Protection of $\psi(CH_2NH)$ Peptide Bond with 2,4-Dimethoxybenzyl Group in Solid-Phase Peptide Synthesis[1)]

Yusuke SASAKI* and Junko ABE

Tohoku College of Pharmacy, 4–1 Komatsushima 4-chome, Sendai 981, Japan.
Received July 1, 1996; accepted September 2, 1996

The reductive alkylation of a resin-bound amine by the Boc-amino aldehyde/$NaBH_3CN$ method is accompanied with undesirable double alkylation at Xaa$\psi(CH_2NH)$Gly sequences. To prevent the double alkylation, the utility of the 2,4-dimethoxybenzyl (Dmb) group for secondary amine protection was investigated. By using this group, Leu-enkephalin and dynorphin (1—8) analogs containing the $\psi(CH_2NH)$ peptide bond between residues Tyr^1/Gly^2 or Gly^2/Gly^3 were synthesized in high yields.

Key words $\psi(CH_2NH)$ peptide bond; double alkylation; 2,4-dimethoxybenzyl group protection; enkephalin analog; dynorphin (1—8) analog

The reductive alkylation of a resin-bound amine using the Boc-amino aldehyde and $NaBH_3CN$ is a convenient method to prepare peptide analogs containing the ψCH_2NH peptide bond isostere in solid-phase peptide synthesis.[2)] This method has been used for the synthesis of the ψCH_2NH pseudopeptide analogs of various biologically active peptides. However, there are some reports of undesirable side reactions, such as acylation of the CH_2NH functional group in subsequent peptide assembly[2,3)] or double alkylation by reductive amination.[4)] The undesirable acylation can be largely excluded by the use of an active ester method for subsequent coupling reaction[2)] or by the use of Cl-Z protection for the pseudobond.[3)] A serious problem is the double alkylation which proceeds during the reductive amination reaction. Such undesirable alkylation has been reported in the synthesis of Fmoc–Xaa$\psi(CH_2NH)$Gly–OH by the solution method as well.[5)] Recently, we have observed the formation of doubly alkylated products during solid-phase synthesis of a series of dynorphin (1—8) analogs containing the pseudobond isostere.[6)] The side reaction occurred at Xaa$\psi(CH_2NH)$Gly sequences, and we could obtain dynorphin (1—8) analogs containing the pseudobond at Tyr^1/Gly^2 (1ψ2) and Gly^2/Gly^3 (2ψ3) only by careful alkylation using 1 eq of the aldehyde/$NaBH_3CN$, in low yields.[6)] More recently, similar alkylations have been reported to occur during solid-phase synthesis of the CH_2NH pseudobond analogs of dynorphin A.[7)]

In the present study, we investigated the possibility of capping the CH_2NH secondary amino function with a 2,4-dimethoxybenzyl (Dmb) group to prevent the double alkylation reaction and demonstrated the usefulness of this protecting group in the synthesis of enkephalin and dynorphin analogs.

Results and Discussion

Initially, a series of Leu-enkephalin (ENK) analogs in which each peptide bond was systematically replaced by the pseudobond was synthesized without Dmb protection using a standard method[2)] for the introduction of the pseudobond. Peptides were constructed on a Merrifield resin using a DIPCI/HOBt-mediated Boc strategy. As shown in Fig. 1c, the syntheses of 1ψ2- (top) and 2ψ3-ENK (bottom) were accompanied with the formation of large amounts of doubly alkylated peptides, resulting in low overall yields of the desired products (24% and <3%, respectively). In contrast, no or negligible formation of the branched peptides was observed in the syntheses of the 3ψ4- and 4ψ5-analogs. These results are in agreement with our recent observation that such double alkylation occurs predominantly at Xaa$\psi(CH_2NH)$Gly sequences.[6)] Accordingly, the utility of the Dmb group for secondary amine protection was investigated in the synthesis of the 1ψ2- and 2ψ3-ENK analogs.

To introduce the Dmb group, two strategically different routes were investigated as shown in Fig 2. One uses N^{α}-Fmoc–N^{α}-Dmb–Gly–OH (method A) and the other uses two-step reductive alkylations, first with 2,4-dimethoxybenzaldehyde and then with Fmoc (or Boc)–Xaa aldehyde, after introduction of the Gly^2 or Gly^3 residue (method B).

Method A The synthesis of 1ψ2-ENK proceeded well, including the condensations of Fmoc–(Dmb)Gly–OH to H–Gly–Phe–Leu–Merrifield or Wang resin and subsequent reductive alkylation with Boc–Tyr(Cl_2-Bzl)–H or Fmoc–Tyr(^{t}Bu)–H. Acidolytic cleavage of the peptides from the Merrifield resin by treatment with HF–anisole (9 : 1) afforded 1ψ2-ENK in an overall yield of 68% (Fig. 1, top a). Similarly, the treatment of the protected peptide Wang resin with a mixture of TFA–phenol (95 : 5) afforded the desired product in a high yield. The synthesis of 2ψ3-ENK on the Merrifield resin also proceeded well, including the condensations of Fmoc–(Dmb)Gly–OH, Fmoc–Gly–H and Boc–Tyr(Cl_2-Bzl)–OH. However, the HF-anisole treatment of the resulting Boc–Tyr(Cl_2-Bzl)–Glyψ[CH_2N(Dmb)]Gly–Phe–Leu–Merrifield resin afforded H–Tyr–Glyψ[CH_2N(Dmb)]Gly–Phe–Leu–OH, in which the Dmb group remained unaffected, as the major product (Fig. 1, bottom a and Table 1, run 1). These results indicate that the acid stability of the Dmb group on Tyr$\psi(CH_2NH)$Gly and Gly$\psi(CH_2NH)$Gly sequences is different, as described below. As shown in Table 2, the Dmb group on 2ψ3-ENK was completely cleaved by the 1 M TFMSA/thioanisole/TFA system.[8)] Next, method A was applied to the synthesis of [2ψ3, D-Leu^8]dynorphin (1—8). The synthesis of H–Tyr(^{t}Bu)–Glyψ[CH_2N(Dmb)]-

* To whom correspondence should be addressed.

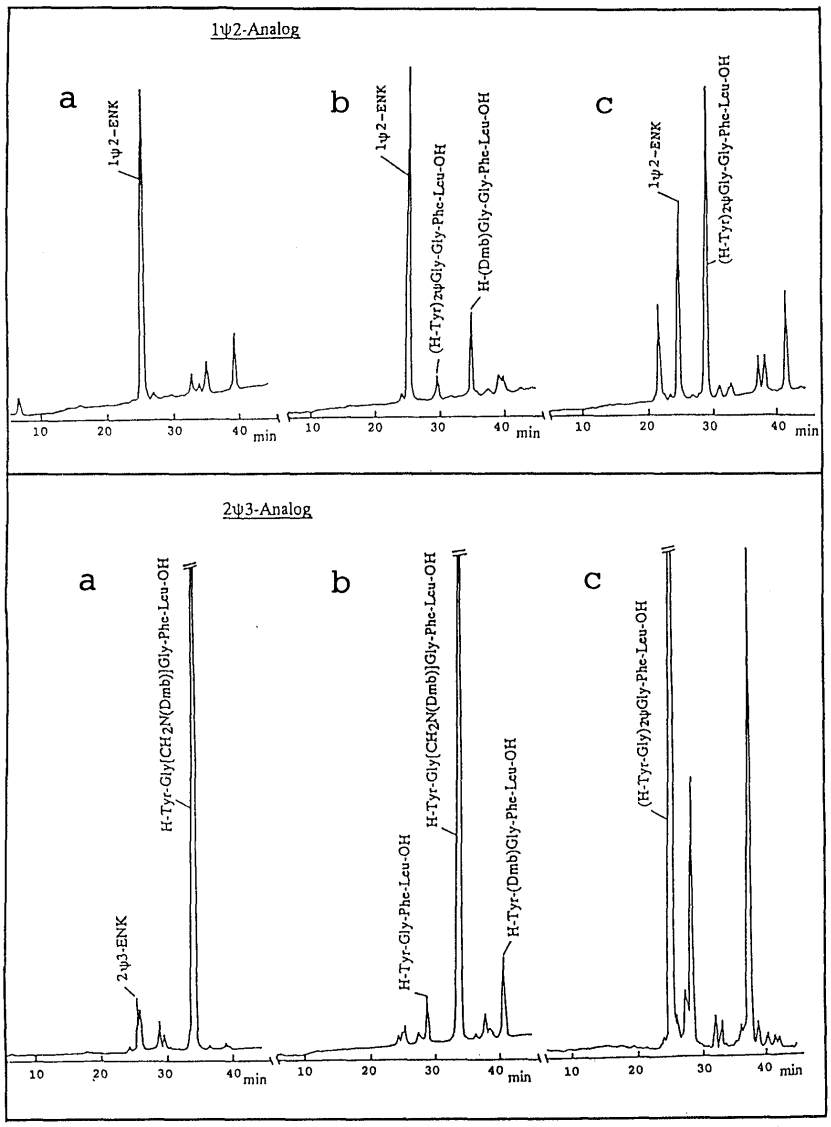

Fig. 1. HPLC Profiles of Crude Peptides of 1ψ2- (Top) and 2ψ3-Analogs (Bottom) Prepared by Methods A (a), B (b) and without Dmb Protection (c)

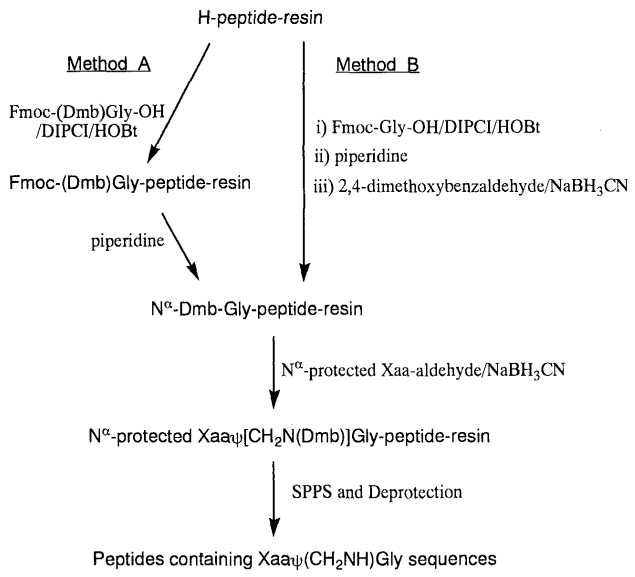

Fig. 2. Synthetic Strategies for Synthesis of $\psi(CH_2NH)$ Pseudopeptides Using the Dmb Protecting Group

Gly–Phe–Leu–Arg(Pmc)–Arg(Pmc)–D-Leu–Rink amide resin proceeded well, including condensations of Fmoc–(Dmb)Gly–OH and Fmoc–Gly–H/$NaBH_3CN$ (10 eq). Direct treatment of the peptide resin with 1.5 M TFMSA/thioanisole/TFA system at room temperature for 2 h and subsequent purification by HPLC afforded the desired peptide in an overall yield of 57%. In this context, without Dmb protection, the target peptide was obtained in a very low yield (7%).[6]

Method B In a preliminary experiment, we observed that the reaction of aromatic aldehydes with a glycyl-peptide resin by the usual reductive alkylation method proceeded slowly and produced the mono-substituted products (unpublished results). These observations led us to examine the utility of method B. For the synthesis of the 1ψ2-ENK, 5 eq of 2,4-dimethoxybenzaldehyde and $NaBH_3CN$ were reacted with the H–Gly–Gly–Phe–Leu–Wang resin for 4 h, followed by the second alkylation using Fmoc–Tyr(^{t}Bu)–H/$NaBH_3CN$ (5 eq) for 2 h. Deprotection and cleavage of the peptide resin with the TFA-phenol reagent afforded the desired product as the major product,

Table 1. Stability of Dmb Group on Various Peptide Resins toward TFA–Phenol Reagent[a]

Run	Peptide resin	Cleavage rate (%)[b]
1	Boc–Tyr(Cl_2-Bzl)–Glyψ[CH_2N(Dmb)]Gly–Phe–Leu–Merrifield resin	<5[c]
2	H–Tyr(^{t}Bu)–Glyψ[CH_2N(Dmb)]Gly–Phe–Leu–Wang resin	<5
3	H–Tyr(^{t}Bu)ψ[CH_2N(Dmb)]Gly–Gly–Phe–Leu–Wang resin	>95
4	H–Pheψ[CH_2N(Dmb)]Gly–Phe–Leu–Wang resin	92
5	H–Valψ[CH_2N(Dmb)]Gly–Phe–Leu–Wang resin	87
6	H–Alaψ[CH_2N(Dmb)]Gly–Phe–Leu–Wang resin	71
7	H–Glyψ[CH_2N(Dmb)]Gly–Phe–Leu–Wang resin	8
8	Ac–Valψ[CH_2N(Dmb)]Gly–Phe–Leu–Wang resin	20

a) TFA–phenol (95:5) at room temperature for 1.5 h. *b*) Cleavage rates were estimated from relative peak areas of the target peptide to those of the Dmb derivatives on analytical HPLC. *c*) Yield after treatment with a mixture of HF–anisole (9:1).

Table 2. Deblocking of Dmb Group of H–Tyr–Glyψ[CH_2N(Dmb)]-Gly–Phe–Leu–OH

Reagent and conditions	2ψ3-ENK (%)[a]
HF/anisole (9:1), 0 °C, 1 h	7
TFA/phenol (95:5), 45 °C, 1 h	<5
TFA/thioanisole (95:5), 45 °C, 2 h	50
1 M Me_3SiBr in TFA/thioanisole (95:5), 45 °C, 2 h	55
1 M TFMSA in TFA/thioanisole (95:5), room temp., 2 h	100

a) Cleavage rates were estimated from peak areas of 2ψ3-ENK and the Dmb derivative on analytical HPLC.

along with small amounts of doubly alkylated peptide and terminated peptide (Fig. 1, top b). Both by-products must be formed by incomplete reaction in the two-step reductive alkylations. For the synthesis of the 2ψ3-analog, 10 eq of 2,4-dimethoxybenzaldehyde/$NaBH_3CN$ was reacted with the H–Gly–Phe–Leu–Wang resin for 4 h. The second reductive alkylation with Fmoc–Gly–H/$NaBH_3CH$ (10 eq) was then performed for 2 h, followed by incorporation of the Tyr[1] residue. The TFA/phenol treatment of the resulting peptide Wang resin again afforded H–Tyr–Glyψ[CH_2N(Dmb)]Gly–Phe–Leu–OH in high yield (Fig. 1, bottom b), along with small amounts of two deletion peptides.

Acid Stability of Dmb Group To gain more information about the stability of the Dmb group, various peptide resins based on the structure of H–Xaaψ[CH_2N(Dmb)]-Gly–Phe–Leu–Wang resin were treated with the TFA–phenol reagent (Table 1, runs 4—8). Interestingly, except when Xaa is Gly, the cleavage of Dmb by the reagent proceeded smoothly to the extent of more than 71% within 2 h. Nevertheless, the Dmb group on the sterically least hindered pseudobond (Xaa = Gly, run 7) resisted the cleavage reaction, suggesting that the stability of the Dmb group is greatly influenced by the presence of a side chain on the Xaa residue. It should be noted that the Dmb group on an N-terminal acetylated derivative (run 8) was resistant to the acidic reagent, while that on the parent compound was cleaved smoothly (run 5). From these results, the easy deprotection of Dmb on H–Xaaψ-(CH_2NH)Gly sequences may be explained, as shown in Fig. 3, by assuming that the primary amino group is well placed to act as an internal base catalyst owing to the steric effect of neighboring side chains (R on Xaa residues), whereas when Xaa is Gly, this amino group arrangement is less favorable due to the weaker steric effect of Gly. The results for the ENK sequences (Table 1, runs 1—3) are also consistent with this mechanism.

The present results demonstrate that the Dmb group on the CH_2NH bond can be successfully cleaved by the 1 M TFMSA/thioanisole/TFA system wherever the pseudobond exists in the molecule, while only the Dmb groups of Xaaψ[CH_2N(Dmb)]Gly sequences (Xaa ≠ Gly) located at the N-terminus can be cleaved under milder acidic conditions, such as with TFA–phenol or TFA–thioanisole.

Opioid Activities of ψCH_2NH Analogs of ENK The *in vitro* biological activities of the $\psi(CH_2NH)$ analogs were evaluated on electrically evoked smooth muscle contractions of guinea pig ileum (GPI) and of mouse *vas deferens* (MVD), and compared with those of the parent peptide (Table 3). Most of these analogs showed drastically reduced activities in both assays. The 4ψ5-analog was the only exception; it showed a slightly higher potency than the parent peptide in the GPI assay and a low but significant potency in the MVD assay. These results suggest that the carbonyl groups of Tyr[1], Gly[2] and Gly[3] of ENK are important for the opioid activity.

Conclusion

From these experiments, it can be concluded that the Dmb group is useful for secondary amine protection to prevent undesirable double alkylations at Xaaψ(CH_2NH)Gly sequences using solid-phase reductive alkylation techniques with the aldehyde/$NaBH_3CN$ method. Although methods A and B are both very effective for minimizing the undesirable branched peptides, the use of Fmoc–(Dmb)Gly–OH (method A) seems to be superior because the reaction of 2,4-dimethoxybenzaldehyde with the resin-bound amine proceeds slowly and it is difficult to complete the two-step reductive alkylation on the resin and to monitor the reaction rate by using Kaiser's ninhydrin test.[9]

Experimental

Melting points were determined on a Yanaco MP-S3 apparatus and are uncorrected. TLC was performed on silica gel plates (Merck, Kiesel gel 60F_{254}, 5 × 10 cm) with the following solvent systems: Rf^1, *n*-BuOH–AcOH–H_2O (4:1:5, upper phase); Rf^2, AcOEt–hexane (1:1). Spots were detected by exposing the plates to iodine vapor. Analytical 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 15 to 60% B over 40 min was used at a flow rate of 1 ml/min and the eluate

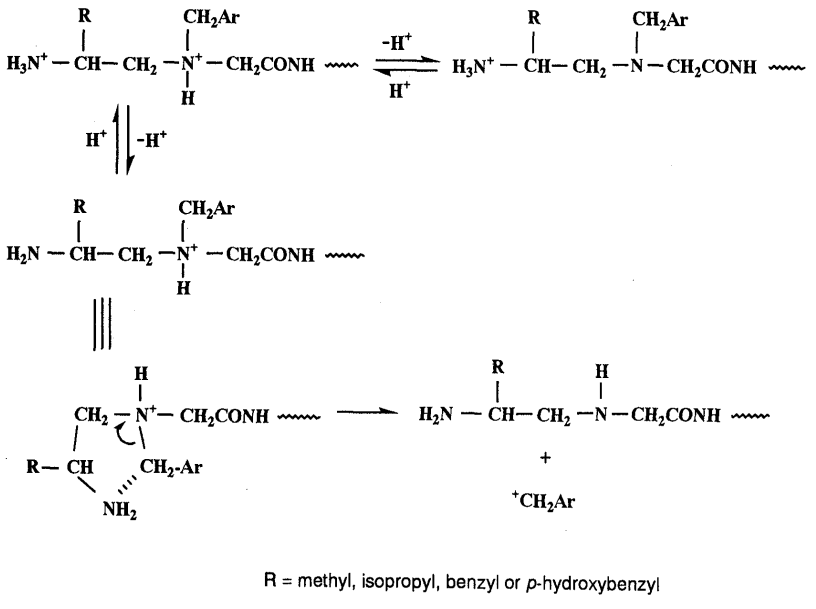

Fig. 3. A Possible Mechanism of Dmb Deprotection from H-Xaaψ[CH_2N(Dmb)]Gly Sequences by TFA–Phenol (95 : 5) Reagent

Table 3. Analytical Data and Opioid Activities of CH_2NH Pseudopeptide Analogs of ENK

Peptide	HPLC (t_R)[a]	FAB-MS (M+H$^+$)	GPI[b]	MVD[b]
ENK	26.5	—	100	100
1ψ2	25.0	542	12	0.03
2ψ3	24.6	542	<0.01	<0.01
3ψ4	22.3	542	<0.01	<0.01
4ψ5	19.5	542	178	0.38

a) Retention time (min) on analytical HPLC (see Experimental). *b*) Relative potency to ENK (ENK = 100).

was monitored at 220 nm. FAB-MS was run on a JEOL JMS-DX303 instrument.

Fmoc–Gly–H Fmoc-aminoacetaldehyde dimethylacetal was obtained from aminoacetaldehyde dimethylacetal and Fmoc-OSu in a usual manner, yield 95%, mp 92—93 °C, Rf^2 0.80. *Anal.* Calcd for $C_{19}H_{21}NO_4$: C, 69.70; H, 6.47; N, 4.28. Found: C, 69.51; H, 6.58; N, 3.86. The product (400 mg) was dissolved in dioxane (3 ml) and 2 N HCl (0.2 ml) was added. The solution was stirred at room temperature for 3 h, then the solvent was evaporated and the residue was extracted with AcOEt. The extract was washed with water, dried over $MgSO_4$ and evaporated to afford an oily residue, yield 235 mg (68%), Rf^2 0.51. This product contained a small amount of the acetal (15% >), but was used without further purification.

Fmoc–(Dmb)Gly–OH A solution of Gly (1.50 g) and 2,4-dimethoxybenzaldehyde (3.35 g) was hydrogenated in 50% aqueous MeOH containing AcOH (1 ml) in the presence of 10% Pd/C (350 mg) for 6 h. After removal of the catalyst and solvents, the residue was extracted with water-saturated *n*-BuOH. The extract was washed with *n*-BuOH-saturated water and evaporated to afford an oil, which was precipitated and triturated with absolute ether, yield 1.7 g (38%), Rf^1 0.38. This product was converted to the Fmoc derivative using Fmoc-OSu in a usual manner to afford a slightly sticky precipitate. *Anal.* Calcd for $C_{26}H_{25}NO_6$: C, 69.78; H, 5.63; N, 3.13. Found: C, 70.03; H, 6.00; N, 2.72. The dicyclohexylamine salt, mp 154—156 °C.

Solid-Phase Method Solid-phase synthesis of pseudopeptide analogs of enkephalin was performed by the DICDI/HOBt-mediated method according to the schedules previously described for the Boc-[10] or Fmoc-strategy,[11] starting with a Boc–Leu–Merrifield resin (0.5 mmol/g) or Fmoc–Leu–Wang resin (0.5 mmol/g). Boc- or Fmoc-amino aldehydes except for Fmoc–Gly–H were prepared *via* the corresponding hydroxamates by the method of Fehrentz and Castro[12] just before use. Fmoc–Gly–H was always used for Glyψ(CH_2NH)Xaa-containing peptides even in the Boc strategy in which the resulting Fmoc group was deblocked with a solution of 30% piperidine/DMF. Unless otherwise mentioned in Results and Discussion, generally 4 equivalents of the corresponding Boc- or Fmoc-amino aldehyde and $NaBH_3CN$ were reacted in DMF containing 1% AcOH for 2 h for the incorporation of the pseudobond. In the Fmoc strategy, the resin-bound free amine was protonated by treatment with a mixture of 5% pyridinium hydrochloride/DCM (10 min) prior to the reaction of the corresponding aldehydes. In some cases, such protonation accelerated the pseudobond formation in Fmoc strategy synthesis (our unpublished results).

Deprotection and Cleavage of Peptides from Resin Generally, 200 mg of protected peptide resin was treated with 5 ml of deblocking reagent for an appropriate time and then most of the reagent was evaporated under reduced pressure. Peptides were extracted with 20% AcOH and the extract was washed with ether and freeze-dried. The peptide was purified by medium-pressure HPLC on a Develosil LOP ODS column (3 × 30 cm) which was eluted with a gradient from 16 to 44% CH_3CN in 0.06% TFA over 150 min at a flow rate of 3 ml/min. The isolated products were analyzed by amino acid analysis and FAB-MS measurements.

Synthesis of [2ψ3, D-Leu^8]dynorphin (1—8)-NH_2 H–Tyr(^{t}Bu)–Glyψ[CH_2N(Dmb)]Phe–Leu–Arg(Pmc)–Arg(Pmc)–D-Leu–NH–Rink amide resin was prepared with the following modifications of the usual method; i) double coupling was employed for incorporation of Arg residues; ii) method A with 10 eq of Fmoc–Gly–H and $NaBH_3CN$ was used for the incorporation of the pseudobond. The peptide resin was treated with a mixture of 1.5 M TFMSA/thioanisole/TFA at room temperature for 2 h. After removal of most reagents, the residue was triturated with absolute ether and purified by preparative HPLC as described above to afford the title peptide along with a small amount of the Dmb derivative (overall yields: 57% and 5 %, respectively).

Title Peptide: Amino acid analysis (6 N HCl): Leu 2.09; Tyr 0.91; Phe 1.00; Glyψ(CH_2NH)Gly 0.96 (eluted at the same position as Lys); Arg 1.98; NH_3 1.20. FAB-MS *m/z*: 967 (M+H)$^+$.

Dmb Derivative: Amino acid analysis (6 N HCl): Leu 2.30; Tyr 0.68; Phe 1.00; Glyψ(CH_2NH)Gly 0.83; Arg 1.83; NH_3 1.60. FAB-MS *m/z*: 1117 (M+H)$^+$.

GPI Assay The GPI and MVD assays were performed according to the methods reported previously[10a] and the activities based on the IC_{50} values of analogs were compared to those of ENK (Table 3).

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: 1ψ2, 2ψ3, 3ψ4, 4ψ5 = ψCH_2NH peptide bond between the numbered residues, Dmb = 2,4-dimethoxybenzyl, Boc = *tert*-butoxycarbonyl, Fmoc = *N*-9-fluorenylmethyloxycarbonyl, Cl_2-Bzl = 2,6-dichloro-

benzyl, Pmc = 2,2,5,7,8-pentamethylchroman-6-sulfonyl, ^{t}Bu = *tert*-butyl, Ac = acetyl, Merrifield resin = chloromethyl resin, Wang resin = *p*-alkoxybenzyl alcohol resin, Rink amide resin = 4-(2,4-dimethoxyphenylaminomethyl)phenoxy resin, DMF = *N*,*N*-dimethylformamide, AcOEt = ethyl acetate, TFMSA = trifluoromethanesulfonic acid, Me_3SiBr = trimethylsilyl bromide, DIPCI = diisopropylcarbodiimide, HOBt = 1-hydroxybenzotriazole, ENK = Leu-enkephalin, HPLC = high-performance liquid chromatography, FAB-MS = fast atom bombardment mass spectroscopy.

2) Sasaki Y., Coy D. H., *Peptides*, **8**, 119—121 (1987); Coy D. H., Hocart S. J., Sasaki Y., *Tetrahedron*, **44**, 835—841(1988).
3) Balasubramaniam A., Stein M., Schriff S., Fischer J. E., "Peptides: Chemistry, Structure and Biology," ed. by Hodges R. S., Smith J. A., ESCOM Sci. Pub., Leiden, 1994, pp. 553—555.
4) Hocart S. J., Nekola M. V., Coy D. H., *J. Med. Chem.*, **31**, 1820—1824 (1988); Harbeson S. L., Shatzer S. A., Le T.-B., Bucks S. H., *J. Med. Chem.*, **35**, 3949—3955 (1992); Kaljuste K., Unden A., *Int. J. Peptide Protein Res.*, **43**, 505—511 (1944); Greiner G., Jezek J., Seyfarth L., Mueller B., Liebmann C., Paegelow I., Reissmann S., "Peptides: Chemistry, Structure and Biology," ed. by Hodges R. S., Smith J. A., ESCOM Sci. Pub., Leiden, 1994, pp. 547—549.
5) Salvi J.-P., Walchshofer N., Paris J., *Tetrahedron Lett.*, **35**, 1181—1184 (1994).
6) Ambo A., Adachi T., Sasaki Y., *Chem. Pharm. Bull.*, **43**, 1547—1550 (1995).
7) Meyer J.-P., Davis P., Lee K. B., Porreca F., Yamamura H. I., Hruby V. J., *J. Med. Chem.*, **38**, 3462—3468 (1995); Meyer J.-P., Gillespie T. J., Hom S., Hruby V. J., Davis T. P., *Peptides*, **16**, 1215—1219 (1995).
8) Yajima H., Fujii N., Ogawa H., Kawatani H., *J. Chem. Soc. Chem. Commun.*, **1974**, 107—110; Yajima H., Fujii N., *J. Am. Chem. Soc.*, **103**, 5867—5871 (1981).
9) Kaiser, E., Colescott R. L., Bossinger C. D., Cook P. I., *Anal. Biochem.*, **34**, 595—598 (1970).
10) *a*) Sasaki Y., Ambo A., Midorikawa K., Suzuki K., *Chem. Pharm. Bull.*, **41**, 1391—1394 (1993); *b*)Sasaki Y., Kohno H., Ohkubo Y., Suzuki K., *Chem. Pharm. Bull.*, **38**, 3162—3163 (1990).
11) Sasaki Y., Chiba T., *J. Med. Chem.*, **38**, 3995—3999 (1995).
12) Fehrentz J.-A., Castro B., *Synthesis*, **1983**, 676—678.