Synthesis and Structure−Activity Relationships of an Orally Available and Long-Acting Analgesic Peptide, N^{α}-Amidino-Tyr-D-Arg-Phe-MeβAla-OH (ADAMB)

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A novel dermorphin tetrapeptide N^{α}-amidino-Tyr-D-Arg-Phe-MeβAla-OH (ADAMB) was designed based on the structures of several dermorphin tetrapeptide analogues, including N^{α}-amidino-Tyr-D-Arg-Phe-Gly-OH (ADA-DER), H-Tyr-D-Arg-Phe-βAla-OH (TAPA), and H-Tyr-D-Arg-Phe-Sar-OH (DAS-DER). These parent compounds were known to show a weak oral analgesic activity in animals and/or to possess a different mechanism of analgesia from other μ-opioid peptides. Six analogues of ADAMB were also synthesized to investigate the effect on potency of *N*-terminal amidination and *N*-methyl-β-alanine (MeβAla) substitution at position 4. Compounds were assessed using the tail pressure test in mice after subcutaneous and oral administration. Among the peptides tested, ADAMB showed the strongest oral antinociceptive activity, with an ED_{50} of 5.8 vs 22.2 mg/kg for morphine, as well as a 38-fold stronger activity after subcutaneous administration. ADAMB also showed long-lasting antinociceptive activity, with 50% of the maximum effect persisting in the tail pressure test at 10 h after oral administration (10 mg/kg). In contrast, orally administered morphine (80 mg/kg) showed a rapid decrease of activity in the same test and its antinociceptive effect disappeared within 4 h. When the antinociceptive effect of ADAMB was compared with that of analogues possessing βAla^4 (**1**) or Sar^4 (**2**), as well as analogues with *N*-substitution (**3**−**6**), it was found that both the N^{α}-amidino substitution and the MeβAla^4 were synergistically involved in creating ADAMB's exceptionally high antinociceptive activity.

Introduction

Since the isolation and identification of enkephalins[1] and other endogenous opioid peptides,[2] numerous peptidic analogues have been synthesized in attempts to develop an analgesic without the serious side effects of narcotics such as morphine.[3] However, these efforts have met with little success because of problems with side effects, low bioavailability, and high cost of production. Dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) was recently isolated from the skin of amphibians[4] and was reported to possess a potent and long-lasting opioid-like activity.[5] The *N*-terminal tetrapeptide of dermorphin is known to be the minimum sequence required for opioid activity,[6] although this fragment shows a lower potency than that of the parent heptapeptide.[7] Synthetic peptides derived from the dermorphin tetrapeptide, which have the sequence Tyr-D-AA^2-Phe-AA^4 (where AA^2 and AA^4 represent a certain amino acid), have been reported to show a potent agonist activity for the μ-opioid receptor.[8] Substitution of D-Ala at position 2 with D-Arg has been widely applied since the studies on D-Arg-kyotorphin were reported,[9] in order to increase the potency and the duration of action of the peptide. For example, Schiller[10] reported that DALDA (D-AA^2 = D-Arg, AA^4 = Lys-NH_2) is a potent and highly selective μ-opioid receptor agonist. Salvadori et al.[11] have extensively studied dermorphin analogues and have reported that N^{α}-amidination strengthens their antinociceptive activity. We recently found N^{α}-amidino-Tyr-D-Arg-Phe-Gly-OH (ADA-DER) as one of the most potent dermorphin tetrapeptides.[12] An analogue with *N*-methylation at position 4, H-Tyr-D-Arg-Phe-Sar-OH (DAS-DER),[13] shows selectivity for the μ_1-opioid receptor subtype and has oral antinociceptive activity in rats without inducing respiratory depression.[14] Sato et al.[15] recently reported that H-Tyr-D-Arg-Phe-βAla-OH (TAPA),[16] in which the amino acid residue at position 4 was substituted with β-alanine, had a more potent and longer-lasting antinociceptive effect than morphine after local or systemic administration and was a selective μ_1-opioid receptor agonist. In addition, the typical withdrawal symptoms that occurred upon cessation of administration or after treatment with naloxone, a classical μ-opioid antagonist, were less severe for TAPA than for morphine.[17] These observations encouraged us to explore the possibility of developing novel and orally active analgesic peptides without the adverse effects of morphine. On the basis of the structures of ADA-DER, DAS-DER, and TAPA, a compound featuring both N^{α}-amidination and *N*-methyl-β-alanine (MeβAla) at position 4 (ADAMB) was designed (Figure 1). This paper reports on the synthesis and the in vivo antinociceptive activity of ADAMB. To assess its potential as an oral analgesic, antinociceptive activity was evaluated in mice by the tail pressure test and was compared with

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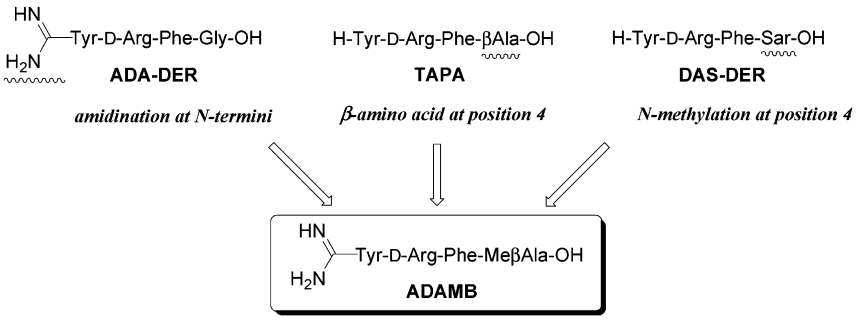
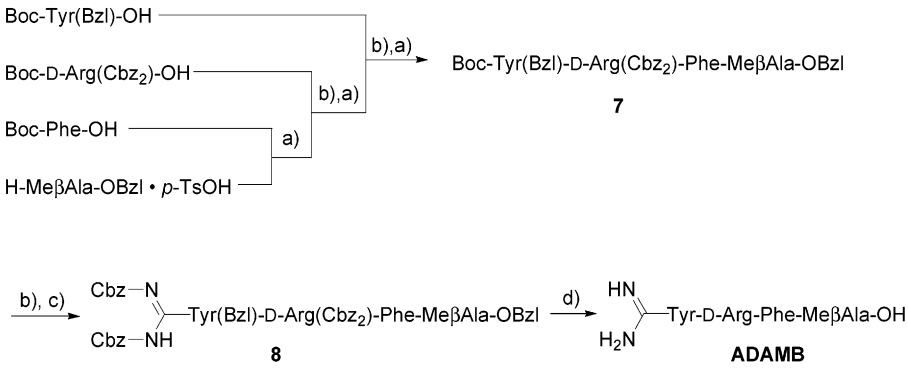


Figure 1. Design of ADAMB based on the structure of several dermorphin tetrapeptide analogues.

Scheme 1[a]



[a] Reagents: (a) WSCI−HOBt. (b) 4 N HCl in EtOAc. (c) 1-(Bis-benzyloxycarbonylguanyl)pyrazole, Et_3N in DMF. (d) H_2/Pd−C in AcOH.

that of morphine. In addition, six analogues of ADAMB were synthesized to clarify the influence of *N*-amidination and Me*β*Ala[4] substitution on antinociceptive activity and the data obtained regarding the structure−activity relationships (SARs) are also discussed here.

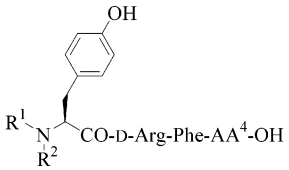
Chemistry

ADAMB was successfully synthesized by both solid phase and solution phase methods. The N^{α}-Fmoc strategy was used for solid phase synthesis, employing a *p*-alkoxybenzyl alcohol resin (Wang resin).[18] N^{α}-Fmoc-protected amino acids were added sequentially, using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) with 1-hydroxybenzotriazole (HOBt) as the coupling reagents in 1-methyl-2-piperidone (NMP). *N*-Terminal amidination was performed on the resin after assembly of the peptide chain by removal of the Fmoc group followed by treatment with a mixture of 1*H*-pyrazole-1-carboxamidine hydrochloride[19] and diisopropylethylamine in dimethylformamide (DMF). Analogues bearing *β*Ala (**1**) or Sar (**2**) at position 4 were also synthesized using the same method. To investigate the effect of *N*-terminal substitution on antinociceptive activity, four other analogues of ADAMB were also synthesized by the solid phase method. These analogues had structures that could be represented by the general formula of R-Xaa-D-Arg-Phe-Me*β*Ala-OH, where R and Xaa are the *N*-terminal substituent and the amino acid residue, respectively. They were designated as compounds **3** (R = H, Xaa = Tyr), **4** [R = H, Xaa = *N*-methyltyrosine (MeTyr)], **5** [R = none, Xaa = *N*,*N*-dimethyltyrosine (Me_2Tyr)], and **6** (R = acetyl, Xaa = Tyr, *β*Ala[4] instead of Me*β*Ala[4]). To obtain a substantial amount of the compound for animal tests, ADAMB was subsequently synthesized by a solution method using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSCI·HCl) with HOBt as the coupling reagent (Scheme 1). The side chain functional groups and the C-terminal carboxylic acid were fully protected by Cbz or Bzl protection groups, which were simultaneously removed by catalytic hydrogenolysis in the presence of Pd on charcoal. Thus, starting with H-Me*β*Ala-OBzl, N^{α}-Boc-protected amino acids were coupled sequentially to give the fully protected tetrapeptide (**7**). An N^{α}-amidino group was introduced in a bis-Cbz-protected form using (benzyloxycarbonylimino-pyrazol-1-yl-methyl)carbamic acid benzyl ester.[20] Then, all of the protecting groups were cleanly removed by catalytic hydrogenation to give the desired product (ADAMB) with a high purity. This method is used to facilitate the large-scale synthesis of compounds containing the highly polar and hydrophilic functional groups. All final products were purified by reverse phase C18 flush column chromatography with elution using aqueous CH_3CN containing 0.1% acetic acid to afford the purified products (>95% purity by high-performance liquid chromatography (HPLC) analysis in two diverse systems). The structure of the final product was confirmed by ^{1}H NMR and by high-resolution mass spectral (HRMS) analysis.

Biological Results

The synthesized compounds were tested to determine their relative opioid receptor binding affinity by displacement of selective radioligands from mice spinal cord or guinea pig brain membrane preparations. [^{3}H][D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]DAMGO), [^{3}H]deltorphin-II, and [^{3}H]U-69593 were used for determining the *μ*-, *δ*-, and *κ*-affinity, respectively. The results of these binding assays are summarized in Table 1. ADAMB showed a high binding affinity for the *μ*-opioid receptor, which was comparable to that of DAMGO in the [^{3}H]DAMGO binding assay. When com-

Table 1. Receptor Binding Assay of ADAMB and Its Analogues



				[3H]DAMGO (μ)		[3H]deltolphin-II (δ)	[3H]U-69593 (κ)
compd	R^1	R^2	AA^4	IC_{50}[a] (nM)	relative potency[b]	IC_{50} (nM)	IC_{50} (nM)
ADAMB	$H_2NC(=NH)-$	H−	Meβ-Ala	12.9 ± 3.4	0.90	>1000	>1000
1	$H_2NC(=NH)-$	H−	β-Ala	20.8 ± 3.2	0.56	>1000	>1000
2	$H_2NC(=NH)-$	H−	Sar	32.9 ± 12	0.35	>1000	>1000
3	H−	H−	Meβ-Ala	10.2 ± 4.2	1.14	>1000	>1000
4	CH_3-	H−	Meβ-Ala	45.0 ± 11	0.26	>1000	>1000
5	CH_3-	CH_3-	Meβ-Ala	101 ± 20	0.11	>1000	>1000
6	CH_3CO-	H−	β-Ala	>1000	<0.01	>1000	>1000
DAMGO				11.6 ± 1.7 (5.07 ± 0.68)[c]	1.00	>1000	>1000
deltorphin-II				nd		13.7 ± 2.3[c]	nd
U-69593				nd		nd	3.17 ± 0.35[c]

[a] Concentration that gives half-maximal effect. Data are given as the mean ± SEM ($n = 3$). [b] Relative potencies are on a molar basis (DAMGO = 1). [c] K_i value.

Table 2. In Vivo Antinociceptive Activity of ADAMB and Its Analogues (Tail Pressure Test)

	MPE %max[a]		ED_{50} (mg/kg)[b]		
compd	sc (1 mg/kg)	po (10 mg/kg)	sc	po	sc/po (%)
ADAMB	100 ± 0.0	82 ± 6.4	0.089 (0.06−0.15)	5.8 (3.6−9.4)	1.5
1	93 ± 6.9	18 ± 4.8	0.32 (0.22−0.46)	19 (9.3−37)	1.7
2	90 ± 5.0	26 ± 11	0.39 (0.25−0.61)	18 (11−29)	2.2
3	91 ± 5.1	31 ± 8.8	0.39 (0.22−0.69)	nd	
4	87 ± 9.7	34 ± 9.7	0.21 (0.13−0.34)	15 (9.0−25)	1.5
5	29 ± 11	13 ± 5.5	nd	nd	
6	<10	<10	nd	nd	
morphine	nd	nd	3.3 (2.2−4.9)	22 (14−36)	15

[a] Data were taken at 1.5 h after drug administration and are given as the mean ± SEM for groups of 10 mice. [b] The ED_{50} value was estimated at the time of peak activity and are given as the mean value with its 95% confidence limit for 10 mice.

pared to a compound without *N*-terminal amidination (**3**), ADAMB had almost the same binding affinity, whereas *N*-terminal methylation (**4**) or dimethylation (**5**) caused a significant decrease of affinity for the μ-opioid receptor. On the other hand, modifications at position 4 caused a decrease of μ-affinity. Thus, each of the analogues in which Meβ-Ala[4] was substituted by β-Ala[4] (**1**) or Sar[4] (**2**) showed a decline of μ-affinity to one-half or one-third of that for ADAMB, respectively. An analogue with *N*-terminal acetylation (**6**) did not show any strong affinity (IC_{50} > 1000 nM) for the μ-opioid receptor. None of the compounds showed any significant affinity (IC_{50} > 1000 nM) for either the δ- or the κ-opioid receptor, which indicated that these analogues show specific affinity for the μ-opioid receptor relative to the δ- and the κ-opioid receptors.

The compounds were tested to assess their in vivo antinociceptive potency (Table 2) using the tail pressure test[21] after subcutaneous (sc) and oral (po) administration to mice. The maximum possible effect (MPE %) was initially measured at fixed doses of 1 subcutaneously and 10 mg/kg orally, after which analogues with a high potency were assessed for antinociceptive activity on the basis of ED_{50} values. ADAMB showed a very strong antinociceptive activity in mice after sc administration, and its ED_{50} value was 37 times higher than that of morphine. ADAMB was significantly antagonized by sc pretreatment with naloxone (data not shown) in the same mouse test. As expected from the design of ADAMB, its po antinociceptive activity was also strong and the ED_{50} value was 3.8 times higher than that of morphine. To assess bioavailability, the ED_{50} dose ratio (sc/po) of ADAMB was calculated and compared with that of morphine. The ratio for ADAMB was 1.5%, while that for morphine was 15%. Substitution of Meβ-Ala at position 4 with either β-Ala (**1**) or Sar (**2**) (corresponding to TAPA and DAS-DER with N^{α}-amidination) caused a significant decrease in potency to one-third of that for ADAMB after both sc and po administration. However, both compound **1** and compound **2** still had a stronger sc antinociceptive activity than morphine and comparable po activity. The compound without *N*-terminal substitution (**3**) also showed a significant decrease of antinociceptive activity when compared with ADAMB. *N*-Terminal monomethylation (**4**) did not affect the antinociceptive activity when compared to the analogue with an *N*-terminal primary amine (**3**), whereas substitution of both hydrogens at the amino terminal with methyl groups (**5**) caused a substantial decrease of activity. Comparison of the ED_{50} ratio (sc/po) for ADAMB with those of compounds **1**, **2**, and **4** showed that the bioavailability was unchanged by modification at the *N*-terminal and position 4. To further investigate the role of the amino terminal, an analogue bearing an *N*-acetyl group (**6**) was synthesized. This modification canceled the basicity and nucleophilicity of the amino group and resulted in no measurable antinociceptive

Table 3. In Vivo Antinociceptive Activity of ADAMB and Selected Analogues (Hot Plate Test)

compd	ED_{50} (sc, mg/kg)[a]	compd	ED_{50} (sc, mg/kg)[a]
ADAMB	0.07 (0.02−0.24)	3	0.15 (0.08−0.21)
1	0.21 (0.12−0.36)	4	0.11 (0.05−0.24)
2	0.08 (0.3−0.21)	morphine	1.7 (0.7−3.8)

[a] The ED_{50} value was estimated at the time of peak activity and was given as the mean value with its 95% confidence limit for 10 mice.

effect, even though the *N*-acetamino group was virtually the same size and shape as the *N*-terminal guanidino group.

ADAMB and several analogues (**1**−**4**), which showed strong antinociceptive activity in the mouse tail pressure test, were assessed for their antinociceptive potency in a mouse hot plate test after sc administration (Table 3). Like the tail pressure test, ADAMB showed the strongest antinociceptive activity among the compounds tested. Morphine was 24 times less potent in terms of the ED_{50} value than ADAMB. The Sar^4 analogue **2** showed a comparable ED_{50} value to that of ADAMB, but **2** was significantly less active than ADAMB in the tail pressure test. With the exception of compound **2**, which is an α-amino acid analogue showing a difference at position 4, the antinociceptive results obtained in the hot plate test showed a good correlation with the data from the tail pressure test.

Figure 2 shows the time course of the antinociceptive effect of sc administered morphine and ADAMB in the mouse tail pressure test. ADAMB (Figure 2B) produced dose-related stronger antinociceptive activity than morphine (Figure 2A). The total duration of action for ADAMB was over 10 h at a maximally active dose of 0.2 mg/kg, whereas the activity of morphine disappeared within 4 h at a dose of 10 mg/kg.

As shown in Figure 3B, oral ADAMB also had dose-related and significant antinociceptive activity, with 50% of MPE being maintained at 10 h after administration at the maximally active dose of 10 mg/kg. The peak effect appeared 4−6 h after administration at this dose. Morphine showed its maximum antinociceptive activity at a dose of 80 mg/kg with a peak at 60 min after administration. Oral ADAMB had a far longer duration of action than morphine, which was effective for less than 4 h after administration (Figure 3A).

To clarify the role of the μ-opioid receptor subtypes, μ_1 and μ_2, in the antinociceptive effect of ADAMB on noxious stimuli, pretreatment (sc) with a μ_1 selective opioid receptor antagonist, naloxonazine, or a nonselective antagonist, naloxone, was done in the mouse tail pressure test (Figure 4). Then, the results were compared with those for morphine (Figure 5). Either naloxonazine (35 mg/kg) or naloxone (0.2 mg/kg) pretreatment at 24 h before administration of ADAMB (0.5 mg/kg) markedly attenuated its antinocieptive action, and the difference in the extent of attenuation was not significant. In contrast, pretreatment with naloxonazine showed only partial attenuation of the antinociceptive effect of morphine (10 mg/kg), while naloxone completely suppressed it.

Discussion

Since the isolation of dermorphin, many analogues have been synthesized and extensively investigated in attempt to develop new peptide analgesics as well as to determine their pharmacological properties.[22] Among them, TAPA and DAS-DER, in which the C-terminal amino acid residue at position 4 is substituted with β-Ala or Sar, respectively, are known to have an antinociceptive activity even after oral administration. Therefore, we expected that a tetrapeptide bearing MeβAla at position 4 could show increased oral antinociceptive activity by combining homologation and *N*-methylation. We have recently synthesized more than 50 compounds with the general structure of N^{α}-amidino-Tyr-D-Arg-Phe-X (where X represents various amino acids, amides, amino esters, and amino alcohols) and have found that they show strong antinociceptive activity comparable to that of morphine after oral administration.[12] Therefore, a combination of these modifications seemed likely to amplify oral activity and to be a promising approach for the development of an orally active analgesic peptide. To confirm our hypothesis, we performed an in vivo test using mice for initial evaluation of the compounds synthesized. As expected, ADAMB showed very potent oral antinociceptive activity and was approximately 4 times as strong as morphine. In our preliminary experiment using a similar mouse test that we employed in this study, the antinociceptive effect of ADAMB was five times stronger than that of TAPA after sc administration and seven times stronger after po administration (based on ED_{50} values). However, the bioavailability of ADAMB may not be sufficient for an oral analgesic since its dose ratio (sc/po) was 10-fold smaller than that of morphine (based on ED_{50} values). Comparison of ADAMB with analogue **3** (no N^{α}-substitution) showed that N^{α}-amidination markedly improved the antinociceptive effect after both sc and po administration, while the dose ratio (sc/po) of the ED_{50} values did not change as compared with that of analogue **4**. This indicated that *N*-amidination is important for antinociceptive activity but not for bioavailability. Likewise, the combination of *N*-methylation and homologation of the amino acid residue at position 4 did not achieve any increase in bioavailability, although significant improvement of antinociceptive activity was observed (ADAMB vs compounds **1** and **2**). Therefore, further structural modification of ADAMB, including alteration of other parts of the molecule, will be necessary before this type of compound can be used as an oral analgesic.

The presence of an *N*-terminal amino group in opioid peptides is crucial for a strong agonist effect on the opioid receptors.[23] *N*-Terminal amidination of dermorphin tetrapeptides is known to decrease the in vitro affinity for opioid receptors, while it strengthens the in vivo antinociceptive acitvity.[24] However, little is known about the SARs of *N*-terminal amidination as well as the effect of this modification on other opioid peptides. In the present study, the antinociceptive activity of the *N,N*-dimethylated analogue (**5**) showed a considerable decrease, whereas the monomethylated analogue (**4**) retained similar activity to that of the unsubstituted analogue (**3**). On the other hand, *N*-acetylation led to a decrease of activity. These observations suggest the importance of an *N*-terminal amino group that retains basicity and, unlike most of the nonpeptide μ-agonists

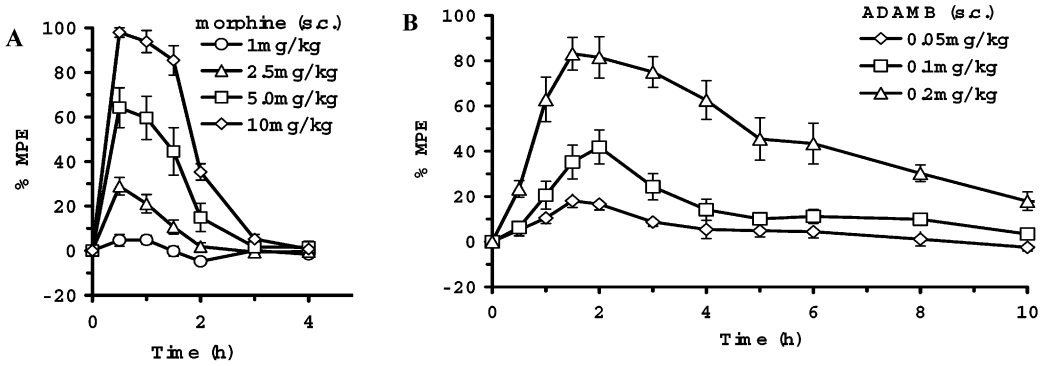


Figure 2. Time course of the antinociceptive effect of subcutaneous morphine (A) and ADAMB (B) in the mouse tail pressure test. The doses used are shown in the figure. Data are given as the mean ± SEM for groups of 10 mice.

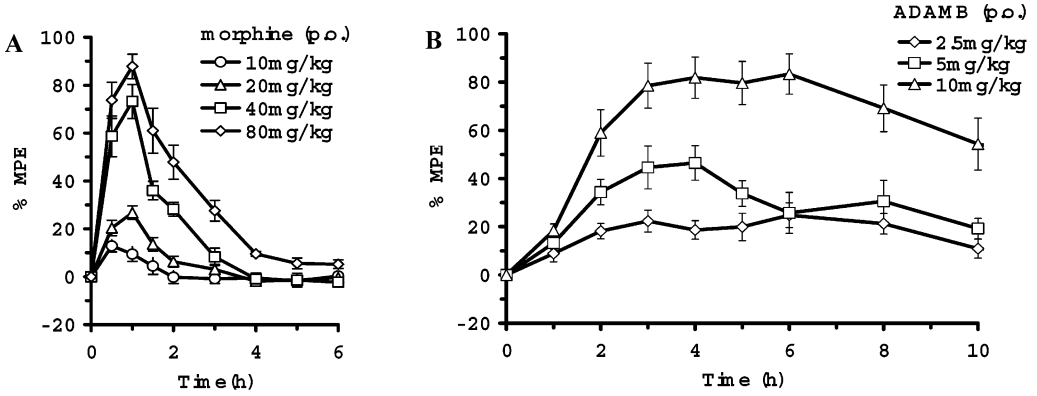


Figure 3. Time course of the antinociceptive effect of oral morphine (A) and ADAMB (B) in the mouse tail pressure test. The doses used are shown in the figure. Data are given as the mean ± SEM for groups of 10 mice.

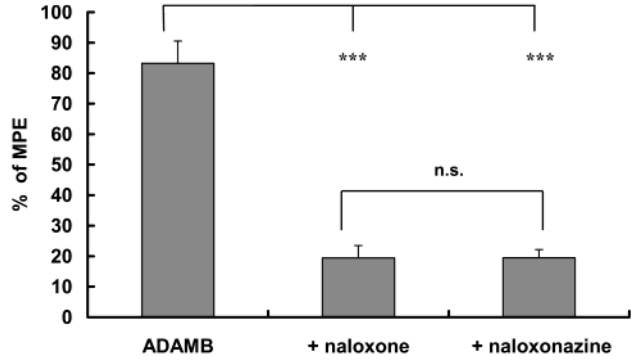


Figure 4. Effect of ADAMB (0.5 mg/kg, sc) after pretreatment with naloxone or naloxonazine in the mouse tail pressure test. Naloxone (0.2 mg/kg, sc) was given 5 min before ADAMB. Naloxonazine (35 mg/kg, sc) was given 24 h before ADAMB. Data are the mean ± SE for 10 mice. *** < 0.001 vs ADAMB alone (Tukey's test).

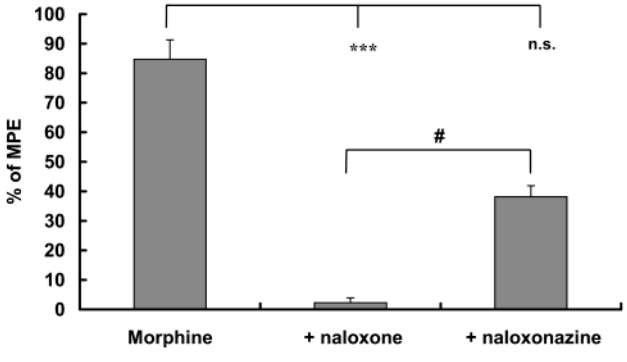


Figure 5. Effect of morphine (10 mg/kg, sc) after pretreatment with naloxone or naloxonazine in the mouse tail pressure test. Naloxone (0.2 mg/kg, sc) was given 5 min before morphine. Naloxonazine (35 mg/kg, sc) was given 24 h before morphine. Data are the mean ± SE for 10 mice. *** < 0.001 vs morphine alone; # < 0.05 for pretreatment with naloxone vs pretreatment with naloxonazine (Tukey's test).

that commonly possess a tertiary amine, at least one unsubstituted amine hydrogen in Tyr[1] as well.

ADAMB has an exceptionally long-lasting antinociceptive activity after both sc and po administration as does TAPA.[15] Comparison of the time−course of the antinociceptive effect of ADAMB with that of morphine showed that the onset was slower for ADAMB after either sc or po administration. The peak time of analgesia after administration was accordingly shifted to later than that for morphine, being 1.5−2 (sc) and 3−4 h (po) for ADAMB vs 0.5 (sc) and 1 h (po) for morphine, respectively. The reason for the stronger and longer antinociceptive activity of ADAMB along with its slow onset is still unclear, although we assume that it is partly due to enhancement of the compound's stability. It could be also explained by the slow and continuous influx of ADAMB into the central nervous system across the blood−brain barrier (BBB) in addition to tight binding of the peptide with the μ-opioid receptors. These properties would be related to a low clearance rate from the blood as well as the peptide's extremely high stability to enzymatic hydrolysis. Nevertheless, this feature may be advantageous in combination with the higher efficacy of the compound relative to morphine, even though slow release morphine formulations such as 12 h oral preparations[3] have been widely accepted for clinical use. The high efficacy and long duration of

action of ADAMB should markedly reduce the effective dose for once or twice daily administration without any manipulation of the drug formulation to extend its duration of action.

Although μ-opioid receptor agonists are very important for the control of pain in the clinical setting, these drugs have several undesirable side effects, including respiratory depression,[25] constipation,[26] and dependence.[27] Some of these effects are thought to be related to one of the μ-receptor subtypes, either the μ_1- or the μ_2-receptor. For example, opioid analgesia has been suggested to be mediated by the μ_1-receptor, whereas the μ_2-receptor seems to be involved in respiratory depression and effects on gastrointestinal transit.[28] TAPA and DAS-DER are known to have a high affinity for the μ_1-opioid receptor subtype since their antinociceptive activity is strongly antagonized by pretreatment with naloxonazine, an irreversible μ_1-opioid receptor antagonist.[29] Therefore, it can be expected that these compounds may show a substantial reduction in the adverse effects of μ-opioid peptides that are elicited through the μ_2-opioid receptor. This seems to be the case for ADAMB. The antinociceptive activity of sc ADAMB in mice was markedly attenuated by pretreatment with naloxonazine, a selective μ_1-antagonist, and the same level of attenuation was achieved as that seen with nonselective naloxone. In contrast, morphine was partially antagonized by naloxonazine but was completely antagonized by naloxone. These results imply that ADAMB has a strong antinociceptive action mainly through interaction with the μ_1-opioid receptor subtype. In fact, we have obtained preliminary experimental results in animals to support our assumption that ADAMB inherits promising characteristics as an analgesic from its parent compounds. Less constipation in comparison to that caused by morphine and negligible psychological dependence were observed in mice, as well as low cross-tolerance for morphine (the details will be reported elsewhere).

In conclusion, ADAMB was synthesized based on the structures of several dermorphin tetrapeptide analogues and showed more potent antinociceptive activity than morphine in the mouse tail pressure test after sc and po administration. This strong and long-lasting activity after oral administration may qualify ADAMB as a candidate for the development as a new analgesic. Further investigations are now underway to confirm whether ADAMB also avoids side effects owing to its μ_1-subtype selective agonism.

Experimental Section

Commercial N^{α}-Fmoc-protected amino acids, N^{α}-Boc-protected amino acids, trifluoroacetic acid (TFA), WSCI·HCl, and HOBt were obtained from Peptide Institute, Inc., Osaka, Japan. HBTU was purchased from Calbiochem-Novabiochem Japan Ltd., Tokyo, Japan. Wang resin (100−200 mesh, 0.37 mmol/g) was obtained from Watanabe Chemical Industries Ltd., Hiroshima, Japan, and 1*H*-pyrazole-1-carboxamidine hydrochloride was purchased from Aldrich Chemicals, WI. Fmoc-MeβAla-OH was prepared according to the reported method[30] with Fmoc-OSu and MeβAla. Boc-D-Arg(Cbz$_2$)OH was prepared according to the procedure reported by Jetten.[31] Thin-layer chromatography was performed on silica plates (0.25 mm; Merck, 60 F_{254}). Flash column chromatography for the intermediates of solution phase synthesis was performed using Merck silica gel 60 (230−400 mesh). Purification of the final products was achieved by the flush chromatography using a C-18 reverse phase silica gel Chromatorex DM1020T, Fuji Silysia Chemical Ltd., Aichi, Japan, eluted by a stepwise gradient of acetonitrile (starting from 1% and stepwise gradient by 2%) in 0.1 M acetic acid. Analytical HPLC was on a reverse phase Nucleosil 5C_{18} column (4.6 mm × 150 mm) in a Shimadzu LC-10A HPLC system. The products were analyzed with the two different eluting systems; A: a linear gradient of 10−70% acetonitrile in 0.1% aqueous TFA over 15 min; B: an isocratic elution with 12% acetonitrile in 0.1% aqueous TFA over 30 min. The flow rate was 1 mL/min, and the chromatogram was recorded by UV detection at 230 and 280 nm. ^{1}H NMR spectra were recorded with a JEOL AL-300 (300 MHz) spectrometer, using tetramethylsilane (TMS) as an internal standard. HRMS were obtained with JEOL mass spectrometer model JMS-700.

Solid Phase Synthesis of N^{α}-Amidino-Tyr-D-Arg-Phe-MeβAla-OH (ADAMB). The peptide was synthesized using an N^{α}-Fmoc strategy and Wang resin. The peptides were carried using a Perkin-Elmer model 430A automated synthesizer on a 0.25 mmol scale. Fmoc-D-Arg(Pmc)OH and Fmoc-Tyr(*t*-Bu) were used for the side chain protection. N^{α}-Fmoc-protected amino acids (4 equiv) were added sequentially, using HBTU with HOBt as coupling reagents in NMP. After the peptide assembly was completed, the Fmoc group was removed from the Fmoc-Tyr(*t*-Bu)-D-Arg(Pmc)Phe-MeβAla-Aliko resin, and the peptide resin was mixed with 1*H*-pyrazole-1-caboxamidine hydrochloride (0.99 g, 6.75 mmol) and diisopropylethylamine (1.29 mL, 7.40 mmol) in DMF (6 mL) and agitated at room temperature overnight. The peptides were cleaved using standard techniques and a cleavage mixture of 90% TFA, 5% anisole, 2.5% methyl sulfide, and 2.5% 1,2-ethandithiol. The aqueous solution of the crude products was neutralized with $NaHCO_3$ and then purified by the method shown above to give the desired compound (60 mg, 39%) as acetic acid salts after lyophilization. $[\alpha]_{25}^{D}$ +28.8° (*c* 0.58, 1 M AcOH). ^{1}H NMR (CD_3OD, 300 MHz): δ 7.23 (m, 5H), 7.05 (dd, J = 8.4, 2.4 Hz, 2H), 6.72 (d, J = 8.4 Hz, 2H), 5.17 (t, J = 7.2 Hz, 1H), 4.41 (m, 1H), 4.26 (m, 1H), 3.59 (m, 1H), 3.45 (m, 1H), 3.10−2.85 (m, 6H), 2.88 (s, 2H), 2.85 (s, 1H), 2.35 (m, 2H), 1.50 (br m, 2H), 1.23 (m, 2H). FAB-HRMS: calcd for $C_{29}H_{42}N_9O_6$ $[M + 1]^+$, 612.3258; found, 612.3233.

Solution Phase Synthesis of ADAMB. Boc-Phe-MeβAla-OBzl. To a stirred suspension of H-MeβAla-OBzl·*p*-TsOH (8.04 g, 22.0 mmol) in EtOAc (20 mL), Boc-Phe-OH (5.31 g, 20.0 mmol), HOBt (2.97 g, 22.0 mmol), and Et_3N (3.07 mL, 22.0 mmol) were added at −10 °C. Then, WSCI·HCl (4.60 g, 24.0 mmol) was added to the solution. The mixture was stirred for 30 min at the same temperature and overnight at room temperature. The reaction mixture was washed with 1 N HCl, saturated $NaHCO_3$, and brine. The organic phase was dried over $MgSO_4$, filtered, and evaporated in vacuo to give colorless oil (8.45 g, 95.9%). $[\alpha]_{25}^{D}$ +7.2° (*c* 1.05, DMF). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.50−7.12 (m, 10H), 5.38 (t, J = 8.7 Hz, 1H), 5.10 (s, 2H), 4.77 (dt, J = 15, 7.5 Hz, 1H), 3.63 (dt, J = 14, 6.6 Hz, 0.67H), 3.46 (dt, J = 6.9, 6.6 Hz, 1H), 3.42 (m, 0.33H), 2.94 (dd, J = 8.1, 3.0 Hz, 2H), 2.81 (s, 1H), 2.67 (s, 2H), 2.53 (dt, J = 13, 6.6 Hz, 1H), 2.42 (m, 0.67H), 2.04 (m, 0.33H), 1.40 (s, 9H). FAB-HRMS: calcd for $C_{25}H_{33}N_2O_5$ $[M + 1]^+$, 441.2389; found, 441.2391.

Boc-D-Arg(Cbz$_2$)Phe-MeβAla-OBzl. Boc-Phe-MeβAla-OBzl (6.54 g, 14.8 mmol) was dissolved into 4 N HCl/EtOAc (20 mL) and stirred for 30 min at room temperature. Et_2O and hexane were added, and the precipitated material was separated by decantation. The residual gum was dissolved in DMF (30 mL) and neutralized with Et_3N at 0 °C. Boc-D-Arg(Cbz$_2$)OH (7.60 g, 14.0 mmol) and HOBt (1.89 g, 14.0 mmol) were added to the solution, and Et_3N (2.10 mL, 15.0 mmol) was added. Then, WSCI·HCl (3.22 g, 16.8 mmol) was added to the solution at −10 °C. This mixture was stirred for 30 min at −10 °C and overnight at 4 °C. The reaction mixture was diluted with EtOAc (150 mL) and washed with 1 N HCl, 10% Na_2CO_3, and brine. The organic phase was dried over $MgSO_4$, filtered, and evaporated in vacuo. The residue was solidified from hexane

to give the tripeptide as white amorphous (11.7 g, 96.7%). $[\alpha]_{25}^{D}$ +3.9° (*c* 1.00, DMF). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.45 (br s, 1H), 9.26 (br s, 1H), 7.43−7.04 (m, 20H), 6.90 (br t, J = 7.0 Hz, 1H), 5.46−4.97 (m, 6H), 5.23 (s, 2H), 5.10 (s, 2H), 4.15 (m, 1H), 3.97 (m, 1H), 3.92 (m, 1H), 3.62(dt, J = 14, 6.9 Hz, 0.67H), 3.40 (m, 0.33H), 3.50 (m, 1H), 2.86 (br d, J = 7.5 Hz, 2H), 2.78 (s, 1H), 2.65 (s, 2H), 2.49 (dt, J = 6.9, 3.0 Hz, 1H), 2.39 (m, 0.67H), 2.18 (m, 0.33H), 1.92 (m, 1H), 1.70 (m, 1H), 1.56 (m, 2H), 1.41 (s, 9H). FAB-MS 865 $[M + 1]^+$. Anal. ($C_{47}H_{56}N_6O_{10}$) C, H, N.

Boc-Tyr(Bzl)-D-Arg(Cbz$_2$)Phe-MeβAla-OBzl (7). Boc-D-Arg(Cbz$_2$)Phe-MeβAla-OBzl (4.76 g, 5.50 mmol) was dissolved into 4 N HCl/EtOAc (20 mL) and stirred for 25 min at room temperature. Et_2O was added to the solution, and the precipitated solid was separated by filtration to give the Boc-removed tripeptide quantitatively. This amine component, Boc-Tyr(Bzl)-OH (1.86 g, 5.00 mmol), and HOBt (743 mg, 5.50 mmol) were dissolved in DMF (10 mL), and WSCI·HCl (1.15 g, 6.00 mmol) was added to the solution at −8 °C. The pH of the solution was adjusted to 5 with Et_3N (1.35 mL). This mixture was stirred for 30 min at −8 °C and overnight at 4 °C. The reaction mixture was diluted with EtOAc (100 mL) and washed with 10% citric acid, saturated $NaHCO_3$, and brine. The organic phase was dried over $MgSO_4$, filtered, and evaporated in vacuo. The residue was solidified from EtOAc and hexane to give white amorphous (4.76 g, 85.8%). $[\alpha]_{25}^{D}$ −3.2° (c 1.04, DMF). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.49 (br s, 1H), 9.29 (br s, 1H), 7.60−7.00 (m, 26H), 7.00 (d, J = 8.7 Hz, 2H), 6.81 (d, J = 8.7 Hz, 2H), 6.79 (br s, 1H), 5.29−4.88 (m, 6H), 5.08 (s, 2H), 4.95 (s, 2H), 4.49 (m, 1H), 4.29 (m, 1H), 3.80 (m, 1H), 3.67 (m, 1H), 3.42 (m, 1H), 3.03−2.79 (m, 5H), 2.76 (s, 1H), 2.65 (s, 2H), 2.46 (br t, J = 7.5 Hz, 1.33H), 2.40 (m, 0.67H), 2.16 (m, 2H), 1.54 (m, 2H), 1.36 (s, 9H). FAB-MS 1119 $[M + 1]^+$. Anal. ($C_{63}H_{71}N_7O_{12}$) C, H, N.

***N*$^\alpha$-(N,N′-Bis-benzyloxycarbonyl)amidino-Tyr(Bzl)-D-Arg(Cbz$_2$)Phe-MeβAla-OBzl (8).** Tetrapeptide **7** (2.43 g, 2.20 mmol) was dissolved in 4 N HCl/dioxane (20 mL) at −8 °C and stirred for 30 min at room temperature. Et_2O (80 mL) was added to the solution, and the precipitated oil was separated by decantation. The residue was solidified from Et_2O (40 mL). The solid was collected by filtration and dried under a reduced pressure to give Boc-removed tetrapeptide (2.23 g, 97.4%) as a white powder. This amine hydrochloride (1.04 g, 1.00 mmol) and (benzyloxycarbonylimino-pyrazol-1-yl-methyl)carbamic acid benzyl ester (416 mg, 1.10 mmol) were dissolved in DMF (2 mL). The pH of the solution was adjusted to 8 with Et_3N (210 μL) at 0 °C. The mixture was stirred for 6 h at room temperature, and Et_3N (50 μL) was added in the middle of the reaction to keep the pH 8. The reaction mixture was poured into 10% citric acid (15 mL), and the precipitated oil was extracted with EtOAc (40 mL). The EtOAc solution was washed with brine, dried over $MgSO_4$, filtered, and evaporated in vacuo. The residual oil was purified by flush chromatography eluted with $CHCl_3$:MeOH (100:1) followed by precipitation from Et_2O to give white amorphous powder (0.93 g, 71.0%). $[\alpha]_{25}^{D}$ +8.9° (*c* 1.01, DMF). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 11.50 (s, 1H), 9.48 (br s, 1H), 9.26 (br s, 1H), 8.81 (t, J = 7.5 Hz, 1H), 7.47−7.09 (m, 35H), 7.06 (d, J = 8.4 Hz, 2H), 6.87 (d, J = 8.1 Hz, 1H), 6.81 (d, J = 8.4 Hz, 2H), 5.23−4.86 (m, 14H), 4.76 (dt, J = 7.2, 7.2 Hz, 1H), 4.42 (dt, J = 7.0, 7.0 Hz, 1H), 3.59 (m, 1H), 3.70 (m, 1H), 3.59 (m, 1H), 3.27 (m, 1H), 2.99 (br t, J = 6.0, 2H), 2.80 (br d, J = 7.2 Hz, 2H), 2.70 (s, 1H), 2.57 (s, 2H), 2.42 (t, J = 7.2 Hz, 1.33H), 2.35 (m, 0.67H), 1.99 (br s, 2H), 1.49 (m, 2H). FAB-MS 1329 $[M + 1]^+$. Anal. ($C_{75}H_{77}N_9O_{14}$) C, H, N.

***N*$^\alpha$-Amidino-Tyr-D-Arg-Phe-MeβAla-OH (ADAMB).** To the solution of protected tetrapeptide **8** (657 mg, 0.50 mmol) in AcOH (10 mL) was added 10% Pd−C (50% wet). This solution was vigorously stirred under H_2 atmosphere for 4 h at room temperature. The catalyst was removed by filtration, and the filtrate was concentrated in vacuo. The residue was precipitated from Et_2O to give a white powder (0.30 g, 91.2%) as acetate salt.

***N*$^\alpha$-Amidino-Tyr-D-Arg-Phe-βAla-OH (1).** The same solid phase procedure for the synthesis of ADAMB was employed, but Fmoc-βAla-Wang resin was used instead of Fmoc-MeβAla-Wang resin as a starting material to give **1** as a white powder (60 mg, 43%). $[\alpha]_{25}^{D}$ +20.5° (*c* 1.11, 1M AcOH). ^{1}H NMR (CD_3OD, 300 MHz): δ 7.24 (m, 5H), 7.06 (d, J = 8.4 Hz, 2H), 6.70 (d, J = 8.4 Hz, 2H), 4.58 (dd, J = 11, 4.2 Hz, 1H), 4.36 (t, J = 7.2 Hz, 1H), 3.97 (t, J = 7.2 Hz, 1H), 3.57−3.32 (m, 3H), 3.07−2.83 (m, 4H), 2.76 (dd, J = 14, 11 Hz, 1H), 2.38 (t, J = 6.3 Hz, 2H), 1.41 (m, 2H), 1.14 (m, 1H), 0.93 (m, 1H). FAB-HRMS: calcd for $C_{28}H_{40}N_9O_6$ $[M + 1]^+$, 598.3102; found, 598.3125.

***N*$^\alpha$-Amidino-Tyr-D-Arg-Phe-Sar-OH (2).** The same solid phase procedure for the synthesis of ADAMB was employed, but Fmoc-Sar-Wang resin was used instead of Fmoc-MeβAla-Wang resin as a starting material to give **2** as a white powder (63 mg, 42%). $[\alpha]_{25}^{D}$ +26.5° (*c* 0.35, 1M AcOH). ^{1}H NMR (CD_3OD, 300 MHz): δ 7.17 (m, 5H), 7.01 (d, J = 8.1 Hz, 2H), 6.67 (d, J = 8.1 Hz, 2H), 5.08 (t, J = 6.3 Hz, 1H), 4.35 (br s, 1H), 4.19 (m, 1H), 3.98 (t, J = 17 Hz, 1H), 3.83 (d, J = 18 Hz, 1H), 3.67 (m, 1H), 3.14−2.73 (m, 9H), 1.51 (m, 1H), 1.40 (m, 1H), 1.16 (m, 2H). FAB-HRMS: calcd for $C_{28}H_{40}N_9O_6$ $[M + 1]^+$, 598.3102; found, 598.3108.

H-Tyr-D-Arg-Phe-MeβAla-OH (3). The same solid phase procedure for the synthesis of ADAMB was employed, but the amidination process was skipped to give **3** as a white powder (76 mg, 53%). $[\alpha]_{25}^{D}$ +47.4° (*c* 0.84, 1 M AcOH). ^{1}H NMR (CD_3OD, 300 MHz): δ 7.25 (m, 5H), 7.11 (d, J = 8.4 Hz, 2H), 6.74 (d, J = 8.4 Hz, 2H), 5.12 (t, J = 7.2 Hz, 1H), 4.22 (m, 1H), 4.09 (m, 1H), 3.53 (br t, J = 7.2 Hz, 2H), 3.17−2.89 (m, 6H), 2.89 (s, 2H), 2.85 (s, 1H), 2.42 (m, 2H), 1.42 (m, 2H), 1.18 (m, 2H). FAB-HRMS: calcd for $C_{28}H_{40}N_7O_6$ $[M + 1]^+$, 570.3040; found, 570.3021.

H-MeTyr-D-Arg-Phe-MeβAla-OH (4). The same solid phase procedure for the synthesis of ADAMB was employed, but Fmoc-MeTyr(*t*-Bu)OH was used instead of Fmoc-Tyr(*t*-Bu)OH, and the amidination process was skipped to give **4** as a white powder (80 mg, 55%). $[\alpha]_{23}^{D}$ +44.4° (*c* 1.01, 1M AcOH). ^{1}H NMR (CD_3OD, 300 MHz): δ 7.21 (m, 5H), 7.00 (d, J = 8.4 Hz, 2H), 6.70 (dd, J = 8.4, 2.7 Hz, 2H), 5.18 (t, J = 7.2 Hz, 0.5H), 5.00 (t, J = 7.5 Hz, 0.5H), 4.24 (dt, J = 6.0, 6.0 Hz, 1H), 3.63 (br m, 1H), 3.42 (br m, 2H), 3.10−2.63 (m, 6H), 2.87 (d, J = 6.3 Hz, 3H), 2.38 (br m, 2H), 2.29 (d, J = 6.6 Hz, 3H), 1.43 (br m, 2H), 1.09 (m, 2H). FAB-HRMS: calcd for $C_{29}H_{42}N_7O_6$ $[M + 1]^+$, 584.3197; found, 584.3203.

Me$_2$Tyr-D-Arg-Phe-MeβAla-OH (5). The same solid phase procedure for the synthesis of ADAMB was employed, but Me$_2$Tyr(*t*-Bu)OH was used instead of Fmoc-Tyr(*t*-Bu)OH, and the amidination process was skipped to give **5** as a white powder (68 mg, 45%). $[\alpha]_{23}^{D}$ +44.4° (*c* 1.08, 1 M AcOH). ^{1}H NMR (CD_3OD, 300 MHz): δ 7.21 (m, 5H), 7.00 (d, J = 8.4 Hz, 2H), 6.70 (dd, J = 8.4, 1.5 Hz, 2H), 5.15 (t, J = 6.9 Hz, 0.5H), 4.99 (t, J = 7.5 Hz, 0.5H), 4.14 (m, 1H), 3.54 (m, 1H), 3.43 (m, 2H), 3.10−2.80 (m, 6H), 2.89 (s, 2H), 2.85 (s, 1H), 2.53 (s, 3H), 2.51 (s, 3H), 2.35 (br m, 2H), 1.38 (m, 1H), 1.24 (m, 1H), 0.95 (m, 2H). FAB-HRMS: calcd for $C_{30}H_{44}N_7O_6$ $[M + 1]^+$, 598.3353; found, 598.3321.

***N*$^\alpha$-Ac-Tyr-D-Arg-Phe-βAla-OH (6).** The same solid phase procedure for the synthesis of **1** was employed, but *N*-terminal was acetylated with acetic anhydride instead of amidination to give **6** as a white powder (84 mg, 56%). $[\alpha]_{25}^{D}$ +30.6° (*c* 0.87, 1M AcOH). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 9.73 (t, 5.1 Hz, 1H), 8.29 (m, 1H), 8.09 (d, J = 8.4 Hz, 1H), 7.89 (t, J = 5.1 Hz, 1H),7.01 (m, 6H), 7.00 (d, J = 8.1 Hz, 2H), 6.64 (d, J = 8.1 Hz, 2H), 4.38 (dt, J = 7.2, 7.2 Hz, 1H), 4.29 (m, 1H), 4.03 (dd, J = 14, 7.5 Hz, 1H), 3.30−3.05 (m, 3H), 2.96 (br d, J = 3.9 Hz, 2H), 2.98−2.60 (m, 3H), 2.14 (m, 2H), 1.77 (s, 3H), 1.57 (m, 1H), 1.42 (m, 1H), 1.22 (m, 2H). FAB-HRMS: calcd for $C_{29}H_{40}N_7O_7$ $[M + 1]^+$, 598.2989; found, 598.2967.

Binding Assays. Synaptosomal fractions were prepared from mice spinal cord and guinea pig cerebella according to the method of Chang et al.[32] Briefly, spinal cord or cerebella was homogenized in a 0.32 M sucrose solution at 0 °C and centrifuged at 6000*g* for 15 min. The supernatant was centri-

fuged at 40 000*g* for 30 min, and the pellets were homogenized in 5 mM Tris-HCl buffer (pH 7.4) at 0 °C. The suspension was centrifuged at 6000*g* for 15 min. The supernatant was then centrifuged twice at 40 000*g* for 30 min each. After the second centrifugation, the membranes were resuspended in 50 mM Tris-HCl buffer (pH 7.4).

[^{3}H]DAMGO, [^{3}H]deltorphin-II, and [^{3}H]U-69593 were used for the determination of relative affinities for μ-, δ-, and κ-receptors, respectively. Binding assays were carried out by incubating an aliquot of the membrane fraction (250 μg for [^{3}H]DAMGO, 350 μg for [^{3}H]deltorphin-II, and 180 μg for [^{3}H]U-69593) containing protease inhibitors and the labeled ligand (3 nM for [^{3}H]DAMGO or [^{3}H]deltorphin-II, and 2 nM for [^{3}H]U-69593) in 50 mM Tris-HCl buffer (pH 7.4). After 60 min at 25 °C, the reaction mixture was filtered over a Whatman GF/B filter soaked with 0.1% polyethyleneimine and the filter was washed twice with cold Tris-HCl buffer. Filters were counted in a liquid scintillation counter after overnight extraction with liquid scintillation fluid (3 mL). Specific binding was determined from the differences between total binding and that in the presence of excess (10 μM) unlabeled ligand. K_d and B_{max} values were obtained using Scatchard analysis.

Antinociceptive Assay. Male mice of ddY strain weighing 10−32 g were used in the experiment. They were purchased from Japan SLC Inc. (Shizuoka, Japan) and housed in cages of 5−6 animals, matched for weight, and placed in a colony room. Animals were given standard food (MM-3, Japan SLC Inc.) and tap water ad libitum in an air-conditioned room at 23 ± 2 °C and 50 ± 10% relative humidity with a standard 12 h light−dark cycle (lights on 6:00−18:00). The tail pressure test was performed according to the reported method by Sakurada et al.[33] with a slight modification. Thus, mechanical pressure was applied to the base of the tail at a rate of 32 g/sec using an automated tail pressure unit (Ugo Basile, Italy). Biting or struggling behavior in mice was used as an indication of response threshold, and only mice responding behaviorally to a tail pressure of 100−300 g were selected for this experiment. The trials were terminated at the level of 500 g to prevent tail tissue damage. The mean ± SEM of the pressure level was plotted. To obtain the response curve, the dose was plotted against percentage of MPE (% MPE = $(P_2 - P_1/500 - P_1) \times 100$, where P_1 is the response pressure before drug administration (g) and P_2 is the response pressure after drug administration (g). In the hot plate test,[34] the animals were placed on a 55 °C hot plate (Ugo Basil, model-DS37) surrounded by a glass tube (ϕ14 × 13 H cm), and the latency of either a jump or a hindpaw lick was measured. Prior to drug administration, the nociceptive response of each mouse was measured and mice showing the response in 10−20 s after placement were chosen for the test. A cutoff time of 40 s was used to minimize tissue damage. The peptides administered were dissolved in saline solution (Fuso Chemical Industries, Osaka, Japan). Saline solution was used as the control. ED_{50} vales were obtained by the method of Litchfield and Wilcoxon.[35]

References

(1) Hughes, J.; Smith, T. W.; Kosterlitz, H. W.; Fothergill, L. A.; Morgan, B. A.; Morris, H. R. Identification of Two Related Pentapeptides from the Brain with Potent Opiate Agonist Activity. *Nature* **1975**, *258*, 577−579.

(2) Roques, B. P.; Noble, F.; Fournie-Zaluski, M.-C. Endogenous Opioid Peptides and Analgesia. In *Opioids Pain Control*; Stein, C., Ed.; Cambridge University Press: Cambridge, 1999; pp 21−45.

(3) Ahmedazi, S. New Approaches to Pain Control in Patients with Cancer. *Eur. J. Cancer* **1997**, *33* (Suppl. 6), S8−S14.

(4) Montecucchi, P. C.; De Castiglione, R.; Erspamer, V. Identification of Dermorphin and 6-Hyp-Dermorphin in Skin Extracts of the Brazilian Frog *Phyllomedusa Rhodei*. *Int. J. Pept. Protein Res.* **1981**, *17*, 316−321.

(5) Erspamer, V.; Melchiorri, P.; Broccardo, M.; Erspamer, G. F.; Falaschi, P.; Improta, G.; Negri, L.; Renda, T. The Brain-Gut-Skin Triangle. New Peptides. *Peptides* **1981**, *2* (Suppl. 2), 7−16.

(6) Broccardo, M.; Erspamer, V.; Erspamer, G. F.; Improta, G.; Linari, G.; Melchiorri, P.; Montecucchi, P. C. Pharmacological Data on Dermorphins, a New Class of Potent Opioid Peptides from Amphibian Skin. *Br. J. Pharmacol.* **1981**, *73*, 625−631.

(7) Sato, T.; Sakurada, S.; Sakurada, T.; Furuta, S.; Chaki, K.; Kisara, K.; Sasaki, Y.; Suzuki, K. Opioid Activities of D-Arg^2-Substituted Tetrapeptides. *J. Pharmacol. Exp. Ther.* **1987**, *242*, 654−659.

(8) de Castiglione, R.; Rossi, A. C. Structure−Activity Relationships of Dermorphin Synthetic Analogues. *Peptides* **1985**, *6* (Suppl. 3), 117−125.

(9) Takagi, H.; Shiomi, H.; Ueda, H.; Amano, H. Morphine-Like Analgesia by a New Dipeptide, L-Tyrosyl-L-Arginine (Kyotorphin) and its Analogue. *Eur. J. Pharmacol.* **1979**, *55*, 109−111.

(10) Schiller, P. W.; Nguyen, T. M.-D.; Chung, N. N.; Lemieux, C. Dermorphin Analogues Carrying an Increased Positive Net Charge in their "Message" Domain Display Extremely High μ-Opioid Receptor Selectivity. *J. Med. Chem.* **1989**, *32*, 698−703.

(11) Salvadori, S.; Marastoni, M.; Tomatis, R. Opioid Peptides. Structure−Activity Relationships in Dermorphin Tetrapeptides. *Il Farmaco Ed. Sci.* **1982**, *37*, 514−518.

(12) Sakurada, S.; Murayama, K.; Nakano, M.; Hongo, K.; Takeshima, S.; Take, N. Peptide Derivatives. PCT Int. Appl. WO 9524421, 1995; *Chem. Abstr.* **1996**, *124*, 146854.

(13) Sasaki, Y.; Matsui, M.; Taguchi, M.; Suzuki, K.; Sakurada, S.; Sato, T.; Sakurada, T.; Kisara, K. D-Arg^2-Dermorphin Tetrapeptide Analogues. A Potent and Long-Lasting Analgesic Activity after Subcutaneous Administration. *Biochem. Biophys. Res. Commun.* **1984**, *120*, 214−218.

(14) Paakkari, P.; Paakkari, I.; Bonhof, S.; Feuerstein, G.; Sirén, A.-L. Dermorphin analogue Tyr-D-Arg^2-Phe-Sarcosine-Induced Opioid Analgesia and Respiratory Stimulation. The Role of Mu_1-receptors? *J. Pharmacol. Exp. Ther.* **1993**, *266*, 544−550.

(15) Sato, T.; Takahashi, N.; Tan-No, K.; Kisara, K.; Sakurada, T.; Sakurada, S. Comparison of Opioid Activity Between a *N*-Terminal Tetrapeptide Analogue of Dermorphin, H-Tyr-D-Arg-Phe-β-Ala-OH and Morphine. *Methods Find Exp. Clin. Pharmacol.* **1998**, *20*, 581−586.

(16) Chaki, K.; Kawamura, S.; Kisara, K.; Sakurada, S.; Sakurada, T.; Sasaki, Y.; Sato, T.; Suzuki, K. Antinociception and Physical Dependence Produced by [D-Arg^2]Dermorphin Tetrapeptide Analogues and Morphine in Rats. *Br. J. Pharmacol.* **1988**, *95*, 15−22.

(17) Chaki, K.; Sakurada, S.; Sakurada, T.; Sato, T.; Kawamura, S.; Kisara, K.; Sasaki, Y.; Suzuki, K. A New Class Opioid Peptide, [D-Arg^2, β-Ala^4]Dermorphin Tetrapeptide. Physical Dependence Liability in Mice. *Neuropeptides* **1989**, *13*, 83−88.

(18) Fields, G. B.; Noble, R. L. Solid Phase Peptide Synthesis Utilizing 9-Fluorenylmethoxycarbonyl Amino Acids. *Int. J. Pept. Protein Res.* **1990**, *35*, 161−214.

(19) Bernatowicz, M. S.; Wu, Y.; Matsueda, G. R. 1*H*-Pyrazole-1-carboxamidine Hydrochloride. An Attractive Reagent for Guanylation of Amines and Its Application to Peptide Synthesis. *J. Org. Chem.* **1992**, *57*, 2497−2502.

(20) Bernatowicz, M. S.; Wu, Y.; Matsueda, G. R. Urethane Protected Derivatives of 1-Guanylpyrazole for the Mild and Efficient Preparation of Guanidines. *Tetrahedron Lett.* **1993**, *34*, 3389−3392.

(21) (a) Sakurada, S.; Takeda, S.; Sato, T.; Hayashi, T.; Yuki, M.; Kutsuwa, M.; Tan-No, K.; Sakurada, C.; Kisara, K., Sakurada, T. Selective Antagonism by Naloxonazine of Antinociception by Tyr-D-Arg-Phe-β-Ala, A Novel Dermorphin Analogue with High Affinity at μ-Opioid Receptors. *Eur. J. Phrmacol.* **2000**, *395*, 107−112. (b) Upton, N.; Sewell, R. D. E.; Spencer, P. S. J. Analgesic Action of μ- and κ-Opiate Agonists in Rats. *Arch. Int. Pharmacodyn.* **1983**, *262*, 199−207. (c) Endoh, T.; Matsuura, H.; Tajima, A.; Izumimoto, N.; Tajima, C.; Suzuki, T.; Saitoh, A.; Suzuki, T.; Narita, M.; Tseng, L.; Nagase, H. Potent Antinociceptive Effects of TRK-820, A Novel κ-Opioid Receptor Agonist. *Life Sci.* **1999**, *65*, 1685−1694.

(22) Negri, L.; Melchiorri, P.; Lattanzi, R. Pharmacology of Amphibian Opiate Peptides. *Peptides* **2000**, *21*, 1639−1647.

(23) Morley, J. S. Structure−Activity Relationships of Enkephalin-Like Peptides. *Annu. Rev. Pharmacol. Toxicol.* **1980**, *20*, 81−110.

(24) Marastoni, M.; Salvadori, S.; Balboni, G.; Borea, P. A.; Marzola, G.; Tomatis, R. Synthesis and Activity Profiles of New Dermorphin-(1−4) Peptide Analogues. *J. Med. Chem.* **1987**, *30*, 1538−1542.

(25) Popio, K. A.; Jackson, D. H.; Ross, A. M.; Schreiner. B. F.; Yu, P. N. Hemodynamic and Respiratory Effects of Morphine and Butorphanol. *Clin. Pharmacol. Ther.* **1978**, *23*, 281−287.

(26) (a) Stewart, J. J.; Weisbrodt, N. W.; Burks, T. F. Central and Peripheral Actions of Morphine on Intestinal Transit. *J. Pharmacol. Exp. Ther.* **1978**, *5*, 547−555. (b) Schulz, R.; Wuster, M.; Herz, A. Centrally and Peripherally Mediated Inhibition of Intestinal Motility by Opioids. *Naunyn Schmiedeberg's Arch Pharmacol.* **1979**, *308*, 255−260.

(27) Martin, W. R. Drug Addiction: Morphine, Sedative/Hypnotic and Alcohol Dependence. In *Handbook of Experimental Pharmacology*; Martin, W. R., Ed.; Springer: Berlin, Germany, 1977; Vol. 45, Pt. 1, pp 1−74.

(28) Pasternak, G. W. Parmacological Mechanisms of Opioid Analgesics. *Clin. Neuropharmacol.* **1993**, *16*, 1−18.

(29) (a) Pasternak, G. W.; Wood, P. J. Multiple μ Opiate Receptors. *Life Sci.* **1986**, *38*, 1889−1898. (b) Ling, G. C. F.; Simantov, S.; Clark, J. A.; Pasternak, G. W. Naloxonazine Actions In Vivo. *Eur. J. Pharmacol.* **1986**, *129*, 33−38.

(30) Milton, R. C. de L.; Becker, E.; Milton, S. C. F.; Baxter, J. E. H.; Elsworth, J. F. Improved Purities for Fmoc-Amino Acids from Fmoc-ONSu. *Int. J. Protein Pept. Res.* **1987**, *30*, 431−432.

(31) Jetten, M.; Co, A.; Peters, C. A. M.; van Nispen, J. W. F. M.; Ottenheijm, H. C. J. A One-Pot N-Protection of L-Arginine. *Tetrahedron Lett.* **1991**, *32*, 6025−6028.

(32) Chang, K. J.; Cuatrecasas, P. Multiple opiate receptors. Enkephalins and Morphine Bind to Receptors of Different Specificity. *J. Biol. Chem.* **1979**, *254*, 2610−2618.

(33) Sakurada, S.; Sakurada, T.; Jin, H.; Sato, T.; Kisara, K.; Sasaki, Y.; Suzuki, K. Antinociceptive Activities of Synthetic Dipeptides in Mice. *J. Pharm. Pharmacol.* **1982**, *34*, 750−751.

(34) Hammond, D. L.; Stapelfeld, A.; Drower, E. J.; Savage, M. A.; Tam, L.; Mazur, R. H. Antinociception Produced by Oral, Subcutaneous or Intrathecal Administration of SC-39566, an Opioid Dipeptide Arylalkylamide, in the Rodent. *J. Pharmacol. Exp. Ther.* **1994**, *268*, 607−615.

(35) Litchfield, J. T.; Wilcoxon, F. A. Simplified Method of Evaluating Dose−Effect Experiments. *J. Pharmacol. Exp. Ther.* **1949**, *96*, 99−113.

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