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Structure–Activity Relationships of Neuromedin U. II. Highly Potent Analogs Substituted or Modified at the N-Terminus of Neuromedin U-8[1,2]

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To develop a highly potent agonist and to examine the structure–contractile activity relationship of neuromedin U-8 (NMU-8), seventeen analogs were synthesized and tested for contractile activity on isolated chicken crop smooth muscle preparations. The analogs were designed to examine the contributions of cyclic structure and acidic function at the N-terminal of NMU-8 and NMU-8 (2—8) to the biological activity. The relative activity (RA) values of NMU-8 analogs were as follows: [D-pGlu^1]-NMU-8, 5.50; [pyrohomoglutamyl (pHgu)^1]-NMU-8, 4.65; [D-pHgu^1]-NMU-8, 4.66; [Asp^1]-NMU-8, 11.4; [acetyl(Ac)-Asp^1]-NMU-8, 9.81; [Ac-Glu^1]-NMU-8, 18.6; [succinyl (Suc)-Tyr^1-NMU-8, 69.3; [3-sulfoalanyl (Sal)^1]-NMU-8, 12.7. The RA values of NMU-8 (2—8) analogs were as follows: α-picolinyl (Pic)-NMU-8 (2—8), 7.96; 2-furoyl (Fur)-NMU-8 (2—8), 9.91; 2-thiophenecarboxyl (Thi)-NMU-8 (2—8), 3.41; 4-hydroxyphenylpropionyl (Hpp)-NMU-8 (2—8), 3.20; *o*-phthalyl (Pht)-NMU-8 (2—8), 11.3; Suc-NMU-8 (2—8), 109; malonyl (Mlo)-NMU-8 (2—8), 17.9; maleyl (Mle)-NMU-8 (2—8), 31.6; glutaryl (Glt)-NMU-8 (2—8), 81.3; The potencies of the analogs were higher than that of p-NMU-8. Suc-NMU-8 (2—8) showed the highest potency among the analogs synthesized. The results reveal that the carboxylic acid group at the N-terminus of NMU-8 makes a major contribution to the activity.

Key words neuromedin U-8; structure–activity relationship; smooth muscle contraction; chicken crop; agonist

Neuromedin U (NMU) is a neuropeptide that was first isolated from porcine spinal cord[3] and subsequently from various other species. Porcine,[3] dog[4] and chicken[5] NMUs were isolated in two molecular forms, *i.e.*, a pentacosapeptide amide and an octa- or a nonapeptide amide (Fig. 1), whereas rat,[6,7] rabbit[8] and guinea pig[9] NMUs were single components with 23, 25 and 9 amino acid residues, respectively. Mammalian NMUs share a common C-terminal heptapeptide amide structure, Phe–Leu–Phe–Arg–Pro–Arg–Asn–NH_2, which appears to be essential for their biological activities. Dog NMU-8 (d-NMU-8) has a pGlu residue in place of Tyr^1 of porcine NMU-8 (p-NMU-8) (Fig. 1). NMU has a potent stimulatory effect on contraction on the isolated rat uterus[3] and chicken gastrointestinal smooth muscle.[10] NMU produces a small but sustained increase in arterial blood pressure in rat[3,7] and reduces blood flow in the superior vascular bed of dog and rat.[11] It also affects intestinal ion transport in porcine jejunum.[12]

We have already reported the structure–activity relationship of p-NMU-8 analogs substituted for each amino acid residue with Gly,[13] suggesting that the amino acid side chains at positions 2—8 are important for the contractile activity on isolated chicken crop assay. The peptide chain length-contractile activity relationships of NMUs[14] revealed that the hexapeptide portion, Phe–Leu–Phe–Arg–Pro–Arg, is essential for the contractile activity and the N-terminal portions of rat and other NMUs are important for the increased activity. Further, NMU analogs with modification at the N-terminal to afford resistance to aminopeptidases showed higher contractile activity than p-NMU-8 in the chicken crop assay.[1]

In this study, in order to develop a highly potent NMU agonist by means of substitution or modification at the N-terminal of NMU-8, seventeen analogs (Fig. 2) were synthesized by solid-phase methodology and tested for contractile activity on isolated chicken crop smooth muscle preparations.[15]

Results and Discussion

In our previous paper,[1] it was suggested that the N-terminal amino acid residue of NMU-8 may not be involved in the receptor activation, since it could be replaced by various amino acids or simple organic acids without significant decrease of the activity, and it was shown that N-terminally modified analogs possessed higher activity than p-NMU-8, due to their greater resistance to aminopeptidases on the isolated chicken crop preparation used. To develop a highly potent agonist, we directed our attention to position 1 of NMU-8. First, various acid-bearing heterocyclic or heteroaromatic ring structures were examined as substituents, because our study[1] revealed that d-NMU-8 in which a pyrrolidone carboxylic acid residue is substituted for Tyr at position 1 of p-NMU-8 possessed five-fold higher activity than p-NMU-8 in chicken crop assay. It can be expected that the cyclic skeleton of the pyrrolidone may be involved in the preferable structure for affinity to NMU receptor in chicken crop. The potencies of analogs [D-pGlu^1]-NMU-8

Porcine	NMU-8	H–Tyr–Phe–Leu–Phe–Arg–Pro–Arg–Asn–NH_2
Dog	NMU-8	pGlu–Phe–Leu–Phe–Arg–Pro–Arg–Asn–NH_2
Chicken	NMU-9	H–Gly–Tyr–Phe–Phe–Phe–Arg–Pro–Arg–Asn–NH_2
Guinea pig	NMU-9	H–Gly–Tyr–Phe–Leu–Phe–Arg–Pro–Arg–Asn–NH_2

Fig. 1. Amino Acid Sequences of Mini NMU Peptides

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$$\overset{1}{\text{X}}\text{-Phe-Leu-Phe-Arg-Pro-Arg-}\overset{8}{\text{Asn}}\text{-NH}_2$$

X =

1	D-pGlu	**10**	Suc (succinyl)-Tyr
2	pHgu	**11**	Suc
3	D-pHgu	**12**	Hpp (4-hydroxyphenylpropionyl)
4	Fur (2-furoyl)	**13**	Mlo (malonyl)
5	Thi (2-thiophencarbonyl)	**14**	Mle (maleyl)
6	Pic (α-picolinyl)	**15**	Glt (glutaryl)
7	Asp	**16**	Pht (*o*-phthalyl)
8	Ac (acetyl)-Asp	**17**	Sal (3-sulfoalanyl)
9	Ac-Glu		

Fig. 2. Structures of NMU-8 Analogs Modified at the N-Terminal

Table 1. Characteristics of Synthetic NMU-8 Analogs and Yields

Analog	$[\alpha]_D^{25}$ ($c=0.5$, 2 M AcOH)	RP-HPLC[a] t_R[b] (min)	HP-TLC[c] Rf^1	HP-TLC[c] Rf^2	Yield (%)
1	−57.8	15.9	0.50	0.57	9.0
2	−56.8	16.4	0.49	0.56	17.1
3	−60.9	16.4	0.48	0.55	10.2
4	−55.8	20.5	0.53	0.56	4.9
5	−60.8	21.9	0.54	0.56	35.6
6	−59.9	21.8	0.52	0.57	39.7
7	−52.7	12.3	0.40	0.51	4.7
8	−70.9	16.8	0.44	0.54	34.4
9	−70.5	19.5	0.53	0.48	21.0
10	−47.8	19.0	0.53	0.58	12.3
11	−59.1	17.3	0.48	0.52	7.6
12	−49.5	20.2	0.54	0.55	16.6
13	−59.8	17.0	0.46	0.51	10.4
14	−59.5	17.8	0.48	0.53	6.0
15	−60.8	17.2	0.50	0.54	10.5
16	−63.8	21.0	0.52	0.55	3.2
17	−51.3	14.3	0.43	0.49	20.4

a) Column, Puresil 5 μ C_{18} 120A; flow rate, 1 ml/min; detection, 210 nm; eluent system, linear gradient from 16% to 40% MeCN (20 min) in 20 mM sodium phosphate buffer (pH 3.0). *b*) Retention time. *c*) Rf^1, *n*-BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24); Rf^2, *n*-BuOH–AcOEt–AcOH–H_2O (1 : 1 : 1 : 1).

(**1**), [pHgu[1]]-NMU-8 (**2**) and [D-pHgu[1]]-NMU-8 (**3**) were 5.50, 4.65 and 4.66 times that of p-NMU-8, and were almost the same as that of d-NMU-8. The replacement of the pyrrolidone ring by a piperidone moiety failed to increase the activity of d-NMU-8. A marked increase of the activity was observed when the pyrrolidone was substituted by furan ring. The RA value of 2-furoyl (Fur)-NMU-8 (2—8) (**4**) was 9.91. The potency of Fur-NMU-8 (2—8) (**4**) was about ten and two times those of p-NMU-8 and d-NMU-8, respectively. The substitution of pyridine and thiophene moieties for the pyrrolidone did not markedly increase the activity. The RA values of α-picolinyl (Pic)- (**6**) and 2-thiophenecarboxyl (Thi)-NMU-8 (2—8) (**5**) were 7.96 and 3.41, respectively. The results indicate that the furan ring may be most advantageous at the N-terminal of NMU-8 analogs.

The high activity of d-NMU-8 may be due not only to the resistance to aminopeptidases,[1] or the ring structure, but also to the decrease of the basicity of the α-amino function by the internal amide formation of the pyrrolidone ring. To decrease the basicity at the N-terminal N^{α}-amino function, an analog, 4-hydroxyphenylpropionyl (Hpp)-NMU-8 (2—8) (**12**), which corresponds to [des-N^{α}-amino-Tyr[1]]-NMU-8, was examined. The relative activity (RA) value of Hpp-NMU-8 (2—8) (**12**) was 3.20. The potency of Hpp-NMU-8 (2—8) (**12**) was almost three times that of the corresponding N^{α}-free p-NMU-8 ($p<0.05$). The results suggests that the loss of the basicity at the N^{α}-amino group of NMU-8 analogs may bring about a higher activity. [Glu[1]]-NMU-8,[1] which may be susceptible to aminopeptidase, had potent activity with the RA value of 7.18.[1] The high activity could be explained by the internal neutralization of the α-amino function by the γ-carboxylic acid function, resulting in loss of the basicity of the N-terminal portion. As can be seen in Table 3 and Fig. 3, [Asp[1]]-NMU-8 (**7**) also had potent activity with the RA value of 11.4. Acetylation of both analogs resulted in further decrease of the basicity at the N-terminal

Table 2. FAB-MS Data and Amino Acid Analyses for Synthetic NMU-8 Analogs

Analog	FAB-MS[a] Found	FAB-MS[a] Formula	Amino acid analysis[b] Asp	Pro	Leu	Phe	Arg	NH_3	Others[c]
1	1059	$C_{50}H_{74}N_{16}O_{10}$	1.00 (1)	0.97 (1)	0.99 (1)	1.98 (2)	1.97 (2)	2.76 (2)	Glu 1.09 (1)
2	1073	$C_{51}H_{76}N_{16}O_{10}$	1.01 (1)	0.96 (1)	1.02 (1)	1.99 (2)	2.01 (2)	2.92 (2)	Hgu 1.02 (1)
3	1073	$C_{51}H_{76}N_{16}O_{10}$	1.03 (1)	0.94 (1)	1.02 (1)	1.98 (2)	2.01 (2)	2.01 (2)	Hgu 1.01 (1)
4	1042	$C_{50}H_{71}N_{15}O_{10}$	1.02 (1)	0.97 (1)	1.02 (1)	1.94 (2)	2.05 (2)	2.37 (2)	—
5	1058	$C_{50}H_{71}N_{15}O_9S_1$	1.04 (1)	1.02 (1)	1.01 (1)	1.99 (2)	1.95 (2)	2.11 (2)	—
6	1053	$C_{51}H_{72}N_{16}O_9$	1.17 (1)	1.05 (1)	1.00 (1)	1.79 (2)	1.99 (2)	2.26 (2)	—
7	1063	$C_{49}H_{74}N_{16}O_{11}$	2.05 (2)	0.99 (1)	1.01 (1)	1.99 (2)	1.95 (2)	2.64 (2)	—
8	1105	$C_{51}H_{76}N_{16}O_{12}$	2.05 (2)	1.03 (1)	1.00 (1)	1.95 (2)	1.97 (2)	1.85 (2)	—
9	1119	$C_{52}H_{78}N_{16}O_{12}$	1.01 (1)	1.01 (1)	1.01 (1)	1.95 (2)	1.97 (2)	1.75 (2)	Glu 1.06 (1)
10	1211	$C_{58}H_{82}N_{16}O_{13}$	1.09 (1)	1.05 (1)	0.96 (1)	1.80 (2)	2.13 (2)	2.00 (2)	Tyr 0.96 (1)
11	1048	$C_{49}H_{73}N_{15}O_{11}$	0.98 (1)	0.90 (1)	0.97 (1)	2.29 (2)	1.86 (2)	1.98 (2)	—
12	1096	$C_{54}H_{77}N_{15}O_{10}$	1.00 (1)	1.01 (1)	1.02 (1)	1.98 (2)	1.99 (2)	2.53 (2)	—
13	1034	$C_{48}H_{71}N_{15}O_{11}$	1.02 (1)	1.00 (1)	1.00 (1)	1.98 (2)	2.00 (2)	2.00 (2)	—
14	1046	$C_{49}H_{71}N_{15}O_{11}$	1.00 (1)	0.99 (1)	0.98 (1)	2.01 (2)	2.02 (2)	2.28 (2)	—
15	1062	$C_{50}H_{75}N_{15}O_{11}$	1.02 (1)	1.02 (1)	1.02 (1)	2.00 (2)	1.94 (2)	1.87 (2)	—
16	1096	$C_{53}H_{73}N_{15}O_{11}$	0.98 (1)	0.99 (1)	1.01 (1)	2.04 (2)	1.98 (2)	2.77 (2)	—
17	1099	$C_{48}H_{74}N_{16}O_{12}S_1$	1.00 (1)	1.03 (1)	1.02 (1)	1.99 (2)	1.95 (2)	1.96 (2)	—

a) For $[M+H]^+$. *b*) Hydrolysis at 130 °C for 3.0 h by vapor of 6 N hydrochloric acid containing phenol (3%); numbers in parentheses are theoretical values. *c*) Other amino acids analyzed.

Table 3. Contractile Activity of NMU-8 Analogs on Isolated Chicken Crop Smooth Muscle Preparations

Peptide No.	RA[a]	Max. contraction[b]	n[c]
1	5.50± 0.81	116± 3	13
2	4.65± 0.97	110± 4	16
3	4.66± 0.64	110± 4	10
4	9.91± 1.53	110± 6	9
5	3.41± 0.59	108± 5	9
6	7.96± 1.47	109± 3	12
7	11.4 ± 3.75	128± 7	7
8	9.81± 2.07	143±13	6
9	18.6 ± 6.86	120± 6	6
10	69.3 ±26.4	126±12	7
11	109 ±37.8	134± 3	6
12	3.20± 0.86	107± 4	5
13	17.9 ± 7.32	129± 9	6
14	31.6 ±14.2	139±15	6
15	81.3 ±24.7	136±10	8
16	11.3 ± 3.43	129± 4	5
17	12.7 ± 1.70	130± 9	7

a) RA was calculated as EC_{50} of p-NMU-8/EC_{50} of each analog. *b*) Maximum contraction was calculated as [(maximal effect of analog/maximal effect of p-NMU-8) × 100]. *c*) Number of experiments.

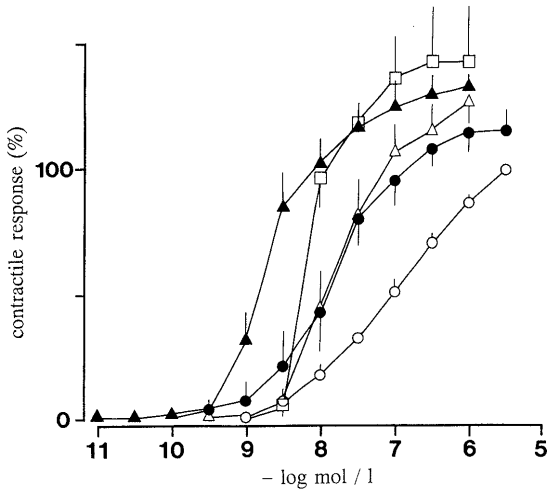

Fig. 3. Concentration–Response Curves of NMU-8 Analogs for the Contraction of Isolated Chicken Crop Smooth Muscle Preparations

●, d-NMU-8; ○, p-NMU-8; △, [Asp[1]]-NMU-8; □, [Ac-Asp[1]]-NMU-8; ▲, Suc-NMU-8 (2—8).

portion, to give [Ac-Asp[1]]-NMU-8 (**8**) and [Ac-Glu[1]]-NMU-8 (**9**), the RA values of which were 9.81 and 18.6, respectively. In this case, the acidity of the carboxylic acid function at the N-terminal portion may favor high potency. Thus, it could be anticipated that the introduction of any acidic function at the N-terminal portion of NMU-8 analogs will produce a potent agonist. Several analogs with a simple acidic function were therefore designed and examined for activity.

The succinylation of p-NMU-8 gave an extremely potent analog, which showed an RA value of 69.3. [Succinyl (Suc)-Tyr[1]]-NMU-8 (**10**) had about seven times higher potency than [Asp[1]]-NMU-8 (**7**). Our study[13] revealed that the side chains of all amino acid residues at positions 2—8 of p-NMU-8 are of importance for the expression of the contractile activity on isolated chicken crop smooth muscle. Further, the elimination of the N^{α}-amino group of [Asp[1]]-NMU-8 (**7**) is expected to give a more potent analog than the parent peptide. Therefore, the Tyr residue deletion analogs of [Suc-Tyr[1]]-NMU-8 (**10**), [des-N^{α}-amino-Asp[1]]-NMU-8, was examined. Suc-NMU-8 (2—8) (**11**) exerted extremely high activity with the RA value of 109. Four other analogs that contain the carboxylic acid function at the N-terminal portion of NMU-8 (2—8) were tested for contractile activity. The potencies of *o*-phthalyl (Pht)-NMU-8 (2—8) (**16**), malonyl (Mlo)-NMU-8 (2—8) (**13**), maleyl (Mle)-NMU-8 (2—8) (**14**) and glutaryl (Glt)-NMU-8 (2—8) (**15**) were at least ten times that of p-NMU-8 and all of them were highly potent agonists. The RA values of Pht-NMU-8 (2—8) (**16**), Mlo-NMU-8 (2—8) (**13**), Mle-NMU-8 (2—8) (**14**) and Glt-NMU-8 (2—8) (**15**) were 11.3, 17.9, 31.6 and 81.3, respectively. The removal of the methylene group of the succinyl function at Suc-NMU-8 (2—8) (**11**) brought about a drastic decrease of the activity, whereas extension of the methylene group did not cause significant depression of the potency. The replacement of the carboxylic acid function of [Asp[1]]-NMU-8 (**7**) by a sulfonic acid group did not bring about a marked increase of the activity. [3-Sulfoalanyl (Sal)[1]]-NMU-8 (**17**) showed an RA value of 12.7.

The present results have revealed that the carboxylic acid function at the N-terminal of NMU-8 analogs and the decrease of the basicity at the N-terminal N^{α}-amino group makes major contributions to the activity. The acid group at the N-terminal may serve to maintain a preferable structure with high affinity for the NMU receptor in chicken crop.

Experimental

General Synthesis of NMU-related peptide amides (Fig. 1, 2) was carried out on a Beckman system 990C peptide synthesizer (Beckman Instruments Ltd., U.S.A.). Semi-preparative reversed-phase (RP)-HPLC was performed on an apparatus equipped with a 590 pump (Waters, U.S.A.), an U6K injector (Waters), a S310 model II UV detector (Soma, Japan) and a 561-3003 recorder (Hitachi, Japan) connected to a column of μ-Bondasphere C_{18} (19 × 150 mm) (Waters) or YMC-pack D-ODS-5-A (20 × 250 mm) (YMC Co., Japan). Analytical RP-HPLC was performed on a system comprising two 510 pumps (Waters), an U6K injector (Waters), a model 680 gradient controller (Waters), a 730 data module (Waters) and a UV-8011 UV detector (Tosoh Co., Japan) using a Puresil 5 μ C_{18} column (4.6 × 250 mm) (Waters). Gel chromatography was performed using a Toyopearl HW-40 (super fine) column. Amino acid analysis of the acid hydrolysate was performed on a 7300 amino acid analyzer (Beckman). HF cleavage reactions were carried out in a Teflon HF apparatus (Peptide Institute Inc., Japan). Fast-atom bombardment mass spectra (FAB-MS) were obtained on a JMS-DX300 mass spectrometer (JEOL Ltd., Japan). Optical rotations of peptides were measured with a DIP-370 digital polarimeter (Nippon Bunko Co., Ltd., Japan). HP-TLC was performed on precoated silica gel plates (Kieselgel 60, E. Merck, Germany). The contractile responses of smooth muscle preparations were recorded using a TD-111T isotonic transducer (Nihon Koden, Japan) with a 561-3003 recorder (Hitachi).

Reagents Unless otherwise stated, all reagents and solvents were obtained as reagent-grade products from Watanabe Chem. Ind., Ltd. or Wako Pure Chem. Ind., Ltd., Japan, and used without further purification. Boc-protected amino acids and benzhydrylamine (BHA) resin HCl (1% divinylbenzene copolymer, 100—200 mesh, amino content; 0.6 meq/g) were purchased from Peptide Institute Inc., Japan.

Peptide Synthesis Peptides were prepared by a standard solid-phase method.[16] All amino acids were protected as α-Boc derivatives. The protecting groups for the amino acid side chains were *p*-tosyl for the guanidino group of Arg, 2,6-dichlorobenzyl for the hydroxyl group of Tyr, benzyl for the γ-carboxyl group of Glu, cyclohexyl for the β-carboxyl group of Asp and Boc for the carboxamide[17] of homoglutamine (Hgn).

Solid-phase peptide synthesis was performed starting from BHA resin HCl. The resin was neutralized with a solution of 10% TEA in DCM, and then washed with DCM (4 times) and DMF (2 times). The first amino acid, Boc–Asn–OH, was anchored on the BHA resin by the DCC coupling method in the presence of HOBt using 0.4 mmol eq of Boc-amino acid per resin nitrogen. After coupling, unreacted amino groups were capped twice by reaction of the resin with acetic anhydride (4 mmol eq)/pyridine (4 mmol eq) in DMF for 20 min. The elongation of the peptide chain was carried out using Boc-amino acids (2 eq) in *N*-methylpyrrolidone with benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP)[18] (2 eq) and *N*-methylmorpholine (4 eq) in dimethylacetamide for the first and third coupling reactions, and DCC (2.5 eq)–HOBt (2.5 eq) for second coupling in DCM–DMF. Each coupling reaction was run for 1.5 h at room temperature. Incorporation of an amino acid was repeated until the Kaiser ninhydrin test[19] became negative. The deprotection of the Boc group during the peptide chain elongation was executed with 33% TFA in DCM for 30 min. The N-terminal acyl group was introduced as follows: acetyl, succinyl, *o*-phthalyl, maleyl and glutaryl groups were introduced with the anhydride (10 eq) and pyridine (10 eq) (3 times) in DMF, and the other acyl groups, with the acid (3 eq), BOP (2 eq) and *N*-methylmorpholine (4 eq) in DMF. Malonic acid was introduced as its mono-*tert*-butyl ester. After the completion of the peptide chain elongation, the peptide-resin was treated with 33% TFA/DCM, washed with ethanol and dried. Peptides were deblocked and cleaved from the resin with anhydrous liquid HF containing 10% anisole. The reaction mixture was kept at -20°C for 30 min and then at -10°C for 60 min. After evaporation of HF *in vacuo* with ice-cooling, the residual mixture was washed with ether prior to extraction of the crude peptide with 12—50% AcOH. The combined extracts were lyophilized. N^{α},N^{ca}-Di-*tert*-butyloxycarbonylhomoglutamine [Boc–Hgn(Boc)–OH][20] was incorporated in the peptide chain instead of pyrohomoglutamic acid (pHgu) for the synthesis of pHgu-peptide. Then, the Hgn residue was cyclized to produce the pHgu moiety.[20] The crude peptide was revealed as a main peak on analytical RP-HPLC.

Peptide Purification The synthetic peptides were highly purified by semi-preparative RP-HPLC on a column (19 × 150 mm) of μ-Bondasphere C_{18} 5 μ—100 Å with 0.1% TFA–MeCN in an isocratic system, followed by gel filtration on a column (1.5 × 47 cm) of Toyopearl HW-40 (super fine) with 25% MeCN/5 mM HCl as an eluent.

Peptide Characterization Homogeneity of the purified peptides was ascertained by analytical RP-HPLC, HP-TLC with two solvent systems, amino acid analysis of acid hydrolysates and/or FAB-MS. HPLC analysis of the purified peptides was carried out using a Puresil 5 μ C_{18} column (4.6 × 250 mm) (Waters) with a linear gradient of 16—40% MeCN over a period of 20 min in 20 mM sodium phosphate buffer (pH 3.0) (flow rate, 1 ml/min; UV detection, 210 nm). For amino acid composition analysis, peptides were hydrolyzed with 6 N HCl vapor. A peptide (50—100 μg) was taken in a test tube (6 × 50 mm) and placed in a vial (40 ml), the bottom of which contained 6 N HCl with 3% phenol (0.5 ml). The vial was evacuated under cooling, then closed with a stopper and kept in a block heater at 130°C for 3 h. The results of amino acid analysis of the acid hydrolysates are shown in Table 2.

Optical rotations of peptides were measured with a 3 × 50 mm cell. A peptide was dissolved in 12% AcOH at a concentration of 0.50%. Values shown in Table 1 were obtained by calculation from the means of 3 successive 30 s integrations. The *Rf* values in HP-TLC refer to the following solvent systems: Rf^1, *n*-BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24) and Rf^2, *n*-BuOH–AcOEt–AcOH–H_2O (1 : 1 : 1 : 1). These data are shown in Table 1.

When single peaks and single spots were observed for a peptide in all chromatographic systems and the amino acid composition (Table 2) was consistent with the calculated values, the peptide was subjected to bioassay. The characteristics of the synthetic peptides are shown in Table 1.

Bioassay The bioasssay methods used for this study were essentially the same as those previously described.[10] The synthetic peptides were tested for contractile activity on isolated chicken crop smooth muscle preparations. The activity of p-NMU-8 was taken as a reference. A chicken (age, 8—14 d) was killed after having been starved for 12 h, the crop was removed rapidly, and vertical strips were cut (about 3 × 20 mm). The tissue was mounted longitudinally at a resting tension of 0.5 g in an organ bath (10 ml) containing Tyrode's solution of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.4, $NaHCO_3$ 12, glucose 5.6. This was kept at 30 °C and bubbled with a mixture of 95% O_2 and 5% CO_2. The tissue preparation was equilibrated for 60 min, challenged twice with carbachol (2 μM) and once with p-NMU-8, and then re-equilibrated for 30—40 min prior to estimation of the contractile activity of each peptide in comparison with that of p-NMU-8. Peptides were added cumulatively and the contractile responses of the tissue preparation were recorded. The pharmacological parameter, relative activity (RA; EC_{50} of p-NMU-8/EC_{50} of each peptide) and maximum contraction (Table 3), was calculated from the concentration-response curves.

References and Notes

1) Part I: Sakura N., Kurosawa K., Hashimoto T., *Chem. Pharm. Bull.*, **43**, 1148 (1995).
2) Amino acids and their derivatives except glycine mentioned in this paper are of L-configuraion unless otherwise indicated. The abbreviations for amino acids and peptides are in accordance with the rules of the IUPAC-IBU Commission on Biochemical Nomenclature in *Eur. J. Biochem.*, **138**, 9 (1984). Other abbreviations used are: BHA, benzhydrylamine; Boc, *tert*- butoxycarbonyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCM, dichloromethane; HOBt, 1-hydroxybenzotriazole; DMF, *N*,*N*-dimethylformamide; TEA, triethylamine; BOP, benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate; MeCN, acetonitrile; AcOH, acetic acid; TFA, trifluoroacetic acid; BuOH, butyl alcohol; AcOEt, ethyl acetate; RP-HPLC, reversed-phase high-performance liquid chromatography; HP-TLC, high-performance thin-layer chromatography.
3) Minamino N., Kangawa K., Matsuo H., *Biochem. Biophys. Res. Commun.*, **130**, 1078 (1985).
4) O'Harte F., Bockman C. S., Abel P. W., Conlon J. M., *Peptides*, **12**, 11 (1991).
5) O'Harte F., Bockman C. S., Zeng W., Abel P. W., Harvey S., Conlon J. M., *Peptides*, **12**, 809 (1991): Domin J., Benito-Orfila M. A., Nandha K. A., Aitken A., Bloom S. R., *Regul. Pept.*, **41**, 1 (1992).
6) Conlon J. M., Domin J., Thim L., DiMarzo V., Morris H. R., Bloom S. R., *J. Neurochem.*, **51**, 988 (1988).
7) Minamino N., Kangawa K., Honzawa M., Matsuo H., *Biochem. Biophys. Res. Commun.*, **156**, 355 (1988).
8) Kage R., O'Harte F., Thim L., Conlon J. M., *Regul. Pept.*, **33**, 191 (1991).
9) Murphy R., Turner C. A., Furness J. B., Parker L., Giraud A., *Peptides*, **11**, 613 (1990).
10) Sakura N., Kurosawa K., Naminohira S., Sakai T., Hashimoto T., "Peptide Chemistry 1992," ed. by Yanaihara N., ESCOM Science Publishers B. V., Leiden, 1993, p. 406.
11) Sumi S., Inoue K., Kogire M., Doi R., Takaori K., Suzuki T., Yajima H., Tobe T., *Life Sci.*, **41**, 1585 (1987).
12) Brown D. R., Quito F. L., *Eur. J. Pharmacol.*, **155**, 159 (1988).
13) Hashimoto T., Masui H., Uchida Y., Sakura N., Okimura K., *Chem. Pharm. Bull.*, **39**, 2319 (1991).
14) Okimura K., Sakura N., Ohta S., Kurosawa K., Hashimoto T., *Chem. Pharm. Bull.*, **40**, 1500 (1992).
15) A part of this work was reported as a preliminary communication; Kurosawa K., Shibata A., Okimura K., Sakura N., Hashimoto T., "Peptide Chemistry 1991," ed. by Suzuki A., Protein Research Foundation, Osaka, 1992, p. 241. Kurosawa K., Ohki K., Okimura K., Sakura N., Hashimoto T., "Peptide Chemistry 1993," ed. by Okada Y., Protein Research Foundation, Osaka, 1994, p. 237.
16) Stewart J., Young J., "Solid Phase Peptide Synthesis," Pierce Chem. Co., Rockford, 1984.
17) Sakura N., Hirose K., Hashimoto T., *Chem. Pharm. Bull.*, **34**, 3506 (1986).
18) Castro B., Dormoy J. R., Evin G., Selve C., *Tetrahedron Lett.*, **14**, 1219 (1975).
19) Kaiser E., Colescott R. L., Bossinger C. S., Cook P. I., *Anal. Biochem.*, **34**, 595 (1970).
20) Sakura N., Hirose K., Nishijima M., Hashimoto T., Okabe T., Miyamori C., Sato T., *Chem. Pharm. Bull.*, **37**, 3125 (1989).