Synthetic Antagonists of in Vivo Responses by the Rat Uterus to Oxytocin[1]

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As part of a program in which we are attempting to synthesize in vivo antagonists of oxytocin, the following four analogues were synthesized and tested for antagonistic activities in rat uterus and rat vasopressor assay systems: [1-(β-mercapto-β,β-diethylpropionic acid),4-threonine]oxytocin (1, dEt_2TOT), [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),4-threonine]oxytocin [2, $d(CH_2)_5TOT$], [1-deaminopenicillamine,2-O-methyltyrosine]oxytocin [3, dPTyr(Me)OT], and [1-deaminopenicillamine,2-O-methyltyrosine,4-threonine]oxytocin [4, dPTyr(Me)TOT]. The required protected intermediates were synthesized by a combination of solid-phase peptide synthesis and by individual 8 + 1 couplings in solution. All four analogues antagonize the actions of oxytocin on the rat uterus (a) in the absence of Mg^{2+}, (b) in the presence of 0.5 mM Mg^{2+}, and (c) in situ. They exhibit, respectively, the following pA_2 values in each of the assay systems a–c: (1) (a) 7.72 ± 0.11, (b) 7.36 ± 0.09, (c) 6.47 ± 0.11; (2) (a) 7.91 ± 0.13, (b) 7.81 ± 0.09, (c) 6.94 ± 0.11; (3) (a) 7.76 ± 0.12, (b) 7.80 ± 0.12, (c) 6.86 ± 0.12; (4) (a) 7.64 ± 0.14, (b) 7.79 ± 0.09, (c) 6.84 ± 0.10. They have the following antivasopressor pA_2 values: (1) 6.30 ± 0.13; (2) 5.86 ± 0.03; (3) 7.59 ± 0.05; (4) 7.32 ± 0.04. Compounds 2–4 are among the most potent in vivo antagonists of oxytocin reported to date.

In a previous report[2] from these laboratories we described the synthesis and some pharmacological properties of [1-deaminopenicillamine,4-threonine]oxytocin (dPTOT). This was shown[2] to be twice as potent as [1-deaminopenicillamine]oxytocin (dPOT)[3] in antagonizing responses to oxytocin by the isolated rat uterus in a Mg^{2+}-free medium. We subsequently ascertained that dPTOT could also antagonize uterine responses to oxytocin in vivo. Its potency is, however, disappointingly low and it can also act as a partial agonist.[1,4] Increasing the size of the lipophilic substituents on the β carbon at position 1 in deaminooxytocin had previously been shown by du Vigneaud and co-workers to give rise to enhanced antagonistic activities in vitro.[5,6] Thus, [1-(β-mercapto-β,β-diethylpropionic acid)]oxytocin (dEt_2OT)[5] and [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid)]oxytocin [$d(CH_2)_5OT$][6] exhibited a progressive enhancement in antagonistic potencies with the latter peptide being equipotent with dPTOT.[2] Since Thr[4] substitution enhanced the in vitro antagonistic potency of dPOT, we speculated[2,7,8] that its substitution in dEt_2OT and in $d(CH_2)_5OT$ might lead to similar enhancements in their respective potencies both in vitro and in vivo. We now report the syntheses and some pharmacological properties of the two analogues designed in this fashion: [1-(β-mercapto-β,β-diethylpropionic acid),4-threonine]oxytocin (1, dEt_2TOT) and [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),4-threonine]oxytocin [2, $d(CH_2)_5TOT$].

[1-Deaminopenicillamine]oxytocin has been shown to have potent antagonistic activity to oxytocin in vitro in the absence of Mg^{2+}.[3,9] It acts as a weak agonist on the rat uterus in vivo but has been shown to antagonize the uterotonic action of infused oxytocin.[9] With the goal of obtaining effective antagonists of in vivo oxytocic responses to oxytocin still in mind, we decided to modify [1-deaminopenicillamine]oxytocin[3] with a substituent that had previously been shown to confer antagonistic properties on oxytocin, namely, 2-O-methyltyrosine substitution.[10-12] [2-O-Methyltyrosine]oxytocin had been shown to be a weak antagonist of in vitro responses to oxytocin.[10,11] Also [N-carbamyl,2-O-methyltyrosine]oxytocin (carbamylmethyloxytocin) had been shown to be an in vivo antagonist, albeit a relatively weak one, of oxytocin.[13,14] We thus also present the synthesis and some pharmacological properties of [1-deaminopenicillamine,2-O-methyltyrosine]oxytocin [3, dPTyr(Me)OT] and its Thr[4] derivative [1-deaminopenicillamine,2-O-methyltyrosine,4-threonine]oxytocin [4, dPTyr(Me)TOT]. In addition, we present here data on the antagonistic properties of dPTOT[2], dEt_2OT[5], and $d(CH_2)_5OT$[6] in vitro with 0.5 mM Mg^{2+} and in vivo.

Peptide Synthesis. Solid-phase peptide synthesis[15-20] was employed to prepare three protected octapeptide amide analogues of the C-terminal protected amide of oxytocin, i.e., Boc-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2, with each intermediate containing the following modifications: (1) Boc-Tyr(Me) in place of Boc-Tyr(Bzl), (2) Thr(Bzl) in place of Gln, and (3) both the Boc-Tyr(Me) and Thr(Bzl) substitutions in the same intermediate. Removal of the N-terminal protecting group from each protected octapeptide amide and coupling 1 and 3 in turn with β-(S-benzylmercapto)-β,β-dimethylpropionic acid[3] and coupling 2 in turn with β-(S-benzylmercapto)-β,β-diethylpropionic acid[5] and β-(S-benzylmercapto)-β,β-cyclopentamethylenepropionic acid[6] by the dicyclohexylcarbodiimide (DCCI)–N-hydroxybenzotriazole (HOBt) preactivation method[21,22] yielded the corresponding protected precursors of dPTyr(Me)OT, dPTyr(Me)TOT, dEt_2TOT, and $d(CH_2)_5TOT$. Deblocking of all four protected intermediates was affected with Na and liquid NH_3[23] as previously described,[17,18] and the resulting disulfhydryl compounds were subjected to oxidative cyclization with $K_3[Fe(CN)_6]$.[24] The analogues were purified by gel filtration on Sephadex G-15.[25]

Bioassay Methods. Antagonistic activities were calculated by Schild's method[26] and expressed as pA_2 values. The pA_2 is the negative logarithm of the molar concentration of a competitive antagonist that reduces the response to $2x$ units of agonist to equal the response to x units in the absence of antagonist.

In vitro pA_2 measurements were on uterine horns from rats pretreated with diethylstilbestrol. The horns were suspended in Munsick's[27] solution containing either 0.5 mM Mg^{2+} or no Mg^{2+}. Isometric contractions were recorded and antagonists were added to the bath solution 1 min prior to the addition of agonist.

Molar concentrations of antagonists at in vivo receptor sites cannot be measured. We have, however, followed the method of Dyckes et al.[28] to estimate in vivo "pA_2" values. This method requires three unjustified assumptions: (1) that the volumes of distribution of the injected antagonists

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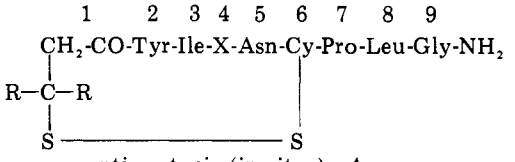
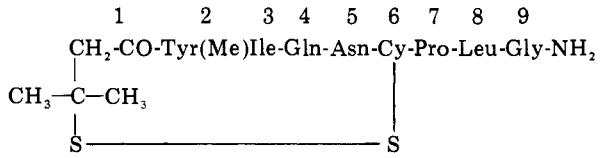
Table I. Effects of Thr[4] Substitution on the Antagonistic Properties of Three 1-β,β-Dialkyl-Substituted Analogues of Deaminooxytocin



peptide	R_2	X	antioxytocic (in vitro) pA_2 no Mg^{2+}	antioxytocic (in vitro) pA_2 0.5 mM Mg^{2+}	antioxytocic (in vivo) pA_2	antivasopressor pA_2
dPOT	$(CH_3)_2$	Gln	7.14 ± 0.05 (31)[a,b] (6.94,[e] 7.04[f])	5.63 ± 0.17 (4)[c,d]		6.27[e]
dPTOT	$(CH_3)_2$	Thr	7.52 ± 0.04 (68)[b]	6.23 ± 0.11 (30)[c,d]	6.31 ± 0.05 (6)[c,d]	6.67 ± 0.09 (7)
dEt_2OT	$(C_2H_5)_2$	Gln	7.55 ± 0.14 (8) (7.24,[e] 7.41[f])	6.82 ± 0.11 (10)	6.20 ± 0.12 (5)	6.83 ± 0.08 (7)
dEt_2TOT	$(C_2H_5)_2$	Thr	7.72 ± 0.11 (18)	7.36 ± 0.09 (17)	6.47 ± 0.11 (17)	6.30 ± 0.13 (5)
$d(CH_2)_5OT$	$(CH_2)_5$	Gln	7.61 ± 0.08 (14) (7.43,[e] 7.53[f])	7.15 ± 0.10 (17)	6.65 ± 0.06 (11)	weak[e]
$d(CH_2)_5TOT$	$(CH_2)_5$	Thr	7.91 ± 0.13 (25)	7.81 ± 0.09 (30)	6.94 ± 0.11 (12)	5.86 ± 0.03 (10)

[a] Means ± SE, number of assay groups in parentheses. [b] Data from Manning et al.[2] [c] Data from Sawyer et al.[4] [d] Also act as partial agonists. [e] From Nestor et al.[6] [f] Calculated from present data by the method of Nestor et al.[6] The abbreviations and their full names are as follows: dPOT, [1-deaminopenicillamine]oxytocin; dPTOT, [1-deaminopenicillamine,4-threonine]oxytocin; dEt_2OT, [1-(β-mercapto-β,β-diethylpropionic acid)]oxytocin; dEt_2TOT, [1-(β-mercapto-β,β-diethylpropionic acid),4-threonine]oxytocin; $d(CH_2)_5OT$, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid)]oxytocin; $d(CH_2)_5$-TOT, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),4-threonine]oxytocin.

Table II. Antagonistic Properties of [1-Deaminopenicillamine,2-*O*-methyltyrosine]oxytocin [dPTyr(Me)OT] and of Its Thr[4] Analogue [dPTyr(Me)TOT].



peptide	dP Tyr(Me)OT antioxytocic (in vitro)[b] pA_2 no Mg^{2+}	dP Tyr(Me)OT antioxytocic (in vitro)[b] pA_2 0.5 mM Mg^{2+}	antioxytocic (in vivo)[b] pA_2	antivasopressor[b] pA_2
dPOT[a]	7.14 ± 0.5	5.63 ± 0.17[c]		6.27[e]
dPTyr(Me)OT	7.76 ± 0.12 (16)	7.80 ± 0.12 (17)	6.86 ± 0.12 (11)	7.59 ± 0.05 (9)
dPTyr(Me)TOT	7.64 ± 0.14 (16)	7.79 ± 0.09 (11)	6.84 ± 0.10 (8)	7.32 ± 0.04 (8)
carbamylmethyl-OT[d]	6.90 ± 0.05 (20)	6.61 ± 0.06 (21)	inhibits	inhibits

[a] From Table I. [b] Numbers of pA_2 determinations in parentheses. [c] Can act as partial agonist as well as antagonist. [d] Data from Bisset et al.[14] [e] Data from Nestor et al.[6]

equal the blood volume, (2) that the antagonists are not rapidly metabolized or redistributed, and (3) that the drugs act as purely competetive antagonists in vivo. Despite these objections, the estimated pA_2 values are useful for comparison with values obtained in vitro and with in vivo pA_2 values for antagonists that have been reported by others.[5,6,28] In any event, the pA_2 values estimated in this manner are negative logarithmic functions of the effective intravenous doses of the antagonists. An in vivo pA_2 of 6.00 would, for example, correspond to an intravenous dose of 67 nmol/kg, while a pA_2 of 7.00 would correspond to a dose of 6.7 nmol/kg.

In vivo rat uterus assays were carried out on anesthetized rats pretreated with diethylstilbestrol by a modification of the assay of Bisset et al.[29] The recorded responses were the integrated intrauterine pressures during 10-min periods following injection of agonist. Vasopressor responses were recorded in anesthetized rats as described by Dekanski.[30] In both in vivo preparations, antagonists were injected 1 min before agonists.

Results and Discussion

The antioxytocic and antivasopressor potencies of the Thr[4]-substituted analogues of dPOT, dEt_2OT, and $d(CH_2)_5OT$ and of the 2-*O*-methyltyrosine analogues of dPOT and dPTOT are presented in Tables I and II, respectively. New measurements of the antioxytocic and antivasopressor properties of dPOT, dET_2OT, and $d(CH_2)_5OT$ together with previously reported in vitro potencies in the absence of Mg^{2+} are also presented in Table I.

Thr[4] Substitution. Thr[4] substitution in analogues with dialkyl substitutions in position 1 led to an enhancement of oxytocic antagonistic potency in all three assay systems. Of particular interest also is the enhancement of antioxytocic activity as measured in vivo, as one goes from the dimethyl to the diethyl to the cyclopentamethylene analogues. Thus, [1-deaminopenicillamine]oxytocin is a partial agonist, the diethyl analogue has a pA_2 of 6.20 and the cyclopentamethylene analogue has a pA_2 of 6.65. Comparable figures for the Thr[4] analogues are 6.31, 6.47, and 6.94. Thus, increasing the size of the alkyl residue at position 1 leads to enhanced in vivo antioxytocic potency, and Thr[4] substitution leads to a further enhancement in each case. Thus, $d(CH_2)_5TOT$ is one of the most potent antagonists of in vivo responses to oxytocin reported to date.

2-*O*-Methyltyrosine Substitution. The incorporation of Tyr(Me)[2] in dPOT to give dPTyr(Me)OT brought about dramatic enhancements in antioxytocic potency in all three assay systems. The enhancement in vivo was particularly striking, a partial agonist being converted to a potent

antagonist with a pA_2 of 6.86. Thr[4] substitution did not lead to an enhancement of antioxytocic potency in any of the three systems. dPTyr(Me)OT and dPTyr(Me)TOT both exhibit 10–12-fold enhancements of antivasopressor potency. In regards to these effects of Tyr(Me)[2] substitution, it may be of interest to recall recent related findings from these laboratories;[31] while this work was in progress, analogues of arginine-vasopressin containing the Tyr(Me)[2] and 1-deaminopenicillamine substitutions individually and in combination were prepared with a view to obtaining potent antivasopressor substances. [2-*O*-Methyltyrosine]arginine-vasopressin [Tyr(Me)AVP] exhibited an antioxytocic pA_2 of 7.44 ± 0.12 in vitro in the absence of Mg^{2+}. Tyr(Me)[2] substitution in [1-deaminopenicillamine]arginine-vasopressin (dPAVP), giving [1-deaminopenicillamine,2-*O*-methyltyrosine]arginine-vasopressin (dPTyr(Me)AVP), in addition to enhancing antivasopressor properties also enhanced the in vitro antioxytocic pA_2 in the absence of Mg^{2+} from 6.93 ± 0.10 to 7.61 ± 0.14.[31]

With in vivo antioxytocic potencies of 6.86 and 6.84, dPTyr(Me)OT and dPTyr(Me)TOT are approximately equipotent with d$(CH_2)_5$TOT (pA_2 = 6.94). Thus, these three compounds, i.e., d$(CH_2)_5$TOT, dPTyr(Me)OT, and dPTyr(Me)TOT, appear to be the most potent antagonists of in vivo oxytocic responses to oxytocin reported to date. These findings may point the way to the design of even more potent in vivo antagonists of oxytocin.

Experimental Section

The procedure of solid-phase peptide synthesis conformed to that published,[15-20] with the exception that the chloroform washes were omitted and a pyridine hydrochloride treatment[32] followed by neutralization was included before isolation of the intermediate-protected peptidyl resins. Chloromethylated resin (Bio-Rad Bio-Beads SX-1) was esterified[33] with Boc-Gly to an incorporation of ~0.63 mmol/g. Amino acid derivatives, including Boc-Tyr(Me), were supplied by Bachem Inc. and Beckman Bio-products Division. The purity of Boc-Tyr(Me) was tested as previously described.[31] Triethylamine and *N*-methylmorpholine (NMM) were distilled from ninhydrin and trifluoroacetic acid (TFA) was distilled from P_2O_5. The acetic acid used for the HCl–acetic acid cleavage reagent and for washings bracketing the TFA cleavage following glutamine incorporation[18,34] was heated under refux with boron triacetate and distilled from the reagent. Dimethylformamide (DMF) was distilled under reduced pressure immediately prior to its use. Methanol was dried with magnesium methoxide and distilled. Other solvents and reagents were of analytical grade. Thin-layer chromatography (TLC) was on silica gel (0.25 mm, Brinkman Silplate) using the following solvent systems: A, cyclohexane–chloroform–acetic acid (2:8:1, v/v); B, propan-1-ol–ammonia (34%) (2:1, v/v); C, ethanol (85%)–ammonia (34%) (3:1, v/v); D, butan-1-ol–acetic acid–water (4:1:5, v/v, upper phase); E, butan-1-ol–water (3.5% in acetic acid, 1.5% in pyridine) (1:1, v/v, upper phase); F, butan-1-ol–acetic acid–water–pyridine (15:3:3:10, v/v). Loads of 10–50 μg were applied and chromatograms were of minimum length 10 cm. Chloroplatinate reagent, ninhydrin, and chlorine–potassium iodide–tolidine were used for detection. For amino acid analysis,[35] peptides (~0.5 mg) were hydrolyzed with constant-boiling hydrochloric acid (400 μL) containing phenol (20 μL) in evacuated and sealed ampules for 18 h at 100 °C. Products containing the *O*-methyltyrosine residue were also hydrolyzed with sulfuric acid[10,31,36] (1.8 M, 400 μL), for 24 h at 130 °C. The analyses were performed using a Beckman automatic amino acid analyzer Model 121. Ratios were referred to Gly = 1.00. Elemental analyses were performed by Galbraith Laboratories, Inc. Samples were dried for a minimum of 6 h in vacuo prior to analysis. Analytical results indicated by the elemental symbols were ±0.4% of theoretical values. Optical rotations were measured with a Bellingham Stanley, Ltd., Model A polarimeter, Type P1.

Boc-Asn-Cys(Bzl)-Pro-Leu-Gly Resin (I). Boc-Gly resin (16.7 g, ~10 mmol of Gly) was subjected to four cycles of deprotection, neutralization, and coupling to yield the protected pentapeptidyl resin (21.85 g, weight gain 5.15 g, 99.5% theory). This protected pentapeptidyl resin served as the common precursor of the four analogues reported here.

Boc-Ile-Thr(Bzl)-Asn-Cys(Bzl)-Pro-Leu-Gly Resin (II). The foregoing pentapeptidyl resin (13 g, ~6 mmol of peptide) was converted in two further cycles of solid-phase peptide synthesis to the Boc-heptapeptidyl resin, II (14.85 g, weight gain 1.85 g, 12% theory). This protected heptapeptidyl resin was employed in the synthesis of the Boc octapeptides III and IV.

Boc-Tyr(Bzl)-Ile-Thr(Bzl)-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (III). Heptapeptidyl resin II (4.85 g, ~2 mmol of peptide) yielded Boc-octapeptide resin (5.3 g, weight gain 450 mg, 90% of theory) in one cycle of solid-phase peptide synthesis with Boc-Tyr(Bzl) as the carboxy component. The resin was ammonolyzed in methanol.[18] Following evaporation of the methanol, the product was extracted into hot DMF and precipitated with water. The crude material was twice reprecipitated from DMF–methanol to yield the protected octapeptide amide III:[37] yield 1.65 g (66% based upon initial Gly content of the resin); mp 230 °C dec; TLC R_f (system D) 0.64, R_f (system E) 0.71. Anal. ($C_{65}H_{88}N_{10}O_{13}S$) C, H, N. Amino acid analysis:[35] Tyr, 1.0; Ile, 1.0; Thr, 1.1; Asp, 1.1; Cys(Bzl), 0.9; Pro, 1.0; Leu, 1.0; Gly, 1.0; NH_3, 2.2.

Boc-Tyr(Me)-Ile-Thr(Bzl)-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (IV). A single cycle of solid-phase peptide synthesis with Boc-Tyr(Me) as carboxy component converted heptapeptidyl resin II (9.9 g, ~4 mmol of peptide) to Boc-octapeptidyl resin (10.6 g, weight gain 700 mg, 98% of theory). The resin was ammonolyzed,[18] and the protected octapeptide amide IV was isolated as was its analogue III above: yield 3.04 g (65% based upon initial Gly content of the resin); mp 231–233 °C dec; TLC (system D) 0.60, R_f (system E) 0.68, a trace of a more mobile contaminant was evident. This product was used successfully in subsequent syntheses. Anal. ($C_{59}H_{89}N_{10}O_{13}S$) C, H, N. Amino acid analysis:[35] Tyr, 0.9; Ile, 0.9; Thr, 1.1; Asp, 1.1; Cys(Bzl), 1.0; Pro, 1.0; Leu, 1.0; Gly, 1.0; NH_3, 2.3. Analysis following hydrolysis with sulfuric acid:[10,36] Tyr(Me)/Tyr/Gly, ~0.7:0.05:1.00.

Boc-Tyr(Me)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (V). Boc-pentapeptidyl resin I (8.74 g, ~4 mmol of peptide) was subjected to three cycles of deprotection, neutralization, and coupling with, successively, Boc-Gln-ONP, Boc-Ile, 0.5H_2O–DCCI, and Boc-Tyr(Me)–DCCI, to yield Boc-octapeptidyl resin (10.4 g, weight gain 1.66 g, 99.3% of theory). Ammonolysis of the resin[18] and product isolation as for compounds III and IV above yielded the protected octapeptide amide V: yield 1.45 g (33% based upon initial Gly content of the resin—a loss was incurred by spillage during reprecipitation); mp 232–236 °C dec; TLC R_f (system D) 0.54, R_f (system E) 0.60. Traces of two contaminants, one more mobile and one less so than the major product, were evident on TLC. The product proved adequate in further synthesis (below). Anal. ($C_{53}H_{79}N_{11}O_{13}S \cdot H_2O$) C, H, N. Amino acid analysis[35] gave Tyr, 0.9; Ile, 1.0; Glu, 1.0; Asp, 1.1; Cys(Bzl), 1.0; Pro, 1.0; Leu, 1.0; Gly, 1.0; NH_3, 3.1. Analysis following hydrolysis with sulfuric acid:[10,36] Tyr(Me)/Tyr/Gly, ~0.7:0.06:1.00.

***S*-Benzyl-β-mercapto-β,β-diethylpropionyl-Tyr(Bzl)-Ile-Thr(Bzl)-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (VI).** The Boc octapeptide amide III (312 mg, 0.25 mmol) was dissolved in cold TFA (3 mL) and left to stand at room temperature for 1 h with occasional vortex mixing. Cold ether (20 mL) was added, and the precipitated material was centrifuged and washed with ether (three times, 10-mL aliquot) by successive centrifugation and decantation, the precipitate being well suspended in each wash by vortex mixing. The product was dried in vacuo over sodium hydroxide pellets. This material was dissolved in DMF (~2 mL), and NMM (75 μL) was added gradually to give a solution of pH ~7 to moist pH paper, the atmosphere above which also gave an alkaline reaction to moist pH paper.[38] A solution of *S*-benzyl-β-mercapto-β,β-diethylpropionic acid,[5,39] (159 mg, 0.63 mmol) and *N*-hydroxybenzotriazole monohydrate[21] (145 mg, 0.95 mmol) in DMF (1.5 mL) was cooled in ice and treated with a solution of DCCI (130 mg, 0.63 mmol) in dichloromethane (0.25 mL). A DMF washing (0.25 mL) brought the total volume of the reaction mixture to 2.5 mL. This mixture was left to stand at room temperature for 1 h with occasional mixing. The precipitated dicyclohexylurea was centrifuged and the supernatant (1.25 mL,

~0.32 mmol of acylating agent) was added to the neutralized solution of the octapeptide derivative.[22] After a reaction time of 2.5 h, a spot of the reaction mixture on filter paper gave only a very faint ninhydrin color. The reaction mixture was set aside at room temperature overnight, during which time the atmosphere within the reaction vessel maintained an alkaline reaction to moist pH paper.[38] The gel which formed was broken by the addition of aqueous acetic acid (5%, 20 mL) and vigorous stirring. The precipitated material was centrifuged and washed with water, ethanol (twice), and ether (twice) (aliquots of 10 mL), by successive centrifugation and decantation, the precipitate being well suspended in each wash. A solution of the crude product in a minimum quantity of hot DMF was diluted tenfold with boiling methanol. The hot solution, upon cooling to room temperature, deposited the acyl octapeptide derivative as a white precipitate, which was filtered and washed with methanol: yield 255 mg (74%); mp 232–233 °C; TLC R_f (system D) 0.63, R_f (system E) 0.69. Anal. ($C_{74}H_{98}N_{10}O_{12}S_2 \cdot CH_3OH$) C, H, N. Amino acid analysis:[35] Tyr, 0.9; Ile, 0.9; Thr, 1.0; Asp, 1.1; Cys(Bzl), 1.0; Pro, 0.9; Leu, 1.0; Gly, 1.0; NH_3, 2.1.

S-Benzyl-β-mercapto-β,β-cyclopentamethylenepropionyl-Tyr(Bzl)-Ile-Thr(Bzl)-Asn-Cys(Bzl)-Pro-Leu-Gly-NH$_2$ (VII). Coupling of the TFA cleavage product of Boc octapeptide amide III (312 mg, 0.25 mmol) and *S*-benzyl-β-mercapto-β,β-cyclopentamethylenepropionic acid[6] in the manner detailed above yielded the acyl octapeptide amide VII: yield 271 mg (78%); mp 232–234 °C; TLC R_f (system D) 0.63, R_f (system E) 0.69. Anal. ($C_{75}H_{98}N_{10}O_{12}S_2 \cdot H_2O$) C, H, N. Amino acid analysis:[35] Tyr, 0.9; Ile, 1.0; Thr, 1.0; Asp, 1.1; Cys(Bzl), 1.0; Pro, 1.0; Leu, 1.1; Gly, 1.00; NH_3, 2.3.

S-Benzyl-β-mercapto-β,β-dimethylpropionyl-Tyr(Me)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH$_2$ (VIII). Boc octapeptide amide V (277 mg, 0.25 mmol) was treated with TFA, and the product was coupled with preactivated *S*-benzyl-β-mercapto-β,β-dimethylpropionic acid[3,39] as above, to yield the acyl octapeptide amide VIII: yield 295 mg (97%); mp 242–244 °C; TLC R_f (system D) 0.49, R_f (system E) 0.55. Anal. ($C_{60}H_{85}N_{11}O_{12}S_2 \cdot 5H_2O$) C, H, N. Amino acid analysis:[35] Tyr, 0.9; Ile, 1.0; Glu, 1.0; Asp, 1.1; Cys(Bzl), 0.9; Pro, 1.0; Leu, 1.0; Gly, 1.00; NH_3, 3.4. Analysis following hydrolysis with sulfuric acid: Tyr(Me)/Tyr/Gly, ~0.7: 0.09:1.00.

S-Benzyl-β-mercapto-β,β-dimethylpropionyl-Tyr(Me)-Ile-Thr(Bzl)-Asn-Cys(Bzl)-Pro-Leu-Gly-NH$_2$ (IX). The TFA cleavage product of Boc octapeptide amide IV (293 mg, 0.25 mmol) was treated with acylating agent derived from *S*-benzyl-β-mercapto-β,β-dimethylpropionic acid,[3,39] and the product was isolated as above: yield 275 mg (86%); mp 239–240 °C; TLC R_f (system D) 0.64, R_f (system E) 0.66. Anal. ($C_{66}H_{90}N_{10}O_{12}S_2 \cdot CH_3OH$) C, H, N. Amino acid analysis:[35] Tyr, 0.9; Ile, 1.0; Thr, 1.0; Asp, 1.1; Cys(Bzl), 0.9; Pro, 1.0; Leu 1.0; Gly, 1.0; NH_3, 2.3. Analysis following hydrolysis with sulfuric acid:[10,36] Tyr(Me)/Tyr/Gly, ~0.7:0.06:1.00.

[1-Deaminopenicillamine,2-(*O*-methyl)tyrosine]oxytocin [dPTyr(Me)OT, X]. A solution of the peptide intermediate VIII (100 mg) in sodium-dried and redistilled ammonia (~300 mL) was treated at the boiling point and with stirring with sodium[23] from a stick of the metal contained in a small-bore glass tube[17,24] until a light blue color persisted in the solution for 15–20 s. The color was discharged by the dropwise addition of dry glacial acetic acid. The solution was evaporated and the residue taken up in aqueous acetic acid (0.2%, 750 mL), a small amount of material remaining undissolved. Following the addition of aqueous ammonia (2 M) to give a solution of pH ~7, an excess of potassium ferricyanide solution (0.01 M, 14 mL) was added gradually, with stirring, during which complete solution was achieved.[24] The yellow solution was stirred a further 10 min, the pH adjusted to ~6 with glacial acetic acid, and for 5 min with anion-exchange resin (Bio-Rad AG-3, chloride form, ~10 g damp weight). The suspension was filtered through a bed of the resin (~80 g damp weight). The bed was washed with aqueous acetic acid (0.2%, 200 mL), and the combined filtrate and washings were lyophilized. The resulting powder was desalted on Sephadex G-15 (column 110 × 2.7 cm), eluting with aqueous acetic acid (50%)[25] with a flow rate of ~6 mL/h. The eluate was fractionated and monitored for absorbance at 280 nm and by TLC. The fractions comprising the major peak of peptide material were pooled and lyophilized, and the residue (57 mg) was further subjected to gel filtration on Sephadex G-15 (column 100 × 1.5 cm), eluting with aqueous acetic acid (0.2 M) with a flow rate ~6 mL/h.[25] The peptide was eluted in a single, symmetrical peak (absorbance 280 nm). Lyophylization of the pertinent fractions gave the oxytocin analogue X: yield 44 mg; TLC R_f (system D) 0.38, R_f (system E) 0.30, R_f (system F) 0.83; $[\alpha]^{22}_D$ −54° (*c* 0.5, 1 M acetic acid). Amino acid analysis: Tyr, 0.9; Ile, 1.0; Glu, 1.0; Asp, 1.1; $^1/_2$-Cys, trace; Pro, 1.0; Leu, 1.0; Gly, 1.00; NH_3, 3.3. A peak superimposed upon the artifact caused by the buffer change (pH 3.25 to 4.25) was presumed to be due to the mixed disulfide of β-mercapto-β,β-dimethylpropionic acid and cysteine.[24] Analysis following hydrolysis with sulfuric acid:[10,36] Tyr(Me)/Tyr/Gly, 0.81:0.05:1.00. Analysis following performic acid oxidation[40] gave a Cys(O_3H)/Gly ratio of 0.91:1.00, the trace of $^1/_2$-Cys and the peak presumed to be due to the mixed disulfide having collapsed.

[1-Deaminopenicillamine,2-(*O*-methyl)tyrosine,4-threonine]oxytocin [dPTyr(Me)TOT, XI). The peptide intermediate IX (100 mg) was reduced by sodium in liquid ammonia and reoxidized with potassium ferricyanide[17,23,24] as detailed above in the preparation of analogue X. The two passes on Sephadex G-15[25] yielded the oxytocin analogue XI: yield 41 mg; TLC R_f (system D) 0.52, R_f (system E) 0.49, F_f (system F) 0.87; $[\alpha]^{22}_D$ −42° (*c* 0.5, 1 M acetic acid). Amino acids analysis:[35] Tyr, 0.9; Ile, 1.0; Thr, 0.9; Asp, 1.0; Pro, 1.0; Leu, 1.0; Gly, 1.0; NH_3, 2.1. Analysis following performic acid oxidation[40] prior to hydrolysis gave a Cys(O_3H)/Gly ratio of 0.94:1.00. Analysis following hydrolysis with H_2SO_4:[10,36] Tyr(Me)/Tyr/Gly, 0.85:0.04:1.00.

[1-(β-Mercapto-β,β-diethylpropionic acid),4-threonine]-oxytocin (XII). Treatment of the intermediate VI (100 mg) as described above[17,23–25] yielded the analogue XII: yield 32 mg; TLC R_f (system D) 0.49, R_f (system E) 0.47, R_f (system F) 0.86; $[\alpha]^{24}_D$ −48° (*c* 0.5, 1 M acetic acid). Amino acid analysis:[35] Tyr, 1.0; Ile, 1.0; Thr, 1.0; Asp, 1.0; Pro, 1.0; Leu, 1.1; Gly, 1.0; NH_3, 2.0. Analysis following performic acid oxidation:[40] Cys(O_3H)/Gly, 1.05:1.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),4-threonine]oxytocin (XIII). The acyl octapeptide derivative VII (100 mg) was reduced and reoxidized as described above[17,23–25] to give the analogue XIII: yield 41 mg; TLC R_f (system D) 0.49, R_f (system E) 0.47, R_f (system F) 0.86; $[\alpha]^{24}_D$ −40° (*c* 0.5, 1 M acetic acid). Amino acid analysis:[35] Tyr, 1.0; Ile, 1.0; Thr, 1.0; Asp, 1.0; Pro, 0.9; Leu, 1.0; Gly, 0.9; NH_3, 2.1. Analysis following performic acid oxidation:[40] Cys(O_3H)/Gly, 0.98:1.00.

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References and Notes

(1) (a) A preliminary report on some of this work was presented at the Meeting of the Endocrine Society, Miami, Fla., June, 1978: W. H. Sawyer, J. Haldar, D. Gazis, J. Seto, K. Bankowski, J. Lowbridge, A. Turan, and M. Manning, *Program Abstr., Annu. Meet. Endocr. Soc., 60th*, p 187. (b) The symbols for the amino acids follow the recommendation of the IUPAC–IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1972).
(2) M. Manning, J. Lowbridge, J. Seto, J. Haldar, and W. H. Sawyer, *J. Med. Chem.*, **21**, 179 (1978).
(3) H. Schulz and V. du Vigneaud, *J. Med. Chem.*, **9**, 647 (1966).
(4) W. H. Sawyer, J. Haldar, D. Gazis, J. Seto, K. Bankowski, J. Lowbridge, A. Turan, and M. Manning, *Endocrinology*, in press.

(5) R. J. Vavrek, M. F. Ferger, G. A. Allen, D. H. Rich, A. T. Blomquist, and V. du Vigneaud, *J. Med. Chem.*, **15**, 123 (1972).
(6) J. J. Nestor, Jr., M. F. Ferger, and V. du Vigneaud, *J. Med. Chem.*, 18, 284 (1975).
(7) M. Manning, J. Lowbridge, and W. H. Sawyer, *Pept.: Chem. Struct. Biol., Proc. Am. Pept. Symp., 4th*, 1976, p 737.
(8) M. Manning, J. Lowbridge, J. Haldar, and W. H. Sawyer, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **36**, 1848 (1977).
(9) W. Y. Chan, R. Fear, and V. du Vigneaud, *Endocrinology*, 81, 1267 (1967).
(10) H. B. Law and V. du Vigneaud, *J. Am. Chem. Soc.*, **82**, 4579 (1960).
(11) K. Jost, J. Rudinger, and F. Sorm, *Collect. Czech. Chem. Commun.*, **26**, 2946 (1961).
(12) J. Rudinger and I. Krejci, *Handb. Exp. Pharmacol.*, **23**, 748 (1968).
(13) G. W. Bisset and D. G. Smyth, unpublished observations; cited in D. G. Smyth, *J. Biol. Chem.*, **242**, 1592 (1967).
(14) G. W. Bisset, B. J. Clark, I. Krejci, I. Polacek, and J. Rudinger, *Br. J. Pharmacol.*, 40, 342 (1970).
(15) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
(16) R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964).
(17) M. Manning, E. Coy and W. H. Sawyer, *Biochemistry*, **9**, 3925 (1970).
(18) M. Manning, *J. Am. Chem. Soc.*, **90**, 1348 (1968).
(19) M. Manning, J. Lowbridge, J. Haldar, and W. H. Sawyer, *J. Med. Chem.*, **19**, 376 (1976).
(20) L. Lowbridge, M. Manning, J. Haldar, and W. H. Sawyer, *J. Med. Chem.*, **20**, 1173 (1977).
(21) W. Konig and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
(22) M. F. Ferger and W. Y. Chan, *J. Med. Chem.*, 18, 1020 (1975).
(23) V. du Vigneaud, C. Ressler, J. M. Swan, P. Katsoyannis, and C. W. Roberts, *J. Am. Chem. Soc.*, **76**, 3115 (1954).
(24) D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *J. Biol. Chem.*, **237**, 1563 (1962).
(25) M. Manning, T. C. Wuu, and J. W. M. Baxter, *J. Chromatogr.*, **38**, 396 (1968).
(26) H. O. Schild, *Br. J. Pharmacol.*, **2**, 189 (1947).
(27) R. A. Munsick, *Endocrinology*, **66**, 451 (1960).
(28) D. F. Dyckes, J. J. Nestor, Jr., M. F. Ferger, and V. du Vigneaud, *J. Med. Chem.*, 17, 969 (1974).
(29) G. W. Bisset, J. Haldar, and J. E. Lewin, *Mem. Soc. Endocrin.*, 14, 185 (1966).
(30) J. Dekanski, *Br. J. Pharmacol.*, **7**, 567 (1952).
(31) K. Bankowski, M. Manning, J. Haldar, and W. H. Sawyer, *J. Med. Chem.*, **21**, 850 (1978).
(32) L. C. Dorman, *Tetrahedron Lett.*, **28**, 2319 (1969).
(33) B. F. Gisin, *Helv. Chim. Acta*, **56**, 1476 (1973).
(34) H. Takashima, R. B. Merrifield, and V. due Vigneaud, *J. Am. Chem. Soc.*, **90**, 1323 (1968).
(35) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
(36) See footnote 33 in ref 31 for a summary of our previous findings relating to the amino acid analysis of *O*-methyltyrosine containing analogues of arginine-vasopressin. Hydrolysis of the products reported here which contained *O*-methyltyrosine with hydrochloric acid in the normal way yielded tyrosine. This demethylation was reportedly avoided by hydrolysis with sulfuric acid.[10] The quantitation of *O*-methyltyrosine reported here was by reference to the TFA cleavage product of Boc-*O*-methyltyrosine,[31] which emerged from the short column before lysine and contained no tyrosine. In samples containing *S*-benzylcysteine, there was overlap, hence, the indication of data as approximate. Hydrolysis of Boc-*O*-methyltyrosine with sulfuric acid[10] under the conditions used for hydrolysis of peptides was shown to allow an 85–90% recovery, 80% as material identical to the TFA cleavage product, and assumed, therefore, to be *O*-methyltyrosine and 5–10% as tyrosine. There was in the case of protected peptides indication that hydrolysis by sulfuric acid of the peptide chain was incomplete, small amounts of material eluting in the void volume of the long column.
(37) A portion of this material was used in the synthesis of [1-deaminopenicillamine,4-threonine]oxytocin.[2]
(38) M. Bodanszky, M. Kondo, C. Y. Lin, and G. F. Sigler, *J. Org. Chem.*, **39**, 444 (1974).
(39) A. gift from Dr. Martha Ferger, Department of Chemistry, Cornell University, New York, N.Y.
(40) S. Moore, *J. Biol. Chem.*, **238**, 235 (1963).

Notes

Synthesis and Chemical Carcinogen Inhibitory Activity of 2-*tert*-Butyl-4-hydroxyanisole

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The title compound 1 was selectively synthesized in its pure isomeric form by means of the hydroxyl-protecting reagent dimethyl-*tert*-butylchlorosilane. Exclusive silylation occurred at the less hindered hydroxyl group of **3**. Dimethyl sulfate methylation of 4 gave **5** in excellent yield. Compound 1 was then obtained by acid hydrolysis of **5**. The two BHA isomers, 1 and **2**, were tested on their inhibitory effects toward benzo[*a*]pyrene-induced neoplasia in the forestomach of the ICR/Ha mouse. Both isomers, when added to the diet, reduced the number of mice with tumors and the number of tumors per mouse. Isomer 1, which has the less hindered free hydroxyl group, showed higher inhibitory effect in the present experimental model.

Butylated hydroxyanisole (BHA) has been widely used as an antioxidant to stabilize fatty foods since 1947.[1] Recently, BHA has been found to protect laboratory animals from chemically induced neoplasia under various experimental conditions.[2] While the detailed mechanism of protection is still under investigation, in vitro observations using benzo[*a*]pyrene (BP) as a model carcinogen indicate the BHA decreases the formation of active BP metabolites as well as the level of BP–DNA binding.[3] Commercially available BHA, which contains two isomers in an approximately 15:85 ratio of **1** and **2**, has been used for tumor protection and mechanistic studies. When

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