Synthesis and Biological Activity of [MeTyr^1,MeArg^7,D-Leu^8]-Dynorphin A(1—9)-NHEt and [D-Cys^2-Cys^5,MeArg^7,D-Leu^8]-Dynorphin A(1—9)-NH_2

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The opioid activities of [MeTyr^1]-Dyn(1—7)-NH_2, [MeTyr^1,D-Leu^8]-Dyn(1—8)-NH_2, [MeTyr^1,D-Leu^8]-Dyn(1—9)-NH_2, [MeTyr^1,D-Leu^8]-Dyn(1—10)-NH_2, [MeTyr^1,D-Leu^8]-Dyn(1—11)-NH_2, and [MeTyr^1,D-Leu^{8,12}]-Dyn(1—13)-NH_2 were examined in the bioassays (guinea pig ileum, mouse *vas deferens* and rabbit *vas deferens*). Because [MeTyr^1,D-Leu^8]-Dyn(1—9)-NH_2 showed the most potent opioid activity of the peptides tested, the biological activities of two kinds of Dyn(1—9) analogues, [MeTyr^1,MeArg^7,D-Leu^8]-Dyn(1—9)-NHEt and [D-Cys^2-Cys^5,MeArg^7,D-Leu^8]-Dyn(1—9)-NH_2 were determined and compared with those of [MeTyr^1,MeArg^7,D-Leu^8]-Dyn(1—8)-NHEt and [D-Cys^2-Cys^5,MeArg^7,D-Leu^8]-Dyn(1—8)-NHEt in the three bioassays, in the receptor binding assays, and in the mouse tail pinch test after subcutaneous administration. The results suggest that the extension of the C-terminal in the peptide chain of [MeArg^7,D-Leu^8]-Dyn(1—8)-NH_2 analogues by Arg is ineffective for increasing the κ-opioid activities, κ-receptor selectivity and/or analgesic effects of the peptides.

Keywords dynorphin A-(1—9) analogue; bioassay; receptor binding assay; analgesic effect; synthesis

Dynorphin A (Dyn) isolated either from porcine pituitary[1] or from porcine duodenum[2] is a 17 amino acid opioid peptide (Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–Lys–Leu–Lys–Trp–Asp–Asn–Gln) containing the sequence of [Leu]enkephalin at its N-terminal. It has been postulated that Dyn is the endogenous ligand for the κ-opioid receptor.[3] Furthermore, its N-terminal fragment, Dyn(1—8)–OH, has also been reported to be the selective ligand for the κ-binding site.[4] Recently, we have reported the synthesis and structure–activity relationships of Dyn(1—8) analogues[5] and demonstrated that [MeTyr^1, MeArg^7,D-Leu^8]-Dyn(1—8)-NHEt (**7**) shows a potent analgesic effect after subcutaneous administration into mice and that [D-Cys^2-Cys^5,MeArg^7,D-Leu^8]-Dyn(1—8)-NHEt (**8**) shows a 3-fold more potent analgesic effect than **7**. Furthermore, **7** displays not only opioid activity similar to that of Dyn in the bioassays [guinea pig ileum (GPI), mouse *vas deferens* (MVD), and rabbit *vas deferens* (RVD)] but also a higher affinity for κ-receptors than for μ- or δ-receptors in the receptor binding assays, while **8** displays extremely potent opioid activity in the three bioassays but different receptor selectivity from that of Dyn in the binding assays.[5b]

As a part of our studies on the biological activities of Dyn fragment analogues, we examined the opioid activities of [MeTyr^1]-Dyn(1—7)-NH_2 (**1**), [MeTyr^1,D-Leu^8]-Dyn(1—8)-NH_2 (**2**),[5b] [MeTyr^1,D-Leu^8]-Dyn(1—9)-NH_2 (**3**), [MeTyr^1, D-Leu^8]-Dyn(1—10)-NH_2 (**4**), [MeTyr^1,D-Leu^8]-Dyn(1—11)-NH_2 (**5**), and [MeTyr^1,D-Leu^{8,12}]-Dyn(1—13)-NH_2 (**6**) in the bioassays (GPI, MVD, and RVD). Furthermore, because nonapeptide **3** showed the most potent opioid activity of the peptides tested, we also examined the opioid activities, opioid receptor selectivity, and analgesic effects of two kinds of Dyn(1—9) analogues, [MeTyr^1,MeArg^7,D-Leu^8]-Dyn(1—9)-NHEt (**9**) and [D-Cys^2-Cys^5,MeArg^7,D-Leu^8]-Dyn(1—9)-NH_2 (**10**), in order to find more desirable analgesic peptides than **7** or **8**.

Synthesis All of the peptides listed in Table I were prepared by the solution method. The synthetic strategy is essentially the same as that used in the preparation of **7** and **8**.[5b] The Gly residue at position 3 was chosen as the racemization-free fragment coupling point. Purification of the HF-deprotected peptides was achieved by high performance liquid chromatography (HPLC) on Nucleosil $5C_{18}$ (2×25 cm) with H_2O–CH_3CN containing 0.015—0.1% HCl. Homogeneity of the purified product was accessed by thin layer chromatography (TLC) and analytical HPLC. Structure identification was achieved by amino acid analysis and fast atom bombardment mass spectroscopy (FAB-MS) (Table I).

Compound **9** was synthesized by the HOBt ester condensation of Boc–MeTyr(Cl_2Bzl)–Gly–Gly–OH[5b] with a TFA treated sample of Boc–Phe–Leu–Arg(Tos)–MeArg(Tos)–D-Leu–Arg(Tos)–NHEt (prepared in a stepwise manner starting from H–Arg(Tos)–NHEt), followed by the HF-deprotection. Compound **10** was synthesized by the HOBt ester condensation of Boc–Tyr(Cl_2Bzl)–D-Cys(MBzl)–Gly–OH[5b] with a TFA-treated sample of Boc–Phe–Cys(MBzl)–Arg(Tos)–MeArg(Tos)–D-Leu–Arg-(Tos)–NH_2 (prepared in a stepwise manner starting from H–Arg(Tos)–NH_2), followed by the HF-deprotection and air oxidation.

Biological Activities and Binding Properties The compounds were tested *in vitro* in three isolated organ preparations (GPI, MVD, and RVD). In the GPI opioid effects are primarily mediated by μ-receptors, whereas

TABLE I. Analytical Data of Dynorphin Fragment Analogues

Compd.	$[\alpha]_D^{20}$ [a] (°)	Rf [b]	Amino acid analysis [c]								FAB-MS [d]
			Tyr	Gly	Phe	Leu	Arg	Pro	Lys	Cys	(MH$^+$)
1	−6.7	0.53		1.98	1.00	1.03	2.30				
3	−7.8	0.64		1.96	1.00	1.98	3.07				
4	−16.2	0.56		1.92	1.00	2.07	3.25	0.99			
5	−25.3	0.46		1.98	1.00	1.96	3.08	1.06	1.01		
6	−31.8	0.50		2.02	1.00	3.16	3.11	1.00	2.04		
9	−31.8	0.62		1.94	1.00	1.90	1.91				1192
10	−29.0	0.56	0.80	1.02	1.00	1.04	2.06			1.83	1184

a) c=0.4 in 0.01 N HCl. *b*) TLC on silica gel. Solvent system: 1-butanol–pyridine–acetic acid–water (15:5:5:8). *c*) The proportions of only the primary protein amino acids were calculated. *d*) Found values are in agreement with calculated values.

TABLE II. Opioid Activities and Analgesic Effects of Dynorphin Fragment Analogues

	Compound	GPI IC_{50} (nM)[a]	MVD IC_{50} (nM)[a]	RVD IC_{50} (nM)[a]	Analgesia (s.c.) ED_{50} (mg/kg)[b]
1	[MeTyr^1]-Dyn(1—7)-NH_2	1.7 ±0.5	4.7 ±0.6	171 ± 9.6	
2	[MeTyr^1, D-Leu^8]-Dyn(1—8)-NH_2	1.4 ±0.3[c]	5.2 ±0.2[c]	14.3 ±12.3[c]	
3	[MeTyr^1, D-Leu^8]-Dyn(1—9)-NH_2	0.2 ±0.03	1.1 ±0.2	0.6 ± 0.1	
4	[MeTyr^1, D-Leu^8]-Dyn(1—10)-NH_2	0.5 ±0.1	3.6 ±0.4	1.7 ± 0.5	
5	[MeTyr^1, D-Leu^8]-Dyn(1—11)-NH_2	0.5 ±0.1	1.8 ±0.4	1.3 ± 0.3	
6	[MeTyr^1, D-Leu^{8,12}]-Dyn(1—13)-NH_2	0.3 ±0.04	1.1 ±0.6	1.2 ± 0.4	
7	[MeTyr^1, MeArg^7, D-Leu^8]-Dyn(1—8)-NHEt	0.3 ±0.03[c]	7.4 ±2.5[c]	2.6 ± 0.4[c]	1.0 (0.4—2.5)[d]
8	[D-Cys^2-Cys^5, MeArg^7, D-Leu^8]-Dyn(1—8)-NHEt	0.007±0.001[c]	0.13±0.01[c]	0.06± 0.01[c]	0.32 (0.13—0.76)
9	[MeTyr^1, MeArg^7, D-Leu^8]-Dyn(1—9)-NHEt	0.18 ±0.04	1.89±0.2	1.5 ± 0.5	0.8 (0.31—2.08)
10	[D-Cys^2-Cys^5, MeArg^7, D-Leu^8]-Dyn(1—9)-NH_2	0.015±0.001	0.08±0.01	0.03± 0.003	0.44 (0.15—1.25)
Dyn		0.2 ±0.03[c]	3.0 ±0.5[c]	17.4 ± 6.7[c]	

a) Results are the means±S.E.M. *b*) Analgesia at four doses of each compound was investigated. Each dose was tested on at least 8 animals. *c*) Taken from ref. 5*b*. *d*) Taken from ref. 5*c*.

TABLE III. Binding Assays of Dyn(1—8)- and Dyn(1—9)-Analogues

Compound	[^{3}H] DAGO K_i^μ, nM[a]	[^{3}H] DPDPE K_i^δ, nM[a]	[^{3}H] U69,593 K_i^κ, nM[a]
7	4.51±0.85[b]	27.24±10.66[b]	1.91±0.22[b]
8	0.85±0.22[b]	1.59± 0.19[b]	3.02±1.01[b]
9	5.61±0.88	66.96±16.05	2.29±0.75
10	0.33±0.10	2.59± 0.58	2.73±0.93
Dyn	7.16±1.96[b]	23.62± 5.93[b]	1.16±0.25[b]

a) Results are the means of five or six determinations±S.E.M. *b*) Taken from ref. 5*b*.

κ-receptors are also present in this tissue. The MVD assay is generally taken as being representative for δ-receptor interactions, even though it also contains μ- and κ-receptors. The RVD is considered to have only κ-receptors. The opioid receptor affinities were determined by displacement of selective radioligands from guinea pig brain membrane binding sites. [^{3}H]DAGO[6] was used as a μ-ligand, [^{3}H]DPDPE[7] was used as a δ-ligand, and [^{3}H]U69,593[8] was used as a κ-ligand. The analgesic effects were examined *in vivo* by mouse tail pinch assay after subcutaneous administration. The results are shown in Tables II and III.

Results and Discussion

[MeTyr^1,D-Leu^8]-Dyn(1—9)-NH_2 (**3**) showed the most potent opioid activity of peptides **1**—**6** (Table II). Surprisingly, its opioid activity on the RVD was about 24-fold more potent than that of [MeTyr^1,D-Leu^8]-Dyn(1—8)-NH_2 (**2**). In addition, McKnight *et al.*[9] reported that the relative affinity of Dyn(1—8) is about 3-fold greater for the κ-site than for μ- or δ-sites but that the relative affinity of Dyn(1—9) is about 17-fold greater for the κ-site than for μ- or δ-sites. Therefore, the biological activities of [MeTyr^1,MeArg^7,D-Leu^8]-Dyn(1—9)NHEt (**9**) and [D-Cys^2-Cys^5,MeArg^7,D-Leu^8]-Dyn(1—9)-NH_2 (**10**) were determined and compared with those of [MeTyr^1,-MeArg^7,D-Leu^8]-Dyn(1—8)-NHEt (**7**) and [D-Cys^2-Cys^5, MeArg^7,D-Leu^8]-Dyn(1—8)-NHEt (**8**), respectively.

[MeTyr^1,MeArg^7,D-Leu^8]-Dyn(1—9)-NHEt (**9**), as compared with octapeptide **7**, showed only 1.7-fold more potent opioid activity on the GPI and RVD (Table II). In the binding assays, compound **9** showed an affinity similar to **7** for μ and κ-receptors (Table III). Its analgesic effect was almost the same as that of **7**.

[D-Cys^2-Cys^5,MeArg^7,D-Leu^8]-Dyn(1—9)-NH_2 (**10**), as compared with octapeptide **8**, showed 2-fold less potent opioid activity on the GPI and 2-fold more potent opioid activity on the RVD. In the binding assays, compound **10** showed a 2.6-fold higher affinity for the μ-receptor, a 1.6-fold lower affinity for the δ-receptor, and a similar affinity for the κ-receptor. Its analgesic effect was almost the same as that of **8**. Since the C-terminal amide moiety of **10** differs from that of **8**, we cannot precisely discuss the effect of the extension of the C-terminal in the peptide chain of octapeptide **8** by Arg. However, the difference in the C-terminal amide moieties between **8** and **10** seems not to have much affect on the opioid activities and receptor selectivity of the peptides because [MeTyr^1,MeArg^7,D-Leu^8]-Dyn(1—8)-NH_2 showed almost the same opioid activity and receptor selectivity as **7**.[5b] Therefore, it seems reasonable to consider that lengthening of the C-terminal of **8** to a nonapeptide does not shift the receptor selectivity toward that of Dyn.

These results suggest that the extension of the C-terminal in the peptide chain of [MeArg^7,D-Leu^8]-Dyn(1—8)-NH_2 analogues, MeArg^7 of which is essential to the duration of the analgesic effects, by Arg is ineffective for increasing the κ-opioid activities, κ-receptor selectivity and/or analgesic effects of the peptides. In addition, it is conceivable that further extension of the C-terminal in the peptide chain of nonapeptide **9** also does not increase the κ-opioid activity of the peptides because [MeTyr^1,D-Leu^8]-Dyn(1—9)-NH_2 (**3**) showed the most potent κ-opioid activity of peptides **1**—**6** in the RVD assay.

Experimental

Optical rotations were measured with a JASCO DIP-140 polarimeter. Amino acid analyses were carried out on a Hitachi 835 amino acid analyzer. Molecular weights of the products were determined by FAB-MS on a JEOL JMS-HX100 mass spectrometer. HPLC was performed with an ALTEX 110A pump and a JASCO UVIDEC 100A ultraviolet detector. A Nucleosil $5C_{18}$ column (4.5 × 150 mm) was used in the analytical HPLC. TLC was performed on precoated silica gel plates ($60F_{254}$, Merck).

Symbols and abbreviations are in accordance with recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature: *Biochem. J.*, **219**, 345 (1984). The following other abbreviations were also used: MeTyr, *N*-methyltyrosine; MeArg, N^α-methylarginine. Boc, *tert*-butoxycarbonyl; Cl_2Bzl, 2,6-dichlorobenzyl; MBzl, 4-methylbenzyl; Tos, tosyl; TFA, trifluoroacetic acid; DCC, dicyclohexylcarbodiimide; HOBt, *N*-hydroxybenzotriazole; MA, mixed anhydride.

TABLE IV. Characterization of Boc–MeTyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–Arg(Tos)–MeArg(Tos)–D-Leu–Arg(Tos)–NHEt (**11**), Boc–Tyr(Cl_2Bzl)–D-Cys(MBzl)–Gly–Phe–Cys(MBzl)–Arg(Tos)–MeArg(Tos)–D-Leu–Arg(Tos)–NH_2 (**12**), and Their Intermediates

Compound	Coupling method	Yield (%)	$[\alpha]_D^{20}$ (°)	*Rf*[a]	Formula	Analysis (%) Calcd			Found		
						C	H	N	C	H	N
Boc–(8—9)–NHEt	MA	88	+1.8 (MeOH)	0.67	$C_{26}H_{44}N_6O_6S \cdot 1/2H_2O$	54.05	7.85	14.55	54.26	7.75	14.43
Z–(7—9)–NHEt	MA	85	−5.8 (MeOH)	0.62	$C_{43}H_{62}N_{10}O_9S_2 \cdot H_2O$	55.17	6.78	14.96	54.75	6.78	14.96
Boc–(6—9)–NHEt	DCC–HOBt	45	−21.6 (MeOH)	0.62	$C_{53}H_{82}N_{14}O_{12}S_3 \cdot H_2O$	52.11	6.93	16.05	51.82	6.85	16.19
Boc–(5—9)–NHEt	MA	80	−27.6 (MeOH)	0.65	$C_{59}H_{93}N_{15}O_{13}S_3 \cdot H_2O$	53.09	7.17	15.74	53.08	7.18	15.58
Boc–(4—9)–NHEt	MA	85	−22.3 (DMF)	0.65	$C_{68}H_{102}N_{16}O_{14}S_3 \cdot 2H_2O$	54.45	7.12	14.94	54.67	7.06	15.06
Boc–(1—9)–NHEt (**11**)	HOBt ester	93	−27.2 (DMF)	0.66	$C_{89}H_{123}Cl_2N_{19}O_{18}S_3 \cdot 1/2H_2O$	55.58	6.50	13.84	55.50	6.72	13.61
Boc–(8—9)–NH_2	MA	92	+13.0 (MeOH)	0.49	$C_{24}H_{40}N_6O_6S \cdot 1/3H_2O$	52.73	7.50	15.37	52.77	7.60	15.14
Z–(7—9)–NH_2	MA	97	−3.4 (MeOH)	0.44	$C_{41}H_{58}N_{10}O_9S_2 \cdot CH_3OH$[b]	54.18	6.71	15.04	54.12	6.62	14.85
Boc–(6—9)–NH_2	DCC–HOBt	45	−16.7 (MeOH)	0.44	$C_{51}H_{78}N_{14}O_{12}S_3 \cdot H_2O$	51.32	6.76	16.43	51.15	6.54	16.48
Boc–(5—9)–NH_2	MA	74	−20.6 (MeOH)	0.51	$C_{62}H_{91}N_{15}O_{13}S_4 \cdot CH_3OH \cdot H_2O$[c]	52.81	6.82	14.66	52.78	6.43	14.29
Boc–(4—9)–NH_2	MA	87	−18.9 (MeOH)	0.56	$C_{71}H_{100}N_{16}O_{14}S_4 \cdot 5/2CH_3OH$[c]	54.83	6.89	13.91	54.62	6.34	13.64
Boc–(1—9)–NH_2 (**12**)	HOBt ester	75	−17.5 (DMF)	0.63	$C_{100}H_{129}Cl_2N_{19}O_{18}S_5 \cdot C_2H_5OC_2H_5 \cdot 3/2CH_3OH$[c]	56.60	6.52	11.89	56.38	6.18	11.72

a) TLC on silica gel. Solvent system: MeOH–$CHCl_3$ (1 : 7). *b*) Precipitated from CH_3OH–H_2O. *c*) Precipitated from CH_3OH–ether.

The bioassays, the binding assays, and the tail pinch tests were performed by the previously described methods.[5b]

Yields, physical constants, and analytical data of Boc–MeTyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–Arg(Tos)–MeArg(Tos)–D-Leu–Arg(Tos)–NHEt (**11**), Boc–Tyr(Cl_2Bzl)–D-Cys(MBzl)–Gly–Phe–Cys(MBzl)–Arg(Tos)–MeArg(Tos)–D-Leu–Arg(Tos)–NH_2 (**12**), and their intermediates are listed in Table IV.

MeTyr–Gly–Gly–Phe–Leu–Arg–MeArg–D-Leu–Arg–NHEt (9) Boc–MeTyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–Arg(Tos)–MeArg(Tos)–D-Leu–Arg(Tos)–NHEt (**11**) (318 mg, 0.166 mmol) was treated for 1 h at −5 °C with anhydrous liquid HF (10 ml) in the presence of anisole (0.2 ml). After removal of the HF *in vacuo*, the residue was dissolved in water and the solution was treated with Amberlite IRA-93 (acetate form) and lyophilized. The crude product was purified by HPLC on Nucleosil $5C_{18}$ (2 × 25 cm) using H_2O–CH_3CN (85 : 15) containing 0.015% HCl as an eluent (105 mg, 53%). Data obtained for characterization are listed in Table I.

Tyr–D-Cys–Gly–Phe–Cys–Arg–MeArg–D-Leu–Arg–NH_2 (10) Boc–Tyr(Cl_2Bzl)–D-Cys(MBzl)–Gly–Phe–Cys(MBzl)–Arg(Tos)–MeArg(Tos)–D-Leu–Arg(Tos)–NH_2 (**12**) (515 mg, 0.245 mmol) was treated for 2 h at −5 °C with liquid HF (10 ml) in the presence of anisole (0.2 ml). After removal of the HF *in vacuo*, the residue was dissolved in water, treated with Amberlite IRA-93 (acetate form), and lyophilized. The free peptide thus obtained was dissolved in water (1.3 l). This solution was adjusted to pH 8 with aqueous NH_3. After air was introduced therein with stirring for 2 d, the solution was readjusted to pH 6 and lyophilized. The crude product was purified by HPLC on Nucleosil $5C_{18}$ (2 × 25 cm) using H_2O–CH_3CN (88 : 12) containing 0.05% HCl as an eluent (140 mg, 48%). Data obtained for characterization are listed in Table I.

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