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Optically Active Aromatic Amino Acids. Part VI. Synthesis and Properties of (Leu^5)-enkephalin Analogues Containing *O*-methyl-L-tyrosine^1 with Ring Substitution at Position 3′

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Abstract: Twelve new [Tyr(Me)^1, Leu^5]-enkephalin analogues with substituents at position 3′ of the Tyr ring have been synthesized using traditional solution methods. The substituents were –CO_2H, –$CONH_2$, –CO_2Me, –(*E*)-CH=NOH, –(*E*)-CH=NOMe and CH_2OH. The analogues were *C*-terminated with methyl esters, amides or as free acids. In the *in vitro* biological assays a remarkable agonist activity to the opiate receptor μ in guinea pig ileum (GPI) relative to Leu-ENK was shown by the following: Leu-ENK, 100; [Tyr(Me)(3′-CO_2Me)^1, Leu-OMe^5]-ENK (I), 8.1; [Tyr(Me)(3′-(*E*)-CH=NOH)^1, Leu-OMe^5]-ENK (VI), 26.2; [Tyr(Me)(3′-(*E*)-CH=NOH)^1, Leu-OH^5]-ENK (VII), 2.9; [Tyr(Me)(3′-(*E*)-CH=NOH)^1, Leu-$NH_2{}^5$]-ENK (VIII), 4.7; and [Tyr(Me)(3′-CH_2OH)^1, Leu-OMe^5]-ENK (X), 5.6. The agonist effect was naltrexone- or naloxone-reversible. The masking of the hydroxyl group in (*E*)-hydroxyiminomethyl group of analogue (VI) by *O*-methylation has totally abolished its GPI agonist activity. It seems that the (*E*)-CH=NOH group shows affinity and plays an analogous role to the phenol group Tyr^1 in leucine–enkephalin and in the tyramine group of the opiate alkaloids. The analogues: [Tyr(Me)(3′-CO_2Me)^1, Leu-OMe^5]-ENK (I), [Tyr(Me)(3′-CO_2H)^1, Leu-OMe^5]-ENK (II), [Tyr(Me)(3′-CO_2Me)^1, Leu-NH_2^5]-ENK (III), [Tyr(Me)(3′-CO_2H)^1, Leu-NH_2^5]-ENK (IV), [Tyr(Me)(3′-$CONH_2$)^1, Leu-NH_2^5]-ENK (V), [Tyr(Me)(3′-(*E*)-CH=NOH)^1, Leu-OMe^5]-ENK (VI), [Tyr(Me)(3′-(*E*)-CH=NOH)^1, Leu-OH^5]-ENK (VII), [Tyr(Me)(3′-(*E*)-CH=NOH)^1, Leu-NH_2^5]-ENK (VIII), [Tyr(Me)(3′-(*E*)-CH=NOMe)^1, Leu-OMe^5]-ENK (IX), [Tyr(Me)(3′-CH_2OH)^1, Leu-OMe^5]-ENK (X), [Tyr(Me)(3′-CH_2OH)^1, Leu-OH^5]-ENK (XI) and [Tyr(Me)(3′-CH_2OH)^1, Leu-NH_2^5]-ENK (XII) under testing had no significant agonist activity to the enkephalinergic receptor in mouse vas deferens (MVD). All methyl esters of synthesized analogues of [Leu^5]-ENK showed higher activity to μ receptors than structurally identical *C*-terminal amides. It is a surprising result since usually *C*-terminate amides are stronger agonists than *C*-terminate esters. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: biological activity; guinea pig ileum assay; [Leu^5]-enkephalin analogues; mouse vas deferens assay; peptide synthesis; tyrosine analogues

Abbreviations: Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; DCC, dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; DMF, dimethylformamide; DPPA, diphenylphosphoryl azide; DCU, dicyclohexylurea; Et, ethyl; EtOAc, ethyl acetate; Et_3N, triethylamine; FIB-MS, fast ion bombardment mass spectrometry; GPI, guinea pig ileum; HPLC, high performance liquid chromatography; IC_{50}, 50% inhibitory concentration; Me, methyl; MVD, mouse vas deferens; NEtM, *N*-ethylmorpholine; NMR, nuclear magnetic resonance; NTI, naltrindole; NTX, naltrexone; TFA, trifluoroacetic acid; TLC, thin layer chromatography.

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INTRODUCTION

The hydroxyl group of tyrosine exerts a particular influence upon the biological activity of many peptides. Its removal, a modification of its chemistry by masking (e.g. methylation, acetylation) or an introduction of suitable substituents into the aromatic ring often gives rise to the formation of peptides with unexpected properties. A methylation of the Tyr^2 hydroxyl group in [Arg^8]-vasopressin totally eliminates its pressor activity and causes a strong increase in antidiuretic activity [1]. [Tyr(Me)^2]-oxytocin and its analogues are antagonists of oxytocin [2,3]. *O*-Methylation of tyrosine in opiate peptides normally results in a remarkable drop in their activity to the receptors (*in vitro*) as well as of the analgesic activity (*in vivo*). [Tyr(Me)^1]-dermorphine has only 2.8% of the activity of dermorphine in guinea pig ileum (GPI) [4]. An even stronger reduction in activity of the receptors in mouse vas deferens (MVD) (to 0.4% of the respective level in unmasked peptides) is shown by [Tyr(Me)^1, Met^5]-enkephalin [5]. A similar result was obtained with the use of GPI receptors [6].

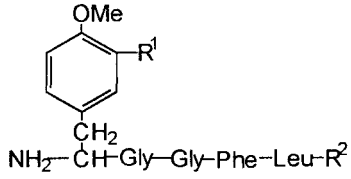
An introduction of electron donor or electron acceptor substituents to aromatic ring Tyr^1-enkephalin did not produce more active analogues. [Tyr(3′-F)^1, D-Ala^2, Met-NH$_2$^{5}]-enkephalin was twice less active than [D-Ala^2, Met-NH$_2$^{5}]-enkephalin. A derivative containing four fluorine atoms in the tyrosine ring was an extreme case, in that the acidity of the OH group (pK_a $\sim$2.2) and, is *in vitro* nearly 300 times less active than the mother compound. One electron donor substituent 3′-Me caused a 10-fold, and two Me substituents at 3′, 5′ positions an over 270-fold decrease in the activity of native enkephalin. A replacement of the hydroxyl group in tyrosine with an amine or a nitro group reduces the activity of methionine–enkephalin by three orders of magnitude [7]. In a cyclic [D-Pen^5]-enkephalin synthesized by Toth *et al.* [8], the introduction of an iodine atom into position 3′ resulted in a slight reduction of the activity in GPI (to 29.6%), whereas the presence of –OCH_3, –NO_2 and –NH_2 at this position reduced the activity down to only a few per cent. Substituents at positions 2′ and 6′ have a different action. The presence of a Me group caused as much as a $\sim$17-fold increase in enkephalin activity.

In this work further solutions were investigated for the interdependence between structure and activity as related to the presence of the Tyr^1 hydroxyl group in enkephalins. Therefore, some substituents not used until now were added to the tyrosine aromatic ring [9]. In order to estimate their own effect on the activity of a respective enkephalin analogue, the phenol group was masked with a methyl group and the following nucleophylic substituents were introduced into position 3′: carboxyl (–CO_2H), hydroxyiminomethyl (–(*E*)-CH=NOH) and hydroxymethyl (–CH_2OH), as well as lipophilic groups, (*E*)-methoxyiminomethyl (–(*E*)-CH=NOMe), carboxymethyl (–CO_2Me) and carboxyamide (–$CONH_2$). The aim of these substituents was to imitate the Tyr^1 hydroxyl group. Structures of the analogues synthesized are shown in Figure 1.

MATERIALS AND METHODS

Biological Assay

The pharmacological data in Tables 2 and 3 were performed in two different laboratories whose assaying methods were also different in details. Even though the results for the GPI and MVD activities are different for [Leu^5]-ENK in the laboratories, the relative potency of the analogues can be compared.



	R^1	R^2
I	-CO_2Me	-OMe
II	-CO_2H	-OH
III	-CO_2Me	-NH_2
IV	-CO_2H	-NH_2
V	-$CONH_2$	-NH_2
VI	-(*E*)CH=NOH	-OMe
VII	-(*E*)CH=NOH	-OH
VIII	-(*E*)CH=NOH	-NH_2
IX	-(*E*)CH=NOMe	-OMe
X	-CH_2OH	-OMe
XI	-CH_2OH	-OH
XII	-CH_2OH	-NH_2

Figure 1 Structures of new [Leu^5]-ENK analogues with ring substitution at position 3′ of Tyr^1.

Table 2 contains an *in vitro* bioassay, i.e. the determination of opiate-agonist activity of the synthesized analogues I, II, IX, X, XI and XII in an electrically stimulated longitudinal muscle strip of GPI and MVD preparations, which was carried out for activity assaying as described before [10,11]. The puffer contained, as a routine, captopril (10^{-5} M) to prevent the *C*-terminal dipeptidyl-carboxypeptidase effect on some of the analogues. To test the opioid sensitivity of the preparations, the experiments were started with the administration of DAMGO ([D-Ala^2, MePhe^4, Gly-ol^5]-ENK; 10^{-7} M) in GPI, and DPDPE (cyclic [D-Pen^2,D-Pen^5]-ENK; 8×10^{-9} M) in MVD. The 'exclusion' concentrations were 10^{-5} M in GPI and 10^{-6} M in MVD. If inhibitory actions were found at $\leq 10^{-5}$ or $\leq 10^{-6}$ M, the opioid antagonist naltrexone (NTX) was used in GPI and naltrindole (NTI) in MVD to prove the opioid receptor-mediated effect.

Table 3 shows the activity assaying results for other [Leu^5]-enkephalin analogues in GPI and MVD. The method of assaying was described earlier [12].

General Procedures

Melting points (uncorrected) were determined on a Boëtius Microscope. ^{1}H- and ^{13}C-NMR (250 MHz) spectra were recorded on a Bruker-HX-72 in ppm (referenced to TMS). Fast ion bombardment mass spectroscopy (FIB-MS) spectra were recorded on an Finnigan MAT 95 instrument. The Cs^+ ion gun was operated at 13 keV, with the temperature of the source of ions at 40°C. Glycerol was used as a matrix solvent. Optical rotations were measured on a Perkin–Elmer Model 241 MC spectropolarimeter with a 10-cm cell. Reaction progress and product purity were routinely monitored by thin layer chromatography (TLC) using precoated Silica gel 60 F_{254} glass plates (from E. Merck) in the following solvent systems: (A) butanol:acetic acid:water (4:1:1) (upper phase); (B) propanol:ammonia (67:33); (C) butanol:acetic acid:water:ethyl acetate (1:1:1:1); (D) ethyl acetate:pyridin:acetic acid:water (5:5:1:3); (E) ethyl acetate:ethanol (10:1); (F) ethyl acetate:acetone (1:1). High performance liquid chromatography (HPLC) was carried out on an apparatus consisting of a Waters 616 Pump, a Waters 600 Controller, a Waters 486 Tunable Absorbance Detector and a Waters 746 Data Module Analytical. Analytical reversed phase (RP) HPLC was performed on a 4 μm, Nova-Pak C_{18} (3.9×150 mm) column and preparative RP-HPLC on a 10 μm μBondapak C_{18} (19×300 mm) column. Solvent A was 0.05% TFA in water and solvent B was 0.038% TFA in 90% acetonitrile in water. Detection was at 220 nm and flow rate 1 ml min^{-1} in analytical chromatography and at 290 nm and 5 ml min^{-1} in preparative chromatography. Compounds were visualized with UV light or ninhydrin solution or with the chlorine gas procedure [13].

Peptide Synthesis

Synthesis of Boc-Gly-Gly-Phe-Leu-OMe (1)

*Z-Phe-Leu-OMe (**2**).* A suspension of H-Leu-OMe·HCl (21.78 g, 119.9 mmol) in EtOAc (360 ml) was stirred with *Z*-Phe-OH (35.88 g, 119.9 mmol) and Et_3N (16.62 ml, 119.9 mmol) at 0°C before the addition of a solution of DCC (25.20 g, 122.1 mmol) in EtOAc (120 ml). After standing at +5°C for 20 h, the reaction mixture was treated with CH_3CO_2H (4.8 ml) and after 1 h more DCU was filtered off and washed with EtOAc. The combined filtrates were evaporated to half volume and washed successively with HCl (1 M) and water, dried (using $MgSO_4$) and evaporated to dryness. Recrystallization from EtOAc/petroleum ether gave the desired product (43.0 g, 110.8 mmol, 84%); m.p. 113–114°C; $[\alpha]_D^{15} = -29.6°$ (*c* 0.7, MeOH). Literature value [14] m.p. 110–111°C; $[\alpha]_D^{23} = -25.0°$ (*c* 2.8, MeOH).

*H-Phe-Leu-OMe·HBr (**3**).* Compound **3** was obtained according to the literature [15].

*Boc-Gly-Gly-OH (**4**).* Compound **4** was obtained according to the literature [14].

*Boc-Gly-Gly-Phe-Leu-OMe (**1**).* To a mixture of the solutions of the hydrobromide (**3**) (8.50 g, 23.0 mmol, 15% excess) in DMF (25 ml) and the dipeptide (**4**) (4.64, 20 mmol) in DMF (25 ml) and DPPA (5.0 ml, 23 mmol) at 0°C, Et_3N (6.0 ml, 43.0 mmol) was added. After stirring for 4 h at 0°C the coupling was carried out overnight at room temperature and the solvent removed under reduced pressure. The semisolid residue was dissolved in EtOAc (1500 ml) and washed with HCl (1 M; 2 ×), saline, $NaHCO_3$ (saturated; 2 ×), water and saline (2 ×). The organic phase was dried ($MgSO_4$) overnight and evaporated. The concentrate was dissolved in boiling EtOAc (40 ml) from which crystalline product was precipitated with addition of petroleum ether (1000 ml) (8.65 g, 17.1 mmol, 86%). M.p. 116–117°C. After purification of the above product (2.0 g) on the column (4.5×37 cm) with Silica gel 60 (70–230 mesh, 300 g) and elution with EtOAc:EtOH (9:1), pure Boc-Gly-Gly-Phe-Leu-OMe was obtained. The yield of purification

was 86%. M.p. 118–122°C, $[\alpha]_D^{20} = -18.0°$ (*c* 1.3, MeOH). TLC: R_F(E) 0.73. HPLC retention time run isocratically of 6.42 min were observed in a 45% solvent B. ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.87 and 0.89 (d, 6H, δ-CH_3-Leu), 1.44 (s, 9H, CH_3-Boc), 1.51–1.61 (m, 2H, β-CH_2Leu), 2.95–3.14 (2m, 2H, β-CH_2Phe), 3.69 (s, 3H, CO_2CH_3), 3.85 (d, 2H, CH_2Gly^2), 3.92 (d, 2H, CH_2Gly^1), 4.53 (m, 1H, α-CHLeu), 4.86 (q, 1H, α-CHPhe), 5.55 (s, 1H, NH-Boc), 7.15–7.26 (m, 5H, aromatic protons), 7.63–7.66 (broad, 3H, 3·CONH). ^{13}C-NMR ($CDCl_3$) δ (ppm): 22.04 and 22.69 (Leu^4-δ), 24.96 (Leu^4-γ), 28.47 (Boc-CH_3), 39.00 (Phe^3-β), 41.21 (Leu^4-β), 43.10 (Gly^2-α), 44.14 (Gly^1-α), 51.03 (Leu^4-α), 52.13 (CO_2**C**H_3), 54.34 (Phe^3-α), 79.95 (Boc**C**(CH_3), 126.83 (Phe^3-ξ), 128.38 (Phe^3-ε), 129.49 (Phe^3-δ), 136.64 (Phe^3-γ), 156.21 (BocC=O), 168.75 (Gly^{2}C=O), 170.05 (Gly^{1}C=O), 171.16 (Phe^{3}C=O), 172.98 (Leu^{4}C=O). FIB-MS, *m*/*z* (+ive): 507 $(M+H)^+$; (−ive): 505 $(M-H)^-$. Found: C, 59.26; H, 7.76; N, 10.62%. $C_{25}H_{38}N_4O_7$ (506.60) requires: C, 59.27; H, 7.56; N, 11.06%.

Synthesis of Boc-Tyr(Me)(3′-CO$_2$Me)-Gly-Gly-Phe-Leu-OMe (5)

*TFA·H-Gly-Gly-Phe-Leu-OMe (**6**).* The protected tetrapeptide (**1**) (3.30 g, 6.50 mmol) was dissolved in TFA (13 ml) at 0°C and the mixture was kept at room temperature for 1 h. TFA was then removed *in vacuo* and the residue triturated (3 ×) with ether, yielding a white solid.

*Boc-Tyr(Me)(3′-CO$_2$Me)-OH (**7**).* DCHA·N-Boc-*O*-Me-(3′-CO_2Me)Tyr-OH (3.47 g, 6.50 mmol) was prepared according to Reference [9], and was dissolved in $CHCl_3$ (10 ml) and cooled to 0°C before the addition of HCl/EtOH (6.8 M, 0.96 ml). The dicyclohexylammonium hydrochloride soon precipitated and was filtered and washed thoroughly with ether. The combined organic phases were evaporated under reduce pressure to yield the title compound as a glassy mass.

*Boc-Tyr(Me)(3′-CO$_2$Me)-Gly-Gly-Phe-Leu-OMe (**5**).* The mixture of the tetrapeptide (**6**) dissolved in DMF (5 ml) and the protected amino acid (**7**) in DMF (10 ml) was cooled to 0°C and DPPA (1.40 ml, 6.50 mmol) was added, followed by NEtM (1.64 ml, 13.0 mmol). The reaction was allowed to run for 1 h at 0°C and overnight (about 20 h) at room temperature and the solvent removed *in vacuo* and replaced with EtOAc for work-up. Work-up consisted of extracting the EtOAc phase with HCl (1 M, 2 ×), NaCl (saturated), $NaHCO_3$ (saturated, 2 ×), water and finally NaCl (saturated). The EtOAc phase was dried with $MgSO_4$, filtered and evaporated. The residue was poured into petroleum ether and filtered. A white solid was obtained, 3.90 g (5.26 mmol, 81%). M.p. 143–150°C, $[\alpha]_D^{20} = -12.4°$ (*c* 2.1, MeOH). TLC: R_F(A) 0.76; R_F(E) 0.59. ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.87 and 0.89 (d, 6H, δ-CH_3-Leu), 1.40 (s, 9H, CH_3-Boc), 1.51–1.66 (m, 2H, β-CH_2Leu), 2.85–3.02 (m, 2H, β-CH_2Tyr), 3.08–3.15 (m, 2H, β-CH_2Phe), 3.71 (s, 3H, CO_2CH_3), 3.80 (s, 1H, OCH_3), 3.81 (s, 3H, CO_2CH_3Tyr), 4.01–4.27 (m, 4H, CH_2Gly^2, CH_2Gly^3), 4.61 (m, 1H, α-CHTyr), 4.63 (m, 1H, α-CHLeu), 5.11 (m, 1H, α-CHPhe), 5.87 (s, 1H, NHBoc), 6.81–7.23 (m, 5H, aromatic protons), 7.57 (broad, 1H, NHPhe), 7.61 (broad, 1H, NHLeu), 7.93 (broad, 1H, NHGly^3), 8.08 (broad, 1H, NHGly^2). FIB-MS, *m*/*z* (+ive): 742 $(M+H)^+$; (−ive): 740 $(M-H)^-$. Found: C, 59.80; H, 7.17; N, 9.37%. $C_{37}H_{51}N_5O_{11}$ (741.84) requires: C, 59.91; H, 6.93; N, 9.44%.

Synthesis of Boc-Tyr(Me)(3′-CO$_2$Me)-Gly-Gly-Phe-Leu-NH$_2$ (8) and Boc-Tyr(Me)(3′-CONH$_2$)-Gly-Gly-Phe-Leu-NH$_2$ (9)

The protected pentapeptide (**5**) (0.37 g, 0.50 mmol) was dissolved in MeOH (10 ml) and treated at −70°C (dry ice and acetone) with liquid ammonia (8 ml). The mixture was stirred at above temperature for 1 h and at room temperature for 40 h. The solvent was removed under reduced pressure and the residue was dissolved in EtOAc. The resulting mixture of unreacted diester (**5**), monoamide (**8**) and diamide (**9**) was separated on the column with Silica gel 60 (70–230 mesh, 300 g) and eluted with EtOAc:EtOH (1:1) mixture. The collected fractions successively contained: unreacted diester (**5**), monoamide (**8**) (0.15 g, 0.21 mmol, 41%) and diamide (**9**) (0.08 g, 0.11 mmol, 22.5%).

Physical properties of monoamide (**8**): m.p. 122–126°C; $[\alpha]_D^{20} = -12.8°$ (*c* 1.5, DMF); TLC: R_F(A) 0.65. FIB-MS, *m*/*z* (+ive): 749 $(M+Na)^+$; 727 $(M+H)^+$; (−ive): 725 $(M-H)^-$. Found: C, 56.63; H, 7.51; N 10.94%. $C_{36}H_{50}N_6O_{10} \times 2H_2O$ (762.86), requires C, 56.68; H, 7.13: N, 11.02%.

Physical properties of diamide (**9**): m.p. 156–160°C; $[\alpha]_D^{20} = -11.8°$ (*c* 1.6, DMF); TLC: R_F(A) 0.52. FIB-MS, *m*/*z* (+ive): 712 $(M+H)^+$; (−ive): 711 $(M)^-$. Found: C, 57.35; H, 7.53: N, 13.13%. $C_{35}H_{49}N_7O_9 \times H_2O$ (729.83), requires C, 57.60; H, 7.04; N, 13.44%.

Synthesis of Boc-Tyr(Me)(3′-CO$_2$H)-Gly-Gly-Phe-Leu-NH$_2$ (10)

The monoamide (**8**) (0.18 g, 0.25 mmol) was suspended in MeOH (2 ml) and NaOH (1 M; 1.2 ml, 1.2 mmol) was added. The saponification was allowed to proceed with stirring for 3 h. The mixture was diluted with water and MeOH was removed under reduced pressure and the residue extracted

with EtOAc. The aqueous basic layer was cooled and acidified with dropwise addition of HCl (4 M), and extracted with EtOAc. The organic phase was washed with NaCl (saturated), dried with $MgSO_4$ and concentrated under reduced pressure. The obtained solid was redissolved in EtOAc and precipitated by the addition of petroleum ether. Pure compound (**10**) was obtained (0.054 g, 0.076 mmol, 30%); m.p. 109–112°C; $[\alpha]_D^{20} = -5.7°$ (*c* 1.1, MeOH). TLC: R_F(B) 0.69. FIB-MS, *m*/*z* (+ive): 713 $(M)^+$; (−ive): 711 $(M)^-$.

Synthesis of Boc-Tyr(Me)(3′-CO$_2$H)-Gly-Gly-Phe-Leu-OH (11). The protected pentapeptide (**5**) (0.30 g, 0.40 mmol) was dissolved in MeOH (3.2 ml) and NaOH (1 M; 1.92 ml, 1.92 mmol) was added. The conditions of saponification and work-up were as described for (**10**). Recrystallizing the resulting solid mass from EtOAc yielded 0.14 g (0.19 mmol, 48%) of compound (**11**); m.p. 133–136°C; $[\alpha]_D^{20} = -3.6°$ (*c* 1.8, MeOH). TLC: R_F(A) 0.66. HPLC retention time run isocratically of 11.30 min was observed in 32% solvent B. FIB-MS, *m*/*z* (+ive): 714 $(M+H)^+$; (−ive): 712 $(M-H)^-$. Found: C, 57.49; H, 7.16; N, 8.98%. $C_{35}H_{47}N_5O_{11} \times H_2O$ (731.80) requires C, 57.44; H, 6.75; N, 9.57%.

Synthesis of Boc-Tyr(Me)(3′-(E)-CH=NOH)-Gly-Gly-Phe-Leu-OMe (12)

*TFA·H-Gly-Gly-Phe-Leu-OMe (**6**)*. Compound (**6**) was obtained from the protected tetrapeptide (**1**) (3.25 g, 6.41 mmol) and TFA (13 ml).

*Boc-Tyr(Me)(3′-(E)-CHNOH)-OH (**13**)*. Compound (**13**) (2.17 g, 6.41 mmol) was prepared according to Reference [9].

*Boc-Tyr(Me)(3′-(E)-CH=NOH)-Gly-Gly-Phe-Leu-OMe (**12**)*. A mixture of the tetrapeptide (**6**) dissolved in DMF (9 ml) and the protected amino acid (**13**) in DMF (9 ml) was cooled to 0°C and DPPA (1.38 ml, 6.41 mmol) was added followed by NEtM (1.62 ml, 12.84 mmol). The conditions of condensation and work-up were as described for compound **5**. Yield: 3.85 g (5.30 mmol, 83%); m.p. 123–128°C; $[\alpha]_D^{20} = -8.3°$ (*c* 1.6, MeOH). TLC: R_F(A) 0.87, R_F(B) 0.85, R_F(E) 0.64. HPLC retention time of 20.37 min was observed in a 35 min 35–60% B gradient. ^{1}H-NMR ($DMSOd_6$) δ (ppm): 0.81 and 0.89 (d, 6H, δ-CH_3Leu), 1.26 (s, 9H, CH_3Boc), 1.48–1.61 (m, 2H, β-CH_2Leu), 2.60–2.79 (m, 2H, β-CH_2Tyr), 2.86–3.05 (m, 2H, β-CH_2Phe), 3.59 (s, 3H, CO_2CH_3), 3.76 (s, 1H, OCH_3), 3.65–3.86 (m, 4H, CH_2Gly^2, CH_2Gly^3), 4.13 (m, 1H, α-CHTyr), 4.27 (m, 1H, α-CHLeu), 4.56 (m, 1H, α-CHPhe), 6.95 (s, 1H, NHBoc), 7.16–7.25 (m, 5H, aromatic protons), 7.56 (broad, 1H, NHPhe), 7.97 (broad, 1H, NHLeu), 8.05 (broad, 1H, $NHGly^3$), 8.36 (broad, 1H, $NHGly^2$), 8.24 (s, 1H, C**H**=NOH), 11.11 (s, 1H, CH=NO**H**). ^{13}C-NMR ($CDCl_3$) δ (ppm): 22.02 and 22.79 (Leu^5-δ), 24.82 (Leu^5-γ), 28.44 ($BocCH_3$), 39.01 (Tyr^1-β, Phe^4-β), 41.08 (Leu^5-β), 43.09 (Gly^3-α), 43.38 (Gly^2-α), 50.88 (Leu^5-α), 52.21 (CO_2**C**H_3), 54.44 (Tyr^1-α), 55.62 (Phe^4-α), 79.66 (Boc**C**(CH_3)), 111.03 (Tyr^1-ε(C-5)), 120.69 (Tyr^1-ε(C-3)), 126.71 (Tyr^1-γ), 128.33 (Phe^4-ξ), 127.44 (Phe^4-ε), 129.20 (Phe^4-δ), 129.60 (Tyr^1-δ), 136.77 (Phe^4-γ), 145.57 (CH=NOH), 155.85 (BocC=O), 156.41 (Tyr^1-ξ), 168.86 (Tyr^1C=O), 169.01 (Gly^3C=O), 171.45 (Gly^2C=O), 172.42 (Phe^4C=O), 173.14 (Leu^5C=O). FIB-MS, *m*/*z* (+ive): 727 $(M+H)^+$; (−ive): 725 $(M-H)^-$. Found: C, 59.68; H, 7.14; N, 11.35%. $C_{36}H_{50}N_6O$ (726.83), requires C, 59.49; H, 6.94; N, 11.56%.

Synthesis of Boc-Tyr(Me)(3′-(E)-CH=NOH)-Gly-Gly-Phe-Leu-NH$_2$ (14). The protected pentapeptide (**12**) (0.36 g, 0.50 mmol) was dissolved in MeOH (10 ml) and treated at −70°C (dry ice + acetone) with liquid ammonia (8 ml). The mixture was stirred at room temperature for 65 h. The solvent was removed under reduce pressure and the obtained solid was crystallized from EtOAc:EtOH (9:1). The yield of pure (**14**) was 0.12 g (0.17 mmol, 35%); m.p. 204–207°C; $[\alpha]_D^{20} = -4.1°$ (*c* 1.3, MeOH). TLC: R_F(A) 0.78. FIB-MS, *m*/*z* (+ive): 712 $(M)^+$; (−ive): 710 $(M-H)^-$. Found: C, 58.68; H, 7.06; N, 13.55%. $C_{35}H_{49}N_7O_9$ (711.81), requires C, 59.06; H, 6.94; N, 13.77%.

Synthesis of Boc-Tyr(Me)(3′-(E)-CH=NOH)-Gly-Gly-Phe-Leu-OH (15). The protected pentapeptide ester (**12**) (0.36 g, 0.5 mmol) was dissolved in MeOH (9.2 ml) and NaOH (1 M; 0.5 ml, 0.5 mmol) added. The saponification was allowed to proceed for 5 h at room temperature. The mixture was diluted with water (40 ml) and extracted with $CHCl_3$ (3 ×). The aqueous layer was cooled and HCl (0.94 M; 0.53 ml, 0.5 mmol) added. The crude **15** precipitated. Reprecipitation from EtOAc–petroleum ether provided 0.16 g (0.22 mmol, 45%); m.p. 124–127°C; $[\alpha]_D^{20} = -3.6$ (*c* 1.6, MeOH). TLC: R_F(A) 0.71. HPLC retention time of 25.70 min was observed in a 30 min 40–60% gradient. FIB-MS, *m*/*z* (+ive): 713 $(M+H)^+$; (−ive): 711 $(M-H)^-$. Found: C, 58.58; H, 6.86: N, 11.34%. $C_{35}H_{48}N_6O_{10}$ (712.80), requires C, 58.97; H, 6.79; N, 11.79%.

Synthesis of Boc-Tyr(Me)(3′-(E)-CH=NOMe)-Gly-Gly-Phe-Leu-OMe (16). The protected pentapeptide (**12**) (0.36 g, 0.5 mmol) was dissolved in 95% EtOH (3 ml) and an etheric solution of diazomethane (from *N*-nitroso-*N*-methylurea, 1.0 g, 10 mmol) was dropped in. After overnight at room temperature the solvents were evaporated and the residue redissolved in EtOAc, washed with $NaHCO_3$ (saturated), water and finally NaCl (saturated). The organic phase was dried with $MgSO_4$, filtered and evaporated; a yellowish powder was obtained (0.30 g, 0.40 mmol, 81%); m.p. 112–113°C; $[\alpha]_D^{20} = -7.9°$ (*c* 1.6, MeOH). TLC: R_F(A) 0.83; R_F(B) 0.85; R_F(E) 0.73. HPLC retention time of 17.58 min was observed in a 40–70% B gradient. ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.87 and 0.89 (d, 6H, δ-CH_3Leu), 1.41 (s, 9H, CH_3Boc), 1.57–1.59 (m, 2H, β-CH_2Leu), 2.85–3.05 (m, 2H, β-CH_2Tyr), 3.09–3.21 (m, 2H, β-CH_2Phe), 3.71 (s, 3H, CO_2CH_3), 3.76 (s, 1H, OCH_3), 3.91 (s, 1H, CH=HO**CH**$_3$), 4.02–4.20 (m, 4H, CH_2Gly^2, H_2Gly^3), 4.49 (m, 1H, α-CHTyr), 4.59 (m, 1H, α-CHLeu), 5.01 (m, 1H, α-CHPhe), 5.67 (s, 1H, NHBoc), 6.73–7.20 (m, 5H, aromatic protons), 7.31 (broad, 1H, NHPhe), 7.46 (broad, 1H, NHLeu), 7.56 (broad, 1H, NHGly^3), 7.75 (broad, 1H, NHGly^2). FIB-MS, *m*/*z* (+ive): 741 $(M+H)^+$; (−ive): 739 $(M-H)^-$. Found: C, 60.12; H, 7.13; N, 10.93%. $C_{37}H_{52}N_6O_{10}$ (740.85), requires: C, 59.99; H 7.07; N, 11.34%.

Synthesis of Boc-Tyr(Me)(3′-CH$_2$OBzl)-Gly-Gly-Phe-Leu-OMe (17)

TFA·H-Gly-Gly-Phe-Leu-OMe ***(6)***. Compound (**6**) was obtained from the protected tetrapeptide (**1**) (0.44 g, 0.87 mmol) and TFA (4 ml).

Boc-Tyr(Me)(3′-CH$_2$OBzl)-OH ***(18)***. Compound (**18**) was obtained from the DCHA salt of (**18**) prepared according to Reference [9]; (0.52 g, 0.87 mmol) in $CHCl_3$ (3 ml) and HCl/EtOH (6.8 M; 0.13 ml, 0.87 mmol HCl). Work-up was as described for compound (**6**).

Boc-Tyr(Me)(3′-CH$_2$OBzl)-Gly-Gly-Phe-Leu-OMe ***(17)***. The mixture of tetrapeptide (**6**) and the protected amino acid (**18**) dissolved in DMF (5 ml) was cooled to 0°C and DPPA (0.19 ml, 0.87 mmol) was added followed by NEtM (0.22 ml, 1.74 mmol). The conditions of condensation and work-up were as described for (**5**). The obtained crude product was dissolved in EtOAc:acetone (7:3) mixture, and purified on the column (2.3 × 15 cm) with Silica gel 60 (230–400 mesh, 100 g) by the flash chromatography method [16]. Pure (**17**) was obtained (0.30 g, 0.37 mmol, 43%); m.p. 126–130°C; $[\alpha]_D^{20} = -13.4°$ (*c* 1.5, MeOH). TLC: R_F(E) 0.86. HPLC retention time run isocratically of 16.4 min was observed in a 55% solvent B. FIB-MS, *m*/*z* (+ive): 804$(M+H)^+$; (−ive): 802 $(M-H)^-$. Found: C, 64.17; H, 7.17; N, 8.77%. $C_{43}H_{57}N_5O_{10}$(803.95) requires: C, 64.24; H, 7.15; N, 8.71%.

Synthesis of Boc-Tyr(Me)(3′-CH$_2$OH)-Gly-Gly-Phe-Leu-OMe (19)

TFA·H-Gly-Gly-Phe-Leu-OMe ***(6)***. Compound (**6**) was obtained from the protected tetrapeptide (**1**) (0.65 g, 1.28 mmol) and TFA (4 ml).

Boc-Tyr(Me)(3′-CH$_2$OH)-OH·H$_2$O ***(20)***. Compound (**20**) (0.44 g, 1.28 mmol) was obtained according to Reference [9].

Boc-Tyr(Me)(3′-CH$_2$OH)-Gly-Gly-Phe-Leu-OMe ***(19)***. The mixture of the tetrapeptide (**6**) and the protected amino acid (**20**) dissolved in DMF (8 ml) was cooled to 0°C and DPPA (0.28 ml, 1.28 mmol) was added followed by NEtM (0.32 ml, 2.56 mmol). The conditions of condensation and work-up were as described for (**5**). The obtained gummy product was poured with petroleum ether. After overnight in the refrigerator a yellowish solid without a sharp m.p. (90–100°C) was obtained. TLC: R_F(A) 0.80; R_F(B) 0.82; R_F(E) two spots 0.54 and 0.68. The resulting mixture of obtaining products were separated by the flash chromatography method [16] on the column (2.3 × 15 cm) with Silica gel 60 (230–400 mesh, 100 g) and eluted with EtOAc:acetone (7:3). The collected second part fractions contained product (**19**) (0.40 g, 0.56 mmol, 44%); m.p. 96–99°C. TLC: R_F(A) 0.80; R_F(B) 0.82; R_F(E) 0.54. The final purification of the product (70 mg) was accomplished by preparative HPLC. The pure product (31 mg) was obtained. $[\alpha]_D^{20} = -11.0°$ (*c* 1.3, MeOH). ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.86 and 0.88 (d, 6H, δ-CH_3Leu), 1.40 (s, 9H, CH_3Boc), 1.54–1.57 (m, 2H, β-CH_2Leu), 2.85–2.96 (m, 2H, β-CH_2Tyr), 3.10 (m, 2H, β-CH_2Phe), 3.68 (s, 3H, CO_2CH_3), 3.76 (s, 1H, OCH_3), 3.89–3.98 (m, 4H, CH_2Gly^2, CH_2Gly^3), 4.43 (m, 1H, α-CHTyr), 4.55 (m, 1H, α-CHLeu), 4.60 (s, 2H, **CH$_2$**OH), 4.87 (m, 1H, α-CHPhe), 5.64 (s, 1H, NHBoc), 6.71–7.23 (m, 5H, aromatic protons), 7.29–7.55 (m, 4H, NHPhe, NHLeu, NHGly^3, NHGly^2). ^{13}C-NMR ($CDCl_3$) δ (ppm): 22.66 and 23.37 (Leu^5-δ), 25.49 (Leu^5-γ), 29.06 (BocCH_3), 39.04 (Tyr^1-β), 39.57 (Phe^4-β), 41.83 (Leu^5-β), 43.71 (Gly^3-α), 43.87 (Gly^2-α), 51.62 (Leu^5-α), 52.86 (CO_2**C**H$_3$), 55.04 (Tyr^1-α), 55.98 (Phe^4-α), 80.66 (Boc**C**(CH_3)), 56.46 (OCH_3), 61.52 (CH_2OH), 110.86 (Tyr^1-ε(C-5)), 120.98 (Tyr^1-ε(C-3)), 127.43 (Tyr^1-γ), 129.01 (Phe^4-ξ), 129.25 (Phe^4-ε), 130.01 (Phe^4-δ), 130.19 (Tyr^1-δ), 137.33

(Phe^4-γ), 156.40 (BocC=O), 156.74 (Tyr^1-ξ), 169.27 (Tyr^{1}C=O), 169.94 (Gly^{3}C=O), 171.68 (Gly^{2}C=O), 173.07 (Phe^{4}C=O), 173.75 (Leu^{5}C=O). FIB-MS, m/z (+ive): 714 $(M+H)^+$; (−ive): 712 $(M-H)^-$. Found: C, 60.14; H, 7.33; N, 9.80%. $C_{36}H_{51}N_5O_{10}$ (713.84) requires: C, 60.58; H, 7.20; N, 9.81%.

Boc-Tyr(Me)(3′-CH$_2$OH)-Gly-Gly-Phe-Leu-OMe (9) obtained by deprotection of Boc-Tyr(Me)(3′-CH$_2$OBzl)-Gly-Gly-Phe-Leu-OMe (17). To a solution of the protected pentapeptide (**17**) (30 mg) in a mixture of MeOH:CH_3CO_2H:H_2O (10:1:1) (2 ml), 15 mg of 10% Pd/C was added. The mixture was shaken at room temperature and hydrogenated (under pressure of 500 mm H_2O) for 16 h. The catalyst was separated and the filtrate was evaporated. The residue (unreacted (**17**) and product (**19**)) was separated by preparative TLC (Silica gel 60) with AcOEt:EtOH (9:1) as a solvent. The identity of the product was confirmed by TLC, HPLC and FIB-MS procedures.

Synthesis of Boc-Tyr(Me)(3′-CH$_2$OH)-Gly-Gly-Phe-Leu-OH (21). The protected pentapeptide ester (**19**) (0.36 g, 0.5 mmol) was dissolved in MeOH (2 ml) and NaOH (1 M; 0.5 ml, 0.5 mmol) was added. The condition of saponification and work-up was as described for (**15**). Crude (**21**) (0.25 g, m.p. 114–116°C) was obtained. Reprecipitation from hot $CHCl_3$ and petroleum ether provided pure product (0.24 g, 0.34 mmol, 69%); m.p. 115–117°C; $[\alpha]_D^{20} = -3.1$) (*c* 1.2, MeOH). TLC: R_F(B) 0.75. HPLC retention time run isocratically of 5.85 was observed in a 50% solvent B. FIB-MS, m/z (+ive): 700 $(M+H)^+$; (−ive): 698 $(M-H)^-$. Found: C, 59.26; H, 7.56; N, 9.72%. $C_{35}H_{49}N_5O_{10} \times 0.5H_2O$ (708.81) requires: C, 59.31; H, 7.11; N, 9.88%.

Synthesis of Boc-Tyr(Me)(3′-CH$_2$OH)-Gly-Gly-Phe-Leu-NH$_2$ (22). The protected pentapeptide (**19**) (0.10 g, 0.14 mmol) was dissolved in NH_3/MeOH (10 M; 2 ml) and 25% NH_3/H_2O (2 ml) was added. The mixture was stirred overnight at room temperature. The solvents were removed under reduce pressure, and the obtained solid dried in a desiccator (P_2O_5, NaOH). The yield of pure (**22**) was 0.09 g (0.127 mmol, 90%); m.p. 122–124°C; $[\alpha]_D^{20} = -9.20$ (*c* 1.6, DMF). TLC: R_F(B) 0.71; R_F(F) 0.24. HPLC retention time run isocratically of 5.41 min was observed in a 50% solvent B. ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.92 and 0.99 (d, 6H, δ-CH_3Leu), 1.40 (s, 9H, CH_3Boc), 1.54–1.67 (m, 2H, β-CH_2Leu), 2.71–3.12 (m, 4H, β-CH_2Tyr, β-CH_2Phe), 3.82 (s, 1H, OCH_3), 3.75–3.92 (m, 4H, CH_2Gly^2, CH_2Gly^3), 4.30 (m, 1H, α-CHTyr), 4.54 (s, 2H, **CH$_2$**OH), 4.63 (m, 1H, α-CHLeu), 5.05 (m, 1H, α-CHPhe), ~6.5 (s, 1H, NHBoc), 6.89–7.38 (m, 5H, aromatic protons), 8.06–8.30 (m, 4H, NHPhe, NHLeu, NHGly^3, NHGly^2). FIB-MS m/z (+ive): 699 $(M+H)^+$; (−ive): 698 $(M)^-$. Found: C, 59.33; H, 7.85; N, 11.45%. $C_{35}H_{50}N_6O_9 \times 0.5H_2O$ (707.82) required: C, 59.39; H, 7.26; N, 11.87%.

Synthesis of unprotected pentapeptides. As a general method, the appropriate Boc-protected pentapeptides (**5**, **8**, **9**, **10**, **11**, **12**, **14**, **15**, **16**, **19**, **21** and **22**) (100 mg) were dissolved or suspended in $CHCl_3$ (1 ml) and TFA (1 ml) was added. After 1 h at room temperature the solvents was removed to the oil under reduced pressure at 20°C and the desirable product was precipitated by the addition of ether (10 ml) and gathered by centrifugation. Suspension in ether and centrifugation was repeated three times. The crude product was then dried *in vacuo* in a desiccator over P_2O_5 and NaOH. As a general procedure of purification, a portion of crude product (70 mg) was subjected to preparative RP HPLC. Fractions from the major product peak were pooled and lyophilized. Physicochemical properties of analogues of enkephalin are listed in Table 1.

RESULTS AND DISCUSSION

Chemical Results

Twelve new analogues of [Leu^5]-enkephalin were synthesized by standard methods in solution in order to investigate structural requirements necessary for binding of opiate receptors by nucleophilic groups containing a nonphenol hydroxyl group in an aromatic Tyr^1 ring. Consequently, *N*-Boc analogues of tyrosine were obtained containing the following groups at the 3′ position: –CO_2Me, –(*E*)-CH=NOH, –(*E*)-CH=NOBzl and –CH_2OH [9]. The phenol group of the amino acid was masked with a methyl substituent to prevent its interaction with opiate receptors. The following compounds were obtained: Boc-Tyr(Me)(3′-CO_2Me)-OH (**7**), Boc-Tyr(Me)(3′-(*E*)-CH=NOH)-OH (**13**), Boc-Tyr(Me)(3′-(*E*)-CH=NOBzl)-OH (**18**) and Boc-Tyr(Me)(3′-CH_2OH)-OH (**20**).

The masked analogues of tyrosine were condensed in solution with H-Gly-Gly-Phe-Leu-OMe (**6**) tetrapeptide, using DPPA as condensing agent. Tetrapeptide **6** was synthesized from two dipeptides described before: Boc-Gly-Gly-OH (**4**) [14] and H-Phe-Leu-OMe (**3**) [15]. The following analogues of leucine enkephalin were obtained: (**5**), (**12**), (**17**) and

Table 1 Physicochemical Properties of Synthetic [Leu^5]-ENK Analogues: Tyr(Me)(3′-R_1)-Gly-Gly-Phe-Leu-R_2

No.	Compound		FIB-MS		HPLC (R_t)		TLC (R_F)			
	R^1	R^2	$[M+H]^+$	$[M-H]^-$	35%B	30%B	A[a]	B[a]	C[a]	D[a]
I	$-CO_2Me$	–OMe	642	640	6.40	13.22	0.28	0.74	0.72	0.93
II	$-CO_2H$	–OH	614	612	2.52	3.73	0.15	0.55	0.63	0.64
III	$-CO_2Me$	$-NH_2$	627	625	2.70	4.20	0.14	0.55	0.61	0.73
IV	$-CO_2H$	$-NH_2$	613	611	2.14	2.92	0.14	0.63	0.63	0.82
V	$-CONH_2$	$-NH_2$	612	610	2.17	2.99	0.12	0.66	0.72	0.85
VI	–(E)CHNOH	–OMe	627	625	6.95	15.34	0.38	0.80	0.85	0.92
VII	–(*E*)CHNOH	–OH	613	611	3.46	6.21	0.36	0.71	0.74	0.87
VIII	–(*E*)CHNOH	$-NH_2$	612	610	2.85	4.64	0.32	0.74	0.69	0.90
IX	–(*E*)CHNOMe	–OMe	641	639	–	–	0.32	0.76	0.81	0.88
X	$-CH_2OH$	–OMe	614	612	4.69	9.65	0.32	0.73	0.70	0.85
XI	$-CH_2OH$	–OH	600	598	2.75	4.29	0.28	0.69	0.67	0.85
XII	$-CH_2OH$	$-NH_2$	599	597	2.33	3.32	0.23	0.71	0.65	0.87

[a] Solvent systems used for TLC are described in the section 'General Procedures'.

(**19**). The Boc-Tyr(Me)(3′-CH_2OH)-Gly-Gly-Phe-Leu-OMe (**19**) analogue was obtained also by reducing deprotection of (**17**). The above masked pentapeptides, after having been appropriately transformed in the side chain of Tyr^1, were used for obtaining other analogues discussed here. The monoamide (**8**) and diamide (**9**) were obtained from a diester protected pentapeptide (**5**) in the reaction with liquid ammonia. The resulting mixture, which contained the input compound and both products, was then separated on a chromatographic column. The assumed structure of the monoamide (**8**) was unequivocally determined by an analysis of the FIB-MS spectrum of positive and negative ions. Amides (**14**) and (**22**) were obtained from esters (**12**) and (**19**), respectively. The ammonolysis of ester (**12**) was carried out as previously, i.e. using liquid ammonia, while that of ester (**19**) was done with ammonia in a methanol-aqueous medium.

Boc pentapeptides containing two (**11**) or one free carboxyl group (**10**, **15** and **21**) were obtained during the saponification of respective esters (**5**, **8**, **12** and **19**) with sodium hydroxide in a methanol-aequous medium. The hydroxyl group of (*E*)-hydroxyiminomethyl substituent in (**12**) was masked with a methyl group, using an ether solution of diazomethane. The product was a diester (**16**). All obtained Boc protected analogues of [Leu^5]-ENK were unmasked with the aid of a TFA: chloroform (1:1) mixture. Crude analogues of leucine enkephalin were purified on a preparation column in the reversed phase of C_{18} HPLC. The purity of intermediate and end products was assayed chromatographically in TLC and analytical RP-HPLC. ^{1}H-NMR spectra were taken of masked tetrapeptide (**1**) and masked analogues (**5**, **12**, **16**, **19** and **22**) [9,17]. Also the ^{13}C-NMR spectra of peptides (**1**), (**12**) and (**19**) were shown [18,19]. The first- and second-order structures of peptides were unequivocally confirmed by an analysis of positive and negative ion mass spectra (FIB-MS) [20].

Physico-chemical characteristics of the products are presented in Table 1.

Biological Results

The introduction of electron-donor or electron-acceptor substituents to the aromatic ring at position 3′ of Tyr^1 enkephalins did not produce more active analogues. In this work, some substituents not used until now were added to the tyrosine aromatic ring at position 3′. The phenol group was masked with a methyl group. They were nucleophylic substituents: carboxyl ($-CO_2H$), hydroxyiminomethyl (–(*E*)-CH=NOH) and hydroxymethyl ($-CH_2OH$), as well as lipophilic groups (*E*)-methoxyiminomethyl (–(*E*)-CH=NOMe), carboxymethyl ($-CO_2Me$) and carboxyamide ($-CONH_2$). The aim of these substituents was to imitate Tyr^1 hydroxyl group.

None of the [Leu^5]-ENK analogues with masked hydroxyl group Tyr^1, and a substituent at position 3′ had any significant effect on the agonist activity in MVD at 10^{-6} M, i.e. no trace was found of a δ agonist action.

Table 2 GPI Assay and MVD Assay of Opioid Peptide Analogues[a]

No.	Compound	GPI				MVD		
		IC_{50} [b] (nM)	Ke of NTX[c] (nM)	Relative potency	(n)[d]	IC_{50} [b] (nM)	Ke of NTI[c] (nM)	(n)[d]
I	Tyr(Me)(3′-CO_2Me)-Gly-Gly-Phe-Leu-OMe	5470	4.4	8.07	3	–	–	2
II	Tyr(Me)(3′-CO_2H)-Gly-Gly-Phe-Leu-OH	– [e]	–	–	3	–	–	2
IX	Tyr(Me)(3′(*E*)CH=NOMe)-Gly-Gly-Phe-Leu-OMe	–	4.6	–	3	–	–	2
X	Tyr(Me)(3′-CH_2OH)-Gly-Gly-Phe-Leu-OMe	7940	0.87	5.56	3	–	–	2
XI	Tyr(Me)(3′-CH_2OH)-Gly-Gly-Phe-Leu-OH	–	–	–	3	–	–	2
XII	Tyr(Me)(3′-CH_2OH)-Gly-Gly-Phe-Leu-NH_2	–	–	–	4	–	–	2
	[Leu^5]-ENK	441.5	0.60	100	4	15.3	0.21	4

[a] The results of Rónai.
[b] Geometric mean and 95% confidence interval.
[c] Equilibrium dissociation constant; geometric mean and 95% confidence interval.
[d] The number of determination.
[e] –, indicates that the peptide has no significant agonist or antagonist activity.

A definite agonist activity in GPI was shown by analogues I, VI, VII, VIII and X. Antagonism with naltrexone gave an expected value of Ke (0.5–1.0 nM) against compound X while the value against Iwas higher than expected (Table 2). Agonist activity of analogues VI, VII and VIII against μ receptors in GPI was naloxone-reversible.

As a consequence of the results presented, only the (*E*)-hydroxyiminomethyl group can be regarded as phenol-like in Tyr^1 of analogues under investigation. Its acidity ($pK_a \sim 10.9$) is comparable with that of tyrosine phenol group ($pK_a \sim 10.1$) and the masking of its hydroxyl group by methylation totally abolished the agonist activity of analogue IX against μ receptors. Therefore, it shows an affinity to the hydroxyl group of a tyramine system of morphine, codeine and other opiate alkaloids [5]. Enkephalin II with a 3′-carboxyl group ($pK_a \sim 4.1$) turned out to be inactive. Another enkephalin, X, with a 3′-hydroxymethyl group ($pK_a \sim 18$) has small activity.

In the obtained enkephalins, an increase in lipophilic character at the *C*-terminal (from acid, amide to ester) results in a stronger affinity to the receptors in GPI (analogues VII, VIII and VI, Table 3). Also methyl esters I and X (Table 2) are active while their amides and acids are not. The result is surprising since usually amides are more active than esters. It is the case, for instance, in [Leu^5]-enkephalins [21], 'small dermorphines' [22] and even [D-Ala^2, Leu^5]-ENK conjugated at the *C*-terminus with a large lipophilic adamantate moiety [23]. It is suspected that factors important here may be the following: special conformation of enkephalin analogues with masked phenol group Tyr^1 different

Table 3 GPI Assay and MVD Assay of Opioid Peptide Analogues[a]

No.	Compound	GPI		MVD	
		IC_{50} [b] (nM)	Relative potency	IC_{50} [b] (nM)	Relative potency
III	Tyr(Me)(3′-CO_2Me)-Gly-Gly-Phe-Leu-NH_2	>10 000	<2.46	>10 000	<0.114
IV	Tyr(Me)(3′-CO_2H)-Gly-Gly-Phe-Leu-NH_2	>10 000	<2.46	>10 000	<0.114
V	Tyr(Me)(3′-$CONH_2$)-Gly-Gly-Phe-Leu-NH_2	>10 000	<2.46	>10 000	<0.114
VI	Tyr(Me)(3′-(*E*)-CH=NOH)-Gly-Gly-Phe-Leu-OMe	939 ± 197	26.2 ± 5.5	>10 000	<0.114
VII	Tyr(Me)(3′-(*E*)-CH=NOH)-Gly-Gly-Phe-Leu-OH	8450 ± 2700	2.91 ± 0.93	>10 000	<0.114
VIII	Tyr(Me)(3′-(*E*)-CH=NOH)-Gly-Gly-Phe-Leu-NH_2	5520 ± 310	4.46 ± 0.25	>10 000	<0.114
	[Leu^5]-ENK	246 ± 39	100	11.4 ± 1.1	100

[a] The results of Schiller.
[b] Mean of three determinations ± S.E.M.

from that of enkephalins themselves, modification of physico-chemical properties, such as lipophility as well as a different capacity of reaching a receptor position in tissues.

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