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SYNTHESIS AND BIOLOGICAL PROPERTIES OF QUATERNIZED N-METHYLATION ANALOGS OF D-ARG-2-DERMORPHIN TETRAPEPTIDE

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Abstract: A series of quaternary N-methylated analogs of D-Arg-2-dermorphin tetrapeptide was synthesized by solid phase method. Two N-methylation analogs, H-Tyr-D-Arg-Phe-Y($^{+}$Me3)-NH_2 [Y = Lys (**1**) and Orn (**2**)], and a compound, H-Tyr-D-Arg-Phe-Dab-NH_2 (**7**), which was obtained as a by-product in this study, showed high *in vitro* biological properties. These compounds also showed potent *in vivo* antinociceptive effects (s. c.) in the mouse writhing test and the *in vivo* effect was antagonized markedly by N-methyllevallorphan, suggesting that they can produce a high degree of peripheral antinociception in mice.

In the search for peripherally acting bioactive substances whose central nervous system-mediated effects are undesirable, enhancement of the molecular polarity is one effective tool to restrict their passage across the blood brain barrier (BBB). The quaternized N-methylation of alkaloids such as morphine[1] or nicotines[2] imparts beneficial properties to these substances as peripherally acting drugs because of their increased polarity and low lipophilicity.

Hardy *et al*.[3] have reported a series of highly polar enkephalin-related peptides having a potent analgesic activity and a high degree of peripheral selectivity imparted by the introduction of D-Arg residue at position 2 or guanidino group at the N-terminus. Recently, Schiller *et al.*[4] have reported that H-Tyr-D-Arg-Phe-Lys-NH_2 (DALDA), an extremely μ-opioid receptor selective and highly polar dermorphin-related tetrapeptide, has a marked peripheral antinociceptive effect in the mouse writhing test. They also suggested that DALDA at high doses may penetrate into the brain.[4]

During the structure-analgesic activity studies of dermorphin-related tetrapeptide analogs containing D-Arg at position 2, we have found some tetrapeptide analogs with very potent analgesic activity even after peripheral administration in mice.[5] The quaternized N-methylation of the D-Arg-2 containing dermorphin-related peptides might produce compounds with highly diminished ability to cross the BBB. In the present study, we synthesized a series of quaternary N-methylated analogs of D-Arg-2 dermorphin tetrapeptide (Table I) and a dimeric tripeptide cross-linked with Dab residue(**7**) which was isolated as a by-product during the synthesis of analog **3** and their *in vitro* and *in vivo* bioactivities were evaluated. In addition, some analogs were assessed for their antinociceptive effects in the periphery from pretreatment with N-methyllevallorphan (NML),[6] a quaternized opiate antagonist unable to cross BBB, in the mouse writhing test.

Peptides were synthesized by the usual solid phase method. The quaternization reaction was accomplished using the $CH_3I/KHCO_3$ method[7] on a solid support. To assess the kinetics of the

quaternization on a solid support, . Boc-Lys-benzhydrylamine(BHA) resin, as a model, was methylated with CH_3I (40 eq.) and powdered $KHCO_3$ (10 eq.) in DMF. As Figure 1 shows, the reaction proceeded rapidly in an early stage (Ca. 75 % /1 hr) and then slowly,[8] probably due to the steric or ionic effects of a quaternized group in the resin matrix. Therefore, the methylation reaction was allowed to proceed for 24 hr with above conditions after construction of the corresponding tetrapeptide on the resin. Analogue **3** was exceptionally prepared by the methylation of Boc-Phe-Dab-BHA resin, which was derived from Boc-Phe-Dab(Fmoc)-BHA resin by 30 % piperidine/DMF treatment, followed by the usual solid phase method to yield Boc-Tyr(Br-Z)-Arg(Tos)-Phe-Dab($^+$Me3)-BHA resin. However this synthetic strategy was accompanied with a minor branched type by-product (**7**) due to the incomplete N-methylation reaction. The protected peptide resin was treated with anhydrous HF containing 10% anisole and the resulting peptide was purified by carboxymethyl cellulose column chromatography and/or preparative HPLC.[9] All peptides possessed satisfactory amino acid and FAB mass spectra analytical data.

Table I summarizes the biological activities of synthetic peptides. In the opioid receptor binding assay,[10] DALDA showed high affinity and selectivity for the μ-receptor in the rat brain. The quaternized N-methylation of Lys-4 side chain afforded **1** with somewhat reduced μ-affinity and selectivity. Shortening of the alkyl side chain of the fourth residue (**2**, **3**) resulted in a tendency to decrease μ-affinity and selectivity. The N-methylation of Tyr-1 residue afforded analogs (**4**-**6**) with very low and no appreciable binding abilities for μ- and δ-receptors, respectively. The very weak binding properties of these analogs may be the result of

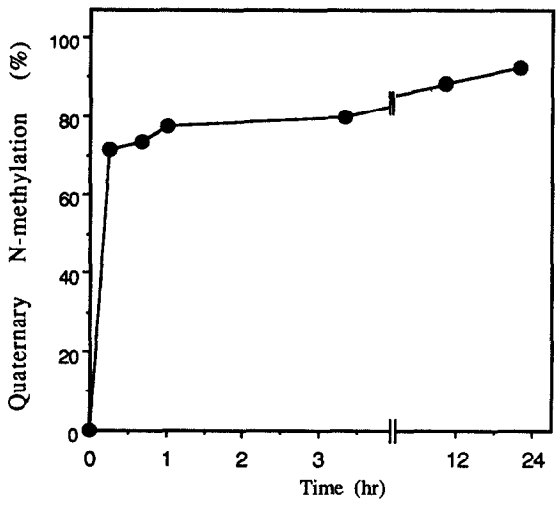

Figure 1. Quaternary N-methylation reaction on a solid support.
Boc-Lys-BHA resin was methylated by CH3I/KHCO3 method in DMF.

Table I. Biological profile of quaternary methylated and branched analogs of D-Arg2-dermorphin tetrapeptide

X-Tyr-D-Arg-Phe-Y-NH_2	Receptor binding assay (*Ki*, nM)[a]			GPI assay	PBQ writhing assay
	[^{3}H]DAGO (μ)	[^{3}H]DADLE (δ)	μ/δ	IC_{50} (nM)[b]	ED_{50} (mg/Kg, s.c.)[c]
DALDA : X = H, Y = Lys	0.38 ± 0.10	2360 ± 1140	6218	50.6 ± 12.8	0.47 (0.39 – 0.59)
1 : X = H, Y = Lys($^+$Me3)	1.12 ± 0.25	3550 ± 862	3167	91.9 ± 17.4	1.18 (0.88 – 1.56)
2 : X = H, Y = Orn($^+$Me3)	2.35 ± 0.63	450 ± 128	193	130 ± 20.8	2.30 (1.64 – 3.24)
3 : X = H, Y = Dab($^+$Me3)	7.52 ± 1.97	5860 ± 862	779	125 ± 32.3	NT[d]
4 : X = $^+$Me3, Y = Lys	207 ± 41.7	> 100000	—	2470 ± 505	NT
5 : X = $^+$Me3, Y = Orn	1920 ± 731	> 100000	—	> 100000	NT
6 : X = $^+$Me3, Y = Dab($^+$Me3)	1300 ± 506	> 100000	—	3120 ± 902	NT
7 : H-Tyr-D-Arg-Phe—┐ H-Tyr-D-Arg-Phe-Dab-NH_2	0.51 ± 0.10	497 ± 196	976	14.2 ± 2.46	0.61 (0.52 – 0.71)

[a]Values are means ± S. E. of 4 to 10 experiments. [b]Values are means ± S. E. [c]The 95 % confidence limits are given in parentheses. [d]Not tested.

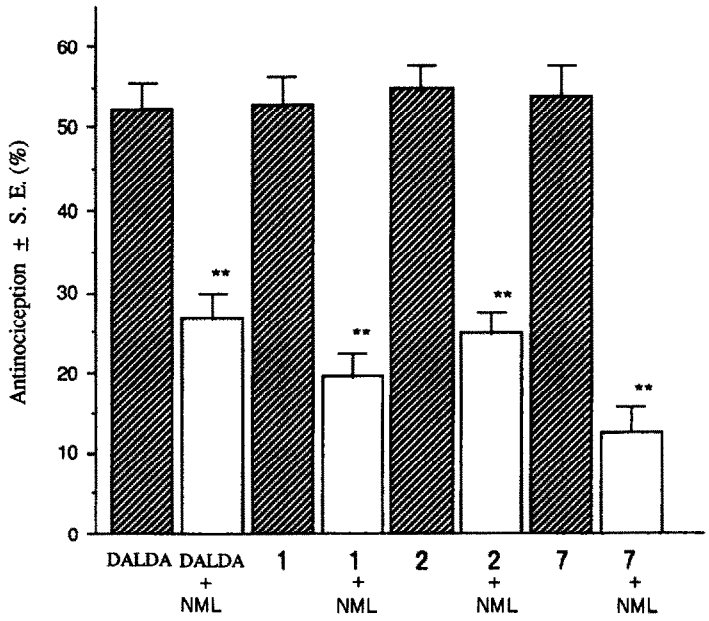

Figure 2. Antinociceptive effects of 1, 2 and 7 in control mice and in N ML-pretreated mice in the PBQ writhing test

Peptide was injected s. c. at a dose of ED50 value (Table I).
**P<0.005 by unpaired t-test.

steric interactions of these compounds due to the quaternization to limited site of the receptors or the lack of an H atom on the N-terminal N atom, which would be required for the interaction with the receptors. The branched type by-product (**7**) possessed μ-affinity nearly comparable to that of DALDA although μ-selectivity decreased to some extent.

The *in vitro* bioactivity of the analogs was evaluated on electrically induced smooth muscle contraction of guinea pig ileum (GPI assay),[11] which is usually used to measure the interaction with μ-receptor in periphery. The quaternary N-methylation of the fourth residue (**1-3**) caused only a slight loss in potency as compared to DALDA, while the N-methylation at the N-terminal amino group resulted in a great loss of activity. It should be noted that the branched type analog **7** was 3 times more potent than DALDA in the smooth muscle assay.

The *in vivo* antinociceptive activity of three selected analogs, **1**, **2**, and **7**, which showed relatively high potencies among compounds tested in the μ-receptor binding and GPI assays, was evaluated in mice by a PBQ writhing test upon s. c. administration.[12] The N-methylated analogs **1** and **2** showed significantly somewhat reduced potencies as compared to DALDA, but **7** showed a high activity comparable to that of DALDA. The reduced potencies of analogs **2** and **3** in the *in vitro* and *in vivo* assays may be based on the

reduced alkyl chains of the fourth residue.

In an attempt to estimate the antinociceptive effect in the periphery, the effect of NML (10 mg/Kg, s. c.) on the antinociceptive activities of these analogs at ED_{50} doses was compared with that of DALDA.[13] As Figure 2 shows, analogs **1** and **2** at 2~5-fold higher doses (ED_{50}) than DALDA on a molar basis were antagonized by NML as high degree as that of DALDA. These results suggest that **1** and **2** have high degrees of antinociceptive effects in periphery although their *in vitro* potencies in the GPI assay are somewhat lower than DALDA. Interestingly, the antinociceptive effect of analog **7** was reversed by NML with an apparently higher degree than that of DALDA. This evidence, taking into account its high *in vitro* potency over that of DALDA, suggests that **7** also can produce a potent and high degree of peripheral antinociception in mice.

In conclusion, the introduction of quaternized N-methylation residues at the fourth position of the D-Arg-2 dermorphin tetrapeptide amide is tolerated without great reduction of biological activities. The quaternized analogs, **1** and **2**, and a non-quaternized but branched type analog **7** among compounds obtained in this study may be useful as high peripherally acting drugs for studying the mechanism of peripheral analgesia[14] although a precise study is needed to determine their access to the brain or centrally-mediated undesirable effects. Regarding their utilities as peripherally acting drugs, further study is in progress.

References and Notes

Abbreviations used in this manuscript : Boc = *t*-butoxycarbonyl, Br-Z = 2-bromobenzyloxycarbony Fmoc = 9-fluorenylmethoxycarbonyl, Dab = L-diaminobutyric acid, DALDA = H-Tyr-D-Arg-Phe-Lys-NH_2, DAGO = [D-Ala^2, $MePhe^4$, Gly-ol^5]enkephalin, DADLE = [D-Ala^2, D-Leu^5]enkephalin, PBQ = phenyl-1,4-benzoquinone, NML = N-methyllevallorphan, DMF = dimethylformamide.

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8) The reaction rate was determined by amino acid analysis after acid hydrolysis of the resin [c.HCl-propionic acid (1 : 1), 130 °C, 2 hr].

9) Most peptides were purified on a medium pressure HPLC as reported previously (Sasaki, Y., Ambo, A., Midorikawa, K., Suzuki, K., *Chem. Pharm. Bull.*, **1993**, 41, 1391.).

10) Receptor binding assay was carried out using radioligands, [^{3}H]DAGO and [^{3}H]DADLE for μ- and δ-receptors, respectively, according to the method as reported previously (*Biochem. Biophys. Res. Commun.*, **1991**, 180, 822.). Inhibition constants (*Ki*) were calculated from the IC_{50} value by the method of Cheng and Prusoff (*Biochem. Pharmacol.*, **1973**, 22, 3099.).

11) GPI assay was carried out according to the method as reported previously (reference in 9) using isolated longitudinal muscle strips from Hartley strain guinea pigs (250-300 g).

12) The antinociceptive activity was evaluated by the standard writhing test using phenyl-1,4-benzoquinone (PBQ) as an irritant. Groups of five to six male ICR strain mice were used. The test compounds were injected s. c. at 10 ml/Kg. After 15 min, aqueous PBQ at 2.5 mg/Kg in a dose volume of 10 ml/Kg was injected intraperitoneally. After an additional 5 min, mice were observed for 10 min for the characteristic writhing syndrome and the antinociceptive activity was compared with that in control (saline) mice. The ED50 was defined as that dose of drug that induced a 50 % reduction in the number of writhes obtained compared to saline administration alone.

13) In the PBQ writhing test, NML at 10 mg/Kg was injected s. c. in a dose volume of 10 ml/Kg 20 min prior to the administration of the test compound at a dose of ED_{50} value, and the antagonism was evaluated in comparison with mice injected with saline instead of NML.

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