Int. J. Peptide Protein Res. 43, 1994, 62–68
Printed in Belgium – all rights reserved

INTERNATIONAL JOURNAL OF
PEPTIDE & PROTEIN RESEARCH
ISSN 0367-8377

Synthesis of methylated phenylalanines via hydrogenolysis of corresponding 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acids

Synthesis and biological activity of oxytocin analogs with methylated phenylalanines in position 2

PAVEL MAJER[1], JIŘINA SLANINOVÁ[1] and MICHAL LEBL[2]

[1] *Institute of Organic Chemistry and Biochemistry, Academy of Science of the Czech Republic, Prague, Czech Republic, and* [2] *Selectide Corporation, Tucson, Arizona, USA*

Received 29 January, accepted for publication 5 June 1993

A new method of synthesizing *ortho*-methylated phenylalanines has been developed. Phenylalanines with at least one free *ortho*-position undergo a Pictet–Spengler cyclization with formaldehyde followed by hydrogenolytic splitting of the endocyclic benzylic C—N bond of 1,2,3,4-tetrahydroisoquinolines and afford corresponding *ortho*-methyl derivatives. Repeating this reaction sequence on the *ortho*-substituted phenylalanines yielded *ortho,ortho*-disubstituted derivatives, and *para*-substituted phenylalanines yielded *ortho,para*-disubstituted analogs. Our modified method of cyclization preserved the configuration at the chiral center: hydrogenolysis, on the other hand, led to racemization. Incorporation of the methylated phenylalanines into position 2 of oxytocin led to, in the case of the D-isomers, potent uterotonic inhibitors.

Key words: hydrogenolysis of 1,2,3,4-tetrahydroisoquinolines; *ortho*-methylated phenylalanines; oxytocin analogs; uterotonic inhibitors

1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid (Tic) is an interesting conformationally restricted analog of phenylalanine and a useful synthon for preparation of analogs of biologically active peptides (1–3), enzyme inhibitors (4, 5) etc. The only suitable synthesis of Tic described so far is Pictet–Spengler cyclization of phenylalanine with formaldehyde in concentrated hydrochloric acid (6, 7). However, racemization of about 30% occurs during this reaction (8). Optically pure Tic has been obtained by crystallization of its derivative (benzyl ester *p*-toluenesulfonate), followed by ester hydrolysis (9).

To avoid this tedious procedure, we have modified the reaction simply by lowering the reaction temperature. After a single recrystallization from aqueous ethanol we have obtained a highly pure optically active product (ee > 96%).

The Tic molecule contains one endocyclic benzylic C—N bond which could be cleaved by reduction, providing *ortho*-methylphenylalanine (oMePhe). We have unsuccessfully attempted to cleave this bond with sodium in liquid ammonia. Finally, we succeeded in catalytic hydrogenolysis on palladium sponge. Subjected repeatedly to this reaction sequence (Pictet–Spengler cyclization and hydrogenolysis) oMePhe afforded *ortho,ortho*-dimethylphenylalanine (diMePhe). This method can also be applied to *para*-substituted derivatives of phenylalanine, thus affording *ortho,para*-disubstituted phenylalanines.

To illustrate the usefulness of substituted phenylalanines in peptide chemistry, *ortho*-methyl- and *ortho,ortho*-dimethylphenylalanine were incorporated into position 2 of oxytocin (as part of our continuing

Abbreviations: AcOH, acetic acid; BHA, benzhydrylamine; Boc, *tert*-butoxycarbonyl; DCHA, dicyclohexylamine; DCM, dichloromethane; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; diMePhe, 2′,6′-dimethylphenylalanine; DMF, dimethylformamide; EtTic, 7-ethyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; HOBt, 1-hydroxybenzotriazole; iPrOH, 2-propanol; 4-MeBzl, 4-methylbenzyl; MeEtPhe, 2′-methyl-4′-ethylphenylalanine; MeTic, 5-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; MeTyr, 2′-methyltyrosine; oMePhe, 2′-methylphenylalanine; OXT, oxytocin; PE, light petroleum; TEA, triethylamine; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Tic(OH), 7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.

effort in mapping the hydrophobic pocket of the uterotonic receptor).

RESULTS AND DISCUSSION

Lowering the reaction temperature in a Pictet–Spengler cyclization of phenylalanine with formaldehyde from the usual 100 °C to 60 °C leads to an increase in α_D of the resulting Tic from *ca.* 120 ° to *ca.* 170 ° (both after a single recrystallization from aqueous ethanol; α_D of optically pure L-Tic is −177 ° in 1 M NaOH). An optical purity in excess of 96% was determined independently by chiral TLC. This relatively small modification to the reaction conditions significantly simplifies the synthesis of optically pure L- or D-Tic.

N-Benzyl groups are known to undergo reductive cleavage with sodium in liquid ammonia. However, the endocyclic benzylic bond of Tic is stable under these conditions. Attempts to cleave the C—N bond of acetyl and Boc derivatives of Tic were also unsuccessful. In all cases, only the starting material was recovered. Nevertheless, the tetrahydroisoquinoline ring of Tic can be opened by catalytic hydrogenolysis. Owing to the stable six-membered cyclic structure the reaction requires relatively drastic conditions in comparison with acyclic benzylamino acids (10) (freshly prepared Pd sponge, 3–5 h in AcOH at 90 °C). Unfortunately, these conditions lead to extensive racemization and complete loss of optical activity of the oMePhe thus obtained. Part of the *o*-methylphenylalanine was *N*-protected with the Boc group for the purpose of peptide synthesis. The second part was cyclized again with formaldehyde in the Pictet–Spengler manner, thus affording 5-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (MeTic). This compound, which is also potentially useful in peptide chemistry, was hydrogenolyzed in the same manner as Tic. In this case higher dilution was necessary due to the lower solubility of MeTic in acetic acid. This reaction also requires longer time to proceed to completion. The cyclization of *p*-ethylphenylalanine gave 7-ethyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (EtTic), hydrogenolysis of which yielded *o*-methyl-*p*-ethylphenylalanine.

7-Hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid [Tic(OH] cannot be prepared directly via cyclization of tyrosine with formaldehyde. Owing to the higher reactivity of hydroxylic *ortho*-positions, only resinous polycondensation products were isolated (7). Tic(OH) can, however, be synthesized either by a tedious multistep procedure (11) or simply via cyclization of 3′,5′-dibromotyrosine followed by catalytic debromination (12). Hydrogenolytic splitting of the benzylic C—N bond of Tic(OH) proceeded normally, affording 2′-methyltyrosine. This hydrogenolysis required a very long reaction time (24 h) due to the low solubility (< 1 g/L) of Tic(OH) in acetic acid.

We can conclude that hydrogenolysis of substituted 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acids represents a useful alternative to classical methods of synthesis of *ortho*-substituted phenylalanines, especially in the case of polysubstituted derivatives. The transformations described are illustrated in Fig. 1. Characteristics of the prepared compounds are summarized in Table 1; conditions used for hydrogenolysis are given in Table 2.

Structure–activity studies in the area of neurohypophysial hormone analogs performed in the Prague laboratory pointed very clearly to the importance of structure and hydrophobicity of the amino acid in position 2 of the oxytocin analog for the design of a potent

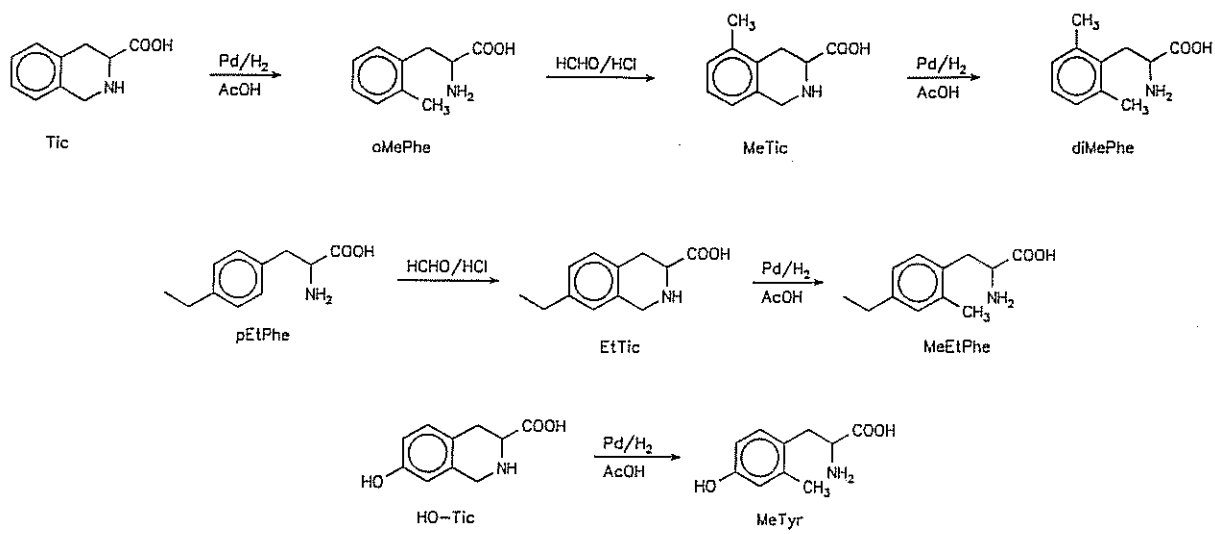

FIGURE 1

Transformations of phenylalanine and substituted phenylalanine.

TABLE I

Characteristics of amino acid derivatives

Compound	M.p. yield	FAB-MS (*m/z*)	Formula, NW calculated/found	%C	%H	%N	^{1}H-NMR
L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (L-Tic)	> 280 °C	178 $M+H^+$	$C_{10}H_{11}NO_2$,	177.2			(D_2O, NaOD) δ 2.7–3.1 (m, 2H, Ar-CH_2-CH), 3.4–3.9 (m, 1H, CH-N), 4.0 (s, 2H, Ar-CH_2-N), 7.0–7.3 (m, 4H, aromatic)
			67.78	6.26	7.90	
	51%		67.70	6.26	8.02	
D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (D-Tic)	> 280 °C	178 $M+H^+$	$C_{10}H_{11}NO_2$,	177.2			See L-enantiomer
			67.78	6.26	7.90	
	51%		67.89	6.37	7.78	
D,L-2-amino-3-(2′-methylphenyl)-propionic acid (oMePhe)	n.d.	180 $M+H^+$	$C_{10}H_{13}NO_2$,	179.2			(D_2O, NaOD) δ 2.29 (s, 3H, CH_3), 2.7–3.0 (m, 2H, CH_2), 3.15–3.7 (m, 1H, CH), 7.15–7.35 (m, 4H, aromatic)
			67.02	7.31	7.82	
	89%		67.16	7.55	7.64	
D,L-5-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (MeTic)	> 280 °C	192 $M+H^+$	$C_{11}H_{13}NO_2$,	191.2			(D_2O, NaOD) δ 2.23 (s, 3H, CH_3), 2.5–3.15 (m, 2H, Ar-CH_2-CH), 3.35–3.55 (m, 1H, CH), 3.95 (s, 2H, Ar-CH_2N), 6.9–7.2 (m, 3H, aromatic)
			69.09	6.85	7.32	
	45%		69.13	6.95	7.22	
D,L-2-amino-3-(2′,6′-dimethylphenyl)-propionic acid (diMePhe)	n.d.	194 $M+H^+$	$C_{11}H_{15}NO_2$,	193.2			(D_2O, NaOD) δ 2.32 (s, 6H, 2 × CH_3), 2.7–3.0 (m, 2H, CH_2), 3.15–3.18 (m, 1H, CH), 7.2–7.35 (m, 3H, aromatic)
			68.37	7.82	7.25	
	77%		68.65	8.13	7.13	
L-2-*tert*-butoxycarbonyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Boc-Tic-OH)	126–128 °C	278 (32%), $M+H^+$, 276 (15%), 232 (8%), 222 (95%), 178 (91%),	$C_{15}H_{19}NO_4$,	277.3			($CDCl_3$) δ 1.35–1.55 (d, 9H, tBu), 3.05–3.25 (m, 2H, Ar-CH_2-CH), 4.3–4.8 (m, 3H, Ar-CH_2-N, CH), 7.0–7.25 (m, 4H, aromatic)
			64.97	6.91	5.05	
	86%	176 (100%), 132 (64%), 130 (30%)	65.12	6.99	4.97	
D,L-2-*tert*-butoxycarbonylamino-3-(2′-methylphenyl)-propionic acid (Boc-oMePhe-OH)	105–108 °C	280 (20%), $M+H^+$, 234 (7M), 224 (100%), 180 (58%), 178 (33%),	$C_{15}H_{21}NO_4$,	279.3			($CDCl_3$) δ 1.2–1.4 (bd, 9H, tBu), 2.37 (s, 3H, CH_3-Ar), 2.9–3.35 (m, 2H, CH_2), 4.3–4.6 (m, 1H, CH), 6.9–7.2 (m, 4H, aromatic)
			64.50	7.58	5.01	
	76%	162 (8%), 134 (48%), 105 (18%)	64.63	7.76	4.89	
D,L-2-*tert*-butoxycarbonyl-5-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Boc-MeTic-OH)	146–148 °C	292 (30%) $M+H^+$, 290 (20%), 236 (95%), 192 (85%), 190 (100%), 146 (55%), 144 (27%)	$C_{16}H_{21}NO_4$,	291.3			($CDCl_3$) δ 1.41–1.51 (bd, 9H, tBu), 2.27 (s, 3H, CH_3-Ar), 2.85–3.4 (m, 2H, Ar-CH_2-CH), 4.35–4.75 (m, 2H, Ar-CH_2-N), 4.75–5.20 (m, 1H, CH), 6.95–7.15 (m, 3H, aromatic)
			65.96	7.27	4.81	
	87%		65.72	7.39	4.77	
D,L-*tert*-butoxycarbonylamino-3-(2′,6′-dimethylphenyl)-propionic acid (Boc-diMePhe-OH)	204–210 °C	294 (15%) $M+H^+$, 238 (90%), 194 (68%), 192 (33%), 148 (43%),	$C_{16}H_{23}NO_4$,	293.4			($CDCl_3$), δ 1.0–1.4 (bd, 9H, t-Bu), 2.39 (s, 6H, 2 × CH_3-Ar), 3.0–3.3 (m, 2H, CH_2), 4.35–4.65 (m, 1H, CH), 6.95–7.15 (m, 3H, aromatic)
			65.51	7.90	4.77	
	75%	119 (61%)	65.28	8.06	4.56	
D,L-7-ethyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (EtTic)	> 280 °C	206 $M+H^+$	$C_{12}H_{15}NO_2$,	205.3			(D_2O, NaOD) δ 1.17 (t, 3H, J = 7.5 Hz, CH_3), 2.60 (q, 2H, J = 7.5 Hz, CH_2-ethyl), 3.00–3.25 (m, 2H, Ar-CH_2-C), 4.40–4.82 (m, 1H, CH), 3.98 (bs, 2H, Ar-CH_2-N), 6.95–7.15 (m, 3H, aromatic)
			70.22	7.37	6.82	
	61%		69.92	7.29	6.77	
D,L-2-amino-3-(2′-methyl-4′-ethylphenyl)-propionic acid (MeEtPhe)	n.d.	208 $M+H^+$	$C_{12}H_{17}NO_2$,	207.3			(D_2O, NaOD) δ 1.18 (t, 3H, J = 7.5 Hz, CH_3 ethyl), 2.29 (s, 3H, CH_3-Ar), 2.62 (q, 2H, J = 7.5 Hz, CH_2-ethyl), 2.72–3.05 (m, 2H, CH_2), 3.18–3.65 (m, 1H, CH), 7.18–7.32 (m, 3H, aromatic)
			69.54	8.27	6.76	
	77%		69.32	8.29	6.89	
D,L-2-*tert*-butoxycarbonyl-7-ethyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Boc-EtTic-OH)	119–120 °C	306 $M+H^+$	$C_{17}H_{23}NO_4$,	305.4			($CDCl_3$) δ 1.21 (t, 3H, J = 7 Hz, CH_3), 1.40–1.52 (bd, 9H, tBu), 2.60 (q, 2H, J = 7.5 Hz, CH_2 ethyl), 3.05–3.25 (m, 2H, Ar-CH_2-C), 4.35–4.75 (m, 2H, Ar-CH_2-N), 4.75–5.20 (m, 1H, CH), 6.95–7.15 (m, 3H, aromatic)
			66.86	7.59	4.59	
	89%		66.62	7.49	4.47	
D,L-2-*tert*-butoxycarbonyl-amino-3-(2′-methyl-4′-ethylphenyl)-propionic acid (Boc-MeEtPhe-OH)	117–119 °C[a]	489 M + DCHA + H^+	$C_{29}H_{48}N_2O_4$,	488.7			(free acid as oil) ($CDCl_3$) δ 1.20 (t, 3H, J 7.5 Hz, CH_3 ethyl), 1.22–1.35 (bd, 9H, tBu), 2.31 (s, 3H, CH_3-Ar), 2.65 (q, 2H, J = 7.5 Hz, CH_2 ethyl), 2.90–3.35 (m, 2H, CH_2), 4.33–4.65 (m, 1H, CH), 7.05–7.20 (m, 3H, aromatic)
	75%		71.27	9.90	5.73	
			71.44	10.17	5.89	
D,L-2-*tert*-butoxycarbonyl-amino-3-(2′-methyl-4′-hydroxyphenyl)-propionic acid (Boc-MeTyr-OH)	159–163 °C[a]	477 (100) M + DCHA + H^+	$C_{27}H_{44}N_2O_5$	476.7			(DMSO-d_6) δ 1.33 (s, 9H, tBu), 2.19 (s, 3H, CH_3), 2.69 and 2.92 (q, 2H, $J_{\alpha\beta}$ = 4.9 and 10.4 Hz, $J_{\beta\beta}$ = 14.1 Hz, β-CH_2), 4.00 (m, 1H, α-CH), 6.96 (d, J_{13} = 8.6 Hz), 6.54 (d, 1H, J_{23} = 1.8 Hz), 6.48 (dd, 1H, Ar), 7.03 (bs, 1H, NH) (dicyclohexylamine not assigned)
	35%		68.04	9.30	5.88	
			68.24	9.06	5.71	

[a] DCHA salt.

TABLE 2

Reaction conditions for hydrogenolysis of isoquinoline derivatives

Starting compound	Product	Amount of starting compound per 500 mL of AcOH	Amount of Pd sponge (g)	Reaction time (h)
Tic	oMePhe	17.7 g/100 mmol	2	2–4
MeTic	diMePhe	1.92 g/10 mmol	1	14–16
EtTic	MeEtPhe	2.05 g/10 mmol	1	10–12
Tic(OH)	MeTyr	0.97 g/ 5 mmol	1	20–26

uterotonic inhibitor (13–15). (For a discussion of very extensive research in this area see e.g. refs. 16–18.) Even minor modifications in the structure of an amino acid in position 2 have a significant impact on the activity of the resulting analog. We have decided to map the hydrophobic pocket of a uterotonic receptor by synthesizing and testing the activities of a number of analogs containing a modified aromatic amino acid in position 2. Since the separation of diastereoisomers resulting from the coupling of a racemic amino acid in this position is relatively simple (19), and since we have described a simple method for the determination of the chirality of the modified aromatic amino acid (20), we decided to prepare modified phenylalanines in the racemic form and couple them as a mixture. Therefore, the procedure described above was convenient for our goal as a source of *ortho*-substituted phenylalanine derivatives.

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

X = L-oMePhe, D-oMePhe, L-diMePhe, D-diMePhe

Synthesis of the analogs was performed by solid-phase methods, using the Boc group for temporary protection. Diastereoisomers obtained after standard deprotection and cleavage from the resin by hydrogen fluoride and oxidation by potassium ferricyanide were separated by reversed-phase HPLC. The chirality of amino acids in position 2 was determined by the method employing chiral TLC separation of the acid hydrolyzate (20). In all cases the diastereomer containing D-amino acid was eluted later.

Biological activities of the oxytocin analogs are given in Table 3. Obviously, substituted phenylalanine in position 2 leads to more potent uterotonic inhibitors than the unsubstituted one. Inhibitory activity of phenylalanines with the L-configuration is observed only in the test performed *in vitro*; however, D-phenylalanines lead to inhibition both *in vitro* and *in vivo*.

The simultaneous presence of two methyl groups in *ortho*-positions of the aromatic ring decreases the potency of the inhibitor. Synthesized analogs have no pressor activity, and they behave as extremely weak agonists in the galactogogic test.

Our study has shown a simple way to prepare *ortho*-substituted phenylalanine derivatives. Application of these amino acid derivatives in structure–activity studies was demonstrated by the example of oxytocin analogs.

TABLE 3

Biological activities of some oxytocin analogs

Compound	Biological activity[a]			
	Uterotonic		Pressor	Galactogogic
	In vitro	*In vivo*		
OXT	450[b]	450[b]	5[b]	450[b]
[L-Phe^2]OXT	~30[b]	168[c]	0.4[b]	~60[b]
[D-Phe^2]OXT	$pA_2 = 6.00$	[d]	0.4[b]	[b]
[L-oMePhe^2]OXT	$pA_2 = 6.6$	0.41[b]	0	2.33[b]
[D-oMePhe^2]OXT	$pA_2 = 7.6$	$pA_2 = 6.7$	0	0
[L-diMePhe^2]OXT	$pA_2 = 6.17$	0	0	[d]
[D-diMePhe^2]OXT	$pA_2 = 7.4$	$pA_2 = 6.2$	0	[d]

[a] Activities were determined in rats.
[b] Agonistic activity in IU/mg.
[c] In cat.
[d] Not determined.

EXPERIMENTAL PROCEDURES

Melting points were determined on a Kofler block and are uncorrected. Thin-layer chromatography was performed on precoated silica gel plates (60F-254, 0.2 mm thick; Merck) with the following solvent systems (v/v): (A) 2-butanol: formic acid: H_2O (75:13.5:11.5), (B) 2-butanol: NH_3 (25% aq.): H_2O (85:7.5:7.5), (C) 1-butanol: AcOH: H_2O (4:1:1) and (D) 1-butanol: pyridine: AcOH: H_2O (15:10:3:6) (A–D were used for free amino acids and peptides); (E) PE: AcOEt (various ratios), (F) $CHCl_3$: CH_3OH (various ratios), (G) AcOEt (neat), (H) PE: toluene: acetone (5:5:1) (E–H were used for protected derivatives). Chiral plates (Machery-Nagel, Durren, Germany) were used for chiral TLC, elution system I – acetonitrile: methanol: water (4:1:1). Paper electrophoresis was performed in a moist chamber in 1 M acetic acid (pH 2.4) 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 analyses were hydrolysed with 6 M HCl at 105 °C for 20 h and analyzed on an amino acid analyzer D-500 (Durrum Corp., Palo Alto, CA). FAB mass spectra were obtained on a ZAB-EQ spectrometer (VG Analytical Ltd., Manchester, UK) with xenon at 8 kV as the bombarding gas. ^{1}H-NMR spectra were measured on a Varian XL-200 (200 MHz, Varian, San Fernando, CA) and Tesla BS-497 (100 MHz, Tesla, Brno, Czech Republic) spectrometers. HPLC analyses and preparations were performed on an SP-8800 instrument equipped with an SP-8450 detector and an SP-4290 integrator (all from Spectra-

Physics, Santa Clara, CA). Optical rotations were determined on a Perkin-Elmer polarimeter type 141 MCA (Perkin-Elmer, Norwalk, CT).

Solid-phase peptide syntheses were performed on semiautomatic apparatus of our own construction (Institute for Organic Chemistry and Biochemistry, Prague, Czech Republic). The progress of each coupling step was followed by the ninhydrin (12) and bromophenol blue (13) tests. In the case of coupling on the secondary amino group of proline the chloranil test was used.

L-1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid (L-Tic)

A mixture of L-Phe (82.5 g, 0.5 mol), 35–40% aqueous formaldehyde (200 mL) and conc. aqueous HCl (600 mL) was intensively stirred at 60 °C for 1 h. After addition of a further 80 mL of formaldehyde and 160 mL of HCl, stirring was continued for an additional 3 h at 60 °C. The mixture was then left to stand overnight in a refrigerator at 5 °C, precipitated Tic hydrochloride was filtered off, washed with a small amount of ice-cold water and dried *in vacuo* over solid KOH. Crude Tic·HCl was dissolved in 3 L of refluxing aqueous ethanol (60%). The hot solution was then neutralized with aqueous ammonia to pH 6–7 and left to stand for 5 h in a refrigerator (at 5 °C). Precipitated Tic was filtered off, washed with aqueous ethanol and dried in dessiccator. Yield 53 g (60%), $\alpha^{25}_D = -160$ to $-162°$ ($c = 1$, 1 M NaOH) m.p. > 280 °C decomp. After recrystallization of Tic from aq. EtOH, α^{25}_D increases to $-172°$ (overall yield 51%). Chiral purity was verified by TLC on Chiralplate. R_F 0.55 (I), D-isomer content < 2%. The same procedure was used for preparation of D-Tic, $\alpha^{25}_D = +172°$ ($c = 1$, 1 N NaOH) after recrystallization, yield 51%. R_F 0.51 (I), L-isomer content < 2%.

General procedure for Pictet–Spengler cyclization of substituted D,L-phenylalanines

A mixture of crude substituted phenylalanine[1] (0.05 mol), 40% aqueous formaldehyde (20 mL) and conc. hydrochloric acid (60 mL) was heated under vigorous stirring to 80–90 °C for 1 h. After addition of further 10 mL of formaldehyde and 20 mL of conc. HCl the mixture was stirred at 80–90 °C for additional 2 h and worked up as described for Tic.

General procedure for hydrogenolysis of 1,2,3,4-tetrahydroisoquinoline derivatives

A corresponding amount of substituted 1,2,3,4-tetrahydroisoquinoline (Table 2) and 500 mL of glacial acetic acid was placed in a 1 L round-bottommed flask with a sidearm provided with a reflux condenser. The mixture was stirred (magnetic stirrer) at 90 °C until it became a clear solution. The apparatus was then flushed with nitrogen[2], and freshly prepared palladium sponge was added (for the amount see Table 2). A continuous stream of hydrogen was then passed through the stirred solution for several hours (Table 2), while the temperature was maintained at 90 °C. The course of the reaction was monitored by paper electrophoresis. The yellow spot of the secondary amine gradually turns to a violet spot of the primary amine. When the reaction was complete, the Pd sponge was filtered off under nitrogen and AcOH was evaporated under reduced pressure. The oily residue was then dissolved in boiling water (5 mL/mmol) decolorized with charcoal, diluted with water and lyophilized. Crude products were used directly for the subsequent reactions.

General procedure for Boc protection of substituted phenylalanines and 1,2,3,4-tetrahydroisoquinoline derivatives

The amino acid (10 mmol) was dissolved in 10 mL of 1 M NaOH and 5 mL of 5% $NaHCO_3$ was added. To the stirred solution, Boc_2O (3 mL) in 10 mL of dioxane was added dropwise during 10 min. The mixture was stirred for 2 h at room temperature; the pH was maintained at 8–9 by addition of 1 M NaOH (pH-state). Dioxane was then evaporated under reduced pressure, the excess of Boc_2O was taken in PE and the pH of the aqueous phase was adjusted to 2–3 with HCl. The Boc-protected amino acid was obtained by extraction of the aqueous phase with EtOAc (3 × 50 mL). The organic extracts were dried over $MgSO_4$ and evaporated. Products were crystallized from a PE:EtOAc mixture. The physical constants of the protected derivatives are given in Table 1.

[D-oMePhe²]oxytocin and [L-oMePhe²]oxytocin

BHA resin (0.7 g, 0.5 mmol) was left to stand for 3 h with 20 mL DCM, neutralized with 7% DIEA/DCM (3 × 12 mL, 3 min) and washed with DMF (3 × 12 mL, 5 min). The first coupling was carried out with 3 equiv. of Boc-Gly-OH (262 mg, 1.5 mmol), HOBt (202 mg, 1.5 mmol) and DCC (367 mg, 1.7 mmol) dissolved in 15 mL DMF for 2 h. The remaining free amino groups were then blocked by acetylation with Ac_2O (2 mL) and TEA (3 mL) in DCM (20 mL) for 2 h. After acetylation the resin was washed with DMF (3 × 12 mL, 2 min), DCM (3 × 12 mL, 2 min), iPrOH (3 × 12 mL, 2 min) and DCM (3 × 12 mL, 2 min). Deprotection was carried out with 50% TFA/DCM, 1% anisole (2 × 12 mL, 2 min and 20 min); the resin was then washed with DCM (4 × 12 mL, 2 min), neutralized with 7% DIEA/DCM (3 × 12 mL, 3 min), washed with DCM

[1] A slightly different procedure was used for D,L-4-ethylphenylalanine because of its lower solubility. The compound was first dissolved in 100 mL of vigorously stirred conc. HCl and then 40% formaldehyde (20 mL) was added.

[2] The reaction mixture must be flushed with nitrogen!

(2 × 12 mL, 2 min) and DMF (4 × 12 mL, 2 min) and was ready for the next coupling step. The subsequent residues were coupled by the same procedure: Boc-Leu-OH·$\frac{1}{2}$ H_2O (360 mg, 1.5 mmol), Boc-Pro-OH (322 mg, 1.5 mmol), Boc-Cys(4MeBzl)-OH (487 mg, 1.5 mmol), Boc-Asn-OH (348 mg, 1.5 mmol), Boc-Gln-OH (263 mg, 1.5 mmol), Boc-Ile-OH·DCHA (700 mg, 1.7 mmol), Boc-D,L-oMePhe-OH (279 mg, 1 mmol) and Boc-Cys(4MeBzl)-OH (487 mg, 1.5 mmol). After the last coupling the peptide resin was deprotected and neutralized as described above, washed with DCM (3 × 12 mL, 2 min) and dried *in vacuo*.

The peptide resin was then treated with HF/thiocresole (95/5) at 0 °C for 1 h. HF was evaporated, and the residue was washed with dry EtOAc (3 × 20 mL) to remove scavenger. The free peptide was extracted from the resin with 50% AcOH (100 mL), and the solution was diluted with water and freeze dried. The lyophilisate was dissolved in water (500 mL), and the pH of the solution was adjusted to 7.0 with 0.1 M NaOH. Potassium ferricyanide (330 mg, 1 mmol in 100 mL of water) was added to this solution in the course of 10 min. During the oxidation (20 min) the pH was maintained at 7.0 by addition of 0.1 M NaOH and then adjusted to 4.5 with acetic acid. The solution was applied on a column of Amberlite CG-50 I (*ca.* 50 mL of ionex), the column was washed with 0.25% AcOH and eluted with 50% AcOH. After freeze-drying, the product (385 mg) was purified by gel filtration on a column of LH-20 (2 × 50 cm, eluent 3 M AcOH). The crude mixture of both diastereoisomers (118 mg) was then separated by HPLC [column Vydac C-18, 12 μm, 25 × 0.8 cm; (A) 0.05% TFA, (B) methanol] using a linear gradient 30–80% of B (0.5%/min). Fractions were collected, pooled and lyophilized, yielding 28 mg (5.5%) of [L-oMePhe^2]OXT and 21 mg (4.1%) of [D-oMePhe^2]OXT, pure according to HPLC.

[L-oMePhe^2]OXT. Amino acid analysis: Cys 1.94, Ile 1.00, Glu 1.11, Asp 1.07, Pro 0.94, Leu 0.97, Gly 0.93. FAB mass: calc. (M + 1) 1021.5, found 1021.5.

[D-oMePhe^2]OXT. Amino acid analysis: Cys 1.95, Ile 0.99, Glu 1.06, Asp 1.11, Pro 1.05, Leu 1.00, Gly 0.97. FAB mass: calc. (M + 1) 1021.5, found 1021.5.

[D-diMePhe^2]oxytocin and [L-diMePhe^2]oxytocin
The analogs were prepared on BHA resin (0.7 g, 0.5 mmol) as described above using Boc-diMePhe-OH (293 mg, 1 mmol) in the eighth coupling step to give 111 mg of a mixture of diastereoisomers after gel filtration. HPLC separation of the diastereoisomers analogous to that described for [D,L-oMePhe^2]OXT gave 21 mg (4.1%) of [L-diMePhe^2]OXT and 19 mg (3.7%) of [D-diMePhe^2]OXT.

[L-diMePhe^2]OXT. Amino acid analysis: Cys 1.93, Ile 1.02, Glu 1.08, Asp 1.05, Pro 0.96, Leu 0.99, Gly 0.96. FAB mass: calc. (M + 1) 1035.5, found 1035.5.

[D-oMePhe^2]OXT. Amino acid analysis: Cys 1.94, Ile 1.02, Glu 1.10, Asp 1.08, Pro 0.94, Leu 1.00, Gly 0.98. FAB mass: calc. (M + 1) 1035.5, found 1035.5.

Determination of the configuration of methylated phenylalanines in oxytocin analogs
The determination was carried out according to the described procedure (20). An aliquot (0.2 mg) of acid hydrolysate (see above) was evaporated *in vacuo*, dissolved in 0.1 M Tris buffer, pH 7.5, (20 μL) and 1% solution (20 μL) of L-amino acid oxidase (snake venom of Crotalus Athrox, Serva) was added. The tube was filled with oxygen, tightly closed and incubated for 24 h at 38 °C. After 24 h the new enzyme (20 μL) was added and the incubation was continued for an additional 24 h. The resulting mixture was either subjected to amino acid analysis that indicated the presence (D-enantiomer) or the absence (L-enantiomer) of the corresponding peak, or spotted directly onto chiral TLC plate (Chiralplate, Macherey-Nagel, Durren, Germany), elution acetonitrile–methanol–water (4:1:1). Enantiomers of substituted phenylalanines are in these cases distinguishable by TLC even without treatment with oxidase in the presence of other amino acids. R_F values: 0.54 (L-oMePhe), 0.43 (D-oMePhe), 0.52 (L-diMePhe), 0.38 (D-diMePhe).

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 (23) in Munsick (24) Mg^{2+} free solution and *in vivo* according to Pliška (25). The galactogogic potency *in vivo* was established according to refs. 26 and 27. The pressor activity was tested on pithed rat preparation according to refs. 28 and 29.

ACKNOWLEDGEMENT

We thank Drs. M. Buděšínský and N.F. Sepetov for NMR spectral measurements. This work was supported by the Academy of Sciences of the Czech Republic under grant no. 45531 (to M.L.).

REFERENCES

1. Kazimierski, 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
2. Hruby, V.J., Cody, W.L., de Lauro Castrucci, A.M. & Hadley, M.E. (1988) *Collect. Czech. Chem. Commun.* **53**, 2549–2572
3. Procházka, Z., Ancans, J.E., Slaninová, J. Machová, A., Barth, T. & Lebl, M. (1990) *Collect. Czech. Chem. Commun.* **55**, 1099–1105
4. Klutchko, S., Blankey, C.J., Fleming, R.W., Hinkley, J.M., Werner, A.E., Nordin, I., Holmes, A., Hoefle, M.L., Cohen D.M., Essenburg, A.D. & Kaplan, H.R. (1986) *J. Med. Chem.* **29**, 1953–1961
5. Grunewald, G.L., Sall, D.J. & Monn, J.A. (1988) *J. Med. Chem.* **31**, 824–830

6. Pictet, A. & Spengler, T. (1911) *Chem. Ber.* **44**, 2030–2036
7. Archer, S. (1951) *J. Org. Chem.* **16**, 430–432
8. Hein, G.E. & Nieman, C. (1962) *J. Am. Chem. Soc.* **84**, 4487–4494
9. Hayashi, K., Ozaki, Y., Nuami, K. & Yoneda, N. (1983) *Chem. Pharm. Bull.* **31**, 312–314
10. Quitt, P., Hellerbach, J. & Vogler, K. (1963) *Helv. Chim. Acta* **46**, 327–333
11. Miyake, A., Itoh, K., Aono, T., Kishimoto, S., Matsushita, Y., Inada, Y., Nishikawa, K. & Oka, Y. (1984) *J. Takeda Res. Lab.* **43**, 53–76
12. Verschueren, K., Toth, G., Tourwe, D., Lebl, M., Van Binst, G. & Hruby, V.J. (1992) *Synthesis*, 458–460
13. Lebl, M., Barth, T., Servítová, L., Slaninová, J. & Jošt, K. (1985) *Collect. Czech. Chem. Commun.* **50**, 132–145
14. Lebl, M., Hill, P., Kazmierski, W., Karászová, L., Slaninová, J., Frič, I. & Hruby, V.J. (1990) *Int. J. Peptide Protein Res.* **36**, 321–330
15. Lebl, M., Toth, G., Slaninová, J. & Hruby, V.J. (1992) *Int. J. Peptide Protein Res.* **40**, 148–151
16. Lebl, M. (1987) in *Handbook of Neurohypophysial Hormone Analogs* (Jošt, K., Lebl, M. & Brtnik, F., eds.), Vol. II, part 1, pp. 17–74, CRC Press, Boca Raton, FL
17. Hruby, V.J. & Smith, C.W. (1987) in *The Peptides* (Smith, C.W., ed.), Vol. 8, pp. 77–207, Academic Press, New York
18. Sawyer, W.H. & Manning, M. (1985) in *Oxytocin: Clinical and Laboratory Studies* (Amico, J.A. & Robinson, A.G., eds.), pp. 423–430, Elsevier, Amsterdam
19. Lebl, M. (1983) *J. Chromatogr.* **264**, 459–462
20. Toth, G., Lebl, M. & Hruby, V. (1990) *J. Chromatogr.* **504**, 450–455
21. Kaiser, E., Colescott, R.L., Bossinger, C.D. & Cook, P.I. (1970) *Anal. Biochem.* **34**, 595–598
22. Krchňák, V., Vágner, J., Šafář, P. & Lebl, M. (1988) *Collect. Czech. Chem. Commun.* **53**, 2542–2548
23. Holton, P. (1984) *Br. J. Pharmacol.* **3**, 328–334
24. Munsick, R.A. (1960) *Endocrinology* **66**, 451–458
25. Pliška, J. (1969) *Eur. J. Pharmacol.* **5**, 253–265
26. Bisset, G.W., Clark, B.J., Haldar, J., Haris, M.C., Lewis, G.P., Rocha e Silva, M. (1967) *Br. J. Pharmacol.* **31**, 537–545
27. Barth, T., Jošt, K. & Rychlík, I. (1975) *Endocrinol. Exp.* **9**, 35–46
28. Shipley, R.E. & Tilden, J.H. (1947) *Proc. Soc. Exp. Biol.* **64**, 453–460
29. Krejči, I., Kupková, B. & Vávra, I. (1967) *Br. J. Pharmacol.* **30**, 497–502

Address:

Michal Lebl, Ph.D., D.Sc.
Selectide Corporation
1580 E. Hanley Blvd.
Tucson, AZ 85737
USA