Articles

Synthesis and Activity Profiles of New Dermorphin-(1–4) Peptide Analogues†

Mauro Marastoni, Severo Salvadori, Gianfranco Balboni, Pier Andrea Borea,‡ Giuliano Marzola,‡ and Roberto Tomatis*

Department of Pharmaceutical Chemistry and Institute of Pharmacology, University of Ferrara, Ferrara, Italy.
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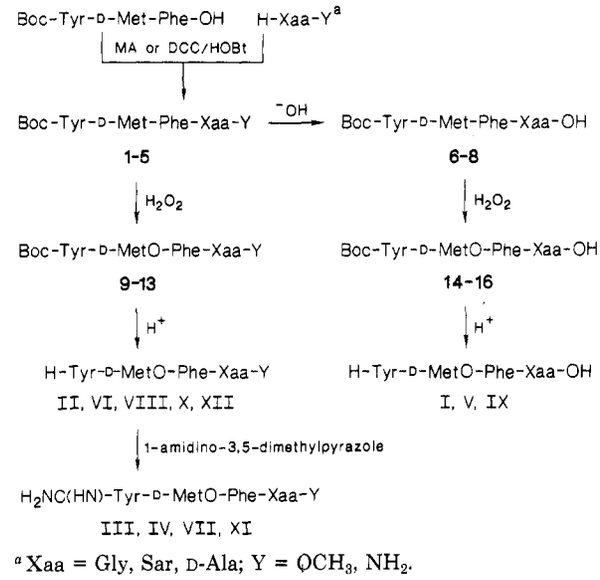
A new series of 12 dermorphin tetrapeptides, W-Tyr-D-MetO-Phe-Xaa-Y (W = H, $H_2NC{=}(NH)$; Xaa = Gly, Sar, D-Ala; Y = OH, OCH_3, NH_2) were prepared by traditional methods in solution and tested for opioid activity. In binding studies based on displacement of μ, δ, and κ opioid receptor selective radiolabels from guinea pig brain membranes, the new analogues showed a negligible affinity for the κ binding site and a preference for μ- over δ-receptors with an evident dependence on N- and/or C-terminal modifications; H-Tyr-D-MetO-Phe-Gly-OCH_3 was shown to be one of the most selective μ-receptor ligands reported to date. All these tetrapeptides display dose-related naloxone-reversible antinociceptive effects following intracerebroventricular (icv) or subcutaneous (sc) administrations in mice. In comparison to morphine, H-Tyr-D-MetO-Phe-Sar-NH_2 and the guanidino derivative $H_2NC{=}(NH)$-Tyr-D-MetO-Phe-Gly-NH_2 showed lower affinity for μ, δ, and κ sites but exceptionally stronger analgesia: respectively they are 560 and 1550 times as potent an analgesic as morphine. Among analogues tested after sc administration, H-Tyr-D-MetO-Phe-Sar-NH_2 and H-Tyr-D-MetO-Phe-D-Ala-OH displayed the highest activities; they were respectively 22 and 30 times more potent than morphine on a molar basis. These results indicate that N- or C-terminal modifications and substitution at position 2 or 4 of dermorphin-(1–4) peptide do not only influence the affinity of the resulting analogues to opioid receptors but also may favorably alter their pharmacokinetic properties.

Since the isolation and identification of dermorphin, H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2, in amphibian skin extracts,[1] a great number of analogues have been synthesized and tested for opioid activity.[2] Studies on the structure–activity relationship have shown that (i) dermorphin-(1–4)-NH_2 displays a higher relative potency in the tests for analgesia than in the assays in vitro,[2b,3] (ii) D-Ala[2] could be successfully replaced by D-Arg[4] or D-MetO,[2c,5] (iii) the substitution of Gly[4] is well tolerated,[2a,6] and (iv) the replacement of the terminal amino group by a guanidino function is advantageous.[2a,7] Recently, in the course of biological studies on tetrapeptides H-Tyr-D-MetO-Phe-Gly-Y (Y = NH_2, NH-alkyl), we observed that, following intracerebroventricular or subcutaneous administration in mice, H-Tyr-D-MetO-Phe-Gly-NH_2 was about respectively 1500 and 17 times as potent an analgesic as morphine.[2c,5]

Therefore modification to the dermorphin-(1–4) sequence could result in oligopeptides with very potent and long-lasting analgesic activity even after systemic administration. For this reason, we synthesized 12 new [D-MetO^2]dermorphin tetrapeptides and investigated their antinociceptive effects as well as their affinity for μ, δ, and κ binding sites in the guinea pig brain.

Attention in the present work was focused on studying the influence of substitution at position 4 and the modifications at the N- and/or C-termini on the opioid character of these analogues.

Scheme I. Synthesis of [D-MetO^2]dermorphin Tetrapeptide Analogues



[a] Xaa = Gly, Sar, D-Ala; Y = OCH_3, NH_2.

† Abbreviations according to IUPAC–IUB Commission on Biochemical Nomenclature, *Eur. J. Biochem.* **1984**, *138*, 9–37, are used throughout. Other abbreviations used are as follows: AcOH, acetic acid; Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DMF, dimethylformammide; Et_2O, diethyl ether; EtOAc, ethyl acetate; EtOH, ethyl alcohol; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MA, mixed anhydride; MeOH, methyl alcohol; mp, melting point; NMM, *N*-methylmorpholine; OMe, methyl ester; OSu, *N*-hydroxysuccinimidyl ester; PE, petroleum ether; Sar, sarcosine; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography.

‡ Institute of Pharmacology.

(1) (a) Montecucchi, P. C.; de Castiglione, R.; Piani, S.; Gozzini, L.; Erspamer, V. *Int. J. Pept. Protein Res.* **1981**, *17*, 275. (b) Montecucchi, P. C.; de Castiglione, R.; Erspamer, V. *Int. J. Pept. Protein Res.* **1981**, *17*, 316.

(2) (a) Tomatis, R.; Salvadori, S.; Sarto, G. P. In *Peptides 1982*; Blaha, K., Malons, P., Eds.; W. de Gruyter: Berlin, 1983; p 495. (b) de Castiglione, R. In *Highlights in Receptor Chemistry*; Melchiorre, C., Giannella M., Eds.; Elsevier Biomedical: Amsterdam, 1984; p 149. (c) Salvadori, S.; Marastoni, M.; Balboni, G.; Sarto, G. P.; Tomatis, R. *J. Med. Chem.* **1986**, *29*, 889 and references cited therein.

(3) (a) Broccardo, M.; Erspamer, V.; Falconieri Erspamer, G.; Improta, G.; Linari, G.; Melchiorri, P.; Montecucchi, P. C. *Br. J. Pharmacol.* **1981**, *73*, 625. (b) Salvadori, S.; Sarto, G. P.; Tomatis, R. *Int. J. Pept. Protein Res.* **1982**, *19*, 536.

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Table I. Physicochemical Properties of Protected Tetrapeptides

no.	Xaa	Y	% yield	mp, °C	crystn solvents	$[\alpha]^{22}_D$	R_f	formula	anal.
				Boc-Tyr-D-Met-Phe-Xaa-Y					
1	Gly	OCH_3	71	173–175	$AcOEt/Et_2O$	+1.2[a]	0.62[c]	$C_{31}H_{42}N_4O_8S$	C, H, N
2	Sar	OCH_3	60	96–98	Et_2O	+20.9[a]	0.69[c]	$C_{32}H_{44}N_4O_8S$	C, H, N, S
3	D-Ala	OCH_3	72	106–108	AcOEt	+18.3[a]	0.65[c]	$C_{32}H_{44}N_4O_8S$	C, H, N
4	Sar	NH_2	61	149–151	AcOEt	+5.3[b]	0.67[c]	$C_{31}H_{43}N_5O_7S$	C, H, N
5	D-Ala	NH_2	75	109–111	AcOEt	−12.1[b]	0.64[c]	$C_{31}H_{43}N_5O_7S$	C, H, N, S
6	Gly	OH	86	116–118	Et_2O	+1.4[a]	0.44[d]	$C_{30}H_{40}N_4O_8S$	C, H, N
7	Sar	OH	81	105–107	Et_2O	+12.5[a]	0.48[d]	$C_{31}H_{42}N_4O_8S$	C, H, N
8	D-Ala	OH	84	123–125	$AcOEt/Et_2O$	+8.6[a]	0.45[d]	$C_{31}H_{42}N_4O_8S$	C, H, N, S
				Boc-Tyr-D-MetO-Phe-Xaa-Y					
9	Gly	OCH_3	91	176–178	$CHCl_3/Et_2O$	+3.5[a]	0.50[c]	$C_{31}H_{42}N_4O_9S$	C, H, N, S
10	Sar	OCH_3	89	102–104	$CHCl_3/Et_2O$	+12.4[a]	0.54[c]	$C_{32}H_{44}N_4O_9S$	C, H, N
11	D-Ala	OCH_3	88	177–179	$CHCl_3/Et_2O$	+19.4[a]	0.55[c]	$C_{32}H_{44}N_4O_9S$	C, H, N
12	Sar	NH_2	87	133–135	$MeOH/Et_2O$	+2.6[b]	0.51[c]	$C_{31}H_{43}N_5O_8S$	C, H, N
13	D-Ala	NH_2	93	117–119	$MeOH/Et_2O$	−13.2[b]	0.50[c]	$C_{31}H_{43}N_5O_8S$	C, H, N, S
14	Gly	OH	86	134–136	$CHCl_3/Et_2O$	+2.1[a]	0.40[d]	$C_{30}H_{40}N_4O_9S$	C, H, N
15	Sar	OH	83	149–151	$CHCl_3/Et_2O$	+14.5[a]	0.45[d]	$C_{31}H_{42}N_4O_9S$	C, H, N, S
16	D-Ala	OH	85	129–131	$CHCl_3/Et_2O$	+6.8[a]	0.39[d]	$C_{31}H_{42}N_4O_9S$	C, H, N

[a] *c* 1.0 (methanol). [b] *c* 1.0 (dimethylformamide). [c] Solvent system (C). [d] Solvent system (B).

Table II. Physicochemical Properties of New Dermorphin-(1–4) Tetrapeptide Analogues (W-Tyr-D-MetO-Phe-Xaa-Y)

no.	W	Xaa	Y	mp, °C	$[\alpha]^{22}_D$	R_f	formula	anal.
I	H	Gly	OH	152–154	+26.1[a]	0.44[f]	$C_{25}H_{32}N_4O_7S\cdot C_2HO_2F_3$	C, H, N, S
II	H	Gly	OCH_3	197–199	+35.2[b]	0.52[f]	$C_{26}H_{34}N_4O_7S$	C, H, N
III	$H_2NC(NH)$	Gly	OCH_3	173–175	−9.8[c]	0.38[g]	$C_{27}H_{36}N_6O_7S\cdot C_2H_4O_2$	C, H, N, S
IV	$H_2NC(NH)$	Gly	NH_2	161–163	+18[d]	0.22[g]	$C_{26}H_{35}N_7O_6S\cdot C_2H_4O_2$	C, H, N
V	H	Sar	OH	164–166	+28.3[a]	0.49[f]	$C_{26}H_{34}N_4O_7S\cdot C_2HO_2F_3$	C, H, N, S
VI	H	Sar	OCH_3	107–109	+13.6[a]	0.54[f]	$C_{27}H_{36}N_4O_7S$	C, H, N
VII	$H_2NC(NH)$	Sar	OCH_3	186–188	−5.3[e]	0.44[g]	$C_{28}H_{38}N_6O_7S\cdot C_2H_4O_2$	C, H, N
VIII	H	Sar	NH_2	135–137	+4.4[c]	0.45[f]	$C_{26}H_{35}N_5O_6S\cdot C_2HO_2F_3$	C, H, N
IX	H	D-Ala	OH	142–144	+30.9[a]	0.58[f]	$C_{26}H_{34}N_4O_7S\cdot C_2HO_2F_3$	C, H, N, S
X	H	D-Ala	OCH_3	109–111	+10.3[d]	0.54[f]	$C_{27}H_{36}N_4O_7S$	C, H, N
XI	$H_2NC(NH)$	D-Ala	OCH_3	158–160	+0.4[d]	0.49[g]	$C_{28}H_{38}N_6O_7S\cdot C_2H_4O_2$	C, H, N
XII	H	D-Ala	NH_2	139–141	−1.1[e]	0.48[f]	$C_{26}H_{35}N_5O_6S\cdot C_2HO_2F_3$	C, H, N

[a] *c* 1.0 (methanol). [b] *c* 1.0 (acetic acid). [c] 1.0 (dimethylformamide). [d] *c* 0.5 (methanol). [e] *c* 0.5 (dimethylformamide). [f] Solvent system A. [g] Solvent system G.

Chemistry. Various conventional methods were tested to obtain the new series of [D-MetO[2]] tetrapeptides (I–XII). Modifications only in position 4 suggested a route in which the invariant segment 1–3 (Boc-Tyr-D-Met-Phe-OH) is first prepared and is subsequently condensed with the appropriate amino acid derivative, Xaa-Y, yielding peptides **1–5** (Scheme I). Under the conditions of this reaction, partial racemization of phenylalanine could not be excluded a priori. We therefore determined the presence of D-Phe in the hydrolysate by gas chromatography of the derivatized amino acids on chiral polysiloxane.[8] Stereochemically uniform Boc-D-Met-Phe-Xaa-amides were used as standards. In all instances substantial amounts of D-Phe enantiomer were not found. Synthetic trials led to the following less favorable observations. (i) Boc- or Z-Phe-Xaa-Y were obtained in relatively good yields after purification by column chromatography: these protected dipeptides are oils or solids difficult to recrystallize. (ii) Cleavage of Boc or Z groups in Sar-containing dipeptides[6] leads to dioxopiperazines when Y = OMe, or OBzl, or even NH_2. As described previously,[2c,9] each Boc-tetrapeptide-sulfide (**1–5** and **6–8**) was oxidized with hydrogen peroxide to yield the corresponding sulfoxides (**9–13** and **14–16**). Treatment of compounds **9–16** with trifluoroacetic acid in methylene chloride containing anisole (0.1%) gave the expected analogues free acids (I, V, IX), esters (II, VI, X), and amides (VIII, XII).

The guanidino derivatives III, IV, VII, and XI were obtained by treating the pertinent peptide acetate with 1-amidino-3,5-dimethylpyrazole acetate.[7] Final purification was accomplished by partition chromatography on Sephadex G-25 or when necessary by HPLC. The homogeneity of intermediate peptides and target analogues was checked by TLC, HPLC, and amino acid and elemental analyses (see Tables I and II).

Analgesic and Binding Assays. The new compounds were tested in vivo by a mouse tail-flick[10] assay after intracerebroventricular and subcutaneous administrations. Relative opioid receptor affinities were determined by displacement of selective radioligands from guinea brain membrane preparations.[11] [^{3}H]DAGO served as a highly selective μ-receptor radioligand, and the somewhat less selective radiolabeled [^{3}H]DADLE and [^{3}H]EKC were used respectively for determining relative δ- and κ-receptor

(4) Sasaki, Y.; Matsui, M.; Taguchi, M., Suzuki, K.; Sakurada, S.; Sato, T.; Sakurada, T.; Kisara, K. *Biochem. Biophys. Res. Commun.* **1984**, *120*, 214.
(5) Salvadori, S.; Marastoni, M.; Balboni, G.; degli Uberti, E.; Tomatis, R. *Peptides* **1985**, *6* (Suppl 3), 127.
(6) Salvadori, S.; Balboni, G.; Marastoni, M.; Sarto, G. P.; Tomatis, R. *Hoppe-Seyler's Z. Physiol. Chem.* **1984**, *365*, 1199.
(7) Salvadori, S.; Sarto, G. P.; Tomatis, R. *Eur. J. Med. Chem.* **1983**, *18*, 489.
(8) Frank, H.; Nicholson, G. J.; Bayer, E. *J. Cromatogr. Sci.* **1977**, *15*, 174.
(9) Fujii, N.; Sasaki, T.; Funakoshi, S.; Irie, M.; Yajima, *H. Chem. Pharm. Bull.* **1978**, *26*, 650.
(10) Janssen, P. A.; Memegeers, C. J.; Dony, J. D. *Arzneim. Forsch.* **1963**, *13*, 502.
(11) Gillan, M. G. C.; Kosterlitz, H. W.; Paterson, S. *Br. J. Pharmacol.* **1980**, *70*, 481.

Table III. Binding Assays of Dermorphin Tetrapeptides (W-Tyr-D-MetO-Phe-Xaa-Y)

				[^{3}H]DAGO (μ)		[^{3}H]DADLE (δ)			
no.	W	Xaa	Y	IC_{50},[a] nM	relative potency[b]	IC_{50},[a] nM	relative potency[b]	$IC_{50}\,\delta/IC_{50}\,\mu$	[^{3}H]EKC (K): IC_{50}, nM
I	H	Gly	OH	61 ± 10	0.07	1980 ± 205	0.07	32	>10000
II	H	Gly	OCH_3	5 ± 0.4	0.80	850 ± 90	0.23	170	>10000
III	$H_2NC(NH)$	Gly	OCH_3	23.5 ± 1.0	0.17	285 ± 10	0.65	12	>10000
IV	$H_2NC(NH)$	Gly	NH_2	5 ± 0.6	0.80	300 ± 56	0.62	60	>10000
V	H	Sar	OH	77 ± 2.4	0.05	2120 ± 52	0.09	27	>10000
VI	H	Sar	OCH_3	6 ± 0.3	0.67	510 ± 20	0.36	85	>10000
VII	$H_2NC(NH)$	Sar	OCH_3	23 ± 0.8	0.17	150 ± 19	1.23	6.5	>10000
VIII	H	Sar	NH_2	6 ± 0.6	0.67	440 ± 23	0.42	73	>10000
IX	H	D-Ala	OH	120 ± 20	0.03	2400 ± 110	0.07	20	>10000
X	H	D-Ala	OCH_3	9 ± 0.7	0.4	1400 ± 210	0.15	155	>10000
XI	$H_2NC(NH)$	D-Ala	OCH_3	280 ± 20	0.01	2700 ± 250	0.07	9.5	>10000
XII	H	D-Ala	NH_2	17 ± 2.0	0.24	900 ± 152	0.21	53	>10000
I′	H	Gly	NH_2	6.3 ± 0.8	0.63	312 ± 20	0.59	50	>10000
D	dermorphin			5.7 ± 0.7	0.70	210 ± 25	0.88	37	>10000
DT	dermorphin-(1–4)-amide			36.7 ± 7.1	0.11	818 ± 118	0.23	22	>10000
	morphine			4 ± 0.8	1	185 ± 25	1	46	530 ± 66

[a] Concentration that gives half-maximal effect. [b] Relative potencies are on a molar basis (morphine = 1).

Table IV. Analgesic Activity of Dermorphin Tetrapeptide Analogues (Tail-Flick Test)

	W-Tyr-D-MetO-Phe-Xaa-Y			analgesia[a]		rel potencies[b]	
				icv:	sc:		
no.	W	Xaa	Y	ED_{50}, pmol/mouse	ED_{50}, μmol/kg	icv	sc
I	H	Gly	OH	20.5 (12.6–41.7)	4.64 (2.69–11.5)	150	2.3
II	H	Gly	OCH_3	10.0 (5.5–16.3)	4.21 (2.60–17.0)	309	2.6
III	$H_2NC(NH)$	Gly	OCH_3	15.5 (11.8–23)	2.39 (1.40–4.89)	199	4.5
IV	$H_2NC(NH)$	Gly	NH_2	2.0 (0.51–9.7)	0.53 (0.31–1.09)	1545	20
V	H	Sar	OH	45.8 (34–72)	4.63 (2.51–17.4)	67	2.3
VI	H	Sar	OCH_3	47.9 (31–85)	4.05 (2.92–8.75)	64	2.7
VII	$H_2NC(NH)$	Sar	OCH_3	38.1 (16.3–87.1)	0.75 (0.48–1.51)	81	14.4
VIII	H	Sar	NH_2	5.5 (2.8–12.6)	0.48 (0.28–0.80)	561	22.5
IX	H	D-Ala	OH	8.4 (6.1–11.4)	0.36 (0.21–1.06)	368	30
X	H	D-Ala	OCH_3	18.2 (14.8–58)	5.16 (3.48–1495)	170	2
XI	$H_2NC(NH)$	D-Ala	OCH_3	209 (75.9–502)	7.23 (4.89–20.3)	14.7	1.4
XII	H	D-Ala	NH_2	33 (27–40.8)	4.38 (2.64–10.0)	93	2.5
I′	H	Gly	NH_2	2.01 (1.35–3.36)	0.66 (0.54–0.83)	1537	16.3
D	dermorphin			11.4 (6.6–27.1)	2.75 (1.73–6.4)	271	3.9
DT	dermorphin-(1–4)-amide			68.8 (46.4–120)	60.3 (26.7–99.1)	45	0.2
	morphine			3090 (2550–4200)	10.8 (7.95–14.5)	1	1

[a] Analgesia of at least four doses of each compound was investigated. Each dose was tested for at least eight animals. The ED_{50} was estimated at the time of peak activity. [b] Relative potencies are on a molar basis (morphine = 1).

affinities. The binding assay data and antinociceptive effects of the 12 new analogues are reported in Tables III and IV and are shown in comparison with the parent H-Tyr-D-Ala-Phe-Gly-NH_2 (DT), dermorphin, and morphine.

Results and Discussion

As previously observed for a different series of [D-MetO^2] tetrapeptides,[12] none of the present analogues 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 the [^{3}H]DAGO and [^{3}H]DADLE binding assays, all new tetrapeptides I–XII, like dermorphin and its shorter homologue DT, displayed a more marked preference for μ-subtypes over δ-receptors. Whereas the substitution of Gly^4 for Sar in the parent peptides I–III and I′ was well tolerated (compounds V–VIII), the introduction of D-Ala^4 (peptides IX–XII) lowered the affinity for both μ and δ sites. Moreover, tetrapeptide esters II, VI, and X or amides VIII, XII, and I′ are more μ-receptor selective than corresponding tetrapeptide acids I, V, and IX, confirming the trend previously observed in the guinea pig ileum and mouse vas deferens for dermorphin-related peptides.[3b,7,13]

Finally the substitution of the N-terminal group by the guanidino function influences the affinity in the binding assay in a different manner. In comparison to the parent peptide II, guanidino derivative III is 5 times less potent in the [^{3}H]DAGO binding assay and, most interestingly, 3 times more potent in the [^{3}H]DADLE binding assay. Consequently, III is relatively nonselective, as indicated by its low $IC_{50}\,\mu/IC_{50}\,\delta$ ratio. Like III, tetrapeptides VII and IX also contain a guanidino function and, in analogy to III, they are about 10 times less selective than the parent compounds VI and X, respectively. A different behavior pertains to the guanidino tetrapeptide IV; it showed, in fact, about the same affinity as its structurally related peptide I′, and therefore analogue IV is still μ-receptor selective. These data are in agreement with our results on isolated organs, where for [D-Ala^2 or D-MetO^2] tetrapeptides, N- and/or C-terminal modifications strongly influence the selective potency in the guinea pig ileum and mouse vas deferens.[2a,7,13]

In the tail-flick test (Table IV), icv administration of new [D-MetO^2] analogues I–XII displayed a greater antinociceptive action than morphine, confirming our previous data concerning different series of [D-MetO^2]dermorphin tetrapeptides.[2c,5] However, some interesting discrepancies

(12) Marastoni, M.; Salvadori, S.; Borea, G. P.; Bertelli, G.; Tomatis, R. *Farmaco Ed. Sci.* **1987**, *42*, 125.

(13) Sarto, G.; Borea, P. A.; Salvadori, S.; Tomatis, R. *Pharmacology* **1984**, *29*, 56.

between binding assay data and icv analgesia of [Sar[4] or D-Ala[4]] analogues were observed: for instance [D-Ala[4]] tetrapeptide acid IX showed a rather high analgesic activity in contrast to its low binding affinity. The discrepancy between the potencies observed in the tail-flick test and the [^{3}H]DAGO binding assay is also pronounced in the case of the tetrapeptide ester VI, which relative to its parent acid V is 13 times more potent in the binding assay but only equipotent in the in vivo test. Better qualitative agreement between icv analgesia and binding assays data is apparently observed with the other analogues. Guanidino derivative IV is not only equipotent to its highly active parent peptide I′ but it is 1500 and 6 times more potent than morphine and dermorphin, respectively.

As recently observed,[12] the data on binding assays reported in Table III and icv analgesia of Table IV indicate that, in comparison to morphine, the enhancement of analgesic action of dermorphin and its analogues was not corelatable to the affinity for μ-, δ, or κ-receptors. When compared to morphine, for example, tetrapeptides IV and VIII displayed a lower affinity for μ, δ, and κ sites but showed the highest icv analgesic activities among the new analogues tested in this study. In the tail-flick test, compounds IV and VIII were respectively 1550 and 560 times more potent than morphine. Evaluation of the antinociceptive effects of I–XII after sc injection in mice (Table IV) revealed additional differences due to structural modifications to dermorphin-(1–4) peptide DT. [D-MetO[2]] analogue I showed remarkably increased activity: 2.3 times as potent as morphine and 14 times more potent than DT, which had two-tenths of the potency of morphine. Tetrapeptide II formed by C-terminal esterification of I exhibited a slightly increased activity but not comparable to the higher potency of parent peptide amide I′.

In comparison with these monosubstituted analogues, comparable or higher activities were obtained with double-substituted analogues V–XII, in which Gly[4] was also replaced by Sar or D-Ala to stabilize the C-terminus against carboxypeptidases. Tetrapeptides VII and IX displayed the highest sc activities among the analogues tested in this study: they were respectively 22 and 30 times more potent than morphine.

Conclusions

Among the new series of [D-MetO[2]]dermorphin tetrapeptides described in this paper, the analogues II and X show a very high preference for μ-receptors over δ-receptors as a consequence of their poor affinity for δ sites and their negligible affinity for κ sites. In the binding assays, peptide II displayed an $IC_{50}\,\delta/IC_{50}\,\mu$ ratio 10 times higher than morphiceptin (H-Tyr-Pro-Phe-Pro-NH_2)[14] and comparable to that of H-Tyr-D-Orn-Phe-Asp-NH_2, a cyclic opioid peptide that seems to be one of the most selective μ-ligands reported to date.[15] In comparison with II and X, the other new tetrapeptides showed a decreased selectivity most likely as a consequence of C- and/or N-terminal modifications.

There was no correlation between the affinity for each type of receptor and the icv antinociceptive activity of dermorphin peptides.

Concerning the relatively greater potency of few Sar[4] or D-Ala[4] derivatives, as compared to the corresponding Gly[4] analogues, it is notable that in each case maximal sc analgesia developed at 60 min, a finding that suggests a differential rate of enzymic degradation does not underlie their differing antinociceptive potencies. Thus, the potent sc analgesia of new analogues can be due to adequate central nervous system permeation, but other factors (diffusion from subcutaneous sites, entry into blood, and binding to plasma proteins) may be more important than metabolic stability.

Despite all this, analogue IX seems to be the most potent analgesic following systemic administration among opioid peptides reported in the literature: it seems, in fact, to bear a very favorable comparison with the excellent analgesics in the dermorphin–enkephalin series such as H-Tyr-D-Arg-Phe-Sar-OH,[4] FK-33824,[16] and H-Tyr-D-MetO-Gly-MePhe-ol.[17] Further work on the new compounds synthesized will be published elsewhere.

Experimental Section

Melting points were determined on a Tottoli apparatus in open capillaries and are uncorrected. Optical rotations were determined with a Perkin-Elmer 141 polarimeter with a 10-cm water-jacketed cell. HPLC analysis was performed on a Bruker liquid chromatograph LC21-C equipped with a Bruker LC313 UV variable-wavelength detector. Recording and quantification were accomplished with a chromatographic data processor (Epson computer FX-80X). A 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 were filtered and degassed prior to use. Analytical determinations for deprotected peptides were carried out by a gradient made up of two solvents: A, 10% (v/v) acetonitrile in water; B, 60% (v/v) acetonitrile in water, both containing 4.5 mM TFA and 4.9 mM TEA. The gradient program used was as follow: linear gradient from 10% to 25% B in 10 min; isocratic 25% B for 5 min; linear gradient from 25% to 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 being monitored 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. In the amino acid analysis of Sar-containing peptides, sarcosine gave a peak with a retention time between aspartic acid and serine. Peptides containing the methionine *S*-oxide residue were hydrolyzed in the presence of an equimolar amount of thioglycolic acid to optimize the recovery of Met. 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 (12:8:3). 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 indicated by the symbols of the elements refer to data within ±0.4% of the theoretical values. 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), unless stated otherwise.

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 was added 1 equiv of *N*-methylmorpholine (NMM); the mixture was cooled to −10 °C, treated with isobutyl chloroformate (IBCF) (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

(14) Chang, K. J.; Killian, A.; Nazum, E.; Cuatrecasas, P.; Chang, J. K. *Science (Washington, D.C.)* **1981**, *212*, 75.
(15) Schiller, P. W.; Nguyen, T. M. D.; Lemieux, C.; Maziak, L. A. *J. Med. Chem.* **1985**, *28*, 1766.
(16) Roemer, D.; Buescher, H. H.; Hill, R. C.; Pless, J.; Bauer, W.; Cardinaux, F.; Closse, A.; Hauser, D.; Huguenim, R. *Nature (London)* **1977**, *268*, 547.
(17) Kiso, Y.; Yamaguchi, M.; Akita, T.; Nakamura, H. *Naturwissenschaften* **1981**, *68*, 210.

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), 1-hydroxybenzotriazole (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 (DCU) was filtered off, and the solution was diluted with EtOAc (100 mL) and worked up as described in method A.

Deprotection. Method C. Boc 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 Et_2O or petroleum ether (PE); the resulting solid peptide was collected and dried.

Method D. The peptide methyl ester was suspended in a 2:1 mixture of MeOH–H_2O (7.5 mL/mmol) and treated with 1.2 molar equiv of 1 N NaOH for 2 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.

Preparation of Boc-Tyr-D-Met-Phe-Xaa-Y (1–8). According to general coupling procedures A or B, Boc-Tyr-D-Met-Phe-OH[2c] was treated with H-Xaa-Y. Crude compounds **1–3**, **5** were crystallized from appropriate solvents, and peptide **4** was purified on a silica gel column (1 × 30 cm) with use of the solvent system F. According to the deprotection procedure D, esters **1–3** (Scheme I and Table I) were treated with NaOH. The resulting peptide acids **6–8** were recrystallized from Et_2O. Characterization of the title products are summarized in Table I.

Preparation of Boc-Tyr-D-MetO-Phe-Xaa-Y (9–16). Each Boc-peptide-sulfide (1–8) (1 mmol) was dissolved in acetic acid (15 mL) and cooled to 0 °C, and 11.2 M H_2O_2 (0.1 mL, 1.1 mmol) was added. After 1 h at 20 °C, the mixture was slowly poured into vigorously stirred Et_2O (150 mL) and the product isolated by filtration. The resulting (*R,S*)-sulfoxide was reprecipitated from appropriate solvents (Table I).

Preparation of Free Tetrapeptides I, II, V, VI, VIII–X, and XII. Each Boc-protected tetrapeptides was deprotected according to procedure C. The resulting free compounds (1 mmol) was dissolved in 0.5 N acetic acid (3 mL) and passed through a 2 × 45 cm Sephadex G-25 column, with solvent system A. The analogues trifluoroacetates I, V, VIII, IX, and XII were crystallized from MeOH–Et_2O (85–90%). The tetrapeptides II, VI, and X were obtained as free bases by water elution of the corresponding trifluoroacetates through Whatman DE52 resin. The fractions containing the peptide were collected and lyophilized to constant weight (80–85%). Amino acid analysis after acid hydrolysis showed the expected composition. Characterizations of the final products are summarized in Table II.

Synthesis of Guanidino Tetrapeptide Acetates. H_2NC=(NH)-Tyr-D-MetO-Phe-Xaa-Y (III, IV, VII, and XI). The title compounds were prepared by amidination of H-Tyr-D-MetO-Phe-Xaa-Y, as free base, (1 mmol) with 1-amidino-3,5-dimethylpyrazole acetate (1.2 mmol) as in ref 7. The crude guanidino derivatives were reprecipitated from EtOH–Et_2O and purified by column chromatography on silica gel (2 × 50 cm) in the solvent system G. The fractions containing the pure compound were evaporated to dryness, and the residue was crystallized from AcOH–Et_2O (50–55%). Physicochemical properties of title analogues are reported in Table II.

Binding Assays. The homogenates of male guinea pig brains were prepared as described by Gillan et al.[11] 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 filteres, and washed three times with ice-cold Tris buffer. [^{3}H][D-Ala^2,D-Leu^5]enkephalin (DADLE) (0.6 nM), 51 Ci/mmol, New England Nuclear, 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 DADLE (100 nM). These concentrations of unlabeled ligands are 100 times larger than their K_d values and suppress μ and δ binding by [^{3}H]EKC without affecting κ binding.[18] From the total binding, specific binding was obtained by deducing the nonspecific 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]DADLE, and [^{3}H]EKC binding) of the peptide under examination, the compounds were added, in triplicate, to the binding assays in at least six different concentrations. The IC_{50} values were calculated by probit analysis. IC_{50} values for morphine were taken from Robson et al.[19]

Pharmacological Assays. The analgesic potency of tetrapeptides and reference compounds was estimated in Swiss–Webster mice weighing 23–25 g. The tail-flick test was essentially that described by Janssen,[10] using water at 55 °C as nociceptive stimulus. Tests were made prior to and at various times after icv and sc 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 $[(T - T_0)/(10 - T_0)] \times 100$ (T = reaction time (seconds) after administration of compound; T_0 = "normal" reaction time before injection of compound; 10 = cutoff time). The specificity of the effects was tested by pretreating the animals with naloxone hydrochloride (0.5–1 mg/kg sc). In all cases, the antagonist prevented any analgesic effect.

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Registry No. **1**, 108269-48-3; **2**, 108269-49-4; **3**, 108269-50-7; **4**, 108269-51-8; **5**, 108269-52-9; **6**, 108269-53-0; **7**, 108269-54-1; **8**, 108269-55-2; **9** (*R*-sulfoxide), 108269-56-3; **9** (*S*-sulfoxide), 108341-70-4; **10** (*R*-sulfoxide), 108269-57-4; **10** (*S*-sulfoxide), 108341-71-5; **11** (*R*-sulfoxide), 108269-58-5; **11** (*S*-sulfoxide), 108341-72-6; **12** (*R*-sulfoxide), 108269-59-6; **12** (*S*-sulfoxide), 108341-73-7; **13** (*R*-sulfoxide), 108269-60-9; **13** (*S*-sulfoxide), 108341-74-8; **14** (*R*-sulfoxide), 108269-61-0; **14** (*S*-sulfoxide), 108341-75-9; **15** (*R*-sulfoxide), 108269-62-1; **15** (*S*-sulfoxide), 108341-76-0; **16** (*R*-sulfoxide), 108269-63-2; **16** (*S*-sulfoxide), 108341-77-1; I, 108269-65-4; I (free base), 108269-64-3; I', 100572-16-5; II, 108269-66-5; II, 108269-68-7; III (free base), 108269-67-6; IV, 108269-70-1; IV (free base), 108269-69-8; V, 108269-72-3; V (free base), 108269-71-2; VI, 108269-73-4; VII, 108269-75-6; VII (free base), 108269-74-5; VIII, 108269-77-8; VIII (free base), 108269-76-7; IX, 108269-79-0; IX (free base), 108269-78-9; X, 108269-80-3; XI, 108269-82-5; XI (free base), 108269-81-4; XII, 108269-84-7; XII (free base), 108269-83-6; BOC-Tyr-D-Met-Phe-OH, 100572-39-2.

(18) Magnan, J.; Paterson, S. J.; Tavani, A.; Kosterlitz, H. W. *Naunyn*-Schmiedeberg's *Arch. Pharmacol.* **1982**, *319*, 197.
(19) Robson, E. L.; Paterson, S. J.; Kosterlitz, H. W. In *Handbook of Psychopharmacology*; Iversen, L. L., Iversen, S. D., Snyder, S. H., Eds.; Plenum: New York, 1983; p 13.