off to give 1.59 g (70%) of 2, m.p. 113–115° (lit.[23] m.p. unlabeled 120°); NMR ($CDCl_3$) δ 1.33 (t, 3H), 1.9–2.2 (m, 3H), 2.05 (s, 3H), 4.28 (q, 2H), 1.02 (2d, 6H), 7.50 (bd, 1H).

DL-[2-^{13}C]*Leucine* (3). A sample of 1.5 g of the above nitrile 2 was hydrolysed with 12 ml 6 N HCl at 120° for 16 hr. The soln was filtered and the filtrate was evaporated to dryness in vacuo at 30°. The residue was dissolved in 8 ml water and the pH was adjusted to 6 with NH_4OH. The mixture was chilled overnight and filtered to give 650 mg of DL-[2-^{13}C]leucine. A second crop of 3 was obtained by diluting the filtrate with an equal volume of EtOH and refrigerating for 2 days. Total yield of product 3, 705 mg (79.1%); m.p. 265–279° (lit.[21] m.p., unlabeled 278°).

N-*Boc*-DL-[2-^{13}C]*Leucine* (4). A mixture of 700 mg of DL-[2-^{13}C]leucine (3) was stirred in 1.8 ml of peroxide-free dioxane and 1.2 ml of H_2O. t-Butylazidoformate (1.2 ml) was added, and the pH was kept at 10.0 using 4.0 N NaOH (Radiometer Autotitrator). After 31 hr additional amounts of water (1.5 ml) and t-butylazidoformate (0.6 ml) were added. The soln was washed with three 30 ml portions of ether. The aqueous phase was adjusted to pH 3 with citric acid, saturated with NaCl and then extracted with five 30-ml of portions of EtOAc. The combined organic phases were dried over Na_2SO_4 and evaporated to dryness in vacuo. The residue was heated with three 50-ml portions of petroleum ether (b.p. 30°) and filtered. The combined organic solns were concentrated to 10 ml and 2 drops of H_2O were added. The mixture was allowed to stand overnight, and then the crystals were filtered off and washed with a little ice-cold petroleum ether to give 1.01 g (87%) of product; m.p. 104–106° (lit.[22] m.p. for Boc-L-leucine·H_2O 78–81°); tlc in solvent systems A, B and C gave single spots identical in R_f to authentic Boc-L-leucine; NMR ($CDCl_3$) δ 0.97 (d, 6H), 1.4–2.0 (m, 3H), 1.44 (s, 9H), 4.16 (m, 1H), 5.97 (m, 1H) and 10.87 (s, 1H). $C_{10}{}^{13}CH_{21}NO_4$ requires: C, 57.32; H, 9.05; N, 6.03. Found: C, 57.16; H, 9.06, N, 6.09%.

Boc-DL-[2-^{13}C]*Leucylglycinamide-resin* (5). A 3.0 g portion of benzhydrylamine resin crosslinked with 1% divinylbenzene and substituted with Boc-glycine at the level of 0.51 mmol/g by the method of Hruby et al.[19] was deprotected and neutralized. Limited coupling was done with 0.301 g (1.2 mmol) of Boc-DL-[2-^{13}C]leucine and an equivalent molar quantity of dicyclohexylcarbodiimide (DCC) for 3 hr. After appropriate washes, the unreacted amino groups were treated with 0.35 g (3.2 mmol) of N-acetylimidazole in methylene chloride for 3 hr to give 3.2 g of 5. At the conclusion, a ninhydrin test was negative. Using modified[43] aldimine[44] test a leucyl substitution level of 0.416 mmol/g was obtained.

Solid phase synthesis of Cys(*DMB*)-*Tyr*-*Ile*-*Gln*-*Asn*-*Cys*(*DMB*)-*Pro*-DL-[2-^{13}C]*Leu*-*Gly*-NH-*Resin* (6). The solid phase synthesis was carried out on a Vega series 95 automated synthesizer, a machine similar to that described by Hruby et al.[42] using 5 as starting material and standard procedures described above. After the last coupling, the peptide-resin was filtered and dried in vacuo and was found to have increased in weight by 0.95 g.

[8-DL-[2-^{13}C]*Leucine*]*oxytocin* (7) *and separation of diastereoisomers* 7a *and* 7b. A portion of the above resin (1 g) was cleaved with 20 ml anhyd HF in the presence of 2 ml of anisole at 0° for 1 hr. After the removal of HF under reduced pressure, the resin was washed with four 25-ml portions EtOAc. The peptide material was extracted into five 25-ml portions of 1 N HOAc. The resin was washed with 200 ml deionized water, and the total volume was brought to 600 ml with deionized water. The soln was adjusted to a pH of 8.5 with 3 N NH_4OH and the compound was oxidized with 60 ml 0.01 N $K_3Fe(CN)_6$ for 30 min. The pH was adjusted to 5 with 20% HOAc and Rexyn 203 (Cl^- form) was added to remove ferro- and excess ferricyanide. The mixture was stirred for 20 min, the resin was filtered off and washed with three 25-ml portions of 20% aqueous HOAc. About 50 ml of 1-BuOH was added to the combined aqueous solns and the soln was concentrated at 20–30° to about 175 ml by rotary evaporation. The soln was lyophilized and the mixture was chromatographed on a Sephadex G-15 column (100 × 1.8 cm) using 30% HOAc as eluent solvent. Fractions corresponding to the products were pooled and lyophilized. The powder was dissolved in 4 ml of upper phase and 2 ml of lower phase of the solvent system 1-BuOH-3.5% aqueous HOAc containing 1.5% pyridine (1:1) and subjected to partition chromatography on a 2.8 × 60 cm column of Sephadex G-25 (block polymerizate, 100–200 mesh) which had been equilibrated with the upper and lower phases.[27] One hundred 5.7-ml fractions were collected. Analyses of the fractions by UV absorbance at 280 nm revealed three peaks, a by-product peak at R_f 0.7, a peak at R_f 0.36 representing the D-7b, and a peak at R_f 0.23 representing the L-7a (Fig. 1). The products were isolated separately, and the highly purified peptides were each further purified by gel filtration chromatography. There was obtained 49 mg of 7b $[\alpha]^{25}_{547}$ +11.7° (c 0.513; 1 N HOAc) (lit.[45] $[\alpha]^{25}_{D}$ +12° (c 0.5, 1 N HOAc). Amino acid analysis gave the following molar ratios: Asp, 1.03; Glu, 1.04; Gly, 1.03; Half-Cys, 1.89; Pro, 0.94; Ile, 1.03; Tyr, 0.93; Leu, 1.10. The compound showed single uniform spots on tlc in solvent systems A, B and C. The milk ejecting activity[30] was about 250 units/mg. There was also obtained 39 mg of 7a; $[\alpha]^{25}_{547}$ −20.7° (c 0.576, 1 N HOAc). Amino acid analysis gave the following molar ratios: Asp, 1.03; Glu, 1.05; Gly, 1.02; Half-Cys, 1.88; Pro, 0.95; Ile, 1.04; Leu, 1.04; Tyr, 0.90. The compound showed single uniform spots on tlc in the solvents system A, B and C identical to authentic oxytocin. The milk-ejecting activity[30] was found to be about 500 units/mg.

Solid phase synthesis of Cys(*DMB*)-*Tyr*-[DL-[2-^{13}C]*Leu*-*Gln*-*Asn*-*Cys*(*DMB*)-*Pro*-*Leu*-*Gly*-NH-*Resin*. Boc-Leucylglycinamido-Resin with a peptide substitution level of 0.41 mmol/g was prepared, and a 2.0 g portion was used to synthesize the title compound. The complete coupling with Boc-DL-[2-^{13}C]leucine at the 3 position was achieved by one 48-hr coupling with 0.247 g (1 mmol) of the amino acid in CH_2Cl_2 and two additional couplings with 0.033 g of the amino acid in DMF. The peptide resin was filtered and dried in vacuo and found to have increased in weight by 0.65 g.

[3-DL-[2-^{13}C]*Leucine*]*oxytocin* (8). 1.3 g of the above resin was cleaved with liquid HF, oxidized with $K_3Fe(CN)_6$ as described earlier, and the salt was removed by chromatography on Sephadex G-15. Attempted separation of the mixture by partition chromatography on Sephadex G-25 using 1-BuOH 3.5% aqueous

HOAc containing 1.5% pyridine (1:1) as eluent solvent gave two fractions corresponding to a by-product, $R_f \sim 0.7$, and 139 mg of the peptide 8, $R_f = 0.24$ (broad peak) (Fig. 3).

Preparative separation of [3-DL-[2-^{13}C]leucine]oxytocin (8) into its diastereoisomers 8a and 8b by reverse phase high pressure liquid chromatography. The preparative separation of [3-DL-[2-^{13}C]leucine]oxytocin into 8a and 8b was accomplished by dissolving 147 mg of the mixture of 8 from the preceding section in 2.0 ml of the hplc solvent system and injecting 100 μl (15–20 mg) samples of the diastereoisomeric mixture per run. Three (3) fractions were collected as shown in Fig. 4. After 147 mg of the mixture 8a had been chromatographed, the pooled fractions were lyophilized and then desalted by gel filtration chromatography on Sephadex G-25 using 0.2 N AcOH as eluent solvent. There was obtained 38.6 mg of 8b. The compound gave a single peak on analytical hplc as shown in Fig. 5. Amino acid analysis gave the following molar ratios: Asp, 1.00; Glu, 1.05; Pro, 1.02; Gly, 1.02; Half-Cys, 1.87; Leu, 1.92; and Tyr, 0.89. A 33.3 mg sample of 8a was obtained. The compound gave single uniform spots on tlc, identical with authentic [3-leucine]oxytocin (see below) using solvent systems A, B and C. The compound 8a also gave a single peak on analytical hplc as shown in Fig. 5 with an identical retention time to authentic [3-leucine]oxytocin (see below). Amino acid analysis gave the following molar ratios: Asp, 1.00; Glu, 1.07; Pro, 1.04; Gly, 1.04; Half-Cys, 1.81; Leu, 1.98; Tyr, 0.87. In addition 14 mg of the mixture 8 was obtained from the intermediate fractions of Fig. 4.

Solid phase synthesis of Cys(DMB)-Tyr(Bzl)-Leu-Gln-Asn-Cys(DMB)-Pro-Leu-Glycinate-Resin. The synthesis was carried out using 2.5 g of Boc-glycinate-resin which had a substitution level of 0.40 mmol/g. The synthetic procedures were similar to those used previously (Table 1).[19,24] At the end of the synthesis the N-terminal Boc protecting group was removed in the usual manner to give 3.4 g of the title compound.

[3-L-Leucine]oxytocin (9). The above resin was ammonolized with anhyd MeOH saturated with anhyd NH_3 (freshly distilled from Na) for 3 days. The peptide was extracted with DMF and precipitated with water. A portion of the protected peptide (320 mg) was treated with Na in liquid NH_3 and oxidized with $K_3Fe(CN)_6$ in the usual manner. Purification of the crude product by partition chromatography on Sephadex G-25 using 1-BuOH–3.5% aqueous HOAc containing 1.5% pyridine (1:1) gave the title compound with an $R_f = 0.22$. The compound was further purified by gel filtration on Sephadex G-25 using 0.2 N HOAc as eluent solvent. The compound showed a single uniform spot on tlc in solvent systems A, B and C. The compound gave a single peak on reverse phase hplc using the same solvent system and conditions used in other analytical hplc experiments reported here. The milk-ejecting activity was 65 units/mg. Amino acid analysis gave the following molar ratios: Asp, 1.00; Glu, 1.02; Pro, 0.92; Gly, 1.00; Half-Cys, 2.06; Leu, 2.01; Tyr, 0.87.

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[1]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 [*J. Biol. Chem.* 247, 977 (1972)] are used. Other abbreviations include: NMR; HMPT, hexamethylphosphorotriamide; hplc, high pressure liquid chromatography; TFA, trifluoroacetic acid; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DMB, 3,4-dimethylbenzyl; HOBT, 1-hydroxybenzotriazole; DMF, dimethylformamide; HOAc, acetic acid.

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