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Synthesis and biological activity of branched enkephalin analogues

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Abstract – The synthesis and biological activity of a new type of enkephalin analogs are reported. A series of branched pentapeptides of the enkephalin sequence with replacement of 2-glycine by D-ornithine and branching of the peptide chain in position 2 by attachment of proline, leucine, asparagine or methionine residues to the δ-amino group of D-ornithine were synthesized by classical solution methodology. Analgesic activity of the new analogs was assayed by the 'tail pinch' method following intracisternal and intravenous administrations to mice. They showed higher analgesic potency and longer duration of action as compared to linear and cyclic pentapeptides with the same amino acid composition. The activity determined in the GPI and MVD bioassays, and in a binding assay, revealed the preference of the branched analogs for the μ-type of opioid receptor over the δ-type. These results raise the possibility to synthesize enkephalin analogs with high analgesic potency and opiate receptor selectivity by varying the chemical character and length of the side chain in the 2-position. © Elsevier, Paris

enkephalin analog / peptide synthesis / analgesic effect / GPI and MVD bioassay / binding assay

1. Introduction

Soon after the discovery of endogenous opioid peptides [1] extensive studies have been performed on structurally modified enkephalins to study structure–activity relationships of peptide opioids. A variety of analogs have been created using different theoretical concepts. Comprehensive information on structures and activity profiles of opioid peptide analogues is contained in numerous review articles (see for example [2, 3]).

This paper describes the synthesis, characterization and opioid activity of new enkephalin analogs with a branched peptide chain in position 2. The new enkephalin pentapeptides were designed [4, 5] on the basis of the tetrapeptide Tyr-D-Orn-Gly-Phe to which proline, leucine, asparagine or methionine residues were attached to the δ-amino group of D-ornithine. In order to evaluate the effect of the branched peptide chains on opioid activity the potencies of the branched analogs with those of the corresponding linear enkephalin analogs where proline, leucine, asparagine or methionine residues are located in position 5 were compared. Since cyclization of enkephalins via side chains (type 2 → 5) has been studied earlier [6, 7] the corresponding cyclic enkephalin analogs obtained by cyclization of the branched analogs, i.e. formation of a peptide chain between the amino group of the amino acid in the side chain and the C-terminal carboxyl group of peptide was also prepared and potencies were compared with those of the branched analogs.

2. Chemistry

The peptides were synthesized by classical methods of peptide chemistry in solution. Synthesis of enkephalin pentapeptides with branched and cyclic structure of peptide chain was performed stepwise using p-nitro-, pentachloro- and pentafluorophenyl esters of

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Abbreviations: DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazol; OPcp, pentachlorophenyl; OPfp, pentafluorophenyl; ONp, *p*-nitrophenyl; RP-HPLC, reversed-phase high performance liquid chromatography; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; DCM, dichloromethane; NH_4OBt, 1-hydroxybenzotriazol ammonium salt; Et_3N, triethylamine; DPPA, diphenylphosphorylazide; HONB, *N*-hydroxy-5-norbornene-2,3-dicarboximide; DCU, dicyclohexyl urea; i.cist., intracisternal; i.v., intravenous; GPI, guinea pig ileum; MVD, mouse vas deferens; OR, opiate receptor; Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl.

Boc-amino acids (*figure 1*). The δ-amino group of D-ornithine was protected by a Z-group. In the case of tyrosine, the Boc group was used for protection of both amino- and hydroxyl groups. This enables removal of protective groups from the synthesized peptide in one step and considerably simplifies the synthesis of peptides. C-terminal carboxylic groups were protected by salt formation. It is necessary to note that to obtain cyclic compounds branched but not linear peptides were used.

Coupling of Boc-Gly-OPcp with phenylalanine sodium salt followed by attachment of Boc-D-Orn(Z)-OPfp provided the protected tripeptide **19**. Coupling with Boc-Tyr(Boc)-OPfp followed by hydrogenolysis afforded tetrapeptide **20a**. The product **20a** was divided into 4 parts and the corresponding fifth amino acid residue was attached to δ-amino group of D-ornithine using activated esters of Boc-protected proline, leucine, asparagine or methionine. After purification on a silica gel column protected pentapeptides **21–24** were obtained. After deprotection (Pd/H_2, TFA), purification (HPLC) and lyophilization branched analogs **1**, **6**, **10**, **13** with a free carboxylic group were obtained.

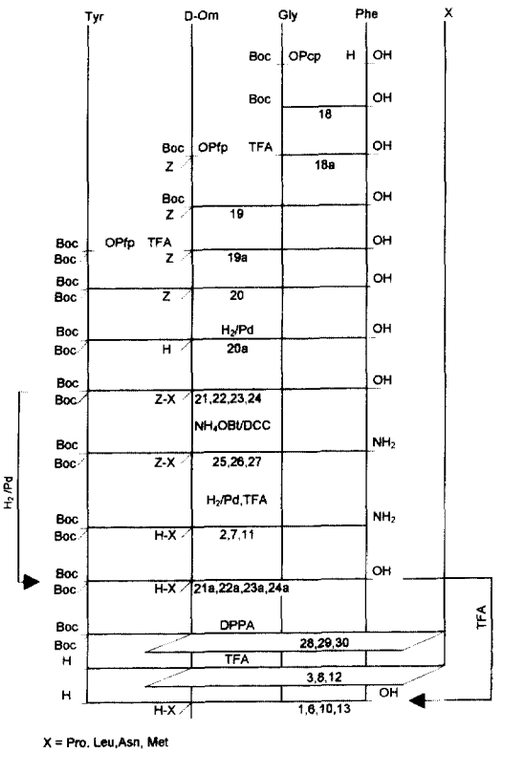

Figure 1. Synthesis of the branched and cyclic enkephalin analogues.

It is known that application of the catalytic hydrogenation method for removal of benzyl type protective groups is limited for the peptides with sulphur-containing amino acids. The deprotections of sulphur-containing peptides **13–14** were done successfully by catalytic hydragenation in the presence of cyclohexylamine (4 equiv.), as proposed by Medzigradsky [8].

Protected pentapeptides **21–23** were transformed into amides **25–27** by the DCC-method using NH_4OBt [9]. After removal of Boc- and Z-groups and purification, HPLC-pure branched pentapeptide amides **2**, **7**, **11** were obtained.

Partially protected pentapeptides **21a–23a** were cyclized by DPPA in DMF solution. Cyclization was performed with DPPA at high dilution (less than 1 mmol/L) in order to minimize competitive reactions [10]. It should be noted that we failed to isolate the reaction product using the 'F' complex for cyclization. The crude cyclic products **28–30** were treated by 50% TFA in DCM at room temperature (30 min) to remove Boc-groups. After semipreparative RP-HPLC, completely homogeneous cyclic peptides **3**, **8**, **12** were obtained. The structure of cyclopeptides were confirmed by mass-spectrometric determination of molecular weight.

Enkephalin analogs with linear structure were synthesized by a [3 + 2] fragment condensation (as shown in *figure 2*). The risk of partial racemization in the coupling steps was minimized by fragment coupling with the achiral glycine residue in the peptide **37** and using a weak base-DIEA.

Coupling of Boc-D-Orn(Z)-OPfp with glycine sodium salt, followed by attachment of Boc-Tyr(Boc)-OPfp provided the protected N-terminal tripeptide **37**. Coupling of Boc-Phe-OPfp with the sodium salt of proline, leucine or methionine afforded C-terminal dipeptides **32–34** which were purified on a silica gel column. Fragment **37** and dipeptides **32a**, **34a–36a** were coupled by the method of activated esters to provide the protected pentapeptides **39–42**, respectively. After deprotection (Pd/H_2,TFA) crude peptides were purified by RP-HPLC. The appropriate peaks containing the main products were collected, re-examined by analytical RP-HPLC and lyophilized. The analytical data of protected and unprotected peptides are shown in *tables I* and *II*.

3. Biological activity and binding property

The pharmacological profile of the compounds was tested in vivo by the mouse 'tail-pinch' assay following intracisternal and intravenous administration and

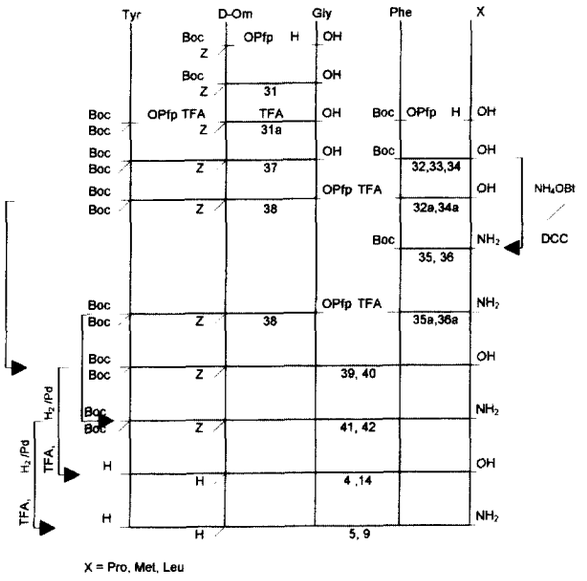

Figure 2. Synthesis of the enkephalin analogues with linear structure.

in vitro in isolated organ preparations the guinea pig ileum (GPI) and mouse vas deferens (MVD). In the GPI opioid effects are primarily mediated by μ-receptors and partly by κ-receptors. The MVD assay is generally taken as being representative for the δ-type of OR even though it also contains μ- and κ-receptors. Activity at the cellular level was evaluated by the ability of the peptides to inhibit the binding in rat brain membranes of [^{3}H]Naloxone as primarily a μ-ligand and [^{3}H]DADLE as a δ-ligand for OR. The results in comparison with [Leu^5]-enkephalin, morphine and corresponding linear and cyclic peptides with the same amino acid composition are shown in *tables III–V*.

4. Results and discussion

The study of analgesic activity by the 'tail pinch' method after intracisternal administration showed that enkephalin analogs with a branched structure possess a pronounced analgesic effect (*table III*) and that they are more active than the respective analogs with linear or cyclic structure.

The branched enkephalin analogs **1**, **6** where proline or leucine residues are attached to the δ-amino group of D-ornithine and where the carboxyl group is not modified were 1.5–10 times more potent than linear peptides **4**, **5**, **9** containing those amino acid residues in position 5.

The Met-containing branched analog **13** obtained by attaching a L-methionine residue to the δ-amino group of D-ornithine was 90 times more potent than the corresponding linear peptide **14**. Analog **13** turned out to be the most active among the branched peptides, 4350 times more potent than [Leu^5]-enkephalin and 27 times more potent than morphine under these conditions (*table III*).

The amidation of the C-terminal carboxyl group increased the activity of branched analogs. These compounds **2**, **7**, **11** were 2–4 times more active than those without carboxyl modification **1**, **6**, **10**. For example, [N$^\delta$-Pro-D-Orn^2,desLeu^5]-enkephalin amide **7** was 7 times more active than morphine and 1000 times more active than [Leu^5]-enkephalin.

Cyclic pentapeptides **3**, **8**, **12**, with a peptide bond between the δ-group of D-ornithine in position 2 and the C-terminal carboxyl group (type 2 → 5 cyclization) demonstrated marked analgesic activity exceeding that of morphine. However, the analgesic effect of cyclopeptides containing a proline residue (**3**) or leucine residue (**8**) in position 5 was weaker than the effect of branched pentapeptide amides **2**, **7**, and practically did not exceed the activity of branched pentapeptides with a free carboxyl group (**1**, **6**). The Asn-containing cyclopeptide **12** seems to be an exception, since it was somewhat more potent than the branched analog with free carboxyl **10**, but nevertheless had considerably shorter duration of action. The branched compound **10** had a duration of action exceeding that of the cyclic compound **12** by 3 times.

The Asn-containing branched pentapeptide amide **11** revealed an analgesic potency practically similar to that of the respective cyclopeptide **12**, and also showed a longer duration of action.

Unlike linear compounds the new branched analogs were active after i.v. administration with an analgesic effect 18–87% that of morphine (*table III*), with the exception of compound **7**.

In the series of branched enkephalin analogs **1**, **6**, **10**, **13** analgesic potency after intravenous administration depended on the nature of amino acid residue attached to the δ-amino group of D-ornithine. While the N$^\delta$-Asn-containing analog **10** displayed more than a half of the analgesic effect of morphine the N$^\delta$-Leu, N$^\delta$-Met and N$^\delta$-Pro-containing enkephalin analogs **6**, **13**, **1** possessed weaker analgesic activity (18–25%). Such difference was not observed after intracisternal administration, since analogs **1**, **6**, **10** possessed practically similar activity. Evidently, pharmacokinetic properties of the molecules come into force, conditioned by various factors, for instance, lipophilicity. The activity of the N$^\delta$-Asn-containing branched analog **10** may be explained by the polar character of the N-terminal part of the molecule due to the aspara-

Table I. Analytical data for protected peptides.

Nc	Compound	Yield (%)	$[\alpha]_D^{22}$(°) DMF, (c)	TLC[a] R_f (A)	R_f (B)	R_f (Other systems)
18	Boc-Gly-Phe	88	+15.7(1)	0.73[b]		0.80 D
19	Boc-D-Orn(Z)-Gly-Phe	67	+3.7(2.04)	0.65[b]	0.72[b]	
20	Boc-Tyr(Boc)-D-Orn(Z)-Gly-Phe	55	+6.3(1)	0.75[b]	0.55[b]	
21	Boc-Tyr(Boc)-D-Orn(Z-Pro)-Gly-Phe	68	-17.0(1)			0.79 D 0.65 E
22	Boc-Tyr(Boc)-D-Orn(Z-Leu)-Gly-Phe	74	-5.0(1)	0.58		0.84 D
23	Boc-Tyr(Boc)-D-Orn(Z-Asn)-Gly-Phe	70	+8.2(1)	0.72		0.83 D
24	Boc-Tyr(Boc)-D-Orn(Boc-Met)-Gly-Phe	30	-0.8(1)	0.55[b]	0.76[b]	
25	Boc-Tyr(Boc)-D-Orn(Z-Pro)-Gly-Phe-NH_2	91		0.48		0.72 E
26	Boc-Tyr(Boc)-D-Orn(Z-Leu)-Gly-Phe-NH_2	75	-10.0(1)	0.68	0.79	
27	Boc-Tyr(Boc)-D-Orn(Z-Asn)-Gly-Phe-NH_2	78		0.53	0.62	
31	Boc-D-Orn(Z)-Gly	80	-41.0(1)	0.75		0.73 E
32	Boc-Phe-Pro	84	-46.8(0.5)c	0.73		0.78 E
33	Boc-Phe-Leu	78	-8.8(1)	0.73	0.71	
34	Boc-Phe-Met	71	-10.2(1)	0.70	0.72	
35	Boc-Phe-Pro-NH_2	72		0.50		0.80 E
36	Boc-Phe-Leu-NH_2	89	-21.0(1)	0.45[b]	0.88[b]	
37	Boc-Tyr(Boc)-D-Orn(Z)-Gly	82		0.68		0.75 E
39	Boc-Tyr(Boc)-D-Orn(Z)-Gly-Phe-Pro	72	-32.5(0.20)	0.66[b]	0.45	
40	Boc-Tyr(Boc)-D-Orn(Z)-Gly-Phe-Met	57	-1.2(0.27)	0.62	0.52	
41	Boc-Tyr(Boc)-D-Orn(Z)-Gly-Phe-Pro-NH_2	64	-14.9(0.17)	0.72[b]	0.53	
42	Boc-Tyr(Boc)-D-Orn(Z)-Gly-Phe-Leu-NH_2	60	-3.6(1)		0.90	0.83 D

[a]TLC was made on 'Merck' plates; [b]TLC was made on 'Silufol' plates; [c]in EtOH.

gine residue, which, evidently, entails the increase in analgesic activity [11].

It should be mentioned that amidation of the C-terminal carboxyl group of branched enkephalin analogs caused a different effect on the analgesic potency following i.v. administration depending on the nature of the N^{δ}-substituting amino acid residue. Amidation of the N^{δ}-Pro-containing analog **1** increased analgesia from 25 to 69% against morphine, while in the case of the N^{δ}-Leu-containing analog **6** amidation considerably weakened the effect (at a dose of 50 mg/kg the effect was 33%, a subsequent dose increase showed no analgesia). Amidation of the N^{δ}-Asn-containing analog **10** also increased analgesia from 57 to 87% as compared with morphine (*table III*).

The potency of the cyclic enkephalin analogs also depended on the nature of the N^{δ}-substituted amino acid residue. Cyclic analogs containing proline **3** and leucine **8** residues in position 5 showed no activity after i.v. administration, whereas analogs containing an asparagine residue **12** produced pronounced analgesia which accounted for a half of the analgesic effect of morphine (57%).

Most of the synthesized analogs produced an analgesic effect of pronounced duration that varied among 15 to 180 min in different analogs, determined at

Table II. Analytical data of enkephalin analogues.

No	Compound	$[a]_D^{22}$(°) 0.2M AcOH (c)	Amino acid analysis					TLC[a]			HPLC	
			Tyr	Gly	Phe	D-Orn	Pro Leu Asn Met	R_f(B)	R_f(D)	R_f(C) R_f(E)	Mobile phase composition	(k')[c]
1	Tyr-D-Orn-Gly-Phe Pro	+41.3 (1)	0.93	1.00	1.10	0.93	1.03	0.30	0.74		10:90	7.5
2	Tyr-D-Orn-Gly-Phe-NH_2 Pro	+20 (1)	0.95	1.00	0.98	0.77	1.21	0.22	0.53		10:90	1.6
3	Tyr-D-Orn-Gly-Phe-Pro	+64 (1)	0.91	1.00	0.91	0.95	0.98	0.66	0.70		20:80	1.5
4	Tyr-D-Orn-Gly-Phe-Pro	+23.7 (0.67)	1.03	1.00	1.13	1.01	0.94	0.44	0.64	0.18(C)	10:90	1
5	Tyr-D-Orn-Gly-Phe-Pro-NH_2	+25 (0.34)	0.83	1.00	1.01	0.87	0.84	0.52		0.51(E)	12:88	2.9
6	Tyr-D-Orn-Gly-Phe Leu	+52.4 (1)	0.95	1.00	1.26	0.98	1.05	0.60[b]	0.50		10:90	5
7	Tyr-D-Orn-Gly-Phe-NH_2 Leu	+32.6 (1)	0.87	1.00	0.97	0.86	1.03	0.74	0.42		15:85	3.6
8	Tyr-D-Orn-Gly-Phe-Leu	+70.3 (1)	0.91	1.00	1.02	0.98	0.92	0.76	0.82		40:60	1
9	Tyr-D-Orn-Gly-Phe-Leu-NH_2	+42.3 (1;EtOH)	0.89	1.00	1.02	0.81	0.93	0.64	0.78		14:86	8.1
10	Tyr-D-Orn-Gly-Phe Asn	+37 (1)	0.89	1.00	1.13	0.97	1.09	0.42[b]	0.18[b]		10:90	2
11	Tyr-D-Orn-Gly-Phe-NH_2 Asn	+53.8 (0.24)	0.98	1.00	0.92	1.07	0.83	0.52	0.48	0.12[b](C)	6:94	4
12	Tyr-D-Orn-Gly-Phe-Asn	+36.2 (0.5;AcOH)	0.93	1.00	0.91	0.95	0.98	0.62	0.78		30:70	0.5
13	Tyr-D-Orn-Gly-Phe Met	+72 (1;AcOH)	1.06	1.21	0.94	1.12	1.00	0.46	0.28		8:92	4.2
14	Tyr-D-Orn-Gly-Phe-Met		0.81	1.00	0.99	1.02	0.73		0.32	0.21[b](C)	10:90	5.2

[a]Thin layer chromatography (TLC) was carried out on Merck precoated 0.25 mm analytical silica-gel plates; [b]TLC was carried out on Silufol plates; [c]capacity factor.

ED_{60-80} and with both administration routes. Some analogs exhibited a duration of analgesic action comparable with that of morphine (60 min). The N^{δ}-Asn-containing branched analog with a free carboxyl group **10** manifested the longest duration, 180 min after i.cist. administration, three times longer than that of morphine under the same conditions.

The pharmacological effects of all the peptides were significantly antagonized by naloxone at a dose of 1.0 mg/kg given subcutaneously 5 min prior to i.cist. administration of these substances, thus confirming that they accomplish their action via an opiate receptor.

The results of the GPI and MVD bioassays are presented in *table IV*. In GPI assays (usually considered as predominantly representing μ-type of OR) the branched analogs with a free carboxyl group (**1**, **6**, **10**, **13**) and analogs with C-terminal carboxamide group (**7**, **11**) demonstrated an activity comparable with that of [Leu^5]-enkephalin. The pentapeptide amide **11** turned out to be the most active compound. Its μ-affinity exceeded the affinity of [Leu^5]-enkephalin by 3 times and was comparable to that of morphine while compound **2** showed lower affinity than that of [Leu^5]-enkephalin. In MVD assays (predominantly reflecting activity at the δ-type of OR) these analogs (**1**, **6**, **10**, **13** and **2**, **7**, **11**) revealed a rather low activity within the range of 0.9–11.2% of the activity of [Leu^5]-enkephalin. Consequently, most of the new compounds (**1**, **6**, **7**, **10**, **11**) were more μ-selective [ratio IC_{50}(MVD)/IC_{50}(GPI) = 1.27–5.79] than [Leu^5]-enkephalin [ratio IC_{50}(MVD)/IC_{50}(GPI) = 0.07]. A

Table III. Analgetic activity of enkephalin analogues ('tail pinch' method in mice).

No.	Enkephalin analogues	Intracisternal administration					Intravenous administration	
					Relative activity			
		ED_{50} nmol/animal	Peak time[a] min	Duration[a] min	[Leu^5]-enkephalin=1	Morphine=1	Duration[a] min	Relative activity Morphine=1
1	Tyr-D-Orn-Gly-Phe-Pro	0.8 (0.5-1.3)	5-30	60	220	1.5	60	0.25
2	Tyr-D-Orn-Gly-Phe-NH_2-Pro	0.2 (0.09-0.44)	5-30	90	870	6	60	0.69
3	Tyr-D-Orn-Gly-Phe-Pro	0.7 (0.3-2.2)	5	15	250	1.7	-	-[c]
4	Tyr-D-Orn-Gly-Phe-Pro	1.2 (0.8-1.6)	15	60	145	1	-	-
5	Tyr-D-Orn-Gly-Phe-Pro-NH_2	2,9 (0.6-5.2)	15	90	59	0, 4	-	-
6	Tyr-D-Orn-Gly-Phe-Leu	0.6 (0.4-1.0)	5-15	30	290	2	30	0.18
7	Tyr-D-Orn-Gly-Phe-NH_2-Leu	0.17 (0.09-0.32)	5	15	1024	7	-	[d]
8	Tyr-D-Orn-Gly-Phe-Leu	0.5 (0.2-1.1)	5-15	60	350	2,4	-	-
9	Tyr-D-Orn-Gly-Phe-Leu-NH_2	6.0 (0.1-11.0)	5-15	15[b]	29	0, 2	-	-
10	Tyr-D-Orn-Gly-Phe-Asn	0.6 (0.3-1.8)	5-15	180	290	2	30	0, 57
11	Tyr-D-Orn-Gly-Phe-NH_2-Asn	0.29 (0.02-5.60)	5-30	90[b]	600	4	45-60[b]	0, 87
12	Tyr-D-Orn-Gly-Phe-Asn	0.30 (0.20-0.50)	5	60	580	4	30	0, 60
13	Tyr-D-Orn-Gly-Phe-Met	0.04 (0.02-0.11)	5	15	4350	27	30	0, 24
14	Tyr-D-Orn-Gly-Phe-Met	3.8 (1.4-10.3)	5		46	0, 3	-	
15	Tyr-Gly-Gly-Phe-Leu	174 (102-281)	5	15	1	0.007	-	-
16	Tyr-Gly-Gly-Phe-Met	154 (90-260)	5-15	15	1 1	0.008	-	-
17	Morphine	1.2 (0.6-2.2)	5-15	60	145	1	60	1

[a]With the ED_{60-80}; [b]with the ED_{50}; [c]substance lacks any activity; [d]in the dose of 50 mg/kg the effect is 33%.

comparison of the branched analogs **1**, **7**, **13** with the corresponding linear analogs **4**, **9**, **14** revealed that the branching of the peptide chain enhanced potency on the GPI and MVD. The N^{δ}-Pro-containing branched analog **2** was more potent on MVD, but slightly less potent on GPI than the corresponding linear analog **5**.

As expected, amidation of the C-terminal carboxyl group of branched analogs of enkephalin augmented the affinity of the branched pentapeptide amides **7**, **11** to the μ- and δ-type of OR ,except in compound **2**.

Leu- and Asn-containing branched pentapeptide amides **7**, **11** manifested an increase in the affinity to both μ- and δ-types of OR by 2–9 times, preserving a preference for the μ-receptor over the δ-receptor, as compared with compounds **6**, **10** with a free carboxyl group.

Table IV. Inhibitory potencies (IC_{50}) of enkephalin analogues in guinea-pig ileum (GPI) and mouse vas deferens (MVD) assays.

No.	Enkephalin analogues	IC_{50} (nM)		Relative activity [Leu^5]-enkephalin=100%		IC_{50} (MVD) / IC_{50} (GPI)
		GPI	MVD	GPI r.a.	MVD r.a.	
1	Tyr-D-Orn-Gly-Phe-Pro	205 ± 29	620 ± 87	139	3,2	3,02
2	Tyr-D-Orn-Gly-Phe-NH$_2$-Pro	1290 ± 270	413 ± 63	22	4,8	0,32
3	Tyr-D-Orn-Gly-Phe-Pro	8860 ± 1700	29000 ± 5000	3,2	0,07	3,4
4	Tyr-D-Orn-Gly-Phe-Pro	19700 ± 6300	8400 ± 750	1,4	0,2	0,42
5	Tyr-D-Orn-Gly-Phe-Pro-NH$_2$	557 ± 139	1760 ± 380	51	1,1	3,16
6	Tyr-D-Orn-Gly-Phe-Leu	364 ± 57	680 ± 90	78	2,9	1,87
7	Tyr-D-Orn-Gly-Phe-NH$_2$-Leu	140 ± 39	178 ± 28	203	11,2	1,27
8	Tyr-D-Orn-Gly-Phe-Leu	354 ± 62	5000 ± 300	80	0,4	14,1
9	Tyr-D-Orn-Gly-Phe-Leu-NH$_2$	700 ± 125	990 ± 270	40	2	1,41
10	Tyr-D-Orn-Gly-Phe-Asn	380 ± 34	2200 ± 540	75	0,9	5,79
11	Tyr-D-Orn-Gly-Phe-NH$_2$-Asn	82 ± 21	238 ± 27	347	8,4	2,9
12	Tyr-D-Orn-Gly-Phe-Asn	200 ± 40	160 ± 23	142	12,5	0,8
13	Tyr-D-Orn-Gly-Phe-Met	340 ± 46	1510 ± 290	84	1,3	4,4
14	Tyr-D-Orn-Gly-Phe-Met	11900 ± 1400	1670 ± 500	2,4	1,2	0,14
15	Tyr-Gly-Gly-Phe-Leu	285 ± 78	20 ± 1	100	100	0,07
16	Tyr-Gly-Gly-Phe-Met	600 ± 150	13 ± 2	47	153	0,02
17	Morphine	78 ± 19	579 ± 244	365	3,4	7,42

The highest potency was displayed by the Asn-containing branched pentapeptide amide **11** which revealed an affinity and selectivity for the μ-type of OR similar to that of morphine.

A comparison of the branched analog with the corresponding cyclic analogs showed that in the group of Pro-containing peptides, the branching of peptide chain enhanced the potency on the GPI (compound **1**), while cyclization decreased the affinity to the μ- and δ-receptors to extremely low values (compound **3**). In the group of Asn- and Leu-containing peptides the differences were not so large, however, the branched pentapeptide amides **7**, **11** demonstrated a higher affinity to the μ- and δ-receptors than the respective cyclopeptides **8**, **12**.

In the binding assay (*table V*) based on displacement of the [^{3}H]Naloxone and [^{3}H]DADLE from rat brain membranes, the branched enkephalin analogs showed the same rank of potency, in general, as in the GPI and MVD assays. In comparison with the cyclic and linear analogs in the group of Pro-containing peptides, increased affinity to the μ-type of OR was observed with the branched pentapeptides **1**, **2** and they were both more potent than [Leu^5]-enkephalin. The N$^\delta$-Leu- and N$^\delta$-Asn-containing branched pentapeptide amides (**7**, **11**) also showed increased affinity

Table V. Opioid receptor binding affinities of enkephalin analogues.

No	Compound	[^{3}H]-Naloxone		[^{3}H]-DADLE		IC_{50} [^{3}H]-DADLE
		IC_{50} nM	Relative potency, %	IC_{50} nM	Relative potency, %	IC_{50} [^{3}H]-Naloxone
1	Tyr-D-Orn-Gly-Phe Pro	8.47± 1.42	122	144.6 ± 31.0	8.1	17.1
2	Tyr-D-Orn-Gly-Phe-NH_2 Pro	5.89 ± 1.28	176	127.10 ± 23.8	9.2	21.6
3	Tyr-D-Orn-Gly-Phe-Pro	1271 ± 377	0.81			
4	Tyr-D-Orn-Gly-Phe-Pro	2657 ± 524	0.05	277 ± 32	4.2	0,1
6	Tyr-D-Orn-Gly-Phe Leu	11.47 ± 2.11	90	13.29 ± 2.29	88	1.2
7	Tyr-D-Orn-Gly-Phe-NH_2 Leu	3.00 ± 0.49	345	82.43 ± 18.29	14.2	27.5
8	Tyr-D-Orn-Gly-Phe-Leu	58.34 ± 11.28	17			
10	Tyr-D-Orn-Gly-Phe Asn	55.41 ± 12.22	18	327.10 ± 53.87	3.6	5.9
11	Tyr-D-Orn-Gly-Phe-NH_2 Asn	17.96 ± 4.84	58	211.50 ± 24.79	5.5	11.8
12	Tyr-D-Orn-Gly-Phe-Asn	30.52 ± 9.80	34			
13	Tyr-D-Orn-Gly-Phe Met	97.31 ± 16.82	10	130.2 ± 26.4	9.0	1.3
15	Tyr-Gly-Gly-Phe-Leu	10.35 ± 1.57	100	11.7 ± 1.7	100	1.1
17	Morphine	10.25 ± 1.45	100			

for the μ-opioid sites in the binding assay as compared with cyclic peptides (type 2 (**5**)) with the same amino acid composition (**8**, **12**). All branched analogs were more μ-selective.

In summary enkephalin analogs with a branched peptide chain in position 2 have pronounced analgesic activity and μ-type opiate receptor selectivity.

5. Experimental protocols

5.1. Chemistry

Amino acid derivatives were received from Reanal (Hungary), except Boc-Tyr(Boc) and Boc-D-Orn(Z) which were prepared in our laboratory by standard methods. Evaporation was performed in vacuo at 40 °C. Melting points (uncorrected) were determined using a digital melting point analyzer 'Fisher', model 355. ^{1}H-NMR spectra were recorded on a Bruker WM-360 spectrometer at 360 MHz in D_2O or DMSO-d_6 (internal standard Me_4Si). The chemical shifts, intensity and form of peaks were in a good agreement with peptide structures. The purity of synthesized compounds was substantiated by TLC on Silufol UV 254 (Czechoslovakia) or Silica gel 60F 254 (Merck, Germany) in systems: A: $CHCl_3$–EtOH–AcOH (85:10:5); B: *n*-BuOH–pyridine–AcOH–H_2O (15:10:3:6); C: *n*-BuOH–AcOH–H_2O (4:1:1); D: $CHCl_3$–MeOH–AcOH–H_2O (30:20:4:6); E (60:18:2:3). Chromatograms were visualized by UV irradiation, by spraying with ninhydrin solution or using chlorine–benzidine reagent. Amino acid analysis was performed in a Biocal BC-200 amino acid analyzer after peptide hydrolysis in a sealed ampoule (24 h at 110 °C).

Protected peptides were purified on chromatographic columns of 'Merck': size B (310–25); size C (440–37). The fractions were monitored with a detector 'Uvicord II' or 'Uvicord III' (LKB, Sweden) at 206, 254 and 280 nm. The following solvent systems were used for elution: chloroform–ethanol–ethylacetate–AcOH–H_2O, 285:5:8:2:0.25 (ch I); 185:5:8:2:0.25 (ch II).

The final purification was by RP-HPLC on a Dupont-830 system with a spectrophotometric detector (λ = 220 nm). The column (22.4 x 250) was packed with reverse-phase sorbent Zorbax C_8 (Dupont). A step gradient elution mode was applied. Mobile phase flow rate was 1.5 mL/min.

HPLC for analytical purposes was performed on a Dupont model 8800 HPLC system, using column 4.6 x 110 packed with reverse-phase sorbent Silasorb C_{18} (Lachema). Different mixtures of CH_3CN and 0.2 M acetate buffer, pH 5.0, served as the mobile phase, and were run at a flow rate of 1.5 mL/min. Optical rotations were determined with a Perkin-Elmer 141 polarimeter with a 10 cm water jacketed cell in solvents and at concentrations specified below. Fast atom bombardment mass spectrometry (FABMS) was performed on a Kratos MS 50 instrument.

5.1.1. Synthesis of branched and cyclic enkephalin analogs (figure 1)

Boc-Gly-Phe ***18****:* 6.6 g (40 mmol) of L-Phe were dissolved in 40 mL of 1 N NaOH solution, added 3.34 g of $NaHCO_3$, 50 mL of DMF and, at stirring and cooling down to –5 °C, 16.94 g (40 mmol) of Boc-Gly-OPcp dissolved in 20 mL of DMF. After stirring during several hours, when the reaction was over (chromatographic control) the mixture was diluted with ethyl acetate and water and acidified with 5% $KHSO_4$ solution to pH 3. Ethyl acetate layer was separated, water phase was extracted with two portions of ethyl acetate additionally. The three organic phases were combined, washed with water, saturated water solution of NaCl and finally dried over anhydrous Na_2SO_4. After solvent evaporation the crystalline precipitate was recrystallized from ethyl acetate with a small addition of petroleum ether. The yield was 11.4 g of dipeptide **18**, m.p. 133–135 °C.

Boc-D-Orn(Z)-Gly-Phe ***19:*** 10.9 g (33.8 mmol) of dipeptide **18** were disssolved in 50 mL of 70% TFA in DCM and kept for 30 minutes. The solvent was removed,the residue was rubbed with ether, filtered, washed with ether on filter and dried in vacuo over potassium hydroxide. Yield was 10.4 g (91%) of dipeptide trifluoroacetate **18a** with R_f 0.40 (B); 0.63 (D).

5.2 g (15.5 mol) of dipeptide trifluoroacetate **18a** were dissolved in 50 mL of DMF, cooled down to 0 °C and upon cooling added 5.3 mL (31 mmol) of DIEA in 5 mL of DMF and 8.2 g (15.5 mmol) of Boc-D-Orn-(Z)-OPfp dissolved in 15 mL of DMF. The reaction was carried out for several hours and left for night. After the reaction was over, the material was treated similarly to compound **18**. The solvent was removed and the product was purified on a silica gel column (C) in system ch II. The respective fractions were collected, evaporated and dried in vacuo. The yield was 5.9 g of protected tripeptide **19** as an oil.

Boc-Tyr(Boc)-D-Orn(Z)-Gly-Phe ***20****:* 20 g (35 mmol) of tripeptide **19** were dissolved in 100 mL of 50% TFA in DCM and the work up continued as for **18a**. The yield was 19.2 g (94%) of tripeptide trifluoroacetate **19a** with R_f 0.62 (D); 0.42 (B).

18.1 g (31 mmol) of tripeptide trifluoroacetate **19a** were dissolved in 100 mL of DMF, cooled to 0 °C, 10.6 mL (62 mmol) of DIEA and 16.6 g (31 mmol) of Boc-Tyr(Boc)-OPfp in 10 mL of DMF were added. The reaction was carried out for 1.5–2 h, and work-up continued as for compound **18**. After solvent evaporation the product was dissolved in ether and cooled down. The precipitate was filtered, washed with ether, dried, and purified by RP-HPLC. Eluation was performed with a mixture of acetonitrile and 0.1 M ammonium acetate solution (40:60). The yield was 14 g of protected tetrapeptide **20** after lyophilization.

Boc-Tyr(Boc)-D-Orn(Z-Pro)-Gly-Phe ***21****:* Palladium black was added to 18 g (22 mol) of protected tetrapeptide **20** in 150 mL of MeOH, 15 mL of AcOH and 20 mL of H_2O and hydrogenated during several hours. After the reaction was over (chromatographic control) the catalyst was filtered, the filtrate was evaporated. The residue was rubbed with ether and filtered, then twice reprecipitated with ether from alcohol, rewashed with ether and dried in vacuo. The yield was 13.8 g of tetrapeptide **20a** with R_f 0.15 (A); 0.72 (D); 0.77 (E).

5.1 g (6.8 mmol) of tetrapeptide **20a** were dissolved in 100 mL of DMF and upon stirring and cooling added 2.32 mL (13.6 mmol) of DIEA in 3 mL of DMF and 2.8 g (6.8 mmol) of Z-Pro-OPfp dissolved in 15 mL of DMF. 2–3 h later the reaction mixture was treated similarly to compound **18**. The product was purified on a silica gel column in system ch I. The yield was 4.3 g of protected pentapeptide **21**.

Boc-Tyr(Boc)-D-Orn(Z-Leu)-Gly-Phe ***22****:* Pentapeptide **22** was obtained similarly to compound **21** from 5.1 g (6.8 mmol) of tetrapeptide **20a** and 3.5 g (6.8 mmol) of Z-Leu-OPcp. The yield was 4.8 g of of protected pentapeptide **22**.

Boc-Tyr(Boc)-D-Orn(Z-Asn)-Gly-Phe ***23****:* Pentapeptide **23** was obtained similarly to compound **21** from 5.1 g (6.8 mmol) of tetrapeptide **20a** and 3.3 g (6.8 mmol) of Z-Asn-ONp. The yield was 4.6 g of protected pentapeptide **23**.

Boc-Tyr(Boc)-D-Orn(Boc-Met)-Gly-Phe ***24****:* Pentapeptide **24** was obtained similarly to compound **21** from 0.9 g (1.2 mmol) of tetrapeptide **20a** and 0.5 g (1.2 mmol) of Boc-Met-OPfp. The yield was 0.4 g of protected pentapeptide **24**.

Boc-Tyr(Boc)-D-Orn(Z-Pro)-Gly-Phe-NH_2 ***25****:* 0.08 g (0.54 mmol) of NH_4OBt and 0.11 g (0.54 mmol) of DCC were added to 0.50 g (0.54 mmol) of compound **21** dissolved in 3 mL of DMF. The reaction mixture was left for night, DCU was filtered. After solvent evaporation the residue was dissolved in ethyl-acetate. Organic phase was washed with 5% $KHSO_4$ solution, water, 5% solution of $NaHCO_3$, water, a saturated aqueous solution of NaCl and finally dried over anhydrous Na_2SO_4. After evaporation of the solvent the product was dried in vacuo. The yield was 0.46 g of protected pentapeptide amide **25**.

Boc-Tyr(Boc)-D-Orn(Z-Leu)-Gly-Phe-NH_2 ***26****:* 0.38 g of protected pentapeptide amide **26** were obtained from 0.50 g (0.53 mmol) of compound **22** similarly to compound **25**.

Boc-Tyr(Boc)-D-Orn(Z-Asn)-Gly-Phe-NH_2 ***27****:* 0.37 g of protected pentapeptide amide **27** were obtained from 0.47 g (0.5 mmol) of compound **23**, 0.076 (0.5 mmol) of NH_4OBt, 0.10 g (0.5 mmol) of DCC similarly to compound **25**.

Boc-Tyr(Boc)-cyclo(-N^{δ}*-D-Orn-Gly-Phe-Pro-)* ***28****:* Palladium black was added to 1.80 g (1.93 mmol) of protected pentapeptide **21** in 160 mL of methanol, 20 mL of AcOH and 4 mL

of water and hydrogenated for 2–3 h. The catalyst was filtered, the filtrate was evaporated and dried in vacuo over KOH. The yield was 1.39 g (90%) of pentapeptide **21a** with R_f 0.08 (E), 0.50 (B).

1090 mg (1.36 mmol) of pentapeptide **21a** were dissolved in 1100 mL of purified DMF, cooled down to –20 °C, Et_3N was added to pH 7.2 and 0.87 mL (4.08 mmol) of DPPA in 50 mL of DMF were slowly dropped on to the mixture. The solution was stirred during 7 days, and pH of the reaction mixture was maintained about 7 by periodic addition of Et_3N. The solvent was removed and 1.5–2 mL of ethyl acetate and a small amount of ether were added to the residue. The precipitate was filtered, washed by ether, dried in vacuo, yielding 400 mg (37%) of a white powder of protected cyclic pentapeptide **28**.

*Boc-Tyr(Boc)-cyclo(-N$^{\delta}$-D-Orn-Gly-Phe-Leu-) **29**:* 2.13 g (2.24 mmol) of protected pentapeptide **22** were hydrogenated similarly to **21**. 1.63 g (97%) of pentapeptide **22a** were obtained.

700 mg (0.86 mmol) of pentapeptide **22a** were cyclized similarly to **28**. 580 mg (84%) of protected cyclic pentapeptide **29** were obtained.

*Boc-Tyr(Boc)-cyclo(-N$^{\delta}$-D-Orn-Gly-Phe-Asn-) **30**:* 1.4 g (1.46 mmol) of protected pentapeptide **23** were hydrogenated similarly to **21**. The yield was 1.12 g (94%) of pentapeptide **23a** with R_f 0.07 (A), 0.70 (D).

800 mg (0.98 mmol) of pentapeptide **23a** were cyclized similarly to **28**. 415 mg (53%) of protected cyclic pentapeptide **30** were obtained.

*Tyr-cyclo(-N$^{\delta}$-D-Orn-Gly-Phe-Pro-) **3**:* 400 mg (0.51 mmol) of protected cyclic pentapeptide **28** were dissolved in 10 mL of 70% TFA in DCM and worked up similarly to **18a**. The product was purified by RP-HPLC. The respective fractions were collected and lyophilized. The yield was 210 mg (70%) of enkephalin cyclopentapeptide **3**. The value of calculated molecular weight coincided with its experimental parameter revealed by FABMS, m.w. 578.

*Tyr-cyclo(-N$^{\delta}$-D-Orn-Gly-Phe-Leu-) **8**:* 580 mg (0.73 mmol) of protected cyclic pentapeptide **29** were treated similarly to compound **3**. The yield was 125 mg (29%) of cyclopentapeptide **8**, m.w. 594.

*Tyr-cyclo(-N$^{\delta}$-D-Orn-Gly-Phe-Asn-) **12**:* 415 mg (0.52 mmol) of protected cyclic pentapeptide **30** were treated similarly to compound **3**. The yield was 120 mg (39%) of cyclopentapeptide **12**, m.w. 595.

*Tyr-(N$^{\delta}$-Pro)-D-Orn-Gly-Phe **1**:* 300 mg (0.32 mmol) of protected pentapeptide **20** were hydrogenated similarly to compound **21a**, the product obtained was dissolved in 5 mL of 70% TFA in DCM and worked up similarly to **3**. The yield was 70 mg (37%) of branched pentapeptide **1**.

*Tyr-(N$^{\delta}$-Leu)-D-Orn-Gly-Phe **6**:* 700 mg (0.73 mmol) of protected pentapeptide **22** were treated similarly to compound **1**. The yield was 205 mg (46%) of branched pentapeptide **6**.

*Tyr-(N$^{\delta}$-Asn)-D-Orn-Gly-Phe **10**:* 700 mg (0.73 mmol) of protected pentapeptide **23** were treated similarly to compound **1**. The yield was 270 mg (60%) of branched pentapeptide **10**.

*Tyr-(N$^{\delta}$-Met)-D-Orn-Gly-Phe **13**:* 0.36 mL (2.92 mmol) of cyclohexylamine, palladium black were added to 700 mg (0.75 mmol) of protected pentapeptide **24** dissolved in 25 mL of methanol and hydrogenated during several days until the reaction was over (chromatoraphic control). The catalyst was filtered, washed by methanol, the combined methanol phase was evaporated, the residue was dissolved in ethyl acetate and washed with 5% solution of $KHSO_4$, water, saturated solution of NaCl and dried over anhydrous Na_2SO_4. Ethyl acetate solution was filtered and evaporated, the residue was dissolved in 6 mL of 50% TFA in DCM and kept for 30 min. The solution was evaporated, residue was rubbed with ether and dried in vacuo. The product was purified by RP-HPLC and lyophilized. The yield was 240 mg (51%) of branched pentapeptide **13**.

*Tyr-(N$^{\delta}$-Pro)-D-Orn-Gly-Phe-NH$_2$ **2**:* 0.28 g (0.30 mol) of protected pentapeptide **25** were treated similarly to compound **1**. The yield was 90 mg (50%) of branched pentapeptide amide **2**.

*Tyr-(N$^{\delta}$-Leu)-D-Orn-Gly-Phe-NH$_2$ **7**:* 0.35 g (0.37 mmol) of compound **26** were treated similarly to compound **1**. The yield was 70 mg (31%) of branched pentapeptide amide **7**.

*Tyr-(N$^{\delta}$-Asn)-D-Orn-Gly-Phe-NH$_2$ **11**:* 0.30 g (0.31 mmol) of compound **27** were treated similarly to compound **1**. The yield was 85 mg (45%) of branched pentapeptide amide **11**.

5.1.2. Synthesis of enkephalin analogs with linear structure (figure 2)

*Boc-Phe-Pro **32**:* Dipeptide **32** was obtained similarly to compound **18** from 4.6 g (40 mmol) of proline, 20 mL 2 N NaOH, 25 mL of DMF, 3.36 g (40 mmol) of $NaHCO_3$ and 15.4 g (40 mmol) of Boc-Phe-ONp dissolved in 15 mL of DMF. The yield was 12.2 g of dipeptide **32**. The analytical sample was purified by RP-HPLC.

*Boc-Phe-Leu **33**:* Dipeptide **33** was obtained similarly to compound **18** from 3.28 g (25 mmol) of leucine, 25 mL of 1 N NaOH solution, 2.1 g (25 mmol) of $NaHCO_3$, 20 mL of DMF and 10.80 g (25 mmol) of Boc-Phe-OPfp dissolved in 25 m of DMF. The precipitate was crystallized from the mixture of EtOAc, petroleum ether and diethyl ether, yielding 7.40 g of dipeptide **33**, m.p. 143–145 °C.

*Boc-Phe-Met **34**:* Dipeptide **34** was obtained similarly to compound **18** from 4.04 g (27 mmol) of methionine, 54 mL of 0.5 N NaOH, 2.30 g of $NaHCO_3$ in 40 mL of DMF and 11.70 g (27 mmol) of Boc-Phe-OPfp in 10 mL of DMF. The solvent was evaporated and crystalline residue was recrystallized from a mixture of EtOAc and petroleum ether. The yield was 7.60 g of dipeptide **34**, m.p. 138–140 °C.

*Boc-Phe-Pro-NH$_2$ **35**:* Dipeptide amide **35** was obtained similarly to compound **25** from 3.98 g (11 mmol) of dipeptide **32**, 1.67 g (11 mmol) of NH_4OBt and 2.26 g (11 mmol) of DCC. The crude product was purified on a silica gel column (C). The yield was 2.89 g of dipeptide amide **35**.

*Boc-Phe-Leu-NH$_2$ **36**:* Dipeptide amide **36** was obtained similarly to compound **25** from 3.02 g (8 mmol) of dipeptide **33**, 1.2 g (8 mmol) of NH_4OBt and 1.65 g (8 mmol) of DCC. The crude product was recrystallized from ethyl acetate. The yield was 2.68 g of dipeptide amide **36**.

*Boc-D-Orn(Z)-Gly **31**:* Dipeptide **31** was obtained similarly to compound **18** from 0.84 g (11.2 mmol) of glycine, 11.2 mL of 1 N NaOH solution, 0.94 g of $NaHCO_3$ in 14 mL of DMF and 6 g (11.2 mmol) of Boc-D-Orn(Z)-OPfp in 12 mL of DMF. The yield was 3.9 g of dipeptide **31** as a white powder.

Boc-Tyr(Boc)-D-Orn(Z)-Gly ***37****:* 3.8 g (9 mmol) of dipeptide **31** were treated with 50% TFA in DCM similarly to compound **18a**. The yield was 3.7 g (92%) of dipeptide trifluoroacetate **31a** with R_f 0.15(A)s; 0.74(D)s.

Tripeptide **37** was obtained similarly to compound **18** from 2.9 g (6.7 mmol) of dipeptide trifluoroacetate **31a** dissolved in 10 mL of DMF, 6.7 mL of 1 N NaOH, 3.63 g (6.7 mmol) of Boc-Tyr(Boc)-ONB dissolved in 10 mL of DMF. The yield was 3.2 g of protected tripeptide **37**.

Boc-Tyr(Boc)-D-Orn(Z)-Gly-ONB ***38****:* 0.58 g (1 mmol) of protected tripeptide **37** and 0.20 g (1.1 mmol) of HONB were dissolved in 10 mL of EtOAc, the solution was cooled down to 0 °C and 0.22 g of DCC dissolved in EtOAc were added upon stirring. The precipitated DCC was filtered, the solvent being distilled. The yield was 0.60 g (80%) of tripeptide **38**.

Boc-Tyr(Boc)-D-Orn(Z)-Gly-Phe-Pro ***39****:* 5.5 g (15.2 mmol) of dipeptide **32** were treated with 50% TFA in DCM similarly to compound **18a**. The yield was 5.6 g (98%) of dipeptide trifluoroacetate **32a** with R_f 0.12(A), 0.70(D).

0.79 g (2.2 mmol) of dipeptide trifluoroacetate **32a** were dissolved in 20 mL of DMF, cooled down to 0 °C, added on stirring 0.68 mL (4 mmol) of DIEA in 5 mL of DMF and 1.5 g (2 mmol) of tripeptide **38**. The reaction mixture was stirred for several hours (chromatographic control), and after the reaction was over it was treated similarly to compound **18**. The product was purified on a silica gel column (C) in system ch II. The analytical sample was purified by RP-HPLC. The yield was 1.34 g (72%) of protected pentapeptide **39**.

Boc-Tyr(Boc)-D-Orn(Z)-Gly-Phe-Met ***40****:* 1.0 g (2.5 mmol) of dipeptide **34** was treated with 50% TFA in DCM similarly to compound **18a**. The yield was 0.95 g (92%) of dipeptide trifluoroacetate **34a** with R_f 0.07(A)s, 0.38(B)s.

Pentapeptide **40** was obtained similarly to compound **39** from 0.50 g (0.6 mmol) of tripeptide **38**, 0.25 g (0.6 mmol) of dipeptide **34a** and 0.20 mL (1.2 mmol) of DIEA. The yield was 0.30 g of pentapeptide **40**.

Boc-Tyr(Boc)-D-Orn(Z)-Gly-Phe-Pro-NH$_2$ ***41****:* 2.6 g (7.2 mmol) of dipeptide **35** were treated with 50% TFA in DCM similarly to compound **18a**. The yield was 2.3 g (85%) of dipeptide amide fluoroacetate salt **35a** with R_f 0.10 (A)s.

Pentapeptide **41** was obtained similarly to compound **39** from 0.50 g (0.6 mmol) of tripeptide **38**, 0.20 mL (1.2 mmol) of DIEA and 0.22 g (0.6 mmol) of dipeptide amide trifluoroacetate **35a**. The yield was 0.35 g of pentapeptide amide **41**.

Boc-Tyr(Boc)-D-Orn(Z)-Gly-Phe-Leu-NH$_2$ ***42****:* 1.13 g (3 mmol) of dipeptide **36** were treated with 50% TFA in DCM similarly to compound **18a**. The yield was 1.13 g (97%) of dipeptide amide trifluoroacetate salt **36a** with R_f 0.51(E)s, 0.78(B). Pentapeptide **42** was obtained similarly to compound **39** from 1.5 g (2 mmol) of tripeptide **38**, 0.68 mL (4 mmol) of DIEA and 0.86 g (2.2 mmol) of dipeptide amide **36a**. The yield was 1.02 g of pentapeptide amide **42**.

Tyr-D-Orn-Gly-Phe-Pro ***4****:* 600 mg (0.64 mmol) of protected pentapeptide amide **39** were hydrogenated and treated by 50% TFA in DCM similarly to pentapeptide **1**. The crude product was purified by RP-HPLC. The yield was 270 mg (70%) of linear pentapeptide **4**.

Tyr-D-Orn-Gly-Phe-Met ***14****:* 800 mg (0.82 mmol) of protected pentapeptide **40** were treated similarly to compound **13**. The yield was 1.10 mg (21%) of linear pentapeptide **14**.

Tyr-D-Orn-Gly-Phe-Pro-NH$_2$ ***5****:* 930 mg (1 mmol) of protected pentapeptide **41** were treated similarly to compound **4**. The yield was 310 mg (52%) of linear pentapeptide **5**.

Tyr-D-Orn-Gly-Phe-Leu-NH$_2$ ***9****:* 600 mg (0.71 mmol) of protected pentapeptide **42** were treated similarly to compound **4**. The yield was 220 mg (50%) of linear pentapeptide **9**.

5.2. Biology

5.2.1. Analgesic activity assay in vivo

Analgesic activity of enkephalin analogs was determined by a 'tail pinch' method [12, 14–16]. Analgesic effect was studied in comparison with morphine, [Leu^5]- and [Met^5]-enkephalins at intracisternal and intravenous administration to outbread male mice weighing 18 to 22 g. Ten mice were used in each experiment.

The studied substances were dissolved in sterile physiological solution and injected in J-shaped needles into the brain of conscious mice at a dose of 10 μL. Control animals received 10 μL of sterile physiological solution. Intravenous administration was into the tail vein. Analgesic activity was tested by pinching the mouse tail with an artery clip with 200 g pressure. The mice who bit the clip within one second after application of pinching were selected for assays. Analgesic activity was defined as a latency on the biting response greater than 6 sec. The analgesic tests were performed 5, 15, 30, 60 and 90 min after administration and then every 30 min until the analgesic reaction was stopped. The analgesic activity of peptides was expressed as a percent of mice not showing the biting response. The results are summarized in *table III*.

5.2.2. GPI and MVD bioassays in vitro

The effect of peptides on peripheral opiate receptors was determined by the capacity to suppress electric stimulation-induced segment contraction of the longitudinal muscle of the guinea pig ileum with mesenteral nervous plexus and of the vas deferens of mice [13, 14–16]. The longitudinal muscle of ileum from guinea pig of any sex (weight 350–500 g) was carefully separated from underlying circular muscle and placed into a small bath with Krebs solution at 36 °C.

The solution was constantly aerated. A constant load of 0.2 g was applied to the tissue. The tissue was stimulated by means of the ring platine electrodes by single impulses of 1 μsec duration with 0.1 Hz frequency. Isometric contractions were registered on a TB-611T recorder connected to a Nihon Kohden polygraph. Vas deferens of mice (weight 27 to 30 g) were placed into a bath with modified Krebs solution (not containing magnesium sulphate) at 31 °C. The substances under study were dissolved in distilled water. The inhibiting activity of the compounds was determined by accumulating doses, adding increasing concentrations of the compounds without washing. The activity of the preparations was expressed as IC_{50} in nmol/L. The results obtained in 8–10 tests were statistically processed using Student's criterion. *Table IV* demonstrates IC_{50} with reliable intervals at $P = 0.05$.

5.2.3. Receptor binding assays

The affinity of the peptides to central opiate receptors was determined by radioreceptor analysis. Adult rats of either sex were killed by decapitation; the brains, without cerebella, were removed and homogenized with a Teflon-glass homogenizer at 4 °C in 50 mM Tris-HCl buffer, pH 7.55. The suspension was centrifuged at 49000 g for 10 min at 4 °C, the supernatant

discarded and the pellet resuspended in 150 mM NaCl in 50 mM Tris-HCl buffer, pH 7.55 at 22 °C. The suspension was incubated for 2 h at 21–23 °C and a second centrifugation was carried out as before. The resulting pellet was finally homogenized in 50 mM Tris-HCl buffer in a volume of 80 mL per triplicate in plastic centrifuge tubes immersed in an ice bath. Each assay contained, in a final volume of 1.06 mL, 2 nM [^{3}H]naloxone (35000–40000 cpm), 50 μg mL^{-1} bacitracin, 1.0 mL of the membrane preparation described above, and various concentrations of a peptide. After of 2 h incubation, the tubes were centrifuged at 15000 g for 5 min at 4 °C, the supernatant discarded, and the pellet washed and thoroughly resuspended in 200 μL water. The radioactivity of an 150 μL aliquot was counted in Kinard's scintillation fluid. Specific binding was measured as the difference between total binding and that in the presence of 10 μL nalorphine. The binding assays with [^{3}H] [D-Ala^2, D-Leu^5]-enkephalin were performed similarly but with the following modifications. In order to increase the selectivity of the interaction between the radioligand and the δ-receptors, the reaction mixture was supplemented with 5 mL $MnCl_2$ and 5 μM morphiceptin, a highly selective μ-receptor specific ligand. The incubation time was 3 h at 21–23 °C, and the nonspecific binding was determined in the presence of 4 μM [Leu^5]-enkephalin. Each peptide was tested in at least three experimental series with different membrane preparations (5–6 various peptide concentrations for each assays). The competition curves were linearized by means of the logit transformation, and the concentration of peptide required to inhibit the specific binding by 50% (IC_{50}) was calculated within 95% confidence limit, by using a computerized weighted non-linear least-squared estimation of all experimental points.

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