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Rational Design of a Low Molecular Weight, Stable, Potent, and Long-Lasting GPR103 Aza-β^3-pseudopeptide Agonist

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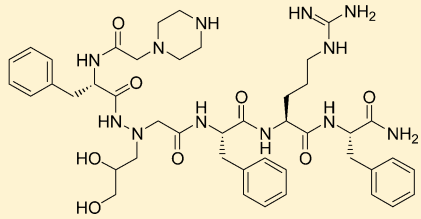
Supporting Information

ABSTRACT: 26RFa, a novel RFamide neuropeptide, is the endogenous ligand of the former orphan receptor GPR103. Intracerebroventricular injection of 26RFa and its C-terminal heptapeptide, 26RFa$_{(20-26)}$, stimulates food intake in rodents. To develop potent, stable ligands of GPR103 with low molecular weight, we have designed a series of aza-β^3-containing 26RFa$_{(20-26)}$ analogues for their propensity to establish intramolecular hydrogen bonds, and we have evaluated their ability to increase $[Ca^{2+}]_i$ in GPR103-transfected cells. We have identified a compound, [Cmpi^{21},aza-β^3-Hht^{23}]26RFa$_{(21-26)}$, which was 8-fold more potent than 26RFa$_{(20-26)}$ in mobilizing $[Ca^{2+}]_i$. This pseudopeptide was more stable in serum than 26RFa$_{(20-26)}$ and exerted a longer lasting orexigenic effect in mice. This study constitutes an important step toward the development of 26RFa analogues that could prove useful for the treatment of feeding disorders.

[Cmpi^{21},aza-β^3-Hht^{23}]26RFa$_{(21-26)}$



INTRODUCTION

A number of neuropeptides exhibiting the Arg-Phe-NH$_2$ motif at their C-terminal extremity, collectively termed RFamide peptides (RFRPs), have been identified in invertebrates.[1] In contrast, only a few RFRPs have been characterized so far in mammals, i.e., neuropeptides FF and AF,[2] prolactin-releasing peptide (PrRP),[3] the RFamide-related peptides RFRP-1 and RFRP-3,[4] and metastin and the derived peptides kisspeptins.[5,6] We have previously isolated from the frog brain a novel 26-amino acid RFamide peptide, designated 26RFa, which exhibits no meaningful similarity with any member of the RFRP superfamily in vertebrates.[1,7] The cDNA encoding the 26RFa precursor has been characterized in human,[7,8] bovine,[8] mouse,[8,9] rat,[7,8] chicken,[10] and quail.[10] The 26RFa peptide and its N-terminally extended form (43RFa/QRFP) have been isolated from the human hypothalamus and spinal cord,[11] and the 26RFa and 43RFa orthologues have been characterized from the quail diencephalon and the rat brain, respectively.[10,12] The search for a receptor target for 26RFa has led to the pairing of this neuropeptide with the formerly orphan G protein-coupled receptor GPR103.[8,9] In the brain, GPR103 mRNA is primarily found in the cerebral cortex, thalamus, hypothalamus, and vestibular nuclei.[9] At the periphery, GPR103 mRNA is expressed in the retina, pituitary, heart, kidney, adrenal gland, testis, and bones.[9] The 26RFa precursor and GPR103 genes are both expressed in neurons located in hypothalamic nuclei involved in the control of feeding behavior notably in the arcuate nucleus, the ventromedial hypothalamic nucleus, and the lateral hypothalamic area.[7,8,11−14] Consistent with this pattern of expression, it has been shown that intracerebroventricular (icv) administration of 26RFa or 43RFa induces a dose-dependent increase in food intake in rodents[7,12,14−17] and that chronic infusion of 43RFa causes obesity in mice.[16] It has also been shown that 26RFa stimulates basal and GnRH-induced luteinizing hormone (LH) release,[18,19] and aldosterone secretion.[8] Interestingly, the orexigenic and the hypophysiotropic effects of 26RFa are mimicked by the C-terminal fragment 26RFa$_{(20-26)}$ (GGFSFRF-NH$_2$), whose sequence is highly conserved from fish to mammals.[15,18,20] In addition,

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26RFa inhibits glucose-induced insulin secretion but does not affect glucagon release.[21] Finally, 26RFa has been recently implicated in bone mineralization,[22,23] in the control of blood pressure,[12,24] and in analgesia.[25−27] All these functions suggest that GPR103 is a suitable target for the development of novel therapeutic agents that could be used, for instance, in the treatment of feeding disorders or osteoporosis.

In vitro, 26RFa increases the intracellular calcium concentration ($[Ca^{2+}]_i$) in GPR103-transfected cells with an EC_{50} in the nanomolar range, while the heptapeptide $26RFa_{(20-26)}$ is about 75-fold less potent.[28] In aqueous solutions, 26RFa adopts a well-defined conformation consisting of an α-helix structure (Pro^4 to Arg^{17}) flanked by two N- and C-terminal disordered regions.[29] However, the C-terminal tail of 26RFa and $26RFa_{(20-26)}$ itself may adopt a local structure stabilized by intramolecular hydrogen bonds for a suitable receptor targeting as often found for regulatory peptides.[30] Introduction of turn mimetics has become a common strategy in pharmacochemistry to induce the conformation of a biologically active peptide and to develop more potent and more stable ligands.[31,32] For instance, incorporation of an aza-β^3-amino acid (Figure 1A),

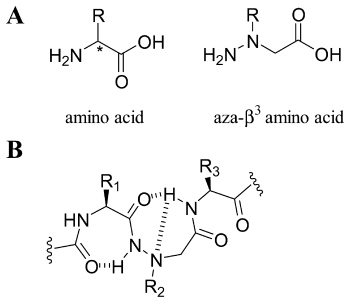

Figure 1. Structure and hydrogen bonds of aza-β^3-pseudopeptides: (A) comparison of an amino acid structure with its aza-β^3-amino acid counterpart, (B) hydrogen bond network induced by an aza-β^3 residue. Adapted from ref 58. Copyright 2005 American Chemical Society.

noted $\Psi[CONHNRCH_2]$ according to the Spatola nomenclature,[33] in a peptide sequence induces the formation of several H-bonds, stabilizing a pseudo-seven-membered ring (γ-turn-like) and a pseudo-eight-membered ring (hydrazino turn) (Figure 1B).[34,35] It has been recently shown that the hydrazino turn can also be surrounded by two β-turns depending on its position in the sequence and on the nature of the side chain harbored by the chiral nitrogen atom of the aza-β^3-amino acid.[36] Moreover, introduction of such an amino acid surrogate in a peptide sequence increases the resistance of the pseudopeptide to proteolysis.[35]

The aim of the present study was to design and evaluate the functional activity of $26RFa_{(20-26)}$ pseudopeptide analogues made up of α- and aza-β^3-amino acid units by assessing the highly sensitive calcium-mobilizing response in GPR103-transfected cells. The binding affinity of selected active analogues was measured, the stability in human serum of the most promising compound was assessed in vitro, and its effect on food intake was determined after icv administration in mice.

RESULTS AND DISCUSSION

Conformational Analysis of $26RFa_{(20-26)}$. We have previously shown that 26RFa encompasses an α-helix structure between Pro^4 and Arg^{17} flanked by two N- and C-terminal disordered regions.[29] To study the proclivity of the C-terminal extremity of 26RFa to adopt a secondary structure, far-UV circular dichroism (CD) spectra of $26RFa_{(20-26)}$ were recorded in four different media, i.e., H_2O, H_2O/TFE (1:1), 100% MeOH, and 60 mM DPC (Figure 2). The CD spectra obtained

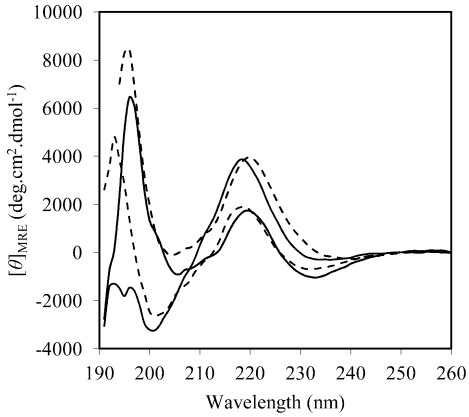

Figure 2. CD spectra of $26RFa_{(20-26)}$ in four different media. CD spectra were recorded in aqueous solution (gray line), in TFE (gray dashed line), in methanol (black dashed line), and in DPC (black line).

in aqueous solution or in H_2O/TFE were characterized by the presence of a negative band around 200 nm and a small positive maximum at 218 nm, indicating a mainly unordered peptide. In MeOH or DPC, the CD spectra exhibited a strong positive band at 196 nm and a second positive band at 220 nm, a pattern consistent with the presence of a turn structure.[37−40] Turns are motifs of secondary structure involved in molecular recognition and often found in neuropeptides and hormones.[31,32,41] They are defined by the number of residues involved in the intramolecular hydrogen bond, i.e., four residues in a β-turn and three residues in a γ-turn. The dihedral angles Φ and Ψ values of these residues distinguish the turn subtype.[41] A possible explanation for the variation of the ellipticity values of the two maxima that were observed in $26RFa_{(20-26)}$ spectra is that the percentages of stabilized turn structures are different in the two media. Although the pattern of the observed spectra is similar to that reported for a type II β-turn by Crisma et al.,[42] one has to be cautious in the assessment of the type of turn present in $26RFa_{(20-26)}$ since the variable nature of turns in polypeptides makes their CD characterization difficult. Moreover, it should be kept in mind that the overall CD spectrum of a peptide represents the combination of the individual spectra of each secondary structure found in the molecule. A short linear heptapeptide such as $26RFa_{(20-26)}$ is expected to adopt in solution a conformation consisting of a mixture of turn and unordered structures. Thus, the pattern of its CD spectrum will depend on the proportion of the different structures. Nevertheless, CD spectroscopy indicated that $26RFa_{(20-26)}$ contains a turn structure in MeOH or DPC. It has been shown that more than 100 peptides bind and activate their receptor by stabilizing β- or γ-turns, including vasopressin, angiotensin II, bradykinin, oxytocin, urotensin II-related peptide, and $cyclo_{1-8}OP$, a potent analogue of the octadecaneuropeptide (ODN).[30,43−48]

Aza-β^3-amino Acid Scan of $26RFa_{(20-26)}$. To favor the formation of intramolecular H-bonds within the $26RFa_{(20-26)}$ sequence, we have synthesized and incorporated the aza-β^3-form of each residue and studied the effect of the obtained

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pseudopeptides on $[Ca^{2+}]_i$ in cultured Gα_{16}-*h*GPR103-transfected CHO cells (Table S1, Supporting Information; compounds LV-2152 to LV-2158; Table 1). The required

Table 1. Effects of Aza-β^3-Substituted 26RFa$_{(20-26)}$ Analogues on Basal $[Ca^{2+}]_i$ and *h*26RFa-Induced $[Ca^{2+}]_i$ Increase in Gα_{16}-*h*GPR103-transfected CHO Cells

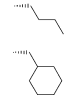
compd	amino acid modified	EC_{50}[a] (nM)	IC_{50}[a] (nM)
LV-2002		10.2 ± 1.1	nd
LV-2021		739 ± 156	nd
LV-2152	Gly^{20}	429 ± 130	nd
LV-2153	Gly^{21}	136 ± 36*	nd
LV-2154	Phe^{22}	2340 ± 1500	nd
LV-2155	$hSer^{23}$	1092 ± 540	nd
LV-2156	Phe^{24}	>10^4	>10^4
LV-2157	Arg^{25}	>10^4	>10^4
LV-2158	Phe^{26}	>10^4	>10^4

[a]Data are the mean ± SEM of at least three distinct experiments performed in triplicate. nd = not determined. An asterisk indicates $p < 0.05$ vs control (LV-2021) as assessed by the Mann−Whitney test.

Fmoc-protected monomers have been synthesized from Fmoc-carbazate as previously described.[49,50] Since it is not possible to prepare aza-β^3-serine, we have decided to use the homoserine (hSer) counterpart. Replacement of the glycine residues at positions 20 (LV-2152) and 21 (LV-2153) enhanced by 2- and 5-fold, respectively, the potency of 26RFa$_{(20-26)}$ on $[Ca^{2+}]_i$. It has been shown that an aza-β^3 residue induces a γ-turn (seven-membered pseudocycle) upstream from the aza-β^3-moiety by establishing an intramolecular hydrogen bond between the NH of the aza-β^3 residue *i* and the C═O of the *i* − 2 residue and a hydrazino turn (eight-membered ring) centered on the aza-β^3-amino acid formed by a bifidic H-bond between the NH of the *i* + 1 residue and the NR of the *i* residue and the C═O of the *i* − 1 residue (Figure 1B).[35] Thus, in both pseudopeptides, a γ-turn cannot be formed. However, the pseudopeptide [aza-β^3-Gly^{21}]26RFa$_{(20-26)}$ (LV-2153) can stabilize a hydrazino turn by two hydrogen bonds (the NH of Phe^{22} with the NR (R = H) of aza-β^3-Gly^{21} and the C═O of Gly^{20}), whereas [aza-β^3-Gly^{20}]26RFa$_{(20-26)}$ (LV-2152) cannot establish a hydrazino turn, resulting in a lower activity of the pseudopeptide. The [aza-β^3-Phe^{22}]26RFa$_{(20-26)}$ (LV-2154) and [aza-β^3-$hSer^{23}$]-26RFa$_{(20-26)}$ (LV-2155) pseudopeptides were 3- and 1.5-fold less potent than 26RFa$_{(20-26)}$, respectively. Finally, the individual substitution of Phe^{24} (LV-2156), Arg^{25} (LV-2157), and Phe^{26} (LV-2158) generated analogues that were totally devoid of an effect on calcium mobilization, indicating that incorporation of intramolecular H-bonds at these points impairs the biological activity. Of note, this Phe-Arg-Phe triad is crucial for the biological activity of 26RFa,[28] as already reported for other RFamide peptides.[51,52] Altogether, these data indicate that the N-terminal part of 26RFa$_{(20-26)}$ likely establishes intramolecular H-bonds in contact with GPR103 for stabilizing a structure close to a hydrazino turn. Conversely, residues from position 22 to position 26 do not seem to harbor γ-turns and/or hydrazino turns. The C-terminal part of 26RFa$_{(20-26)}$ may also exhibit a different kind of turn such as an inverse γ-turn or a particular subtype of β-turn, as found in other RFamide peptides. For instance, NPAF adopts, in solution, an α-helical conformation between Asn^6 and Ala^{14}, while the C-terminal tetrapeptide is unstructured. In sodium dodecyl sulfate (SDS) solution, this C-terminal region forms a type I β-turn, which is involved in receptor recognition.[53] Similarly, $PrRP_{20}$ encompasses a C-terminal α-helix and a flexible N-terminal domain which tends to form a β-turn structure in a biomimetic environment.[54]

Aza-β^3-amino Acid Substitutions of 26RFa$_{(20-26)}$ at Position 23. We have recently demonstrated that the Ser^{23} residue of 26RFa$_{(20-26)}$ tolerates particularly well its substitution by a D-serine or an alanine with minor loss of activity.[28] Indeed, replacement of the Ser^{23} residue by a norvaline (Nva) significatively enhances the potency of the heptapeptide.[28] On the basis of this previous structure−activity relationship study in which Ser^{23} was substituted by differently branched aliphatic residues, the native Ser^{23} was replaced herein by different aza-β^3-moieties selected on the basis of the ability of their amino acid counterparts to improve the biological activity of 26RFa$_{(20-26)}$ (Table S1, Supporting Information; compounds LV-2159 to LV-2163). The pseudopeptides synthesized were tested for their ability to mobilize $[Ca^{2+}]_i$ in *h*GPR103-transfected cells (Table 2). The analogue [aza-β^3-

Table 2. Effect of Ser^{23} Residue−Aza-β^3-Substituted 26RFa$_{(20-26)}$ Analogues on Basal $[Ca^{2+}]_i$ and *h*26RFa-Induced $[Ca^{2+}]_i$ Increase in Gα_{16}-*h*GPR103-Transfected CHO Cells

Compd	Side-chain		EC_{50} (nM)[a]	IC_{50} (nM)[a]
	Name	Formula		
LV-2002	-		10.2 ± 1.1	nd
LV-2021	Ser		739 ± 156	nd
LV-2155	hSer		1092 ± 540	nd
LV-2159	Hht		341 ± 77	nd
LV-2160	Ala		1095 ± 267	nd
LV-2161	Nva		>10^4	>10^4
LV-2162	Nle		29800 ± 18000**	nd
LV-2163	Cha		1367 ± 112*	nd

[a]Data are the mean ± SEM of at least three distinct experiments performed in triplicate. nd = not determined. A single asterisk indicates $p < 0.05$ and two asterisks indicate $p < 0.01$ vs control (LV-2021) as assessed by the Mann−Whitney test.

Nva^{23}]26RFa$_{(20-26)}$ (LV-2161) was totally devoid of agonistic and antagonistic activity on calcium mobilization, suggesting that the shifting of the propyl side chain from C^α to N^α impairs a local hydrophobic interaction within the binding pocket. Concurrently, substitution by an aza-β^3-Ala (LV-2160), aza-β^3-Cha (LV-2163), or aza-β^3-Nle (LV-2162) led to compounds that were 1.5−40 times less potent than 26RFa$_{(20-26)}$ (LV-2021). In contrast, replacement of the Ser^{23} residue by an aza-β^3-hydroxyhomothreonine (aza-β^3-Hht; LV-2159) improved by 2-fold the potency of the heptapeptide in the calcium mobilization assay. As a matter of fact, the Ser^{23} residue of 26RFa is not fully conserved across vertebrate species.[20] For instance, the 26RFa sequence harbors an Ala residue at position 23 in quail and chicken and a Gly residue in zebrafish and goldfish.[10,55] The fact that mutation of this residue does not alter the recognition, binding, and activation of the receptor suggests either that its side chain is not an anchoring point or

that GPR103 has concomitantly evolved to maintain an interaction at this point. Altogether, these observations lead us to propose that the γ,δ-dihydroxypropyl side chain of the aza-β³-Hht residue may stabilize a novel interaction in the binding pocket to improve its biological activity.

Gly²⁰–Gly²¹ Modifications of 26RFa(20–26). To further investigate the local requirement of the N-terminal extremity of 26RFa(20–26), we have designed analogues modified on the Gly²⁰–Gly²¹ peptide bond or on the nitrogen atom of Gly²⁰ and Gly²¹ (Table S1, Supporting Information; Figure 3A; compounds LV-2043 to LV-2048 and LV-2111). The pseudopeptides [Cmp²¹]26RFa(21–26) (LV-2044) and [Gly²⁰Ψ-(CH₂NH)Gly²¹]26RFa(20–26) (LV-2045), the N-methylated peptides [Sar²⁰]26RFa(20–26) (LV-2046) and [Sar²¹]-26RFa(20–26) (LV-2047), and the acylated peptide [Ampc²¹]-26RFa(21–26) (LV-2048) were 2–13 times less potent than 26RFa(20–26) (LV-2021) (Figure 3B). Of note, all these compounds have a lower capacity to form hydrogen bonds. For instance, the *N*-methyl group of sarcosine precludes the formation of inter- or intramolecular H-bonds and enhances the flexibility of the pseudopeptide.[56] As a result, incorporation of this residue into nociceptin at position 2 or 3 decreases the binding and activation of the OP4 receptor.[57] In 26RFa(20–26), the ability of the N-terminal residues to form H-bonds seems to play an important role in GPR103 interaction. For instance, acetylation of Gly²¹ of 26RFa(21–26) generated an analogue totally devoid of an effect on $[Ca^{2+}]_i$. Interestingly, replacement of the Gly residues at positions 20 and 21 by the constrained 4-(carboxymethyl)piperazine motif (Cmpi²¹, LV-2043; EC_{50} = 138 ± 96 nM) led to an analogue 5-fold more potent than 26RFa(20–26) and 22-fold more potent than LV-2044 (Figure 3B), suggesting that the nitrogen atom in position 4 of the heterocycle plays a crucial role in the local conformation of the pseudopeptide.

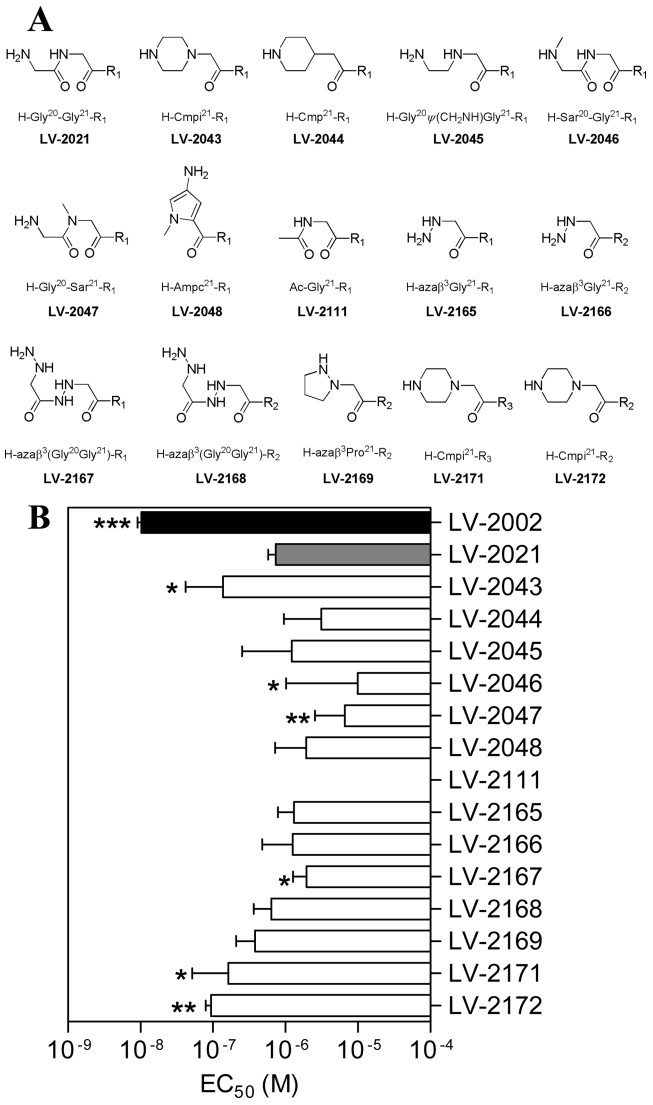


Figure 3. Structures of the N-terminal radicals and effects on basal $[Ca^{2+}]_i$ of N-terminally modified analogues and combined pseudopeptides: (A) developed formulas of the H-Gly²⁰-Gly²¹ modifications, R_1 = Phe-Ser-Phe-Arg-Phe-NH_2, R_2 = Phe-(aza-β³)Hht-Phe-Arg-Phe-NH_2, R_3 = Phe-(aza-β³)Nva-Phe-Arg-Phe-NH_2, (B) comparison of the EC_{50} value ± SEM of peptides and pseudopeptides on $[Ca^{2+}]_i$ mobilization in $G\alpha_{16}$-*h*GPR103-transfected CHO cells. Each EC_{50} value refers to the compound concentration yielding 50% of the maximal Ca^{2+} response. Key: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ vs control (26RFa(20–26), LV-2021) as assessed by the Mann–Whitney test.

Combined 26RFa(20–26) Pseudopeptides. To enhance the biological activity of 26RFa(20–26), the most effective modifications at positions 20, 21, and 23 were combined within the same pseudopeptide (Tabls S1, Supporting Information; Figure 3; compounds LV-2165 to LV-2169, LV-2171, and LV-2172). Unfortunately, double or triple modifications, i.e., [aza-β³-(Gly²¹,Hht²³)] (LV-2166), [aza-β³-(Gly²⁰,Gly²¹)] (LV-2167), and [aza-β³-(Gly²⁰,Gly²¹,Hht²³)] (LV-2168) did not lead to additivity of their individual contributions to the biological activity (Figure 3B). In fact, it has been recently shown that multiple incorporation of aza-β³-moieties in a peptide sequence is not a straightforward strategy to improve the biological activity of a peptide inasmuch as the succession of aza-β³ residues introduces an important intramolecular H-bond network that leads to massive rigidification of the peptide, limiting the dynamic process of ligand/receptor recognition.[58] Deletion of Gly²⁰ and introduction of aza-β³-Gly in position 21 led to a compound (LV-2165) which was 12 times less potent than [aza-β³-Gly²¹]26RFa(20–26) (LV-2153). This decreased activity could be accounted for by the loss of a hydrogen bond between the C=O moiety of residue Gly²⁰ and the NH moiety of residue Phe²², which destabilizes a hydrazino-turn-like structure. In contrast, the compounds [aza-β³-(Pro²¹,Hht²³)]26RFa(21–26) (LV-2169; EC_{50} = 382 ± 173 nM) and [Cmpi²¹,aza-β³-Nva²³]26RFa(21–26) (LV-2171; EC_{50} = 164 ± 112 nM) exhibited a 2- and 4.5-fold enhanced potency as compared to 26RFa(20–26), respectively. More importantly, the aza-β³-Hht²³ modification combined with the Cmpi²¹ incorporation generated a pseudopeptide (LV-2172) that was 8 times more potent than 26RFa(20–26) to increase $[Ca^{2+}]_i$ (EC_{50} = 93.6 ± 13.4 nM) (Figure 3B). These data show that the backbone constraint brought by the Cmpi moiety and the intramolecular hydrogen bond(s) promoted by the aza-β³-Hht moiety could stabilize a conformation close to the bioactive one. Finally, it is interesting to note that the two combined pseudopeptides encompassing the Cmpi radical exhibited a higher potency than their Cmpi-free counterparts (LV-2171 vs LV-2161 and LV-2172 vs LV-2159). The potencies of the two compounds were in the same range, suggesting that their higher biological activity can be ascribed to the Cmpi moiety.

Affinity of Selected Pseudopeptides. Binding assays were performed for compounds selected on the basis of the

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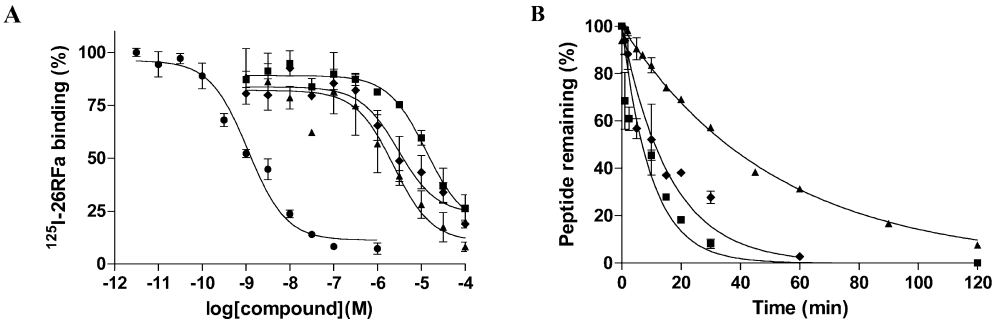

Figure 4. Affinity and stability of selected compounds: (A) Competition curves showing displacement of ^{125}I-labeled 26RFa by increasing concentrations of 26RFa (LV-2002; ●), 26RFa$_{(20-26)}$ (LV-2021; ■), [aza-β^3-Gly^{21}]26RFa$_{(20-26)}$ (LV-2153; ◆), and [Cmpi^{21},aza-β^3-Hht^{23}]26RFa$_{(21-26)}$ (LV-2172; ▲) on membrane preparation from *h*GPR103-transfected HEK193 cells. Data are expressed as a percentage of the specific binding of ^{125}I-26RFa in the absence of competitive ligands. Data represent the mean ± SEM of at least three independent experiments performed in duplicate, (B) degradation kinetics of 26RFa (LV-2002; ●), 26RFa$_{(20-26)}$ (LV-2021; ◆), and [Cmpi^{21},aza-β^3-Hht^{23}]26RFa$_{(21-26)}$ (LV-2172; ▲) evaluated by HPLC or LC−MS analysis after incubation of the peptide in human serum. Each point is the mean ± SEM of two independent experiments.

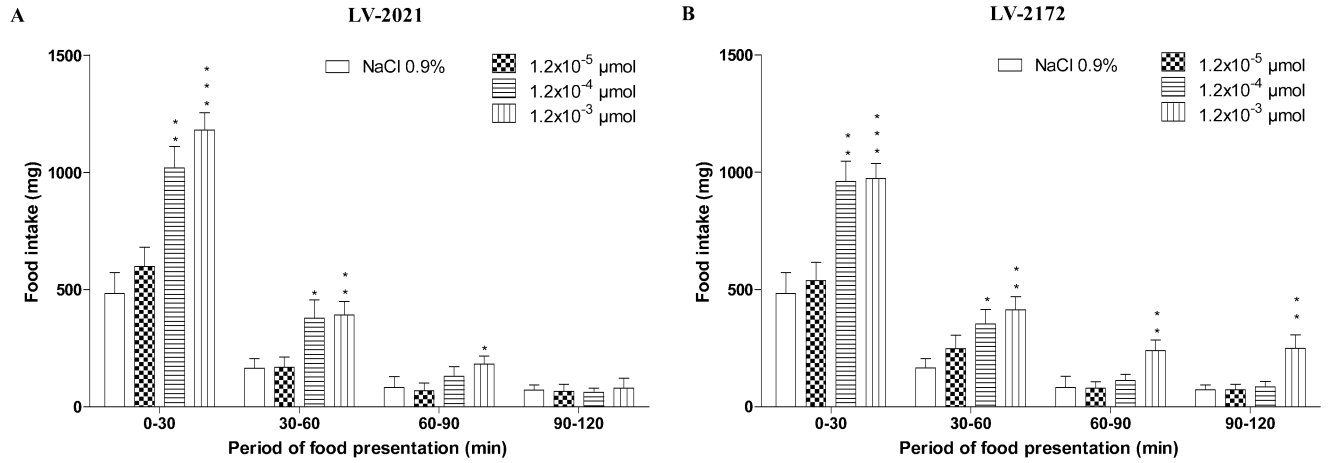

Figure 5. Time course of the effect of LV-2021 and LV-2172 on food intake. Mice, partially food-deprived, were injected icv (10 µL) with saline, 26RFa$_{(20-26)}$ (LV-2021; A), or [Cmpi^{21},aza-β^3-Hht^{23}]26RFa$_{(21-26)}$ (LV-2172; B) (0.012, 0.12, and 1.2 nmol each). Ten minutes after icv injection, each animal had access to a weighed food pellet, and its food consumption was measured during the period indicated. Mean ± SEM of data from 12 mice per group. Key: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ vs saline as assessed by the Kruskal−Wallis test.

functional characteristics described above. The binding affinity of [aza-β^3-Gly^{21}]26RFa$_{(20-26)}$ (LV-2153) and [Cmpi^{21},aza-β^3-Hht^{23}]26RFa$_{(21-26)}$ (LV-2172) was determined by competition experiments using human GPR103-transfected cell membrane preparations (Figure 4A). In agreement with the results obtained by Fukusumi et al.,[8] 26RFa (LV-2002) displaced [^{125}I]-26RFa with an IC$_{50}$ of 1.26 ± 0.23 nM, whereas 26RFa$_{(20-26)}$ (LV-2021) was a poor competitor (IC$_{50}$ = 8730 ± 4330 nM). Consistent with an increase in their agonistic activity, the [aza-β^3-Gly^{21}]26RFa$_{(20-26)}$ (LV-2153) and [Cmpi^{21},aza-β^3-Hht^{23}]26RFa$_{(21-26)}$ (LV-2172) pseudopeptides were 4 and 5 times more potent in displacing [^{125}I]-26RFa than 26RFa$_{(20-26)}$ with IC$_{50}$ values of 2987 ± 1091 and 1994 ± 487 nM, respectively. This increased affinity can likely be ascribed to a decrease in the entropy penalty associated with the constrained analogues.

Susceptibility of LV-2172 to Enzymatic Degradation. When incorporated into a peptide, aza-β^3-amino acids are known to enhance the resistance of the pseudopeptide to enzymatic degradation.[35] To compare the metabolic stability of [Cmpi^{21},aza-β^3-Hht^{23}]26RFa$_{(21-26)}$ (LV-2172) to those of 26RFa and 26RFa$_{(20-26)}$, the compounds were incubated in vitro with human serum as described before.[59] Intact compounds and their metabolites were separated by RP-HPLC and analyzed by MALDI-TOF mass spectrometry. As most natural peptides, 26RFa (LV-2002) and 26RFa$_{(20-26)}$ (LV-2021) had a short half-life, i.e., 6.9 and 11.2 min, respectively. The half-life of LV-2172 in human serum (35.6 min) was 5 and 3 times higher than those of 26RFa and 26RFa$_{(20-26)}$, respectively (Figure 4B). RP-HPLC analysis revealed the formation of two novel compounds, i.e., the fragment [Cmpi^{21},aza-β^3-Hht^{23}]26RFa$_{(21-25)}$, which appeared after 2 min of incubation, and the fragment [Cmpi^{21},aza-β^3-Hht^{23}]-26RFa$_{(21-24)}$, which appeared after 15 min, resulting from cleavage of the Arg^{25}−Phe^{26} and Phe^{24}−Arg^{25} peptide bonds, respectively. No breakdown product from the N-terminal region of LV-2172 was detected, suggesting that the Cmpi moiety efficiently protected this region from the action of peptidases. Consistent with these data, a previous report has shown that aza-β^3-analogues of peptide 88−99 of histone H4 exhibit a longer half-life than the native peptide.[35]

Effect on Feeding Behavior. To compare the effects of 26RFa$_{(20-26)}$ (LV-2021) and [Cmpi^{21},aza-β^3-Hht^{23}]-26RFa$_{(21-26)}$ (LV-2172) on feeding behavior, both compounds were icv injected in food-restricted mice. As previously reported,[15] icv administration of graded doses of 26RFa$_{(20-26)}$

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(0.012−1.2 nmol) induced a transient increase in food intake during the first 30 min, and this orexigenic effect rapidly declined. During the 60−90 min period, only the highest dose of $26RFa_{(20-26)}$ (1.2 nmol) slightly stimulated food consumption (Figure 5). At the same doses, [$Cmpi^{21}$,aza-β^3-Hht^{23}]$26RFa_{(21-26)}$ mimicked the dose-dependent food-intake-stimulating effect of $26RFa_{(20-26)}$, but at the highest dose (1.2 nmol), the orexigenic response was still observed during the 90−120 min period (Figure 5). The 2 h cumulative food intakes induced by LV-2021 and LV-2172 were 1833 ± 454 and 1873 ± 427 mg, respectively. The long-lasting orexigenic effect of [$Cmpi^{21}$,aza-β^3-Hht^{23}]$26RFa_{(21-26)}$ can be likely ascribed to its higher affinity and higher potency, its relatively high lipophilicity, which may improve its central nervous system (CNS) bioavailability, a higher stability in the brain, and/or a lower desensitization effect. Whether 26RFa analogues can cross the blood−brain barrier (BBB) is currently unknown.

CONCLUSIONS

The novel RFamide peptides 26RFa and 43RFa/QRFP are potent stimulators of food intake, and GPR103-deficient mice suffer from severe osteopenia.[60,61] Thus, the development of small molecules which act as selective ligands of GPR103 may prove useful for the treatment of feeding disorders or osteoporosis. It has previously been shown that the C-terminal heptapeptide $26RFa_{(20-26)}$ mimics the orexigenic effect of 26RFa in mice. In the present study, rational design of GPR103 agonists has led to the identification of an analogue of 26RFa, [$Cmpi^{21}$,aza-β^3-Hht^{23}]$26RFa_{(21-26)}$ (LV-2172), that is 8 times more potent than $26RFa_{(20-26)}$ in mobilizing $[Ca^{2+}]_i$ in GPR103-transfected cells, that possesses a longer half-life than $26RFa_{(20-26)}$ in human serum, and that exhibits a prolonged orexigenic effect as compared to $26RFa_{(20-26)}$. The design of such a low molecular weight, lipophilic, metabolically stable, and long-lasting GPR103 ligand provides a new pharmacological tool for investigations of the physiological role and pathophysiological implication of 26RFa and represents a crucial step toward the development of drug candidates for the treatment of related disorders.

EXPERIMENTAL SECTION

Materials. All Fmoc amino acid residues, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), and 1-hydroxybenzotriazole (HOBt) were purchased from Fluka Chemicals (Buchs, Switzerland) or NovaBiochem (Darmstadt, Germany). The Rink amide 4-methylbenzhydrylamine (MBHA) resin was from VWR International (Fontenay-sous-Bois, France). *N*,*N*-Diisopropylethylamine (DIEA), piperidine, trifluoroacetic acid (TFA), and triisopropylsilane (TIS) were supplied from Acros Organics (Geel, Belgium). *N*,*N*-Dimethylformamide (DMF), dichloromethane (DCM), the nutrient mixture F-12 HAM, L-glutamine, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), penicillin−streptomycin solution, probenecid, lactoperoxidase, and other reagents were from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Acetonitrile was from Fisher Scientific (Illkirch, France). Phenol, Geneticin (G-418 sulfate), hygromycin B, Hank's balanced salt solution (HBSS), and pluronic F-127 were from Gibco Life Technologies (Cergy-Pontoise, France). Fetal bovine serum was from Lonza (Viviers, Belgium). The human GPR103 receptor membrane target system was from Perkin-Elmer (Boston, MA).

Fmoc-amino Acid Synthesis. Fmoc-aza-β^3-amino acids were prepared as previously described.[49,50] Briefly, the general route consists of the formation of a substituted hydrazone by condensation between the corresponding aldehyde and Fmoc-carbazate. The hydrazone was then reduced by $NaBH_3CN$ to give the expected Fmoc-substituted hydrazine. The $-CH_2COOH$ moiety was introduced by reductive amination of glyoxilic acid with the Fmoc-substituted hydrazine or by nucleophilic substitution of methyl or benzyl bromoacetates.

Pseudopeptide and Peptide Synthesis. All pseudopeptides and peptides were synthesized on a Liberty microwave-assisted automated peptide synthesizer (CEM, Orsay, France) using the standard manufacturer's procedures (0.1 mmol scale) on a Rink amide MBHA resin. All Fmoc-amino acids (0.4 mmol, 4 equiv) were coupled by in situ activation with TBTU/HOBt (0.4 mmol:0.4 mmol, 4 equiv) and DIEA (0.8 mmol, 8 equiv) in DMF. Reactive side chains were protected as follows, unless otherwise stated: Thr, Ser, and Tyr, *tert*-butyl (*t*Bu) ether; Lys, *tert*-butyloxycarbonyl (Boc) carbamate; Glu, *O*-*tert*-butyl (O*t*Bu) ester; Asn, trityl (Trt) amide; Arg, pentamethyldihydrobenzofuran (Pbf) sulfonylamide.

Peptide and Side Chain Cleavage and Purification. All pseudopeptides and peptides were cleaved from the resin and/or deprotected by adding 10 mL of an ice-cold mixture of TFA/TIS/H_2O (9.5:0.25:0.25, v/v/v) and agitating at 0 °C for 5 min and at room temperature for 3 h as previously described.[28] Crude peptides were purified by semipreparative reversed-phase HPLC (RP-HPLC) on a Vydac 218TP1022 C_{18} column (2.2 × 25 cm; Alltech, Templemars, France) using a linear gradient (10−50% over 50 min) of acetonitrile/TFA (99.9:0.1, v/v) at a flow rate of 10 mL/min. Absorbance was monitored at 215 and 280 nm using a UV detector (Gilson, Villiers Le Bel, France). Analytical RP-HPLC analysis, performed on a Vydac 218TP54 C_{18} column (0.46 × 25 cm; Alltech), revealed that the purity of all peptides was higher than 98.6% (Tabls S1, Supporting Information). Absorbance was monitored at 215 and 280 nm. The authenticity of each peptide was verified by MALDI-TOF-MS on a Voyager DE-PRO (Applera-France) in the reflector mode with α-cyano-4-hydroxycinnamic acid as a matrix.

Circular Dichroism Spectroscopy. Circular dichroism spectra were acquired on a CD6 dichrograph (Jobin-Yvon, Longjumeau, France) calibrated using *d*-10-camphorsulfonic acid. Peptides were dissolved in H_2O, TFE/H_2O (50:50, v/v), MeOH, or 60 mM DPC at a final concentration of 0.325 mg/mL. Data points were collected from 260 to 194 nm at room temperature with a 0.5 mm path length quartz cell. For each spectrum, five scans were accumulated and then averaged. The baseline was obtained by recording a spectrum of the solvent, and the mean residue molar ellipticity ($[\theta]_{MRE}$), deg cm^2 $dmol^{-1}$, was calculated from the observed ellipticity after baseline correction.

Ca^{2+} Mobilization Assays. The establishment of a cell line stably expressing *h*GPR103 and the $G\alpha_{16}$ protein has been previously described.[28] Changes in $[Ca^{2+}]_i$ concentrations induced by 26RFa analogues in $G\alpha_{16}$-*h*GPR103-transfected CHO cells were measured on a benchtop scanning fluorometer Flexstation III (Molecular Devices, Sunnyvale, CA) as previously described.[28] Briefly, 96-well assay black plates with a clear bottom (Corning International, Avon, France) were seeded at a density of 40 000 cells/well 24 h prior to assay. For agonist experiments, cells were loaded with 2 μM fluo-4AM (Invitrogen) for 1 h, washed three times, and incubated for 30 min with standard HBSS containing 2.5 mM probenecid and 5 mM HEPES. Compounds to be tested were added at final concentrations ranging from 10^{-12} to 10^{-5} M, and the fluorescence intensity was measured for 2 min. Antagonist experiments were conducted using the same protocol except that compounds were manually injected at concentrations of 10^{-6}, 3.16×10^{-6}, 10^{-5}, and 3.16×10^{-5} M 30 min prior to the injection of the agonist, i.e., *h*26RFa, at a single concentration of 10^{-7} M. A xenon lamp was used as the excitation source. The wavelengths of excitation (485 nm) and emission (525 nm) of fluo-4AM were selected by two monochromators included in the device equipped with a bottom reading probe.

Binding Experiments. Synthetic human 26RFa (3 μg) was radiolabeled with 0.5 mCi of $Na^{125}I$ by means of the lactoperoxidase method as previously described.[13] Monoiodinated 26RFa, labeled on the Tyr^{15} residue, was purified by reversed-phase HPLC on a 0.46 × 25 cm Adsorbosphere C_{18} column by using a linear gradient (25−60% over 35 min) of acetonitrile/TFA (99.9:0.1, v/v) at a flow rate of 1 mL/min. Nonlabeled 26RFa and monoiodinated [^{125}I]26RFa eluted at

39% and 41% acetonitrile, respectively. Membrane preparations from *h*GPR103-transfected HEK293 cells (9 μg) were incubated at room temperature in MultiScreen-FC, previously coated with 0.3% poly(ethylenimine) and 0.5% BSA, for 1 h with 0.2 nM ^{125}I-*h*26RFa in assay buffer (0.5% BSA, 50 mM HEPES, 1 mM $CaCl_2$, and 5 mM $MgCl_2$) in the presence of variable concentrations of *h*26RFa or analogues. After incubation, the wells were rinsed with buffer (10 mM HEPES and 0.5 M NaCl), and radioactivity was quantified with a γ counter (LKB Wallac, Mt. Waverley, Australia).

Susceptibility of Peptides to Enzymatic Degradation. The stability of 26RFa (LV-2002), 26RFa$_{(20-26)}$ (LV-2021), and [Cmpi^{21},aza-β^3-Hht^{23}]26RFa$_{(21-26)}$ (LV-2172) was tested in vitro as previously described.[35,59,62] Peptides or pseudopeptides (250 μg/mL) were incubated at 37 °C with pooled human serum (healthy donors). The reaction was stopped after different times by adding TFA (10% of the final volume), and the samples were diluted five times in PBS (pH 7.4). After centrifugation, the supernatants were collected and analyzed by RP-HPLC on a Vydac 218MS54 C_{18} column (0.46 × 25 cm) using a linear gradient (10−60% over 50 min) of acetonitrile/TFA (99.9:0.1, v/v) at a flow rate of 1 mL/min. For 26RFa and [Cmpi^{21},aza-β^3-Hht^{23}]26RFa$_{(21-26)}$, HPLC peak areas were used to calculate the percentage of intact compound remaining at the various time points during the incubation. Half-life times ($t_{1/2}$) were calculated with the Prism software (Graphpad Software, San Diego, CA) from exponential decay curves. Mass spectra of the intact peptide and its metabolites were obtained by MALDI-TOF-MS on a Voyager DE-PRO (Applera-France) in the reflector mode with α-cyano-4-hydroxycinnamic acid as a matrix. For 26RFa$_{(20-26)}$, which coeluted with its metabolites, the collected HPLC fraction was submitted to a quantitative analysis on a nano-LC 1200 system coupled to a 6340 ion trap mass spectrometer equipped with an HPLC-chip cube interface (Agilent Technologies, Massy, France). Peptides were separated on an Agilent Zorbax 300SB-C_{18} 5 μm separation chip column (43 mm × 75 μm) and a 4 mm 40 nL Zorbax 300SB-C_{18} 5 μm enrichment column using a linear gradient (3−80% over 7 min) of acetonitrile/H_2O/formic acid (90.0:9.9:0.1, v/v/v) at a flow rate of 0.4 μL/min. Optimized ionization parameters used were as follows: capillary voltage, 1885 V; drying gas flow rate, 5 L/min; drying temperature, 300 °C. Trap MS data acquisition was performed in full scan mode from 150 to 1300 *m*/*z*, in standard enhanced mass range mode, and with positive ion polarity, a scan delay of 0 μs, a trap maximum accumulation time of 150000 μs, and an ion charge control (ICC) target of 500 000. An external standard calibration curve was prepared with concentrations of 26RFa$_{(20-26)}$ ranging from 10 to 1000 fmol/μL in duplicate, and the curve coefficient R^2 obtained was >0.99. Four dilutions of each sample were analyzed (each dilution point injected twice) in the same conditions as the standard used. For quantification, the QuantAnalysis software version 1.8 (Agilent Technologies) was used, and the half-life time was calculated as described above.

Animals. Male Swiss albino CD1 mice (IFFA-CREDO/Charles River, Saint-Germain sur l'Arbresle, France), weighing 22−25 g, were housed in Makrolon cages (length 40 cm, width 25 cm, height 18 cm; 20 animals in each one), with free access to standard semisynthetic laboratory diet and tap water, under controlled temperature (22 ± 1 °C) and lighting (light on from 7:00 a.m. to 7:00 p.m.). Each animal was used once. Animal manipulations were performed according to the European Community Council Directive of Nov 24, 1986 (86/609/EEC), and were approved by the local Ethical Committee (authorization numbers N/10-04-04-12 and N/13-04-04-15).

Intracerebroventricular Injection. Intracerebroventricular (icv) injections (10 μL) were performed in the left ventricle of manually immobilized mice, within about 3 s, according to the procedure of Haley and McCormick,[63] by using a Hamilton microsyringe (50 μL) connected to a needle (diameter 0.5 mm) equipped with a guard at 3.5 mm from the tip to control its penetration into the brain. Compounds (LV-2021 and LV-2172) were injected at doses of 12, 120, and 1200 pmol/mouse.

Food Intake. Mice were isolated in individual cages (length 24 cm, width 10 cm, height 7 cm) 2 days before the experiments with free access to water, and pellets of food were laid down on the floor of the cages, to accustom the animals to the test conditions. Eighteen hours before the experiments (3:00 p.m. to 9:00 a.m.), each mouse had access to only 3 g of food (which represented approximately half of their daily consumption) with tap water ad libitum. The day of testing, at 8:00 a.m., unconsumed food was removed, and the test was performed at 9:00 a.m. After icv administration (10 min before testing) mice had access to a weighed food pellet (5 g) deposited on the floor of the cage. During the test, every 30 min, the pellet was briefly (<20 s) removed with forceps and weighed.

Statistical Analysis. Calcium measurement experiments were performed in triplicate, and data, expressed as the mean ± SEM, were analyzed with the Prism software. The EC_{50} values were determined from concentration−response curves using a sigmoidal dose−response fit with variable slope. Differences between 26RFa or 26RFa$_{(20-26)}$ and analogue activities were analyzed by the Mann−Whitney test.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Chemical data for peptides and pseudopeptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED[64]

Ampc, 4-amino-1-methylpyrrole-2-carboxylic acid; Cha, cyclohexylalanine; Cmp, 4-(carboxymethyl)piperidine; Cmpi, 4-(carboxymethyl)piperazine; DPC, dodecylphosphocholine; Hht, hydroxyhomothreonine; icv, intracerebroventricular; Sar, sarcosine; TFE, trifluoroethanol

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