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Studies on Analgesic Oligopeptides. III.[1,2] Synthesis and Analgesic Activity after Subcutaneous Administration of [D-Arg^2]dermorphin and Its N-Terminal Tetrapeptide Analogs

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[D-Arg^2]dermorphin and nineteen N-terminal tetrapeptide analogs were synthesized by a conventional solution method and their analgesic activities after subcutaneous administration to mice were examined. The analgesic effect was assessed by means of the tail pressure test. [D-Arg^2]dermorphin was found to have analgesic potency equal to or slightly greater than that of dermorphin. The N-terminal tetrapeptide, H–Tyr–D-Arg–Phe–Gly–OH (I), showed a potency 4.8 times that of morphine and comparable with that of dermorphin on a molar basis. Among the tetrapeptide analogs, several analogs in which Gly^4 was replaced by sarcosine or D-Ala exhibited very potent activity more than that of I. On the other hand, replacement of Gly^4 by Pro, Leu or D-Leu resulted in a marked decrease in potency. Replacement of either Phe^3 by other aromatic amino acid or D-Arg^2 by other basic D-amino acid gave analogs with greatly decreased activities. However, one analog whose guanidino functionality on D-Arg^2 was blocked by a nitro group, showed activity one-third that of the parent peptide (I). On the basis of these results, the structure–activity relationship for the tetrapeptide is discussed.

Keywords——[D-Arg^2]dermorphin; N-terminal tetrapeptide analog; peptide synthesis; analgesic activity; subcutaneous administration; tail pressure test; structure–activity relationship

Dermorphin (H–Tyr–D-Ala–Phe–Gly–Tyr–Pro–Ser–NH$_2$), isolated from the skin of South American frogs, has potent opioid activity.[3] Studies on its structure–activity relationship have revealed that the N-terminal tetrapeptide is the minimum sequence for the opioid activity.[4] On the other hand, it has been reported that enkephalin analogs having the Tyr^1–D-Arg^2 sequence possess potent analgesic activities after intracisternal[5] or intravenous[6] administration. We have therefore become interested in the effect of the introduction of a basic amino acid, D-Arg, into the dermorphin molecule on the analgesic properties. Thus, a tetrapeptide analog of dermorphin, H–Tyr–D-Arg–Phe–Gly–OEt, has been found to possess a very potent analgesic activity after intracerebroventricular administration in mice[7] and some of its analogs were reported in a preliminary communication to have outstandingly potent analgesic activities exceeding that of morphine even after subcutaneous administration.[8]

In the present paper, we describe the synthesis and the analgesic activity of [D-Arg^2]dermorphin and nineteen N-terminal tetrapeptide analogs, and discuss the structure–activity relationship for the tetrapeptide. All the analogs were synthesized by the conventional solution method. Synthesis of [D-Arg^2]dermorphin was carried out according to the route illustrated in Fig. 1.

Boc–D-Arg(Tos)–OH was coupled with H–Phe–Gly–OEt by the DCC–HOBt method[9] to give Boc–D-Arg(Tos)–Phe–Gly–OEt (**1**) which, after treatment with 4 N hydrochloride in

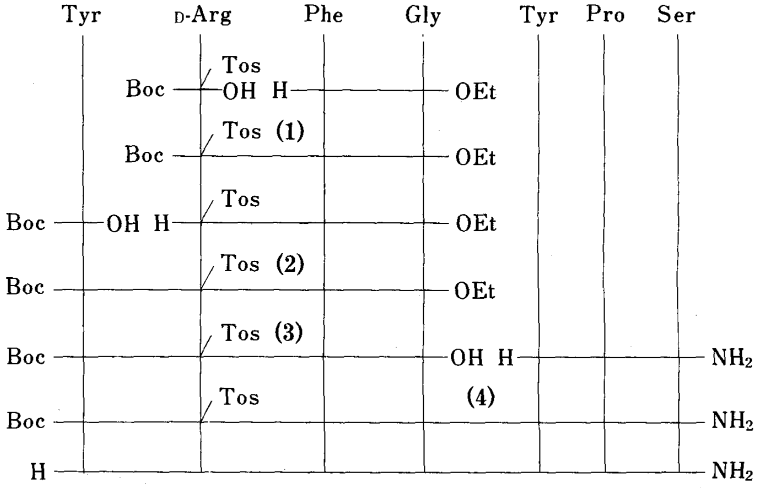

Fig. 1. Synthetic Route to [D-Arg^2]dermorphin

dioxane, was coupled with Boc–Tyr–OH by the same method to give Boc–Tyr–D-Arg(Tos)–Phe–Gly–OEt (**2**). Compound **3** was coupled with H–Tyr–Pro–Ser–NH_2[10] by means of WSCI and HOBt to give Boc–Tyr–D-Arg(Tos)–Phe–Gly–Tyr–Pro–Ser–NH_2 (**4**). Deprotection of **4** was carried out by using the trifluoromethanesulfonic acid–thioanisole system as a deblocking reagent.[11] Crude peptide thus obtained was purified by column chromatography on carboxymethyl cellulose and partition chromatography on Sephadex G-25.[12]

Nineteen analogs of the N-terminal tetrapeptide were synthesized by stepwise addition of single Boc-amino acids starting from the corresponding amino acid methyl, ethyl or benzyl ester in essentially the same manner as described for the preparation of **2**. The tetrapeptide analogs synthesized were as follows, H–Tyr–D-Arg–Phe–X–OY (I: X = Gly, Y = H. II: X = Gly, Y = Et. III: X = Gly, Y = *n*-Pr. IV: X = Sar, Y = H. V: X = Sar, Y = Me. VI: X = Sar, Y = Et. VII: X = D-Ala, Y = H. VIII: X = Pro, Y = H. IX: X = Leu, Y = H. X: X = D-Leu, Y = Me), H–Tyr–D-Arg–X–Gly–OY (XI: X = Phe(NO_2), Y = Et. XII: X = Tyr, Y = H. XIII: X = Trp, Y = Et), H–Tyr–D-Arg–D-Phg–Sar–OEt (XIV), H–Tyr–X–Phe–Gly–OY (XV: X = L-Arg, Y = H. XVI: X = D-Arg(NO_2), Y = Et. XVII: X = D-Har, Y = Et. XVIII: X = D-Lys, Y = Et), H–Tyr(Et)–D-Arg–Phe–Gly–OEt (XIX). For the synthesis of six analogs (I—VI), two tetrapeptide intermediates, **2** and **7**, were built up as described above and saponified with 1 N sodium hydroxide, affording **3** and **8**. The resulting tetrapeptide intermediates were led to the esters, **9** and **10**, by treatment with corresponding alkyl halide in the presence of potassium fluoride.[13] These six intermediate tetrapeptides (**2**, **3** and **7**—**10**) were deprotected by treatment with the trifluoromethanesulfonic acid–thioanisole system. In the case of the syntheses of analogs VII, VIII, IX, XII and XV, a nitro group for the guanidino function of the L- or D-Arg side chain and a benzyl group for the carboxyl function of the corresponding C-terminal amino acid were used; these groups can be removed simultaneously by catalytic hydrogenolysis. For the synthesis of analog XIX, a nitro group for the D-Arg side chain was used and deprotection of the tetrapeptide intermediate (**41**) was carried out by treatment with 4 N hydrochloride in dioxane followed by catalytic hydrogenolysis. The other analogs having D-Arg at position 2 were prepared with the tosyl group for side chain protection and by deprotection of the tetrapeptide intermediates with the trifluoromethanesulfonic acid–thioanisole system. For the synthesis of analog XIII, **31** was treated first with the deblocking reagent described above and then with the methanesulfonic acid–thioanisole system[14] to complete removal of the N^{i}-formyl group on the Trp residue. Analog XVII was prepared by guanidylation of Boc–Tyr–D-Lys–Phe–Gly–OEt, derived from **40** by catalytic hydrogenolysis, with 3,5-dimethylpyrazole-1-carboxamidine in dimethylformamide followed by treatment with 4 N hydrochloride in

Table I. Physicochemical Properties of Intermediates

Compound No.	Structure	mp (°C)	$[\alpha]_D$[a)]	Rf^1[b)]	Formula	Analysis (%) Calcd (Found)		
						C	H	N
5	H–Phe–Sar–OEt·HCl	121—123	−0.2°	0.23	$C_{14}H_{20}N_2O_3 \cdot HCl$	55.40 (55.89	7.13 7.04	9.45 9.31)
6	Boc–D-Arg(Tos)–Phe–Sar–OEt	85—89	−0.5°	0.62	$C_{32}H_{46}N_6O_8S$	56.45 (56.95	7.18 6.87	11.95 12.46)
7	Boc–Tyr–D-Arg(Tos)–Phe–Sar–OEt	114—118	+7.4°	0.76	$C_{41}H_{55}N_7O_{10}S$	58.57 (58.76	6.59 6.62	11.34 11.70)
8	Boc–Tyr–D-Arg(Tos)–Phe–Sar–OH	127—132	+8.0°	0.52	$C_{39}H_{51}N_7O_{10}S$	57.91 (57.83	6.11 6.35	11.81 12.11)
9	Boc–Tyr–D-Arg(Tos)–Phe–Gly–O–*n*-Pr	168—169	−8.4°	0.87	$C_{41}H_{55}N_7O_{10}S$	58.59 (58.76	6.45 6.62	11.51 11.70)
10	Boc–Tyr–D-Arg(Tos)–Phe–Sar–OMe	111—114	+8.1°	0.68	$C_{40}H_{53}N_7O_{10}S$	57.70 (58.31	6.39 6.48	12.20 11.90)
11	Boc–Phe–D-Ala–OBzl	83—84	+12.8°	0.74	$C_{24}H_{30}N_2O_5$	67.58 (67.58	7.06 7.09	6.60 6.57)
12	Boc–D-Arg(NO₂)–Phe–D-Ala–OBzl	85—88	−4.3°	0.58	$C_{30}H_{41}N_7O_8$	57.80 (57.40	6.89 6.58	15.03 15.62)
13	Boc–Tyr–D-Arg(NO₂)–Phe–D-Ala–OBzl	97—100	+7.3°	0.71	$C_{39}H_{50}N_8O_{10}$	58.94 (59.23	6.77 6.37	13.80 14.17)
14	H–Phe–Pro–OBzl·HCl	135—137	−28.6°	0.49	$C_{22}H_{24}N_2O_4 \cdot HCl$	63.32 (63.37	6.13 6.04	6.50 6.72)
15	Boc–D-Arg(NO₂)–Phe–Pro–OBzl	98—101	+40.8°	0.49	$C_{32}H_{43}N_7O_8$	58.62 (58.79	6.49 6.63	14.41 15.00)
16	Boc–Tyr–D-Arg(NO₂)–Phe–Pro–OBzl	119—121	−12.0°	0.63	$C_{41}H_{52}N_8O_{10}$	59.85 (60.28	6.38 6.42	13.40 13.72)
17	H–Phe–Leu–OBzl·HCl	154—156	−15.6°	0.76	$C_{22}H_{28}N_2O_3 \cdot HCl$	65.32 (65.24	7.28 7.22	6.80 6.92)
18	Boc–D-Arg(NO₂)–Phe–Leu–OBzl	96—100	−27.3°	0.65	$C_{33}H_{47}N_7O_8$	59.31 (59.18	7.24 7.07	14.22 14.64)
19	Boc–Tyr–D-Arg(NO₂)–Phe–Leu–OBzl	107—109	−11.4°	0.82	$C_{42}H_{56}N_8O_{10}$	59.99 (60.56	6.80 6.78	13.07 13.45)
20	Boc–Phe–D-Leu–OMe	134—136	+23.3°	0.63	$C_{21}H_{32}N_2O_5$	63.94 (64.26	8.06 8.22	7.27 7.14)
21	Boc–D-Arg(NO₂)–Phe–D-Leu–OMe	106—109	+6.3°	0.47	$C_{27}H_{43}N_7O_8$	54.18 (54.62	7.26 7.30	16.12 16.52)
22	Boc–Tyr–D-Arg(NO₂)–Phe–D-Leu–OMe	114—117	+26.4°	0.67	$C_{36}H_{52}N_8O_{10}$	56.70 (57.13	7.00 6.93	14.42 14.81)
23	Boc–Phe(NO₂)–Gly–OEt	128—129	−1.9°	0.57	$C_{18}H_{25}N_3O_7$	54.57 (54.67	6.41 6.37	10.62 10.63)
24	Boc–D-Arg(Tos)–Phe(NO₂)–Gly–OEt	117—118	−15.8°	0.55	$C_{31}H_{43}N_7O_{10}S$	52.49 (52.75	6.14 6.14	13.76 13.89)
25	Boc–Tyr–D-Arg(Tos)–Phe(NO₂)–Gly–OEt	125—130	+2.3°	0.71	$C_{40}H_{52}N_8O_{12}S$	54.97 (55.29	6.05 6.03	12.63 12.90)
26	Boc–Tyr(Bzl)–Gly–OBzl	91—93	+2.2°	0.73	$C_{30}H_{34}N_2O_6$	69.11 (69.48	6.72 6.61	5.64 5.40)
27	Boc–D-Arg(NO₂)–Tyr(Bzl)–Gly–OBzl	89—92	−8.1°	0.65	$C_{36}H_{45}N_7O_9$	61.21 (61.07	6.53 6.30	12.98 13.62)
28	Boc–Tyr–D-Arg(NO₂)–Tyr(Bzl)–Gly–OBzl	102—106	+9.9°	0.81	$C_{45}H_{56}N_8O_{12} \cdot H_2O$	60.37 (59.99	6.19 6.27	12.06 12.44)
29	Boc–Trp(CHO)–Gly–OEt	59—61	−2.3°	0.58	$C_{21}H_{27}N_3O_6$	60.60 (60.42	6.57 6.52	10.23 10.07)
30	Boc–D-Arg(Tos)–Trp(CHO)–Gly–OEt	102—104	−10.9°	0.55	$C_{34}H_{45}N_7O_9S$	56.62 (56.10	6.39 6.23	13.20 13.47)

TABLE I. (continued)

Compound No.	Structure	mp (°C)	$[\alpha]_D$ a)	Rf^1 b)	Formula	Analysis (%) Calcd (Found) C	H	N
31	Boc–Tyr–D-Arg(Tos)–	136—140	−0.7°	0.62	$C_{43}H_{54}N_8O_{11}S$	58.44	6.40	12.40
	Trp(CHO)–Gly–OEt					(57.96	6.11	12.50)
32	Boc–D-Phg–Sar–OEt	113—114	−3.2°	0.53	$C_{18}H_{26}N_2O_5$	61.59	7.60	8.00
						(61.70	7.48	8.00)
33	Boc–D-Arg(Tos)–	95—97	+7.3°	0.54	$C_{31}H_{44}N_6O_8S$	56.79	7.09	12.19
	D-Phg–Sar–OEt					(56.34	6.71	12.72)
34	Boc–Tyr–D-Arg(Tos)–	119—122	+5.8°	0.57	$C_{40}H_{53}N_7O_{10}S$	57.95	6.49	11.53
	D-Phg–Sar–OEt					(58.31	6.48	11.90)
35	Boc–Arg(NO_2)–Phe–Gly–	87—90	−20.1°	0.51	$C_{29}H_{39}N_7O_8$	56.60	6.35	16.36
	OBzl					(56.76	6.41	15.98)
36	Boc–Tyr–Arg(NO_2)–	112—114	−20.5°	0.50	$C_{38}H_{48}N_8O_{10}$	58.35	6.21	14.55
	Phe–Gly–OBzl					(58.75	6.23	14.43)
37	Boc–D-Arg(NO_2)–Phe–	95—100	−18.2°	0.47	$C_{24}H_{37}N_7O_8$	52.79	6.67	17.21
	Gly–OEt					(52.26	6.76	17.78)
38	Boc–Tyr–D-Arg(NO_2)–	124—127	+3.9°	0.59	$C_{33}H_{46}N_8O_{10}$	55.44	6.69	15.46
	Phe–Gly–OEt					(55.45	6.49	15.68)
39	Boc–D-Lys(Z-Cl)–Phe–	86—87	−7.6°	0.64	$C_{32}H_{43}ClN_4O_8$	59.02	6.86	8.55
	Gly–OEt					(59.38	6.70	8.66)
40	Boc–Tyr–D-Lys(Z-Cl)–	124—126	+2.0°	0.72	$C_{41}H_{52}ClN_5O_{10}$	60.62	6.28	8.64
	Phe–Gly–OEt					(60.76	6.47	8.64)
41	Boc–Tyr(Et)–D-Arg(NO_2)–	105—110	+1.1°	0.62	$C_{35}H_{50}N_8O_{10}$	56.79	6.99	15.15
	Phe–Gly–OEt					(56.59	6.79	15.09)

a) Optical rotations were measured in MeOH ($c=1$) at 20—23 °C. b) See Experimental.

dioxane. Purification of tetrapeptide analogs was performed by column chromatography on carboxymethyl Sepharose and Toyopearl HW-40 and/or partition column chromatography on Sephadex G-25. The homogeneity of each peptide was checked by thin-layer chromatography (TLC), elemental analysis and amino acid analysis. Analytical data for intermediates and target peptides are shown in Tables I and II, respectively.

The analgesic activities of [D-Arg^2]dermorphin and the tetrapeptide analogs were measured in mice by means of the tail pressure test[15] after subcutaneous administration and compared with those of morphine and the parent peptides. As shown in Table III, [D-Arg^2]dermorphin was found to have analgesic potency equal to or slightly greater than that of dermorphin in the present assay system. Although only a few analogs have been prepared for structure–activity studies relating to position 2 in dermorphin,[16] the D-Ala^2 residue has been considered to be of great importance for the activity. This is the first report of a replacement of D-Ala^2 that retains the potency comparable to dermorphin.

Analgesic activities of the tetrapeptide analogs are listed in Table IV. The N-terminal tetrapeptide of [D-Arg^2]dermorphin, I, showed a very pronounced analgesic activity which is slightly higher than that of dermorphin or [D-Arg^2]dermorphin and approximately 5 times that of morphine. The potency of analogs in which Gly^4 of I is replaced by Sar or D-Ala was considerably increased, presumably due to enhanced stability against peptidases. However, replacing Gly^4 of I by Pro, Leu or even D-Leu gave analogs (VIII—X) with markedly low potency. These results seem to suggest that the presence of an amino acid having a small side chain is needed at position 4 for high activity and conversely a bulkier amino acid at position 4 induces lower potency. A similar phenomenon was also observed regarding the effects of the C-terminal substituent groups of analogs V and VI; both of them (formed by esterification of

TABLE II. Physicochemical Properties of Tetrapeptide Analogs

Analog No.	$[\alpha]_{\mathrm{D}}$ a)	Rf^1 b)	Rf^2 b)	Formula	Analysis (%) Calcd (Found)			Amino acid anal.			
					C	H	N				
I	+36.0°	0.28	0.57	$C_{26}H_{35}N_7O_6\cdot$	51.34	6.67	14.15	Gly	1.00	Tyr	0.83
				$2CH_3COOH\cdot 2H_2O$	(51.64	6.79	14.05)	Phe	0.98	Arg	0.92
II	+31.9°	0.52	0.74	$C_{28}H_{39}N_7O_6\cdot$	54.30	7.25	13.37	Gly	1.00	Tyr	0.86
				$2CH_3COOH\cdot H_2O$	(54.30	6.98	13.85)	Phe	0.94	Arg	1.05
III	+26.2°	0.57	0.74	$C_{29}H_{41}N_7O_6\cdot$	54.97	7.16	13.87	Gly	1.00	Tyr	0.82
				$2CH_3COOH\cdot H_2O$	(54.91	7.12	13.58)	Phe	1.00	Arg	0.92
IV	+45.2°	0.30	0.59	$C_{27}H_{37}N_7O_6\cdot$	52.43	7.01	14.22	Sar	1.03 c)	Tyr	0.83
				$2CH_3COOH\cdot 2H_2O$	(52.31	6.94	13.78)	Phe	1.09	Arg	0.93
V	+38.2°	0.41	0.72	$C_{28}H_{39}N_7O_6\cdot$	54.33	7.02	14.36	Sar	0.94	Tyr	0.89
				$2CH_3COOH\cdot H_2O$	(54.30	6.98	13.85)	Phe	1.00	Arg	1.09
VI	+41.0°	0.48	0.73	$C_{29}H_{41}N_7O_6\cdot$	54.21	7.43	13.75	Sar	1.01	Tyr	0.91
				$2CH_3COOH\cdot \frac{5}{2}H_2O$	(54.08	7.43	13.38)	Phe	1.00	Arg	1.05
VII	+39.7°	0.33	0.72	$C_{27}H_{37}N_7O_6\cdot$	52.20	6.71	14.18	Ala	1.05	Tyr	0.92
				$CH_3COOH\cdot 3H_2O$	(52.00	7.07	14.64)	Phe	1.05	Arg	0.97
VIII	+22.0°	0.34	0.64	$C_{30}H_{39}N_7O_6\cdot$	53.76	6.90	13.42	Pro	1.06	Tyr	0.92
				$2CH_3COOH\cdot \frac{5}{2}H_2O$	(53.82	6.91	12.92)	Phe	1.00	Arg	0.98
IX	+22.6°	0.43	0.73	$C_{30}H_{43}N_7O_6\cdot$	54.99	6.88	13.37	Leu	1.00	Tyr	0.86
				$2CH_3COOH\cdot H_2O$	(55.50	7.26	13.34)	Phe	0.99	Arg	0.98
X	+50.9°	0.46	0.81	$C_{31}H_{45}N_7O_6\cdot$	56.20	7.32	13.32	Leu	1.00	Tyr	0.96
				$2CH_3COOH\cdot H_2O$	(56.06	7.39	13.08)	Phe	0.99	Arg	0.99
XI	+33.8°	0.46	0.81	$C_{28}H_{38}N_8O_8\cdot$	47.79	6.39	13.28	Tyr	0.97	Phe(NO$_2$)	0.93 c)
				$\frac{5}{2}CH_3COOH\cdot 4H_2O$	(47.36	6.74	13.39)	Gly	1.04	Arg	0.99
XII	+33.7°	0.26	0.58	$C_{26}H_{35}N_7O_7\cdot$	50.28	6.32	13.95	Gly	1.08	Tyr	1.97
				$2CH_3COOH\cdot 2H_2O$	(50.48	6.64	13.74)	Arg	0.95		
XIII	+12.1°	0.41	0.67	$C_{30}H_{40}N_8O_6\cdot$	55.53	6.66	15.05	Gly	1.02	Tyr	1.00
				$2CH_3COOH\cdot \frac{1}{2}H_2O$	(55.35	6.69	15.19)	Arg	0.98	Trp	0.84 d)
XIV	+57.4°	0.37	0.72	$C_{28}H_{39}N_7O_6\cdot$	53.76	7.29	13.42	Sar	0.93	Phg	1.10
				$2CH_3COOH\cdot \frac{3}{2}H_2O$	(53.62	7.03	13.68)	Tyr	0.92	Arg	1.10
XV	+7.0°	0.26	0.57	$C_{26}H_{35}N_7O_6\cdot$	54.01	6.67	16.21	Gly	1.00	Tyr	0.91
				$CH_3COOH\cdot H_2O$	(54.27	6.67	15.82)	Phe	0.98	Arg	1.01
XVI	+26.0°	0.64	0.86	$C_{28}H_{38}N_8O_8\cdot$	52.31	5.84	15.99	Gly	1.13	Tyr	0.91
				$CH_3COOH\cdot H_2O$	(52.01	6.40	16.18)	Phe	0.98	Arg	0.97
XVII	+26.6°	0.43	0.77	$C_{29}H_{41}N_7O_6\cdot$	55.58	7.24	14.00	Gly	1.00	Tyr	0.95
				$2CH_3COOH\cdot \frac{1}{2}H_2O$	(55.60	7.07	13.76)	Phe	1.00	Har	0.98 c)
XVIII	+21.4°	0.39	0.72	$C_{28}H_{39}N_5O_6\cdot$	57.70	7.41	10.46	Gly	1.04	Tyr	0.94
				$2CH_3COOH\cdot \frac{1}{2}H_2O$	(57.30	7.21	10.44)	Phe	1.00	Lys	0.96
XIX	+27.7°	0.41	0.79	$C_{30}H_{43}N_7O_6\cdot$	55.97	6.78	13.74	Gly	1.00	Tyr	0.60 e)
				$2CH_3COOH\cdot \frac{1}{2}H_2O$	(56.18	7.21	13.49)	Phe	0.95	Arg	0.98

a) Optical rotations were measured in H_2O ($c=1$) at 20—23 °C. *b*) See Experimental. *c*) See ref. 20. *d*) Value after hydrolysis with 4 M CH_3SO_3H. *e*) The low recovery of Tyr is due to incomplete hydrolysis of H–Tyr(Et)–OH (see ref. 20).

IV) showed a significant decrease in potency as compared with IV. Introduction of a nitro substituent at the *para*-position of the Phe^3 aromatic ring resulted in a considerably reduced activity in accord with the results for [Phe(NO$_2$)^3]dermorphin in *in vitro* assays.17) Analogs in which Phe^3 is replaced by other aromatic amino acids also exhibited greatly reduced activities, suggesting that the Phe residue at position 3 is of crucial importance for the activity. Replacement of D-Arg^2 by its L-antipode, XV, resulted in complete loss of the activity even at higher doses (up to 20 mg/kg). Blocking of the guanidino functionality on D-Arg^2 gave analog XVI, which was 3 times less potent than the parent peptide (II). The analgesic effect of this

TABLE III. Analgesic Activity of [D-Arg^2]dermorphin after Subcutaneous Administration to Mice

Compound	ED$_{50}$$^{a)}$ (mg/kg, *s.c.*)	Relative potency$^{b)}$
Morphine·HCl	6.2 (4.1—9.4)	1.0
Dermorphin	4.6 (3.4—6.3)	3.3
[D-Arg^2]dermorphin	3.5 (2.8—4.4)	5.1

a) 95% confidence limits are given in parentheses. *b*) Relative potency is on a molar basis.

TABLE IV. Analgesic Activities of Tetrapeptide Analogs after Subcutaneous Administration to Mice

Analog No.	Peptide	Peak time$^{a)}$	Relative potency$^{b)}$	ED$_{50}$$^{c)}$ (mg/kg, *s.c.*)	Relative potency$^{d)}$
	Morphine·HCl	30	1.00	6.20 (4.08—9.42)	1.0
	H–Tyr–D-Ala–Phe–Gly–OH$^{e)}$	30	0.25	21.00 (13.73—31.46)	0.4
I	H——D-Arg——————OH	45	>1.37	2.40 (1.46—3.96)	4.8
II	H——D-Arg——————OEt	45	>1.37	2.40 (1.20—4.80)	4.9
III	H——D-Arg——————OPr	45	>1.37	1.60 (0.98—2.62)	7.5
IV	H——D-Arg————Sar–OH	45	>1.37	0.55 (0.33—0.92)	21.4
V	H——D-Arg————Sar–OMe	45	>1.37	1.15 (0.59—2.23)	10.1
VI	H——D-Arg————Sar–OEt	45	>1.37	1.30 (0.94—1.81)	9.3
VII	H——D-Arg———D-Ala–OH	45	>1.37	1.45 (0.99—2.10)	7.6
VIII	H——D-Arg———Pro–OH	30	0.15	—	—
IX	H——D-Arg———Leu–OH	45	0.11	—	—
X	H——D-Arg——D-Leu–OMe	30	0.36	—	—
XI	H——D-Arg–Phe(NO$_2$)–OEt	30	0.19	—	—
XII	H——D-Arg–Tyr———OH	30	0.28	—	—
XIII	H——D-Arg–Trp———OEt	30	0.39	—	—
XIV	H——D-Arg–D-Phg–Sar–OEt	15	0.09	—	—
XV	H——L-Arg——————OH	—	0	—	—
XVI	H——D-Arg(NO$_2$)———OEt	30	0.81	8.00 (5.71—11.20)	1.6
XVII	H——D-Har——————OEt	15	0.36	—	—
XVIII	H——D-Lys——————OEt	45	0.19	—	—
XIX	H–Tyr(Et)–D-Arg———OEt	15	0.11	—	—

a) Time (min) to the onset of the highest % of maximum possible effect (%MPE). *b*) The highest %MPE of each peptide was compared with that of morphine at a dose of 10 mg/kg, *s.c.* (morphine = 1.00). *c*) 95% confidence limits are given in parentheses. *d*) ED$_{50}$ value of each peptide was compared with that of morphine on a molar basis. *e*) This peptide was also synthesized as a reference peptide by essentially the same method as described for the preparation of analog I; $[\alpha]_D^{21}$ +65.2° (c = 1, 2 N AcOH), *Anal.* Calcd for $C_{23}H_{28}N_4O_6 \cdot 2H_2O$: C, 56.09; H, 6.55; N, 11.38. Found: C, 56.38; H, 6.32; N, 11.40.

analog in the tail flick test is 1.8 times as potent as that of morphine (ED$_{50}$: 2.20 mg/kg, *s.c.*, unpublished data) and this potency is nearly equal to that of H–Tyr–D-Met(O)–Phe–Gly–OEt in the tail flick test reported by Kiso *et al.*[18] These results are very suggestive of a profound contribution of the positively charged guanidino group on D-Arg^2 to high potency. Moreover, the facts that analogs in which D-Arg^2 is replaced by D-Har or D-Lys exhibited short-lived or very weak activity suggest that not only the guanidino function but also the side chain length of the D-Arg residue is of critical importance for the potency. Ethylation of the phenolic hydroxyl group on Tyr^1 gave analog XIX with a very weak activity, analogously with a result obtained for [Tyr(Me)^1]dermorphin.[19]

Since the analgesic effect of the most active tetrapeptide analog (IV) is completely abolished by pretreatment with naloxone (0.5 mg/kg intraperitonially), the potent activity produced by this series of tetrapeptide analogs seems to be due to their direct action on the opiate receptors in the brain. Details of the pharmacological studies will be reported in a following paper.

Experimental

All melting points are uncorrected. Optical rotations were determined with a JASCO DIP-140 polarimeter. Amino acid analyses were performed on a Hitachi model 835 amino acid analyzer using a high-separation column.[20] TLC was performed on silica gel plates (Kieselgel GF_{254}, Merck) with the following solvent systems: Rf^1, 1-BuOH–AcOH–H_2O (4:1:5, upper phase); Rf^2, 1-BuOH–pyridine–AcOH–H_2O (15:10:3:12). The Boc group of all intermediates was removed by 4 N HCl–DOX treatment before TLC.

Boc–D-Arg(Tos)–Phe–Gly–OEt (1)——DCC (1.8 g) was added to a solution of Boc–D-Arg(Tos)–OH (3.4 g), HOBt (1.2 g) and HBr·H–Phe–Gly–OEt [derived from Z–Phe–Gly–OEt[21] (3.1 g) by 15% HBr–AcOH] in DMF (10 ml) containing TEA (1.2 ml) at 0 °C, and the mixture was stirred at 5 °C overnight. A few drops of AcOH were added and the whole was stirred further for 30 min at room temperature; DC-urea that appeared was filtered off. The filtrate was diluted with H_2O (80 ml) and extracted twice with EtOAc (40 ml). The extract was washed well with 1 N citric acid, 1 N $NaHCO_3$ and H_2O, dried over $MgSO_4$, and then evaporated to dryness *in vacuo*. The resulting residue was recrystallized from EtOAc; yield 4.5 g (86%), mp 110—112 °C, $[\alpha]_D^{26}$ $-20.5°$ ($c=1$, MeOH). Rf^1 0.66. *Anal.* Calcd for $C_{31}H_{44}N_6O_8S$: C, 56.35; H, 6.71; N, 12.72. Found: C, 56.56; H, 6.65; N, 12.66.

Boc–Tyr–D-Arg(Tos)–Phe–Gly–OEt (2)——Compound **1** (1.52 g) was treated with 4 N HCl–DOX (8 ml) at room temperature for 30 min, then the solution was evaporated to dryness *in vacuo*. The excess HCl was removed by repeated evaporation with fresh DOX *in vacuo*. The resulting residue was dissolved in DMF (5 ml) containing TEA (0.32 ml), then Boc–Tyr–OH (0.62 g) and HOBt (0.30 g) were added, followed by DCC (0.47 g) at 0 °C. After being stirred at 5 °C overnight, the mixture was worked up in the same manner as described for the preparation of **1**. The resulting product was recrystallized from 2-propanol; yield 1.71 g (95%), mp 166—169 °C, $[\alpha]_D^{26}$ $-7.5°$ ($c=1$, MeOH). Rf^1 0.78. *Anal.* Calcd for $C_{40}H_{53}N_7O_{10}S$: C, 58.31; H, 6.48; N, 11.90. Found: C, 58.29; H, 6.61; N, 11.43.

Boc–Tyr–D-Arg(Tos)–Phe–Gly–OH (3)——A solution of **2** (5.53 g) in H_2O (3 ml)–MeOH (10 ml) was treated with 2 N NaOH (3.4 ml) and the mixture was stirred at room temperature for 40 min, then diluted with H_2O (60 ml) and washed twice with EtOAc. The aqueous phase was chilled, acidified with solid citric acid and extracted twice with EtOAc (50 ml). The extract was washed well with H_2O, dried over $MgSO_4$ and evaporated to dryness *in vacuo*. The resulting product was reprecipitated from EtOAc–abs. ether; yield 4.6 g (86%), mp 134—140 °C, $[\alpha]_D^{24}$ $-3.6°$ ($c=1$, MeOH). Rf^1 0.54. *Anal.* Calcd for $C_{38}H_{49}N_7O_{10}S$: C, 57.34; H, 6.21; N, 13.32. Found: C, 56.96; H, 6.20; N, 11.92.

Boc–Tyr–D-Arg(Tos)–Phe–Gly–Tyr–Pro–Ser–NH_2 (4)——WSCI (80 mg) was added to a solution of **3** (320 mg), CF_3COOH·H–Tyr–Pro–Ser–NH_2[10] (191 mg) and HOBt (54 mg) in DMF (2 ml) containing TEA (0.06 ml) at 0 °C and the mixture was stirred at 5 °C for 40 h, diluted with H_2O (20 ml) and extracted twice with 1-BuOH (10 ml). The extract was washed with 1-BuOH–saturated 1 N AcOH (×3) and 1-BuOH–saturated H_2O (×3), then evaporated to dryness *in vacuo* to give an oily residue, which was crystallized from abs. ether and precipitated from MeOH–EtOAc–abs. ether; yield 405 mg (84%), mp 155—157 °C, $[\alpha]_D^{20}$ $-15.7°$ ($c=1$, MeOH). Rf^1 0.45. *Anal.* Calcd for $C_{55}H_{71}N_{11}O_{14}S$: C, 57.83; H, 6.27; N, 13.49. Found: C, 57.54; H, 6.11; N, 12.94.

H–Tyr–D-Arg–Phe–Gly–Tyr–Pro–Ser–NH_2——A solution of **7** (230 mg), *o*-cresol (50 mg) and thioanisole (1 ml) in CF_3COOH (3 ml) was treated with CF_3SO_3H (0.3 ml) and the mixture was stirred at room temperature for 100 min, then concentrated to a small volume *in vacuo*. To this solution, abs. ether (30 ml) was added. The resulting oil was washed well with abs. ether, and dried *in vacuo*. The residue was dissolved in H_2O (3 ml), and the solution was treated with Dowex 1×2 (AcOH form) resin (8 g by wet weight) for 30 min. After removal of the resin by filtration, the filtrate was lyophilized. The product was dissolved in H_2O (0.5 ml) and the solution was applied to a column (2×11 cm) of CM-cellulose. The column was eluted first with 0.1 M pyridinium acetate buffer (pH 5.10, 150 ml) and then with a linear gradient formed from 0.35 M pyridinium acetate buffer (pH 5.10, 300 ml) through a mixing chamber containing 0.1 M pyridinium acetate buffer (pH 5.10, 300 ml). Fractions of 5.5 ml each were collected and tubes No. 90—105 (numbering from the starting point of gradient elution) were pooled and lyophilized. The product thus obtained was applied to a column (2.5×45 cm) of Sephadex G-25 preequilibrated with the lower phase of 1-BuOH–AcOH–H_2O (4:1:5) and eluted with the upper phase of the same solvent system. Fractions of 5.3 ml each were collected and tubes No. 28—40 were pooled, and evaporated to dryness. The residue was lyophilized from H_2O; yield 95 mg, $[\alpha]_D^{18}$ $-16.5°$ ($c=1$, H_2O). Rf^1 0.32, Rf^2 0.60. *Anal.* Calcd for $C_{43}H_{57}N_{11}O_{10}\cdot 2CH_3COOH\cdot 4H_2O$: C, 52.26; H, 6.81; N, 14.27. Found: C, 52.66; H, 6.56; N, 13.80. Amino acid analysis (6 N HCl): Ser 0.92; Gly 1.00; Tyr 1.86; Phe 0.97; Arg 1.03; Pro 1.05; NH_3 1.06 (recovery 88%).

Boc–Tyr–D-Arg(Tos)–Phe–Gly–O–*n*-Pr (9)——A solution of **2** (177 mg) and KF (30 mg) in DMF (2 ml) was treated with *n*-propylbromide (0.04 ml) and the mixture was stirred at room temperature for 2 d, then extracted twice with EtOAc (10 ml). The extract was washed well with 5% Na_2CO_3, H_2O, dried over $MgSO_4$ and evaporated to dryness. The product was reprecipitated from EtOAc–abs. ether; yield 110 mg (61%).

Boc–Tyr–D-Arg(Tos)–Phe–Sar–OMe (10)——This compound was prepared from **8** (405 mg), KF (60 mg) and methyliodide (0.07 ml) in the same manner as described for the preparation of **9**; yield 290 mg (70%).

H–Tyr–D-Arg–Phe–Gly–OH (I)——Compound **3** (150 mg) was treated with the CF_3SO_3H–thioanisole system in the same manner as described for the preparation of [D-Arg^2]dermorphin. The crude peptide thus obtained was applied to a column (2 × 10 cm) of CM-cellulose, which was eluted with a linear gradient formed from 0.12 M pyridinium acetate buffer (pH 5.10, 300 ml) through a mixing chamber containing H_2O (300 ml). Fractions of 6 ml each were collected and tubes No. 54—60 were pooled and lyophilized; yield 76 mg.

Analog IV was prepared in essentially the same manner as described above. Analogs II, III, V, VI, XI and XIV were also prepared from the corresponding tetrapeptide intermediates, **2**, **9**, **10**, **7**, **25** and **34**, in essentially the same manner as described above, and the purification of these peptides was performed in the same manner as described for [D-Arg^2]dermorphin.

H–Tyr–D-Arg–Phe–D-Ala–OH (VII)——Compound **13** (300 mg) was treated with 4 N HCl–DOX (5 ml) at room temperature for 30 min, then the solution was evaporated to dryness. The resulting residue was dissolved in H_2O (15 ml) and hydrogenated overnight in the presence of 10% Pd–C (100 mg). After removal of the catalyst through celite, the solution was treated with Dowex 1 × 2 (AcOH form) resin (10 g by wet weight) and lyophilized. The product was applied to a column (2 × 9 cm) of CM-cellulose, which was eluted with a linear gradient formed from 0.15 M pyridinium acetate buffer (pH 5.0, 300 ml) through a mixing chamber containing H_2O (300 ml). Fractions of 5 ml each were collected and tubes No. 67—78 were pooled and lyophilized. The partially purified product was applied to a column (2.5 × 45 cm) of Toyopearl HW-40, which was eluted with 2% AcOH. Fractions of 7 ml each were collected and tubes No. 33—50 were pooled and lyophilized; yield 100 mg.

Analogs VIII, IX, XII, XV, XVIII and XIX were prepared from the corresponding tetrapeptide intermediates, **16**, **19**, **27**, **36**, **40** and **41**, in essentially the same manner as described above.

H–Tyr–D-Arg–Trp–Gly–OEt (XIII)——Compound **31** (89 mg) was treated with the CF_3SO_3H–thioanisole system as described for the preparation of [D-Arg^2]dermorphin. The resulting oily residue was dissolved in CH_3SO_3H (0.7 ml) containing thioanisole (0.08 ml) and ethanedithiol (0.04 ml) and the whole was stirred at room temperature for 90 min, then abs. ether was added. The oily residue thus obtained was washed with abs. ether and dried *in vacuo*. The crude peptide was purified by column chromatography on CM-cellulose in a manner similar to that used for [D-Arg^2]dermorphin, and then on Toyopearl HW-40 in a manner similar to that described for analog VII; yield 15 mg.

H–Tyr–D-Arg(NO_2)–Phe–Gly–OEt (XVI)——Compound **38** (200 mg) was treated with 4 N HCl–DOX (8 ml) at room temperature for 30 min, then the solution was evaporated to dryness *in vacuo*. The resulting residue was treated with Dowex 1 × 2 (AcOH form) resin (5 g) in 40% EtOH (20 ml) and then applied to a CM-Sepharose column. The column (2 × 11 cm) was eluted with a linear gradient formed from 0.15 M pyridinium acetate in 30% EtOH (pH 5.30, 300 ml) through a mixing chamber containing 30% EtOH (300 ml). Fractions of 4 ml each were collected and tubes No. 60—90 were pooled and lyophilized. The product was applied to a column (2.5 × 35 cm) of Sephadex LH-20, which was eluted with 2% AcOH in 50% EtOH. Fractions of 6.2 ml each were collected and tubes No. 28—33 were pooled, evaporated to dryness and lyophilized from H_2O; yield 130 mg.

H–Tyr–D-Har–Phe–Gly–OEt (XVII)——Compound **40** (405 mg) was dissolved in MeOH (20 ml) and hydrogenated in the presence of 10% Pd–C in the usual manner. The de-carbobenzoxylated compound (270 mg) was dissolved in DMF (1 ml) and combined with a solution of 3,5-dimethylpyrazole-1-carboxamidine nitrate (201 mg) in DMF (1 ml) containing TEA (0.16 ml). The resulting mixture was stirred at room temperature for 3 d, then worked up as described for the preparation of **7**; yield 275 mg. The product (180 mg) thus obtained was de-*tert*-butoxycarbonylated by treatment with 4 N HCl–DOX in the usual manner, treated with Dowex 1 × 2 (AcOH form) resin and then purified by column chromatography on CM-cellulose and Toyopearl HW-40 as described for the preparation of analog XIII; yield 50 mg.

Analgesic Assay——Male Std-ddy strain mice (20—25 g) were used. Mice were injected subcutaneously with a test compound dissolved in Ringer's solution. The analgesic effect was assessed by means of the tail pressure test as described previously.[8,15] Changes in responsive tail pressure were expressed as a percentage of maximum possible effect (%MPE) as follows: $\%\text{MPE}=(P_t-P_0/100-P_0)$ where P_0 is pre-drug responsive pressure (mmHg) and P_t is responsive pressure (mmHg) at t time after drug administration. The ED_{50} values and 95% confidence limits were determined by the method of Litchfield and Wilcoxon.[22]

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2) Amino acids, peptides and their derivatives in this study are of L-configuration unless otherwise stated.

Abbreviations used are: Boc = *tert*-butoxycarbonyl, Z = carbobenzoxy, Me = methyl, Et = ethyl, *n*-Pr = *n*-propyl, Bzl = benzyl, Tos = tosyl, Sar = sarcosine, Har = homoarginine, Phe(NO$_2$) = *p*-nitrophenylalanine, Phg = phenylglycine, DCC = dicyclohexylcarbodiimide, WSCI = water-soluble carbodiimide, HOBt = 1-hydroxybenztriazole, ONSu = succinimide ester, TFA = triethylamine, DMF = dimethylformamide, EtOAc = ethyl acetate, DOX = dioxane, CM- = carboxymethyl-, TLC = thin-layer chromatography.

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