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Intestinal Absorption of Fluorescence-derivatized Cationic Peptide 001-C8-NBD via Adsorptive-mediated Transcytosis

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Abstract—The intestinal absorption of an intact oligopeptide was investigated in rats using a synthetic cationic peptide, 001-C8 (H-MeTyr-Arg-MeArg-D-Leu-NH$(CH_2)_8NH_2$). The peptide was coupled with 4-nitrobenzo-2-oxa-1,3-diazole (NBD) to prepare a fluorescence-labeled derivative 001-C8-NBD (H-MeTyr-Arg-MeArg-D-Leu-NH$(CH_2)_8$NH-NBD) for the purpose of quantification. The degradation half-life of 001-C8-NBD in jejunal homogenate (1 mg/mL) was 99.5 min, which was significantly longer than that of natural leucine enkephalin (1.14 min). The absorption of 001-C8-NBD was evaluated by the vascular-perfusion method. Intact 001-C8-NBD appeared in the blood time-dependently and the absorption volume at 30 min (2.75 ± 0.14 μL/cm intestine) was significantly larger than that of [^{3}H]PEG 900 (0.88 ± 0.13 μL/cm intestine), of which membrane permeability is very low. The absorption of 001-C8-NBD was greatly reduced by an adsorptive-mediated endocytosis inhibitor, protamine (10 mM). No inhibition of the absorption of [^{3}H]PEG 900 by protamine was observed. The intestinal absorption was also measured by an in vivo loop method. The absorption clearance of 001-C8-NBD measured by this method (0.083 ± 0.008 μL/min/cm intestine) was comparable to that obtained by the vascular perfusion method (0.092 ± 0.005 μL/min/cm intestine). All of these data suggested that 001-C8-NBD was absorbed as the intact oligopeptide in the intestine in vivo. Adsorptive-mediated transcytosis is suggested to have enormous potential as an oral delivery system for peptide and/or protein drugs.

Introduction

The therapeutic utility of pharmacologically active proteins and peptides is limited because of their short degradation half-lives in the gastrointestinal tract and low transepithelial membrane permeability, necessitating administration by iv injection. However, di- or tripeptides, which are major intestinal luminal digestive products of proteins, are taken across intestinal epithelial cells by carrier-mediated transport systems, including the oligopeptide transporter PepT1.[1,2] PepT1 transports only oligopeptides containing up to three amino acid residues, and cannot transport larger peptides.[3–5] Endocytosis could be an alternative for larger peptides. Receptor-mediated endocytosis mechanisms are involved in the transport of various immunoglobulins and growth factors.[6] Fluid-phase endocytosis has also been reported using horseradish peroxidase as a protein model in the human colon carcinoma cell line Caco-2.[7] However, very little information is available on the intestinal adsorptive-mediated endocytosis (AME), which is initiated by the binding of cationic moiety of substances to negative charges on the plasma membrane.

In a previous study,[8] we prepared a novel peptide, 001-C8 (H-MeTyr-Arg-MeArg-D-Leu-NH$(CH_2)_8NH_2$), and its derivatives with different numbers of cationic

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and neutral amino acids and with various carboxyl-terminal structures, in order to clarify the structural specificity of the AME in brain capillary endothelial cells (BCECs). 001-C8 consists of a partial amino acid sequence of E-2078, a dynorphin-like analgesic peptide,[9,10] and the carboxyl-terminal structure of ebiratide, an ACTH analogue,[11,12] with two arginine residues and an octanediamine residue, and has been confirmed to be efficiently taken up via the AME mechanism.[8] Very recently, we have demonstrated that an AME mechanism may operate in the intestine by using enterocyte-like Caco-2 cells in vitro.[13] However, it remains unknown whether 001-C8 is actually absorbed from the intestine in vivo. In the present study, we examined in vivo absorption of 001-C8 in rats by means of the intestinal vascular perfusion method and the loop method. For the purpose of quantification, we coupled the relatively small fluorescence label 4-nitrobenzo-2-oxa-1,3-diazole (NBD) to 001-C8.

Results and Discussion

Stability of the 001-C8-NBD in rat jejunum

The stability of 001-C8-NBD (100 μM) was assessed in rat jejunal mucosal homogenate (1 mg protein/mL) at 37 °C. The degradation of 001-C8-NBD at the initial concentration of 100 μM followed first-order kinetics (Figure 1). The degradation half-life of 001-C8-NBD was 99.5 min, which was 87 times longer than that of leucine enkephalin (1.14 min). The stability of 001-C8 is presumably due to the methylation of Tyr and Arg, substitution with the D-isomer of Leu and the modification of the carboxyl-terminal structure.

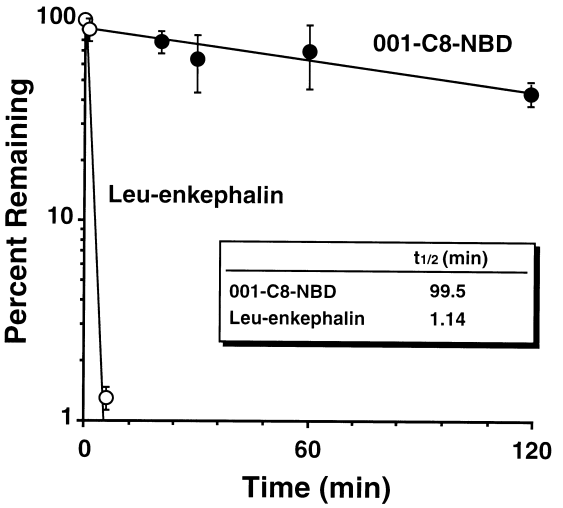


Figure 1. Time course of degradation of 001-C8-NBD and leucine enkephalin in rat jejunum homogenate. 001-C8-NBD (100 μM) and leucine enkephalin (1 mM) were each incubated in rat jejunum homogenate (1 mg/mL) at 37 °C. Degradation of the peptides was analyzed by HPLC as described in 'Results and Discussion'. Each point represents the mean ± SEM of three experiments. Inset: The degradation half-lives of 001-C8-NBD and leucine enkephalin were estimated assuming that the intact peptides decreased according to a first-order process.

In vitro intestinal absorption of 001-C8-NBD in rats

The intestinal absorption of 001-C8-NBD in rats was evaluated by the vascular-perfusion method. That is, the small intestine was excised and single-pass perfusion of both the lumen and the vasculature was performed. The test compounds were applied into the intestinal loop (25 cm in length) and the vascular perfusate was analyzed. This system is advantageous for evaluating the absorption of compounds which are easily metabolized in plasma and/or the liver and which have low absorbability. Figure 2(A) shows the time course of the absorption of 001-C8-NBD. The absorption occurred in a time-dependent manner and the absorption volume at 30 min (2.75 ± 0.14 μL/cm intestine) was significantly higher than that of [^{3}H]PEG 900 (0.88 ± 0.13 μL/cm intestine), suggesting the involvement of specialized mechanism(s). The absorption of 001-C8-NBD was greatly reduced by a polycationic peptide and an adsorptive-mediated endocytosis inhibitor, protamine (10 mM), which had no effect on the absorption of [^{3}H]PEG 900. Although because of the difference of electric charge between 001-C8-NBD and PEG900, which have almost same molecular weight, their paracellular permeabilities may not beidentical, the results strongly suggest that 001-C8-NBD is absorbed by both of paracellular and transcellular pathways, including adsorptive-mdiated endocytosis.

The viability and the integrity of the intestinal tissues used in the experiments were confirmed by comparing the absorption of 3-*O*-[^{3}H]methylglucose ([^{3}H]3OMG), which is known to be actively transported by a sodium-dependent glucose transporter, SGLT1,[14] and of phenolsulfonphthalein (PSP), a water-soluble membrane-impermeable marker, with the reported values. Figure 2(B) shows the time course of the absorption of both compounds. The absorption clearance of [^{3}H]3OMG and the extent of absorption of PSP in 1 hr were 1.9 μL/min/cm intestine and 5.6% of the dose, which are comparable to the reported values obtained by an in situ perfusion technique, 3.0 μL/min/cm intestine[15] and 6.4% of dose,[16] respectively.

In situ intestinal absorption of 001-C8-NBD in rats

Intestinal absorption was also measured by an in situ loop method. The absorption clearance of 001-C8-NBD

measured by this method (0.083 ± 0.008 μL/min/cm intestine) was comparable to that obtained by the vascular perfusion method (0.092 ± 0.005 μL/min/cm intestine) (Figure 3). It appears that the in vitro clearance measured by the vascular perfusion method reflects in vivo absorption of the peptide.

Metkephamid (Tyr-D-Ala-Gly-Phe-Me-Met-NH_2·CH_3-COOH, MW 660.8) is a stable analogue of natural methionine enkephalin. When it is administered orally, only a small fraction (0.53%) is absorbed as an intact form because of its extensive degradation by intestinal luminal- and enterocyte-associated enzymes.[17] However, puromycin, an aminopeptidase inhibitor, enhanced metkephamid absorption to 3.5% of the administered dose, though the intestinal absorption mechanism of Metkephamid has not been clarified yet.[17] The absorption of 001-C8-NBD (7.58 ± 0.78% at 30 min) was about 15 times and two times greater than that of metkephamid in the absence or presence of puromycin, respectively.

It remains unclear what is the precise mechanism underlying the absorption of this peptide and what is the subcellular fate of the absorbed peptide. Fluorescence derivatization of the peptide offers the advantage of rapid and direct observation of the distribution in the tissue of interest by using fluorescence microscopy. We are now addressing these points by means of visual three-dimensional analysis using high-resolution confocal microscopy. Our preliminary data suggest that 001-C8-NBD is transported by cells of a human colon carcinoma cell line, Caco-2, via the AME[13]/AMT[18] mechanism. Being similar to the transport across the intestinal epithelial barrier, 001-C8-NBD seems to have potential as a carrier for the oral delivery of drugs to the brain, because this peptide has been shown to be transported across the blood–brain barrier via the AME[8]/AMT[19] mechanism.

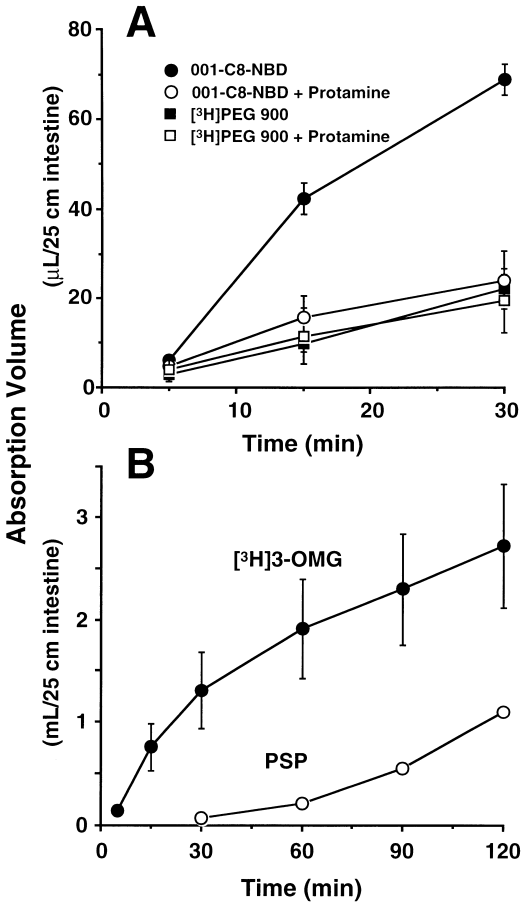


Figure 2. Time courses of appearance of 001-C8-NBD and [^{3}H]PEG 900, in the presence or absence of an endocytosis inhibitor (A) or [^{3}H]3-*O*-methylglucose or phenolsulfonphthalein (B), in the vascular outflow measured by the intestinal vascular perfusion method in rats. 001-C8-NBD (100 μM) with or without 10 mM protamine, [^{3}H]PEG 900 (1 μCi/mL), [^{3}H]3-*O*-methylglucose (1 nM) or phenolsulfonphthalein (1 mM) was introduced into the jejunal segment by luminal single-pass perfusion, and the vascular outflow was analyzed as described in 'Results and Discussion'. Each point represents the mean ± SEM of three–seven experiments.

Conclusion

In the present study, we have prepared a novel fluorescence-derivatized peptide, 001-C8-NBD. It was relatively stable in jejunum homogenate and was efficiently absorbed through intestinal epithelial cells in vivo.

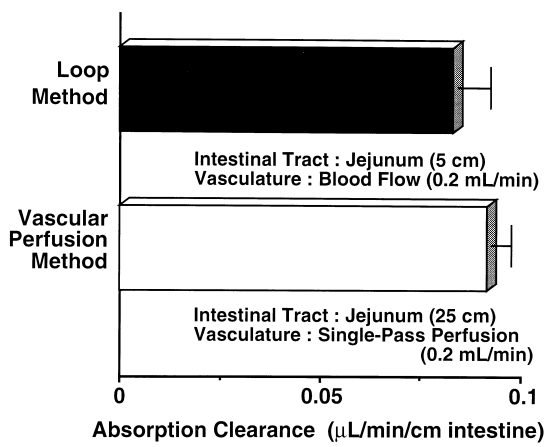


Figure 3. Comparison of the vascular perfusion method and the loop method to evaluate the absorption rates of 001-C8-NBD in rats. 001-C8-NBD (100 μM) was introduced into in vitro (vascular perfusion method) or in vivo (loop method) intestinal preparations and the venous blood was analyzed as described in 'Results and Discussion'.

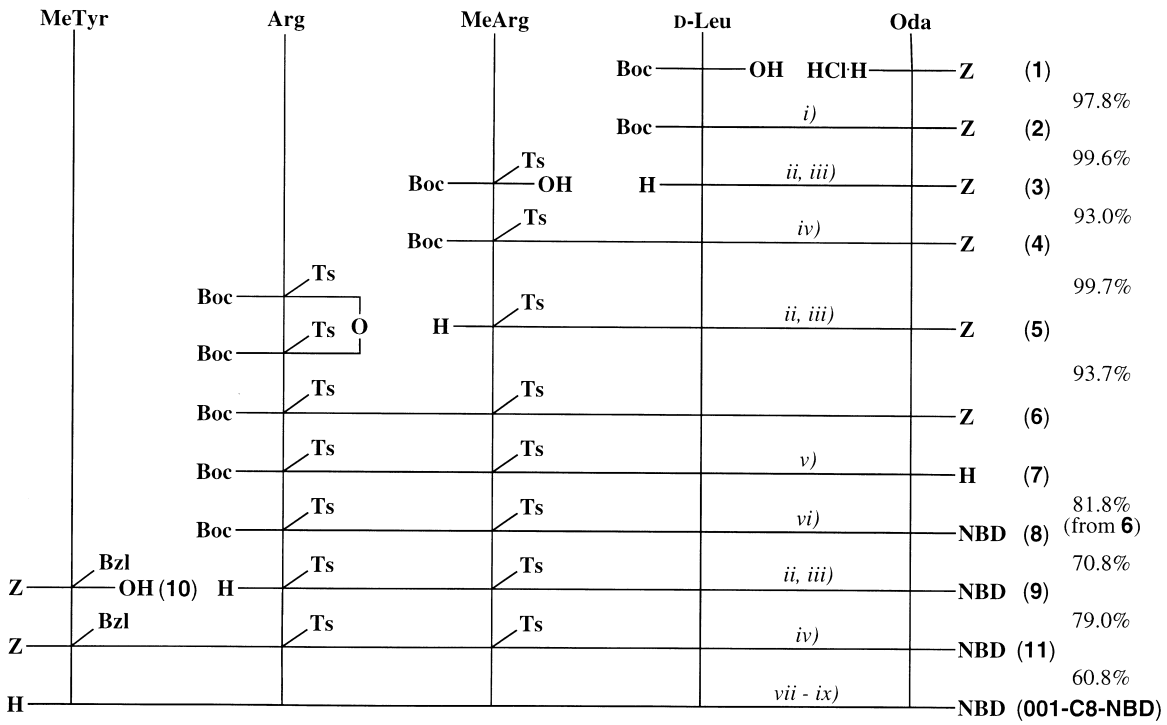
Adsorptive-mediated transcytosis is suggested to have enormous potential as an oral delivery system for peptide and/or protein drugs.

Experimental

Preparation of 001-C8-NBD

All of the melting points are uncorrected, and were measured on a Yanaco MP-J3 (Yanaco Co., Ltd., Kyoto, Japan) (Scheme 1). ^{1}H NMR spectra were recorded on a Jeol GX-270 spectrometer (Jeol Co., Ltd., Tokyo, Japan), and the chemical shifts are given in δ values from TMS used as the internal standard.[20] Fast-atom bombardment mass spectra (FAB-MS) were obtained on a Jeol JMS HX-100 mass spectrometer (Jeol Co., Ltd.). All specific rotations were measured by a SEPA-200 polarimeter (Horiba Ltd., Kyoto, Japan). Silica gel column chromatography was carried out with Merck silica gel 60 (Art. 7734, 70-230 mesh). Preparative reversed-phase high-performance liquid chromatography (RPHPLC) was performed on Cosmosil 5C_{18}AR 20×250 mm (Nacalai Tesque, Kyoto, Japan). HF treatment was carried out by use of the HF-reaction apparatus developed by Peptide Institute Inc., Osaka, Japan. Boc-D-Leu-OH·H_2O, Boc-Arg(Ts)-OH·3/4AcOEt·1/4H_2O, and Z-Tyr(Bzl)-OH were purchased from Peptide Institute, Osaka, Japan, and Boc-MeArg(Ts)-OH was purchased from Watanabe Chemical Industry Ltd., Hiroshima, Japan.

***N*-Benzyloxycarbonyl-1,8-octanediamine hydrochloride (Z-Oda-H·HCl) (1).** A solution of benzyloxycarbonyloxy succinimide (ZOSu) (5.00 g, 20.1 mmol) and triethylamine (TEA) (2.80 mL, 20.2 mmol) in diethyl ether (750 mL) was added dropwise to a solution of 1,8-octanediamine (Oda) (5.38 g, 37.4 mmol) in diethyl ether (500 mL) over a period of 26 h at room temperature. The mixture was stirred overnight, then evaporated in vacuo, and the residue was dissolved in MeOH (240 mL) and 6 M HCl (15 mL). The mixture was diluted with water (1 L), and insoluble material was filtered off. The filtrate was then applied to a Diaion® HP20 column. The column was thoroughly washed with water to remove unreacted Oda, and the desired product **1** was eluted with 50% MeOH. The eluate containing **1** was evaporated in vacuo, and the crystalline residue was recrystallized from MeOH and diethyl ether. Yield 3.26 g (51.5% from ZOSu); mp 183–185.5 °C (dec.); ^{1}H NMR (CD_3OD) = δ 1.34 [broad s, 8H, Oda/-CH_2CH_2-(CH_2)$_4$$CH_2CH_2$-], 1.49 (m, 2H, Oda/-$CH_2CH_2$-$NH_2$), 1.63 (m, 2H, Oda/Z-NH-CH_2CH_2-), 2.89 (t, 2H, -CH_2-NH_2), 3.09 (t, 2H, Z-NH-CH_2-), 5.05 (s, 2H, Ph-CH_2)



Scheme 1. Synthetic scheme of 001-C8-NBD. (i) DCC-HOBt/TEA/DMF; (ii) TFA-CH_2CI_2; (iii) sat. $NaHCO_3$ aq; (iv) EDC·HCl-HOBt/DMF; (v) H_2/Pd; (iv) NBD-CI/$NaHCO_3$; (vii) HF-thioanisole; (viii) Dowex 1×8 (AcO^- form); (ix) RPHPLC.

and 7.30 (m, 5H, Ph-CH_2); FAB-MS: m/z = 279.1 $(M+H)^+$ (calcd for $C_{16}H_{26}O_2N_2+H=279.2$). Found: C, 59.33; H, 8.73; N, 8.62; Cl, 11.18 %. Calcd for $C_{16}H_{26}O_2N_2\cdot HCl\cdot 0.5H_2O$: C, 59.34; H, 8.71; N, 8.65; Cl, 10.95%.

N^1-Benzyloxycarbonyl-N^8-(N^α-*t*-butoxycarbonyl-D-leucyl)-1,8-octanediamine (Boc-D-Leu-Oda-Z) (2). *N*,*N*′-Dicyclohexylcarbodiimide (DCC) (2.51 g, 12.2 mmol) and TEA (1.55 mL, 11.2 mmol) were added to a solution of Z-Oda-H·HCl (**1**) (3.20 g, 10.2 mmol), Boc-D-Leu-OH·H_2O (3.04 g, 12.2 mmol) and 1-hydroxybenzotriazole (HOBt) (1.65 g, 12.2 mmol) in DMF (30 mL) at 0 °C. The reaction mixture was stirred overnight at room temperature, acetic acid (234 mL, 4.09 mmol) was added and stirring was continued for a further 1 h. The precipitated *N*,*N*′-dicyclohexylurea (DCUrea) was filtered off, and the filtrate was concentrated in vacuo. The residue was dissolved in ethyl acetate (AcOEt) (50 mL), and insoluble material was filtered off again. The filtrate was subjected to general work up: (1) the organic solution was successively washed with 10% citric acid (20 mL×3), brine (20 mL×1), saturated aqueous sodium hydrogencarbonate (20 mL×3), and brine (20 mL×2); (2) the organic layer was dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crystalline residue was recrystallized from diethyl ether and hexane. Yield 4.91 g (97.8%); mp 53–56 °C; $[\alpha]_D^{24}$ +20.4° (*c* 1.04, $CHCl_3$); ^{1}H NMR (DMSO-d_6) = δ 0.86 (two d, 6H, D-Leu/δ-CH_3×2), 1.23 [m, 8H, Oda/ -CH_2CH_2-(CH_2)$_4$-CH_2CH_2-], 1.37 [s, 9H, $(CH_3)_3$C-], 1.41 [m, 6H, Oda/-CH_2CH_2-(CH_2)$_4$-CH_2CH_2-, D-Leu/β-CH_2], 1.57 (m, 1H, D-Leu/γ-CH), 3.02 [m, 4H, Oda/-CH_2-(CH_2)$_6$-CH_2-], 3.91 (m, 1H, D-Leu/α-CH), 5.00 (s, 2H, Ph-CH_2), and 7.33 (m, 5H, Ph-CH_2); FAB-MS: *m*/*z* = 492.4 $(M+H)^+$ (calcd for $C_{27}H_{45}O_5N_3+H$: 492.3). Found: C, 66.00; H, 9.29; N, 8.57%. Calcd for $C_{27}H_{45}O_5N_3$: C, 65.96; H, 9.23; N, 8.55%.

N^1-Benzyloxycarbonyl-N^8-D-leucyl-1,8-octanediamine (H-D-Leu-Oda-Z) (3). A solution of **2** (3.00 g, 6.10 mmol) in CH_2Cl_2 (10 mL) was treated with trifluoroacetic acid (TFA) (10 mL). The reaction mixture was stirred for 2 h, and then concentrated in vacuo. The residue was dissolved in AcOEt (30 mL), and the solution was washed with saturated sodium hydrogencarbonate (20 mL×3) and brine (20 mL×2), and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure to obtain **3** as a crystalline substance. Yield 2.39 g (99.6%). The product was used for the next coupling reaction without further purification.

N^1-Benzyloxycarbonyl-N^8-(N^α-*t*-butoxycarbonyl-N^g-tosyl-N^α-methylarginyl-D-leucyl)-1,8-octanediamine [Boc-MeArg(Ts)-D-Leu-Oda-Z] (4). A solution of the amine segment **3** (2.05 g, 5.23 mmol), Boc-MeArg(Ts)-OH (2.43 g, 5.49 mmol) and HOBt (740 mg, 5.49 mmol) in DMF (30 mL) was treated with EDC·HCl (1.05 g, 5.49 mmol) at 0 °C. After having been stirred for 10 min at 0 °C, the reaction mixture was further stirred overnight at room temperature, and the solvent was removed under reduced pressure. The residue was dissolved in AcOEt (50 mL), and worked up according to the general procedure as described for the preparation of **2**. The oily product was purified by silica gel column chromatography ($CHCl_3$: MeOH = 19:1). The fractions containing the desired product were combined and concentrated in vacuo. The residue was lyophilized from dioxane to obtain **4** as a colorless powder. Yield 3.97 g (93.0%); $[\alpha]_D^{24}$ +2.2° (*c* 1.1, MeOH); ^{1}H NMR (DMSO-d_6) = δ 0.85 (two d, 6H, D-Leu/δ-CH_3×2), 1.22 [m, 8H, Oda/-CH_2CH_2-(CH_2)$_4$-CH_2CH_2-], 1.38 [s, 9H, $(CH_3)_3$C-], 1.38–1.52 [m, 9H, Oda/-CH_2CH_2-(CH_2)$_4$-CH_2CH_2-, D-Leu/β-CH_2, MeArg/γ-CH_2, D-Leu/γ-CH], 1.52–1.70 (m, 2H, MeArg/β-CH_2), 2.37 (s, 3H, Ts/Ph-CH_3), 2.69 (s, 3H, MeArg/NCH_3), 2.98 [m, 6H, Oda/-CH_2-(CH_2)$_6$-CH_2-, MeArg/δ-CH_2], 4.27 (m, 1H, D-Leu/α-CH), 4.27 and 4.45 (m, total 1H, MeArg/α-CH), 5.00 (s, 2H, Z/CH_2-Ph), 7.28 and 7.63 (d, each 2H, Ts/Ph), and 7.34 (m, 5H, Z/Ph); FAB-MS: *m*/*z* = 816.9 $(M+H)^+$ (calcd for $C_{41}H_{65}O_8N_7S+H$: 816.5). Found: C, 59.10; H, 8.13; N, 11.81%. Calcd for $C_{41}H_{55}O_8N_7\cdot H_2O$: C, 59.04; H, 8.10; N, 11.75%.

N^1-Benzyloxycarbonyl-N^8-(N^g-tosyl-N^α-methylarginyl-D-leucyl)-1,8-octanediamine [H-MeArg(Ts)-D-Leu-Oda-Z] (5). Compound **5** with a free amino group was prepared from **4** (3.86 g, 4.73 mmol) by the same method as described for preparing **3**, and used without further purification. Yield 3.38 g (99.7%).

N^1-Benzyloxycarbonyl-N^8-(N^α-*t*-butoxycarbonyl-N^g-tosylarginyl-N^g-tosyl-N^α-methylarginyl-D-leucyl)-1,8-octanediamine [Boc-Arg(Ts)-MeArg(Ts)-D-Leu-Oda-Z] (6). A solution of Boc-Arg(Ts)-OH·3/4AcOEt·1/4H_2O (2.37 g, 4.92 mmol) in DMF (15 mL) was treated with EDC·HCl (945 mg, 4.92 mmol) at −20 °C, and the mixture was stirred for 100 min at −20 °C. To this chilled mixture was added dropwise a solution of the amine segment **5** (950 mg, 1.26 mmol) in DMF (10 mL) over a period of 10 min. The reaction mixture was stirred overnight at room temperature, and concentrated in vacuo. The residue was dissolved in AcOEt (50 mL), and worked up according to the general procedure. The oily residue was purified by silica gel column chromatography ($CHCl_3$:MeOH = 24:1). The fractions containing the desired product were combined and concentrated in vacuo. The residue was lyophilized from dioxane to give **6** as a colorless powder. Yield 1.33 g (93.7%); $[\alpha]_D^{24}$ −17.6° (*c* 1.15, MeOH); ^{1}H NMR (DMSO-d_6) = δ 0.84 (two d, 6H, D-Leu/δ-CH_3×2), 1.21 [m, 8H, Oda/-CH_2CH_2-(CH_2)$_4$-CH_2CH_2-], 1.34 [s, 9H, $(CH_3)_3$C-],

1.43–1.74 (m, 2H, MeArg/β-CH_2), 2.33 (s, 6H, Ts/Ph-CH_3×2), 2.56 and 2.91 (s, total 3H, MeArg/NCH_3), 2.93–3.15 [m, 8H, Oda/-CH_2-(CH_2)6-CH_2-, MeArg/δ-CH_2, Arg/δ-CH_2], 4.25 (m, 2H, D-Leu/α-CH, Arg/α-CH), 4.53 and 4.83 (m, total 1H, MeArg/α-CH), 5.00 (s, 2H, Z/Ph-CH_2), 7.27 and 7.63 (d, each 4H, Ts/Ph×2), and 7.33 (m, 5H, Z/Ph); FAB-MS: $m/z = 1127.0$ $(M+H)^+$ (calcd for $C_{54}H_{83}O_{11}N_{11}S_2+H$: 1126.6). Found: C, 56.48; H, 7.47; N, 13.48%. Calcd for $C_{54}H_{83}O_{11}N_{11}S_2 \cdot H_2O$: C, 56.67; H, 7.49; N, 13.46%.

N^1-(4-Nitrobenzo-6-oxa-5,7-diazolyl)-N^8-(N^α-*t*-butoxycarbonyl-N^g-tosylarginyl-N^g-tosyl-N^α-methylarginyl-D-leucyl)-1,8-octanediamine [Boc-Arg(Ts)-MeArg(Ts)-D-Leu-Oda-NBD] (8). Hydrogen gas was introduced into a mixture of compound **6** (1.77 g, 1.57 mmol), Pd-black (200 mg), and acetic acid (180 μL, 3.15 mmol) in methanol (30 mL) for 1.5 h. The catalyst was filtered off, and the filtrate was concentrated in vacuo. The residue was dissolved in AcOEt (50 mL), and this solution was washed with saturated sodium hydrogencarbonate (30 mL×3), and brine (30 mL×2). The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo.

To a solution of the above-obtained segment **7** in anhydrous THF (25 mL) and MeOH (25 mL) was added $NaHCO_3$ (396 mg, 4.71 mmol). Next, a solution of NBD-Cl (628 mg, 3.14 mmol) in anhydrous THF (25 mL) was added at 0 °C over a period of 15 min; the reaction was carried out in the dark. The mixture was stirred overnight at room temperature and concentrated in vacuo. The brown residue was dissolved in AcOEt (50 mL), and worked up according to the general procedure. The crude product was purified by silica gel column chromatography ($CHCl_3$:MeOH = 24:1). The fractions containing fluorescence-labeled peptide **8** were combined, and concentrated in vacuo. The residue was lyophilized from dioxane to give **8** as a brown powder. Yield 1.48 g (81.8 %); $[\alpha]_D^{25}$ −14.7° (*c* 0.770, MeOH); ^{1}H NMR (CD_3Cl) = δ 0.86 (two d, 6H, D-Leu/δ-CH_3×2), 1.26 [m, 8H, Oda/-CH_2CH_2-$(CH_2)_4$-CH_2CH_2-], 1.37 [s, 9H, $(CH_3)_3$C-], 2.36 (s, 6H, Ts/Ph-CH_3×2), 2.74 and 3.05 (s, total 3H, MeArg/NCH_3), 6.16 (d, 1H, NBD/Ar×1/2), 7.20 and 7.68 (d, each 4H, Ts/Ph×2), and 8.42 (d, 1H, NBD/Ar×1/2); FAB-MS: $m/z = 1155.5$ $(M+H)^+$ (calcd for $C_{52}H_{78}O_{12}N_{14}S_2+H$: 1155.5). Found: C, 52.57; H, 6.78; N, 14.99%. Calcd for $C_{52}H_{78}O_{12}N_{14}S_2 \cdot 1.2C_4H_8O_2$ (dioxane)$\cdot 1.7H_2O$: C, 52.81; H, 7.10; N, 15.18%.

N^α-*t*-Benzyloxycarbonyl-*O*-benzyl-N^α-methyltyrosine [Z-MeTyr(Bzl)-OH] (10). A solution of Z-Tyr(Bzl)-OH (208 mg, 0.500 mmol) in anhydrous tetrahydrofuran (THF) (1.5 mL) was treated with CH_3I (116 μL, 4.00 mmol) and NaH (60% in oil) (36 mg, 0.90 mmol) at 0 °C. The mixture was stirred for 15 min at 0 °C, and overnight at room temperature, then it was acidified with 1 M HCl (3 mL), and the THF was evaporated in vacuo. The resulting aqueous layer was extracted with AcOEt (10 mL×3), and the combined AcOEt layer was extracted with saturated aqueous $NaHCO_3$ (10 mL×3). The alkaline extract was acidified with citric acid, and then extracted with AcOEt (10 mL×3) again. The AcOEt extract was dried over anhydrous $MgSO_4$, and concentrated in vacuo. The oily residue was crystallized by trituration with hexane, and the crude product was recrystallized from AcOEt–hexane to give **10** as fine prisms. Yield 152 mg (72.5%): mp 93–94 °C; $[\alpha]_D^{22}$ −45.5° (*c* 1.05, $CHCl_3$); ^{1}H NMR ($CDCl_3$) = δ 2.79 and 2.85 (s, total 3H, NCH_3), 2.89–3.34 (m, 2H, β-CH_2), 4.84 (m, 1H, α-CH), 5.02 (s, 2H, Bzl/Ph-CH_2-), 5.11 (s, 2H, Z/Ph-CH_2-), 6.86 and 7.08 (d, each 2H, MeTyr/Ph), 7.21 (m, 5H, Bzl/Ph), and 7.44 (m, 5H, Z/Ph); FAB-MS: $m/z = 420.0$ $(M+H)^+$ (calcd for $C_{25}H_{25}O_5N+H$: 420.2). Found: C, 71.36; H, 5.87; N, 3.30%. Calcd for $C_{25}H_{25}O_5N$: C, 71.58; H, 6.01; N, 3.34%.

N^1-(4-Nitrobenzo-6-oxa-5,7-diazolyl)-N^8-(N^α-benzyloxycarbonyl-*O*-benzyl-N^α-methyltyrosyl-N^g-tosylarginyl-N^g-tosyl-N^α-methylarginyl-D-leucyl)-1,8-octanediamine [Z-MeTyr(Bzl)-Arg(Ts)-MeArg(Ts)-D-Leu-Oda-NBD] (11). Compound **8** (124 mg, 0.107 mmol) was dissolved in TFA (125 μL) and CH_2Cl_2 (125 μL), and the solution was stirred for 15 min at room temperature, then worked up by the same procedure as described for preparing **4**. The amine segment, H-Arg(Ts)-MeArg(Ts)-D-Leu-Oda-NBD (**9**), was obtained in a 70.8% yield (80.0 mg).

A solution of **9** (80.0 mg, 75.8 μmol), **10** (35.0 mg, 83.4 μmol), and HOBt (11.3 mg, 83.4 μmol) in DMF (1.0 mL) was treated with EDC·HCl (16.0 mg, 83.4 μmol). The reaction mixture was stirred for 10 min at 0 °C, and 2 h at room temperature, followed by concentration in vacuo. The residue was dissolved in AcOEt (20 mL), and worked up according to the general procedure. The crude product was purified by silica gel column chromatography ($CHCl_3$:MeOH = 12:1). The fractions containing **11** were combined, and concentrated in vacuo. The residue was then lyophilized from dioxane to give **11** as a brown powder. Yield 86.9 mg (79.0 %); $[\alpha]_D^{20}$ −17° (*c* 0.13, MeOH); ^{1}H NMR ($CDCl_3$) = δ 0.88 (two d, 6H, D-Leu/δ-CH_3×2), 1.26 [m, 8H, Oda/CH_2CH_2-$(CH_2)_4$-CH_2CH_2], 2.36 (s, 6H, Ts/Ph-CH_3×2), 2.71 and 3.08 (d, total 3H, MeArg/NCH_3), 2.78 (s, 3H, MeTyr/NCH_3), 6.18 (d, 1H, NBD/Ar×1/2), 6.85 and 7.20 (d, each 2H, MeTyr/Ph), 7.23 (m, 5H, Bzl/Ph), 7.26 (m, 5H, Z/Ph), 7.37 and 7.71 (d, each 4H, Ts/Ph×2), and 8.41 (d, 1H, NBD/Ar×1/2); FAB-MS (positive) m/z 1456.3 $(M+H)^+$ (calcd for $C_{72}H_{93}O_{14}N_{15}S_2+H$: 1456.6). Found: C, 57.61; H, 6.43; N,

12.93%. Calcd for $C_{72}H_{93}O_{14}N_{15}S_2 \cdot 1.4C_4H_8O_2$ (dioxane)$\cdot 1.7H_2O$: C, 57.87; H, 6.73; N, 13.04%.

N^1-(4-Nitrobenzo-6-oxa-5,7-diazolyl)-N^8-(N^α-methyltyrosyl-arginyl-N^α-methyl-arginyl-D-leucyl)-1,8-octanediamine (001-C8-NBD). Anhydrous HF (4 mL) was added to **12** (60.0 mg, 41.2 μmol) and thioanisole (359 μL, 3.29 mmol) at −70 °C. The reaction mixture was stirred for 1 h at 0 °C, and then HF was removed under reduced pressure. The residue was dissolved in 4% acetic acid (10 mL), and the solution was washed with diethyl ether (5 mL). The aqueous layer was passed through a Dowex 1×8 column (AcO^- form), and the eluate was lyophilized. The crude product was purified by preparative RPHPLC (gradient elution: 25–45% (0.5%/min) CH_3CN-0.1% TFA aq, 8 mL/min), and the fractions containing 001-C8-NBD were lyophilized to give a brown powder. Yield 37.3 mg (60.8%); $[\alpha]_D^{20}$ −18° (*c* 0.55, MeOH); ^{1}H NMR (CD_3OD) = δ 0.93 (two d, 6H, D-Leu/δ-CH_3×2), 1.40 [m, 8H, Oda/-CH_2CH_2-(CH_2)$_4$-CH_2CH_2-], 2.65 (s, 3H, MeTyr/α-NCH_3), 2.81 and 2.96 (s, total 3H, MeArg/α-NCH_3), 4.11 (1H, m, MeTyr/α-CH), 4.36 (m, 1H, D-Leu/α-CH), 4.91 (m, 1H, Arg or MeArg/α-CH),[21] 6.33 (d, 1H, NBD/Ar×1/2), 6.74 and 7.07 (d, each 1H, MeTyr/Ph), and 8.52 (d, 1H, NBD/Ar×1/2); FAB-MS: m/z = 924.7 $(M+H)^+$ (calcd for $C_{43}H_{69}O_8N_{15}$ + H: 924.5).

Stability of the peptide

The peptide was dissolved in 500 μL of Krebs–Henseleit bicarbonate buffer (pH 6.0) (KHBB, 118 mM NaCl, 4.74 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, 1.27 mM $CaCl_2$, 24.9 mM $NaHCO_3$) containing 10 mM D-glucose and 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), and incubated with an equal volume of rat jejunal homogenate (2 mg protein/mL) at 37 °C. At the designated time, 2 mL of MeOH was added to the mixture to terminate the reaction. The whole was cooled on ice and centrifuged at 1500×*g* for 15 min to remove proteins. Peptide analysis was carried out by reversed-phase HPLC. The HPLC conditions were as follows: column, ODS-80TM analytical column (particle size 5 μm, 15×0.46 cm, Tosoh, Tokyo, Japan); mobile phase, a mixture of water, acetonitrile and trifluoroacetic acid (85:15:0.1 for 001-C8-NBD, and 70:30:0.1 for leucine enkephalin); column temperature, 40 °C; flow rate, 1.0 mL/min; detection, fluorescence at excitation and emission wavelengths of 480 nm and 550 nm, respectively, for 001-C8-NBD and absorbance at 278 nm for leucine enkephalin.

Intestinal vascular perfusion method

Vascular perfusion of the rat small intestine was performed as described by Yamashita et al.[22] with some modifications. In brief, male Wistar rats (Sankyo Laboratory, Toyama, Japan) which had been starved overnight were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), and a midline incision was made to expose the small intestine (upper jejunum, about 25 cm). The mesenteric arcades to adjacent portions were carefully tied off, and polyethylene tubing (i.d. 3 mm) was placed on both ends of the intestinal segment for luminal single-pass perfusion. Polyethylene tubing (i.d. 0.5 mm) was also used to cannulate the superior mesenteric artery and the portal vein, and perfused with heparin and then KHBB (pH 7.4) containing 3% bovine serum albumin, 10 mM D-glucose and 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) at a perfusion rate of 0.2 mL/min. The intestinal segment was single-pass-perfused with test compounds at a perfusion rate of 2.5 mL/min, isolated from other portions and placed in a serosal bath containing 100 mL of KHBB (pH 7.4) warmed to 37 °C with a water jacket. Vascular outflow was collected and analyzed by HPLC as described above or with a liquid scintillation counter (LSC-700, Aloka Co., Ltd., Tokyo, Japan).

Intestinal loop method

The in vivo intestinal loop was prepared as described by Barr and Riegelman,[23] with minor modifications. In brief, a 5-cm-long segment of jejunum, approximately 3 cm distal to the ligament of Treitz, was cut and both ends were ligated. 001-C8-NBD dissolved in 0.2 mL of KHBB (pH 6.0), was injected into the loop, and all venous blood was collected for 30 min from the portal vein cannulated with polyethylene tubing. The blood lost was continuously replaced with heparinized blood previously collected from donor animals from the femoral vein at a perfusion rate of 0.2 mL/min.

Data analysis

The absorption (μL/cm intestine) was calculated by dividing the amount that appeared in the blood sample (peak area in HPLC or dpm per cm intestine used) by the initial concentration of test compound in the intestinal lumen (peak area in HPLC analysis or dpm per μL). All data are expressed as means ± SEM, and statistical analysis was performed by using Student's one-tailed *t* test. The criterion of significance was taken to be $P < 0.05$.

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