SYNTHESIS, BIOASSAY AND NMR STUDY OF METHYLENEOXY ISOSTERS OF OXYTOCIN AND VASOPRESSIN

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Syntheses of pseudodipeptides H-Tyrψ[CH_2O]Ile-OH and H-Tyrψ[CH_2O]Phe-OH were carried out using the intramolecular Williamson reaction of *O*-benzyltyrosinol with ethyl chloroacetate followed by N-protection and aldol reaction of the corresponding morpholin-3-one in position C_2 with butanone or benzaldehyde, elimination of the hydroxy group to give derivatives with a double bond either as the *E*/*Z* (1 : 1) diastereomeric mixture in the case of the former derivative or as the *Z*-isomer only in the case of the latter one. Stereoselective hydrogenation and hydrolysis of both the lactams yielded the corresponding pseudodipeptides lacking the carbonyl group as a hydrogen bond donor. The introduction of the pseudodipeptides into positions 2 and 3 of oxytocin and vasopressin caused total absence of all biological activities in the formed analogues. The results of the bioassay and NMR study confirmed the importance of the H-bond between the backbone carbonyl of the Tyr^2 and NH proton of the Asn^5 residues for stabilization of the β-turn in the cyclic hexapeptide part of both the hormones and for their biological activity.
Keywords: Peptides; Peptidomimetics; Methyleneoxy isosters; Solid phase synthesis; Biological activity; ^{1}H and ^{13}C NMR spectroscopy.

Isosteric bond introduction into peptides is motivated by the limited usage of peptides as potential therapeutic agents because of their fast enzymatic degradation and a poor transport through biomembranes. The replacement of a peptide bond with a suitable isostere (surrogate) can increase the stability of a peptide toward enzymatic degradation, prolong the half-time of peptide action and improve peptides transport into a cell[1]. On the other hand, the substitution with surrogate of larger flexibility or rigidity can influence noncovalent interactions in a given peptide molecule and, consequently, its space structure and interaction with a corresponding receptor and finally lead to a changed biological potency.

We have been interested in CH_2O and CH_2S surrogates for several years[2–6] since they offer polar and flexible structures resistant to proteolytic en-

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zymes. Contrary to the CH_2S, the geometry of the CH_2O surrogate is very similar to *trans*-amide bond and resembles the peptide bond in extended conformation[7,8]. In addition, the CH_2O surrogate exhibits negligible nucleophilicity and resistance to oxidation.

An introduction of both the surrogates into bioactive peptide molecules can thus influence biological activities in a different way. For example, the CH_2O surrogate significantly accelerated and increased biological activity of oostatic peptides after their application in the flesh fly *Neobellieria bullata* contrary to the CH_2S surrogate, which evoked a moderate effect only[6]. The replacement of the Leu-Gly peptide bond in oxytocin (**1a**) either maintained (CH_2O) or partially dissociated (CH_2S) its biological activities[2,4] and the introduction of the CH_2O surrogate into molecules of substance P-(6-11)-peptide and [Leu[8]]enkephalin amide resulted in agonists with affinity to corresponding receptors[7] and with selective activity.

1a, X = Ile, Y = Leu; **1b**, X = Phe, Y = Arg

2a, X = Ile, Y = Leu; **2b**, X = Phe, Y = Arg

3a, X = Ile; **3b**, X = Phe

In the present study we were interested in synthesis of oxytocin (**1a**) and vasopressin (**1b**) analogues **2a** and **2b** containing the CH_2O surrogate as a substitute for Tyr^2-Ile^3 and Tyr^2-Phe^3 peptide bonds, respectively. Our intention was to evaluate the importance of intramolecular hydrogen bond between the carbonyl oxygen of Tyr^2 residue and the amide proton of Asn^5 residue. This bond was designed to stabilize the β-turn important for the interaction of both the hormones with receptors[9,10]. The Asn^5 residue has been proposed to be necessary for biological activity of oxytocin and

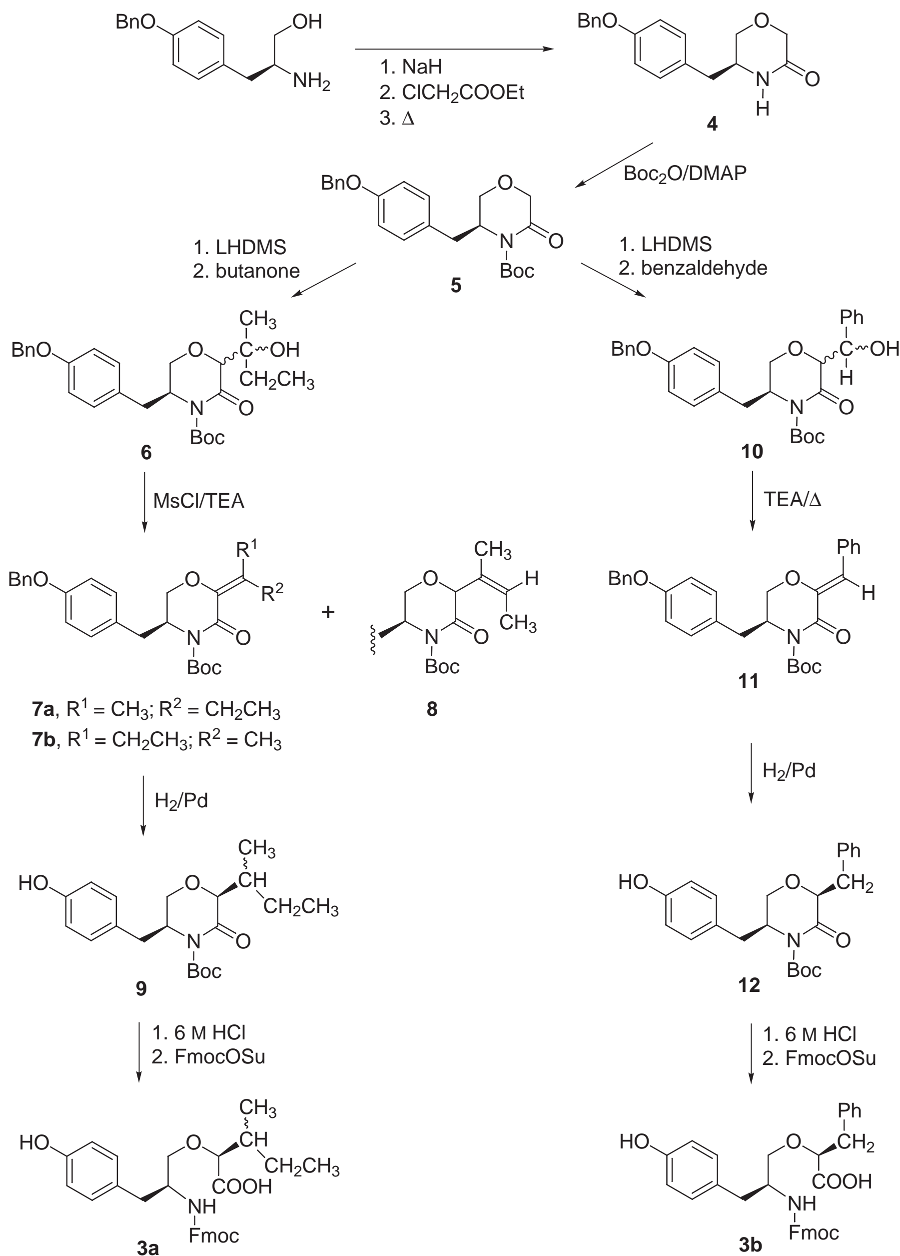
vasopressin[11] due to its side-chain interaction with the receptor binding site (even though some findings[12,13] proposed a more extensive exploration of structure–function relations at this residue because of the potency found in Asp^5 containing analogues). Also the Tyr^2 residue was postulated to exhibit an essential function in the interaction of both the hormones with corresponding receptors and the side-chain of this residue was designed to play an important role in the bioactive conformation[14–16]. Therefore, the introduction of the CH_2O surrogate between positions 2 and 3 could increase relative flexibility of the cyclic hexapeptide parts in corresponding hormone analogues and thus interfere with designed conformations, excluding both the analogues from a proper interaction with corresponding receptor system. The increase in the flexibility of the cyclic part could also worsen conditions for stacking of Tyr^2 and Phe^3 aromatic rings in vasopressin, designed for its biologically active model[10].

RESULTS AND DISCUSSION

Our synthetic strategy was based on preparation of corresponding pseudodipeptides H-Tyrψ[CH_2O]Ile-OH (**3a**) and H-Tyrψ[CH_2O]Phe-OH (**3b**) and their further utilization as the building blocks in the solid-phase synthesis of both the hormone isosteric analogues **2a** and **2b**.

In the synthesis of the pseudodipeptides **3a** and **3b** we utilized the intramolecular modification of the Williamson reaction, providing stereochemically defined compounds[17]. The starting compound for both the C_2-alkylation reactions, (5*S*)-5-[4-(benzyloxy)benzyl]-4-(*tert*-butoxycarbonyl)morpholin-3-one (**5**), was prepared by the reaction of *O*-benzyltyrosinol with ethyl chloroacetate affording the (5*S*)-5-[4-(benzyloxy)-benzyl]morpholin-3-one (**4**), followed by N-protection with di-*tert*-butyl dicarbonate.

The condensation of **5** with butanone provided a mixture of stereoisomers of **6** in good yield. Elimination of the hydroxy group led to a mixture of two regioisomers (7 : 3) with the double bond in different positions. The major product was isolated as a mixture of *E*- and *Z*-stereoisomers **7a** and **7b** in the 1 : 1 ratio, according to ^{1}H NMR and HPLC analyses; the minor product **8** was *Z*-isomer only. The separation of the diastereomers using flash chromatography was not successful and therefore we decided to continue with this mixture and to separate the diastereomers later on. The catalytic hydrogenation of the **7a**/**7b** mixture provided a mixture of the diastereomers of the N-protected 2,5-substituted morpholin-3-one **9** due to the presence of Ile and aIle side-chains. Hydrolysis of the lactam ring fol-



BnO
OH
NH2
1. NaH
2. ClCH2COOEt
3. Δ
4
Boc2O/DMAP
5
Boc
1. LHDMS
2. butanone
1. LHDMS
2. benzaldehyde
6
10
MsCl/TEA
TEA/Δ
7a, R1 = CH3; R2 = CH2CH3
7b, R1 = CH2CH3; R2 = CH3
8
11
H2/Pd
9
12
1. 6 M HCl
2. FmocOSu
3a
3b
COOH
Fmoc

lowed by introduction of the Fmoc protecting group provided the desired pseudodipeptide **3a** which was used for the synthesis of the oxytocin analogue **2a**.

The quenching of the enolate generated from **5** with benzaldehyde afforded **10**, followed by elimination of the hydroxy group providing substituted morpholin-3-one **11**, only as the *Z*-isomer. This compound, after catalytic hydrogenation, afforded stereoselectively (2*S*,5*S*)-2-benzyl-4-(*tert*-butoxycarbonyl)-5-(4-hydroxybenzyl)morpholin-3-one (**12**) (d.e. > 99%). The stereoselectivity of this reaction is induced by the benzyl substituent at the C_5 position which adopts the pseudoaxial conformation as a consequence of minimizing the pseudoallylic $A_{1,3}$ strain caused by N-Boc protecting group[18]. Finally, acid hydrolysis of the morpholin-3-one ring and introduction of the Fmoc protecting group provided H-Tyrψ[CH_2O]Phe-OH (**3b**) which was used in the synthesis of [8-arginine]vasopressin analogue **2b**.

Solid-phase synthesis of the methyleneoxy analogues **2a** and **2b** of oxytocin **1a** and [8-arginine]vasopressin **1b** was carried out on a Rink amide resin[19] using N^{α}-Fmoc protected amino acids in three-fold excess: Gly, Leu or Arg(Mtr), Pro, Cys(Trt), Asn(Trt), Gln(Trt). DIC with HOBt as condensation agents in DMA were used, in the presence of DIEA. In each step, the Fmoc deprotection was performed with 20% piperidine in DMF. In both the syntheses, the N-protected pseudodipeptides **3a** or **3b**, with their phenolic hydroxy groups unprotected, were coupled in two-fold excess with the growing peptide chains on the resin using a mixture of TOTU, HOBt, and DIEA. The peptides were detached from the resin by treatment with a TFA–TIS mixture in DCM. The side-chain deprotection in oxytocin analogue **2a** was performed with a TFA–water–EDT–TIS mixture and in vasopressin analogue **2b** with a TFA–thioanisole–phenol–water–EDT–TIS mixture for a prolonged time due to the presence of the Arg(Mtr) residue. Closure of the disulfide bonds in both the peptide analogues was carried out by oxidation with $K_3Fe(CN)_6$ at pH 8. After desalting and purification, both the analogues were analyzed and characterized by analytical HPLC, AAA, FAB MS, and ^1H NMR and ^{13}C NMR spectroscopies. In the oxytocin analogue **2a**, however, we obtained a non-separable mixture of two stereoisomers that differ in configuration at β-carbon of the side chain in position 3 of the peptide and correspond to oxytocin and [3-alloisoleucine]-oxytocin isosteric analogues.

The uterotonic, pressoric and antidiuretic activities of the newly synthesized methyleneoxy analogues **2a** and **2b** were tested and compared with the activities of the parent neurohypophyseal hormones **1a** and **1b** (Table I).

Both the analogues were entirely inactive in all the tests used in bioassay. These results are in accordance with those obtained from the experiments on analogues with methyleneamino surrogate in the same position[20] (Table I). As we indicated above, in the case of oxytocin analogue **2a**, a mixture of two non-separable stereoisomers differing in configuration at β-carbon of the side chain in position 3 and corresponding to oxytocin and [3-alloisoleucine]oxytocin analogues was, in fact, bioassayed. Even though the uterotonic activity of the latter is by about one order of magnitude lower than that of oxytocin[21], it is still significant (moreover, only 30% of the *allo*-form was present in the mixture). The absence of the biological potency in analogue **2a** can be therefore explained similarly to analogue **2b** by a significant conformational change caused by introduction of the flexible methyleneoxy peptide bond surrogate, unable to form hydrogen bond between the Asn^5 amide proton and Tyr^2 carbonyl oxygen. In the proposed conformational model of oxytocin, based on NMR studies, this H-bond is assumed to stabilize a β-turn, involving the Tyr-Ile-Gln-Asn sequence. This β-turn structure has been suggested to be one of building elements in overall conformation necessary for recognition by corresponding receptors and transduction of biological activities, as well[9–12,22–25]. By introducing the methyleneoxy surrogate, formation of this hydrogen bond was avoided and the flexible twenty-membered disulfide ring could not adopt a proper conformation for the interaction with the receptor.

TABLE I
Biological activities of oxytocin (**1a**), [8-arginine]vasopressin (**1b**) and their analogues

Peptide	UT[a]	BP[b]	AD[c]	Ref.
OT[d] (**1a**)	450[e]	5	5	38
[$aIle^3$]OT	27	~0.18	0.046	21
[$Tyr^2\psi[CH_2O]Ile^3$]OT (**2a**)	0	0	0	
[$Tyr^2\psi[CH_2NH]Ile^3$]OT	0	0	0	20
[Arg^8]VP[f] (**1b**)	17	412	465	38
[$Tyr^2\psi[CH_2O]Phe^3,Arg^8$]VP (**2b**)	0	0	0	
[$Tyr^2\psi[CH_2NH]Phe^3,Arg^8$]VP	0	0	0	20

[a] Uterotonic activity; [b] pressor activity; [c] antidiuretic activity; [d] OT, oxytocin; [e] biological activity values are given in IU mg^{-1}; [f] VP, vasopressin.

The active conformation of the twenty-membered ring of [8-arginine]-vasopressin (**1b**) is close to the active conformation of oxytocin[26–28]. Consequently, in the case of vasopressin analogue **2b**, the β-turn involving the Tyr-Phe-Gln-Asn sequence also cannot be formed due to the presence of the methyleneoxy isosteric bond and the resulting conformation of the ring is disfavored for interaction with receptor.

The NMR measurement of both the analogues **2a** and **2b** was performed in DMSO since this solvent was described to prevent a conformational averaging in the peptide backbone of the oxytocin hexapeptide ring in water. Also β-turn regions of conformers of [1-(3-mercaptopropanoic acid)]-oxytocin in the crystal structure were described to correspond to the β-turn regions proposed in the model for oxytocin and its 1-deamino analogue in DMSO (ref.[29]).

The NMR analysis (Tables II and III) confirmed our previous presumption that the flexibility of the twenty-membered rings in the analogues **2a** and **2b** has increased by introducing the methyleneoxy peptide bond surrogate. Temperature coefficients of amide proton chemical shifts of methyleneoxy analogs and parent peptides are compared in Table IV. The observed higher negative values of the temperature coefficient of the Asn^5 amide proton in the methyleneoxy analogues, when compared with those of the parent hormones **1a**, **1b**, indicate that the amide proton of Asn^5 in the methyleneoxy analogues is not involved in the hydrogen bond that leads to the increased flexibility of the twenty-membered ring. Moreover, the NOE contact between $^{\alpha}$CH of Cys^1 and Cys^6 observed in [8-arginine]vasopressin **1b** disappeared in the case of the methyleneoxy analog **2b**. In the same manner, the NOE contact between $^{\alpha}$CH of Tyr^2 and Asn^5 of oxytocin **1a** was no longer observed in the corresponding methyleneoxy analogue **2a**. Thus, the essentiality of the hydrogen bond in the twenty-membered ring stabilizing biologically active conformation of oxytocin and vasopressin was confirmed.

It is evident that increasing the peptides metabolic stability to enzymatic degradation by introduction of the methyleneoxy isosteric bond was not effective in the isosteric analogues **2a** and **2b** of oxytocin and [8-arginine]-vasopressin. It is, in contrast, to flexible linear oostatic peptides[30] where the introduction of this surrogate increased their stability and caused the acceleration and enhancement of their biological activity[6]. However, the same structure change, made in the backbone of peptides with relatively ordered conformation like that in the cyclic part of neurohypophyseal hormones, apparently hampers a proper conformation of peptides and their recognition by the corresponding receptor system. This consequently leads to loss of biological activity of the peptides. From the synthetic point of view, our

TABLE II
Proton NMR data of peptides **1a**, **1b** and **2a**, **2b** in DMSO-d_6

Residue	Parameter	**1a**	**2a** major	**2a** minor	**1b**	**2b**
Cys-1	NH_2	8.27	8.21		7.51	8.22
	αH	4.00	4.05		3.51	4.07
	$βH_2$(*J*αβ)	3.37 (5.7); 3.08 (7.1)	3.40 (a);	3.11 (a)	3.02 (5.6); 2.66 (8.0)	3.43 (4.3); 3.10 (7.5)
Tyr-2	NH	8.48	8.47	8.50	8.60	8.50
	αH(*J*Nα)	4.62 (8.5)	3.96 (7.8)	3.95 (8.0)	4.35 (a)	3.81 (8.1)
	$βH_2$(*J*αβ)	3.17 (3.8); 2.76 (10.8)	2.88 (7.3)	2.71 (7.0)	2.85 (5.2); 2.62 (9.6)	2.65 (8.1); 2.53 (6.8)
	o-H	7.12	7.03		6.91	6.65
	m-H	6.68	6.69		6.62	6.58
	OH	9.20	9.12		~9.20	9.09
	ψ[CH_2O]	–	3.52; 3.41	3.57; 3.43	–	3.44; 3.28
Ile-3	NH	8.02	–	–	8.39	–
(Phe)	αH (*J*Nα)	3.88 (a)	3.53 (a)	3.60 (a)	4.27 (7.0)	3.91 (a)
	βH (αβ)	1.79 (5.9)	1.74		3.18 (4.7); 2.92 (10.1)	3.03 (4.2); 2.90 (8.7)
	$γH_2$	1.45; 1.12	1.23		–	–
	β-CH_3	0.90	0.88		–	–
	γ-CH_3	0.86	0.88		–	–
	o-H	–	–		7.22	7.26
	m-H	–	–		7.30	7.33
	p-H	–	–		7.21	7.24
Gln-4	NH	8.24	8.04	8.00	8.08	8.19
	αH (*J*Nα)	3.95 (5.4)	4.24 (7.7)	4.28 (~8.0)	4.02 (6.5)	4.20 (7.5)
	$βH_2$(*J*αβ)	1.83–1.90	1.91 (5.0, 9.0)		1.87	1.92 (4.8, 9.0)
	$γH_2$	2.16	2.18		2.12	2.17
	$CONH_2$	7.26	7.29		7.34	7.31
		6.74	6.79	6.81	6.84	6.82

TABLE II
(*Continued*)

Residue	Parameter	**1a**	**2a** major	**2a** minor	**1b**	**2b**
Asn-5	NH	7.76	8.08	8.12	7.97	8.10
	αH (*J*Nα)	4.49(7.4)	4.52 (7.2)		4.48 (7.0)	4.53 (7.8)
	βH_2 (*J*αβ)	2.63 (6.7)	2.60 (6.6)	2.57 (6.6)	2.56 (6.5)	2.62 (6.4)
		2.59 (6.7)	2.53 (5.6)		2.52 (6.5)	2.52 (5.5)
	$CONH_2$	7.33	7.46	7.43	7.23	7.50
		6.86	7.01		6.72	7.02
Cys-6	NH	8.28	8.02	8.07	8.29	7.91
	αH (*J*Nα)	4.72 (77.6)	4.79 (8.6)		4.75 (7.8)	4.76 (8.2)
	βH_2 (*J*αβ)	3.18 (6.5); 3.03 (6.7)	3.10 (3.0);	2.93 (9.5)	3.12 (5.8); 2.95 (7.6)	3.07 (3.2); 2.90 (9.9)
Pro-7	αH	4.32	4.31		4.33	4.32
	βH_2 (*J*αβ)	2.03 (3.0); 1.83 (9.0)	2.06 (4.0);	1.86 (8.6)	2.02 (3.6); 1.87 (8.4)	2.07 (4.1); 1.86 (8.5)
	γH_2	1.80–1.90	1.91		1.87	1.90; 1.86
	δH_2	3.59; 3.44	3.58		3.64; 3.56	3.63; 3.56
Leu-8	NH	7.96	7.95		8.20	8.09
(Arg)	αH_2 (*J*Nα)	4.18 (7.6)	4.18 (7.6)		4.19 (7.6)	4.18 (7.5)
	βH_2 (*J*αβ)	1.51	1.51 (5.4, 9.4)		1.76 (5.0; 8.6)	1.75 (6.1, 8.7)
	γH	1.64	1.62		1.58; 1.52	1.52
	δ-CH_3	0.89; 0.83	0.89; 0.84		–	–
	δ-CH_2	–	–		3.07	3.10
	δ-NH	–	–		7.51	7.50
Gly-9	NH	7.81	7.81		8.07	7.87
	αH_2 (*J*Nα)	3.65 (6.0); 3.54 (5.6)	3.65 (6.2)	3.55 (5.6)	3.66 (5.9); 3.60 (5.6)	3.67 (5.9); 3.61 (5.7)
	$CONH_2$	7.07 6.98	7.05 6.98		7.13 6.98	7.14 7.00

[a] The *J*-value could not be determined.

TABLE III
^{13}C chemical shifts of peptides **1a**, **1b** and **2a**, **2b** in DMSO-d_6

Residue	Parameter	**1a**	**2a**		**1b**	**2b**
			major	minor		
Cys-1	CO	166.85	166.30	166.39	168.03	166.08
	Cα	51.95	52.18		54.43	52.03
	Cβ	40.35	41.35		44.05	41.43
Tyr-2	CO	171.98	–		170.96	–
	Cα	54.95	51.55		54.84	51.28
	Cβ	36.73	35.93		36.73	36.18
	i-C	127.71	128.49		127.73	128.21
	o-C	130.15	130.13		129.92	129.96
	m-C	115.29	115.37		115.03	115.15
	p-C	156.10	155.95		155.86	155.72
	ψ[CH_2O]	–	69.99	70.53	–	69.55
Ile-3	CO	171.54	172.05		171.83	172.15
(Phe)	Cα	59.56	84.96	83.57	55.46	82.73
	Cβ	35.81	37.45	37.76	36.62	39.60
	Cγ	25.06	24.64		–	–
	β-CH_3	15.60	15.11	14.68	–	–
	γ-CH_3	11.16	11.38	11.85	–	–
	i-C	–	–		138.04	137.59
	o-C	–	–		129.16	129.36
	m-C	–	–		128.21	128.31
	p-C	–	–		126.33	126.51
Gln-4	CO	171.25	171.28		170.82	170.76
	Cα	54.42	52.67		53.21	52.87
	Cβ	26.46	27.36		27.25	27.19
	Cγ	31.61	31.93		31.52	31.66
	$CONH_2$	174.48	174.35	174.38	174.18	174.26

TABLE III
(*Continued*)

Residue	Parameter	**1a**	**2a**	**1b**	**2b**
			major minor		
Asn-5	CO	170.84	171.23	173.48	172.27
	Cα	50.38	49.70	50.09	49.51
	Cβ	36.37	36.58	35.92	35.50
	$CONH_2$	171.89	171.79	171.21	171.01
Cys-6	CO	167.91	168.51	170.82	168.28
	Cα	51.64	50.10	50.96	50.00
	Cβ	41.20	40.20	40.94	38.92
Pro-7	CO	172.03	171.30	171.79	171.74
	Cα	60.29	60.06	59.96	59.86
	Cβ	29.05	29.19	28.96	29.12
	Cγ	24.59	24.55	24.48	24.46
	Cδ	46.94	46.93	46.84	46.87
Leu-8	CO	172.34	172.26	171.77	171.49
(Arg)	Cα	51.53	51.55	52.36	52.52
	Cβ	40.10	40.00	28.47	28.48
	Cγ	24.36	24.39	24.92	25.06
	Cδ	23.18	23.19	40.00	40.49
		21.66	21.69		
	(N>C=N)	–	–	157.34	156.86
Gly-9	$CONH_2$	171.10	171.01	171.67	171.10
	Cα	42.17	42.13	42.05	41.97

work shows compatibility of methyleneoxy pseudodipeptide building blocks with methods commonly used in the solid-phase peptide synthesis.

EXPERIMENTAL

Protected amino acids were purchased from Bachem (Bubendorf, Switzerland) or were prepared in our laboratory following general protocols[31]. In the N^{α}-Fmoc-amino acid preparations, the prescribed pH of the reaction mixtures was maintained using pH meter with an automatic titrator (Radiometer, Copenhagen, Denmark). *O*-Benzyltyrosinol, {(2*S*)-2-amino-3-[4-(benzyloxy)phenyl]propan-1-ol}, was purchased from Advanced ChemTech (Louisville, U.S.A.) and the Rink amide resin from Calbiochem–Novabiochem AG (Läufelfingen, Switzerland). Melting points were measured on a Kofler block and were not corrected. Column chromatography was carried out with Silica gel 60 (Merck, Darmstadt, Germany). Organic solutions immiscible with water were dried over anhydrous Na_2SO_4. Solvents were evaporated *in vacuo* on a rotary evaporator (bath temperature 30 °C); DMF was evaporated at 30 °C and 150 Pa. An average degree of substitution on the resin was determined from a resin weight increase after loading the Fmoc-Gly-OH, spectroscopically by the measurement of the dibenzofulvene–piperidine complex absorption after the Fmoc-group cleavage with 5% piperidine in DCM–DMF 1 : 1 for 10 min and 20% piperidine in DMF for 15 min, and by the quantitative AAA of glycine loaded on the resin. Optical rotations were measured on a Perkin–Elmer 141 MCA polarimeter at 22 °C and $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. For measurement of IR spectra (ν, cm^{-1}), a Bruker IFS 88 instrument (Karlsruhe, Germany) was used. The samples for amino acid analysis were hydrolyzed with 6 M HCl containing 3% of phenol at 110 °C for 20 h. AAA were performed on a Biochrom 20 instrument (Pharmacia, Sweden). Molecular weights of the peptides were determined using mass spec-

TABLE IV
Temperature coefficients of backbone amide protons chemical shifts (in ppb/°C) of oxytocin (**1a**), [8-arginine]vasopressin (**1b**) and their isosteric analogues **2a**, **2b**

Residue	**1a**	**2a**		Residue	**1b**	**2b**
		major	minor			
Tyr-2	–1.2	–3.3	–3.6	Tyr-2	–5.3	–4.3
Ile-3	–5.7	–		Phe-3	–7.1	–
Gln-4	–3.1	–4.0	–1.0	Gln-4	–1.5	–1.6
Asn-5	**–1.5**	**–2.6**	**–2.8**	**Asn-5**	**+0.5**	**–3.2**
Cys-6	–6.2	–2.7	–5.4	Cys-6	–8.2	–7.4
Leu-8	–4.4	–3.6	–3.6	Arg-8	–4.4	–3.7
Gly-9	–4.0	–3.7	–3.7	Gly-9	–4.4	–3.7

troscopy with FAB technique (Micromass, Manchester, England). For HPLC, a Spectra Physics instrument with an SP 8800 pump, an SP 4290 integrator and a Thermo Separation Products Spectra 100 UV detector were used. The compounds were purified by preparative HPLC on a 25 × 1 cm column, 10 μm Vydac RP-18 (The Separations Group, Hesperia, CA, U.S.A.), flow rate 3 ml min^{-1}, detection at 220 nm using 0–100% gradient of ACN in 0.05% aqueous TFA, 60 min. The analytical HPLC was carried out on a 25 × 0.4 cm column: 5 μm LiChrospher WP-300 RP-18 (Merck, Darmstadt, Germany), flow rate 1 ml min^{-1}, detection at 200 nm, using the 0–90% gradient of ACN in 0.05 aqueous TFA, 60 min, unless otherwise stated. ^{1}H NMR spectra were measured on a Varian UNITY-200 (at 200 MHz) and/or UNITY-500 (at 500 MHz) spectrometer. ^{13}C NMR spectra were measured on a Varian UNITY-500 spectrometer (at 125.7 MHz) at room temperature unless otherwise stated.

(5*S*)-5-[4-(Benzyloxy)benzyl]morpholin-3-one (**4**)

A mixture of *O*-benzyltyrosinol (5.0 g, 20 mmol) in THF (300 ml) and NaH (1.0 g, 25 mmol; 60% dispersion in mineral oil) was stirred at room temperature under nitrogen for 30 min, and then ethyl chloroacetate (2.44 g, 20 mmol) was added dropwise in 5 min. After 30 min of stirring, the reaction mixture was refluxed for 3 h, cooled and poured into 1 M HCl (300 ml). The product was extracted with EtOAc (3 × 100 ml), the combined extracts were washed with water (3 × 100 ml) and brine (3 × 100 ml), dried and the solvent was evaporated. The crude product was crystallized from a PE–EtOAc mixture affording a yield 2.2 g (51%) of white solid, m.p. 120–123 °C; $[\alpha]_D$ –5.21 (*c* 0.3, MeOH). ^{1}H NMR ($CDCl_3$, 200 MHz): 2.61 dd, 1 H, *J* = 13.7, 9.1 and 2.85 ddd, 1 H, *J* = 13.7, 5.2, 1.4 (C(5)-CH_2); 3.55 dd, 1 H, *J* = 11.6, 7.0 and 3.94 ddd, *J* = 11.6, 3.7, 1.4 (C(6)-CH_2); 3.72 m, 1 H, *J* = 9.1, 7.0, 5.2, 3.7 (C(5)H); 4.14 d, 1 H, *J* = 16.0 and 4.22 d, 1 H, *J* = 16.0 (C(2)H_2); 5.06 s, 2 H (CH_2 (Bn)); 5.78 bs, 1 H (NH); 6.94 m, 2 H and 7.10 m, 2 H (C_6H_4); 7.30–7.50 m, 5 H (C_6H_5). For $C_{18}H_{19}NO_3$ (297.3) found FAB MS, *m/z*: 298.1 (M^+ + 1).

(5*S*)-5-[4-(Benzyloxy)benzyl]-4-(*tert*-butoxycarbonyl)morpholin-3-one (**5**)

To a solution of **4** (2.0 g, 6.7 mmol) in DCM (30 ml), di-*tert*-butyl dicarbonate (4.5 g, 20 mmol) and DMAP (2.5 g, 20 mmol) were added at 0 °C and the reaction mixture was stirred at room temperature for 3 h, washed with 20% citric acid (3 × 50 ml) and brine (3 × 50 ml), dried and evaporated to dryness. The crude product was purified on a Silica gel 60 column using a PE–EtOAc (65 : 35) mixture giving a yield of 1.6 g (67%) of **5**, m.p. 81–83 °C; $[\alpha]_D$ –2.2 (*c* 0.46, MeOH). ^{1}H NMR ($CDCl_3$, 200 MHz): 1.59 s, 9 H (Boc); 2.98 m, 2 H (C(5)-CH_2); 3.57 dd, 1 H, *J* = 12.5, 2.5 and 3.80 ddd, 1 H, *J* = 12.5, 1.5, 1.0 (C(6)H_2); 4.15 m, 1 H (C(5)H); 4.16 d, 1 H, *J* = 17.1 and 4.35 d, *J* = 17.1 (C(2)H_2); 5.05 s, 2 H (CH_2 (Bn)); 6.93 m, 2 H and 7.18 m, 2 H (C_6H_4); 7.30–7.50 m, 5 H (C_6H_5). For $C_{23}H_{27}NO_5$ (397.4) found FAB MS, *m/z*: 398.2 (M^+ + 1).

(2*RS*,5*S*)-5-[4-(Benzyloxy)benzyl)-4-(*tert*-butoxycarbonyl)-2-(*RS*)-2-hydroxybutan-2-yl)-morpholin-3-one (**6**)

To a solution of LHDMS (2.5 ml of 1 M solution in THF, 2.5 mmol) in THF (5 ml), protected morpholin-3-one **5** (1.0 g, 2.5 mmol) in THF (5 ml) was added dropwise at –78 °C under nitrogen. The resulting mixture was stirred for 30 min, butanone (0.23 ml, 2.5 mmol) was added at this temperature and stirring continued for another 2 h. The reaction mixture was

poured into 20% citric acid (50 ml) and the aqueous phase was extracted with EtOAc (3 × 50 ml). The collected organic extracts were washed with brine, dried and EtOAc was evaporated. The crude product was purified on a Silica gel 60 column using a PE–EtOAc (7 : 3) mixture to obtain 0.8 g (68%) of colorless oily **6** as a mixture of diastereomers (*ca* 3 : 2) which was used for next reaction without purification. ^{1}H NMR ($CDCl_3$, 200 MHz), major isomer: 0.97 t, 3 H, *J* = 7.3 and 1.70 m, 2 H (CH_3CH_2); 1.31 s, 3 H (CH_3); 1.58 s, 9 H (Boc); 2.96 m, 2 H (C(5)-CH_2); 3.58 m, 1 H and 3.84 m, 1 H (C(6)H_2); 4.13 m, 1 H (C(5)H); 4.47 s, 1 H (C(2)H); 5.05 s, 2 H (CH_2 (Bn)); 6.94 m, 2 H and 7.15 m, 2 H (C_6H_4); 7.30–7.50 m, 5 H (C_6H_5); minor isomer: 0.94 t, 3 H, *J* = 7.3 and 1.70 m, 2 H (CH_3CH_2); 1.21 s, 3 H (CH_3); 1.55 s, 9 H (Boc); 2.80 dd, 1 H, *J* = 13.0, 9.6 and 3.05 dd, 1 H, *J* = 13.0, 4.0 (C(5)-CH_2); 3.60 m, 1 H and 3.87 m, 1 H (C(6)H_2); 4.26 m, 1 H (C(5)H); 4.29 s, 1 H (C(2)H); 5.05 s, 2 H (CH_2 (Bn)); 6.94 m, 2 H and 7.15 m, 2 H (C_6H_4); 7.30–7.50 m, 5 H (C_6H_5). For $C_{26}H_{35}NO_6$ (469.6) found FAB MS, *m/z*: 470.2 (M^+ + 1). IR ($CHCl_3$): ν(OH) 3 467.

(2*E*)- and (*Z*)-(5*S*)-5-[4-Benzyloxy)benzyl]-2-(butan-2-yliden)-4-(*tert*-butoxycarbonyl)-morpholin-3-one (**7a** and **7b**)

To a solution of the **6** (0.8 g, 1.7 mmol) and TEA (1.2 ml, 5.1 mmol) in DCM (20 ml), methanesulfonyl chloride (0.6 ml, 5.1 mmol) was added dropwise at 0 °C. The reaction mixture was then allowed to warm up to room temperature and stirred for 2 h after which it was washed with 20% citric acid (2 × 50 ml) and brine (2 × 50 ml). The organic phase was dried and evaporated to give a white solid. Under these conditions a spontaneous elimination of corresponding mesylate proceeded. The purification on a Silica gel 60 column using a PE–EtOAc (9 : 1) afforded a mixture of *E*- and *Z*-isomers (*ca* 1 : 1) with a yield 0.28 g (36%) of **7a** and **7b**; $[\alpha]_D$ −52.1 (*c* 0.36, MeOH). ^{1}H NMR ($CDCl_3$, 500 MHz): 1.04 t, 3 H, *J* = 7.4 and 2.25 m, 2 H (Et (*Z*)); 1.10 t, 3 H, *J* =7.2, 2.38 dq, 1 H, *J* = 12.4, 7.2 and 2.75 dq, 1 H, *J* = 12.4, 7.2 (Et (*E*)); 1.84 s, 3 H (Me (*E*)); 2.15 s, 3 H (Me (*Z*)); 1.56 s, 9 H (*t*-But); 2.90 m, 1 H, *J* = 13.0, 10.6, 2.2 and 2.96 m, 1 H, *J* = 13.0, 4.6, 1.6, 1.0 (C(5)-CH_2); 3.78 m, 1 H, *J* = 11.8, 8.0, 2.2, 1.0 and 3.90 m, 1 H, *J* = 11.8, 4.2, 1.6 (C(6)H_2); 4.25 m, 1 H (C(5)H); 5.05 s, 2 H (CH_2 (Bn)); 6.93 m, 2 H and 7.16 m, 2 H (C_6H_4); 7.43 m, 2 H, 7.39 m, 2 H and 7.33 m, 1 H (C_6H_5). ^{13}C NMR ($CDCl_3$, 125.7 MHz, APT): 11.76 and 12.84 (CH_3 (Et)); 17.82 and 18.71 (CH_3); 27.18 and 27.08 (CH_2 (Et)); 28.08 (3 × CH_3 (Boc)); 36.10 (C(5)-CH_2); 56.83 and 56.81 (C(5)); 64.33 and 64.28 (CH_2 (Bn)); 70.05 (C(6)); 83.27 and 83.22 (C(Boc)); 94.76 (C(2)=**C**<); 138.67 (C(2)); 115.07 (2 × CH); 130.46 (2 × CH); 129.65 and 157.74 (C_6H_4); 136.69 (C); 128.58 (2 × CH); 127.42 (2 × CH) and 127.96 (CH) (C_6H_5); 151.20 (C=O (Boc)); 159.35 (C(3)). For $C_{27}H_{33}NO_5$ (451.6) found FAB MS, *m/z*: 452.2 (M^+ + 1). IR ($CHCl_3$): ν(C=C) 1 617.

In the chromatography purification described above, an isomer (2*RS*,5*S*)-5-[4-(benzyloxy)-benzyl]-2-[(*Z*)-(but-2-en-2-yl)-4-*tert*-(butoxycarbonyl)morpholin-3-one (**8**) was separated (0.1 g). ^{1}H NMR ($CDCl_3$, 200 MHz): 1.56 d, 3 H, *J* = 6.7 (CH_3); 1.58 s, 9 H (Boc); 1.69 bs, 3 H (CH_3); 2.91 dd, 1 H, *J* = 13.4, 10.4 and 3.05 dd, 1 H, *J* = 13.4, 4.3 (C(5)-CH_2); 3.56 dd, 1 H, *J* = 12.2, 2.5 and 3.79 dd, 1 H, *J* = 12.2, 2.7 (C(6)H_2); 4.14 m, 1 H (C(5)H); 4.58 bs, 1 H (C(2)H); 5.05 s, 2 H (CH_2 (Bn)); 5.52 bq, 1 H, *J* = 6.7 (=CH); 6.92 m, 2 H and 7.18 m, 2 H (C_6H_4); 7.30–7.46 m, 5 H (C_6H_5).

(2*S*,5*S*)-2-(*RS*)-(Butan-2-yl)-4-(*tert*-butoxycarbonyl)-5-(4-hydroxybenzyl)-morpholin-3-one (**9**)

To a solution of the **7a** and **7b** (0.2 g, 0.44 mmol) in MeOH (200 ml), Pd-black (10 mg) was added under argon and hydrogenation was carried out at room temperature for 4 h. The catalyst was removed by filtration and the solvent was evaporated to give 0.16 g (99%) of colorless oil, which was a mixture of stereoisomers due to the presence of Ile and aIle side-chains in the 7 : 3 ratio. ^{1}H NMR ($CDCl_3$, 500 MHz; only signals of the major isomer which could be fully assigned are given): 0.96 t, 3 H, *J* = 7.2 and 1.45 m, 2 H (CH_3CH_2); 1.09 d, 3 H, *J* = 6.8 and 2.26 m, 1 H (CH_3CH); 1.58 s, 9 H (Boc); 2.98 m, 2 H (C(5)-CH_2); 3.54 dd, 1 H, *J* = 12.4, 2.5 and 3.80 dd, 1 H, *J* = 12.4, 1.0 (C(6)H_2); 4.03 d, 1 H, *J* = 2.4 (C(2)H); 4.10 m, 1 H (C(5)H); 4.96 bs, 1 H (OH); 6.80 m, 2 H and 7.14 m, 2 H (C_6H_4). ^{13}C NMR ($CDCl_3$, 125.7 MHz; only signals of the major isomer which could be fully assigned are given): 12.29 and 26.13 (CH_3CH_2); 15.88 and 37.75 ($CH_3CH<$); 28.05 (3 × CH_3 (Boc)); 37.35 (C(5)-CH_2); 57.36 (C(5)); 63.98 (C(6)); 83.68 (>C< (Boc)); 83.99 (C(2)); 115.52 (2 × CH); 130.72 (2 × CH); 129.57 (C) and 154.51 (C) (C_6H_4); 151.49 (C=O (Boc)); 170.15 (C(3)). For $C_{20}H_{29}NO_5$ (363.2) found FAB MS, *m/z*: 364 (M^+ + 1).

Fmoc-Tyrψ[CH_2O]Ile-OH (**3a**)

A mixture of substituted morpholin-3-one **9** (0.16 g, 0.44 mmol) and 6 M HCl (10 ml) was refluxed for 6 h and then heated in a sealed tube at 100 °C for 4 h to complete the hydrolysis. After cooling, the aqueous phase was washed with Et_2O (2 × 5 ml) and evaporated to dryness to give 72 mg (59%) of the epimer mixture of hydrochlorides of H-Tyrψ[CH_2O]Ile-OH and H-Tyrψ[CH_2O]aIle-OH. ^{1}H NMR (D_2O, 200 MHz): 0.89 t, *J* = 7.6 and 0.92 t, *J* = 7.6 (γ-CH_3 (Ile + aIle)); 0.91 d, *J* = 7.6 and 0.97 d, *J* = 7.0 (β-CH_3 (Ile + aIle)); 1.30 m, 1 H and 1.48 m, 1 H (γ-H_2 (Ile + aIle)); 1.86 m, 1 H (β-H, (Ile + aIle)); 2.97 m, 2 H (β-H_2 (Tyr)); 3.53 dd, 1 H, *J* = 11.6, 7.0 and 3.70 m, 1 H (ψ-CH_2-O); 3.70 m, 1 H (α-H (Tyr)); 3.82 d, *J* = 5.2 and 3.94 d, *J* = 3.7 (α-H (Ile + aIle)); 6.89 m, 2 H and 7.20 m, 2 H (C_6H_4 (Tyr)). FAB MS, *m/z*: 282 (M^+ + 1).

To a suspension of the pseudodipeptide hydrochlorides (72 mg, 0.26 mmol) in water (4 ml), a Fmoc-OSu (95 mg, 0.28 mmol) in dioxane (4 ml) was added at 0 °C, the solution was adjusted to pH 8.5 with 10% Na_2CO_3 and maintained at room temperature for 3 h. Dioxane was evaporated, the aqueous phase was washed with Et_2O (3 × 15 ml), acidified with 1 M HCl to pH 3 and the separated product was extracted with EtOAc (3 × 15 ml). The collected organic extracts were washed with brine (3 × 15 ml), dried and EtOAc was evaporated to yield 0.11g (84%) of the colorless oily N-protected epimer mixture of pseudodipeptides **3a**; $[\alpha]_D$ +14.3 (*c* 0.1, MeOH). For $C_{30}H_{33}NO_6$ (503.6) found FAB MS, *m/z*: 504.2 (M^+ + 1).

(2*RS*,5*S*)-5-[4-(Benzyloxy)benzyl]-4-(*tert*-butoxycarbonyl)-2-(*RS*)-[hydroxy(phenyl)methyl]-morpholin-3-one (**10**)

The compound was prepared from **5** (1 g, 2.5 mmol) in THF (5 ml) and LHMDS (2.5 ml of 1 M solution in THF, 2.5 mmol) in THF (5 ml) according to the procedure described for **6** by quenching the enolate with benzaldehyde (0.27 ml, 2.5 mmol). The crude product was purified on a Silica 60 column using a PE–EtOAc (75 : 25) to yield 0.98 g (78%) of pure **10**. ^{1}H NMR ($CDCl_3$, 200 MHz): 1.55 s, 9 H (Boc); 2.08 dd, 1 H, *J* = 13.1, 4.0 and 2.54 ddd, 1 H,

J = 13.1, 4.0, 1.5 (C(5)-CH_2); 3.53 ddd, 1 H, *J* = 12.5, 2.5, 1.5 and 3.72 dd, 1 H, *J* = 12.5, 1.0 (C(6)H_2); 3.62 d, 1 H, *J* = 4.3 (OH); 3.99 m, 1 H, *J* = 11.0, 4.0, 2.5, 1.0 (C(5)H); 4.42 d, 1 H, *J* = 4.6 (C(2)H); 5.02 s, 2 H (CH_2 (Bn)); 5.27 dd, 1 H, *J* = 4.6, 4.3 (C(2)-CH); 6.87 m, 2 H and 6.98 m, 2 H (C_6H_4); 7.25–7.52 m, 10 H (2 × C_6H_5). For $C_{30}H_{33}NO_6$ (503.6) found FAB MS, *m/z*: 504.2 (M^+ + 1). IR ($CHCl_3$): ν(OH) 3 606, 3 458.

(*Z*)-(5*S*)-2-Benzylidene-5-[4-(benzyloxy)benzyl]-4-(*tert*-butoxycarbonyl)-morpholin-3-one (**11**)

A reaction of **10** (0.98 g, 1.95 mmol) with methanesulfonyl chloride (0.3 ml, 3.9 mmol) in the presence of TEA (0.54 ml, 3.9 mmol), according to the procedure described in the **7a**, **7b** preparation, afforded the corresponding methanesulfonyl derivative which, on heating with TEA (0.47 ml, 3.4 mmol) to 100 °C for 30 min, underwent elimination to give 0.78 g (83%) of **11** as a white solid, m.p. 103–106 °C; $[\alpha]_D$ –52.1 (*c* 0.4, MeOH). ^{1}H NMR ($CDCl_3$, 200 MHz): 1.58 s, 9 H (Boc); 2.94 dd, 1 H, *J* = 12.5, 10.2 and 3.06 dd, 1 H, *J* = 12.5, 5.5 (C(5)-CH_2); 4.05 dd, 1 H, *J* = 11.6, 1.8 and 4.16 dd, 1 H, *J* = 11.6, 1.5 (C(6)H_2); 4.41 m, 1 H (C(5)H); 5.05 s, 2 H (CH_2 (Bn)); 6.95 s, 1 H (C(2)=CH); 6.95 m, 2 H and 7.20 m, 2 H (C_6H_4); 7.30–7.50 m, 8 H and 7.77 m, 2 H (2 × C_6H_5). For $C_{30}H_{31}NO_5$ (485.6) found FAB MS, *m/z*: 486.2 (M^+ + 1). IR ($CHCl_3$): ν(C=C) 1 617.

(2*S*,5*S*)-2-Benzyl-4-(*tert*-butoxycarbonyl)-5-(4-hydroxybenzyl)-morpholin-3-one (**12**)

The morpholin-3-one **11** (0.78 g, 1.62 mmol) was hydrogenated in MeOH (200 ml) using conditions described in the **9** preparation to give 0.57 g (89%) of colorless oily **12**. $[\alpha]_D$ –55.3 (*c* 0.2, MeOH). ^{1}H NMR ($CDCl_3$, 500 MHz): 1.56 s, 9 H (Boc); 2.20 dd, 1 H, *J* = 13.4, 10.7 and 2.56 ddd, 1 H, *J* = 13.4, 4.2, 1.5 (C(5)-CH_2); 3.24 dd, 1 H, *J* = 14.0, 5.6 and 3.27 dd, 1 H, *J* = 14.0, 4.5 (C(2)-CH_2); 3.54 ddd, 1 H, *J* = 12.5, 2.5, 1.5 and 3.69 dd, 1 H, *J* = 12.5, 1.2 (C(6)-CH_2); 4.00 m, 1 H, *J* = 10.7, 4.2, 2.5, 1.2 (C(5)H); 4.40 dd, 1 H, *J* = 4.5, 5.6 (C(2)H); 4.78 bs, 1 H (OH); 6.74 m, 2 H and 6.96 m, 2 H (C_6H_4); 7.23 m, 1 H and 7.32 m, 4 H (C_6H_5). For $C_{23}H_{27}NO_5$ (397.5) found FAB MS, *m/z*: 398.2 (M^+ + 1). IR ($CHCl_3$): ν(OH) 3 597.

Fmoc-Tyrψ[CH_2O]Phe-OH (**3b**)

Compound **12** (0.57 g, 1.44 mmol) was hydrolyzed with 6 M HCl (30 ml) under conditions used in preparation of the **3a** to give 0.25 g (52%) of pseudodipeptide hydrochloride, m.p. 162–163 °C; $[\alpha]_D$ –7.03 (*c* 0.2, MeOH). ^{1}H NMR (D_2O, 200 MHz): 2.78 d, 2 H, *J* = 7.6 (β-H_2 (Tyr)); 3.00 dd, 1 H, *J* = 14.0, 8.7 and 3.20 dd, 1 H, *J* = 14.0, 4.3 (β-H_2 (Phe)); 3.34 dd, 1 H, *J* = 10.7, 4.6 and 3.66 dd, 1 H, *J* = 10.7, 3.0 (ψ-CH_2-O); 3.54 m, 1 H (α-H (Tyr)); 4.20 dd, 1 H, *J* = 8.7, 4.3 (α-H (Phe)); 6.82 m, 4 H (C_6H_4); 7.30–7.50 m, 5 H (C_6H_5). FAB MS, *m/z*: 316 (M^+ + 1). This compound (0.32 g, 1 mmol) in water (15 ml) was reacted with Fmoc-OSu (0.34 g, 1.0 mmol) in dioxane (15 ml) using the same procedure as described in preparation of **3a** to yield 0.44 g (86%) of the colorless oily N-protected pseudodipeptide **3b**; $[\alpha]_D$ –41.3 (*c* 0.2, MeOH). For $C_{33}H_{31}NO_6$ (537.6) found FAB MS, *m/z*: 538.2 (M^+ + 1).

[$Tyr^2\psi[CH_2O]Ile^3$]oxytocin (**2a**) and [$Tyr^2\psi[CH_2O]Phe^3$]vasopressin (**2b**)

The solid phase synthesis of oxytocin and vasopressin analogues **2a** and **2b** was carried out on a Rink amide resin[19] (1 g) using, with regard to an average substitution of the Gly residue (0.52 mmol g^{-1}) on the resin, a three-fold excess (1.56 mmol) of N^{α}-Fmoc protected amino acids: Gly, Leu, Arg(Mtr), Pro, Cys(Trt), Asn(Trt) and Gln(Trt) assembled stepwise to the resin. The coupling of the amino acids was mediated with 3 equivalents of DIC, HOBt and DIEA in DMA (20 ml) until the Kaiser test[32] was negative. In each step, the Fmoc deprotection was performed with 20% piperidine in DMF (2 × 20 min) and washing was carried out with DMF (3 × 20 ml) and DMA (3 × 20 ml). In the last step, the protected pseudodipeptides **3a** or **3b** in two-fold excess were coupled to the growing peptide chains using a 2.5-fold molar excess of a TOTU, HOBt, and DIEA mixture. Finally, the peptide resin was washed three times with 20 ml of DMA, isopropyl alcohol, DMF, Et_2O and was dried in a desiccator. The peptides were detached from the resin by treatment with a TFA (10%)–TIS (1%) mixture in DCM (30 ml) for 10 min and with 5% TFA in DCM at room temperature for 20 min. After evaporation to dryness, the side-chain deprotection of the oxytocin analogue **2a** was performed by treatment of the residue with a TFA (94%)–water (2.5%)–EDT (2.5%)–TIS (1%) mixture for 1.5 h, and that of the vasopressin analogue **2b** by treatment with TFA (81.5%)–thioanisole (5%)–phenol (5%)–water (5%)–EDT (2.5%)–TIS (1%) mixture at room temperature for 6 h. The reaction mixtures were concentrated to 10% of their original volumes, the crude peptides were precipitated by addition of Et_2O and separated by filtration. A closure of disulfide bonds in both the peptide analogues was carried out by oxidation of their solutions in 1 M AcOH with $6 \cdot 10^{-3}$ M $K_3Fe(CN)_6$, at pH 8 adjusted with NH_4OH, to a permanent yellow color. The oxidation was followed by desalting on an Amberlite CG-50 column and lyophilization, yielding the crude oxytocin (40 mg) and vasopressin (43 mg) analogues, which were purified by preparative HPLC. Oxytocin analogue **2a** (25 mg, 0.025 mmol): HPLC retention time 23.2 min; m. p. 170–175 °C; AAA: Asp 0.9, Glu 1.0, Gly 1.0, Leu 1.0, Pro + Cys 1.4, Cys 0.5. ^1H and ^{13}C NMR data are given in Tables II and III. For $C_{43}H_{67}N_{11}O_{12}S_2$ (994.2) found FAB MS, *m/z*: 994 (M^+ + 1). Vasopressin analogue **2b** (24 mg, 0.022 mmol): HPLC retention time 24.7 min; m.p. 153–157 °C; AAA: Asp 1.0, Glu 1.0, Gly 1.0, Arg 1.0, Pro + Cys 1.5, Cys 0.5. ^1H and ^{13}C NMR data are given in Tables II and III. For $C_{46}H_{66}N_{14}O_{12}S_2$ (1 071.2) found FAB MS, *m/z*: 1 071 (M^+ + 1).

Biological Activity Assessment

Peptides were tested for uterotonic activity *in vitro* in the previously described rat uterotonic test for oxytocin according to Holton[33] and Munsick[34], either in the absence of Mg^{2+} or in the presence of 1 mM Mg^{2+} in the bathing solution on uteri from 3–5 different rats (Wistar, Konárovice, Czech Republic). Female rats were estrogenized 24–48 h before the experiment. Cumulative dose response curves were constructed using data from experiments in which doses were added successively to the organ bath in doubling concentrations and in 1 min intervals without the fluid being changed until the maximal response was obtained. The activity was determined by comparing the threshold doses of the standard and the analogue (IU mg^{-1} or EC_{50}). In the case of the antagonistic activity, the dose of the analogue was applied to the organ bath 1 min prior to the standard dose of oxytocin. The antagonistic activity was expressed as EC_{50} or pA_2, *i.e.* the concentration of the analogue, which reduced the effect of the double dose of agonist, in our case oxytocin, to the effect of dose *x*, and the negative decadic logarithm of the EC_{50}, respectively. The peptides were tested for pressor po-

tency in the pressor test according to Dekanski[35]. The test was performed using 4-aminophenyl phenyl ether-treated male rats under urethane anaesthesia (doses applied *i.v.*). Tests to estimate antidiuretic or diuretic properties were conducted on conscious hydrated male rats in the modified Burn test[36,37] in a standard way. The animals fasted for 16 h were weighed and then given a water load (4% of body weight). Immediately after the water load, the tested substances were applied *s.c.* The rats were then placed into individual metabolic cages, and their urine was collected over a 5 h period. The time was determined in which the rats excreted half of the water load. Synthetic oxytocin and arginine vasopressin were used as standards in the relevant tests.

NMR Spectra Measurement

NMR spectra of oxytocin **1a**, vasopressin **1b** and their analogues **2a** and **2b** were measured on a Varian UNITY-500 spectrometer (^{1}H at 500 MHz, ^{13}C at 125.7 MHz) in DMSO. Standard algorithm based on combination of 2D-COSY, 2D-TOCSY and 2D-ROESY spectra was used for complete structure assignment of protons. Temperature coefficients of amide NH protons were determined from 1D-proton spectra measured in the interval 20–40 °C. Carbon signals were assigned using the correlation *via* J(C,H) couplings observed in 2D-HMQC and 2D-HMBC spectra. NMR parameters are summarized in Tables II–IV.

Abbreviations Used

AAA, amino acid analysis; ACN, acetonitrile; APT, attached proton test; Boc, *tert*-butoxycarbonyl; Bn, benzyl; COSY, correlation spectroscopy; DCM, dichloromethane; DIC, *N*,*N*-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMA, *N*,*N*-dimethylacetamide; DMAP, 4-(dimethylamino)pyridine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDT, ethane-1,2-dithiol; Et_2O, diethyl ether; EtOAc, ethyl acetate; FAB MS, fast atom bombardment mass spectrometry; Fmoc, [(fluoren-1yl)methoxy]carbonyl; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; HOBt, 1-hydroxybenzotriazole; LHMDS, lithium bis(trimethylsilyl)amide; MsCl, methanesulfonyl chloride; Mtr, 2,3,6-trimethyl-4-methoxybenzenesulfonyl; OSu, succinimid ester; PE, light petroleum; ROESY, rotating frame NOESY spectroscopy; TIS, triisopropylsilane; TOCSY, total correlation spectroscopy; TOTU, *O*-cyanoethoxycarbonylmethylidene amino-1,1,3,3,-tetramethyl uronium tetrafluoroborate; TFA, trifluoroacetic acid; TEA, triethylamine; THF, tetrahydrofuran; Trt, triphenylmethyl. The nomenclature and symbols of amino acids follow published recommendations of the IUPAC/IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9–37).

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