Synthesis of the Parallel Dimer of Oxytocin[1,2]

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Abstract: The cyclic parallel dimer of oxytocin has been synthesized by the coupling of bis(*t*-butyloxycarbonyl)-L-cystine with N,N′-bis(L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl)-L-cystinyl-bis(L-prolyl-L-leucylglycinamide) by the mixed anhydride method and the subsequent removal of the *t*-butyloxycarbonyl groups. The N,N′-bis(L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl)-L-cystinyl-bis(L-prolyl-L-leucylglycinamide) was obtained by treatment of N-benzyloxycarbonyl-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide with sodium in liquid ammonia for removal of the benzyloxycarbonyl and benzyl groups and subsequent oxidation with potassium ferricyanide. The parallel dimer was isolated and purified by partition chromatography and gel filtration on Sephadex G-25. The synthetic parallel dimer was found to be identical with the α dimer of oxytocin, one of the two 40-membered ring isomeric dimers of the hormone previously described. The parallel dimer is readily converted to oxytocin by reduction with sodium in liquid ammonia and subsequent oxidation.

Two cyclic isomeric dimers of the posterior pituitary hormone oxytocin, designated as α dimer and β dimer, were recently isolated as by-products in the synthesis of oxytocin.[3] The unresolved mixture of dimers was separated from the hormone by partition chromatography on Sephadex G-25; the separation of α dimer from β dimer was then effected by gel filtration on Sephadex G-25. The isomeric dimers apparently are the products of intermolecular oxidation reactions between pairs of oxytoceine molecules that occur to a minor extent compared with the intramolecular reaction leading to oxytocin. The two isomeric dimers were also obtained through disulfide interchange by treatment of the hormone with triethylamine.[3]

Parallel and antiparallel structures are possible for these cyclic dimers of oxytocin, each containing a 40-membered ring with two disulfide bridges as shown in Figure 1. The synthesis of one of these dimers should establish the structure of either the α dimer or the β dimer by direct comparison.

The synthesis of the parallel dimer was undertaken by the series of reactions shown in Figure 2. This method of synthesis should lead unambiguously to the parallel dimer unless evidence of disulfide interchange should be encountered. If disulfide interchange should take place, one would then expect to obtain both parallel and antiparallel dimers. However, only one dimer was isolated; no trace of a second dimer was detected. We therefore conclude that the synthetic dimer is the parallel dimer.

To form the 40-membered ring, N,N′-bis(*t*-butyloxycarbonyl)-L-cystine (II) was coupled with the polypeptide disulfide III. The yield was low but a sufficient amount of synthetic parallel dimer was obtained to establish that it is identical with the α dimer. Thus the α dimer possesses the parallel structure shown in Figure 1. It then follows that the β dimer possesses the antiparallel structure.

The protected octapeptide N-benzyloxycarbonyl-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide[4] (I) was treated with sodium in liquid ammonia to remove the N-benzyloxycarbonyl and S-benzyl groups[5] and the O-benzyl group.[6] The reduced material was then oxidized in aqueous solution with potassium ferricyanide[7] and the resulting symmetrical disulfide, bis(L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl)-L-cystinyl-bis(L-prolyl-L-leucylglycinamide) (III), was isolated by partition chromatography on Sephadex G-25. The disulfide III was coupled with N,N′-bis(*t*-butyloxycarbonyl)-L-cystine in dimethylformamide by the mixed anhydride method.[8] The resulting material was treated with anhydrous trifluoroacetic acid for removal of the N-*t*-butyloxycarbonyl groups.[9] The crude product was subjected to partition chromatography on Sephadex G-25 in the solvent system 1-butanol–benzene–pyridine–0.1% aqueous acetic acid (6:2:1:9). Under these conditions the α dimer and β dimer of oxytocin are not separable and emerge with an R_f of 0.32, while oxytocin has an R_f of about 0.24.[3] The synthetic material gave a sharp symmetrical peak with R_f 0.32; no peak corresponding to the position of oxytocin was detected. The isolated material was subjected to gel filtration on a short Sephadex G-25 column (1.38 × 82 cm) in 0.2 *N* acetic acid. Under these conditions the α dimer and β dimer of oxytocin emerge as one peak well ahead of oxytocin. Only one peak was obtained at the position expected for a dimer of oxytocin. An acid hydrolysate of this synthetic product had the amino acid compositon expected of a dimer of oxytocin. The synthetic product traveled as a single spot on paper chromatography and paper electrophoresis.

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(2) Reprint requests should be sent to Professor Vincent du Vigneaud at the Department of Chemistry, Cornell University, Ithaca, N. Y. 14850.

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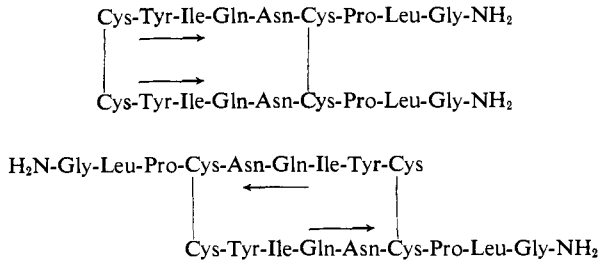

Figure 1. Upper: cyclic parallel dimer of oxytocin. Lower: antiparallel dimer. The arrows indicate the direction of peptide linkages.

The α dimer travels faster than the β dimer upon gel filtration on block polymerizate Sephadex G-25 in 0.2 *N* acetic acid when a column of suitable efficiency is employed. When samples of the synthetic dimer were compared on a long column (1.24 × 159 cm) with samples of the α dimer and β dimer the synthetic dimer emerged at the same effluent volume as the α dimer. No trace of β dimer was detected in the synthetic preparation. The specific rotation of the synthetic dimer was $[\alpha]^{19}D$ −105° (*c* 0.5, 1 *N* acetic acid) as compared with values of −106 and −78° for α dimer and β dimer, respectively, under comparable conditions. It is thus apparent that the synthetic dimer is identical with the α dimer isolated previously[3] and that the α dimer possesses the parallel structure shown in Figure 1.

Previous work has shown that the disulfide bridge in the 20-membered ring of oxytocin is reduced by sodium in liquid ammonia to give oxytoceine.[10] In the studies on the isomeric dimers of oxytocin, reduction of either α dimer or β dimer also liberated oxytoceine, since subsequent oxidation gave, in each case, oxytocin.[3] When a sample of the synthetic parallel dimer was treated with sodium in liquid ammonia, measurement of the sulfhydryl content of the reduced material showed that 82% of the amount of sulfhydryl groups theoretically obtainable from a dimeric structure containing two disulfide linkages had been liberated. The reduced material was oxidized, and subsequent isolation by partition chromatography gave oxytocin in high yield.

Experimental Section

Methods and Materials. Sephadex G-25 medium block polymerizate (Pharmacia Fine Chemicals, Inc., New Market, N. J.) was used for partition chromatography and gel filtration as described previously.[3] Peptides were detected by the Folin-Lowry procedure.[11] The Ellman method was used for the determination of sulfhydryl content.[12]

N,N′-Bis(L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl)-L-cystinyl-bis(L-prolyl-L-leucylglycinamide) (III). N-Benzyloxycarbonyl-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide[4] (I) (3.00 g) was dissolved in 450 ml of liquid ammonia (distilled from sodium) and treated at the boiling point with sodium until a blue color persisted throughout the solution for 2 min. The solution was evaporated *in vacuo* to low volume and lyophilized. The residue was dissolved in deaerated water (500 ml) containing trifluoroacetic acid (1.14 ml). The resulting solution (pH 8.5) was titrated with 0.1 *N* potassium ferricyanide[7] (24.6 ml) and stirred for 2 hr. The solution was then stirred with 80 ml (wet volume) of the chloride form of AG 3-X4 resin (Bio-Rad Lab., Richmond, Calif.) for 30 min and the resin was filtered off. The filtrate was evaporated *in vacuo* to a volume of 20 ml and subjected to gel filtration on a 2.82 × 62.2 cm column in 0.2 *N* acetic acid. A flow rate of 27 ml/hr was maintained and the eluates were collected in fractions of 4.2 ml. The chromatogram contained one peak with a maximum at effluent volume 294 ml. The material from the eluates represented by the central portion of the peak was isolated by lyophilization and dissolved in 20 ml of the organic phase of 1-butanol–ethanol–pyridine–0.2 *N* aqueous acetic acid (8:1:1:10) and subjected to partition chromatography in this solvent system under the following conditions: column size, 4.68 × 112.4 cm; hold-up volume, 495 ml; fraction volume, 11.2 ml; flow rate, 67 ml/hr; regenerating solvent, pyridine–0.1% aqueous acetic acid (1:4). The chromatogram showed one sharp peak with R_f 0.34, and isolation of the material from the eluates represented by this peak gave 1.29 g of the polypeptide disulfide.

A sample (30 mg) was dissolved in 0.2 *N* acetic acid (1.0 ml) and subjected to gel filtration on a 1.38 × 82 cm column in 0.2 *N* acetic acid at a flow rate of 5 ml/hr. The eluates were collected in fractions of 1.04 ml. The chromatogram showed one sharp symmetrical peak with a maximum at effluent volume 85.6 ml. Isolation by lyophilization gave 26.5 mg, $[\alpha]^{20}D$ −114° (*c* 1, 1 *N* acetic acid). For analysis a sample was dried at 100° over phosphorous pentoxide *in vacuo* with a loss in weight of 12.5%.

Anal. Calcd for $C_{80}H_{124}N_{22}O_{22}S_2 \cdot 2CH_3COOH$: C, 53.2; H, 7.01; N, 16.2. Found: C, 52.8; H, 7.02; N, 16.6.

A sample was hydrolyzed in 6 *N* HCl at 110° for 24 hr and analyzed[13] on a Beckman-Spinco amino acid analyzer. The following molar ratios of amino acids and ammonia were found, with the value of leucine taken as 1.0; aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.0; glycine, 0.9; cystine, 0.5; leucine, 1.0; isoleucine, 1.0; tyrosine, 0.9; and ammonia, 3.0.

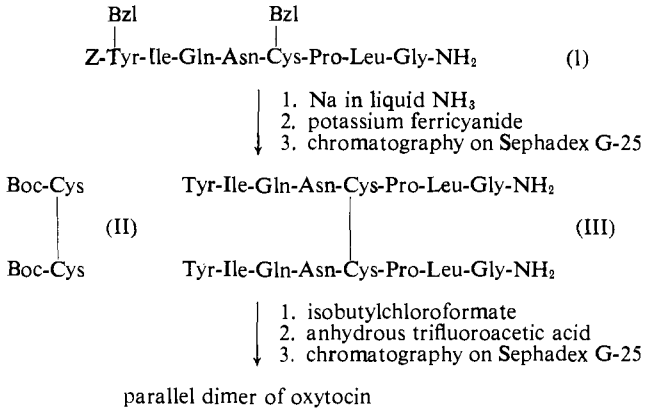

Figure 2. Scheme for the synthesis of the parallel dimer.

Parallel Dimer of Oxytocin. Bis(*t*-butyloxycarbonyl)-L-cystine (II) (134 mg) (Cyclo Chemical Corp., Los Angeles, Calif.) was dissolved in dimethylformamide (6.5 ml) containing triethylamine (0.080 ml). The solution was cooled to −15° and isobutylchloroformate (0.082 ml) was added. The mixture was warmed to −5° over a period of 15 min and mixed with a solution of N,N′-bis-(L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl)-L-cystinyl-bis(L-prolyl-L-leucylglycinamide) (III) (606 mg) in dimethylformamide (6.5 ml) containing triethylamine (0.080 ml) at −5°. The mixture was stirred at 4° for 6 hr and at room temperature for 12 hr, and was then evaporated *in vacuo* to a thick oil. Water (100 ml) was added and the mixture was allowed to stand at 4° for 2 hr. The gelatinous precipitate was isolated by centrifugation, washed with two 80-ml portions of absolute ethanol and two 80-ml portions of ethyl acetate, and dried *in vacuo*. Anhydrous trifluoroacetic acid (6.0 ml) was added and the solution stirred for 30 min. The solution was evaporated *in vacuo* to a thick oil; water (10 ml) was added and the resulting mixture was lyophilized.

The product was extracted from the lyophilisate with 15 ml of the organic phase of 1-butanol–benzene–pyridine–0.1% aqueous acetic acid (6:2:1:9) and subjected to partition chromatography in this solvent system under the following conditions: column size, 4.32 × 58.4 cm; hold-up volume, 216 ml; fraction volume, 11 ml; flow rate, 66 ml/hr; regenerating solvent, pyridine–0.1% aqueous

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acetic acid (3:5). The chromatogram showed a large symmetrical peak at R_f 0.32, corresponding to the position established for the dimers of oxytocin.[3] Isolation of the material from the eluates represented by this peak gave 18.3 mg of the synthetic material. The product was dissolved in 0.2 *N* acetic acid (1.0 ml) and subjected to gel filtration on a 1.38 × 82 cm column in 0.2 *N* acetic acid. A flow rate of 5 ml/hr was maintained and the eluates were collected in fractions of 1.04 ml. Only one sharp peak was obtained with a maximum at effluent volume 85.6 ml, corresponding to the position for the dimers of oxytocin under the same conditions. Isolation by lyophilization gave 14.1 mg of the synthetic parallel dimer.

A sample of the parallel dimer was hydrolyzed in 6 *N* HCl at 110° for 24 hr and analyzed[13] on a Beckman-Spinco amino acid analyzer. The following molar ratios of amino acids and ammonia were found, with the value of leucine taken as 1.0: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.0; glycine, 1.0; cystine, 0.9; leucine, 1.0; isoleucine, 1.0; tyrosine, 0.9; and ammonia, 3.1. Paper chromatography of the material on Whatman No. 1 in 1-butanol–acetic acid–water (5:1:5) gave only one spot, R_f 0.28, when color was developed with Pauly reagent, as compared with previously reported values of 0.28 and 0.23 for α dimer and β dimer, respectively.[3] On paper electrophoresis in 0.1 *M* sodium acetate buffer at pH 5.5 (18 hr at 4° and 300 V) the material migrated as one spot toward the cathode (color development with Pauly reagent).

Comparisons of Parallel Dimer with α Dimer. Subjection of the parallel dimer (0.83 mg) to gel filtration on a 1.24 × 159-cm column in 0.2 *N* acetic acid at a flow rate of 5.7 ml/hr gave a chromatogram showing only one sharp symmetrical peak with a maximum at effluent volume 127 ml, identical with that reported for the α dimer under these conditions.[3] The β dimer has a maximum at effluent volume 137 ml.[3] Gel filtration of a mixture of the parallel dimer (0.51 mg) and the α dimer (0.56 mg) gave a chromatogram showing only one sharp symmetrical peak with a maximum at effluent volume 127 ml. The parallel dimer exhibited the specific rotation $[\alpha]^{19}D$ −105° (*c* 0.5, 1 *N* acetic acid) as compared to $[\alpha]^{20}D$ −106° (*c* 0.5, 1 *N* acetic acid) reported for the α dimer. The β dimer exhibits the specific rotation $[\alpha]^{20}D$ −78° (*c* 0.5, 1 *N* acetic acid).[3] It is interesting that both the parallel dimer and the α dimer are readily soluble in 6 *N* HCl at room temperature; the β dimer is not soluble under these conditions.[3]

Conversion of Parallel Dimer to Oxytocin. A sample (7.24 mg, 3.40 μmol) of the parallel dimer was dissolved in 25 ml of liquid ammonia (distilled from sodium) and treated at the boiling point with sodium until a blue color persisted throughout the solution for 1 min. The solution was evaporated *in vacuo* to low volume and lyophilized. The residue was dissolved in deaerated water (30 ml) containing 0.1 *N* trifluoroacetic acid (0.30 ml). The sulfhydryl content of the solution was 11.1 μmol. The pH of the solution was adjusted to 7.5 with 0.1 *N* trifluoroacetic acid. The solution was aerated for 2.3 hr; no thiols were detected after this period. The solution was acidified with glacial acetic acid and lyophilized.

The product was dissolved in 2 ml of the organic phase of 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9) and subjected to partition chromatography in this solvent system under the following conditions: column size, 0.95 × 55.05 cm; hold-up volume, 10.3 ml; fraction volume, 1.07 ml; flow rate, 2.8 ml/hr; regenerating solvent, pyridine–0.1% aqueous acetic acid (3:5). The chromatogram showed a major peak with R_f 0.28 corresponding to the position of oxytocin under these conditions. Isolation of the oxytocin gave 3.2 mg possessing an oxytocic potency[14] of about 540 units/mg.

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(14) The assays for oxytocic activity were performed on isolated rat uteri from rats in natural estrus according to the method of P. Holton, *Brit. J. Pharmacol.*, **3**, 328 (1948), as modified by R. A. Munsick, *Endocrinology*, **66**, 451 (1960), with the use of magnesium-free van Dyke–Hastings solution. Oxytocic activity was measured against the USP posterior pituitary reference standard.

Communications to the Editor

Chemistry of Singlet Oxygen. X. Carotenoid Quenching Parallels Biological Protection[1]

Sir:

β-Carotene efficiently quenches singlet oxygen, generated either by dye sensitization or by the NaOCl–H_2O_2 reaction.[2] This quenching bears on the mechanism of the protective action of carotenoids against photodynamic damage in living organisms; this protective action may be the "universal function of carotenoid pigments."[3]

We now report that *the rate of quenching is a sensitive function of the length of the conjugated polyene chain and parallels the protective action of natural compounds.* The techniques used were similar to those previously reported.[2,4] The compounds used as quenchers were all-*trans*-retinol (**1**, 5 conjugated C=C) and two synthetic carotene analogs, a C_{30} hydrocarbon (**2**, 7 conjugated C=C) and a C_{35} hydrocarbon (**3**, 9 conjugated C=C).[5]

In the previous paper, it was shown that when $[AO_2]^{-1}$ is plotted against $[A]^{-1}$ at constant [Q], straight lines result; the intercept on the $[AO_2]^{-1}$ axis is a measure of the amount of singlet oxygen formed, and the increase in ratio of slope to intercept compared to plots with no Q is a measure of quenching of singlet oxygen (a kinetic scheme for this system is given in the accompanying communication).[6] Thus quenching of

(1) Paper IX: S. Mazur and C. S. Foote, *J. Amer. Chem. Soc.*, **92**, 3225 (1970). Contribution No. 2557; supported by a grant from the USPHS-NAPCA (No. AP-00681).

(2) C. S. Foote and R. W. Denny, *J. Amer. Chem. Soc.*, **90**, 6233 (1968); C. S. Foote, R. W. Denny, L. Weaver, Y. Chang, and J. Peters, *Ann. N. Y. Acad. Sci.*, in press.

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(4) Solutions in benzene–methanol (80:20) containing known amounts of the photooxygenation acceptor 2-methyl-2-pentene (A) and the quencher (Q) were irradiated for a constant time, sufficient to produce a readily measurable amount of product (AO_2) without oxidizing a significant fraction of A (<7%). The sensitizer was methylene blue (MB); solutions under pure O_2 were irradiated with a tungsten–halogen lamp through a $K_2Cr_2O_7$ filter (3 g/100 ml of H_2O, 2.5-cm path length) with shortwave cutoff (1% transmission) of 510 nm, which ensured that light was absorbed only by the sensitizer, not by the quencher. Although this filter was not used in previous experiments,[2] controls and subsequent experiments with filtered light established that light absorbed by β-carotene had no effect on the observed quenching or on the production of AO_2. Photooxygenated solutions were reduced with $NaBH_4$, internal standard was added, and the product alcohols were determined gas chromatographically.

(5) The C_{30} and C_{35} hydrocarbons were kindly supplied by Dr. H. Pommer, BASF, Ludwigshafen, Germany. For the purposes of this discussion, the endocyclic double bonds in all carotenoids are counted as conjugated.