Synthesis and Opioid Activities of [D-Leu^8]Dynorphin(1—8) Analogs Containing a Reduced Peptide Bond, $\Psi(CH_2NH)$[1)]

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[D-Leu^8]Dynorphin(1—8)-NH_2 analogs, in which each peptide bond was systematically replaced with a $\psi(CH_2NH)$ peptide bond, were synthesized by the solid-phase method. The $\psi(CH_2NH)$ bond was introduced by the Boc-amino acid aldehyde/$NaCNBH_3$ method on a solid support. In the syntheses of the analogs, undesirable double alkylation took place at the sequences of Tyr$^1\psi(CH_2NH)$Gly^2 and Gly$^2\psi(CH_2NH)$Gly^3, possibly due to the low steric hindrance of the glycine residue. To suppress the double alkylation, a minimum amount of aldehydes was employed. In the receptor binding assay, the $\psi(CH_2NH)$ replacement of N-terminal peptide bonds which led to $^1\psi^2$- (2) and $^2\psi^3$-analogs (3) resulted in a marked reduction in binding affinities for μ-, δ-, and κ-opioid receptors, while that of the other peptide bonds afforded analogs with a high κ-receptor affinity. A $^3\psi^4$-analog (4) showed extremely high κ-receptor selectivity (μ/κ K_i ratio = 339, δ/κ K_i ratio = 24104). In the *in vitro* bioactivity assay (guinea pig ileum assay), 2 showed a very low IC_{50} ratio (2.0) in the presence and absence of peptidase inhibitors whereas those of other analogs were >27, suggesting that the introduction of the CH_2NH isostere at Tyr^1–Gly^2 greatly enhanced the enzymatic stability of the parent peptide. Furthermore, analogs 2 and 3 showed a very low sensitivity to the inhibitory effect of NaCl plus 5′-guanylylimidodiphosphate on their binding at a κ-receptor site as compared with the other analogs and the parent peptide. These results suggest that the two analogs (2 and 3) have partial κ-antagonist properties.

Key words dynorphin(1—8) analog; reduced peptide bond; κ-antagonist property; receptor-binding assay; guinea pig ileum assay

Dynorphin A (Dyn) has been isolated from porcine pituitary[2)] and gut.[3)] The 17-amino acid-peptide is thought to be an endogenous ligand for the κ-opioid receptor.[4)] A shorter peptide, Dyn(1—8), has also been identified in porcine hypothalamus and found to be the minimum sequence for κ-receptor preference.[5)]

Tachibana *et al.* have reported a metabolically stable Dyn(1—8) analog, [MeTyr^1, MeArg^7, D-Leu^8]Dyn(1—8)-NHEt (E-2078), which not only retains κ-receptor selectivity similar to that of Dyn, but also produces a 2.5-fold more potent analgesia than morphine in the mouse tail-pinch test after i.v. administration.[6)]

On the other hand, the replacement of the peptide bond by the isosteric $\psi(CH_2NH)$ bond has been proved to be an effective modification for the design of antagonists of various biologically active peptides: *i.e.*, gastrin,[7)] bombesin,[8)] luteinizing hormone releasing hormone (LH-RH),[9)] secretin,[10)] substance P,[11)] growth hormone-releasing hormone,[12)] angiotensin,[13)] and casomorphin.[14)] Such modification can offer higher resistance to enzymatic degradation.[15)] In the present study we examined this modification to obtain Dyn(1—8) analogs with κ-receptor antagonist activity. We chose [D-Leu^8]Dyn(1—8)-NH_2 as the lead because [MeTyr^1, D-Leu^8]Dyn(1—8)-NH_2 still has a high κ-receptor selectivity, comparable to that of Dyn.[6)]

The present paper describes i) the synthesis of $\psi(CH_2$-NH) analogs of the lead peptide, in which each peptide bond was systematically replaced by the CH_2NH isostere, ii) receptor-binding properties of the synthetic analogs at the μ-, δ-, and κ-receptors, iii) the sensitivity of their binding at the κ-receptor site to Na^+ ions and guanine nucleotide (Gpp(NH)p), and iv) their *in vitro* bioactivities on guinea pig ileum (GPI) in the presence or absence of enzyme inhibitors.

Experimental

Optical rotations were measured with a JASCO DIP-140 polarimeter. Amino acid analysis was carried out on a Hitachi L-8500 amino acid analyzer after 6 N HCl hydrolysis of the peptide at 110 °C for 20 h. Thin-layer chromatography (TLC) was performed on silica gel plates (Merck, Kiesel gel $60F_{254}$, 5 × 10 cm) with the following solvent systems: A, *n*-BuOH–AcOH–H_2O (4 : 1 : 5, v/v, upper layer); B, *n*-BuOH–AcOH–pyridine–H_2O (15 : 3 : 10 : 12, v/v). Analytical high-performance liquid chromatography (HPLC) was performed on a YMC octadecyl silica (ODS) column (AM-303-10, 4.6 × 250 mm) using the following solvent systems: A, 0.06% TFA; B, 0.06% TFA in 80% acetonitrile. A linear gradient from 20 to 70% B over 50 min was used at a flow rate of 1 ml/min and the eluate was monitored at 220 nm. Fast atom bombardment mass spectrometry (FAB-MS) was run on a JEOL JMS-DX303 instrument. N^{α}-Boc amino acids were used with the following side chain protections: tosyl or NO_2 for Arg, 2,6-dichlorobenzyl for Tyr. Boc-amino acid aldehydes were obtained by reduction of the Boc-amino acid *N,O*-dimethylhydroxamate with $LiAlH_4$ according to the method of Fehrentz and Castro.[16)]

Synthesis of Peptides [D-Leu^8]Dyn(1—8) analogs were constructed on benzhydrylamine resin (BHA-resin, 0.85 meq/g) by the solid-phase technique using the *N,N′*-diisopropylcarbodiimide/1-hydroxybenztriazole-mediated Boc strategy as described previously.[17)] The $\psi(CH_2NH)$ peptide bonds were introduced by the method of Sasaki and Coy.[18)] The formation of the reduced peptide bond was checked by amino acid analysis after hydrolysis of the resin in concentrated HCl : propionic acid (1 : 1) at 130 °C for 2.5 h. Typically, for the synthesis of [$^5\psi^6(CH_2NH)$, D-Leu^8]Dyn(1—8)-NH_2 (6), the reduced peptide bond isostere was formed by the reductive alkylation of MSA·Arg(Tos)-Arg(Tos)-D-Leu-BHA-resin with Boc-leucinal (4 eq) in the presence of $NaCNBH_3$ (4 eq) in 1% acetic acid/DMF for 2 h. Then protected target peptide was constructed on the resin. The peptide was cleaved from the resin and deprotected by treatment with 10% anisole/HF at 0 °C for 60 min. After the removal of excess HF *in vacuo*, the resulting residue was extracted with 5% acetic acid. The extract was washed with ether and evaporated to dryness *in vacuo*. The crude peptide was then purified by medium-pressure HPLC on a Develosil LOP ODS column (3 × 30 cm) which was eluted with a linear gradient from 12 to 28% acetonitrile in

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Table 1. Analytical Data of Synthetic Dynorphin(1—8) Analogs

Analog	$[\alpha]_D$[a] (°)	TLC[a] *Rf* (A)	TLC[a] *Rf* (B)	FAB-MS $(M+H)^+$	Amino acid analysis[a] Tyr	Gly	Phe	Leu	Arg
[D-Leu^8]Dyn(1—8)-NH_2 (**1**)	−6.0	0.37	0.75	981	0.85	2.00	1.00	2.14	2.03
[$^1\psi^2$(CH_2NH), D-Leu^8]Dyn(1—8)-NH_2 (**2**)	−19.0	0.28	0.71	966[b]	—	1.04	1.00	2.02	1.98
[$^2\psi^3$(CH_2NH), D-Leu^8]Dyn(1—8)-NH_2 (**3**)	−5.2	0.21	0.66	967	0.92	—	1.00	2.10	2.02
[$^3\psi^4$(CH_2NH), D-Leu^8]Dyn(1—8)-NH_2 (**4**)	+1.0	0.20	0.67	967	0.91	1.00	—	1.93	2.01
[$^4\psi^5$(CH_2NH), D-Leu^8]Dyn(1—8)-NH_2 (**5**)	+10.2	0.23	0.72	966[b]	0.75	1.97	—	1.03	2.00
[$^5\psi^6$(CH_2NH), D-Leu^8]Dyn(1—8)-NH_2 (**6**)	+12.2	0.29	0.75	966[b]	0.72	2.03	1.00	1.06	1.02
[$^6\psi^7$(CH_2NH), D-Leu^8]Dyn(1—8)-NH_2 (**7**)	+12.4	0.30	0.70	967	0.89	2.07	1.00	2.01	—
[$^7\psi^8$(CH_2NH), D-Leu^8]Dyn(1—8)-NH_2 (**8**)	−8.2	0.26	0.65	967	0.95	2.01	1.00	1.03	0.99
Branched peptide (**9**)[c]	−6.0	0.36	0.75	1116	—	1.01	1.00	1.94	1.80
Branched peptide (**10**)[c]	+1.8	0.25	0.71	1173	1.67	—	1.00	2.12	2.02

a) See Experimental. *b*) Value shows M^+. *c*) See Fig. 1.

0.06% TFA over 220 min at a flow rate of 2.5 ml/min. Eluated material was detected at 280 nm. Fractions around the main peak were checked by analytical HPLC and the identifiable fractions were pooled appropriately and lyophilized to give a yield of 27.6% based on the starting resin.

For the syntheses of analogs **2**, **3**, **7**, and **8**, a modified method of $\psi(CH_2NH)$ bond formation was employed as mentioned in Results and Discussion.

Other peptides were also synthesized by the above-mentioned method. Physicochemical data of synthetic peptides are given in Table 1.

Receptor-Binding Assays The opioid receptor-binding assay was carried out by the same method as described previously.[19] [^{3}H]-DAGO,[20] [^{3}H]DADLE,[21] and [^{3}H]U-69593[22] were used as μ-, δ-, and κ-radioligands, respectively. The competitive experiments were carried out in the presence of peptidase inhibitors, bacitracin, bestatin, and soybean trypsin inhibitor. Bindings of μ- and δ-ligands were measured in rat brain homogenate. The κ-receptor binding assay was done with guinea pig brain homogenate.[19a] The values of inhibitory constant (K_i) of the synthetic peptides were calculated according to the equation of Cheng and Prusoff.[23] The K_d values of [^{3}H]DAGO, [^{3}H]DADLE, and [^{3}H]U-69593 used were 0.42, 1.14, and 0.57 nM, respectively.

To examine the sensitivity of the binding at the κ-receptor site to NaCl and Gpp(NH)p, binding assays of synthetic analogs and nor-BNI[24] were performed by using guinea pig cerebellum membranes and [^{3}H]dipre-norphin[25] (0.3 nM) as the radiolabeled ligand in the presence of 120 mM NaCl plus 50 μM Gpp(NH)p, according to the method of Frances *et al.*[25] Nonspecific binding was determined in the presence of excess nor-BNI (1 μM).

GPI Assay The myenteric plexus-longitudinal muscle was prepared from the ileum of anesthetized male Hartley guinea pigs weighing 300—350 g. The GPI assay was performed without or with a mixture of amastatin[26] (1 μM), captopril[27] (10 μM), and soybean trypsin inhibitor[28] (800 μg/20 ml) in the same manner as reported previously.[29] The IC_{50} is the concentration of a compound necessary to inhibit the amplitude of the electrically induced contraction by 50% (Table 3).

Results and Discussion

Peptide Synthesis Introduction of the reduced peptide bond $\psi(CH_2NH)$ was performed by reductive alkylation of the corresponding resin-bound amine with Boc-amino acid aldehyde and $NaCNBH_3$ according to the method of Sasaki and Coy.[18] All the target peptides except for **2** and **3** were synthesized in satisfactory yields of 14—31.6% based on the starting resin. In the case of the syntheses of **2** and **3**, however, the main product was found to be a doubly alkylated product (**9** and **10**) from the FAB-MS and amino acid analysis data. Although this method is now widely used for the $\psi(CH_2NH)$ analogs of a variety of biologically active peptides, there are some reports of double alkylation with this alkylation method on a solid support.[30] It may depend upon the sequence, because the Glyψ(CH_2NH)Phe analog (**4**) was obtained as the main product without any problem. Our present results showed that the undesirable double alkylation takes place dominantly at the sequence of Xψ(CH_2NH)Gly (X= amino acid), possibly due to the low steric hindrance of the Gly residue. Thus we attempted the synthesis of **2** and **3** by using 1 eq of Boc-amino acid aldehyde (crude). The alkylation was repeated 3 times. Figure 2 shows HPLC profiles of the crude peptides, **2** and **3**, obtained by this modified procedure. The desired peptides were obtained, though the yields were less than 10%. It is clear that the problem of simultaneous double alkylation, especially in the case of -Xψ(CH_2NH)Gly- or -Glyψ(CH_2NH)Gly- sequence, is serious. The best method to avoid the side reaction seems to be to employ an appropriate protecting group. In this context, further studies are in progress.

Another problem with the use of the reductive alkylation in the solid-phase method is the protection of the argininal side chain. Protection of the guanidino function with Tos has been shown to be unsuitable.[30a,31] In this study we

9: Tyrψ(CH_2>N)Gly–Gly–Phe–Leu–Arg–D-Leu–NH_2
Tyrψ(CH_2>N)

10: Tyr–Glyψ(CH_2>N)Gly–Phe–Leu–Arg–D-Leu–NH_2
Tyr–Glyψ(CH_2>N)

Fig. 1. Branched Peptides **9** and **10**

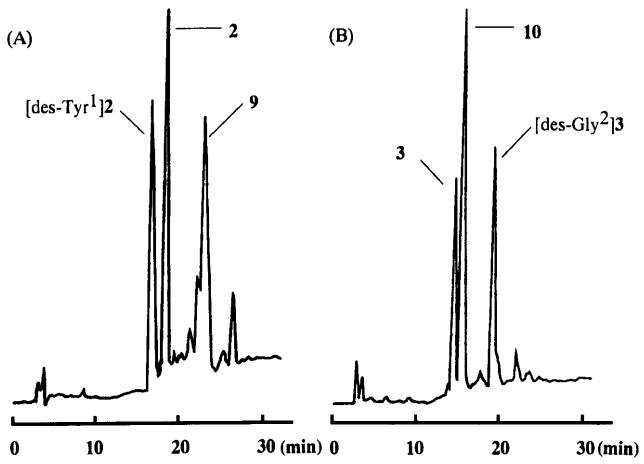

Fig. 2. HPLC Profiles of the Crude Peptides **2** (A) and **3** (B)

Table 2. Receptor Binding Assays of Synthetic Dynorphin(1—8) Analogs

Analog	K_i (nM)[a]			K_i ratio	
	[^{3}H]U-69593 (κ)	[^{3}H]DAGO (μ)	[^{3}H]DADLE (δ)	μ/κ	δ/κ
1	0.042±0.013	0.331±0.109	2.73±0.38	7.88	65
$^1\psi^2$ (**2**)	1.74±0.17	5.95±1.96	163±40	3.32	91.1
$^2\psi^3$ (**3**)	72.9±19.0	450±98	$IC_{50} \geq 10000$	6.20	—
$^3\psi^4$ (**4**)	0.067±0.024	22.8±6.1	1615±410	339	24104
$^4\psi^5$ (**5**)	0.062±0.010	3.31±1.20	25.3±3.7	53.4	408
$^5\psi^6$ (**6**)	0.043±0.011	0.04±0.01	28.3±9.4	0.93	658
$^6\psi^7$ (**7**)	0.241±0.092	6.36±2.51	7.20±2.47	26.4	29.9
$^7\psi^8$ (**8**)	0.0042±0.0025	0.071±0.031	6.44±1.24	16.8	1533
9	37.1±1.5	88.8±22.5	154±41	2.39	4.15
10	75.0±19.4	80.0±12.2	1199±217	1.07	16.0
Dyn(1—8)	0.674±0.232	2.00±0.46	1.98±0.62	2.97	2.94
Dyn(1—13)	0.074±0.021	0.813±0.273	1.41±0.19	11.0	19.1
E-2078	1.01±0.19	4.22±1.46	3.57±0.56	4.18	3.53

a) Competitive experiments were carried out in the presence of peptidase inhibitors (see Experimental).

used the NO_2 group for the side chain protection, though a low yield of aldehyde formation from Boc-Arg(NO_2)-N(Me)OMe by $LiAlH_4$ reduction due to the formation of a cyclic δ-lactam derivative has been pointed out.[31] For the syntheses of analogs **7** and **8**, a single reaction of 3 eq of Boc-Arg(NO_2)-al (crude) with the corresponding resin-bound amine failed to give complete alkylation as checked by Kaiser's test.[32] But three repetitions of the reaction gave a negative Kaiser's test and subsequently **7** and **8** were obtained in overall yields of 17.5 and 20.9%, respectively. In this connection, it has recently been reported that the use of Boc-Arg$^{\omega,\omega'}$(Z)$_2$-OH[31] or Fmoc-Arg$^{\omega,\omega'}$(Boc)$_2$-OH[33] is promising for the synthesis of argininals.

Biological Activity The receptor-binding properties of analogs were determined by competition experiments with selective radioligands, [^{3}H]DAGO and [^{3}H]DADLE, for μ- and δ-receptors, respectively, using a rat brain homogenate, and with [^{3}H]U-69593 for κ-receptor using a guinea pig brain homogenate in the presence of peptidase inhibitors as described previously.[19] As Table 2 shows, the lead compound (**1**) showed a high κ-affinity and selectivity roughly comparable to those of Dyn(1—13). The $\psi(CH_2NH)$ replacement of the N-terminal peptide bonds (**2** and **3**) resulted in a marked reduction in binding affinities for all receptors, and the latter reduced the binding potency dramatically. These results suggest that the two N-terminal peptide bonds, especially the Gly^2–Gly^3 bond, are of critical importance for the high binding potency of the parent peptide at the receptors. The other $\psi(CH_2NH)$ analogs (**4**—**8**) retained a high κ-affinity comparable to that of **1** or Dyn(1—13), except for **7** which showed a somewhat reduced κ-affinity. Two analogs, **6** and **8**, showed a higher μ-affinity than the parent peptide while other analogs showed one order of magnitude lower μ-affinity than **1**. It is noteworthy that the $^3\psi^4$-analog (**4**) retains a high κ-affinity and extremely high κ-selectivities with μ/κ and δ/κ ratios of 339 and 24104, respectively. The binding properties of the two branched by-products, **9** and **10**, were also examined. They showed a markedly reduced κ-affinity and selectivity.

The *in vitro* biological activities of the $\psi(CH_2NH)$ analogs (**2**—**8**) were evaluated on electrically evoked smooth muscle contraction of GPI, which is considered to contain μ- and κ-receptors.[34] The GPI potency was measured in the presence and absence of peptidase inhibitors, amastatin, captopril and trypsin inhibitor, to assess the stability of these peptides to degradative enzymes. As Table 3 shows, the potency profile of these analogs in the presence of peptidase inhibitors correlated well with that of the binding affinities for the κ-receptor, except for **2**. Analog **2** showed a very low potency in view of its binding affinity. Interestingly, this analog showed a very low IC_{50} ratio of 2.0 in the presence and absence of peptidase inhibitors whereas those of other analogs were >27, suggesting that the introduction of the CH_2NH isostere at Tyr^1–Gly^2 greatly enhanced the stability as compared with that of the parent peptide. E-2078 also showed a high potency and a good enzymatic stability with an IC_{50} ratio of 1.2 in the GPI assay.

It has been demonstrated that Na^+ ions and Gpp(NH)p, an enzymatically stable analog of GTP, selectively inhibit equilibrium binding of opioid agonists but not that of antagonists in cerebellum membranes of GPI.[25] To assess

Table 3. GPI Assay of Synthetic Dynorphin(1—8) Analogs in the Presence and Absence of Peptidase Inhibitors

Analog	IC_{50}, nM[a]		IC_{50} ratio (−)/(+)
	Peptidase inhibitors (+)[b]	(−)	
1	0.120±0.013	4.66±0.60	38.8
$^1\psi^2$ (**2**)	161±66	318±63	2.0
$^2\psi^3$ (**3**)	150±41	>4000	>26.6
$^3\psi^4$ (**4**)	1.92±0.31	52.9±12.7	27.5
$^4\psi^5$ (**5**)	1.58±0.20	73.9±15.5	46.8
$^5\psi^6$ (**6**)	0.409±0.154	16.0±5.14	39.1
$^6\psi^7$ (**7**)	0.708±0.271	28.4±6.90	40.1
$^7\psi^8$ (**8**)	0.073±0.010	3.67±0.72	50.1
E-2078	0.234±0.038	0.291±0.02	1.2
DAGO	1.85±0.27	4.43±1.28	2.3

a) The inhibitory effects (IC_{50}) of the ligands were determined from the concentrations required to give a reduction in the electrically induced contraction of GPI tissue of 50%. b) Amastatin (1 μM), captopril (10 μM), and soybean trypsin inhibitor (800 μg/20 ml) were included in the buffered solution.

Table 4. Competitive Binding Properties of ψCH_2NH Analogs with Respect to [^{3}H]Diprenorphin Binding in the Guinea Pig Cerebellum

	Ligand	IC_{50}, nM		IC_{50} ratio
		120 mM NaCl (−)	120 mM NaCl + 50 μM Gpp(NH)p (+)	(+)/(−)
κ-Agonist	Dyn(1—8)	12.2±4.0	837±234	68.6
κ-Antagonist	nor-BNI	1.03±0.10	0.488±0.04	0.47
	1	0.566±0.17	31.3±3.8	55.3
	$^1\psi^2$ (**2**)	18.3±6.6	177±32	9.7
	$^2\psi^3$ (**3**)	539±150	3560±340	6.6
	$^3\psi^4$ (**4**)	10.6±5.0	568±128	53.6
	$^4\psi^5$ (**5**)	3.43±1.28	414±62	120
	$^5\psi^6$ (**6**)	0.622±0.133	183±38	294
	$^6\psi^7$ (**7**)	1.24±0.29	269±66	217
	$^7\psi^8$ (**8**)	0.228±0.09	61.5±16.9	270

the antagonist activity, we examined the effects of the two allosteric effectors on the binding of analogs at the κ-receptor by means of competition experiments against [^{3}H]diprenorphin. As Table 4 shows, the IC_{50} value of the κ-agonist, Dyn(1—8), was sensitive to Na^+ ions and Gpp(NH)p (IC_{50} ratio value of 68.6) while that of a typical κ-antagonist, nor-BNI, was not. Among the analogs, **2** and **3** showed low values of the IC_{50} ratio of 9.7 and 6.6, respectively, while other analogs were greatly affected by the effectors, with IC_{50} ratios of 55—294. The obviously reduced sensitivity to the inhibitory effects of the effectors suggests that the two analogs (**2** and **3**) are partial κ-antagonists, though further studies are needed to clarify the antagonist properties. The partial antagonist property of **2** could account for the remarkably low GPI potency (Table 3) in view of its binding affinity (Table 2), as compared to those of other analogs.

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References and Notes

1) Amino acids and peptides are of L-configuration unless otherwise noted. Abbreviations used are: Boc = *tert*-butoxycarbonyl, Tos = tosyl, MSA = methenesulfonic acid, DMF = dimethylformamide, DAGO = [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin, DADLE = [D-Ala^2, D-Leu^5]enkaphalin, U-69593 = (5α,7α,8β)-(+)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5] dec-8-yl]benzeneacetamide, nor-BNI = nor-binaltorphimine, Gpp(NH)p = 5′-guanylylimidodiphosphate, HPLC = high-performance liquid chromatography.
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