Novel [D-Arg^2]dermorphin(1—4) Analogs with μ-Opioid Receptor Antagonist Activity[1)]

Akihiro AMBO, Takanori TERASHIMA, and Yusuke SASAKI*

Tohoku Pharmaceutical University; 4–4–1 Komatsushima, Aoba-ku, Sendai 981–8558, Japan.
Received May 31, 2002; accepted July 30, 2002; published online August 7, 2002

Ten Tyr-D-Arg-Phe-βAla-NH$_2$ (YRFB) analogs in which specific amino acid side chains are shifted to the N^{α}-position were synthesized, and the binding to these analogs to the μ receptor and their *in vitro* biological properties were evaluated. Some analogs in which a *N*,*N*-bis(*p*-hydroxybenzyl)-Gly residue was substituted for Tyr^1 exhibited μ receptor antagonist activities (pA$_2$) between 5.3 and 6.1. Of these analogs, [*N*,*N*-bis(*p*-hydroxybenzyl)-Gly^1]YRFB was found to be the most potent specific antagonist for the μ-opioid receptor.

Key words dermorphin; opioid peptide; peptoid; antagonist; opioid receptor binding

It is now well established that there are three main types of opioid receptors: μ, δ, and κ, and agonists or antagonists useful in the study of these opioid receptors have been discovered.[2)] However, in efforts to enhance potency and receptor selectivity, the search continues for ligands specific for each type of receptor. Based upon the extensive study of the structure–activity relationships of dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH$_2$), a naturally occurring peptide isolated from the skin of *Phyllomedusa sauvagei*, we have synthesized and described a shorter analog, Tyr-D-Arg-Phe-βAla-NH$_2$ (YRFB), which is a potent μ-receptor agonist and elicits analgesia even after peripheral administration in mice.[3)] Simon *et al.*[4)] have described a peptide mimic they call "peptoid." In peptoid the amino acid side chain on the α-carbon is shifted to the nitrogen atom on the peptide backbone.[4)] Peptoids are synthesized using *N*-alkylated glycine units in which the alkyl group corresponds to the amino acid side chain. The *N*-substituted glycine has been used to enhance bioavailability and proteolytic stability for some peptidic enzyme inhibitors[5,6)] and for an analog of a fragment from parathyroid hormone-related peptide.[7)] We have described deltorphin analogs containing *N*-substituted glycine (*i.e.*, *N*-*n*-butyl-Gly for Nle, *N*-iso-butyl-Gly for Leu, and *N*-*sec*-butyl-Gly for Ile) and have demonstrated that such modifications produce significant differences in biological response, including antagonist and mixed agonist/antagonist activities.[8)] In the present study, ten YRFB analogs (Fig. 1) were synthesized in which the Tyr^1, D-Arg^2, or Phe^3 residues were replaced by *N*-substituted glycines, and their binding to receptor and biological activities were tested.

Results and Discussion

All analogs were synthesized by the solid-phase method using Boc chemistry as described previously.[9)] *N*-substituted glycine residues were introduced on the solid support by combining the usual introduction of the Gly residue and the reductive alkylation of the glycyl peptide resin using the corresponding alkylaldehyde and NaCNBH$_3$ in 1% AcOH/DMF.[10)] Table 1 presents the analytical data for the synthetic peptides.

Table 2 shows the receptor binding assay data and *in vitro* bioassay data for the synthetic analogs on guinea pig ileum (GPI) tissues. Single replacement of Tyr^1, D-Arg^2, or Phe^3 by corresponding *N*-substituted glycines yielded analogs **1**—**3**, which exhibited dramatic decreases in μ-receptor affinity (15000-fold lower than the parent peptide) and were effectively inactive in the GPI assay. The losses in activity are likely to be due to changes in the particular orientations of Tyr^1 and Phe^3 and/or the distance between the two residues, which are of crucial importance in the opioid agonist

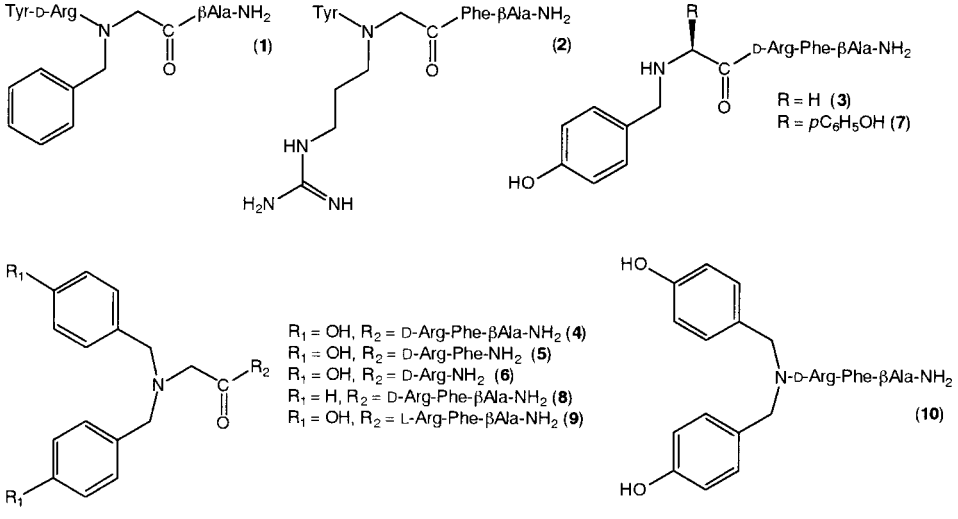

Fig. 1. Structures of the Synthetic Peptide

* To whom correspondence should be addressed. e-mail: ysasaki@tohoku-pharm.ac.jp

Table 1. Analytical Data for Synthetic Peptides

Analogs	$[\alpha]_D$ (°)[a]	TLC[b] Rf(A)	TLC[b] Rf(B)	FAB-MS ($M+H^+$)
[*N*-Benzyl-Gly^3]YRFB (**1**)	+46.7	0.33	0.67	555
[*N*-Guanidinopropyl-Gly^2]YRFB (**2**)	+17.1	0.32	0.69	555
[*N*-*p*-Hydroxybenzyl-Gly^1]YRFB (**3**)	+2.8	0.27	0.67	555
[*N*,*N*-Bis(*p*-hydroxybenzyl)-Gly^1]YRFB (**4**)	+7.2	0.41	0.81	661
[*N*,*N*-Bis(*p*-hydroxybenzyl)-Gly^1, desβAla^4]YRFB (**5**)	+5.7	0.53	0.83	590
[*N*,*N*-Bis(*p*-hydroxybenzyl)-Gly^1, desPhe^3, desβAla^4]YRFB (**6**)	+3.2	0.38	0.78	443
[*N*-*p*-Hydroxybenzyl-Tyr^1]YRFB (**7**)	+13.7	0.48	0.81	661
[*N*,*N*-Bis(benzyl)-Gly^1]YRFB (**8**)	−0.4	0.54	0.84	629
[*N*,*N*-Bis(*p*-hydroxybenzyl)-Gly^1, L-Arg^2]YRFB (**9**)	+7.2	0.42	0.82	661
[desTyr^1, N^α,N^α-bis(*p*-hydroxybenzyl)-D-Arg^2]YRFB (**10**)	+5.8	0.66	0.78	604

a) Optical rotation in 1% AcOH at 20 °C. *b*) See Experimental.

Table 2. Opioid Receptor Binding and GPI Assays of Synthetic Peptides

No.	[^{3}H]DAMGO K_i (μ), nM	[^{3}H]DPDPE K_i (δ), nM	$\frac{K_i(\delta)}{K_i(\mu)}$	GPI assay IC_{50}, nM	Antagonism activity (pA_2)[a] *vs.* YRFB	Antagonism activity (pA_2)[a] *vs.* EM-1
YRFB	0.18±0.06	369±236	2050	3.15±1.10	NA	NA
1	2710±307	>27536	>12.1	>10000	NA	NA
2	3844±327	>27536	>7.16	≈10000	NA	NA
3	5355±774	>27536	>5.14	>10000	NA	NA
4	529±132	7202±2684	13.6	>10000	6.0±0.3	6.1±0.2
5	580±121	1482±529	2.56	≈10000	5.6±0.2	5.9±0.3
6	1661±591	493±138	0.30	>10000	NA	NA
7	25.4±9.1	1156±531	45.4	49.6±1.2	NA	NA
8	5485±1030	>27536	>5.02	≈10000	NA	NA
9	204±27	538±205	2.64	>10000	5.5±0.4	5.9±0.2
10	328±133	209±20	0.64	>10000	5.3±0.2	5.5±0.3

a) The pA_2 value against YRFB or endomorphin-1 (EM-1) as μ-agonist. NA, no antagonist activity at 10 μM. Data are means±S.E. (n=6—8).

activity.[11] Replacing of Tyr^1 with bis-*p*-hydroxybenzylated Gly (**4**, Fig. 1) resulted in a 10-fold increase in binding affinity for the μ receptor compared with the mono-*p*-hydroxybenzylated compound (**3**). Eliminating the C-terminal βAla residue of **4** yielded analogs **5** but low μ receptor selectivity. The additional deletion of the Phe^3 residue (**6**) resulted in a significant loss in μ receptor affinity; however, this deletion analog demonstrated a significantly increased affinity for the δ receptor. Analog **7** is a regioisomer of **4** and possessed a potent affinity for the μ receptor and GPI potency. Deletion of the hydroxyl groups at the bis-*p*-hydroxybenzyl groups of **4** yielded analogs **8**, which exhibited loss of activity in both assays. The L-Arg^2 enantiomer of **4** (analog **9**) and [des-Tyr^1, N^α,N^α-bis(*p*-hydroxybenzyl)-D-Arg^2]YRFB (**10**) still exhibited moderate affinities for both the μ and δ receptors. All of these analogs were significantly inactive as μ receptor agonists in the GPI assay except for **7**, which as mentioned above, is a potent μ agonist.

Next, attempts were made to assess the antagonist activity of these analogs at the μ receptor on GPI tissue, using YRFB and endomorphin-1 (EM-1)[12,13] as agonists. Analogs **4**, **5**, **9**, and **10** exhibited antagonist activity values (pA_2) between 5.3 and 6.1, whereas the other analogs were inactive. Of these four compounds, **4** was the most potent and specific μ antagonist with a pA_2 of 6.0 to 6.1. These results suggest that the βAla residue at position 4 and the chirality of Arg^2 are not important elements for μ antagonist activity. As indicated in

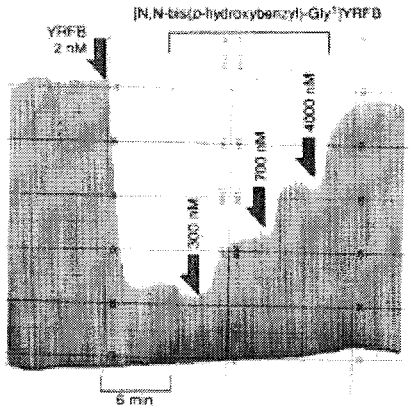

Fig. 2. Dose–Response Dependence of Antagonism of [*N*,*N*-Bis(*p*-hydroxybenzyl)-Gly^1]YRFB (**4**) against YRFB in the GPI Assay

Fig. 2, analog **4** counteracted the inhibitory effects of YRBF in a dose-dependently manner. The antagonistic effect of **4** (10 μM) was also tested against the δ agonist DPDPE in the mouse vas deferens assay and against the κ agonist dynorphin(1—13) in the presence of the μ antagonist CTAP[14,15] in the GPI assay. Analog **4** exhibited no antagonist activity against either the δ or κ receptor, thus, **4** appears to be a specific μ receptor antagonist.

In conclusion, our present results lend support to other reports that the introduction of *N*-substituted Gly residues into

biologically active peptides significantly alters biological activity.[4,8] The diversity of conformational states that such a substitution produces may be responsible for this effect and/or the removal of an amide hydrogen may prevent formation of the hydrogen bond required for maintaining an active conformation or interacting with the receptor.[16] Using the μ agonist YRFB as the starting compound in the present study, we have created some novel antagonists to the μ receptor. Analog **4** is a moderately potent μ antagonist compared with the typical μ antagonist naloxone (pA_2=8.4—8.8). It is possible that further analysis of **4** and the structure–activity relationships reported in this study may lead to novel peptide or nonpeptide antagonists that are even more useful for the study of the μ-opioid receptor.

Experimental

TLC carried out on silica gel plate (Merck, Kiesel gel $60F_{254}$, 5×10 cm) with the following solvent systems: Rf(A), BuOH–AcOH–H_2O (4 : 1 : 5, upper phase); Rf(B), BuOH–AcOH–pyridine–H_2O (15 : 3 : 10 : 12). All protected-amino acids and methoxybenzohydrylamine resin (MBHA resin) were purchased from Watanabe Chemical Ind., Ltd. (Hiroshima). Peptides were hydrolyzed with 6 N HCl at 110 °C for 22 h, amino acids were then analyzed with a Hitachi L-8500 amino acid analyzer. FAB-MS was determined with a JEOL JMS-DX303 spectrometer. Analytical HPLC was conducted on a column of YMC-Pack ODS-AM-302, using solvent systems A (0.06% TFA) and B (0.06% TFA in 80% acetonitrile). Peptides were eluted with a linear gradient of 10% to 50% B over 40 min at a flow rate of 1 ml/min. Peptides were purified by elution on a Develosil LOP ODS column (24×360 mm, Nomura Kagaku) with a linear 10—50% gradient of solvent B over 180 min at a flow rate of 3.0 ml/min. The eluate was monitored at 280 nm.

Peptide Synthesis Peptides were synthesized by the solid-phase technique using a DICDI/HOBt-mediated Boc strategy as previously described.[9] *N*-substituted glycine residues were also introduced on the solid support in a two-step process. As usual, the Gly residue was introduced first; then the glycyl-peptide resin was subjected to reductive alkylation using the corresponding alkylaldehyde and $NaCNBH_3$ in 1% AcOH/DMF.[10] For synthesis of [*N*-guanidinopropyl-Gly^2]YRFB (**2**), the H-Gly-Phe-βAla-MBHA resin was alkylated with three equivalents of and $NaCNBH_3$ in 1% AcOH/DMF as is usual[10] and then reacted with Fmoc-Tyr-OH in the presence of PyBOP/HOBt[17] in DMF. After the removal of Boc-group with 0.5 M methanesulfonic acid/DCM, the amino group was guanylated using 3,5-dimethylpyrazole-1-carboxamidine nitrate in DIPEA/DMF.[18] The peptide resin was treated with anhydrous HF containing anisole to give a crude product, which was then purified by reverse phase-HPLC. For the syntheses of **3** and **4**, two equivalents of *p*-hydroxybenzaldehyde and $NaCNBH_3$ were reacted with the H-Gly-D-Arg(Tos)-Phe-βAla-MBHA resin for 6 h at 60 °C. Deprotection and cleavage of the peptide from the resin yielded analogs **3** and **4** in a ratio of 1 : 1, which were separated by reverse phase-HPLC. The purity of synthetic peptides was determined to be greater than 95% by analytical HPLC. The analytical data for the synthetic peptides is summarized in Table 1.

Receptor Binding Assay Receptor binding was assayed using rat brain synaptosomal membrane fractions as described elsewhere.[19] [^{3}H]DAMGO and [^{3}H]DPDPE were radioligands for μ- and δ-opioid receptors, respectively. The IC_{50} values were determined from log dose-displacement curves. K_i values were calculated by the equation of Cheng and Prusoff.[20] The K_d values of [^{3}H]DAMGO and [^{3}H]DPDPE were 0.73 nM and 0.76 nM, respectively.

GPI Assay We assayed the biological activity towards the μ receptor by means of the guinea pig ileum (GPI) assay as reported in detail previously.[9] The myenteric plexus-longitudinal muscle was obtained from male Hartley strain guinea pig (250—300 g) ileum according to Rang.[21] Dose–response curves were constructed and IC_{50} values were calculated graphically.

pA_2 values were calculated according to the procedure of Arunlakshana and Schild.[22]

References and Notes

1) Amino acids and peptides are of L-configuration unless otherwise noted. Amino acids and peptides used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature in *Eur. J. Biochem.*, **139**, 9 (1984). Other abbreviations used are: YRFB=Tyr-D-Arg-Phe-βAla-NH_2, DMF=dimethylformamide, DICDI=diisopropylcarbodiimide, HOBt=1-hydroxybenzotriazole, DIPEA=diisopropylethylamine, DAMGO=[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin, DPDPE=[D-Pen^2, D-Pen^5]enkephalin, Tos=tosyl, Fmoc=*N*-9-fluorenylmethyloxycarbonyl, PyBOP=benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate, MBHA resin=methoxybenzohydrylamine resin, GPI=guinea pig ileum.
2) Dhawan B. N., Cesselin F., Raghubir R., Reisine T., Bradley P. B., Portoghese P. S., Hamon M., *Pharmacol. Rev.*, **48**, 567—592 (1996).
3) Sasaki Y., Ambo A., Suzuki K., *Chem. Pharm. Bull.*, **39**, 2316—2318 (1991).
4) Simon R. J., Kania R. S., Zuckermann R. N., Huebner V. D., Jewell D. A., Banbille S., Ng S., Wang L., Rosenberg S., Marlowe C. K., Spellmeyer D. C., Tan R., Frankel A. D., Santi D. V., Cohen F. E., Bartlett P. A., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 9367—9371 (1992).
5) Suh J. T., Skiles J. W., Williams B. E., Youssefyeh R. D., Jones H., Loev B., Neiss E. S., Schwab A., Mann W. S., Khandwala A., Wolf P. S., Weinryb I., *J. Med. Chem.*, **28**, 57—66 (1985).
6) Skiles J. W., Fuchs V., Miao C., Sorcek R., Grozinger K. G., Mauldin S. C., Virous J., Mui P. W., Jacober S., Chow G., Matteo M., Skoog M., Weldon S. M., Possanza G., Keirns J., Letts G., Rosenthal A. S., *J. Med. Chem.*, **35**, 641—662 (1992).
7) Heizmann G., Felder E. R., *Peptide Res.*, **7**, 328—332 (1994).
8) Sasaki Y., Chiba T., *J. Med. Chem.*, **38**, 3995—3999 (1995).
9) Sasaki Y., Ambo A., Midorikawa K., Suzuki K., *Chem. Pharm. Bull.*, **41**, 1391—1394 (1993).
10) Sasaki Y., Coy D. H., *Peptides*, **8**, 119—121 (1987).
11) Castiglione-Morelli M. A., Lelj F., Pastore A., Salvadori S., Tancredi T., Tomatis R., Trivellone E., Temussi P. A., *J. Med. Chem.*, **30**, 2067—2073 (1987).
12) Champion H. C., Zadina J. E., Kastin A. J., Hackler L., Ge L. J., Kadowitz P. J., *Biochem. Biophys. Res. Commun.*, **235**, 567—570 (1997).
13) Champion H. C., Zadina J. E., Kastin A. J., Kadowitz P. J., *Life Sci.*, **61**, PL409—415 (1997).
14) Pelton J. T., Kazmierski W., Gulya K., Yamamura H. I., Hruby V. J., *J. Med. Chem.*, **29**, 2370—2375 (1986).
15) Kazmierski W., Wire W. S., Lui G. K., Knap R. J., Shook J. E., Burks T. F., Yamamura H. I., Hruby V. J., *J. Med. Chem.*, **31**, 2170—2177 (1988).
16) Hansen P. E., Morgan B. A., "The Peptides," Vol. 6 Chap. 8, eds. by Udenfriend S., Meienhofer J., Academic Press, London, 1984, pp. 269—321.
17) Frerot E., Coste J., Pantaloni A., Dufour M.-N., Jouin P., *Tetrahedron*, **47**, 259—270 (1991).
18) Habbeb A., *Methods Enzymol.*, **25B**, 558—566 (1972).
19) Sasaki Y., Ambo A, Suzuki K., *Biochem. Biophys. Res. Commun.*, **180**, 822—827 (1991).
20) Cheng Y., Prusoff W. H., *Biochem. Pharmacol.*, **22**, 3099—3108 (1973).
21) Rang H. P., *Br. J. Pharmacol.*, **22**, 356—365 (1964).
22) Arunlakshana O., Schild H. O., *Br. J. Pharmacol.*, **14**, 48—58 (1959).