Chem. Pharm. Bull. 38(2) 404—406 (1990) Vol. 38, No. 2

Synthesis and Structure–Activity Relationships of [MeTyr^1, MeArg^7]-Dynorphin A(1—8)–OH Analogues with Substitution at Position 8

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A series of [MeTyr^1, MeArg^7]-Dynorphin A (Dyn)(1—8)–OH analogues, modified at position 8 with various amino acids, is described. Their biological activities were determined in the three bioassays [guinea pig ileum (GPI), mouse vas deferens (MVD), and rabbit vas deferens (RVD)] and in the mouse tail-pinch test after subcutaneous administration. None of the analogues tested displayed more potent κ-opioid activity in the RVD than [MeTyr^1, MeArg^7, D-Leu^8]-Dyn(1—8)–NHEt (1), which is a potent analgesic peptide with similar opioid receptor selectivity to that of Dyn. However, [MeTyr^1, MeArg^7, MeIle^8]-Dyn(1—8)–OH (11) showed about a twofold more potent analgesic effect than 1. Based on the obtained results it is conceivable that in the case of Dyn(1—8)–OH analogues both a lipophilic L-amino acid in position 8 and an unchanged 7–8 amide bond are essential to maintain potent κ-opioid activity.

Keywords dynorphin A; octapeptide; synthesis; structure–activity relationship; bioassay; analgesic effect

Since the discovery of dynorphin A (Dyn),[1] which has been postulated to be the endogenous ligand for the κ-opioid receptor,[2] a number of analogues of its fragments have been synthesized and evaluated for biological activity.[3] Recently, we have demonstrated that a metabolically stable analogue of Dyn(1—8)–NH_2, MeTyr–Gly–Gly–Phe–Leu–Arg–MeArg–D-Leu–NHEt (**1**), not only retains opioid receptor selectivity similar to that of Dyn but also produces a more potent analgesic effect than morphine even when administered subcutaneously into mice.[4] In an earlier paper, we have reported the synthesis and structure–activity relationships of Dyn(1—8)–NH_2 analogues, all of which are terminated with D-Leu amide moieties.[5]

With respect to Dyn(1—8)–OH, which has a carboxyl group at the C-terminal, Goldstein *et al.* reported that the activity of Dyn(1—8)–OH was sevenfold less potent on the guinea pig ileum (GPI) than that of Dyn(1—8)–OMe and suggested that the lower activity of the former was possibly due to the effect of a negatively charged C-terminal acid group.[3d] On the other hand, we recently indicated that Dyn(1—8)–OH still shows opioid activity comparable to that of Dyn in the presence of peptidase inhibitors in the GPI, mouse vas deferens (MVD), and rabbit vas deferens (RVD) assays.[4b] Therefore, the present investigation was undertaken to inquire into the effect of conversion of the C-terminal ethylamide of **1** to a carboxyl group. In addition, the effects of replacing D-Leu at position 8 of the resulting [MeTyr^1, MeArg^7, D-Leu^8]-Dyn(1—8)–OH (**2**) with various amino acids on opioid activity in the bioassays (GPI, MVD, and RVD) and on analgesic activity were also examined.

Synthesis All of the Dyn(1—8)–OH analogues listed in Table I were synthesized by the classical solution method by a combination of stepwise elongation with fragment condensation. The Gly residue in position 3 was chosen as the racemization-free fragment coupling point. The mixed-anhydride and *N*,*N*′-dicyclohexylcarbodiimide (DCC)–*N*-hydroxybenzotriazole (HOBT) coupling methods were employed. α-Amino groups were protected with benzyloxycarbonyl groups. The carboxyl group of the amino acid at position 8 of each peptide was protected with a *tert*-butyl group. Side chain protecting groups were as follows: Tyr, Cl_2Bzl; Arg, Tos; D-Glu, Bu^t; Asp, Bu^t. All the protecting groups were removed with HF/anisole in the usual manner. Purification was achieved by high-performance liquid chromatography (HPLC) on Nucleosil $5C_{18}$ (2 × 25 cm) and homogeneity of the purified product was evaluated by thin

TABLE I. Analytical Data of Dyn(1—8) Analogues: MeTyr–Gly–Gly–Phe–Leu–Arg–R

Compound No.	R	$[\alpha]_D^{20}$ [a]	Rf [b]	Amino acid analysis[c]					FAB-MS[d] (MH^+)
				Gly	Phe	Leu	Arg		
1	MeArg–D-Leu–NHEt	−21.8	0.70	1.87	1.00	1.96	0.95		1036
2	MeArg–D-Leu–OH	−32.4	0.68	1.95	1.00	1.95	0.99		1009
3	Arg–D-Leu–NH_2	−7.5	0.62	1.95	1.00	1.96	1.97		994
4	Arg–D-Leu–OH	−1.8	0.65	2.01	1.00	2.00	1.99		995
5	MeArg–D-Ala–OH	−35.1	0.54	1.95	1.00	1.00	0.99	1.01 (Ala)	967
6	MeArg–β-Ala–OH	−29.2	0.56	1.96	1.00	1.01	1.01		967
7	MeArg–MeAla–OH	−64.7	0.54	1.92	1.00	1.04	0.99		981
8	MeArg–Asp–OH	−35.4	0.48	1.96	1.00	0.99	0.99	1.01 (Asp)	1011
9	MeArg–D-Glu–OH	−30.8	0.51	1.93	1.00	1.00	1.00	1.01 (Glu)	1025
10	MeArg–Ile–OH	−10.6	0.63	1.96	1.00	1.05	1.01	0.99 (Ile)	1009
11	MeArg–MeIle–OH	−67.1	0.63	1.95	1.00	0.97	0.96		1023

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.

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TABLE II. Biological Activities of Dyn(1—8) Analogues

Compound	GPI IC_{50} (nM)[a]	MVD IC_{50} (nM)[a]	RVD IC_{50} (nM)[a]	Analgesia (s.c.)[b] ED_{50} (mg/kg)
1	0.3±0.03	7.4±2.5	2.6±0.4	1.0 (0.4—2.5)
2	0.6±0.08	7.0±0.7	21.4±7.4	0.92 (0.08—10.73)
3	1.4±0.3	5.2±0.2	14.3±12.3	
4	0.6±0.1	10.9±1.6	39.0±6.9	
5	1.0±0.2	8.8±1.7	14.2±3.2	1.92 (1.01—3.73)
6	1.6±0.2	13.8±2.7	21.0±4.1	0.61 (0.23—1.91)
7	1.3±0.3	33.4±10.0	32.5±12.6	1.22 (0.54—2.79)
8	2.8±0.5	21.2±2.9	37.8±14.1	
9	2.2±0.1	32.8±5.2	42.0±6.6	
10	0.4±0.06	5.5±0.5	7.6±0.7	>10.0
11	0.7±0.16	9.8±1.0	14.8±4.8	0.42 (0.17—1.01)
Dyn	0.2±0.03	3.0±0.5	17.4±6.7	
Morphine	71.6±5.0	736±135	Inactive	2.1 (1.2—3.5)

a) Results are the means±SEM. *b*) Analgesia at four doses of each compound was investigated. Each dose was tested on at least 8 animals.

layer chromatography (TLC) and analytical HPLC. Structural identification was achieved by amino acid analysis and fast atom bombardment mass spectroscopy (FAB-MS).

Biological Activity The analogues listed in Table I were tested *in vitro* in three isolated organ preparations (GPI, MVD, and RVD) and *in vivo* by the mouse tail-pinch assay after subcutaneous administration. The biological activities of Dyn and morphine were taken as references. The results are given in Table II. In the GPI, opioid effects are primarily mediated by μ-receptors, though κ-receptors are also present in this tissue. The MVD assay is generally taken as being representative for δ-receptor interactions, even though the vas deferens also contains μ- and κ-receptors. The RVD has been considered to have only κ-receptors, since κ-receptor agonists such as ethylketocyclazocine and bremazocine inhibit its contraction, whereas μ-, δ-, σ-, and ε-receptor agonists, such as morphine, FK33-824, enkephalin, [D-Ala^2, D-Leu^5]-enkephalin, SKF-10047, and β-endorphin have no effect.[6]

Results and Discussion

As shown in Table II, conversion of **1** to the corresponding carboxylic acid (**2**) reduced the potency about twofold on the GPI and eightfold on the RVD. However, the analgesic activity after subcutaneous administration into mice was unchanged. A similar tendency was also observed on the RVD in the case of the conversion of [MeTyr^1, D-Leu^8]-Dyn(1—8)–NH_2 (**3**) to the corresponding carboxylic acid (**4**). These results indicate that a negatively charged C-terminal carboxyl group is detrimental to κ-opioid activity for the case of Dyn(1—8)–OH analogues containing C-terminal D-Leu. Therefore, the effects of replacement of D-Leu at position 8 of **2** by various amino acids on opioid activities and analgesic activities were next examined.

Introduction of less bulky D-Ala at position 8 of **2** to give [MeTyr^1, MeArg^7, D-Ala^8]-Dyn(1—8)–OH (**5**) induced a 1.6-fold decrease in potency on the GPI, whereas it increased the potency 1.5-fold on the RVD. The analgesic activity was half that of **2**. Substitution of β-Ala in position 8 of **2** to give [MeTyr^1, MeArg^7, β-Ala^8]-Dyn(1—8)–OH (**6**) induced two to threefold decreases in potency on the GPI and MVD, whereas it caused no change in potency on the RVD. The analgesic activity was slightly more potent than that of **2**. Introduction of MeAla at position 8 of **2** to give [MeTyr^1, MeArg^7, MeAla^8]-Dyn(1—8)–OH (**7**) reduced the potency about twofold on the GPI, about fivefold on the MVD, and 1.5-fold on the RVD. Both [MeTyr^1, MeArg^7, Asp^8]-Dyn(1—8)–OH (**8**) and [MeTyr^1, MeArg^7, D-Glu^8]-Dyn(1—8)–OH (**9**), which have two carboxyl groups at the C-terminals, showed about one-fourth of the activity of **2** on the GPI and about half of the activity of **2** on the RVD. Introduction of Ile in position 8 of **2** to give [MeTyr^1, MeArg^7]-Dyn(1—8)–OH (**10**) increased the potency 1.5-fold on the GPI and about threefold on the RVD. Because **10** can be degraded by enzymes during the bioassays, **10** may have a more potent opioid activity in nature than the observed values. This interpretation is compatible with the fact that its analgesic activity was less than one-tenth of that of **2**. Therefore, the results obtained with **10** appear to indicate again that C-terminal carboxyl groups of Dyn(1—8)–OH analogues are not necessarily detrimental to κ-opioid activity. Substitution of MeIle at position 8 of **2** to give [MeTyr^1, MeArg^7, MeIle^8]-Dyn(1—8)–OH (**11**) caused no change in potency on the GPI and MVD, and increased the potency 1.5-fold on the RVD. However, compound **11** produced a very potent analgesic effect (ED_{50}=0.42 mg/kg), which is about twice that of **1** or **2** and about ninefold that of morphine.

Based on these results, it is conceivable that in the case of Dyn(1—8)–OH analogues both the lipophilic L-amino acid at the C-terminal and an unchanged 7–8 amide bond are essential in order to maintain potent κ-opioid activity. For this reason it seems difficult to obtain a metabolically stable Dyn(1—8)–OH analogue that shows κ-opioid activity comparable to that of **1**. On the other hand, we found some potent analgesic Dyn(1—8)–OH analogues (**2**, **6**, **7**, and **11**) that differ from **1** in the opioid receptor selectivity. These peptides may be worthy of further examination.

Experimental

General Methods 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. Analytical and preparative HPLC was performed using an ALTEX 110A pump and a JASCO UVIDEC 110A 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 (60F254, Merck) using the following solvent systems (all v/v): (A) MeOH–$CHCl_3$ (1:9), (B) MeOH–$CHCl_3$ (1:7).

A mixed anhydride was prepared as follows: Ethyl chloroformate (1 eq) was added at about −20 °C to a solution of a carboxy component (1 eq) and NMM (1eq) in THF or DMF. The mixture was stirred for 5 min and a solution of an amine component was added.

Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature: *Biochem. J.*, **219**, 345 (1984). The following other abbreviations are also used: MeTyr, *N*-methyltyrosine; MeArg, N^{α}-methylarginine; MeAla, *N*-methylalanine; β-Ala, β-alanine; MeIle, *N*-methylisoleucine; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; Cl_2Bzl, 2,6-dichlorobenzyl; Tos, tosyl; Bu^t, *tert*-butyl; NMM, *N*-methylmorpholine; THF, tetrahydrofuran; DMF, dimethylformamide; DCU, *N*,*N*′-dicyclohexylurea.

The bioassays (GPI, MVD, and RVD) and the tail-pinch test were performed by the methods described elsewhere.[6,7]

Z–MeArg(Tos)–MeIle–OBu^t (12) Z–MeArg(Tos)–OH[8] (14.3 g, 30 mmol) and MeIle–OBu^t[9] (6.1 g, 30 mmol) were dissolved in DMF (40 ml). To this solution, HOBT (4.86 g, 36 mmol) and DCC (6.80 g, 33 mmol) were added at 0 °C. The mixture was stirred at 4 °C for 4 d, then

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the precipitated DCU was filtered off and the solvent was removed *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$–MeOH, 30 : 1) (5.6 g, 28%): $[\alpha]_D^{20}$ −77.2° (c = 1.0, DMF); TLC, *Rf* (A) 0.80. *Anal.* Calcd for $C_{33}H_{49}N_5O_7S$: C, 60.07; H, 7.49; N, 10.61. Found: C, 59.69; H, 7.45; N, 10.37.

Z–Arg(Tos)–MeArg(Tos)–MeIle–OBu^t (13) HCl·MeArg(Tos)–MeIle–OBu^t (5.40 g, 9.61 mmol), prepared by catalytic hydrogenation (Pd/C) of **12** at atmospheric pressure, and Z–Arg(Tos)–OH (4.89 g, 10.57 mmol) were dissolved in DMF (15 ml). To this solution, HOBt (1.71 g, 12.68 mmol) and DCC (2.40 g, 11.62 mmol) were added at 0 °C. The mixture was stirred at 4 °C overnight and at room temperature for 2 d, then the precipitated DCU was filtered off and the solvent was removed *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$–MeOH, 20 : 1) (2.78 g, 30%): $[\alpha]_D^{20}$ −75.9° (c = 1.0, DMF); TLC, *Rf* (A) 0.51. *Anal.* Calcd for $C_{46}H_{67}N_9O_{10}S_2 \cdot 1/2H_2O$: C, 56.42; H, 7.00; N, 12.87. Found: C, 56.27; H, 7.00; N, 12.70.

Z–Leu–Arg(Tos)–MeArg(Tos)–MeIle–OBu^t (14) HCl·H–Arg(Tos)–MeArg(Tos)–MeIle–OBu^t (2.22 g, 2.54 mmol), prepared by catalytic hydrogenation (Pd/C) of **13**, was dissolved in DMF (10 ml) containing NMM (0.31 ml, 2.8 mmol). This solution was added to a mixed anhydride prepared from Z–Leu–OH (743 mg, 2.80 mmol) in DMF (5 ml). The mixture was stirred at 0 °C for 2 h, then the solvent was evaporated off and the residue was dissolved in AcOEt. The solution was washed with 5% $NaHCO_3$ and water and concentrated to dryness (2.31 g, 83%): $[\alpha]_D^{20}$ −79.2° (c = 1.0, DMF); TLC, *Rf* (A) 0.39. *Anal.* Calcd for $C_{52}H_{78}N_{10}O_{11}S_2 \cdot H_2O$: C, 56.71; H, 7.32; N, 12.72. Found: C, 56.80; H, 7.22; N, 12.69.

Z–Phe–Leu–Arg(Tos)–MeArg(Tos)–MeIle–OBu^t (15) HCl·H–Leu–Arg(Tos)–MeArg(Tos)–MeIle–OBu^t (2.07 g, 2.1 mmol), prepared by catalytic hydrogenation (Pd/C) of **14**, was dissolved in DMF (5 ml) containing NMM (0.32 ml, 2.94 mmol). This solution was added to a mixed anhydride prepared from Z–Phe–OH (692 mg, 2.31 mmol) in DMF (5 ml). The mixture was stirred at 0 °C for 2 h, then the solvent was evaporated off and the residue was dissolved in AcOEt. The solution was washed with 5% $NaHCO_3$ and water and concentrated to dryness (2.34 g, 90%): $[\alpha]_D^{20}$ −71.2° (c = 1.0, DMF); TLC, *Rf* (B) 0.76. *Anal.* Calcd for $C_{61}H_{87}N_{11}O_{12}S_2 \cdot H_2O$: C, 58.68; H, 7.19; N, 12.34. Found: C, 58.80; H, 7.09; N, 12.14.

Boc–MeTyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–Arg(Tos)–MeArg(Tos)–MeIle–OBu^t (16) HCl·H–Phe–Leu–Arg(Tos)–MeArg(Tos)–MeIle–OBu^t (2.00 g, 1.765 mmol), prepared by catalytic hydrogenation (Pd/C) of **15**, was dissolved in DMF (10 ml) containing NMM (0.233 ml, 2.12 mmol). This solution was added to a mixed anhydride prepared from Boc–MeTyr(Cl_2Bzl)–Gly–Gly–OH[5] (1.104 g, 1.942 mmol) in DMF (8 ml). The mixture was stirred at 0 °C for 4 h, then the solvent was evaporated off and the residue was purified by column chromatography on silica gel ($CHCl_3$–MeOH, 20 : 1) (1.68 g, 58%): $[\alpha]_D^{20}$ −63.6° (c = 1.0, DMF); TLC, *Rf* (B) 0.76. *Anal.* Calcd for $C_{79}H_{110}Cl_2N_{14}O_{16}S_2 \cdot H_2O$: C, 56.99; H, 6.66; N, 11.78. Found: C, 57.00; H, 6.74; N, 11.70.

MeTyr–Gly–Gly–Phe–Leu–Arg–MeArg–MeIle–OH (11) Compound **16** (1.56 g, 0.95 mmol) was treated for 1 h at −5 °C with anhydrous liquid HF (20 ml) in the presence of anisole (1.5 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 (90 : 10) containing 0.015% HCl as an eluent (240 mg, 24%). The results of characterization of the product are summarized in Table I.

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