Discovery of Potent Hexapeptide Agonists to Human Neuromedin U Receptor 1 and Identification of Their Serum Metabolites

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S *Supporting Information*

ABSTRACT: Neuromedin U (NMU) and S (NMS) display various physiological activities, including an anorexigenic effect, and share a common C-terminal heptapeptide-amide sequence that is necessary to activate two NMU receptors (NMUR1 and NMUR2). On the basis of this knowledge, we recently developed hexapeptide agonists **2** and **3**, which are highly selective to human NMUR1 and NMUR2, respectively. However, the agonists are still less potent than the endogenous ligand, hNMU. Therefore, we performed an additional structure—activity relationship study, which led to the identification of the more potent hexapeptide **5d** that exhibits similar NMUR1-agonistic activity as compared to hNMU. Additionally, we studied the stability of synthesized agonists, including **5d**, in rat serum, and identified two major biodegradation sites: Phe^2-Arg^3 and Arg^5-Asn^6. The latter was more predominantly cleaved than the former. Moreover, substitution with 4-fluorophenylalanine, as in **5d**, enhanced the metabolic stability at Phe^2-Arg^3. These results provide important information to guide the development of practical hNMU agonists.

KEYWORDS: *Agonist, biodegradation site, neuromedin U, neuromedin U receptor, peptide*
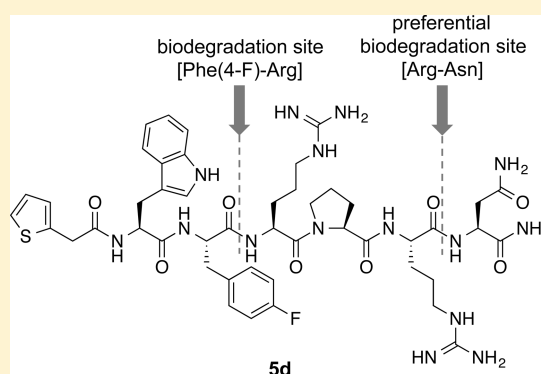
Neuromedin U (NMU)[1] and S (NMS)[2] exhibit various biological behaviors, including appetite suppression,[3,4] reduction in body weight,[5] regulation of energy homeostasis,[6] increase in body temperature,[5] and regulation of glucose homeostasis.[7] Recently, two-types of neuromedin U receptors (NMUR1 and NMUR2) were shown to be involved in the anorexigenic activity of peripherally administrated human NMU (hNMU) conjugated with high molecular weight poly(ethylene)glycol (PEG) or human serum albumin (HSA).[8,9] Accordingly, the pursuit of NMU-based drug development to treat obesity is considered promising. Mammalian NMU and NMS share the C-terminal heptapeptide-amide **1a** (H-Phe^0-Leu^1-Phe^2-Arg^3-Pro^4-Arg^5-Asn^6-NH$_2$, Figure 1A), which activates both receptors.[1,2] In 1995, Hashimoto et al. reported that N-terminal succinylated **1a**, which was synthesized as an aminopeptidase-resistant derivative, showed potent contractile activity toward chicken crop smooth muscle expressing avian NMU receptors.[10] Recently, we performed a structure—activity relationship (SAR) study of heptapeptide **1a** to develop receptor-selective agonists using cultured cells expressing human NMU receptors. As shown in Figure 1A, the results indicated that (1) the α-amino group at the N-terminal Phe[0] residue in **1a** was not important for the agonistic activity because the N-terminal *des*-amino derivative,

1b, had similar activity to **1a**; (2) N-terminal 3-pyridylpropionyl (**1c**) and 3-cyclohexylpropionyl (**1d**) structures were important for selective agonistic activities toward NMUR1 and NMUR2, respectively; (3) bulky aromatic amino acid residues, e.g., Trp and naphthylalanine (Nal), at residue 1 (Leu[1]) were important for both agonistic activity and selectivity toward NMUR1; (4) aliphatic amino acids, e.g., Leu, at residue 2 (Phe[2]), were important for both agonistic activity and selectivity toward NMUR2; and (5) α,β-diaminopropanoic acid (Dap) and α,γ-diaminobutyric acid (Dab) at residue 3 (Arg[3]) led to higher agonistic activity and selectivity toward NMUR2.[11] Furthermore, the structure of the C-terminal three amino acid residues, e.g., Pro[4], Arg[5], and Asn[6]-amide, was critical for the activation of both NMUR1 and NMUR2.[11] These results implied that the N-terminal structure could be modified to develop more potent and selective peptidomimetic agonists. Consequently, SAR studies based on the N-terminal structures of **1c** and **1d** successfully led to the discovery of hexapeptide derivatives **2** and **3** (Figure 1B), which selectively
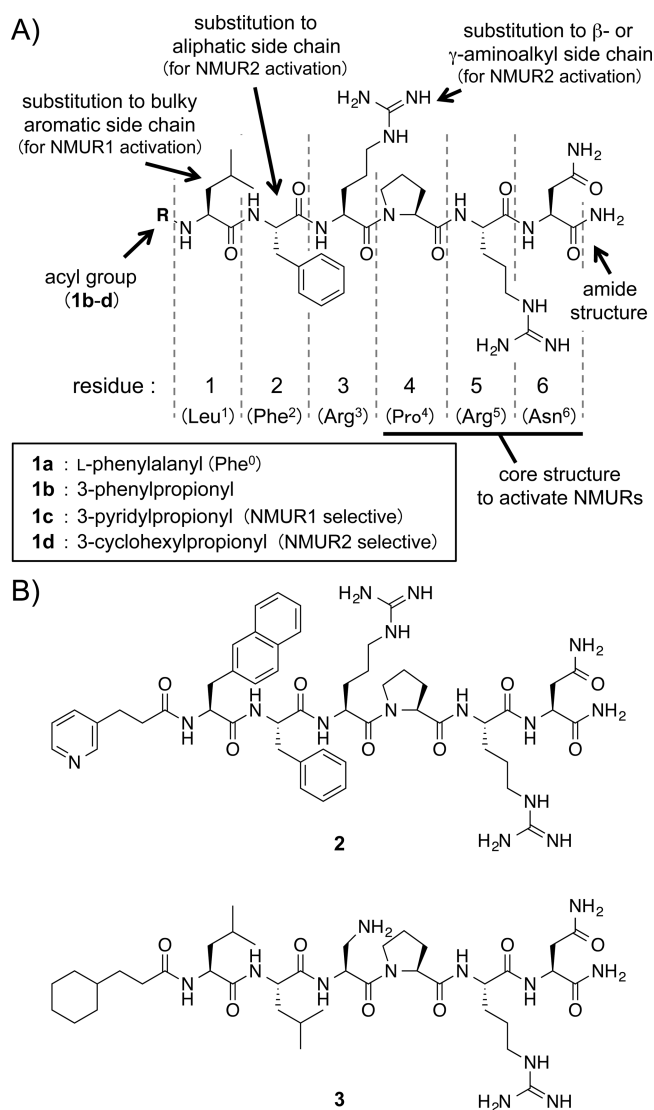
Figure 1. (A) Structures of human neuromedin U-derived peptides **1a**−**d** and the SAR results from our previous study.[11] Arrows indicate the structural entities required for potent biological activity of the derivatives. (B) Structures of the NMUR1-selective agonist **2** and the NMUR2-selective agonist **3**.[11]

important information to guide the development of a more metabolically stable peptidic hNMU agonist.

All peptide derivatives were synthesized by 9-fluorenylme-thoxycarbonyl (Fmoc)-based solid-phase peptide synthesis as reported previously.[11] Constructed protected peptide resins were treated with trifluoroacetic acid (TFA)-*m*-cresol-thioani-sole-1,2-ethanedithiol (EDT) (4.0 mL, 40:1:1:1) for 150 min at room temperature, and the resultant crude peptides were purified by preparative reversed-phase (RP)-HPLC. All peptide derivatives were characterized by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) and key peptides were analyzed by NMR (see the Supporting Information). Each peptide had a purity of >95% as assessed by RP-HPLC analysis at 220 or 230 nm and was solubilized as a 20 mM stock solution in DMSO for subsequent bioassays. The agonistic activities of peptide derivatives for human NMUR1 and NMUR2 stably expressed in Chinese hamster ovary (CHO) cells were evaluated by calcium mobilization assays (see the Supporting Information). The results for each peptide are presented as a relative value (%) compared to the activity of hNMU at a concentration of 1000 nM.

In the present study, to decrease the interexperimental deviation in the screening of potent agonists toward human NMUR1 and NMUR2, we adopted a system in which these receptors were stably expressed in CHO cells. In our previous study employing a transient receptor expression system, one of the potent derivatives, **4a** (Figure 2A, derivative **7a** in ref 11), which was derivatized from **1c** by substituting the Leu[1] residue with a Phe, displayed higher selectivity to NMUR1.[11] This result was reproduced in the stable receptor expression system (Figure 2B). Hence, we focused on derivative **4a** and began by modifying its N-terminal 3-pyridylpropionyl group. When we introduced 3-(2-thienyl)propionyl and 2-thienylacetyl groups (Figure 2A), the corresponding derivatives, **4b** and **4c**, respectively, exhibited more potent agonistic activities toward NMUR1 and NMUR2 as compared to **4a** (Figure 2B). However, the derivatives with other acyl groups, such as 3-phenylpropionyl (**S1a**), phenylacetyl (**S1b**), and acetyl (**S1c**), showed decreased activities (Figure S1, Supporting Information). These results suggested that the N-terminal thiophene ring played an important role for the activation of human NMU receptors. Therefore, we chose derivative **4c** with a lower molecular weight for further modifications.

Next, we focused on residue 1 (Phe) of a **4a**-type agonist. In the previous study, introducing the bulky aromatic 2-naphtylalanine (2-Nal) at residue 1 of derivative **2** improved both agonistic activity and selectivity toward NMUR1 as compared to derivative **1c** (Figure 1A), which had Leu at that position.[11] To further increase the agonistic activity, we synthesized three **4c** derivatives, **4d** through **4f**, bearing Trp (**4d**), 1-Nal (**4e**), and 2-Nal (**4f**) with a side chain similar or identical to residue 1 in derivative **2** (Figure 2A). As shown in Figure 2B, the Trp-bearing derivative, **4d**, exhibited the highest agonistic activities to both receptors among this series of derivatives, including the original hexapeptide lead, **1b**, and the NMUR1-selective agonist, **2**. In particular, the agonistic activity of **4d** to NMUR1 was very similar to that of hNMU.

Next, we modified residue 2 (Phe[2]) based on the structure of derivative **4d**. Kurosawa et al. reported that substituting Phe with Tyr typically increased the contractile activity of avian smooth muscle.[12] However, in our previous evaluation of human NMU receptors, substitution with three kinds of aromatic amino acids, including Tyr, phenylglycine, and

activate NMUR1 and NMUR2, respectively.[11] Particularly, the agonistic activity of hexapeptide **3** was amazingly selective to NMUR2, with no NMUR1 activation detected below 10^{-6} M; however, the agonistic activity of hexapeptide **3** was 3-fold less active than hNMU. However, the NMUR1 selectivity and potency of hexapeptide derivative **2** still had room for improvement, with an NMUR1-agonistic activity of about 10-fold less than that of hNMU. Therefore, in the present study, in order to develop more potent agonists, we performed additional SAR work and found agonist **5d**, with potent activity and similar NMUR1-agonistic activity to hNMU. Moreover, we performed stability studies in rat and human serum to assess the biodegradation of the synthesized agonists and found that they were cleaved at two positions: Phe[2]-Arg[3] and Arg[5]-Asn[6]. The amide bond of the Arg[5]-Asn[6] site was more preferentially cleaved. In addition, a substitution of Phe[2] with the 4-fluorophenylalanine [Phe(4-F)[2]] in **5d** enhanced metabolic stability at the Phe[2]-Arg[3] site. These results will provide

A)

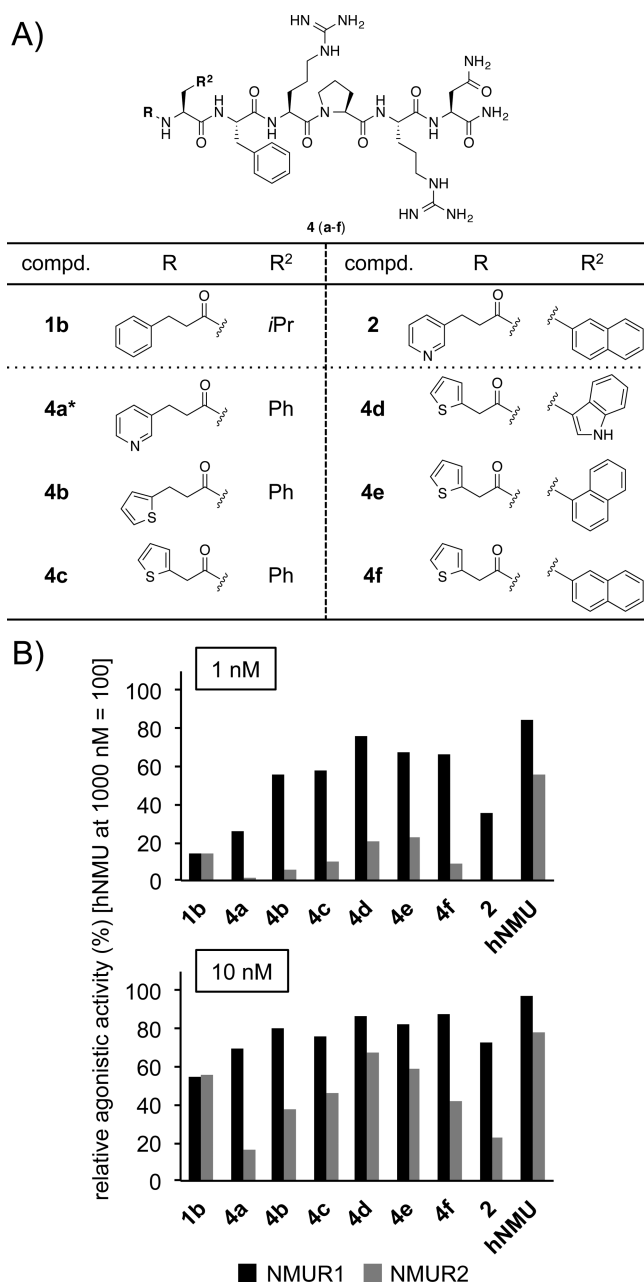| compd.	R	R^2	compd.	R	R^2
1b	(3-phenylpropanoyl)	iPr	2	(3-pyridylpropanoyl)	(2-naphthyl)
4a*	(3-pyridylpropanoyl)	Ph	4d	(2-thienylacetyl)	(3-indolyl)
4b	(3-thienylpropanoyl)	Ph	4e	(2-thienylacetyl)	(1-naphthyl)
4c	(2-thienylacetyl)	Ph	4f	(2-thienylacetyl)	(2-naphthyl)

B)

Figure 2. (A) Structures of **4a–f**. The asterisk indicates that **4a** was previously reported.[11] (B) Effect of substitutions at the N-terminus (residue 0) and residue 1 on the agonistic activity in CHO cells toward stably expressed human NMUR1 (black bar) and NMUR2 (gray bar) as determined by the calcium mobilization assay. Peptide concentrations, 1 and 10 nM; positive control, hNMU (activity at 1000 nM = 100%).

homoPhe, at residue 2 decreased the agonistic activity.[11] Moreover, substitution with cyclohexylalanine (an aliphatic amino acid) increased selectivity for NMUR2 rather than NMUR1.[11] Therefore, derivatives **5a–d**, bearing a series of β-aromatic amino acids at residue 2, i.e., 2-thienylalanine (**5a**), His (**5b**), 4-pyridylalanine (**5c**), and Phe(4-F) (**5d**), were synthesized (Figure 3A). The agonistic activity of derivative **5a** toward NMUR1 slightly decreased by comparison with **4d**, whereas its activity toward NMUR2 was nearly maintained (Figure 3B). Derivatives **5b** and **5c**, bearing nitrogen-heteroaromatic amino acids at residue 2, showed lower
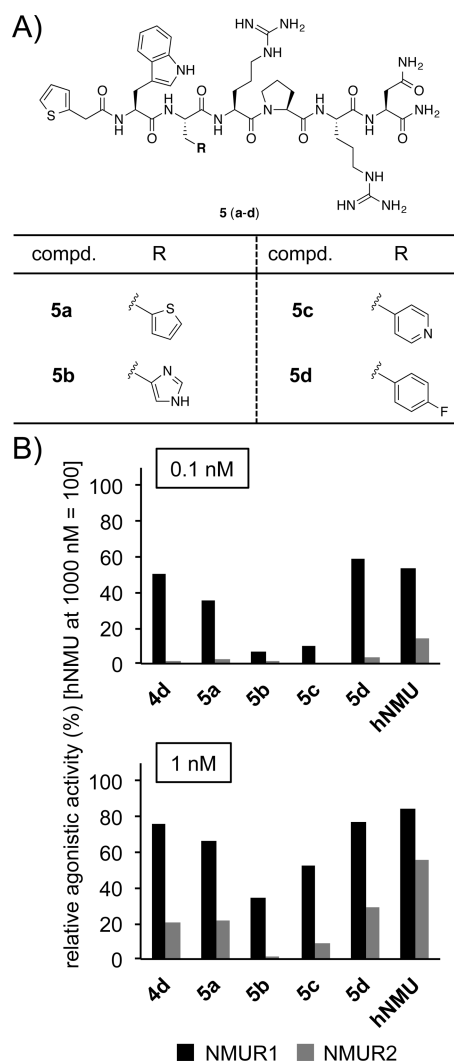
A)

compd.	R	compd.	R
5a	(2-thienyl)	5c	(4-pyridyl)
5b	(imidazolyl)	5d	(4-fluorophenyl)

B)

Figure 3. (A) Structures of **5a–d**. (B) Effect of substitutions at residue 2 on the agonistic activity in CHO cells toward stably expressed human NMUR1 (black bar) and NMUR2 (gray bar) as determined by the calcium mobilization assay. Peptide concentrations, 0.1 and 1 nM; positive control, hNMU (activity at 1000 nM = 100%).

agonistic activities toward both receptors as compared to **4d** (Figure 3B). Interestingly, introduction of a fluorine atom (**5d**) to the 4-position of Phe^2 in **4d** slightly increased the agonistic activities toward NMUR1 and NMUR2. Specifically, the activity toward NMUR1 was slightly higher as compared to hNMU at 0.1 nM (Figure 3B).

Then, to precisely compare the agonistic activities of derivative **5d** and hNMU, we evaluated their dose-dependencies and calculated EC$_{50}$ values and intrinsic activities (i.a.) toward both human NMU receptors (Figure 4). The EC$_{50}$ values of derivative **5d** toward NMUR1 and NMUR2 were 0.083 ± 0.01 nM (i.a. = 95%) and 2.6 ± 0.3 nM (i.a. = 82%), respectively. This represented a ~1.7-fold increase and a ~2.8-fold decrease in potency as compared to hNMU, which gave values of 0.14 ± 0.02 and 0.92 ± 0.2 nM, respectively. These results indicated that derivative **5d** was the most potent hexapeptide agonist to activate human NMUR1 to date. In addition, the observed relatively potent agonistic activity of hexapeptide **5d** to NMUR2 would also be an important feature for the development of practical antiobesity drugs, which may require the engagement of both human NMU receptors to
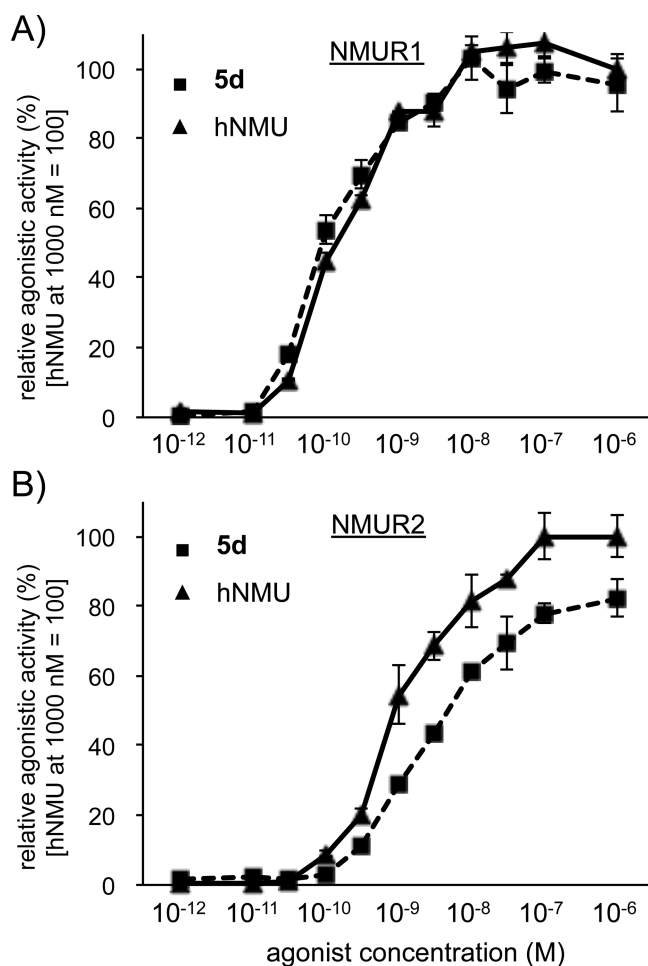
Figure 4. *In vitro* agonistic activity in CHO cells of hexapeptide derivative **5d** toward stably expressed human NMUR1 (A) and NMUR2 (B) as determined by the calcium mobilization assay. Peptide concentrations, $10^{-12}-10^{-6}$ M; reference compound, hNMU. Data were determined in triplicate.
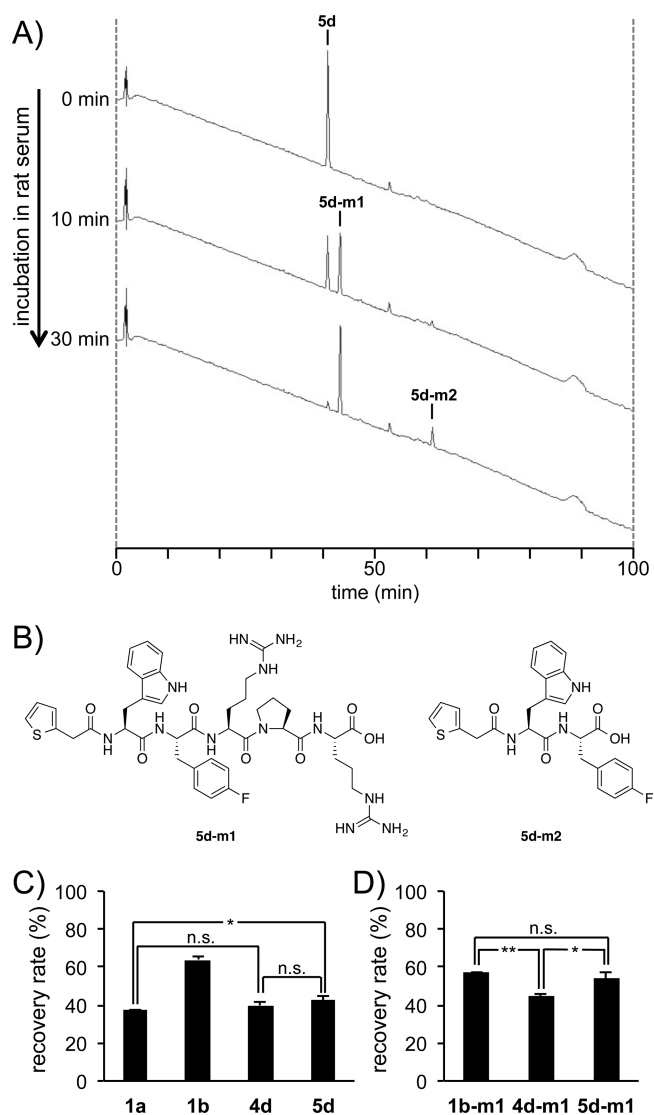

Figure 5. (A) Analytical RP-HPLC chromatograms showing the time-dependent metabolic degradation of **5d** in 25% rat serum. An aliquot of each sample extracted by Sep-Pak C18 Plus cartriges was analyzed using a C18 reverse-phase column detected by UV at 220 nm. (B) The structures of the two major metabolites, **5d-m1** and **5d-m2**. (C) Recovery rates of intact peptides **1a**, **1b**, **4d**, and **5d** after incubation for 10 min in 25% rat serum. (D) Recovery rates of intact peptides, **1b-m1**, **4d-m1**, and **5d-m1** after incubation for 60 min in 25% rat serum. Data were determined in triplicate. n.s., not significant; *$p <$ 0.05 and **$p <$ 0.005.

induce full anorexigenic activity from a peripheral administration of PEG- or HSA-conjugated hNMU.[8,9]

Next, we examined the *in vitro* stability of derivative **5d** in rat serum to understand its metabolic degradation profile. As comparators, we also examined the derivatives **1a**, **1b**, and **4d**. These peptides were incubated in 25% rat serum at 37 °C,[13,14] and metabolites at different time points were identified by RP-HPLC after extraction with a Sep-Pak C18 Plus cartridge (see the Supporting Information). RP-HPLC chromatograms showing a representative **5d** stability time course are shown in Figure 5A. After 10 min, a new peak appeared at a retention time of 43.7 min (**5d-m1**); after 30 min, another new peak, with a retention time of 61.2 min, was observed (**5d-m2**). We successfully determined the structures of these two metabolites by mass spectrometric analysis [**5d-m1** m/z 903.4091 (M + H)$^+$ (calcd for $C_{43}H_{56}FN_{12}O_7S$ 903.4099); and **5d-m2** m/z 494.1552 (M + H)$^+$ (calcd for $C_{26}H_{25}FN_3O_4S$ 494.1550); Figure 5B]. The results suggested that in rat serum the amide bond of derivative **5d** seemed to be preferentially cleaved at the C-terminal Arg^5-Asn^6 site and subsequently at the Phe^2-Arg^3 site. A similar metabolic profile was observed for derivatives **1b** and **4d** (Figure S2, Supporting Information). Their respective metabolites were **1b-m1** (3-phenylpropionyl-Leu^1-Phe^2-Arg^3-Pro^4-Arg^5-OH), **1b-m2** (3-phenylpropionyl-Leu^1-Phe^2-OH),

4d-m1 (2-thienylacetyl-Trp^1-Phe^2-Arg^3-Pro^4-Arg^5-OH), and **4d-m2** (2-thienylacetyl-Trp^1-Phe^2-OH) (Figure S2, Supporting Information). No metabolites of **1a** were detected probably due to sequential degradation from the N-terminal-free amino acid residue by aminopeptidase. Additionally, we noted that a cleavage at the C-terminal Arg-Asn site was observed in hNMU under the same conditions (data not shown). Moreover, we examined the stability of derivative **5d** in human serum and observed a similar degradation pattern as that shown in rat serum (Figure S3, Supporting Information).

To better understand the metabolism, the recovery rates (%) of intact peptide derivatives **1a**, **1b**, **4d**, and **5d** at 10 min were determined (Figure 5C). At first, the N-terminal *des*-amino hexapeptide derivative, **1b**, which was designed to resist

aminopeptidase degradation,[11] showed improved serum stability (64.0 ± 1.5% recovery) as compared to the free N-terminal heptapeptide derivative, **1a** (37.0 ± 0.7% recovery). Contrary to our expectations, however, the recovery rate of the N-terminal acylated derivative, **4d**, like **1b**, was low (39.8 ± 2.1%) and similar to that of heptapeptide **1a**. However, the recovery rate of **5d** was slightly better (42.7 ± 1.9%) as compared to **1a**. These results suggested that the effect of the fluorine atom toward the metabolic stabilization of **4d** was not observed due to probable rapid degradation at the Arg^5-Asn^6 site.

Thus, to investigate the effect of the $Phe(4\text{-}F)^2$-Arg^3 bond on metabolic stability, three metabolites, **1b-m1**, **4d-m1**, and **5d-m1**, which did not include the metabolically unstable Arg^5-Asn^6 bond, were chemically synthesized and evaluated. As shown in Figure 5D, the recovery rate of **5d-m1** at 60 min (54.0 ± 2.9%) was significantly improved as compared to **4d-m1** (44.7 ± 0.6%) and was similar to **1b-m1** (56.9 ± 0.4%). These results suggested that (1) the cleavage rate of the Phe^2-Arg^3 bond was slower than that of the Arg^5-Asn^6 bond; (2) substitution with $Phe(4\text{-}F)$ at residue 2 in **4d** enhanced the stability of the original Phe^2-Arg^3 bond; and (3) the structural changes in the three N-terminal moieties (acyl group, residues 1 and 2) from **1b** to **5d** promoted the cleavage rate of the Arg^5-Asn^6 bond because **5d** was more rapidly degraded than **1b** in spite of similar recovery rates between **1b-m1** and **5d-m1**.

In conclusion, this study clarified that (1) the novel hexapeptide derivative, **5d** (designated CPN-170; CPN, C-terminal core peptide derivative of neuromedin U) displayed potent agonistic activity toward human NMU receptors, especially NMUR1; (2) our hexapeptide derivatives were hydrolyzed at two major cleavage sites (the Arg^5-Asn^6 and Phe^2-Arg^3 bonds) in rat serum; (3) the Arg^5-Asn^6 bond was preferentially cleaved as compared to the Phe^2-Arg^3 bond; (4) introduction of a fluorine atom at the 4-position of Phe in **4d** significantly stabilized the Phe^2-Arg^3 bond; and (5) N-terminal structural modifications at the acyl group, residues 1 and 2 influenced not only the agonistic activity but also the metabolic stability of both cleavage sites. In practical terms, stabilization of the Arg^5-Asn^6 bond will be necessary to exert a long-lasting anorexigenic effect *in vivo*. Furthermore, to achieve innovative translational research based on NMUR1, the discovery of more potent and selective agonists beyond derivative **2** will be expected from future SAR studies based on hexapeptide derivative **5d**.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials, experimental procedure, analytical data for all peptide derivatives, analytical HPLC chromatograms, NMR spectra, and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

α-MEM, Alfa Modified Eagle Minimum Essential Medium; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CPN, C-terminal core peptide derivative of neuromedin U; Dab, α,γ-diaminobutyric acid; Dap, α,β-diaminopropanoic acid; DMSO, dimethyl sulfoxide; DIPCI, diisopropylcarbodiimide; DMF, dimethylformamide; EDT, 1,2-ethanedithiol; ESI, electrospray ionization; FCS, fetal calf serum; Fmoc, 9-fluorenylmethoxycarbonyl; HBSS, Hank's Balanced Salt Solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOBt, 1-hydroxybenzotriazole; HSA, human serum albumin; i.a., intrinsic activity; Nal, naphtylalanine; NMS, neuromedin S; NMR, nuclear magnetic resonance; NMU, neuromedin U; NMUR1, neuromedin U receptor type 1; NMUR2, neuromedin U receptor type 2; Phe(4-F), 4-fluorophenylalanine; RP-HPLC, reversed phase high-performance liquid chromatography; SAR, structure−activity relationship; TFA, trifluoroacetic acid; TMS, tetramethylsilane; TOF MS, time-of-flight mass spectrometry

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