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Original paper

Opioid peptides. Synthesis and biological properties of dermorphin related hexapeptides

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Summary – The Gly[4] and / or Tyr[5] residues in dermorphin hexapeptide (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-OH) were replaced by Nα-methyl- or D-amino acids in order to examine the effect on opioid activity. Two pseudopeptides (H-Tyr-D-Ala-Phe-Gly-ψ(NHCO)-Xaa-Pro-OH, Xaa – Tyr or Phe) in which the Gly[4]-Xaa bond is reversed, were also prepared. Metabolic stability, analgesia and selectivity of these compounds for different receptor populations have been investigated. Results suggest that the 12 new analogues showed a negligible affinity for the *K* binding site and some selectivity for μ- or δ receptors. In some cases the analgesic potencies seems to be related to enzymatic stability of the peptides.

Résumé – **Peptides opioides: synthèse et propriétés biologiques d'hexapeptides apparents à la dermorphine.** *Des hexapeptides H-Tyr-D-Ala-Phe-Xaa-Xbb-Pro-OH (Xaa = Gly, Sar; Xbb = L ou D-Phe, Tyr, Phe(NMe), Tyr(NMe) et des pseudopeptides présentant la rétroinversion de la liaison peptidique Xaa-ψ(NHCO)-Xbb ont été synthétisés; leur activité opioïde et leur résistance à la biodégradation ont été évaluées. Les résultats suggèrent que les 12 nouveaux analogues présentent de très faibles affinités pour les récepteurs K et quelque sélectivité pour les récepteurs μ ou δ. Dans quelques cas, il semble exister une corrélation entre le nouveau pouvoir analgésique des peptides et leur résistance aux peptidases.*

dermorphin analogs / opioids / peptide synthesis / biodegradation / analgesia / binding

Introduction

Since the isolation and identification of dermorphin [1] (DER), H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2, a great number of analogues have been synthesized and tested for opioid activity [2–4]. Several studies concerning the relationship between structure and biological activity of DER have demonstrated that a progressive reduction in analgesic activity follows the decrease in the number of aminoacids in the chain and that the N-terminal tetrapeptide amide of DER is the minimum sequence retaining some antinociceptive effect after subcutaneous (s.c.) administration [5, 6]. In comparison to DER, its shorter hexapeptide homologue showed comparable activity in the isolated guinea-pig ileum (GPI) and mouse vas deferens (MVD) [3–6], slightly lower analgesia in rat [5] and mouse [6] after intracerebroventricular (i.c.v.) injection, but negligible analgesic effects following s.c. administration [3].

Since this apparent discrepancy may result from unfavourable pharmacokinetic or transport parameters and / or low stability in plasma, we synthesized a number of DER (1–6)–OH analogues containing Nα-methyl- or D-amino acids at the postulated scissible amide bonds [7, 8]. The pseudopeptides, (H-Tyr-D-Ala-Phe-Gly-ψ(NHCO)-Xaa-Pro-OH, Xaa = Tyr or Phe), in which the Gly[4]-Xaa[5] bond is reversed, were also prepared. The new peptides were evaluated with respect to resistance towards enzymic degradation and tested for opioid activity.

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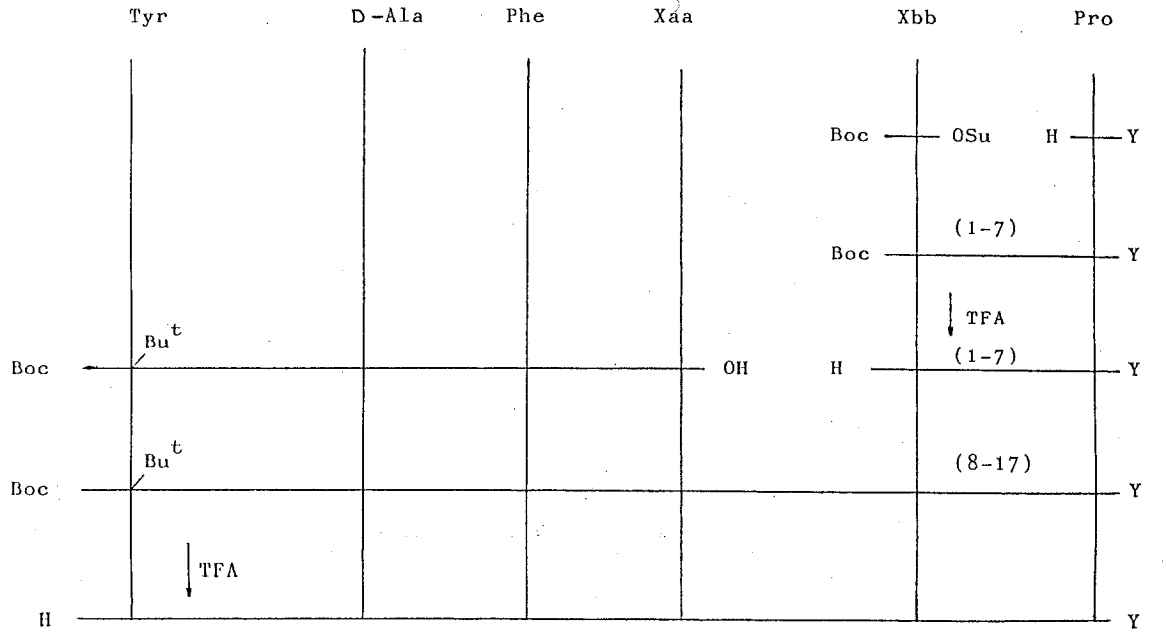
Abbreviations: These follow the rules of the IUPAC-IUB Commission on Biochemical Nomenclature (see [1984] Eur. J. Biochem. 138, 9–37). Other abbreviations are; **Boc:** *t*-butyloxycarbonyl; **Bu[t]:** *t*-butylether; **DAGO:** H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol; **DPDPE:** H-Tyr-D-Pen-Gly-Phe-D-Pen-OH; **DCC:** dicyclohexylcarbodiimide; **DMF:** dimethylformamide; **HOBt:** 1-hydroxybenzotriazole; **HPLC:** high-performance liquid chromatography; **MA:** mixed anhydride; **NMM:** *N*-methylmorpholine; OBu[t]: *t*-butyl ester; **OSu:** *N*-hydroxysuccinimidyl ether; **PE:** petroleum ether; **Pen:** penicillamine; **Phe(NMe):** Nα-methylphenylalanine; **TEA:** triethylamine; **TFA:** trifluoroacetic acid; **THF:** tetrahydrofuran; **TLC:** thin layer chromatography; **Z:** benzyloxycarbonyl.

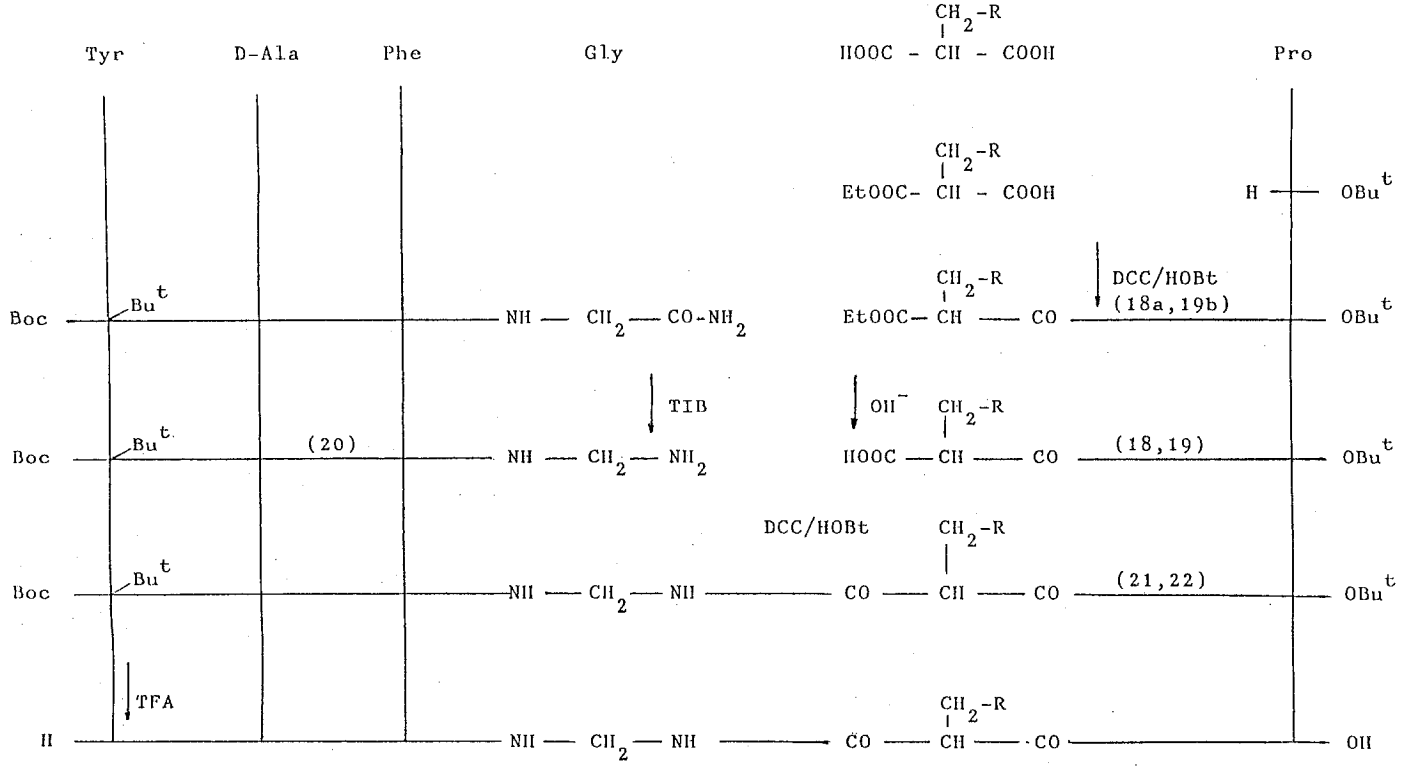
Chemistry

The peptides were synthesized by conventional methods in solution. The hexapeptide analogues **(I–X)** were prepared by 4 + 2 coupling (Scheme 1), a similar method to that described previously for DER(1-6) homolog [6].

The DER pseudopeptides **XI** and **XII** embodying partially retro-inverso modifications (PMRI) were prepared as depicted in the Scheme 2. The gem-diaminoalkyl moiety **20** was obtained by treating the corresponding tetrapeptide amide [9] with *bis*-(trifluoroacetoxy-iodobenzene) (TIB) [10] under conditions known to proceed



Scheme 1. Synthesis of hexapeptide dermorphin analogues **(I–X).** Xaa = Gly, Sar; Xbb = Tyr, D-Tyr, Phe, D-Phe, Phe(NMe), Tyr(NMe); Y $=OCH_3$, OH, NH_2.



Scheme 2. Synthesis of PMRI analogues: [$^4\psi^5$, NHCO]DER hexapeptides (**XI** and **XII**), R = C_6H_5 or C_6H_4OH.

without racemization [11]. Acylation of H-Pro-OBu^t with the pertinent 2-alkylmalonyl derivatives resulted in the C-terminal pseudopeptides **18a** and **19a.** Deprotection of the ethyl esters **18a** and **19a,** followed by coupling with the N-terminal fragment, **20,** yielded the fully protected PMRI analogs **21** and **22.** Treatment of **21** and **22** with trifluoroacetic acid gave the desired analogs XI and XII. Pseudopeptides **XI** and **XII** were obtained as diastereomeric mixtures, due to the racemic nature of both 2(4-hydroxy-benzyl)- and 2(benzyl)malonic acid monoethylesters, but the low analgesic activities observed did not justify attempts at resolution.

Final purification was accomplished by partition chromatography with solvent system (D) on Sephadex G25. The purity of **I–XII** was checked by TLC, HPLC, amino acid analysis and elemental analysis. The characterization of DER hexapeptide analogues is reported in Table I.

Results and Discussion

Relative opioid receptor affinities were determined by displacement of selective radioligands from guinea brain membrane preparations [12]. [^{3}H]DAGO served as a selective μ-receptor radioligand, and the radiolabelled [^{3}H]DPDPE and [^{3}H]EKC were used for determining relative δ- and κ-receptor affinities, respectively. Analgesia was tested by a mouse tail-flick [13] assay after s.c. administration. Enzymic degradation of peptides was evaluated after exposure to rat plasma, brain and kidney homogenates. The binding assay data, antinociceptive effects and half-lives of the 12 new analogs are reported in Tables II and III and are shown in comparison with DER and the parent H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-NH_2 (DH).

As previously observed for a different series of DER peptides [4], none of the present analogs displaced the binding of [^{3}H]EKC to any significative degree. The high IC_{50} values (> 10 μM) indicate that they have a negligible affinity for the κ binding site.

In comparison to Der and DH, all new hexapeptides I–XII were less potent in the [^{3}H]DAGO binding assay. Whereas the substitution of Tyr^5 for L-Phe (compound **III**) was tolerated, the introduction of D-amino acids (peptides **II** and **IV**) or Nα-methyl-residues (compounds V–X) was detrimental. Moreover, hexapeptide amide **VI** or ester **VIII** are more potent than corresponding hexapeptide acid **VII** (see also DH *vs* I) confirming the trend previously observed for enkephalin analogs [14–16] and DER-related peptides [3, 4, 17].

Finally, analogs **XI** and **XII**, containing the retro-inversion of peptide bond between the positions 4 and 5, also show a low affinity: they retain about 10% of the potency of the parent peptides **I** and **III.**

[^{3}H]DPDPE binding assays show a dual trend of the relative potencies. In some cases, the effect of structural changes at positions 4 and 5 parallels that found in [^{3}H]DAGO binding assay, in other cases there is a partial dissociation between potencies on [^{3}H]DAGO and

Table I. Data of dermorphin-related peptides.

No.	Structure	mp (°C) (cryst. solv.)	$[\alpha]_D^{o\,a}$	TLC (system)	HPLC Rt min	Amino acid analysis[b]
I	H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-OH	174–176 (MeOH/Et_2O)	+ 7.6	0.55 (A)	26.88	Tyr: 1.96; Ala: 1.01; Phe: 1.02; Gly: 1.00; Pro: 0.97.
II	H-Tyr-D-Ala-Phe-Gly-D-Tyr-Pro-OH	178–180 (MeOH/Et_2O)	+ 15.5	0.56 (A)	28.94	Tyr: 1.95; Ala: 1.03; Phe: 0.99; Gly: 1.00; Pro: 0.98.
III	H-Tyr-D-Ala-Phe-Gly-Phe-Pro-OH	208–210 (MeOH/Et_2O)	+ 2.6	0.60 (A)	32.35	Tyr: 0.97; Ala: 1.02; Phe: 2.03; Gly: 1.00; Pro: 0.99.
IV	H-Tyr-D-Ala-Phe-Gly-D-Phe-Pro-OH	196–198 (AcOEt)	+ 19.1	0.60 (A)	36.30	Tyr: 0.99; Ala: 1.01; Phe: 2.05; Gly: 1.00; Pro: 0.97.
V	H-Tyr-D-Ala-Phe-Gly-Phe(NMe)-Pro-OH	135–137 (MeOH/Et_2O)	− 21.9	0.62 (A)	37.69	Tyr: 0.98; Ala: 1.04; Phe: 1.01; Gly: 1.00; Phe(NMe): 0.95; Pro: 0.98.
VI	H-Tyr-D-Ala-Phe-Gly-Tyr(NMe)-Pro-NH_2	162–164 (MeOH/Et_2O)	− 21.5	0.58 (A)	38.93	Tyr: 0.99; Ala: 1.03; Phe: 1.03; Gly: 1.00; Tyr(NMe): 0.94; Pro: 0.97.
VII	H-Tyr-D-Ala-Phe-Gly-Tyr(NMe)-Pro-OH	163–165 (Et_2O)	− 18.6	0.57 (A)	33.64	Tyr: 0.98; Ala: 1.02; Phe: 1.01; Gly: 1.00; Tyr(NMe): 0.95; Pro: 0.99.
VIII	H-Tyr-D-Ala-Phe-Gly-Tyr(NMe)-Pro-OCH_3	148–150 (Et_2O)	− 25.5	0.61 (A)	39.68	Tyr: 0.97; Ala: 0.98; Phe: 1.02; Gly: 1.00; Tyr(NMe): 0.93; Pro: 0.97.
IX	H-Tyr-D-Ala-Phe-Sar-Tyr(NMe)-Pro-OCH_3	160–162 (Et_2O)	− 8.9	0.62 (A)	41.12	Tyr: 1.01; Ala: 1.00; Phe: 1.03; Sar: 0.97; Tyr(NMe): 0.96; Pro: 0.98
X	H-Tyr-D-Ala-Phe-Sar-Tyr-Pro-OH	156–158 (Et_2O)	− 11.4	0.59 (A)	34.88	Tyr: 1.99; Ala: 1.00; Phe: 1.01; Sar: 0.96; Pro: 0.97.
XI	H-Tyr-D-Ala-Phe-Gly-ψ(NHCO)-Tyr-Pro-OH	169–171 (AcOEt/Et_2O)	− 17.1	0.62 (A)	52.63/53.68[c]	Tyr: 0.99; Ala: 1.00; Phe: 1.01; Pro: 0.97; NH_3: 1.04;
XII	H-Tyr-D-Ala-Phe-Gly-ψ(NHCO)-Phe-Pro-OH	158–160 (AcOEt/Et_2O)	− 6.7	0.63 (A)	46.53/48.10[c]	Tyr: 0.98; Ala: 1.00; Phe: 1.02; Pro: 0.99; NH_3: 1.06.

[a]Optical rotations; C = 1 in methanol, temperature: 22°C.
[b]In the aminoacid analysis of peptides, Nα-methylamino acids gave only one peak; gem-diamine moiety yields 2 mol ammonia; a malonyl residue is not detectable (see *Experimental protocols*).
[c]Retention times of 2 diastereomers.

Table II. Binding assays of opioid peptide analogs[a].

No.	Structure	[^{3}H]DAGO (μ)		[^{3}H]DPDPE (δ)		$IC_{50}\delta / IC_{50}\mu$	[^{3}H]EKC (κ)
		IC_{50} (nM)	relative potency	IC_{50} (nM)	relative potency		IC_{50} (nM)
DH	H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-NH_2	10 ± 0.9	1	680 ± 91	1	68	> 10 000
I	H ——— OH	40 ± 5.6	0.25	720 ± 120	0.9	18	> 10 000
II	H ——— D-Tyr — OH	145 ± 6.2	0.07	150 ± 31	4.5	1	> 10 000
III	H ——— Phe — OH	24 ± 0.3	0.42	205 ± 27	3.3	8.5	> 10 000
IV	H ——— D-Phe — OH	100 ± 11	0.1	105 ± 20	6.5	1	> 10 000
V	H ——— Phe(NMe) — OH	1050 ± 145	0.01	2150 ± 200	0.3	2	> 10 000
VI	H ——— Tyr(NMe) — NH_2	398 ± 60	0.03	1700 ± 250	0.4	4.3	> 10 000
VII	H ——— OH	505 ± 54	0.02	2300 ± 350	0.3	4.5	> 10 000
VIII	H ——— OCH_3	95 ± 12	0.11	790 ± 180	0.8	8.3	> 10 000
IX	H — Sar-Tyr(NMe) —— OCH_3	800 ± 105	0.01	6500 ± 1000	0.1	8.1	> 10 000
X	H — Sar-Tyr ——— OH	265 ± 33	0.04	890 ± 75	0.7	3.3	> 10 000
XI	H-Tyr-D-Ala-Phe-Gly-ψ(NHCO)-Tyr-Pro-OH	315 ± 42	0.03	45 ± 4.2	15	0.1	> 10 000
XII	H ——— Gly-ψ(NHCO)-Phe-Pro-OH	240 ± 33	0.04	610 ± 80	1	2.5	> 10 000
DER	H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	5.7 ± 0.7	1.75	335 ± 40	2	59	> 10 000

[a]Mean of 3 determinations ± SEM.

Table III. Analgesic and stability data of peptides.

No.	Structure	Analgesia[a] ED_{50} (μmol / kg, s.c.)	Relative potency[b]	Half-life (min)		
				Plasma	Brain	Kidney
DH	H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-NH_2	308 (164 −706)	1	2.8	15.4	2.2
I	H ——— OH	406 (238 −894)	0.7	2.0	3.7	2.2
II	H ——— D-Tyr — OH	107 (61 −174)	3	> 180	> 180	31.3
III	H ——— Phe — OH	320 (171 −728)	1	1.9	3.7	2.1
IV	H ——— D-Phe — OH	90 (56 −161)	3.5	> 180	> 180	31
V	——— Phe(NMe) — OH	950 (530 −2500)	0.3	> 180	n.d.	n.d.
VI	H ——— Tyr(NMe) — NH_2	600 (350 −1400)	0.5	> 180	> 180	10.3
VII	H ——— OH	620 (385 −1550)	0.5	> 180	n.d.	n.d.
VIII	H ——— OCH_3	68 (61 −154)	4.5	> 180	> 180	10.1
IX	H — Sar-Tyr(NMe) —— OCH_3	980 (540 −2450)	0.3	> 180	n.d.	10.8
X	H — Sar-Tyr ——— OH	53 (39 −88)	6	2.3	7.9	2.6
XI	H-Tyr-D-Ala-Phe-Gly-ψ(NHCO)-Tyr-Pro-OH	190 (89 −329)	1.5	> 180	> 180	9.9
XII	H ——— Gly-ψ(NHCO)-Phe-Pro-OH	220 (122 −351)	1.4	> 180	> 180	n.d.
DER	H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	2.75 (1.73− 6.42)	112	> 180	21.1	2.4

[a]Analgesia was tested on at least 7 mice, and evaluated 30 min after administration.
[b]Relative potencies are on molar basis (DH = 1).

[^{3}H]DPDPE binding assays: for instance, change of Tyr^5 in DH by D-Tyr, Phe or D-Phe (compounds **II**−**IV**) increased the affinity for δ-sites. The difference between the potencies observed in the [^{3}H]DPDPE and the [^{3}H]DAGO assays is particularly pronounced in the case of the pseudopeptide **XI.** Consequently, in comparison to DER and DH, analogues **II**−**IV** are nonselective, and the PMRI analog **XI** show an opposite affinity pattern.

An objective of this work had been to obtain hexapeptide analogs with sufficient metabolic stability to show good s.c. activity. It has previously suggested that stability to kidney peptidases is a good indicator for *in vivo* stability [18] and hence the stability of the peptides in rat plasma, brain and kidney was examined *in vitro* (Table III).

It can be noted that DH, though quite resistant to degradation in brain, was rapidly destroyed by plasma and kidney enzymes. Gradient elution RP-HPLC analysis revealed the presence of a Der catabolite which coeluted with the N-terminal tetrapeptide, DER(1-4)-OH [8]. As expected, whereas compounds **I** and **III** were quickly cleaved, peptides containing D- or *N*-methyl-residues at position 5 were very resistant to degradation in brain and plasma and relatively stable to kidney homogenate. A markedly increased resistance towards different tissue enzymes, pertained even to pseudopeptide **XI** in which the Gly-Tyr bond was reversed. On the contrary [Sar^4]DER(1-6)-OH (compound **X**) is rapidly destroyed producing H-Tyr-D-Ala-Phe-Sar-OH as the main degradation product, in the three preparations studied. The metabolite proved to be chromatographically identical to the synthetic [Sar^4] DER(1-4)-OH on RP-HPLC using isocratic elution (mobile phase composition, 20%B; flow rate, 1 ml / min).

Table IV. Physicochemical properties of protected peptides[a].

No.	Structure	mp (°C)	Crystall. solvents	$[\alpha]_D^{o\,b}$	TLC (system)
1	Boc-Tyr-Pro-OH	174–176	AcOEt/Et_2O	− 76.6	0.79 (B)
2	Boc-D-Tyr-Pro-OH	176–177	AcOEt/Et_2O	− 60.4	0.78 (B)
3	Boc-Phe-Pro-OH	180–182	Et_2O	− 56.7	0.82 (B)
4	Boc-D-Phe-Pro-OH	177–179	AcOEt/Et_2O	− 48.4	0.80 (B)
5	Boc-Phe(NMe)-Pro-OCH_3	oil	–	−120.7	0.24 (H)
6	Boc-Tyr(NMe)(Bzl)-Pro-OCH_3	oil	–	− 93.3	0.39 (H)
7	Boc-Tyr(NMe)(Bzl)-Pro-NH_2	66– 68	Et_2O/PE	− 79.8	0.41 (H)
8	Boc-Tyr(But)-D-Ala-Phe-Gly-Tyr-Pro-OH	144–146	MeOH/Et_2O	− 6.8	0.53 (G)
9	Boc-Tyr(But)-D-Ala-Phe-Gly-D-Tyr-Pro-OH	152–154	AcOEt/Et_2O	− 11.7	0.52 (G)
10	Boc-Tyr(But)-D-Ala-Phe-Gly-Phe-Pro-OH	216–218	MeOH/Et_2O	+ 7.9	0.63 (G)
11	Boc-Tyr(But)-D-Ala-Phe-Gly-D-Phe-Pro-OH	133–135	AcOEt/Et_2O	− 13.8	0.60 (G)
12	Boc-Tyr(But)-D-Ala-Phe-Gly-Tyr(NMe)-Pro-OCH_3	110–112	AcOEt/Et_2O	− 47.9	0.52 (D)
13	Boc-Tyr(But)-D-Ala-Phe-Gly-Phe(NMe)-Pro-OH	103–105	AcOEt/Et_2O	− 39.4	0.67 (G)
14	Boc-Tyr(But)-D-Ala-Phe-Gly-Tyr(NMe)-Pro-OH	131–133	Et_2O	− 37.1	0.84 (A)
15	Boc-Tyr(But)-D-Ala-Phe-Gly-Tyr(NMe)-Pro-NH_2	146–148	MeOH/Et_2O	− 37.1	0.56 (C)
16	Boc-Tyr(But)-D-Ala-Phe-Sar-Tyr(NMe)-Pro-OCH_3	125–127	Et_2O	− 19.2	0.58 (C)
17	Boc-Tyr(But)-D-Ala-Phe-Sar-Tyr-Pro-OH	133–134	Et_2O	− 22.3	0.61 (C)

[a]All the peptides showed correct elemental analyses.
[b]Optical rotations: c = 1 in methanol, temperature = 22°C.

All investigated DER analogues showed antinociceptive effects (Table III) that were completely abolished by s.c. administration of naloxone hydrochloride (0.5–1 mg/kg, 5 min prior to the testing procedure).

In comparison to DH, the peptides with sufficient metabolic stability (compounds **II, IV** and **VIII**) displayed an increased analgesic activity in spite of a lower binding affinity. On the other hand, the antinociceptive effect of **X** is surprising in view of the instability of this peptide. A possible explanation is that **X** is particularly effective at crossing the blood-brain barrier so that pharmacologically active brain levels of the peptide are obtained despite short plasma and kidney half-lives. Alternatively, the analgesic activity of **X** might be due, at least in part, to its stable metabolic product H-Tyr-D-Ala-Phe-Sar-OH (unpublished data). This is in line with the observed properties of [Sar^4] DER tetrapeptides, as compared to the corresponding Gly^4 analogues [4]. The pseudopeptide **XI**, in spite of its metabolic stability and high potency in [^{3}H] DPDPE binding assay, exhibited a relatively weak analgesia. Thus, as observed previously [9], in addition to a number of factors connected with pharmacokinetic properties, high affinity for μ-receptors seems to be a critical requirement for potent s.c. analgesics. Further work on the new compounds synthesized is in progress.

Experimental protocols

Materials and methods

HPLC analysis was performed on a Bruker liquid chromatograph L21-C equipped with a Bruker LC313 UV variable-wavelength detector. Recording and quantification were accomplished using a chromatographic data processor (Epson computer FX-80X). An IBO1 C-18 column (250 × 4.5 mm) i.d. 5 μm particle size) was used in the HPLC system. All solvents used were UV spectroscopic grade and filtered and degassed prior to use. Analytical determinations for deprotected peptides were carried out by a gradient made up of 2 solvents: A, 10% (v/v) acetonitrile in water; B, 60% (v/v) acetonitrile in water; both contained 4.5 mM trifluoroacetic acid and 4.9 mM triethylamine. The gradient programme used was as follow: linear gradient from 10–25% B in 10 min; isocratic 25% B for 5 min; linear gradient from 25–40% B in 8 min. Chromatography was performed at a flow rate of 1 ml/min: all analogues showed by analytical HPLC, less than 1% impurities while monitoring at 210 and 277 nm. The amino acid composition was determined with a Carlo Erba 3A29 amino acid analyzer, after acid hydrolysis in constant-boiling HCl containing 1% phenol. TLC was performed on precoated plates of silica gel F254 (from E. Merck) with use of the following solvent systems: A, 1-butanol/AcOH/H_2O (6:1:5); B, EtOAc/pyridine/AcOH/H_2O (60:20:6:11); C, $CHCl_3$/MeOH/benzene (85:10:5); D, $CHCl_3$/MeOH (1:1); E, $CHCl_3$/MeOH/30% ammonia (12:83). Ninhydrin 1% (Merck), fluorescamine (Hoffman-La Roche), and/or chlorine reagent were used as spray reagents. Samples were considered pure when they showed single spots with more than one solvent system. Elemental analyses were carried out after the products were dried for 12 h at 50°C (0.2 torr). Open column chromatography was run on silica gel 60 (70-230 mesh, Merck).

Coupling procedures

Method A
To a stirred solution (0.5–0.8 M) of Boc-protected amino acid or Boc-protected peptide (1 mmol) in DMF, 1 equiv of NMM was added; the mixture was cooled to −10°C, treated with isobutyl chloroformate (1 equiv), and allowed to react for 2–3 min. A precooled solution of amino component hydrochloride or trifluoroacetate (1.1 mmol) in DMF (0.4–0.6 M) was added to the mixture, followed by NMM (1.1 equiv). The reaction mixture was stirred for 1 h at −10°C and 2–3 h at 0–10°C and then diluted with EtOAc (100 ml). The solution or suspension was washed consecutively with brine, 0.5 N $KHSO_4$, brine, 5% $NaHCO_3$, and brine. The organic phase was dried ($MgSO_4$), filtered, and evaporated to dryness. The residue was crystallized from appropriate solvents or purified by column chromatography.

Method B
To a solution of the carboxy component (2 mmol) in DMF (10 ml) were added the amino acid component (2 mmol), NMM (2 mmol if the amino component was in the protonated form), HOBt (2 equiv), and DCC (2.1 mmol) in the above order at 0°C. The reaction mixture was stirred for 2 h at 0°C and 24 h at room temperature; *N,N'*-dicyclohexylurea was

filtered off, and the solution was diluted with EtOAc (100 ml) and worked up as described in *Method A*.

Method C
To a cooled solution of the carboxy component (8 mmol) in NaOH 1N (8 ml), Boc-protected amino acid-succinimidyl ester (4 mmol) in dioxan (6 ml) was added. The reaction mixture was stirred for 2 h at 0°C and 12 h at room temperature, evaporated and diluted with a solution of citric acid 1N (50 ml). The product was extracted into EtOAc (3 × 30 ml), back-washed with saturated aqueous NaCl and then worked up as described in *Method A*.

Deprotection procedures

Method D
Boc and/or Bu^t protecting groups were removed by treating the peptide with aqueous 90% TFA (1:10, w : v) containing anisole (1 ml) for 30–40 min. The solvent was evaporated *in vacuo* at 0°C, and the residue was triturated with ether or petroleum ether; the resulting solid peptide was collected and dried.

Method E
Hydrogenations were carried out in methanol at atmospheric pressure and room temperature, in the presence of 10% palladized charcoal (catalyst to peptide ratio, 1:9 w:w). The reaction mixture was filtered through a Celite bed, and evaporated to dryness. The residue was treated as described above in *Method D*.

Method F
The peptide methyl ester was suspended in a 2:1 mixture of MeOH-H_2O (7.5 ml/mmol) and treated with 1.2 mol-equiv 1 N NaOH for 3 h at room temperature. The solution was then diluted with water, concentrated *in vacuo* to remove the methanol, and washed with EtOAc. After cooling at 0°C, it was acidified with 1 N HCl and the product extracted with EtOAc. The organic solution was washed with brine, dried ($MgSO_4$), filtered and evaporated to dryness. The resulting solid peptide acid was crystallized from appropriate solvents.

HO-m(R, S)*Tyr(Bu^t)-Pro-OBu^t* **(18)**
According to general *Method B*, EtO-*m(R, S)*Tyr(Bu^t)-OH [21] (10 mmol) in DMF (15 ml) was reacted with H-Pro-OBu^t·HCl (11 mmol) in the presence of NMM (11 mmol). The crude ethylester was dissolved in EtOH (10 ml) and treated with 1 M NaOH (10 ml) checking the progress of hydrolysis by TLC, solvent system (D) (deprotection *Method F*). The resulting acid was purified using a column (1 × 40 cm) and solvent system (D): (78%), R_f 0.64 (D). A sample of the above oil (1 mmol) was dissolved in ether (15 ml) containing dicyclohexylamine (0.2 ml, 9 mmol); precipitation of title compound as DCHA salt occurred on standing (90%), mp 147–149°C, $[\alpha]_D^{25}$ −32.3 (c 1.0, MeOH). Anal. calcd. for $C_{35}H_{56}N_2O_6$: C: 69.96; H: 9.40; N: 4.66; found: C: 69.81; H: 9.36; N: 4.70.

HO-m(R, S)*Phe-Pro-OBu^t* **(19)**
19 was prepared as **18** from EtO-*m-(R, S)*Phe-OH [21] and H-Pro-OBu^t. Compound **19** as DCHA, was recrystallized from EtOAc (86%): mp: 128–130°C. $[\alpha]_D^{25}$ −41.4 (c 1.0, MeOH). Anal. calcd. for $C_{31}H_{48}N_2O_5$: C: 70.42; H: 9.15; N: 5.30; found: C: 70.29; H: 9.11; N: 5.36.

Boc-Tyr-(Bu^t)-D-Ala-Phe-gGly-H·CF$_3$COOH **(20)**
To a stirred suspension of Boc-Tyr(Bu^t)-D-Ala-Phe-Gly-NH_2 [21] (3.0 g, 5 mmol) in 14 ml of acetonitrile-water (6:4) was added TIB [10] (2.58 g, 6 mmol) in acetonitrile (6 ml). The resulting solution was stirred for 3–4 h under N_2 at room temperature, following the reaction by TLC (solvent system B) and then the mixture was evaporated. The resulting diamine derivative was triturated with Et_2O and then recrystallized twice from EtOH-Et_2O (3.14 g, 90%): mp: 135–137°C. $[\alpha]_D^{21}$ + 10.6 (c 1.0, MeOH); R_f(C) 0.29. Anal. calcd. for $C_{33}H_{48}N_6O_8F_3$: C: 55.53; H: 6.77; N: 11.77; found: C: 55.38; H: 6.73; N: 11.82.

Boc-Tyr(Bu^t)-D-Ala-Phe-Gly-ψ(NHCO)Tyr-Pro-OBu^t **(21)**
According to *Method B*, compound **20** (0.698 g, 1 mmol) in THF (20 ml) was reacted with HO-*m(R, S)*Tyr(Bu^t)-Pro-OBu^t in the presence of NMM (0.11 ml, 1 mmol). The pseudohexapeptide **21** was recrystallized from EtOac (59%); mp: 217–219°C: $[\alpha]_D^{21}$ −22.8 (c 1.0, MeOH); R_f: C: 0.72. Amino acid analysis: Tyr: 0.97; Ala: 1.00; Phe: 1.01; Pro: 0.98; NH_3: 2.04.

Boc-Tyr(Bu^t)-D-Ala-Phe-Gly-ψ(NHCO)Phe-Pro-OBu^t **(22)**
22 was prepared as **21** from Boc-Tyr(Bu^t)-D-Ala-Phe-gGly-H and HO-*m(R, S)*Phe-Pro-OBu^t. The title compound was recrystallized from EtOAc (65%), mp: 236–238°C; $[\alpha]_D^{21}$ −19.5 (c 1.0, DMF); R_f: C: 0.69. Amino acid analysis: Tyr: 0.96; Ala: 1.00; Phe: 1.03; Pro: 0.97; NH_3: 2.06.

Preparation of free hexapeptides ***I–XI***
Each Boc-hexapeptide was deprotected according to *Method D*. The resulting free compound (1 mmol) was dissolved in 0.5 N acetic acid (4 ml) and passed through a 2 × 30 cm Sephadex G25 column, with solvent system (A). The peptide trifluoroacetates were converted into corresponding acetates through anion exchanger resin DE52 Whatman (acetate form) using 0.2 N acetic acid as eluting solvent. The fractions containing the peptide were collected and lyophilized to a constant weight (80–85%). Characterization of free peptides **I–XI** is reported in Table I.

Binding assays
The homogenates of male guinea pig brains were prepared as described by Gillan *et al.* [12]. Briefly, animals were sacrificed by cervical dislocation, brains were removed, and after excision of the cerebellum the brain tissue was homogenized in a 50 mM Tris buffer (ph 7.4 at 0°C) and centrifuged at 48 000 rpm for 10 min; the pellet was then resuspended in Tris buffer, incubated at 37°C for 45 min, and centrifuged again.

For the binding assays, 1.8 ml of final homogenate corresponding to 18 mg of brain tissue was used and the volume made up to 2.0 ml with solutions of the inhibitory cold ligands and of the tritiated ligand. The mixture was incubated for 40 min at 25°C, filtered through Whatman GF/B glass filters, and washed 3 times with ice-cold Tris buffer. [^{3}H]D-Pen^2, D-Pen^5 enkephalin (DPDPE) (1 nM), 51.5 Ci/mmol (NEN Research Products), was used as δ ligand and [^{3}H] [D-Ala^2, MePhe^4, Gly-ol^5]-enkephalin (DAGO) (0.6 nM), 45 Ci/mmol (Amersham), was used as μ ligand. (±)-[^{3}H] ethylketazocine (EKC) (0.6 nM), 20 Ci/mmol (New England Nuclear), was used as κ ligand. The binding of [^{3}H]EKC was measured in the presence of unlabeled DAGO (100 nM) and unlabeled DPDPE (100 nM). These concentrations of unlabelled ligands are 100 times larger than their K_d values and suppress μ and δ binding by [^{3}H]EKC without affecting κ binding [21]. From the total binding, specific binding was obtained by deducing the non-specific binding which was not inhibited by 10^{-3} M bremazocine. To determinate IC_{50} values (*i.e.*, values for 50% inhibition of specific [^{3}H]DAGO, [^{3}H]DPDPE and [^{3}H]EKC binding) of the peptide under examination, the compounds were added, in triplicate, to the binding assays in at least 6 different concentrations. The IC_{50} values were calculated by probit analysis.

Analgesic assay
The analgesic potency of peptides was estimated in Swiss mice weighing 27–30 g. The tail-flick test was essentially that described by Janssen [13] using water at 55°C as nociceptive stimulus. Tests were made prior to and at various times after s.c. administration of each compound in saline (4 μl). The average reaction time in control animals was 1 s. Complete analgesia was assumed to be present when no reaction appeared 10 s after application of noxious stimulus. Percent analgesia was calculated according to the formula (T−To/10−To)·100 (T = reaction time (s) after administration of compound, To = "normal" reaction time before injection of compound; 10 = cut off time). The specificity of the effects was tested by pretreating the animals with naloxone hydrochloride 0.5–1.0 mg/kg s.c. In all cases, the antagonist prevented any analgesic effect.

Metabolism of peptides
The peptide degradation was studied in rat plasma, brain or kidney obtained from male Sprague–Dawley rats (250–300 g). Either the brain or kidney was homogenized in 5 vol (w/v) Tris/HCl (50 mM, pH 7.4, at 0°C) with an ultra-Turrax (Janke & Kundel, Staufen, FRG) using 3 × 15 s bursts. The supernatant obtained after centrifugation (3 000 *g* for 15 min, at 4°C) was decanted off and used for the degradation studies. A 50 μl volume of peptide (2 mg/ml) was added to 0.5 ml of plasma or supernatant and mixed on a vortex mixer. The solution was incubated at 37°C for varying periods of time up to 180 min; the incubation was terminated by addition of ethanol (1.0 ml). After centrifugation (5 000 rpm for 5 min), an aliquot (10 μl) of the clear supernatant was injected into the RP-HPLC column. The degradation half-life ($T_{1/2}$) was

obtained by a least-squares linear regression analysis of a plot of logarithmic peptide concentration *versus* time, using a minimum of 5 points. Where an error limit (standard error) is indicated, values were determined in at least 3 separated assays.

[Leu^5]enkephalin (2 mg/ml) was used as a standard compound to check the degradative activity of plasma and homogenate preparations.

HPLC of analogues **(I–XII)** and related fragments were achieved as previously described for the analysis of DER and metabolites [8].

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