

Solid-Phase Synthesis and Biological Activity of the Parallel Dimer of Deamino-Oxytocin

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INTRODUCTION

The neurohypophyseal hormones oxytocin and vasopressin, as well as numerous analogues of these molecules, promote a range of important biological activities (9,10,12,16). These hormones all contain a 20-membered heterodetic ring with a single disulfide bridge, which can occasionally be altered (e.g., carba substitution) without significantly affecting activities. The long-standing synthetic challenge in this field has been to optimize yields and purities of the desired monomeric, monocyclic peptides, while minimizing the formation of dimers and higher oligomers. However, it is also of interest to prepare intentionally and/or to isolate these dimers (parallel or anti-parallel) (24,25) and determine their activities. When such activities are observed (typically 0.25% to 4% that of the parent hormone), the question remains whether these are due to a true interaction of the dimer with a receptor, or to monomers that are present as contaminants to the dimer.

An alternative explanation for observed activities involves re-equilibration of dimer to monomer under the conditions of biological testing. Such an explanation is consistent with the earlier observation from one of our laboratories that a synthetic *carba* analogue of the deamino-oxytocin anti-parallel dimer was completely lacking biological activities (21). Attempted syntheses of the deamino-oxytocin parallel dimer (two disulfides) in solution were unsuccessful, with formation of monomer at low concentration and polymer at higher concentrations. These difficulties can be attributed to the relative ease of oxidation of cysteinyl (free α -amino group) residues with respect to mercaptopropionyl residues [formation of oxytocin is achieved readily by air oxidation but deamino-oxytocin requires more powerful agents to achieve cyclization (8)]. The present communication shows how a solid-phase approach can be used to obtain the desired dimer (structure given in Scheme 1), and continues with a description of the biological properties of the synthetic product.

MATERIALS AND METHODS

Materials, solvents, instrumentation and general methods were essentially as summarized in previous publications from one of our laboratories (1,2,19). Acidolytic cleavage/deprotection of peptidyl tris(alkoxy)benzylamide (PAL)-resins was carried out with trifluoroacetic acid (TFA)-dichloromethane-triethylsilane-water (95:4:0.5:0.5), at 25°C for 2 h, and gave the corresponding peptide amides in high yields (90%–97%). Analytical HPLC (Figure 1) was performed on a Vydac C-18 reversed-phase column (The Separations Group, Hesperia, CA) (5 μ , 4.6 \times 250 mm); linear gradient over 20 min using CH₃CN and 0.01 *N* aqueous HCl from 1:9 to 6:4, flow rate 1.2 ml/min.

ABSTRACT

The parallel dimer of deamino-oxytocin has been synthesized by a novel solid-phase route. Successive orthogonal deprotection and oxidation reactions, carried out while the peptide remained anchored to a polymeric support, resulted in the formation of two disulfide bridges and conversion, with minimal side reactions, of the linear monomeric precursor to the dimer. The purified dimer showed approximately 0.1% to 2% of the biological activities of the monomer, as well as prolonged action. The time course of response to the deamino-oxytocin dimer differed from that of the monomer (and of oxytocin), and is probably due to slow formation of monomer under the conditions of biological testing.

Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in 1972, *J. Biol. Chem.* 247:977-983. The following additional abbreviations are used: AcM, acetamidomethyl; β Mpa, β -mercaptopropionic acid; DIPCDI, *N,N'*-diisopropylcarbodiimide; DMF, *N,N*-dimethylformamide; FABMS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; MBHA, 4-methylbenzhydrylamine (resin); NMP, *N*-methylpyrrolidinone; PAL, tris(alkoxy)benzylamide linker of reference 2; PS or @, copoly(styrene-1%-divinylbenzene) polymeric support; TFA, trifluoroacetic acid; Tmob, 2,4,6-trimethoxybenzyl; T1(tfa)₃, thallium (III) trifluoroacetate. Amino acid symbols denote the L-configuration. All solvent ratios and percentages are volume/volume.

Preparation of S-2,4,6-Trimethoxybenzyl-L- β -Mercaptopropionic Acid [β Mpa(Tmob)-OH]

A solution of 3-mercaptopropionic acid (0.47 g, 4.4 mmol) in CH_2Cl_2 (8 ml) was stirred at 5°C , and TFA (0.35 ml, 2.2 mmol) was added portionwise over 5 min. A solution of 2,4,6-trimethoxybenzyl alcohol (19) (0.89 g, 4.4 mmol) in CH_2Cl_2 (20 ml) was added dropwise over approximately 5 min and, after an additional 5 min, the reaction mixture was concentrated *in vacuo*. The residue was chased with anhydrous diethyl ether (Et_2O , ca. 40 ml), triturated further with Et_2O (ca. 150 ml) and filtered to provide a white solid (1.0 g, 75%); mp 114°C ; TLC (chloroform-acetic acid, 19:1) R_f 0.47; ^1H NMR (CDCl_3) δ 6.12 (s, 2H, aromatic), 3.81 (s, 9H, OCH_3), 3.75 (s, 2H, CH_2), 2.71 (s, 4H, $2 \times \text{CH}_2$); ^{13}C NMR (CDCl_3) δ 178.2 (COOH), 159.9 (aryl C4), 158.4 (aryl C2 and C4), 107.4 (aryl C1), 90.2 (aryl C3 and C5), 55.3 (ortho, $2 \times \text{CH}_3\text{O}$), 54.9 (para, CH_3O), 34.5 (SCH_2Ar), 25.8 ($\alpha\text{-CH}_2$), 22.9 ($\beta\text{-CH}_2$); Anal. Calcd. for $\text{C}_{13}\text{H}_{18}\text{O}_5\text{S}$, MW 286.34; C, 54.53; H, 6.34; S, 11.19. Found: C, 54.75; H, 6.36; S, 10.96.

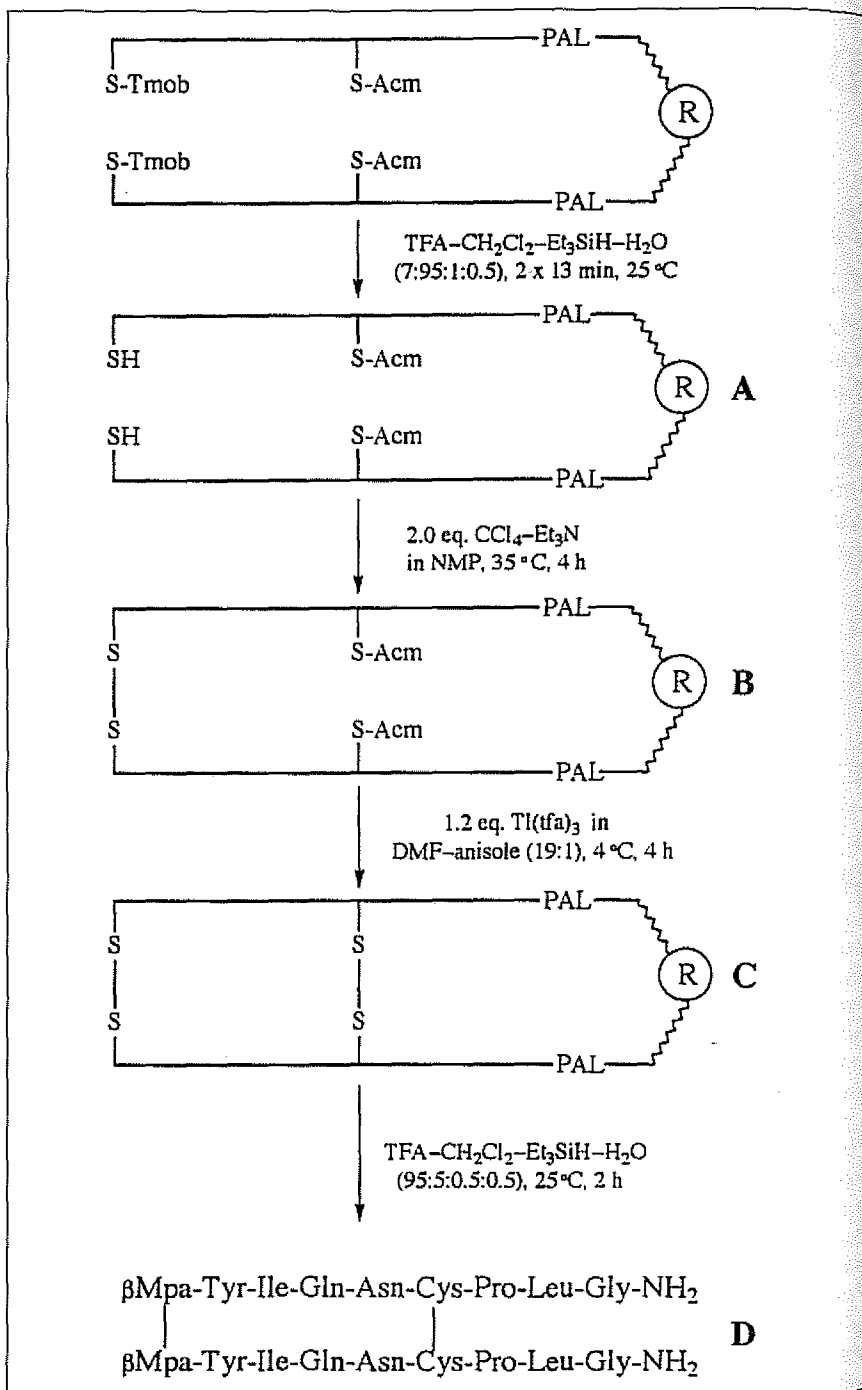
Solid-Phase Synthesis of β Mpa (Tmob)-Tyr(tBu)-Ile-Gln-Asn-Cys (Acm)-Pro-Leu-Gly-PAL-Resin

The linear peptide was assembled on a MilliGen/Biosearch 9600 Automated Peptide Synthesizer (Bedford, MA), starting with a PAL-Nle-MBHA-PS resin (1.0 g, initial loading ca. 0.3 mmol/g) (2). Couplings of all N^α -Fmoc amino acid derivatives were mediated by N,N' -diisopropylcarbodiimide (DIPCDI)/1-hydroxybenzotriazole (HOBt) in N,N -dimethylformamide (DMF) for 1 h, except Fmoc-Asn and Fmoc-Gln were incorporated as their pentafluorophenyl active esters in the presence of HOBt, coupled for 1 h in DMF. Upon completion of chain assembly, an aliquot of peptide-resin was cleaved and analyzed by HPLC (Figure 1A, major component t_R 16.1 min, >90%). The crude linear mono-acetamidomethyl (Acm) peptide was characterized further by fast atom bombardment mass spectrometry (FAB-MS): calculated for $\text{C}_{46}\text{H}_{72}\text{N}_{12}\text{O}_{13}\text{S}_2$ mass 1064.47; found, m/z 1065.5 [MH^+] and 1087.6 [$(\text{M} + \text{Na})^+$]. Amino

Table 1. Activities of Oxytocin and Analogues in Rat (IU/mg)

Peptide	Uterotonic	Galactogogic	Pressor	References
Oxytocin	450/450*	450	5	12
Deamino-oxytocin	800	400	1.5	8,12
Deamino-carbo-1-oxytocin anti-parallel dimer	0/0*	0.03	0	21
Deamino-oxytocin parallel dimer	2.8/10.8*	0.6	0	this work

The numbers followed by an asterisk reflect *in vivo* uterotonic activity (Figure 2).



Scheme 1. Orthogonal solid-phase synthesis of the parallel dimer of deamino-oxytocin.

acid analysis of crude cleaved peptide: Asp, 1.01; Gly, 1.03; Glu, 1.01; Ile, 0.92; Leu, 1.06; Pro, 1.10; Tyr, 0.97.

Synthesis of Resin-Bound Monocyclic Intermediate (Scheme 1B)

Peptide-resin (ca. 25 mg) was swelled in CH_2Cl_2 (5 min) and then treated with $\text{TFA}-\text{CH}_2\text{Cl}_2-\text{Et}_3\text{SiH}-\text{H}_2\text{O}$ (2 ml; 7:92:0.5:0.5), 2×13 min, 25°C , to remove selectively the 2,4,6-trimethoxybenzyl (Tmob) group (19). Following washes with CH_2Cl_2 , DMF and *N*-methylpyrrolidinone (NMP) (5×2 ml each), oxidation with 20 mM $\text{CCl}_4-\text{Et}_3\text{N}$ (2 eq each) in NMP (0.4 ml), 35°C for 4 h, gave the monocyclic resin-bound intermediate. For further characterization, the peptide-resin was cleaved to show a single major component by HPLC (Figure 1B, t_R 19.7 min, ca. 75% purity) and FABMS: calculated for $\text{C}_{92}\text{H}_{142}\text{N}_{24}\text{O}_{26}\text{S}_4$ mass

2126.94; found, largest peak at m/z 2150.84 (includes one C^{13}) [$(\text{M} + \text{Na})^+$].

Synthesis of Fully Oxidized Dimer (Scheme 1C)

Peptide-resin (ca. 25 mg) containing bound monocyclic intermediate (Scheme 1B) was swelled in DMF (5 min) and then treated with ca. 0.025 M thallium (III) trifluoroacetate [$\text{Tl}(\text{tfa})_3$] (2 eq) in DMF-anisole (0.3 ml; 19:1) at 4°C . After 4 h, the thallium reagent was removed with DMF washes (5×2 ml) and the peptide released from the resin with the standard TFA cocktail. HPLC (Figure 1C) showed two primary products in a 3:1 ratio; the more abundant of these (t_R 20.5 min) was assigned to the desired bicyclic peptide, whereas the lesser (t_R 17.2 min) co-migrated with authentic deamino-oxytocin, the monocyclic monomer.

Preparative Experiment

The two optimized oxidation steps given above were carried out on a larger scale of peptide-resin (ca. 150 mg), with amounts of reagents and solvent volumes scaled up proportionally. The crude cleaved product (ca. 50 mg, ca. 50 μmol) was dissolved in 0.1 M Na_2HPO_4 , 6 M guanidine HCl, pH 5.0 (1.5 ml) and applied to gel filtration on Sephadex G-25 (Pharmacia Biotech, Piscataway, NJ) developed with the same buffer. The major fraction was collected and desalted through an Amicon (Beverly, MA) stirred ultrafiltration cell (43 mm, molecular weight cut-off >500 daltons). The resultant material (ca. 10 mg) was the desired parallel dimer, of approximately 93% purity (no monomer present) by HPLC (Figure 1D), and characterized further by FABMS: calculated for $\text{C}_{86}\text{H}_{130}\text{N}_{22}\text{O}_{24}\text{S}_4$ mass 1982.85; found, quasi-molecular ion cluster, largest peak at m/z 2007.2 (includes one C^{13}) [$(\text{M} + \text{Na})^+$]. The overall yield, based on starting Fmoc-Gly-PAL-resin, was approximately 15%. Since the yields of chemical steps were high, the cited yield may reflect the sparing solubility of the title peptide.

Activity Studies

Biological activities (Table 1) were determined as described in the literature (3,7,13,17,20) by the following procedures: uterotonic *in vitro* tests in magnesium-free solution, uterotonic *in vivo* tests using ethanol-anesthetized rats, galactogogic tests also using ethanol-anesthetized rats and pressor tests using pithed rat preparations. Wistar rats weighing 200–250 g were used in all tests. Dose-response curves were constructed and threshold doses were compared. Synthetic oxytocin (450 IU/mg) was used as a standard for oxytocin assays; synthetic arginine vasopressin (400 IU/mg) was a standard for the pressor assay.

RESULTS

This paper reports a novel, efficient, solid-phase method (Scheme 1) to synthesize the parallel dimer of $\beta\text{Mpa-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH}_2$ (deamino-oxytocin). Our approach applied recent discoveries in the protection and manipulation of cysteine residues (1,18,19) and took advantage of the *pseudo-dilution*

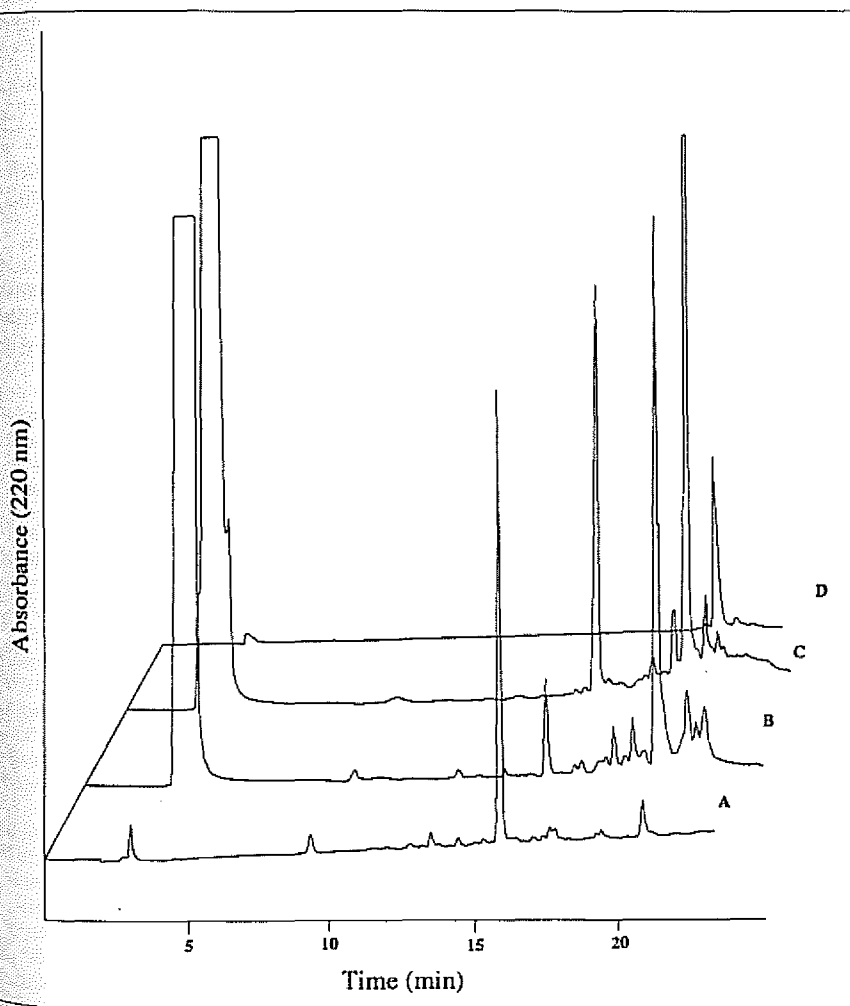


Figure 1. HPLC at stages A, B, C directly after cleavage from the support (correlate to Scheme 1). D represents the purified parallel dimer of deamino-oxytocin. HPLC conditions are given in Materials and Methods.

phenomenon, which applies to the chemistry of polymer-bound species (1,14, 15). The monomeric linear precursor was synthesized by standard stepwise Fmoc chemistry (1,2,4). Treatment with dilute TFA in the presence of suitable scavengers selectively removed the Tmob group protecting the N-terminal β -mercaptopropionyl residue, and gave the monothiol mono(Acm) intermediate, which was retained on the support with negligible loss of chains. Since intramolecular cyclization is precluded, intermolecular oxidation of the thiols in proximal chains gave the monocyclic parallel dimer, with two attachment points (*via* the C-terminus) to the resin. Oxidation conditions with triethylamine and carbon tetrachloride, adapted (18) from Wenschuh et al. (23), proved optimal and provided the first disulfide bridge in >90% yield.

A reciprocal protection scheme, using Acm for β Mpa¹ and Tmob for Cys⁶, was also tried. After Tmob removal, the intramolecular oxidation step was very slow (ca. 20% oxidation over the 4-h time period that was sufficient for oxidizing completely the intermediate with a free Tmob at β Mpa¹). This result points out sequence-dependent subtleties in the applications of this chemistry.

Returning to the preferred route (Scheme 1), a further step with Fujii's thallium reagent (1,5) simultaneously removed Acm and closed the second disulfide bridge to provide the complete, cyclized parallel dimer. TFA at moderate concentration now released peptides from the support, with the desired dimer being the major product. These final steps were accompanied by some formation (ca. 25%) of the monocyclic monomer, i.e., deamino-oxytocin.

It is not known whether the monocycle is due to an intramolecular side reaction of the thallium oxidation step, or to disulfide exchange of the dimer at a later stage, e.g., cleavage. The desired bicyclic parallel dimer was obtained readily in high purity (>90%; no monomer present) and 15% overall yield by simple gel filtration.

The parallel deamino-oxytocin dimer showed low activity in three assays; values were 2.8, 10.8 and 0.6 IU/mg for uterotonic *in vitro*, uterotonic *in vivo* and galactogogic tests, respectively (Table 1). No activity was found in the pressor test. Activities determined in the *in vivo* uterotonic test (Figure 2) and galactogogic test were *extremely prolonged*. In the *in vitro* test, the tissue continued to contract after removal of the dimeric peptide from the bath by several washes. This result is unusual, because in similar experiments with oxytocin, the response fades quickly due to dilution of peptide in the bathing medium.

DISCUSSION

In contrast to the anti-parallel dimer of carba-1-oxytocin (21), the newly available parallel dimer of deamino-oxytocin has been found to possess a low but significant level of activity in several assays. The responses in the uterotonic *in vivo* and galactogogic tests were extremely prolonged and differed from that of oxytocin. It is expected that any activities due to small amounts of monomer present in the original synthetic sample or arising during sample preparation would be short-lasting.

Enhanced and prolonged activity of deamino-oxytocin in the *in vivo* uterotonic test (but not in antidiuretic, galac-

togogic and pressor tests) was studied in detail experimentally by Gazis et al. (6) and described theoretically by Wanner and Pliska (22). Prolongation of activity can be explained by an accumulation of deamino-oxytocin in the uterus, and its subsequent slow release. Such a phenomenon might also partly account for the activity of the deamino-oxytocin dimer, if it could be shown that the dimer accumulates. However, the time course of the effects *actually* observed is consistent with the slow generation of an active compound. Comparisons of activities of carba analogues with activities of bis(disulfide) dimers are also suggestive of the absence of intrinsic activities in dimers; hence activities observed are most likely due to slow formation of the monomer. Consequently, we believe that the dimer acts as a hormonogen (11).

Further chemical studies are directed at unambiguous synthesis of the anti-parallel dimer of deamino-oxytocin, and the preparation of both the parallel and anti-parallel dimers of additional analogues. It bears mentioning that the title chemistry of this paper gave the parallel dimer of oxytocin in similar yield. The parallel dimer of oxytocin has been made intentionally, by low yield solution chemistry (24), and also isolated indirectly (along with the anti-parallel dimer) as a by-product from the oxidation of the reduced dithiol form of oxytocin to the monocyclic monomer (25).

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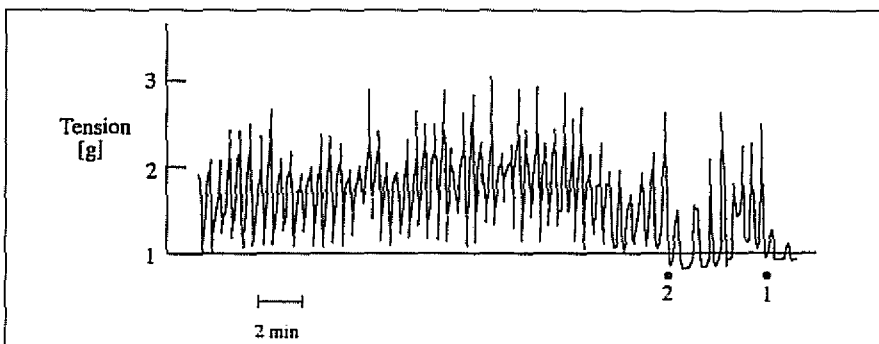


Figure 2. Contractions of rat uterus after intravenous administration of oxytocin and the parallel dimer of deamino-oxytocin. Timeline is right to left. At point 1, 5.5×10^{-6} mg of oxytocin was added, and at point 2, 2×10^{-3} mg of the dimer was added.

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