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Conformationally restricted analogs of oxytocin; stabilization of inhibitory conformation†

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Analogs of oxytocin containing tetrahydroisoquinoline carboxylic acid (Tic) of L or D configuration in position 2 were synthesized and their biological activities were tested. Both analogs showed negligible agonist activity in uterotonic, galactogogic, and pressor assays, but they are *in vitro* uterotonic inhibitors. In comparison with oxytocin analogs containing L- or D-phenylalanine in position 2, the analog with the D-configuration of the conformationally fixed aromatic residue has significantly increased inhibitory activity which suggests that the proper conformation for the interaction with the receptor, but not for its activation, was stabilized. ^{1}H NMR and CD studies, supported by theoretical calculations, suggest that the conformational properties of the analog containing D-tetrahydroisoquinoline carboxylic acid are similar to those of [2-D-phenylalanine]oxytocin.

Key words: circular dichroism; conformation; conformational constraint; NMR; oxytocin inhibitors

Until now the direct determination of a peptide hormone conformation during its interaction with the receptor (the so-called "biologically active conformation") has not been possible. Interaction with enzymes or other proteins can be studied more easily, since they can be isolated in a pure form. At the same time, knowledge of possible biologically active conformations can simplify the design of new compounds with desirable properties, for example superagonists, inhibitors, or compounds with a prolonged time course of action. A model for a biologically active conformation would allow the synthesis of conformationally restricted peptides that may interact only with the desired receptor and not with the alternative one, thus leading to high specificity of action. Moreover, in favorable cases, the analog may be stable to the enzymes normally responsible for inactivation of the parent hormone, thus providing an additional useful property for *in vivo* biological activity.

More than 1000 analogs of neurohypophyseal hormones have been synthesized and studied (1, 2). The design of most of these compounds was based on previous knowledge of structure-activity relationships and only a few were synthesized primarily for conformational studies. Conformational restriction can be achieved by various methods (for a review see ref. 3), including introduction of sterically demanding amino acids or by modification of peptide bonds. Only a very limited number of analogs of oxytocin have been conformationally restricted by the introduction of additional intramolecular covalent bonds (4–7), even though this method has proven to be rather effective in constraining the structure of linear peptides (3, 8–16).

Introduction of tetrahydroisoquinoline carboxylic acid (Tic) residues into a peptide leads to analogs which generally will possess only one of the possible side chain conformers (17–19). This substitution, when made in a residue position originally occupied by a phenylalanine residue, is rather conservative in

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* Nomenclature and abbreviations follow published recommendations (*European J. Biochem.* (1984) 138, 9–37). Other abbreviations: OXT, oxytocin; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TFE, trifluoroethanol; Cle, cycloleucine (1-aminocyclopentanecarboxylic acid); Tcp, 2,4,5-trichlorophenyl; Tmb, 2,4,6-trimethylbenzyl; pMB, *p*-methylbenzyl; NSu, *N*-hydroxysuccinimidyl; DVB, divinylbenzene.

TABLE 1

Biological data (I.U./mg) of oxytocin analogs

	Uterotonic *in vitro*	Uterotonic *in vivo*	Galactogogic	Pressor
OXT[a]	450	450	450	3
[L-Phe^2]OXT	22.2	168	175	0.56
[D-Phe^2]OXT	$pA_2 = 7.3$		1.2	< 0.04
[L-Tic^2]OXT	$pA_2 < 5.6$	$pA_2 < 6.0$	0.01	nil
[D-Tic^2]OXT	$pA_2 = 6.7$		0.17	< 0.05

[a]OXT = oxytocin = H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2.

terms of overall polarity and hydrophobicity. The aromatic residue in position 2 of oxytocin plays an important role in its biological activity. The hydroxyl group in the p-position of the aromatic residue is important for full activity, but even analogs containing phenylalanine instead of tyrosine have modest potency. Recently we have demonstrated that the introduction of an aromatic amino acid of D-configuration into position 2 of oxytocin and vasopressin can lead to antagonist analogs (20, 21) though not always (1, 2), and we suggest that the interaction of aromatic side chain and sulfur in position 6 is of importance for the proper conformation of the molecule of oxytocin (22, 23). We have therefore decided to substitute the tyrosine residue by the conformationally restricted Tic residue, and to study the biological and physicochemical consequences of this substitution. In a related study we have described the synthesis of vasopressin analogs containing Tic in position 2 or 3; these analogs were shown to be devoid of any biological activity (24).

Synthesis of ([L-Tic^2]OXT (Ia) and [D-Tic^2](OXT) (Ib)) was performed in both laboratories, using solid phase synthesis. In one case Nps and Boc protecting groups and coupling by active esters were used, and in the other case N^{α}-Boc protection and DCCI/HOBt coupling methods were employed.

Products were purified by gel filtration and reversed phase HPLC and were found identical by chromatographic and electrophoretic techniques and by ^{1}H NMR spectroscopy.

The biological activities of both analogs are given in Table 1. As can be seen, generally agonist activities (if any) were dramatically decreased, or weak inhibitory activities were observed. However, the inhibitory activity in the *in vitro* uterotonic test of the D-amino acid containing analog Ib was comparable to the inhibitory activity of [2-D-phenylalanine]oxytocin. This means that an analog with the appropriately fixed side chain conformation is well suited for interaction with the receptor for inhibitory action. Therefore we decided to study its properties more thoroughly in different systems. Binding of neurohypophyseal hormones by neurophysin is very sensitive to a change made in position 2. We found that the affinity of [L-Tic^2]OXT was decreased 25 times in comparison with oxytocin and that [D-Tic^2]OXT was not bound at all (25).

We compared the two analogs by HPLC on two reversed phase systems. It is well known that a change of configuration of aromatic residue in position 2 of oxytocin leads to pronounced differences in HPLC retention times, which may be due to changes of peptide conformation (26–28). Interestingly, the D-Tic- and L-Tic-containing analogs had only slightly different k′ values in both systems (Table 2). On the other hand, the D-Phe- and L-Phe-containing compounds differed considerably, suggesting that the fixed

TABLE 2

RP HPLC data of oxytocin analogs

	Vydac C_{18}[a]	EDMA-40-AB[b]
[L-Phe^2]OXT	0.92	2.65
[D-Phe^2]OXT	2.17	6.51
[L-Tic^2]OXT	1.37	3.00
[D-Tic^2]OXT	1.50	3.45

[a]25 × 0.4 cm, 25% acetonitrile in 0.1% TFA in water.
[b]8 × 0.8 cm (Tessek, Prague), 20% acetonitrile in 0.1% TFA in water.

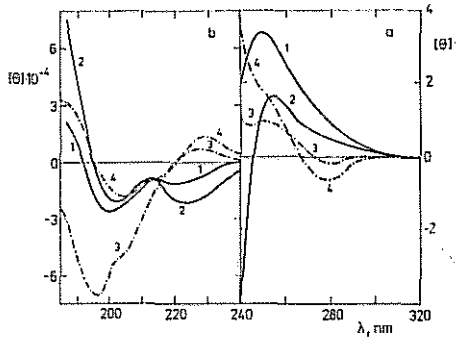

FIGURE 1

CD spectra of L-amino acid containing oxytocin analogs. *1* — [L-Tic^2]OXT (Ia) in 0.02 M phosphate buffer pH 7.2, *2* — [L-Tic^2]OXT (Ia) in TFE, *3* — OXT in 0.02 M phosphate buffer pH 7.2, *4* — OXT in TFE.

TABLE 3

Chemical shifts of the α and β protons in oxytocin analogs

		Position in peptide chain								
		1	2	3	4	5	6	7	8	9
OXT	α-CH	4.28	4.77	≠4.05	4.12	4.73	4.86	4.60	4.30	3.84, 3.94
	β-CH_2	3.37	3.00	' 1.88	2.08	2.80	2.98	2.20	1.64	
	β'-CH_2	3.47	3.23			3.30				
[L-Phe^2]OXT	α-CH	4.27	4.87	4.12	4.11	4.76	4.95	4.45	4.31	388, 3.94
	β-CH_2	3.22	3.05	1.97	2.05	2.86	2.99	1.94	1.65	
	β'-CH_2	3.37	3.32			2.88	3.24	2.32		
[D-Phe^2]OXT	α-CH	4.76	5.02	3.90	4.39	4.62	4.92	4.43	4.31	3.87, 3.94
	β-CH_2	2.96	3.12	1.70	2.04	2.79	2.96	1.97	1.65	
	β'-CH_2	3.34				2.82	3.28	2.32		
[L-Tic^2]OXT	α-CH	5.01	5.13	4.28	3.93	4.72	4.94	4.42	4.30	3.86, 3.91
	β-CH_2	3.50	3.32	1.76	1.94	2.57	3.20	2.28	1.68	
	β'-CH_2	3.41	3.22			2.65	3.14	2.02		
	N-CH_2		4.82							
[D-Tic^2]OXT	α-CH	4.86	5.27	3.97	4.14	4.42	4.85	4.46	4.30	3.86, 3.96
	β-CH_2	3.01	3.42	1.70	2.01	2.94	3.38	2.28	1.68	
	β'-CH_2	3.47					2.88			
	N-CH_2		4.64							
			4.79							

TABLE 4

^1H NMR data for isoleucine residue in oxytocin analogs

	α-CH	β-CH	δ-CH_2	δ-CH_3	δ-CH_3	NH	$J_{NH-\alpha CH}$	$J_{C\alpha H-C\beta H}$
[L-Phe^2]OXT	4.12	1.97	1.29	0.93	0.89	8.00	6.21	6.0
		1.07						
[D-Phe^2]OXT	3.90	1.70	1.22	0.73	0.78	8.33	6.95	6.6
		1.02						
[L-Tic^2]OXT	4.28	1.76	0.96	0.34	0.72	6.77	9.2	3.6
		0.24						
[D-Tic^2]OXT	3.97	1.70	0.95	0.76	0.67	8.57	10.5	8.0

of oxytocin constrained in position 2, [L-Cle^2]OXT, possesses CD properties similar to that of [L-Tic^2]OXT (33).

The CD spectra of [D-Tic^2]OXT (Ib, Fig. 3b) do not indicate any marked effect of the sterically constrained residue to stabilize an ordered conformation of the peptide backbone. The spectra in both solvents show as a single prominent feature, a strong negative $\pi - \pi^*$ band at about 220 nm which is characteristic of a more unordered conformation, very similar to that of [D-Phe^2]OXT (IIb, Fig. 3b). This similarity indicates a similar conformation for the peptide backbone of Ib and IIb. The only difference between these two compounds is the positive $n - \pi^*$ band of IIb at 220 nm probably originating in a rigid local conformation which includes the aromatic amino acid residue.

We also have employed ^1H NMR spectroscopy to examine the conformational properties of the new analogs. A comparison of the chemical shifts of α-protons can be seen in Table 3. Significant differences can be observed in the shifts of protons of amino acids which make up the residues of the heterodetic ring of the hormone. In the upper part of the spectrum of [L-Tic^2]OXT, an extremely high upfield shift of the methyl protons of isoleucine is evident (Table 4). The upfield shift of these protons in [D-Tic^2]OXT is approximately the same as observed in [D-Phe^2]oxytocin, in agreement with the CD derived conclusions about the similar conformations of these compounds. The chemical shifts of amide protons and their temperature dependencies and NH-CαH coupling constants are given in the Table 5. When we compare the

aromatic moiety in Tic cannot assume a proper orientation for interaction with the stationary phase.

A sensitive tool for studying peptide conformation is CD spectroscopy. Free L-Tic shows (in 0.1 M HCl) a CD spectrum which does not differ greatly from that of L-phenylalanine. There is a very weak multiple band of the aromatic B_{2u} transition at 267 nm and a somewhat more intense narrow B_{1u} band at 214 nm. Both bands are negative. A possibly stronger band of the E_{1u} transition is superimposed on bands of the carboxyl chromophore (not shown).

These weak aromatic CD bands thus contribute negligibly to the spectra of analogs Ia and Ib. The long wavelength spectral region of Ia and Ib (Figs. 1a, 3a) is then formed solely by bands of the $n \rightarrow \sigma^*$ transitions of the disulfide chromophore. For [L-Tic^2]OXT (Ia) (Fig. 1a) in neutral water solution an unusually intense positive band at 250 nm which merges with another positive band lying at longer wavelengths is observed. The intensity of 3400 ellipticity units of the 250 nm band is the highest ever observed in oxytocin analogs (29), and indicates that the flexibility of the disulfide group in Ia is substantially reduced in comparison with oxytocin. Oxytocin appears to possess equal populations of enantiomeric disulfide conformers (30), but in Ia a single helicity appears to be favored. The 250 nm band belongs to rectangular, and the long wavelength band to strained, conformers of the disulfide group (29). The change in sign of the long wavelength band (relative to oxytocin, Fig. 1a) suggests a change in the torsional angle of strained (non rectangular) disulfide conformers. Assuming that the observed CD is due to righthanded conformers, this would mean a change from transoid (in oxytocin) to cisoid conformers in [L-Tic^2]OXT. In trifluoroethanol the overall character remains unchanged. Also a comparison of [L-Tic^2]OXT with the parent compound [L-Phe^2]OXT (IIa, Fig. 2a), where the tyrosine CD bands do not interfere, shows a marked difference in the intensity and solvent dependence of both disulfide bands. The disulfide bands of [D-Tic^2]OXT (Ib) do not exhibit any unusual parameters (Fig. 3a).

At short wavelengths, compounds Ia, b and IIa, b show two CD bands with maxima lying between 215 and 230 nm due to the $n \rightarrow \pi^*$ transition of the amide bond as well as a negative band at about 200 nm and a positive one at shorter wavelengths due to the amide $\pi \rightarrow \pi^*$ transition.

[L-Tic^2]OXT also differs markedly in the CD properties from comparable compounds bearing no sterically constrained side chain in position 2 in the short wavelength region (185–240 nm). There is a prominent negative $n \rightarrow \pi^*$ band at 220 nm, an unusually weak negative $\pi \rightarrow \pi^*$ band at 200 nm, and a short wavelength positive $\pi \rightarrow \pi^*$ band of appreciable intensity (Fig. 1b). The CD does not change significantly when going from one solvent to the other. The CD spectrum of oxytocin in buffer solution (Fig. 1b), differs substantially from that of [L-Tic^2]OXT (Fig. 1b). However, in trifluoroethanol, the CD of oxytocin changes and becomes similar to that of [L-Tic^2]OXT.

The weak response of amide CD bands of [L-Tic^2]OXT to a change of solvent suggests that the peptide backbone of this analog is, relative to oxytocin, rigid. The pattern of a new negative $n \rightarrow \pi^*$ and positive $\pi \rightarrow \pi^*$ bands indicates an enhanced population of an ordered, probably a reverse turn, conformation. The L-Tic might well be responsible for this tendency, functioning as a residue in the putative β-turn. The similarity of the CD spectra in trifluoroethanol of [L-Tic^2]OXT and oxytocin suggests that the stabilized conformation of [L-Tic^2]OXT may be one of the energetically accessible conformations of oxytocin. It is worth mentioning that another analog

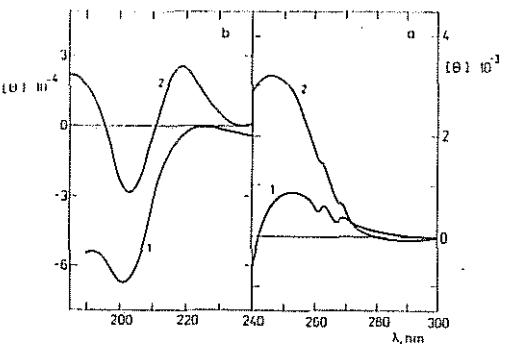

FIGURE 2

CD spectra of [L-Phe^2]OXT (IIa) in *1* — 0.02M phosphate buffer pH 7.2, *2* — TFE.

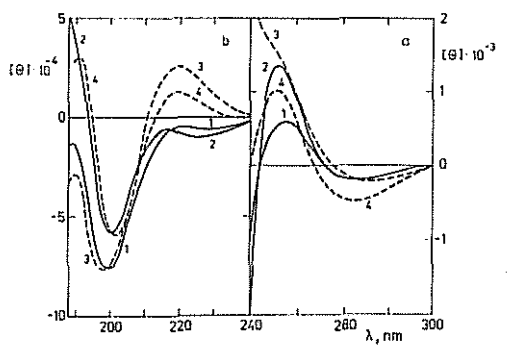

FIGURE 3

CD spectra of D-amino acid containing oxytocin analogs. *1* — [D-Tic^2]OXT (Ib) in 0.02 M phosphate buffer pH 7.2, *2* — [D-Tic^2]OXT (Ib) in TFE, *3* — [D-Phe^2]OXT (IIb) in 0.02 M phosphate buffer pH 7.2, *4* — [D-Phe^2]OXT (IIb) in TFE.

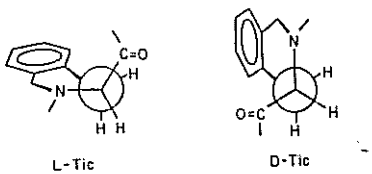

FIGURE 4

Projection of Tic residue conformation in [L-Tic^2]OXT (left) and [D-Tic^2]OXT (right).

solvent in both analogs, and in almost all cases lower temperature dependencies were found in comparison to oxytocin, suggesting a more rigid structure. Coupling constants between the α and β protons (Table 6) in the neighborhood of the Tic residue differ significantly from those of oxytocin due to side chain-side chain interactions between the Tic aromatic moiety and isoleucine. From the CαH-CβH coupling constants of the Tic residue we can deduce the stereochemistry of this residue, which is schematically shown on Fig. 4. It is evident that in both cases the side chain of this residue is the gauche(+) conformation (17, 18), but in [L-Tic^2]OXT the tetrahydroisoquinoline carboxylic acid residue is much more strained than in [D-Tic^2]OXT. Results of the NMR studies can be used to construct a conformational model of the Tic-Ile fragment in both molecules. In this model the α-CH of Ile^3 is outside the shielding cone of the aromatic ring, and therefore it is deshielded. On the other hand, the NH proton and the γ-methyl group of Ile is inside the shielding cone and therefore highly shielded. Rigidization of the Ile side chain conformation in [L-Tic^2]OXT is evidenced also by the very small $C_\alpha H$-$C_\beta H$ coupling constant. The reason for the large coupling constant of NH-αCH of Ile is a fixed ϕ angle for this residue, which is also the reason for its proton solvent inaccessibility. In [D-Tic^2]OXT the NH proton of Ile is deshielded and more accessible to the solvent, and the methyl groups of Ile are further from the aromatic ring and thus not so dramatically shielded.

To further examine the conformational consequences of the D and L-Tic^2 residues in oxytocin analogs, we have performed energy minimization calculations (using CHARMM (35)) on both the model dipeptides (Ac-L-Tic-Ile-NHMe and Ac-D-Tic-Ile-NHMe), and on each of the analogs studied in this paper. The energy minimized conformation for Ac-L-Tic-Ile-

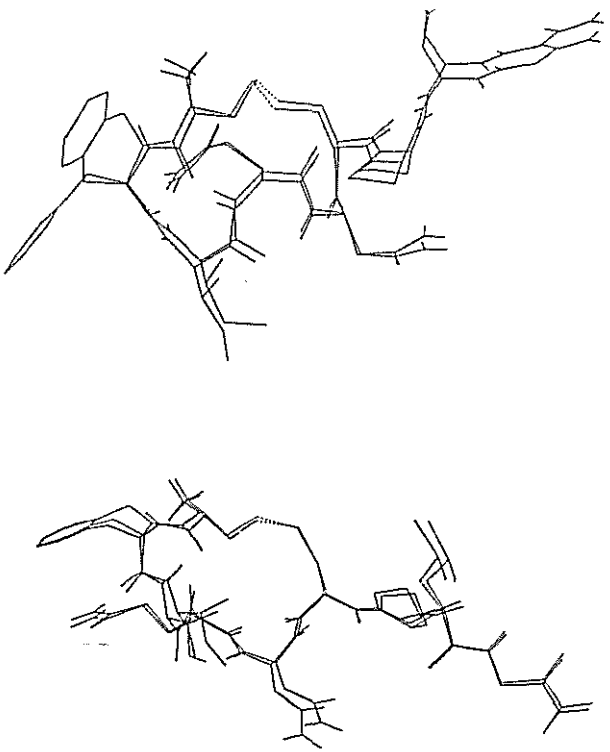

FIGURE 5

Superposed energy minimized structures of [D-Tic^2]OXT and [D-Phe^2]OXT.

chemical shifts of protons in Ia to those of oxytocin in aqueous solution (34), an high upfield shift (1.3 ppm) is observed for the amide proton of isoleucine, and a significant upfield shift (0.5 ppm) also is observed for the Asn peptide amide proton. Notable also is the relatively large coupling constant observed for the Ile[3] amide proton, which is further evidence for a more stabilized conformation. Comparison of the temperature dependencies of NH protons between analogs containing an L-amino acid in position 2 (Ia, IIa) reveals decreased solvent accessibility of NH protons in the analog with the constrained amino acid (Ia). In analogs containing a D-amino acid in position two, significant differences are seen (with the exception of NH of cysteine in position 6). The amide proton of isoleucine is probably less accessible to the

TABLE 5

^1H NMR data for amide protons in oxytocin analogs

		Position in peptide chain						
		2	3	4	5	6	8	9
OXT	δ(NH)	8.99	7.96	8.22	8.34	8.21	8.45	8.36
	$-d\delta/dT$ ($\times 10^3$)	6.5	7.0	5.5	5.0	5.5	9.5	7.0
	$J_{NH-C_\alpha H}$	6.4	6.0	4.0	8.0	6.5	6.0	6.0
[L-Phe^2]OXT	δ(NH)	9.03	8.00	8.28	8.34	8.23	8.47	8.38
	$-d\delta/dT$ ($\times 10^3$)	4.7	5.3	5.4	3.8	3.9	8.0	5.8
	$J_{NH-C_\alpha H}$	7.7	6.2	4.0	8.4	6.6	6.9	5.9
[D-Phe^2]OXT	δ(NH)	8.75	8.33	7.75	8.52	8.84	8.45	8.39
	$-d\delta/dT$ ($\times 10^3$)	6.4	5.4	3.0	5.2	4.7	7.7	6.0
	$J_{NH-C_\alpha H}$	7.7	6.9	8.0	5.8	6.9	6.6	5.9
[L-Tic^2]OXT	δ(NH)		6.77	8.13	7.78	8.45	8.49	8.38
	$-d\delta/dT$ ($\times 10^3$)		3.0	3.3	2.5	5.6	6.5	5.8
	$J_{NH-C_\alpha H}$		9.2	4.2	8.6	7.3	6.2	5.9
[D-Tic^2]OXT	δ(NH)		8.57	8.45	9.18	8.15	8.58	8.47
	$-d\delta/dT$ ($\times 10^3$)		5.0	3.5	6.8	2.9	6.3	3.5
	$J_{NH-C_\alpha H}$		10.5	6.1	8.8	7.2	6.3	7.4

TABLE 6

$^3J_{\alpha,\beta}$ and $^2J_{\beta,\beta'}$ coupling constants of ring constituting amino acids in oxytocin analogs

		Position of amino acid in peptide chain					
		1	2	3	4	5	6
OXT	$^3J_{\alpha,\beta}$	3.8	8.1	6.0	6.6	7.5	9.6
	$^3J_{\alpha,\beta'}$	6.0	6.2		6.8	7.5	3.0
	$^2J_{\beta,\beta'}$	15	14			15.9	
[L-Phe^2]OXT	$^3J_{\alpha,\beta}$	6.6	9.1	6.0	a	4.6	9.6
	$^3J_{\alpha,\beta'}$	5.0	6.1			7.9	3.8
	$^2J_{\beta,\beta'}$	14.4	14.2			15.2	14.6
[D-Phe^2]OXT	$^3J_{\alpha,\beta}$	9.6	7.8	6.6	a	5.8	9.1
	$^3J_{\alpha,\beta'}$	4.3				8.7	3.7
	$^2J_{\beta,\beta'}$	14.9				15.6	15.0
[L-Tic^2]OXT	$^3J_{\alpha,\beta}$	5.6	2.8	3.6	a	7.8	9.4
	$^3J_{\alpha,\beta'}$	8.6	5.8			6.6	3.3
	$^2J_{\beta,\beta'}$	14.4	13.9				15.2
[D-Tic^2]OXT	$^3J_{\alpha,\beta}$	3.2	4.0	8.0	a	a	8.5
	$^3J_{\alpha,\beta'}$	5.6	3.5				3.9
	$^2J_{\beta,\beta'}$		17.3				

[a]Not determined.

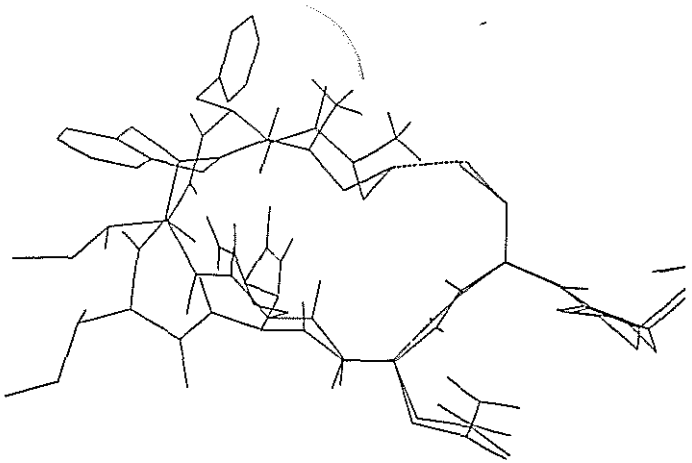

FIGURE 6
Superposed energy minimized structures of [L-Tic^2]OXT and [L-Phe^2]OXT.

NHMe (no constraints imposed during the calculations) reveals that indeed a δ-methyl group of isoleucine is in the vicinity of the shielding cone of the neighboring aromatic ring. However, the most striking result came from a comparison of the energy minimized conformations of [D-Tic^2]OXT and [D-Phe^2]OXT. These two structures can be essentially superimposed (Fig. 5, top). The only exception is the side chain of phenylalanine, which, however, can be rotated by 120° to attain an essentially identical superposition (Fig. 5, bottom). No such fit was found in the region Cys^1 to Gln^4 for [L-Tic^2]OXT and [L-Phe^2]OXT (Fig. 6). These results are compatible with conclusions drawn from the CD and NMR spectra. A D-Tic residue in position 2 does not change the conformation of the oxytocin peptide backbone which was already modified by the introduction of D-phenylalanine; it only stabilizes it and "freezes out" one of the possible rotamers of the phenylalanine side chain.

In conclusion, substitution of a D-Tic^2 residue for the Tyr^2 residue in oxytocin appears to stabilize a conformation of oxytocin which has the ability to bind to the uterotonic receptor in its antagonist conformation. This conformation may be similar to that accessible to [D-Phe^2]OXT. Substitution of Tyr^2 by L-tetrahydroisoquinoline carboxylic acid leads to a conformation that is different from those accessible to oxytocin, and thus this analog binds very poorly to the uterotonic receptor in its agonist state.

EXPERIMENTAL PROCEDURES

General methods

Thin-layer chromatography (TLC) was carried out on silica gel coated plates (Silufol, Kavalier, Czechoslovakia) in the following systems: 2-butanol–98% formic acid–water (10:3:8) (S1); 2-butanol–25% aqueous ammonia–water (85:7.5:7.5) (S2); 1-butanol–acetic acid–water (4:1:1) (S3); 1-butanol–pyridine–acetic acid–water (15:10:3:6) (S4). 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. Samples for amino acid analysis were hydrolyzed with 4 M MSA at 105° for 48 h and analyzed on a T339 amino acid analyzer (Mikrotechna, Prague, Czechoslovakia) or on a D-500 analyzer (Dionex Corp., Sunnyvale, CA, USA). Fast atom bombardment mass spectra were obtained on a ZAB-EQ spectrometer (VG Analytical Ltd., Manchester) with Xenon at 8 kV as the bombarding gas. High performance liquid chromatography (HPLC) was carried out on an SP-8700 instrument equipped with an SP-8400 detector and SP-4100 integrator (all from Spectra Physics, Santa Clara, USA). Before use, all amino-acid derivatives were subjected to ninhydrin test (36).

Solid phase synthesis

Protected peptide resin precursors to [L-Tic^2]OXT and [D-Tic^2]OXT. a) Benzhydrylamine resin (1% DVB, 1 g) was substituted to the level of 0.6 mmol/g by Boc-Gly using dicyclohexylcarbodiimide and *N*-hydroxybenzotriazole as the coupling reagent. Washing protocols, cleavage of N$^{\alpha}$-Boc groups, and neutralization reactions were performed as previously described (24). In the next step we coupled Nps-Leu with the use of dicyclohexylcarbodiimide (3 equiv.). The Nps group was cleaved by a mixture of 6 M HCl in MeOH-dichloromethane (1:3, 20 mL) for 4 min. Next we coupled Nps-Pro-OTcp (this and all other

couplings using active esters needed 24 h to reach completion according to ninhydrin test), Boc-Cys(Tmb), Nps-Asn-OTcp, Nps-Gln-OTcp, Nps-Ile-ONsu, Boc-L-Tic, and Boc-Cys(Tmb). The protected peptide resin A (1.64 g) was obtained.

b) Chloromethylated polystyrene resin (1% DVB, 2.25 g) with a capacity of 1 mmol Cl/g was substituted with Boc-Gly to a level of 0.44 mmol/g resin. Then Boc-Leu, Boc-Pro, Boc-Cys(pMB), Boc-Asn, Boc-Gln, Boc-Ile, Boc-L-Tic, and Boc-Cys(pMB) were added using the protocol previously described (24). Coupling of L-Tic to Ile and Cys(pMB) to L-Tic had to be repeated three times. Trifluoroethanol (5 mL) was added during the coupling. The N^{α}-Boc protecting group was cleaved, and the resin was washed and dried. Peptide-resin B (2.64 g) was obtained.

c) Synthesis of protected [D-Tic^2]OXT-resin was performed with the use of N^{α}-Boc protecting groups on the same resin as used for the protected peptide-resin A, except that Boc-Cys(Tmb) was used and Boc-D-Tic replaced Boc-L-Tic. Resin C was obtained.

d) The synthesis was performed on a *p*-methylbenzhydrylamine resin (1.05 g, 1.08 mmol NH_2/g) in the same way as in the case of resin C, with the exception that Boc-Cys(pMB) was used instead of Boc-Cys(Tmb); 1.92 g of resin D was obtained.

[2-L-Tetrahydroisoquinoline carboxylic acid]oxytocin ([L-Tic^2]OXT, Ia). a) The protected peptide resin (0.5 g) was treated with 10 mL anhydrous hydrogen fluoride containing 1 mL anisole. After 1 h stirring at 0°, the mixture was evaporated to dryness *in vacuo*, triturated with EtOAc, and extracted with 3 M acetic acid. The powder obtained after lyophilization was dissolved in 1 L of degassed water, the pH was brought to 8 by aqueous ammonia solution, and 0.01 M $K_3Fe(CN)_6$ was added until yellow color persisted for 30 min. The pH of the solution was brought to 4 by acetic acid, and the solution was filtered over an Amberlite IRC-50 (10 × 2 cm) column. The peptide was eluted by a gradient of AcOH (0.25% to 50%). The main peak was lyophilized, part of the product (30 mg) was purified on a column of Separon SI-C-18 (250 × 8 mm) in a gradient of methanol in 0.05% trifluoroacetic acid (40–60% in 30 min, flow rate, 4 mL/min). According to the analytical HPLC the product was contaminated with a byproduct with a higher k′ (k′ = 7.62, Separon SI-C-18, 25 × 0.4 cm, 50% MeOH 0.05% TFA) than the main product. This contamination was removed by gel filtration on Bio-gel P-4 (100 × 1 cm) in 3 M AcOH where it was eluted slightly later than [L-Tic^2]OXT. Amino acid analysis revealed that this product contained no Gln. After lyophilization of the main peak, 8 mg of the analog was obtained. Amino acid analysis (4 M MSA, 48 h); Asp 0.98, Glu 0.93, Pro 1.12, Cys 1.78, Gly 1.00, Ile 0.93, Leu 1.06, Tic 0.96. HPLC: k′ 6.66 (Separon SGX C-18, 25 × 0.4 cm 50% MeOH — 0.05% TFA). TLC: R_F 0.15 (S1), 0.03 (S2), 0.01 (S3), 0.59 (S4). Electrophoresis: $E^{His}_{5.7}$ 0.33, $E^{Gly}_{2.4}$ 0.80. Anal. calc. for $C_{44}H_{66}N_{12}O_{11}S_2 \cdot 1.5\ CH_3COOH \cdot 2\ H_2O$ (1129.3): C 49.99%, H 6.78%, N 14.88%. Found: C 49.90%, H 7.06%, N 14.88%. MS: 1005 $(M + 2H + H)^+$.

b) Peptide resin B was suspended in anhydrous methanol saturated with NH_3 at −7° and then was stirred at room temperature for 4 days. The protected peptide was extracted from the resin by DMF (100 mL, 65°, 24 h). The DMF extract was concentrated to 10 mL *in vacuo*, and the product was precipitated by water (125 mL), filtered, and dried (0.74 g). Part of the product (304 mg) was dissolved in anhydrous liquid ammonia (250 mL) and treated with sodium until the blue color persisted for 45 s. The oxidation and purification was performed as for a) above, and an identical product was obtained.

[2-D-Tetrahydroisoquinoline carboxylic acid]oxytocin ([D-Tic^2]OXT, Ib). Cleavage of the protected peptide resin C (or D) and purification was performed in the same way as for [L-Tic^2]OXT (case A above). The product was pure according to HPLC (k′ 7.67, Separon SGX C-18, 25 × 0.4 cm 50% MeOH — 0.05% TFA); TLC (R_F 0.14(S1), 0.03(S2), 0.01(S3), 0.59(S4)), and electrophoresis ($E^{His}_{5.7}$ 0.33, $E^{Gly}_{2.4}$ 0.80). Amino acid analysis: Asp 1.01, Glu 0.94, Pro 0.96, Cys 1.80, Gly 1.00, Ile 0.93, Leu 0.99, Tic 1.02.
Anal. calc. for $C_{44}H_{66}N_{12}O_{11}S_2 \cdot 1.5\ CH_3COOH \cdot 1\ H_2O$ (1111.2): C 50.80%, H 6.71%, N 15.12%. Found: C 50.92%, H 6.99%, N 15.32%. MS: 1005 $(M + 2H + H)^+$.

Spectroscopic methods

CD spectra were obtained on a Jobin-Yvon Dichrographe Mark V equipped with data processor using software DICHROSOFT version A written by Dr. P. Malon from the Laboratory of Peptide Synthesis, Institute of Organic Chemistry and Biochemistry, Prague. Spectra were recorded in cells of optical path of 1 and 0.02 cm at room temperature and hormone concentration of about 0.3 mg/mL ($3 \cdot 10^{-4}$ mol/L). The CD data are expressed in terms of molar ellipticity (degree $cm^2 dmol^{-1}$). Solvents used were 0.05 M phosphate buffer pH 7.3 and 2,2,2-trifluoroethanol.

^{1}H NMR spectra were measured on Bruker WM-250 or AM-250 spectrometer in D_2O and/or a D_2O/H_2O (1:1) mixture. The pH was adjusted to 3.5 with perdeuterated acetic acid. The solvent signal was reduced by employing a selective solvent suppression program (37). Resonances referenced to sodium 3-trimethylsilyltetradeuteriopropionate (TSP) were assigned by 2-D-COSY, 2-D-relayed COSY, double resonance, and comparison with the literature (34, 38).

Theoretical calculations

Energy minimization studies were carried out with the

CHARMM molecular mechanics software, version 19 (35). The empirical energy functions used in minimization included potential energy terms for bond angles, length, dihedral and improper dihedral angles, as well as van der Waals, and electrostatic terms for non-bonded interactions (35). The starting conformations in all cases were extended structures with *trans* peptide bond, except for those involving secondary amino acids (D-Tic, L-Tic, Pro), that were allowed to optimize. An adopted basis set Newton-Raphson (ABNR) minimization algorithm was employed with 2000 steps to assure convergence.

Pharmacological methods

All pharmacological tests were performed using Wistar rats weighing 200–300 g. The uterotonic potency *in vitro* was evaluated using the Holton procedure (39, 41) in Munsick (38) solution, and *in vivo*, according to Pliška (41). The galactogogic potency *in vivo* was established using published methods (42, 43). Pressor activity was tested on pithed rat preparation using published methods (44, 45).

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REFERENCES

1. Lebl, M., Jošt, K. & Brtnik, F. (1987) in *Handbook of Neurohypophyseal Hormone Analogs*, (Jošt, K., Lebl, M. & Brtnik, F., eds.), Vol. II, Part 2, pp. 127–267, CRC Press, Boca Raton
2. Hruby, V.J. & Smith, C.W. (1987) in *The Peptides* (Smith, C.W., ed.), Vol. 8, pp. 77–207, Academic Press, New York
3. Hruby, V.J. (1982) *Life Sci.* **31**, 189–199
4. Zaoral, M. & Krchňák, V. (1977) *Collect. Czech. Chem. Commun.* **42**, 3500–3509
5. Kaurov, O.A. (1978) Ph.D. Thesis, Leningrad University
6. Skala, G., Smith, C.W., Taylor, C.J. & Ludens, J.H. (1984) *Science* **226**, 443–445
7. Hill, P.S., Slaninová, J. & Hruby, V.J. (1988) in *Peptides, Chemistry and Biology* (Marshall, G.R., ed.), pp. 468–470, ESCOM, Leiden
8. Rose, G.D., Gierasch, L.M. & Smith, J.A. (1985) *Adv. Protein Chem.* **37**, 1–109
9. Schiller, P.W. & Di Maio, J. (1983) in *Peptides: Structure and Function* (Hruby, F.J. & Rich, D.H., eds.), pp. 269–278, Pierce, Rockford, IL
10. Schiller, P.W., Eggimann, B., Di Maio, J., Lemieux, C. & Nguyen, T.M.-D. (1981) *Biochem. Biophys. Res. Commun.* **101**, 337–343
11. Mosberg, H.I., Hurst, R., Hruby, V.J., Gulligan, J.J., Burks, T.F., Gee, K. & Yamamura, H.I. (1982) *Biochem. Biophys. Res. Commun.* **106**, 506–512
12. Cody, W.L., Hadley, M.E. & Hruby, F.J. (1988) in *The Melanotropic Peptides* (Hadley, M.E., ed.), pp. 75–92, CRC Press, Boca Raton
13. Chipens, G.I., Mutulis, F.K., Myshlyakova, N.V., Misina, I.P., Vitolina, R.O., Klusha, V.T. & Katayev, B.S. (1985) *Int. J. Peptide Protein Res.* **26**, 460–468
14. Mutulis, F.K., Mutula, I.E., Maurops, G.H. Sekacis, I.P., Grigoryeva, V.D., Kukaina, E.M., Golubeva, V.V. Myshlyakova, N.V., Klusha, V.J. & Chipens, G.I. (1985) *Bioorg. Khim.* **11**, 1276–1278
15. Sawyer, T.K., Hruby, V.J., Darman, P.S. & Hadley, M.E. (1982) *Proc. Natl. Acad. Sci. USA* **19**, 1751–1755
16. Al Obeidi, F., Hadley, M.E., Pettit, B.M. & Hruby, V.J. (1989) *J. Am. Chem. Soc.* **111**, 3413–3416
17. Kazmierski, W. & Hruby, V.J. (1988) *Tetrahedron* **44**, 697–710
18. Kazmierski, W., Wire, W.S., Lui, G.K., Knapp, R.J., Shook, J.E., Burks, T.F., Yamamura, H.I. & Hruby, V.J. (1988) *J. Med. Chem.* **31**, 2170–2177
19. Kazmierski, W., Yamamura, H.I., Burks, T.F. & Hruby, V.J. (1989) in *Peptides 1988* (Jung, G. & Bayer, E., eds.), pp. 643–645, W. de Gruyter, Berlin
20. Lebl, M., Barth, T., Servitova, L., Slaninová, J. & Jošt, K. (1985) *Collect. Czech. Chem. Commun.* **50**, 132–145
21. Zaoral, M., Bláha, I., Lebl, M. & Barth, T. (1987) in *Peptides 1986* (Theodoropoulos, D., ed.), pp. 465–468, De Gruyter, Berlin
22. Lebl, M., Sugg, E.E. & Hruby, V.J. (1987) *Int. J. Peptide Protein Res.* **29**, 40–45
23. Lebl, M., Frič, I., Sugg, E.E., Cody, W.L. & Hruby, V.J. (1987) in *Peptides 1986* (Theodoropoulos, D., ed.), pp. 341–344, De Gruyter, Berlin
24. Procházka, Z., Ancans, J.E., Slaninová, J., Machová, A., Barth, T. & Lebl, M. (1990) *Collect. Czech. Chem. Commun.* **55**, 1099–1105
25. Fassina, G., Lebl, M. & Chaiken, I. (1987) *Collect. Czech. Chem. Commun.* **53**, 2627–2636
26. Lebl, M. & Hruby, V.J. (1987) in *Handbook of Neurohypophyseal Hormone Analogs* (Jošt, K., Lebl, M. & Brtnik, F., eds.), Vol. I, Part 1, pp. 155–159, CRC Press, Boca Raton
27. Lebl, M. (1983) *J. Chromatogr.* **264**, 459–462
28. Larsen, B., Fox, B.L., Burke, M.F. & Hruby, V.J. (1979) *Int. J. Peptide Protein Res.* **13**, 12–21
29. Frič, I. (1987) in *Handbook of Neurohypophyseal Hormone Analogs* (Jošt, K., Lebl, M. & Brtnik, F., eds.), Vol. I, Part 1, p. 159, CRC Press, Boca Raton
30. Wood, S.P., Tickle, I.J., Treharne, A.M., Pitts, J.E., Mascarenhas, Y., Li, J.Y., Husain, J., Cooper, S., Blundell, T.L., Hruby, V.J., Buku, A., Fischman, A.J. & Wyssbrod, H.R. (1986) *Science* **232**, 633–636
31. Gierasch, L.M., Deber, C.M., Madison, V., Niu, C.-H. & Blout, E.R. (1981) *Biochemistry* **20**, 4730–4738
32. Woody, R.W. (1985) in *The Peptides, Vol. 7, Conformation in Biology and Drug Design* (Hruby, V.J., ed.), pp. 15–114, Academic Press, New York
33. Hlaváček, J., Frič, I. & Hruby, V.J. (1990) *J. Protein Chem.*, **9**, 9–15.
34. Brewster, A.I.R. & Hruby, V.J. (1973) *Proc. Natl. Acad. Sci. US* **70**, 3806–3809
35. Brooks, B.R., Bruccoleri, R.E., Olafson, B.D., States, D.J., Swaminathan, S. & Karplus, M. (1983) *J. Comput. Chem.* **4**, 187–217
36. Kaiser, E., Colescott, R.L., Bossinger, C.D. & Cook, P.I. (1970) *Anal. Biochem.* **34**, 595–598
37. Kao, L.-F. & Hruby, V.J. (1986) *J. Magn. Resonance* **70**, 394–407
38. Hruby, V.J. & Lebl, M. (1987) in *Handbook of Neurohypophy-*

ated Hormone Analogs (Jošt, K., Lebl, M. & Brtník, F., eds.), Vol. I, Part I, pp. 105–155, CRC Press, Boca Raton
39. Holton, P. (1964) *Brit. J. Pharmacol.* **3**, 328
40. Munsick, R.A. (1969) *Endocrinology* **66**, 451
41. Pliška, V. (1969) *Eur J. Pharmacol.* **5**, 253
42. Bisset, G.W., Clark, B.J., Haldar, J., Harris, M.C., Lewis, G.P., Rocha, e Silva, M. (1967) *Brit. J. Pharmacol. Chemother.* **31**, 537
43. Barth, T., Jošt, K. & Rychlik, I. (1975) *Endocrinol. Exp.* **9**, 35
44. Shipley, R.E. & Tilden, J.H. (1947) *Proc. Soc. Exp. Biol.* **64**, 453
45. Krejči, I., Kupková, B. & Vávra, I. (1967) *Brit. J. Pharmacol. Chemother.* **30**, 497

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