tetramethylpiperidinyl carbonate, 58229-35-9; 1-oxido-2,2,6,6-tetramethylpiperidinol, 58229-36-0; p-nitrophenyl chloroformate, 7693-46-1.

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Synthesis of Specifically Deuterated *S*-Benzylcysteines and of Oxytocin and Related Diastereomers Deuterated in the Half-Cystine Positions[1,2]

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S-Benzylcysteine derivatives specifically deuterated at the α carbon only, the β carbon only, and at both the α and β carbons have been synthesized. These labeled compounds have been enzymatically resolved and the enantiomers and reacemates have been converted to the *N-tert*-butyloxycarbonyl derivatives. The deuterium labels were not exchanged under the conditions of the syntheses. Condensation of the sodium salt of diethyl α-acetamidomalonate with benzyl chloromethyl sulfide followed by hydrolysis with DCl afforded *S*-benzyl-DL-[α-$^2H_1$]cysteine. Acetylation followed by treatment with hog renal acylase separated the stereoisomers. A Mannich reaction with [$^2H_2$]methylene diacetate, diethyl α-acetamidomalonate, and dimethylamine followed by quaternization of the amino nitrogen with methyl iodide gave diethyl α-acetamido-α-dimethylamino[$^2H_2$]methylmalonate methiodide (**15**). Treatment of **15** with sodium benzylmercaptide gave diethyl α-acetamido-α-benzylthio[$^2H_2$]methylmalonate, which was hydrolyzed with HCl to yield *S*-benzyl-DL-[β,β-$^2H_2$]cysteine or with DCl to afford *S*-benzyl-DL-[α,β,β-$^2H_3$]cysteine. These compounds were resolved as before. The preparation of *S*-benzyl-DL-[α,β,β-$^2H_3$]cysteine required an efficient source of ethanol-*d*. This deuterated solvent was prepared in quantitative yield in 2 h from tetraethoxysilane, D_2O, and a catalytic amount of thionyl chloride. The protected deuterated amino acids were used in the preparation of several oxytocin analogues in which the specific deuteration appears in either the 1-hemicystine or the 6-hemicystine residues.

Specific deuterium labels in amino acids, peptides, and proteins are very useful for studying the chemical, biological, and especially the physical properties of these molecules. If the label is introduced at nonexchangeable positions, it provides a durable marker which does not significantly alter the properties of the molecule.[3-13]

The usefulness of such labels has been demonstrated for proteins in studies of structure and folding.[14-19] Unambiguous proton[8-11,20-22] and carbon-13[12,13,23] nuclear magnetic resonance assignments for many peptides have been made using specifically deuterated derivatives. The development of deuteron magnetic resonance spectroscopy has opened the way for a direct study of the microdynamical behavior of specific segments of the neurohypophyseal peptide hormones and their binding to the neurophysins, their biological carrier proteins.[24,25] These studies have begun to provide insights into the conformational aspects of these biologically active compounds, giving more direct evidence for structure–activity relationships than has been available from amino acid substitutions, which are more likely to perturb the structure of the molecule.[26-29]

In order to perform these experiments it is necessary to prepare the appropriate specifically deuterium labeled amino acid or amino acid derivative by a synthetic route which maintains the integrity of the deuterium label throughout the synthesis.

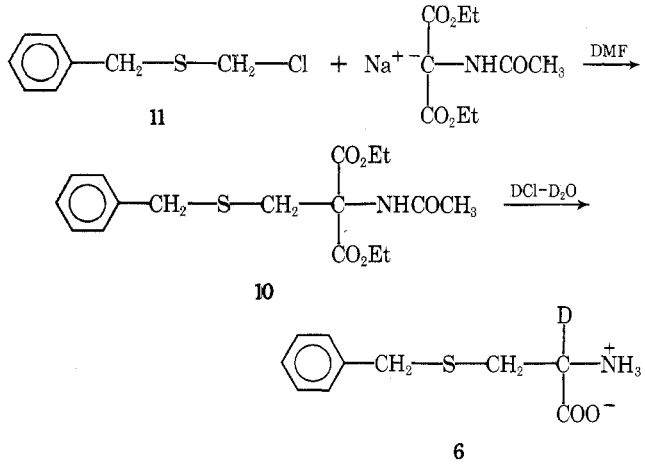
We report here the total synthesis of *S*-benzylcysteine deuterated specifically in the α position only, the β positions only, and in both the α and β positions. The specifically deuterated cysteine derivatives were also resolved into their enantiomeric pairs without loss of the label, and one was used for the synthesis of the specifically labeled oxytocin [6-hemi[β,β-$^2H_2$]cystine]oxytocin (**2**). The racemic compounds were used to incorporate specifically deuterated cysteine into the half-cystine positions of the oxytocin derivatives [6-hemi-DL-[α-$^2H_1$]cystine]oxytocin (**3**), [1-hemi-DL-[β,β-$^2H_2$]cystine]oxytocin (**4**), and [1-hemi-DL-[α,β,β-$^2H_3$]cystine]oxytocin (**5**) and the diastereomeric pairs were separated by partition chromatography on Sephadex G-25.[31,32,45]

H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2
1 2 3 4 5 6 7 8 9
1

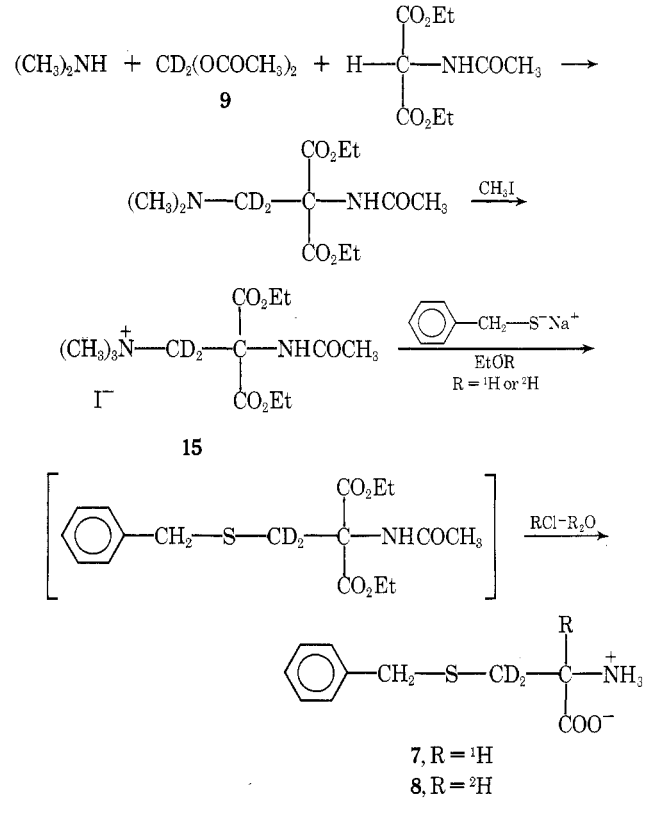
The synthesis of each of the partially deuterated *S*-benzylcysteine derivatives was accomplished using different approaches. The *S*-benzyl-DL-[α-$^2H_1$]cysteine (**6**) was made by displacement of chloride from benzyl chloromethyl sulfide by sodium diethyl acetamidomalonate followed by hydrolysis of the adduct with 11 N DCl in D_2O (Scheme I).

The *S*-benzyl-DL-[β,β-$^2H_2$]cysteine (**7**) and *S*-benzyl-DL-[α,β,β-$^2H_3$]cysteine (**8**) were made using a modification of the methods of Cornelius[30] and Atkinson, et al.[33] This approach involved making the Mannich adduct of diethyl acetamidomalonate, formaldehyde, or its equivalent, and

Scheme I



Scheme II



dimethylamine. The adduct was quaternized with methyl iodide, and the product was treated with sodium benzylmercaptide. Upon hydrolysis, the resulting product gave *S*-benzyl-DL-cysteine (Scheme II).

In place of formaldehyde we used [α,α-$^2H_2$]methylene diacetate (**9**). Scrupulously anhydrous conditions were required before **9** could be isolated in good yield. Since the Mannich adduct is deuterated, one expects to obtain **7** when HCl is used in the hydrolysis step, and **8** when DCl is used. This approach gave **7** as expected with >95% deuteration at the β positions. However, when the synthesis of **8** was attempted in this manner, we obtained the *S*-benzyl-DL-cysteine derivative which was >90% **7** (virtually undeuterated in the α position). The reaction of sodium benzylmercaptide with the quaternary salt was done for 6 days in refluxing ethanol. Isolation and characterization (NMR) of the reaction product revealed that it was not the expected adduct but was ethyl *N*-acetyl-*S*-benzyl-DL-[β,β-$^2H_2$]cysteinate (>90%). Apparently, during the course of the reaction, ethanolysis, decarboxylation, and subsequent proton abstraction from the solvent by the attendant carbanion lead to the unexpected product. When the displacement reaction was run in ethanol-*d* (**12**), however, the same ethanolysis and decarboxylation occurred, but this time a deuteron was abstracted from the solvent rather than a proton. After DCl hydrolysis **8** was obtained with >95% deuteration at both the α and β positions. These experiments required a rapid and inexpensive source of ethanol-*d*. The published[34] preparation of ethanol-*d* from tetraethoxysilane and D_2O gave a 90% yield after 24 h reaction and distillation of the product. We modified this approach by the addition of a catalytic amount (ca. 0.3%) of thionyl chloride which produced a slight concentration of D^+ in solution.[35] We have obtained ethanol-*d* in 99.9% yield after only 2 h reaction time which was pure as judged by NMR and gas chromatography. *S*-Benzyl-DL-[β,β-$^2H_2$]cysteine (**7**) was readily resolved into its enantiomers by N-acetylation followed by reaction with hog renal acylase.[36] A similar treatment of **6** and **8** separated the enantiomers without loss of label at either the α or β positions. The resulting *S*-benzyl-L-[β,β-$^2H_2$]cysteine (**7a**) as well as **6**, **7**, and **8** were readily converted to *N*-*tert*-butyloxycarbonyl (N-Boc) derivatives using the general method employing *tert*-butyl azidoformate.[37] No exchange of deuterium was noted in either of the above procedures.

The solid-phase synthesis[38] of the oxytocin derivatives was carried out on an automated Vega Model 95 synthesizer, a solid-state version of our automated instrument,[39] or on a semiautomated apparatus designed and built in our laboratory. The methodology used is shown in Table I. Each coupling was >99.4% complete as judged by the ninhydrin test.[40] It was of considerable interest that the active ester couplings of asparagine and glutamine catalyzed by the presence of 1 molar equiv of 1-hydroxybenzotriazole were complete after 3.5 and 5 h, respectively. Without the catalyst, these couplings normally require 8–14 h. Similar catalysis of nitrophenyl ester coupling in solution peptide synthesis has been reported.[41] At the conclusion of the synthesis, the N-terminal Boc group was removed and the protected nonapeptide amide was obtained by ammonolysis (in methanol) of the corresponding peptide-resin ester.

The nonapeptide was deprotected with sodium in liquid ammonia[42] and then oxidized under nitrogen[43] with 0.01 N $K_3Fe(CN)_6$.[44] The isomers were separated from each other and from by-products by partition chromatography on Sephadex G-25 using the solvent system 1-butanol–3.5% HOAc in 1.5% aqueous pyridine (1:1).[45] At R_f 0.32, the peak for the hemi-D-cysteine derivatives of oxytocin appeared, and at R_f 0.24, the peak for the oxytocin derivatives appeared, both R_f values in close agreement with our previous experience with diastereomeric mixtures.[45] The diastereomers were isolated and further purified by gel filtration on Sephadex G-25 using 0.2 N HOAc as eluting solvent. The purity of the products was checked by thin layer chromatography in three solvent systems, quantitative amino acid analysis, and optical rotation determination.

The milk ejecting activities[46] of the partially deuterated oxytocin derivatives were indistinguishable within experimental error from those of the native hormone. The partially deuterated hemi-D-cystine derivatives all had milk ejecting activities greatly reduced compared to those of oxytocin. These derivatives have been used for the assignment of α,β and carbonyl carbons in ^{13}C NMR spectra,[13,47] and except for these carbon atoms the ^{13}C NMR spectra were indistinguishable from those of authentic oxytocin.

Experimental Section

Thin layer chromatography (TLC) was done on silica gel G plates using the following solvent systems: (A) 1-butanol–acetic

Table I. Solid-Phase Peptide Synthesis Methodology

	Normal DCC Coupling			Nitrophenyl ester coupling (for Asn and Gln only)			
Step	Solvent or reagent	Time, min	No. of times	Step	Solvent or reagent	Time, min	No. of times
1	CH_2Cl_2	1	4	1	CH_2Cl_2	1	4
2	Ninhydrin test		1	2	Ninhydrin test		1
3	TFA–CH_2Cl_2–anisole (25:73:2)	2	1	3	TFA–CH_2Cl_2–anisole (25:73:2)	2	1
4	TFA–CH_2Cl_2–anisole (25:73:2)	20	1	4	TFA–CH_2Cl_2–anisole (25:73:2)	20	1
5	CH_2Cl_2	1	3	5	CH_2Cl_2	1	3
6	DIEA–CH_2Cl_2 (10:90)	2	2	6	DIEA–CH_2Cl_2 (10:90)	2	2
7	CH_2Cl_2	1	4	7	CH_2Cl_2	1	4
8	Amino acid[a] (1.5 equiv)–CH_2Cl_2		1	8	DMF	1	5
9	DCC (1.2 equiv)–CH_2Cl_2	20	1	9	Amino acid[a] (4 equiv)–DMF, 1-Hydroxybenzotriazole (4 equiv)	210–300	1
10	CH_2Cl_2	1	2				
11	100% EtOH	1	2	10	DMF	1	3
12	CH_2Cl_2	1	3	11	CH_2Cl_2	1	2
13	Amino acid (1.5 equiv)–CH_2Cl_2		1	12	100% EtOH	1	2
14	DCC (1.2 equiv)–CH_2Cl_2	20	1				
15	CH_2Cl_2	1	2				
16	100% EtOH	1	2				

[a] All amino acids are N^{α}-Boc protected.

acid–water (4:1:5, upper phase only); (B) 1-butanol–acetic acid–pyridine–water (15:3:10:12); (C) 1-pentanol–pyridine–water (35:35:30). Capillary melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were obtained using a Varian T-60 NMR spectrometer or a Bruker WH-90 NMR spectrometer. Amino acid analyses were obtained by the method of Spackman, Stein, and Moore[48] on a Beckman 120 C amino acid analyzer after hydrolysis in 6 N HCl for 22 h. Optical rotation values were measured at the mercury green line (547 nm) using a Zeiss Old 4 polarimeter. Elemental analyses were performed by Spang Microanalytical Laboratory or Heterocyclic Chemical Corp., and deuterium analyses were performed by Joseph Nemeth, Urbana, Ill.

Benzyl Chloromethyl Sulfide (11). The title compound was made in 59% yield following the reported procedure:[49] bp 102 °C (2 mm) [lit. 102 °C (2 mm)]; NMR (neat) δ 3.55 (s, 2 H), 4.10 (s, 2 H), 7.00 (s, 5 H).

Diethyl α-Acetamido-α-benzylthiomethylmalonate (10). A 1.73-g portion of sodium hydride (50% in oil, 0.036 mol) was placed in a dry, nitrogen-filled reaction flask, covered with dry dimethylformamide (DMF), and cooled to 0 °C. A solution of 7.83 g (0.036 mol) of diethyl α-acetamidomalonate in 40 ml of DMF was added to the cold hydride mixture over 20 min. Stirring was continued until hydrogen evolution ceased (ca. 1 h). Benzyl chloromethyl sulfide (6.15 g, 0.036 mol) was added, and the flask was placed in a 90–100 °C bath for 66 h. The mixture was cooled, filtered, and concentrated in vacuo to a brown oil. Trituration with 20 ml of water was followed by three 5-ml chloroform extractions. The combined organic layers were dried (K_2CO_3) and filtered, and the filtrate concentrated in vacuo to a brown solid which was treated eight times with 20-ml portions of boiling hexane. Each time the clear hexane layer was decanted from an oily lower layer. As the hexane cooled, white needles were deposited: yield 6.68 g (53% of theory); mp 87.3–88 °C; NMR ($CDCl_3$) δ 1.25 (t, 6 H), 2.05 (s, 3 H), 3.55 (s, 2 H), 3.75 (s, 2 H), 4.25 (q, 4 H), 6.95 (s, broad, 1 H), 7.30 (s, 5 H). Anal. Calcd for $C_{17}H_{23}NS$: C, 57.79; H, 6.53; N, 3.98. Found: C, 57.35; H, 6.54; N, 4.13.

S-Benzyl-DL-[α-$^2H_1$]cysteine (6). A 6.0-g portion of 10 was treated with ca. 11 M DCl in D_2O (made by adding 13 ml of $SOCl_2$ dropwise to 31.3 ml of cold D_2O) at reflux for 6 h. After cooling slightly, Norit was added and the mixture was briefly brought to boiling. The mixture was rapidly filtered and the filtrate was evaporated to dryness in vacuo. The residue was taken up in about 25 ml of water, and concentrated NH_4OH was added to pH 5.5. After filtration of the resulting crystals and drying over NaOH in vacuo, there was obtained 2.33 g (65% of theory) of 6: mp 215.5–217 °C (lit. for protio compound,[49] 215–216 °C); NMR (CF_3COOH) δ 2.75 (s, 2 H), 3.40 (s, 2 H), 3.60–3.80 (α-CH, undetectable), 6.90 (s, 5 H).

N-Boc-S-benzyl-DL-[α-$^2H_1$]cysteine (13). The title compound was made in 70% yield following the reported procedure,[37] mp 110.5–111.0 °C (lit.[45] 111–111.3 °C, lit.[37] 63–65 °C). Single, uniform spots were obtained on TLC using systems A, B, and C with R_f values identical with those of the protio analog: NMR ($CDCl_3$) δ 1.45 (s, 9 H), 2.90 (s, 2 H), 3.75 (s, 2 H), 4.40–4.60 (α-CH, undetectable), 5.35 (broad, 1 H), 7.30 (s, 5 H). Quantitative deuterium analysis indicated 96% deuteration (calcd 4.75 atom %, found 4.55 atom %).

S-Benzyl-L-[α-$^2H_1$]cysteine (6a). The resolution of 6 was done using the reported procedure for the protio compound[36] with minor modifications. The starting material, N-acetyl-S-benzyl-DL-[α-$^2H_1$]cysteine, was obtained by adding 1.25 g of 6 to 3 ml of H_2O and treating the resulting slurry with 0.9 g of Na_2CO_3 and 1.15 ml of acetic anhydride. After 20 min, the homogenous mixture was acidified with concentrated HCl to pH 1. There was obtained 1.42 g (95%), mp 154.5–155.2 °C (lit.[36] for protio analog, mp 157 °C). The acetylated amino acid was slurried in 90 ml of water and the pH was adjusted to 7.5 with NH_4OH. The total volume was brought to 110 ml and acylase I (hog kidney, Calbiochem, 0.23 g) was added and the mixture was stirred at 38 °C for 2 days maintaining the pH. An additional 57 mg of enzyme was added and the mixture stirred 2 days more. The pH was lowered to 5 with acetic acid, a few milliliters of butanol were added and the mixture was concentrated to about 20 ml. The mixture was filtered and the filtrate was acidified with concentrated HCl to give 640 mg of N-acetyl-S-benzyl-D-[α-$^2H_1$]cysteine (90.5%), mp 140–141 °C. The residue on the filter was transferred to a flask where it was boiled with 1 N HCl and filtered. This process was repeated and the combined filtrates were neutralized with NH_4OH, giving 390 mg of S-benzyl-L-[α-$^2H_1$]cysteine (64%), mp 215–218 °C, $[\alpha]^{21}_{547}$ −17.4° (c 1, 5 N HCl) [lit.[36] $[\alpha]^{25}_{D}$ −19.5° (c 1, 5 N HCl), commercial amino acid (Fox 4978) $[\alpha]^{24}_{547}$ −17.1°]. A quantitative deuterium analysis showed the compound to be 97% deuterated (calcd 7.69 atom %, found 7.52 atom %).

Dibromo[$^2H_2$]methane (14).[28] Dibromomethane (55 g) was successively exchanged with protions of 10% NaOD in D_2O by rapidly stirring at reflux for 24-h periods. After five exchanges using 22-, 13-, 12-, 11-, and 7-ml portions of base, the exchange was judged to be about 80% complete by NMR. Three additional exchanges employing 12 ml each of 10% NaOD in D_2O gave 20.2 g of 14 which was >95% deuterated.

[α,α-$^2H_2$]Methylene Diacetate (9).[33] A 20-g portion of 14 was mixed with 66 ml of anhydrous acetic acid (distilled from triacetyl-

borate), 6.7 ml of acetic anhydride (freshly distilled after standing over P_2O_5), and 32.2 g of anhydrous potassium acetate under dry nitrogen in a flask equipped with a mechanical stirrer. After all the reagents were added, the nitrogen inlet was replaced with a condenser, and the mixture was refluxed for 24 h. Stirring was continued while the mixture cooled to room temperature. Ether (200 ml) was added and the mixture was filtered. The filter cake was washed with four 50-ml portions of ether. The ether and acetic acid were distilled from the mixture at atmospheric pressure. At 54–55 °C (10 mm) [lit.[33] 61–63 °C (12 mm)] there was obtained 7.63 g (49% of theory) of **9** which was >95% deuterated.

Diethyl α-Acetamido-α-dimethylamino[$^2H_2$]methylmalonate Methiodide (15). An 11.2-g portion of **9** (0.083 mol) was cooled to −10 °C and 27.8 ml of a 40% aqueous solution of dimethylamine was added dropwise such that the temperature did not exceed 0 °C. Then 14.9 g (0.083 mol) of diethyl α-acetamidomalonate was added in one portion. The flask was sealed and the mixture was stirred at 40 °C for 1 h. The flask was cooled to −10 °C and 20% aqueous NaOH was added to pH 11. The cold mixture was extracted with two 50-ml portions of ether. The combined organic extracts were dried over Na_2SO_4, filtered, and freed of solvent giving a semisolid residue. The residue was taken up in 150 ml of dry ether and 25 ml of methyl iodide was added. The mixture was stirred at 40 °C for 24 h, and then allowed to stand at room temperature for 24 h. The white product was filtered and dried in vacuo to give 24.24 g (70% of theory) of **15**: mp 174–175 °C (lit.[30] 171–173 °C); NMR (D_2O) δ 1.20 (t, 6 H), 2.10 (s, 3 H), 3.15 (s, 9 H), 4.35 (q, 4 H), 4.35 (s, absent).

***S*-Benzyl-DL-[β,β-$^2H_2$]cysteine (7).** Under dry nitrogen, 0.32 g (0.014 mol) of sodium was added to 30 ml of absolute ethanol. When all the sodium had reacted, 1.74 g (0.014 mol) of benzyl mercaptan was added followed by 5.84 g (0.014 mol) of **15**. The mixture was refluxed for 6 days, cooled, and freed of solvent. The residue was disolved in 20 ml of chloroform and 10 ml of cold water was added. The mixture was shaken and separated, and the organic layer was dried over anhydrous K_2CO_3. The drying agent was filtered off and the solvent removed leaving a residue which solidified on standing. Without further purification, the residue was treated with concentrated HCl at reflux for 5.5 h. The mixture was cooled, treated with Norit, and again boiled. The mixture was filtered hot and the filtrate was evaporated to dryness in vacuo. The residue was dissolved in about 20 ml of H_2O and the pH was adjusted to 5.5 with NH_4OH. After filtering and drying, there was obtained 2.14 g (72% from **15**) of white crystals melting at 209–211 °C: NMR (CF_3COOH) δ 2.75 (β,β-CH_2, undetectable), 3.20 (s, 2 H), 3.40–3.60 (1 H), 6.80 (s, 5 H). Quantitative deuterium analysis indicated 97% deuteration (calcd 15.40 atom %, found 14.95 atom %).

***N*-Boc-*S*-benzyl-DL-[β,β-$^2H_2$]cysteine (16).** Employing the same method used to convert **6** to **13**, 3.00 g (0.014 mol) of **7** gave 3.80 g of **16** (87%). The white crystals melted at 110.5–111.0 °C; NMR ($CDCl_3$) δ 1.45 (s, 9 H), 2.90 (β,β-CH_2, undetectable), 3.75 (s, 2 H), 4.40–4.60 (1 H), 5.35 (broad, 1 H), 7.25 (s, 5 H). A single spot was obtained in TLC using systems A, B, and C with R_f values identical with those of the protio analogue.

***S*-Benzyl-L-[β,β-$^2H_2$]cysteine (7a).** A sample of **7** was acetylated and resolved with hog renal acylase as described above for compound **6**. The resolved L isomer (**7a**) had mp 215–218 °C $[\alpha]^{21}_{547}$ −20.7° (*c* 1, 5 N HCl).

***N*-Boc-*S*-benzyl-L-[β,β-$^2H_2$]cysteine (17).** Employing the same method used to convert **6** to **13**, 0.75 g (0.0025 mol) of **7** was converted to 1.01 g of **17** (92%) which remained an oil: NMR ($CDCl_3$) δ 1.50 (s, 9 H), 2.90 (β,β-CH_2, undetectable), 3.75 (s, 2 H), 4.40–4.60 (1 H), 5.40 (broad, 1 H), 7.30 (s, 5 H), 11.60 (s, 1 H); $[\alpha]^{22.5}_{547}$ −48.8° (*c* 1.0, CH_3COOH), for all-protio analogue (Biosynthetica 6145) $[\alpha]^{22.5}_{547}$ −52.2° (*c* 1.0, CH_3COOH). A single spot was obtained on TLC using systems A, B, and C, with R_f values identical with those of the protio analogue.

Ethanol-*d* (12). Under a nitrogen atmosphere, 104.2 g (0.50 mol) of tetraethoxysilane was mixed with 40 g (2.0 mol) of D_2O and a catalytic amount of thionyl chloride (ca. 0.5 ml). After stirring at room temperature for 2 h, the mixture was distilled at 2 mm. The last traces of product were obtained by increasing the vacuum and warming the distillation flask to about 30 °C. There was obtained 93.9 g (99.9% of theory) of **12**. No starting materials contaminated the product as ascertained by gas chromatography and NMR. The infrared spectrum was consistent with spectra of other deuterated alcohols.[50]

***S*-Benzyl-DL-[α,β,β-$^2H_3$]cysteine (8).** The reaction of 13.30 g (0.032 mol) of **15** with sodium benzylmercaptide in ethanol-*d* gave ethyl *N*-acetyl-*S*-benzyl-DL-[α,β,β-$^2H_3$]cysteinate, which was then treated with 11 N DCl in D_2O to give 5.55 g of **8** (81% from **15**). The white crystals melted at 211–212 °C; NMR (CF_3COOH) δ 2.75 (β,β-CH_2, undetectable), 3.40 (s, 2 H), 3.60–3.80 (α-CH, undetectable), 6.90 (s, 5 H).

***N*-Boc-*S*-benzyl-DL-[α,β,β-$^2H_3$]cysteine (18).** A 2.50-g portion (0.0117 mol) of **8** was converted to 3.45 g of **18** (94% of theory) using the same method as used to convert **6** to **13**. The white crystals melted at 111.5–111.8 °C; NMR ($CDCl_3$) δ 1.50 (s, 9 H), 2.90 (β,β-CH_2, undetectable), 3.75 (s, 2 H), 4.40–4.60 (α-CH, undetectable), 5.35 (broad, 1 H), 7.30 (s, 5 H). A single spot was obtained on TLC using systems A, B, and C, with R_f values identical with those of the protio analogue. Quantitative deuterium analysis indicated 95% deuteration (calcd 14.28 atom %, found 13.50 atom %).

Boc-Leucylglycinate-Resin (19). A 5.00-g portion of Boc-glycinate-resin (polystyrene crosslinked with 1% divinylbenzene, LS 601 Merrifield Resin, Lab Systems, Inc., San Mateo, Calif.) substituted with Boc-glycine at the level of 0.64 mmol/g by the method of Gisen[51] was deprotected and neutralized as described in steps 1–7 of Table I. Limited coupling was done with 0.585 g (2.35 mmol) of Boc-leucine·H_2O and an equivalent molar quantity of dicyclohexylcarbodiimide (DCC). The reaction was allowed to proceed for 30 min. After the appropriate washes (see Table I, steps 10–12), the unreacted amino groups were treated with 0.35 g (3.2 mmol) of *N*-acetylimidazole in CH_2Cl_2 for 3 h. At the conclusion, a ninhydrin test was negative. The average of four modified[52] aldimine[53] determinations gave a leucyl substitution level of 0.495 mmol/g.

Solid-Phase Synthesis of *S*-3,4-Dimethylbenzylcysteinyl-*O*-benzyltyrosylisoleucylglutaminylasparaginyl-*S*-benzyl-DL-[α-$^2H_1$]cysteinylprolylleucylglycinate-Resin (20). The solid-phase synthesis of **20** was performed as a Vega Model 95 synthesizer, an automated machine similar to that described by Hruby et al.[39] The preparation was done on a 1.5-mmol scale, which required 3.19 g of the resin **19**. The synthetic cycles are outlined in Table I. All reactions and washes were carried out with 30-ml portions. After the last coupling, the terminal Boc group was removed (Table I, steps 1–6). The peptide-resin was filtered and dried in vacuo and found to have increased in weight by 1.42 g (85% of theory). The peptide-resin **20** was ammonolyzed and the peptide was extracted into DMF and precipitated with water as described elsewhere.[45] There was obtained 1.44 g (74% of theory) of the protected nonapeptide amide H-Cys(DMB)-Tyr(Bzl)-Ile-Gln-Asn-DL-[α-$^2H_1$]Cys(Bzl)-Pro-Leu-Gly-NH_2 (**21**), mp 210–214 °C. Anal. Calcd for $C_{66}H_{90}N_{12}O_{12}S_2 \cdot 4H_2O$: C, 57.47; H, 6.82. Found: C, 57.30; H, 6.95.

[6-Hemi-DL-[α-$^2H_1$]cystine]oxytocin (3). The protecting groups were removed by treatment of 325 mg (0.25 mmol) of **21** with sodium in liquid ammonia (freshly distilled from sodium) and the sulfhydryl groups were oxidized under nitrogen with 50 ml of 0.01 N $K_3Fe(CN)_6$ solution.[44] The product [6-hemi-DL-[α-$^2H_1$]cystine]oxytocin (**3**) was purified by partition chromatography using the solvent system 1-butanol–3.5% aqueous acetic acid in 1.5% pyridine (1:1).[45] Analysis by the Folin–Lowry method[54] showed a small by-product peak at R_f 0.61, and a large, poorly resolved peak at R_f 0.38–0.20 of the incompletely separated diastereomeric oxytocin derivatives. The fractions corresponding to the diastereomeric mixture were pooled and the peptide mixture obtained after isolation was subjected to the same solvent system as above. As found previously[45] the diastereomers were nicely separated with peaks at R_f 0.32 and 0.24 corresponding to [6-hemi-D-[α-$^2H_1$]cystine]oxytocin (**3b**) and [6-hemi[α-$^2H_1$]cystine]oxytocin (**3a**), respectively. The oxytocin peak (R_f 0.24) was isolated to give 70 mg of uncontaminated product. The 6-hemi-D oxytocin derivative as isolated contained small amounts of oxytocin. Repurification by partition chromatography gave 51.4 mg of the pure 6-hemi-D diastereomer **3b**. Both **3a** and **3b** were separately purified by gel filtration chromatography on Sephadex G-25 (200–270 mesh) using 0.2 N acetic acid as eluent solvent. Final purified yields were 64 mg of **3a** and 50 mg of **3b**. Each of the isomers gave single spots on TLC in solvent systems A, B, and C, identical with those of authentic protio analogues. Diastereomer **3a** exhibited a carbon-13 spectrum identical with that of authentic oxytocin except for the absence of the peak corresponding to the 6-hemicystine α carbon. It had $[\alpha]^{22}_{547}$ −22.6° (*c* 0.5, 1 N HOAc). A sample of **3a** was hydrolyzed in 6 N HCl for 24 h at 110 °C. Amino acid analysis gave the following molar ratios: aspartic acid, 1.0; glutamic acid, 1.1; proline, 0.9; glycine, 1.0; half-cystine, 1.9; isoleucine, 1.0; leucine, 1.1; tyrosine, 0.9. Milk-ejecting activity determined using mouse-mammary tissue in vitro[46] was identical with that of authentic oxytocin within experimental error.

Diastereomer **3b** had $[\alpha]^{22}_{547}$ −108.1° (*c* 0.5, 1 N HOAc) [lit.[55] $[\alpha]^{20}D$ −81° (*c* 0.5, 1 N HOAc)]. Amino acid analysis gave the following molar ratios: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.0; glycine, 1.0; half-cystine, 2.0; isoleucine, 0.9; leucine, 1.0; tyrosine, 0.9. Milk-ejecting activity was ca. 34.7 units/mg. Anal. Calcd for $C_{43}H_{65}DN_{12}O_{12}S_2 \cdot CH_3CO_2H$: C, 50.75; H, 6.39; N, 15.79. Found: C, 50.78; H, 6.37; N, 16.19.

Solid-Phase Synthesis of [6-Hemi[β,β-$^2H_2$]cystine]oxytocin (2). Boc-leucylglycinate-resin with a substitution level of 0.27 mmol/g was prepared in an analogous manner to that described for the preparation of **19**. A 3.80-g portion of this resin was used to synthesize the title compound on a 1-mmol scale. A semiautomated apparatus designed and built in our laboratory was used which allowed the reaction vessel to be filled and emptied by application of vacuum at the top and bottom, respectively, of the vessel, and to be shaken mechanically. For each step, 35 ml of solvent was used except for the DIEA neutralization step in which case 40 ml was used. For this synthesis 0.8 equiv of DCC was used for each equivalent of Boc-amino acid.[56] The N-terminal Boc group was removed. The resin was dried and found to have increased in weight by 1.08 g (105%). The protected nonapeptide was cleaved from the resin by ammonolysis. After extraction into DMF, precipitation, filtering, and drying, there was obtained 725 mg of nonapeptide, mp 215–217 °C, and a second crop weighing 390 mg, mp 173–178 °C. A portion of the first crop (325 mg, 0.25 mmol) was deprotected with sodium in liquid ammonia and oxidized with 0.01 N aqueous $K_3Fe(CN)_6$. Purification by partition chromatography using the solvent system 1-butanol–3.5% aqueous acetic acid in 1.5% pyridine (1:1) gave a peak centered at R_f 0.23 corresponding to the title compound **2**. The fractions corresponding to the peak were pooled and lyophilized. Final purification by gel filtration chromatography afforded 63 mg of [6-hemi[β,β-$^2H_2$]cystine]oxytocin. It had $[\alpha]^{24.5}_{547}$ −23.7° (*c* 0.5, 1 N HOAc). Analysis on TLC in solvent systems A, B, and C gave single spots identical with those of an authentic protio analogue. A sample was hydrolyzed in 6 N HCl for 24 h at 110 °C. Amino acid analysis gave the following molar ratios: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.1; glycine, 1.0; half-cystine, 2.0; isoleucine, 0.9; leucine, 1.0; tyrosine, 0.9. Milk-ejecting activity was identical with that of authentic oxytocin within experimental error.

Solid-Phase Synthesis of Boc-*O*-benzyltyrosylisoleucylglutaminylasparaginyl-*S*-3,4-dimethylbenzylcysteinylprolylleucylglycinate-Resin (22). Boc-leucylglycinate-resin with a substitution level of 0.36 mmol/g was prepared in an analogous manner to that described for the preparation of **19**. A 7.50-g portion of this resin was used to synthesize the title compound on a 2.7-mmol scale using the semiautomated apparatus described above in the preparation of **2**. In each step 50 ml of solvent was used except for the DIEA neutralization step in which case 60 ml was used. Again, 0.8 equiv of DCC was used for each equivalent of Boc-amino acid.[56] After each coupling series, a ninhydrin test indicated >99.4% coupling for all but Boc-*O*-benzyltyrosine; in this case a third coupling using 0.5 equiv was performed, but the reaction was only about 94% complete as judged by the ninhydrin test. The unreacted amino groups were acetyl terminated with *N*-acetylimidazole in CH_2Cl_2. The peptide-resin was filtered and dried in vacuo and found to have increased in weight by 2.89 g.

[1-Hemi-DL-[β,β-$^2H_2$]cystine]oxytocin (4) and Separation of the Diastereomers. A 4.75-g portion of the Boc-octapeptide resin **22** was deprotected and neutralized in the usual way (Table I, steps 1–6) and then treated with 0.55 g (1.74 mmol) of **16** and 0.32 g (1.57 mmol) of DCC in CH_2Cl_2 for 30 min. Following the reaction, a ninhydrin test indicated that the reaction was ca. 94% complete. A second coupling with 0.27 g (0.85 mmol) of **16** and 0.16 g (0.80 mmol) of DCC gave >99.4% coupling as judged by the ninhydrin test. The Boc group was removed and the resin dried to give 4.87 g of the protected peptide-resin precursor of **4**. After cleavage from the resin by ammonolysis, extraction of the peptide into DMF, and precipitation with water, 1.43 g of the protected nonapeptide amide was obtained, mp 220–225 °C. A 325-mg portion was deprotected with sodium in liquid ammonia and oxidized in the manner described for compound **21**. After purification by partition chromatography, the uncontaminated L diastereomer was gel filtered on Sephadex G-25 using 0.2 N acetic acid as eluent, and lyophilized to give 40 mg of [1-hemi[β,β-$^2H_2$]cystine]oxytocin (**4a**). It had $[\alpha]^{22}_{547}$ −22.2° (*c* 0.5, 1 N HOAc). A sample of **4a** was hydrolyzed in 6 N HCl for 24 h at 110 °C. Amino acid analysis gave the following molar ratios: aspartic acid, 1.0; glutamic acid, 1.0; proline, 0.9; glycine, 1.0; half-cystine, 2.0; isoleucine, 1.0; leucine, 1.0; tyrosine, 1.0. Development on TLC in systems A, B, and C gave single, uniform spots identical with those of the protio analogue. Milk-ejecting activity was identical with that of authentic oxytocin within experimental error.

A sample of [1-hemi-D-[β,β-$^2H_2$]cystine]oxytocin had $[\alpha]^{22}_{547}$ −68.9° (*c* 0.5, 1 N HOAc) [lit.[32] $[\alpha]^{20}D$ −56° (*c* 0.5, 1 N HOAc)]. Amino acid analysis following hydrolysis for 24 h at 110 °C in 6 N HCl gave the following molar ratios: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.0; glycine, 1.0; half-cystine, 2.0; isoleucine, 1.0; leucine, 1.0; tyrosine, 0.9. Development on TLC in systems A, B, and C gave single, uniform spots identical with those of the protio analogue. Milk-ejecting activity was ca. 38.6 units/mg.

[1-Hemi-DL-[α,β,β-$^2H_3$]cystine]oxytocin (5) and Separation of the Diastereomers. The same process described for the preparation of **4** was repeated, substituting **18** for **16**. There was obtained 4.87 g of nonapeptide-resin which after ammonolysis, extraction, and precipitation gave 1.24 g of the protected nonapeptide amide, mp 227–228 °C. Anal. Calcd for $C_{66}H_{87}D_3N_{12}O_{12}S_2 \cdot 2H_2O$: C, 58.86; H, 7.26; N, 12.48. Found: C, 58.82; H, 6.86; N, 12.49. A 325-mg sample was deprotected and oxidized as described for compound **21**. After the usual chromatographic purifications, there was obtained 77.5 mg of [1-hemi-L-[α,β,β-$^2H_3$]cystine]oxytocin (**5a**) and 72.2 mg of [1-hemi-D-[α,β,β-$^2H_3$]cystine]oxytocin (**5b**). Compound **5a** had $[\alpha]^{22}_{547}$ −24.5° (*c* 0.5, 1 N HOAc). A sample of **5a** was hydrolyzed in 6 N HCl for 24 h at 110 °C. Amino acid analysis gave the following molar ratios: aspartic acid, 1.0; glutamic acid, 1.1; proline, 1.0; glycine, 1.1; half-cystine, 2.0; isoleucine, 1.0; leucine, 1.0; tyrosine, 0.88. On TLC, **5a** gave single, uniform spots in systems A, B, and C. Milk-ejection activity was identical with that of authentic oxytocin within experimental error.

Compound **5b** had $[\alpha]^{22}_{547}$ −68.4° (*c* 0.5, 1 N HOAc). Amino acid analysis gave the following molar ratios: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.1; glycine, 1.0; half-cystine, 1.9; isoleucine, 0.9; leucine, 1.0; tyrosine, 1.0. Milk-ejecting activity was ca. 32.6 units/mg.

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Registry No.—**2**, 57866-58-7; **3**, 57866-59-8; **3a**, 57866-60-1; **3b**, 57866-61-2; **4**, 57866-62-3; **4a**, 57866-63-4; **4b**, 57866-64-5; **5**, 51548-05-1; **5a**, 51493-96-0; **5b**, 51548-06-2; **6**, 57866-70-3; **6a**, 57866-71-4; **7**, 57866-72-5; **7a**, 57866-73-6; **8**, 51494-04-3; **9**, 22117-87-9; **10**, 57866-74-7; **11**, 3970-13-6; **13**, 57866-75-8; **14**, 22117-86-8; **15**, 57866-76-9; **16**, 57866-77-0; **17**, 57866-78-1; **18**, 51493-98-2; **19** resin free, 32991-17-6; **21**, 57866-68-9; **22** resin free, 57866-69-0; diethyl α-acetamidomalonate, 1068-90-2; *N*-acetyl-*S*-benzyl-DL-[α-$^2H_1$]cysteine, 57866-79-2; *N*-acetyl-*S*-benzyl-D-[α-$^2H_1$]cysteine, 57866-80-5.

References and Notes

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(2) 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 DCC, dicylohexylcarbodiimide; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; DMB, 3,4-dimethylbenzyl.
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Carbon-13 Nuclear Magnetic Resonance Spectra of the Streptovaricins and Related Compounds[1,2]

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Absorptions of the 40 carbon atoms of streptovaricin C have been assigned in the carbon magnetic resonance spectrum of that ansamycin antibiotic. Carbon absorptions of other streptovaricins, including streptovaricin D, whose biosynthesis has recently been studied, have also been assigned. Methods employed in assigning the individual carbons include off-resonance decoupling, specific proton decoupling, and comparison of the streptovaricins' spectra with spectra of one another, of compounds derived from the streptovaricins, and of model compounds.

During the course of our extensive studies of the chemistry and biochemistry of the streptovaricins,[1,3] members of the ansamycin class of antibiotics,[3] it has become necessary to assign the chemical shifts of individual carbon atoms in their carbon magnetic resonance (^{13}C NMR) spectra, both for studying the biosynthesis of streptovaricins[2] and for the characterization of new, related compounds. In the present paper, we report these ^{13}C NMR assignments.

Discussion

Chemical shifts for the carbon atoms of streptovaricins A–E, G, and J (SvA–SvE, SvG, and SvJ, respectively)[4] and for compounds derived from the streptovaricins (all of whose structures are shown in Figure 1) were determined on proton decoupled spectra and are summarized in Table I. Assignments were made by comparison of the spectra with proton off-resonance decoupled spectra, by single-frequency proton decoupling experiments, from standard chemical shift data, and by comparison with chemical shifts of model compounds, as discussed below. The most abundant component of the complex, SvC, was employed as the reference compound for the assignments and the spectra of other compounds were compared with that of SvC. This was especially valuable since SvC occupies a central position in the streptovaricin family (Figure 1). Thus, SvD is 14-deoxy-SvC, SvB is SvC 11-acetate, SvJ is SvC 7-acetate, SvG is 6-hydroxy-SvC, and SvE is 7-oxo-7-deoxy-SvC. Streptovaricin A is SvG 11-acetate and SvF is *O*-demethyl-SvG-7-lactone. The spectrum of SvD is of special significance, since it is the component isolated in our biosynthetic studies employing ^{13}C-labeled precursors.[2]

Carbon atom absorptions were divided initially into the groups shown in Table I according to the number of attached hydrogen atoms (i.e., methyl, methylene, methine, quaternary carbons) by observations of the off-resonance decoupled spectra. Following this, some of the carbons—the methoxy carbon, the quaternary aliphatic C-14, the methylenedioxy carbon, and the quinonoid carbonyl carbon (C-21)[5]—were assigned unambiguously from their off-resonance multiplicities and characteristic chemical shifts[6]