Expedited Articles

Structure−Function Studies on the New Growth Hormone-Releasing Peptide, Ghrelin: Minimal Sequence of Ghrelin Necessary for Activation of Growth Hormone Secretagogue Receptor 1a[⊥]

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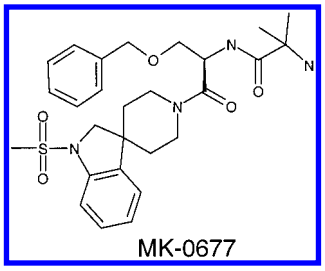
The recently discovered growth hormone secretagogue, ghrelin, is a potent agonist at the human growth hormone secretagogue receptor 1a (hGHSR1a). To elucidate structural features of this peptide necessary for efficient binding to and activation of the receptor, several analogues of ghrelin with various aliphatic or aromatic groups in the side chain of residue 3, and several short peptides derived from ghrelin, were prepared and tested in a binding assay and in an assay measuring intracellular calcium elevation in HEK-293 cells expressing hGHSR1a. Bulky hydrophobic groups in the side chain of residue 3 turned out to be essential for maximum agonist activity. Also, short peptides encompassing the first 4 or 5 residues of ghrelin were found to functionally activate hGHSR1a about as efficiently as the full-length ghrelin. Thus the entire sequence of ghrelin is not necessary for activity: the Gly-Ser-Ser(*n*-octanoyl)-Phe segment appears to constitute the "active core" required for agonist potency at hGHSR1a.

Introduction

The pulsatile release of growth hormone (GH) from the pituitary somatotrophs is regulated by two hypothalamic neuropeptides: growth hormone-releasing hormone (GHRH) and somatostatin (SST). The first peptide stimulates release of GH, whereas SST inhibits secretion of the same hormone.[1,2] Release of GH from the pituitary somatotrophs can also be controlled by the synthetic growth hormone-releasing peptides (GHRPs). A hexapeptide, His-D-Trp-Ala-Trp-D-Phe-Lys-amide (GHRP-6), was the first synthetic peptide which released GH from somatotrophs in a dose-dependent manner in several species including humans.[3] From the subsequent chemical studies on GHRP-6, other potent growth hormone secretagogues (GHSs) resulted: GHRP-1, GHRP-2, and hexarelin, also extensively studied in vitro and in vivo.[4−6]

GHRP-1	Ala-His-D-(2')-Nal-Ala-Trp-D-Phe-Lys-NH_2
GHRP-2	D-Ala-D-(2')-Nal-Ala-Trp-D-Nal-Lys-NH_2
Hexarelin	His-D-2-MeTrp-Ala-Trp-D-Phe-Lys-NH_2

These synthetic GHSs stimulate secretion of GH by a mechanism different from that of GHRH, but like GHRH, they functionally antagonize release of SST from the pituitary and hypothalamus.[3−6] The low oral bioavailability (<1%) of the peptidyl GHSs stimulated a search for nonpeptide compounds mimicking the action of GHRP-6 in the pituitary. Shortly thereafter, several benzolactams and spiroindans were reported to stimulate GH release in various animal species and in humans.[7−9] A small spiroindan with improved oral bioavailability, *N*-[1(*R*)-[(1,2-dihydro-1-methanesulfonylspiro[3*H*-indole-3,4′-piperidin]-1′-yl)carbonyl]-2-(phenylmethoxy)ethyl]-2-amino-2-methylpropanamide methanesulfonate (MK-0677), emerged as one of the most potent synthetic GHSs.[8]



The actions of the above-mentioned GHSs (both peptides and nonpeptides) are mediated by a specific GHS receptor.[10,11] This receptor is present in the pituitary and hypothalamus of various mammalian species (GHSR1a) and it is distinct from the GHRH receptor. The GHS receptor was also detected[9−13] in other areas of the central nervous system (CNS) and in peripheral tissues, for instance adrenal and thyroid glands, heart, lung, kidney, and skeletal muscles. A

[⊥] Dedicated to Prof. Dr. Miklos Bodanszky on the occasion of his 85th birthday.

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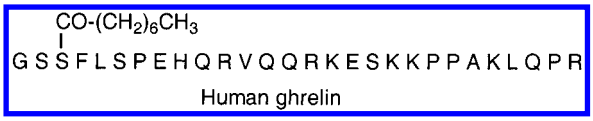
truncated version of GHSR1a was subsequently reported by scientists from Merck.[10]

The GHS receptors belong to the family of G-protein-coupled receptors. Their activation leads to depolarization and inhibition of potassium channels, to an increase in intracellular concentrations of inositol trisphosphate (IP_3), and to a transient increase in the concentrations of intracellular calcium.[11−13] Thus, it was speculated that the peptidyl and nonpeptidyl GHSs could mimic the actions of yet unidentified endogenous ligand(s) for the GHS receptor(s).

Recently, a 28-amino acid peptide has been isolated from human gut extract.[14] This peptide, ghrelin, was able to induce GH release from primary cultured pituitary cells in a dose-dependent manner without stimulating the release of other pituitary hormones. Injected intravenously into anesthetized rats, ghrelin stimulated pulsatile release of GH.[14] These observations suggested[14] that ghrelin is a specific endogenous ligand for the GHS receptor(s).



Ghrelin is also the first peptide isolated from natural sources which possesses the hydroxyl group of one of its serine residues acylated by *n*-octanoic acid. This until now unreported posttranslational modification appears to be necessary for the GH-releasing potency of both human and rat ghrelin.[14] In the GH-releasing assay, the desoctanoyl form of the hormone is at least 100-fold less potent than the parent peptide.[14,15]

The present study aimed at the elucidation of the structural features of human ghrelin which are critical for its interaction with GHSR1a. First, a role of the *n*-octanoyl group in binding to and activation of the cloned hGHSR1a was investigated in detail by evaluation of several analogues of ghrelin in which the hydroxyl group of Ser^3 was acylated with various aliphatic or aromatic acids. Furthermore, the biological importance of the ester group in the side chain of residue 3 was studied through an analogue of ghrelin in which *n*-octanoic acid was coupled to the β-amino group of 2,3-diaminopropionic acid replacing Ser^3. In intact cells, this new analogue of ghrelin, with the *n*-octanoyl group attached to the peptide by an amide bond, should be less susceptible than the parent compound to esterases and acyl migration. Also, analogues esterified at the other three serine residues of ghrelin were tested for their ability to activate hGHSR1a.

Additionally, in an effort to elucidate the smallest segment of human ghrelin which could still activate hGHSR1a, several truncated analogues of this hormone were prepared and evaluated for binding to and activation at hGHSR1a. At this receptor, the agonist potency of the short peptide Gly-Ser-Ser(*n*-octanoyl)-Phe-Leu-NH_2, derived from the N-terminal region of human ghrelin, was equal to that of the full-length ghrelin.

Results

Analogues of human ghrelin (Tables 1−4) were prepared by solid-phase syntheses as described in the Experimental Section. The chromatographically homogeneous peptides **1**−**26** were analyzed for their chemical integrity by electrospray mass spectrometry, ^{1}H NMR spectrometry (peptides **21**−**26**), and chemical sequencing (Edman degradation, peptides **1**−**22, 24**) (see Experimental Section). In the mass spectra of compounds **1**−**26,** only molecular ions corresponding to peptides with one *n*-octanoyl group attached were observed. Additionally, the ^{1}H NMR experiments (COSY and ROESY) on compounds **21**−**26** showed that *n*-octanoic acid has been coupled to the side chain of Ser^3. This was further supported by the characteristic downfield shift of the signals corresponding to the β-protons of Ser^3, in comparison to the signals of the equivalent protons of Ser^2. Furthermore, chemical sequencing of compounds **1**−**14**, **18**−**22**, and **24** yielded negligible amounts of serine in cycle 3 of Edman degradation, thus confirming that Ser^3 in these peptides has been modified. Similarly, upon sequencing of compounds **15**−**17**, negligible amounts of serine were observed in cycle 2 for compound **15**, in cycle 6 for compound **16**, and in cycle 18 for compound **17**, again due to acylation of the hydroxyl group of the respective serine residues. Taken together, the above observations confirm the presence and exact (expected) location of a single *n*-octanoyl group in the compounds studied.

Peptides **1**−**26** were evaluated for their binding affinities to the cloned hGHSR1a in a competitive binding assay with [^{35}S]MK-0677 as the radiolabeled ligand and also for their ability to stimulate inositol trisphosphate-coupled mobilization of intracellular calcium in HEK-293 cells expressing hGHSR1a.

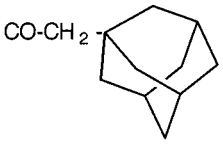
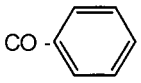
The role of the *n*-octanoyl group in interaction of human ghrelin with hGHSR1a was examined by testing compounds **1**−**11** (see Table 1) in which the hydroxyl group in the side chain of Ser^3 was acylated by various aliphatic or aromatic acids. Acylation of Ser^3 with hydrophobic acids resembling in size *n*-octanoic acid, such as the unsaturated 2,4,6-octatrienoic acid or the branched 2-propylpentanoic acid or the longer chain 11-undecanoic acid or palmitic acid, yielded compounds **1**−**4** with agonist potencies similar to that of ghrelin. In contrast, replacement of the *n*-octanoyl group with the substantially smaller acetyl group led to compound **5** which was 20-fold less potent than human ghrelin in the hGHSR1a activation assay. Ghrelin without the *n*-octanoyl group (desoctanoylghrelin) poorly activated hGHSR1a even at micromolar concentrations (compound **6** in Table 1).

To explore possible nonhydrophobic interactions with the receptor, the hydroxyl group of Ser^3 in compounds **7**−**9** was acylated with aliphatic acids which mimic the extended hydrophobic chain of *n*-octanoic acid, but they also contain bromo, amino, or amido groups. In the hGHSR1a activation assay, analogues with amido and amino groups in the side chain of residue 3 were respectively 5- and 20-fold less potent as agonists at hGHSR1a (compounds **8** and **9** in Table 1), whereas the analogue **7** with a 8-bromooctanoyl group attached to the side chain of Ser^3 retained the potency of the parent compound.

Interestingly, compound **10** with the bulky and rigid hydrophobic 1-adamantaneacetyl group in place of the *n*-octanoyl group in position 3 activated hGHSR1a as

Table 1. Analogues of Human Ghrelin Modified at Position 3

X
G S S F L S P E H Q R V Q Q R K E S K K P P A K L Q P R
1 28

No.	X	Binding Assay* IC_{50} (nM)	Functional Assay** EC_{50} (nM)	Functional Assay** % activation at 10 μM relative to ghrelin
human ghrelin	CO-$(CH_2)_6CH_3$	0.25 ± 0.07	32 ± 4.5	100
1	CO-CH=CH-CH=CH-CH=CH-CH_3	0.98 ± 0.36	39 ± 10	108 ± 1
2	CO-$CH(CH_2CH_2CH_3)_2$	0.96 ± 0.05	38 ± 11	103 ± 1
3	CO-$(CH_2)_9CH_3$	0.12 ± 0.03	9.1 ± 6.2	104 ± 3
4	CO-$(CH_2)_{14}CH_3$	0.87 ± 0.17	8.3 ± 0.6	96 ± 11
5	CO-CH_3	> 2000	2000 ± 480	59 ± 13
6	---------	> 10,000	>10,000	41 ± 4
7	CO-$(CH_2)_6CH_2Br$	0.08 ± 0.0	18 ± 0.9	88 ± 7
8	CO-$(CH_2)_2$CO-NH-$(CH_2)_2CH_3$	1020 ± 202	410 ± 120	86 ± 10
9	CO-$(CH_2)_6NH_2$	> 2000	1200 ± 370	68 ± 3
10		0.12 ± 0.05	24 ± 9.5	95 ± 7
11		11 ± 1.5	53 ± 3.2	85 ± 2

*[^{35}S]MK-0677 binding assay. IC_{50} reflects concentration of peptide at 50% specific binding. **Aequorin bioluminescence assay. ED_{50} is the concentration of peptide at 50% maximum calcium accumulation.

efficiently as ghrelin, but compound **11** with a smaller benzoyl group in the same position was 2-fold less potent.

In compounds **12** and **14**, 2,3-diaminopropionic acid was incorporated in place of Ser^3 and the β-amino group of this new residue was acylated with *n*-octanoic acid. Peptides **12** and **14** (Table 2), with the *n*-octanoyl group attached to the side chain of residue 3 through an amide bond, activated hGHSR1a as efficiently as the parent compounds, ghrelin and compound **13** (peptide encompassing 1−14 residues of ghrelin)

Of analogues of ghrelin with the hydroxyl group of Ser^2 or Ser^6 or Ser^{18} selectively acylated by *n*-octanoic acid, compound **15,** with a modified Ser^2, was as potent as ghrelin in the functional assay, whereas compounds **16** and **17**, with a modified Ser^6 and Ser^{18}, respectively, were inactive even at micromolar concentrations (Table 3).

Binding and functional data for truncated analogues of human ghrelin are compiled in Table 4. Shortening of the chain of ghrelin by omission of 5, 10, 14, 18, 23, or 24 residues from its C-terminal end yielded peptides **13** and **18**−**26** with agonist properties similar to that of the parent compound. The relative binding affinities of the same peptides for the cloned hGHSR1a, however, gradually decreased with the extent of the C-terminal deletions. For example, analogue **13** encompassing residues 1−14 of ghrelin was bound 40-fold weaker than human ghrelin, whereas analogue **21** consisting of only the first 5 residues of ghrelin was 200-fold weaker ligand at the same receptor. The short peptides encompassing residues 1−3, 2−5, 3−5, or 2−4 of ghrelin (compounds **23**−**26** in Table 4) were poor activators even at micromolar concentrations.

Discussion and Conclusions

Several peptide hormones such as secretin, glucagon, the vasoactive intestinal peptide, and cholecystokinin were detected in and then isolated from the gastrointestinal tract.[16] Of these, cholecystokinin was posttranslationally modified by sulfonylation of the hydroxyl group of Tyr^{27}. It is noteworthy therefore that the recently isolated peptide from gut extracts, ghrelin, is also posttranslationally modified, through acylation of the hydroxyl group of Ser^3 by *n*-octanoic acid. In biological systems, modification of serine residues by

Table 2. Analogues of Human Ghrelin with an Amide Bond in the Side Chain of Residue 3

No.	Compound	Binding Assay* IC_{50} (nM)	Functional Assay** EC_{50} (nM)	Functional Assay** % activation at 10 μM relative to ghrelin
human ghrelin	G S NH-CH(CH$_2$-O-CO(CH$_2$)$_6$CH$_3$)-CO-F L S P E H Q R V Q Q R K E S K K P P A K L Q P R (3)	0.25 ± 0.07	32 ± 4.5	100
12	G S NH-CH(CH$_2$-NH-CO(CH$_2$)$_6$CH$_3$)-CO-F L S P E H Q R V Q Q R K E S K K P P A K L Q P R (3)	0.42 ± 0.12	31 ± 9.8	105 ± 5
13	G S NH-CH(CH$_2$-O-CO(CH$_2$)$_6$CH$_3$)-CO-F L S P E H Q R V Q Q (3)	9.6 ± 1.5	17 ± 4	97 ± 9
14	G S NH-CH(CH$_2$-NH-CO(CH$_2$)$_6$CH$_3$)-CO-F L S P E H Q R V Q Q (3)	8 ± 2.7	38 ± 1.8	102 ± 3

*,**See corresponding footnotes in Table 1.

Table 3. Analogues of Human Ghrelin *n*-Octanoylated at Other Serine Residues

No.	Compound	Binding Assay* IC_{50} (nM)	Functional Assay** EC_{50} (nM)	Functional Assay** % activation at 10 μM relative to ghrelin
human ghrelin	G S S(CO(CH$_2$)$_6$CH$_3$) F L S P E H Q R V Q Q R K E S K K P P A K L Q P R (3)	0.25 ± 0.07	32 ± 4.5	100
15	G S S(CO(CH$_2$)$_6$CH$_3$) F L S P E H Q R V Q Q R K E S K K P P A K L Q P R (2)	48 ± 7.2	42 ± 14	81 ± 14
16	G S S F L S(CO(CH$_2$)$_6$CH$_3$) P E H Q R V Q Q R K E S K K P P A K L Q P R (6)	> 1000	> 10.000	36 ± 1
17	G S S F L S P E H Q R V Q Q R K E S(CO(CH$_2$)$_6$CH$_3$) K K P P A K L Q P R (18)	> 5000	> 10,000	46 ± 2

*,**See corresponding footnotes in Table 1.

acetylation of the hydroxyl group has been occasionally detected,[17] but until now posttranslational acylation of the hydroxyl group with an extended acid such as hydrophobic *n*-octanoic acid has not been reported. This unusual structural feature appears to be necessary for the GH-releasing activity of ghrelin,[14,15] thus suggesting that a hydrophobic interaction between the *n*-octanoyl group and the GHSR1a plays a defining role in molecular recognition. This was supported in this study by agonist potency similar to that of ghrelin of analogues with the unsaturated or branched octanoyl group in position 3 and also of analogues with longer aliphatic chains than the *n*-octanoyl group. For example, peptides with the hydroxyl group of Ser^3 acylated by 11-undecanoic acid or by a 16-carbon chain of palmitic acid (twice as long as *n*-octanoic acid) activated the GHSR1a as efficiently as the parent compound. In contrast, the analogue with the small and thus less hydrophobic acetyl group replacing the *n*-octanoyl group was already a 20-fold less effective agonist than ghrelin, confirming that for efficient activation of hGHSR1a a large hydrophobic group is required in position 3. Kojima and coworkers similarly reported[15] that in the GH-releasing assay, the ghrelin analogue with a *n*-hexanoyl group instead of the *n*-octanoyl group was less potent than ghrelin, whereas in the same assay, the 11-undecanoyl analogue was as potent as the parent compound. The crucial role of hydrophobic interaction in the recognition

Table 4. Truncated Analogues of Human Ghrelin

No.	Compound	Binding Assay* IC_{50} (nM)	Functional Assay** EC_{50} (nM)	Functional Assay** % activation at 10 μM, relative to ghrelin
human ghrelin	$CO(CH_2)_6CH_3$ G S S F L S P E H Q R V Q Q R K E S K K P P A K L Q P R (1–28)	0.25 ± 0.07	32 ± 4.5	100
18	$CO(CH_2)_6CH_3$ G S S F L S P E H Q R V Q Q R K E S K K P P A-NH_2 (1–23)	0.16 ± 0.02	15 ± 4.5	100 ± 4
19	$CO(CH_2)_6CH_3$ G S S F L S P E H Q R V Q Q R K E S-NH_2 (1–18)	0.77 ± 0.18	22 ± 16	92 ± 16
13	$CO(CH_2)_6CH_3$ G S S F L S P E H Q R V Q Q (1–14)	9.6 ± 1.5	17 ± 4	97 ± 9
20	$CO(CH_2)_6CH_3$ G S S F L S P E H Q-NH_2 (1–10)	7.1 ± 5.7	20 ± 6	89 ± 17
21	$CO(CH_2)_6CH_3$ G S S F L-NH_2 (1–5)	55 ± 10	11.5 ± 2.3	96 ± 7
22	$CO(CH_2)_6CH_3$ G S S F-NH_2 (1–4)	889 ± 72	72 ± 29	91 ± 4
23	$CO(CH_2)_6CH_3$ Ac- S S F L-NH_2 (2–5)	> 2000	1150 ± 120	30 ± 5
24	$CO(CH_2)_6CH_3$ G S S-NH_2 (1–3)	> 10,000	>10,000	28 ± 1
25	$CO(CH_2)_6CH_3$ Ac- S F L-NH_2 (3–5)	> 10,000	2500 ± 1200	29 ± 7
26	$CO(CH_2)_6CH_3$ Ac- S S F-NH_2 (2–4)	> 10,000	>10,000	28 ± 1

*,**See corresponding footnotes in Table 1.

of ghrelin peptides by GHSR1a was reemphasized by the activity of our analogue of ghrelin in which the *n*-octanoyl group was replaced by a 1-adamantaneacetyl group. This peptide with this rigid hydrophobic substituent at position 3 elicited a response similar to that of analogous peptides with flexible extended hydrocarbon groups. Acylation of the hydroxyl group of Ser^3 with a smaller benzoyl group yielded an analogue about one-half as potent as ghrelin.

Interaction between ghrelin and hGHSR1a seems to be weaker when Ser^3 is modified by acids which mimic the extended hydrophobic chain of *n*-octanoic acid but also possess an amino or amide group which allows formation of ionic or hydrogen bonds, respectively, with the receptor. At least 10-fold lower agonist potency of compounds **8** and **9** at hGHSR1a strongly indicated that polar interaction between the side chain of residue 3 and the receptor is unfavorable for ligand binding and recognition. The corresponding 8-bromooctanoyl analogue was as active as ghrelin.

To evaluate a role of the ester bond in the side chain of residue 3, an analogue isosteric with ghrelin was prepared. The *n*-octanoyl group was attached to the side chain of residue 3 through an amide bond. This experiment was prompted by the expectation that an amide bond in the side chain of residue 3 should be less susceptible to enzyme-catalyzed cleavage than an ester bond. Thus, 2,3-diaminopropionic acid was incorporated in position 3 of the peptide and its β-amino group acylated with *n*-octanoic acid. The agonist potency of this "isosteric ghrelin" was found to be equal to that of ghrelin itself. This indicates that neither detachment nor migration of the *n*-octanoyl group play a role in the mechanism of activation of hGHSR1a.

Of the four serine residues present in the sequence of human ghrelin, only one in position 3 is acylated by *n*-octanoic acid, whereas the hydroxyl groups of the neighboring Ser^2 and the distant Ser^6 and Ser^{18} are free. This suggested that residues close to Ser^3 probably direct the esterification. Yet, residues next to Ser^3 might

also be involved in formation of a ligand−receptor complex, either by directly interacting with the receptor or by contributing to the formation of a biologically active geometry of the N-terminal segment of ghrelin. To test a role of the C-terminal portion of ghrelin in binding and receptor activation, the hydroxyl group of Ser^6 or Ser^{18} was selectively acylated with *n*-octanoic acid. Analogues with the *n*-octanoyl group attached to the side chain of Ser^6 or Ser^{18} had poor activity, even at micromolar concentrations. This suggested that mainly the N-terminal part of ghrelin is involved in molecular recognition. The *n*-octanoyl Ser^2 analogue, however, was equipotent to ghrelin, revealing that the GHS receptor does not recognize the exact location of the *n*-octanoyl group in the N-terminal segment of ghrelin; it does not distinguish between Ser(*n*-octanoyl) in position 2 or 3. The same receptor did not recognize peptides unrelated to ghrelin, even when they contained the Ser(*n*-octanoyl) residue. For example, compounds Tyr-Ser(*n*-octanoyl)-Tyr-Arg-NH_2 and Pro-Lys-Phe-Glu-Ala-Val-Glu-Lys-Pro-Gln-Ser(*n*-octanoyl)-NH_2 did not activate hGHSR1a even at micromolar peptide concentrations.

To determine the smallest segment of ghrelin that can elicit a biological response at hGHSR1a similar to that of the parent compound, truncated analogues of ghrelin were evaluated for binding to and activation of hGHSR1a. The N-terminal part of ghrelin with Ser(*n*-octanoyl) in position 3 was preserved, but the chain was gradually shortened by the omission of blocks of several amino acids from the C-terminal end of ghrelin. Interestingly, a peptide encompassing only the first 5 residues of ghrelin, Gly-Ser-Ser(*n*-octanoyl)-Phe-Leu-NH_2, activated the hGHSR1a as efficiently as the full-length ghrelin. The still shorter tetrapeptide, Gly-Ser-Ser(*n*-octanoyl)-Phe-NH_2, was somewhat less potent. Further omission of amino acids from the C-terminus or N-terminus of the N-terminal tetrapeptide yielded compounds inactive at hGHSR1a, even at micromolar concentrations. Thus, the short segment of ghrelin, Gly-Ser-Ser(*n*-octanoyl)-Phe, appears to constitute the essential part of the chain required for activity at hGHSR1a. Although the N-terminal pentapeptide activated hGHSR1a as efficiently as ghrelin, in competition with [^{35}S]MK-0677 for binding to the cloned hGHSR1a, its affinity was about 200-fold lower than that of the full-length compound. This seems to imply that ghrelin(1−5) and MK-0677 bind differently to the cloned hGHSR1a.

Our studies thus confirmed that the *n*-octanoyl group of ghrelin is one of the principal structural features determining its potency at hGHSR1a. For maximum activity bulky, flexible, or rigid hydrophobic groups are needed in the side chain of residue 3, whereas the hydrophilic groups in the same position significantly reduce agonist activity. The ester group, however, is not essential for binding and activity: it can be replaced by an amide group. Our study also showed that the entire sequence of ghrelin is not necessary for activation of hGHSR1a. The short peptides encompassing the first 4 or 5 residues of ghrelin were found to activate the hGHSR1a about as efficiently as the full-length ghrelin, thus implying that the N-terminal Gly-Ser-Ser(*n*-octanoyl)-Phe segment constitutes the "essential core" required for efficient binding to and activation of hGHSR1a.

In summary, we report here the synthesis of short peptides which are potent agonists at the hGHSR1a. The ghrelin-derived peptides investigated in this study should be useful in further studies on the physiological role of ghrelin and also in the design of new GHSs.

Experimental Section

Materials. Fmoc-protected amino acids were obtained from AnaSpec (San Jose, CA), 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin from PE Biosystems (Foster City, CA), Boc-7-aminoheptanoic acid from Bachem (King of Prussia, PA) and *n*-octanoic acid, 2,4,6-octatrienoic acid, 2-propylpentanoic acid, 11-undecanoic acid, palmitic acid, 8-bromooctanoic acid, 1-adamanteneacetic acid and benzoic acid from Aldrich (Milwaukee, WI).

Peptide Synthesis, Purification, and Characterization. Elongation of peptidyl chains on 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin was performed on a 431A ABI peptide synthesizer. Manufacturer-supplied protocols were applied for coupling of the hydroxybenzotriazole esters of amino acids in *N*-methylpyrrolidone (NMP). The fluorenylmethyloxycarbonyl (Fmoc) group was used as a semipermanent α-amino protecting group, whereas the side chain protecting groups were: *tert*-butyl for serine, trityl for serine in position 3 and for histidine and glutamine, *tert*-butyl ester for glutamic acid, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for arginine, *tert*-butyloxycarbonyl (Boc) for lysine and for α-amino group of glycine in position 1. The peptidyl resin was then transferred into a vessel and the trityl group from the side chain of Ser^3 was manually removed with 1% trifluoroacetic acid (TFA) in dichloromethane (45 min at room temperature). The peptidyl resin was thoroughly washed and then agitated for 4 h with the 6-fold excess of each 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) and a selected acid in dichloromethane (DCM) or NMP, in the presence of a catalytic amount of 4-(dimethylamino)pyridine (ca. 10 mg). The peptidyl resin was again washed with DCM, NMP, and methanol, dried, and treated with TFA in the presence of scavengers (ca. 3% total of the mixture of water−anisole−triethylsilane, 1:1:1, v/v/v). After 1.5 h, the resin was filtered off, TFA was removed in vacuo and the residue was triturated with ether. The precipitate which formed was filtered off, washed thoroughly with ether, and dried. The crude peptide was analyzed by analytical reverse-phase high-pressure liquid chromatography (RP HPLC) on a C18 Vydac column attached to a Waters 600E system with automatic Wisp 712 injector and 991 photodiode array detector. A standard gradient system of 0−100% buffer B in 30 min (G1) or a gradient of 20−80% buffer B in 30 min (G2) was used for analysis; buffer A was 0.1% trifluoroacetic acid in water and buffer B was 0.1% trifluoroacetic acid in acetonitrile. HPLC profiles were recorded at 210 and 230 nm. Preparative separations were performed on a Waters Delta Prep 4000 system with a semipreparative C18 RP Vydac column. The above-described solvent system of water and acetonitrile, in a gradient of 20−80% buffer B in 60 min (G3) or in a gradient of 0−60% buffer B, was used for separations. The chromatographically homogeneous compounds (purity > 94%) were analyzed by electrospray mass spectrometry (Hewlett-Packard series 1100 MSD spectrometer) and by peptide sequencing (ABI 494 cLC protein sequencer).

The ^{1}H NMR spectra (400 MHz) of compounds **21**−**26** were recorded on Varian Unity 400 (Varian Inc., California) in CD_3OH (0.13 mL) at 25 °C in 3-mm NMR tubes using a 3-mm indirect detection gradient probe (Nalorac Corp., California). The data processing was performed on the spectrometer. Chemical shifts were reported on the δ scale (ppm) by assigning the residual solvent peak at 3.30 ppm to ^{1}H of methanol. The COSY spectra were acquired with a spectral width of 3597.1 Hz into 1K data points in f_2, with 357

increments in the f_1 dimension, and the 90° pulse was 7.75 μs. The ROESY spectra were acquired with a spectral width of 3597.1 Hz into 1K data points in f_2 and with 358 increments in the f_1 dimension. The delay between the successive pulses was 2 s, the mixing time used was 0.3 s, and the 90° pulse was 7.75 μs.

Filter Binding Assay. Binding of [^{35}S]MK-0677 to crude membranes prepared from HEK 293-aequorin stable cell lines was performed as described in detail in refs 9 and 10. For a 96-well filter binding assay, 0.05 nM [^{35}S]MK-0677 (specific activity ~ 1200 Ci/mmol) was bound to 4 μg of membrane protein/well with or without competing test ligand. The bound membranes were filtered on 0.5% polyethylenimine prewet filters (UniFilter 96 GF/C, Packard #6005174, Meriden, CT). Filters were washed 8 times, dried, and counted with Microscint 20 (Packard #6013621, Meriden, CT). IC_{50} values were determined from three separate assays performed in triplicate.

Aequorin Bioluminescence Functional Assay. The aequorin bioluminescence assay is a reliable test for identifying G-protein-coupled receptors which couple through the Gα protein subunit family consisting of G_q and G_{11} which leads to the activation of phospholipase C, mobilization of intracellular calcium, and activation of protein kinase C. A stable cell line expressing the hGHSR1a and the aequorin reporter protein were used.[18] The assay was performed using a Luminoskan RT luminometer (Labsystems Inc., Gaithersburg, MD) controlled by custom software written for a Macintosh PowerPC 6100. 293AEQ17/GHSR1a cells were cultured for 72 h and the apo-aequorin in the cells was charged for 1 h with coelenterazine (10 μM) under reducing conditions (300 μM reduced glutathione) in ECB buffer (140 mM NaCl, 20 mM KCl, 20 mM HEPES−NaOH, pH 7.4, 5 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mg/mL bovine serum albumin). The cells were harvested, washed once in ECB medium, and resuspended to 500 000 cells/mL. 100 mL of cell suspension (corresponding to 5×10^4 cells) was then injected into the test plate containing the ghrelin peptides, and the integrated light emission was recorded over 30 s, in 0.5-s units. 20 μL of lysis buffer (0.1% final Triton X-100 concentration) was then injected and the integrated light emission recorded over 10 s, in 0.5-s units. The "fractional response" values for each well were calculated by taking the ratio of the integrated response to the initial challenge to the total integrated luminescence including the Triton X-100 lysis response. The functional EC_{50} values were measured in three separate assays.

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Supporting Information Available: Table S1 with RP HPLC, MS, and peptide sequencing data for compounds **1**−**26**; Tables S2 and S3 with ^{1}H NMR chemical shift assignments for compounds **21**−**26**. This material is available free of charge via the Internet at http://pubs.acs.org.

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