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The Synthesis and Biological Properties of [1-Deaminopenicillamine]-Oxytocin Deuterated in the 1-Position[1,2]

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This paper describes the synthesis of 3-*S*-benzylmercapto-3-methyl-d_3-butanoic-2,2,4,4,4-d_5 acid (*S*-benzyl-deaminopenicillamine-d_8) and [1-deaminopenicillamine-d_8]-oxytocin. Within experimental error the deuterated oxytocin analog possessed the same anti-oxytocic activity as the protio derivative [1-deaminopenicillamine]-oxytocin.

[1-Deaminopenicillamine]-oxytocin (Fig. 1) was found by Schulz and du Vigneaud (*1*) to be devoid of avian vasodepressor, oxytocic, and pressor activities but to be a potent inhibitor of the oxytocic activity of oxytocin. This compound may be regarded as an analog of the highly active oxytocic agent deamino-oxytocin, [1-β-mercaptopropionic acid]-oxytocin, in which the two hydrogens on the β carbon of the β-mercaptopropionic acid residue are replaced with methyl groups. Deamino-oxytocin is a

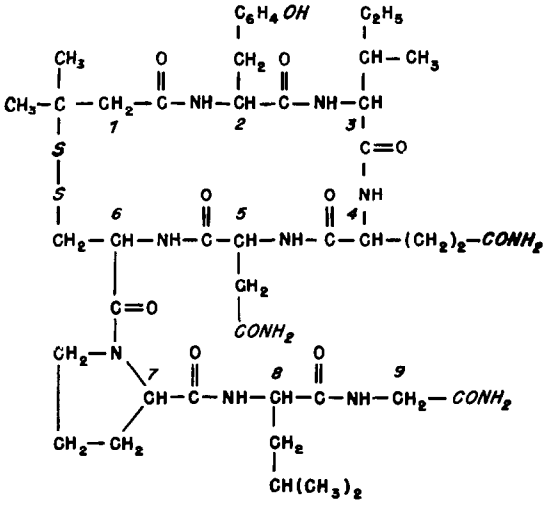

FIG. 1. Structure of [1-deaminopenicillamine]-oxytocin, with numbers indicating the position of the individual amino acid residues.

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[2] All optically active amino acid residues are of the L variety.

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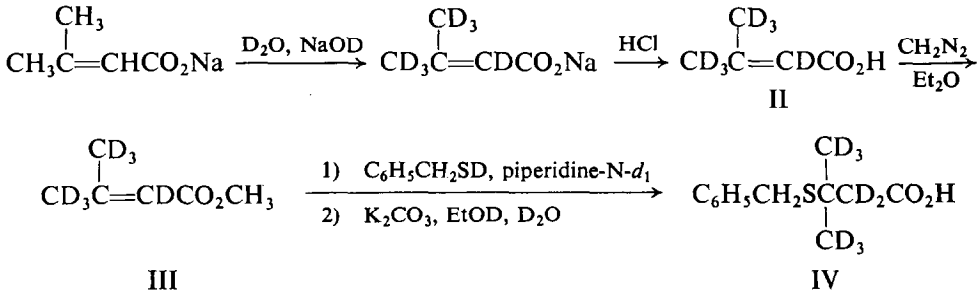
much more potent oxytocic agent than the parent natural hormone, oxytocin, which has a half-cystine residue bearing a free amino group at position 1. Deamino-oxytocin possesses 803 ± 36 units per mg of oxytocic activity (*2*), whereas oxytocin possesses 546 ± 18 units per mg (*3*). Thus, the formal replacement of the aforementioned hydrogens with methyl groups converts deamino-oxytocin from a highly active oxytocic agent to a potent anti-oxytocic compound.

As an initial step in a program designed to evaluate the effect of replacing non-exchangeable hydrogens in the various residues of oxytocin and deamino-oxytocin by deuterium on biological activity, [1-β-mercaptopropionic-α,α-d_2 acid]-oxytocin, [1-β-mercaptopropionic-β,β-d_2 acid]-oxytocin, and [1-β-mercaptopropionic-α,α,β,β-d_4 acid]-oxytocin were synthesized and compared by extensive bioassay for their oxytocic activity with protio deamino-oxytocin, [1-β-mercaptopropionic acid]-oxytocin (*4*). Within the limits of the method of biological assay it was not possible to detect any significant difference in the oxytocic activity of the four compounds. Although the replacement of hydrogen by deuterium in this series did not affect the oxytocic potency of deamino-oxytocin, we thought it would be interesting to see whether the replacement of hydrogen by deuterium in the deaminopenicillamine residue would have an effect on the anti-oxytocic activity of [1-deaminopenicillamine]-oxytocin.

For the synthesis of [1-deaminopenicillamine-d_8]-oxytocin, the desired protected polypeptide precursor, *S*-benzyl-deaminopenicillaminyl-d_8-O-benzyltyrosylisoleucyl-glutaminyl asparaginyl-*S*-benzylcysteinylprolylleucylglycinamide (I), was prepared by the Merrifield solid-phase method of peptide synthesis (*5–7*) as modified by Manning et al. (*8, 9*) using *S*-benzyl-deaminopenicillamine-d_8 (3-*S*-benzylmercapto-3-methyl-d_3-butanoic-2,2,4,4,4-d_5 acid) (IV) as the final residue to be incorporated in the chain. The latter compound was obtained as described in the experimental section through the following series of reactions:



After incorporation of the deuterated acid (IV), the completed polypeptide was cleaved from the resin with NH_3 in MeOH to yield the desired protected polypeptide amide I. The latter compound was treated with Na in liquid NH_3 according to the method of Sifferd and du Vigneaud (*10*) to remove the protecting groups, and the resulting dithiol compound was oxidized to the corresponding cyclic disulfide compound, [1-deaminopenicillamine-d_8]-oxytocin, with an aqueous solution of $K_3Fe(CN)_6$ (*11*). The analog was purified by partition chromatography (*12*) followed by gel filtration (*13*) on Sephadex G-25.

The purified deuterated deaminopenicillamine-oxytocin was compared with the corresponding protio compound by extensive bioassay for anti-oxytocic activity as

described in the experimental section. Within experimental error the anti-oxytocic potencies were the same.

EXPERIMENTAL SECTION[5]

3-*Methyl*-d_3-2-*butenoic*-2,4,4,4-d_4 *acid* (*II*). Following the reported procedure (*14*), 50 g of the Na salt of β,β-dimethylacrylic acid, 200 ml of D_2O, and 1 g of Na were heated in a stainless-steel bomb for 24 hr at 150°C. The spent water was removed *in vacuo* and fresh D_2O added. The exchange was repeated four times. The resulting sodium 3-methyl-d_3-2-butenoate-2,4,4,4-d_4 was dissolved in a minimum amount of H_2O and the aqueous solution acidified with concentrated HCl. The precipitate was collected by filtration, dried, and sublimed to give 25.7 g (59%) of pure acid II: mp 69–70°C ir (CCl_4) 3540, 3440–2500, 2260, 2230, 2200, 2120, 2050, 1692, 1635, 1359, 1290, and 1255 cm^{-1}.

Anal. Calcd for $C_5H_1D_7O_2$: C, 56.03; H, 8.24; D, 87.50 at. %. Found: C, 55.86; H, 8.01; D, 87.70 at. % (100% D).

Methyl 3-*methyl*-d_3-2-*butenoate*-2,4,4,4-d_4 (*III*). The acid II (5.0 g, 0.047 mole) was converted to the methyl ester III by stirring it with an excess of diazomethane in Et_2O solution for 12 hr. The solvent was removed, and 5.1 g (91%) of the pure III was obtained by distillation: bp 69–72°C (112 mm); ir (CCl_4) 3010, 2990, 2945, 2900, 2830, 2260, 2230, 2200, 2120, 2090, 2050, 2030, 1725, 1635, 1435, 1280, 1228, 1105, 1055, and 928 cm^{-1}; nmr ($CDCl_3$) τ 6.32 (s, CH_3O), no other peaks observed.

Anal. Calcd for $C_6H_3D_7O_2$: C, 59.46; H, 8.97; D, 70 at. %. Found: C, 59.16; H, 9.12; D, 70 at. % (100% D).

Benzylmercaptan-S-d_1. Benzylmercaptan (50 ml) and D_2O (50 ml) containing 0.2 g of Na were refluxed for 24 hr under N_2. The organic layer was separated, and the exchange repeated twice. The deuterated benzylmercaptan was separated and distilled at reduced pressure [75°C (10 mm)] under N_2. The nmr spectrum showed the S–H position to be 97–98% exchanged.

Piperidine-N-d_1. In a glove bag, piperidine (50 ml) in 100 ml of Et_2O was washed five times with 20 ml of a saturated solution of Na_2CO_3 in D_2O. The Et_2O layer was dried ($MgSO_4$), the Et_2O removed, and the deuterated piperidine distilled (bp 100–106°C) under N_2. The nmr spectrum showed the N–H position to be approximately 90% exchanged.

S-*Benzyl-deaminopenicillamine*-d_8 (IV). Benzylmercaptan-*S*-d_1 (8.5 g, 0.069 mole), the ester III (2.9 g, 0.024 mole) and piperidine-*N*-d_1 (1 ml) were heated between 120–130°C for 48 hr under N_2. The solution was cooled, acidified with HCl, and extracted with Et_2O. The Et_2O layer was evaporated to a yellow oil which was dissolved in a solution of 4 g of K_2CO_3, 30 ml of D_2O, and 60 ml of EtOD and refluxed under N_2 for 24 hr. The solvent was distilled off until the vapors reached 100°C and the distillate was clear. The residue was acidified, extracted with Et_2O, and the organic layer dried over $MgSO_4$. The Et_2O was removed, and the residue distilled to give 4.1 g (75%) of a clear oil IV: bp 96–100°C (0.1 mm). The oil was crystallized from pentane at –30°C

[5] Deuterium analyses were performed by J. Nemeth, Urbana, Illinois, using the falling drop technique. Infrared (ir) spectra were obtained on a Perkin–Elmer Infracord or Perkin–Elmer 257 Grating Spectrophotometer; nuclear magnetic resonance (nmr) data were recorded on a Varian Associates model A-60 or model A-60A spectrometer; optical rotations were obtained on a Perkin–Elmer model 141 Polarimeter; amino acid analyses were obtained by the method of Spackman, Stein, and Moore, *Anal. Chem.* **30**, 1190 (1958), on a Beckman Spinco model 116 amino acid analyzer. Unless otherwise noted, melting points are uncorrected.

to give pure IV: mp 43.5–44.5°C; ir (CCl_4) 3080, 3060, 3030, 2920, 2660, 2550, 2220, 1710, 1605, 1499, 1458, 1410, 1292, and 682 cm^{-1}; nmr ($CDCl_3$) τ 2.70 (s, 5, C_6H_5) and 6.20 (s, 2, CH_2S).

Anal. Calcd for $C_{12}H_8D_8O_2S_1$: C, 62.02; H, 7.33; S, 13.80; D, 50 at. %. Found: C, 61.79; H, 7.18; S, 13.83; D, 47.6 at. % (96% D).

[1-*Deaminopenicillamine*-d_8]-*oxytocin.* Chloromethylcopolystyrene–2% divinylbenzene (50 g) was added to a solution of Boc-glycine (17.0 g) and Et_3N (9.8 g) in 100 ml absolute EtOH and stirred at reflux for 24 hr. The resin was filtered off, washed three times each with absolute EtOH, 95% EtOH, H_2O, and CH_3OH, and dried *in vacuo*: wt 57.1 g. Amino acid analysis of an acid hydrolysate showed 0.76 mmole/g of esterified resin.

This Boc-glycine resin (3.14 g, 2.39 mmole) was placed in a Merrifield reaction vessel, washed with CH_3OH and CH_2Cl_2, and allowed to swell in CH_3OH overnight. It was then subjected to six cycles of deprotection, neutralization, and coupling as described for the synthesis of [8-phenylalanine]-oxytocin by Baxter, Manning, and Sawyer (*8*), using the following amino acid derivatives: Boc-leucine, Boc-proline, Boc-*S*-benzylcysteine, Boc-asparagine nitrophenyl ester, Boc-glutamine nitrophenyl ester, Boc-isoleucine. After washing with EtOH, HOAc, EtOH, and CH_2Cl_2 and drying, the protected heptapeptide resin weighed 4.3 g.

A portion of the latter (0.41 mmole) was placed in a reaction vessel and allowed to swell in CH_2Cl_2 before beginning the cycles for addition of the last two residues, Boc-*O*-benzyltyrosine and *S*-benzyl-deaminopenicillamine-d_8. In each of the cycles the coupling step was carried out twice to ensure complete reaction (*9*). After incorporation, the deprotection and neutralization steps were repeated as described by Baxter et al. (*8*).

The polypeptide resin from the above synthesis was allowed to swell in 80 ml of anhydrous CH_3OH overnight. The resulting mixture was cooled to –6°C in an ice–salt bath, and Na-dried NH_3 was slowly bubbled into the cooled mixture for 1.5 hr. The mixture was stirred overnight at 2°C and the CH_3OH and NH_3 were then removed under vacuum. The residue was extracted three times with 30 ml portions of DMF and twice with 10 ml portions of CH_3OH, and the combined extracts were evaporated to dryness *in vacuo*. The residue was triturated with 30 ml of CH_3OH and evaporated to dryness, then triturated with 30 ml of 95% EtOH, washed on a filter with EtOH and Et_2O, and finally dried at 56° *in vacuo*. The product, *S*-benzyl-deaminopenicillaminyl-d_8 - *O* - benzyltyrosylisoleucylglutaminylasparaginyl - *S* - benzylcysteinylprolylleucyl - glycinamide (I), weighed 215 mg; mp 228.5–235°C (cor); $[\alpha]_D^{24}$ –46.6° (*c* 0.9, DMF).

The protected polypeptide I (160 mg, 0.121 mmole) was dissolved in 120 ml of Na-dried liquid NH_3. A freshly cut Na stick was intermittently introduced into the boiling solution until a blue color persisted for 40 sec; NH_4Cl was added to discharge the blue color, and the NH_3 was allowed to evaporate spontaneously. The reaction mixture was subsequently evaporated under aspirator vacuum to ensure complete removal of NH_3, and the residue was stirred with 200 ml of 0.05% F_3CCOOH. The pH of this mixture was adjusted to 8.1 with dilute NH_4OH, and an excess (4.7 ml) of 0.1 *N* $K_3Fe(CN)_6$ was added. After stirring the reaction for 15 min, AG3-X4 resin (F_3CCOO^- cycle) was added to remove ferrocyanide and excess ferricyanide ions. The resin was removed by filtration, and the solution was lyophilized.

The product from lyophilization was dissolved in 10 ml of the upper phase and 1 ml of the lower phase of the solvent system *n*-butanol–benzene–0.5% aqueous AcOH containing 0.1% pyridine (3:2:5) and applied to a Sephadex G-25 column (2.8 × 70 cm) that had been equilibrated with lower and upper phases (*12*). The column was eluted with the upper phase, and the chromatogram obtained by plotting the Folin–

Lowry (*15*) color values of the fractions showed a major peak with an R_f of 0.49. The fractions corresponding to this peak were combined and diluted with twice their volume of H_2O. The resulting mixture was concentrated on a rotary evaporator and lyophilized: yield 40 mg.

The powder obtained from lyophilization was dissolved in 8 ml of 2.4 *N* AcOH and subjected to gel filtration (*13*) on a Sephadex G-25 column that had been equilibrated with 0.2 *N* AcOH. The column was eluted with 0.2 *N* AcOH, and the fractions containing the product were combined and lyophilized to give a white powder with the expected amino acid composition: yield, 37 mg; $[\alpha]_D^{24}$ −52.1° (*c* 0.5, 1 *N* AcOH).

Anal. Calcd for $C_{45}H_{61}N_{11}O_{12}S_2D_8 \cdot H_2O$: C, 51.65; H, 6.92; N, 14.72. Found: C, 51.71; H, 6.82; N, 14.65.

Biological activity determinations. The oxytocic response to oxytocin of isolated uteri from 200–250 g Sherman albino rats in natural estrus (*3*) was measured by the method of Holton (*16*), as modified by Munsick (*17*), with the use of Mg-free van Dyke–Hastings solution as the bathing fluid. Isotonic contractions were recorded with a Harvard heart/smooth muscle transducer and Grass polygraph Model 5.

The experimental plan used was based on that outlined by Schild (*18*) for assessing the relative potencies of inhibitory substances. In each test the average response of the tissue to a medium dose (x units) of oxytocin is first determined. Subsequently, two amounts of inhibitor are injected immediately prior to injections of $2x$ units of oxytocin at levels which will inhibit the responses to slightly more than and slightly less than the average response to x units of oxytocin given without inhibitor. Extrapolation between these points is then used to calculate the concentrations of inhibitor necessary to reduce the response to $2x$ units of oxytocin to that of x units. The negative logarithm to base 10 of this concentration is defined by Schild as the pA_2 value.

In these studies the inhibitory potency of [1-deaminopenicillamine-d_8]-oxytocin was compared to that of [1-deaminopenicillamine]-oxytocin in an extensive series of assays. Within experimental error, no difference could be established. Both compounds gave pA_2 values in the range 6.8–7.0.

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