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Conformationally biased analogs of oxytocin

MICHAL LEBL[1,a], GEZA TOTH[2], JIŘINA SLANINOVÁ[1] and VICTOR J. HRUBY[2]

[1] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia and* [2] *Department of Chemistry, University of Arizona, Tucson, USA*

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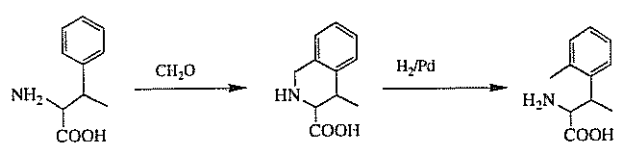
Four diastereomeric analogs of oxytocin containing substituted phenylalanine in position 2 were synthesized. This modified phenylalanine side chain contained one methyl group attached to the β-carbon and the second one at the 2′ position of the aromatic ring. All analogs were found to be inhibitors of uterotonic activity of oxytocin with pA_2 values ranging from 6.0 to 8.3; the most potent one ($pA_2 = 8.3$) contained dimethylphenylalanine of the D-erythro configuration

Key words: alkyl-substituted phenylalanines; conformational restriction; inhibitory activity; oxytocin analogs

The quest for analogs of neurohypophyseal hormones having very high inhibitory activity at various receptors has led to the synthesis of several therapeutically promising compounds. However, still more potent and/or prolonged antagonists are being obtained in studies in several laboratories all over the world (for recent reviews see 1–3). Recently described inhibitors of oxytocin that do not resemble the structure of neurohypophyseal hormones (either isolated from natural sources (4) or arrived at by well targeted synthetic efforts (5)) provide evidence that this area of research can bring surprises.

It is known from numerous structure-activity studies that modification of position 2 is of extreme importance for modulation of neurohypophyseal hormones' biological activities. We have studied the importance of hydrophobic amino acids of D-configuration for the inhibitory potency of these analogs (6). There is suggestive evidence that hydrophobicity of the receptor pocket may play a substantial role in a nonproductive interaction of a hormone analog with the target site. The surprising finding that a methyl group on the β-carbon of the aromatic amino acid in position 2 of the peptide chain may function in a manner similar to the hydroxyl group in the p-position of aromatic ring (7) prompted us to explore further the importance of a β-carbon modification in combination with additional substitution of the aromatic ring of the aromatic amino acid in position 2.

Several syntheses of β-methylphenylalanine have been described (for a review see 8, for recent asymmetric synthesis see 9). For the preparation of β-2′-dimethylphenylalanine we have employed a simple method recently developed in our laboratory (10). This method consists in forming a tetrahydroisoquinoline ring by the action of formaldehyde on phenylalanine (11) and opening the ring by energetic hydrogenolysis (see Scheme 1). Unfortunately, this method leads to partial racemization. However, in this case the racemization was a favorable event since it provided an easy way to obtain all four diastereoisomeric peptide analogs. The synthesis of a mixture of stereoisomers is, of course, a feasible solution for the preparation of peptide analogs only when reliable methods for separation of the isomers and for configuration determination are available. We were fortunate to find that all four peptide isomers are easily separable on a Vydac-C18 chromatographic column and that the configuration of



SCHEME 1
Simple synthesis of β-2′-dimethylphenylalanine.

[a] Present address: Selectide Corporation, Tucson, AZ 85737, USA.

the aromatic amino acid can be determined after acid hydrolysis of the sample followed by separation of the aromatic amino acid and determination of configuration using published chiral TLC methods (12).

Partially racemized amino acid was N^{α}-protected by the Boc group and this derivative was used for coupling to the properly protected heptapeptide-resin prepared by standard solid phase procedures (see Experimental Procedures). After the last coupling, the protecting groups were cleaved off the peptide simultaneous with splitting the peptide from the resin, by the action of liquid HF. The mixture of diastereoisomers was oxidized with potassium ferricyanide, desalted and purified by HPLC on a reversed phase column. The first purification did not afford completely pure analogs, and thus for biological and analytical evaluation, all fractions had to be repurified on the same column. The second chromatographic run afforded pure compounds. Four fractions of 6, 28, 16 and 12 mg were obtained. This represents, most probably, the ratio in which the diastereoisomers of N^{α}-Boc-3,2′-dimethylphenylalanine coupled to the growing peptide chain. It probably does not reflect directly the content of the individual isomers in the starting amino acid derivative, since the coupling of each stereoisomer will be influenced strongly by the chiral, sterically demanding *N*-terminal amino acid isoleucine. The excess amino acids used in the coupling may have allowed for preferential condensation of D-amino acid. Samples of each fraction were hydrolyzed, applied to a reversed phase column and eluates containing the aromatic acid were spotted on chiral TLC plates. Comparison with standards in which the stereochemistry was determined previously (12) enabled us to assign the configuration of the relevant amino acid in all analogs.

Cys-X-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

oxytocin, X = Tyr

I. X = (S,R)-3,2′-dimethylphenylalanine (L-threo)
II. X = (R,S)-3,2′-dimethylphenylalanine (D-threo)
III. X = (S,S)-3,2′-dimethylphenylalanine (L-erythro)
IV. X = (R,R)-3,2′-dimethylphenylalanine (D-erythro)

Biological activities of the analogs I–IV are summarized in Table 1. As can be seen, all analogs are uterotonic antagonists, the most potent inhibitor being analog IV which contains a D-*β*,2′-dimethylphenylalanine residue of erythro (2R,3R) configuration in the 2 position. The least potent antagonist is the compound with L-dimethylphenylalanine of the threo (2S,3R) configuration. Interestingly, the analog with an L-erythro-(2S,3S)-dimethylphenylalanine residue is less potent than that with D-erythro-dimethylphenylalanine. These findings show clearly that the configuration of the aromatic amino acid in position 2 of oxytocin is important for the potency of an inhibitor. However, the appropriate positioning of the hydrophobic substituents can be of even greater importance. The additional methyl substitution on the aromatic ring is of crucial importance for the appearance of the inhibitory activity since oxytocin analogs containing *β*-methylphenylalanine of the L-threo and D-threo configuration in position 2 are fairly potent uterotonic agonists (13). In this paper, we show that [(2S,3R)-3,2′-dimethylphenylalanine^2]oxytocin is a very weak uterotonic antagonist whereas the [(2R,3R)-3,2′-dimethylphenylalanine^2]-oxytocin is a quite potent antagonist, and the other two diastereoisomers are intermediate in antagonist potency.

TABLE 1

Biological activities (rat) of oxytocin analogs

Compound	Biological activity	
	Uterotonic *in vitro*	Pressor
I L-threo	$pA_2 = 6.0$	0
II D-threo	$pA_2 = 7.25$	0
III L-erythro	pA_2-7.35	0
IV D-erythro	$pA_2 = 8.3$	$pA_2 = 6.1$

0 means inactive up to doses of $2 \cdot 10^{-2}$ mg.

EXPERIMENTAL PROCEDURES

General methods

Thin-layer chromatography (TLC) was carried out on silica gel coated plates (Silufol, Kavalier, Czechoslovakia) in the following systems: 1-butanol-formic acid-water (10:3:8) (S1); 2-butanol-25% ammonia-water (85:7.5:7.5) (S2); 1-butanol-acetic acid-water (4:1:1) (S3); and 1-butanol-acetic acid-pyridine-water (15:3:10:6) (S4). Chiral TLC was performed on Chiralplate (Macherey Nagel) in the system acetonitrile-methanol-water (4:1:1) (S5). Paper electrophoresis was performed in a moist chamber in 1 M acetic acid (pH 2.4) and in pyridine-acetate buffer (pH 5.7) on Whatman 3MM paper at 20 V/cm for 60 min. Spots in TLC and electrophoresis were detected with ninhydrin or by the chlorination method (14). Samples for amino acid analysis were hydrolyzed with 6 M HCl at 105° for 20 h and analyzed on an Amino Acid Analyzer T339 (Mikrotechna Praha, Czechoslovakia) or a D-500 Analyzer (Durrum Corp., USA). Fast atom bombardment mass spectra were obtained on a ZAB-EQ spectrometer (VG Analytical Ltd., Manchester) using Xenon at 8 kV as the bombarding gas. High performance liquid chromatography (HPLC) was carried out on a Spectra Physics SP-8800 instrument equipped with an SP-8450 detector and SP-4290 integrator (all from Spectra Physics, Santa Clara, USA). HPLC purities of products were determined on the column of Vydac 218TP54. Preparative liquid chromatography was carried out on the above described equipment using a Vydac 218TP510 column (5 μm, 250 × 10 mm). Before use, all amino acid derivatives were subjected to the ninhydrin test (15).

A cycle for incorporating each amino acid residue

into the growing peptide chain consisted of the following: 1. cleavage of the N^{α}-Boc group by 50% trifluoroacetic acid in dichloromethane containing 5% anisole, one treatment for 2 min, a second one for 20 min; 2. washing with dichloromethane (3 ×, 1 min/wash); 3. washing with isopropanol (3 ×, 1 min/wash); 4. washing with dichloromethane (3 ×, 1 min/wash); 5. neutralization by 5% diisopropylethylamine in dichloromethane (3 × 2 min); 6. washing with dichloromethane (2 ×, 1 min/wash); 7. washing with dimethylformamide (3 ×, 1 min/wash); 8. addition of the N^{α}-Boc-protected amino acid derivative in dimethylformamide, followed by HOBt, bromophenol blue solution, and by diisopropylcarbodiimide (DIC) and stirring until the color of bromophenol blue has disappeared (16) (2–20 min); 9. washing with dimethylformamide (3 ×, 1 min/wash); 10. washing with dichloromethane (3 ×, 1 min/wash); 11. washing with isopropylalcohol (3 ×, 1 min/wash); 12. washing with dichloromethane (3 ×, 1 min/wash).

Erythro-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid

Erythro-3-methylphenylalanine hydrochloride (3.15 g, 14.6 mmol) was refluxed in a mixture of concentrated HCl (20 mL) and 37% formaldehyde solution (5 mL) for 30 min, and after addition of further HCl (5 mL) and formaldehyde (2.5 mL), for another 1 h. The mixture was re-evaporated from water and crystallized from water. Yield 2.4 g (72.2%). R_f 0.24 (S1, yellow spot in ninhydrin detection), 0.45 and 0.51 (S5, Chiralplate). HPLC: R_T 7.94 (Vydac C18, 25 × 1 cm, 3 mL/min, 15–25% of CH_3CN in 0.05% trifluoroacetic acid in 10 min).

3,2′-Dimethylphenylalanine

The above prepared derivative (1.1 g, 4.8 mmol) was dissolved in 300 mL of 70% acetic acid and after addition of palladium freshly prepared from 1 g of $PdCl_2$, hydrogen was bubbled through at 60° until the reaction was completed according to TLC. R_f 0.28 (S1, violet spot in ninhydrin detection). The mixture was filtered and evaporated to dryness. According to the ^1H NMR spectra, the crude reaction mixture contained products of the erythro and threo configurations in the ratio 7:2. The oily residue was dissolved in hot water (30 mL) and the crystals formed in 3 days were filtered off: 450 mg (40.9%). According to TLC on a Chiralplate, it contained the title compound of the erythro configuration (R_f 0.33 and 0.57 (S5)). The filtrate was lyophilized: 700 mg (63.6%) and analyzed by chiral TLC. This product contains the title compound of both erythro and threo configurations in an approximately equimolar ratio (R_f 0.57 erythro-L, 0.55 threo-L, 0.48 threo-D, and 0.33 erythro-D; all in S5).

N^{α}-*Boc-3,2′-dimethylphenylalanine*

The mixture of stereoisomers of 3,2-dimethylphenylalanine (650 mg, 3.36 mmol) was dissolved in water (40 mL) and *tert.*-butylalcohol (20 mL); the pH of the solution was adjusted to 10.3 by 20% NaOH and Boc_2O (810 mg, 3.7 mmol) in *tert.*-butylalcohol (15 mL) was added in 5 portions while the pH was maintained at 10.3. After 2 h at room temperature, the mixture was concentrated to a small volume, diluted by water, extracted by petroleum ether, acidified by the addition of $KHSO_4$ solution, and the product was extracted by ethyl acetate. Drying over Na_2SO_4 and following evaporation afforded 0.66 g (67%) of an oily product homogeneous on TLC. This product was used directly for solid phase synthesis.

[β-Me-2′-MePhe]²]OXT (I–IV)

The protected heptapeptide of the oxytocin sequence bound to the *p*-methylbenzhydrylamine resin (0.68 g, 0.3 mmol) was prepared by standard methods (17), and placed into a shaker vessel of a solid phase synthesizer. Two steps of solid phase coupling using the protected phenylalanine derivative prepared as described above (90 mg) followed by N^{α}-Boc-Cys(pMB)-OH (290 mg) were performed (DIC/HOBt). The first coupling was performed using only a very small excess of the amino acid derivative and therefore, the condensation was catalyzed by the addition of dimethylaminopyridine (50 mg). The protected nonapeptide-resin was washed, dried and placed into a vessel of a HF apparatus together with 0.5 mL of anisole and 0.5 mL of ethanedithiol. Hydrogen fluoride (10 mL) was distilled in and after stirring for 1 h at 0°, the solvents were evaporated off. The residue was stirred with ethyl acetate, filtered, and the peptide was extracted by 30% acetic acid. The white powder afforded after lyophilization was dissolved in water, the pH was adjusted to 8 by 5% ammonia solution, and 0.01 M potassium ferricyanide was added until the yellow color of the solution persisted (plus 50% more). After stirring for 30 min, Amberlite IR45 (Cl^- form) was added, the solution was stirred for another 30 min and filtered. Lyophilization afforded 214 mg of a white powder. Part of this mixture (100 mg) was introduced onto an HPLC column (Vydac C18, 25 × 2.5 cm) and a gradient elution (0–15% CH_3CN in 0.1% trifluoroacetic acid in 2 min and 15–40% in 60 min) was performed. The fractions obtained were lyophilized and analyzed. Four fractions were obtained. Fraction 1: 6 mg, R_t 34.58 min, FAB MS: 1020, Chiral TLC (for methodology see ref. 10): R_f 0.55 (S5-L-threo-3,2′-dimethylphenylalanine); fraction 2: 28 mg, R_t 35.64 min, FAB MS: 1020, Chiral TLC: 0.33 (S5 – D-erythro-3,2′-dimethylphenylalanine); fraction 3: 16 mg, R_t 37.34 min, FAB MS: 1020, Chiral TLC: 0.57 (S5 – L-erythro-3,2′-dimethylphenylalanine); fraction 4: 12 mg, R_t 48.20 min, FAB MS: 1020, Chiral TLC: 0.48 (S5 – D-threo-3,2′-dimethylphenylalanine). All fractions exhibited the same amino acid analysis. For the biological testing each fraction was purified once more on the same column.

Pharmacological methods

The uterotonic activity *in vitro* was determined on an isolated strip of rat uterus in the absence of magnesium (18, 19). The pressor activity was determined on pithed rats as described previously (20). The inhibitory potencies are expressed as their pA_2 values (21, 22).

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Address:

Dr. *Michal Lebl*
Selectide Corporation
10900 N. Stallard Place #122
Tucson, AZ 85737
USA