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Structure–Activity Relationship of Ghrelin: Pharmacological Study of Ghrelin Peptides

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Ghrelin, a novel peptide purified from the stomach, is the endogenous ligand of the growth hormone secretagogue receptor. The Ser[3] residue of ghrelin is modified with a lipid *n*-octanoic acid, a modification necessary for hormonal activity. To clarify the role of acyl modification and to identify the active core of ghrelin, we examined the activities of partially digested ghrelin and synthetic ghrelin derivatives. The activities confirmed that the N-terminal portion is the active core. Moreover, synthetic ghrelin derivatives demonstrated that octanoic acid is not the only modification of the Ser[3] side chain to sustain the activity of ghrelin; other acyl acid modifications maintained activity. Amino acid replacement of Ser[3] indicated that an L-configuration of the third residue is critical for ghrelin activity. In addition, more stable ether or thioether bonds are capable of replacing the octanoyl ester bond in ghrelin, advantageous for the generation of pharmaceuticals with longer stability. © 2001 Academic Press

Key Words: **ghrelin; growth hormone; growth hormone secretagogue; peptide modification; lipid modification; acyl acid; peptide synthesis.**

Abbreviations used: GHS, growth hormone secretagogue; GH, growth hormone; GHS-R, growth hormone secretagogue receptor; $[Ca^{2+}]_i$, intracellular calcium concentration; Nal, β-naphthyl-L-alanine; Nle, norleucine; Fmoc, 9-fluorenylmethoxycarbonyl; Boc, *tert*-butyloxycarbonyl; β-Nal, 2-naphtylalanine; tBu, *tert*-butyl; Trt, trityl; HOBt, 1-hydroxybenzotriazole; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIPEA, diisopropylethylamine; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; DMAP, 4-dimethylaminopyridine; Tips, triisopropylsilane; TFA, trifluoroacetic acid.

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Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor (GHS-R), has potent growth hormone releasing activity both *in vitro* and *in vivo* (1–4). Purified from both rat and human stomachs, ghrelin is modified by *n*-octanoic acid at the hydroxyl group of Ser[3]. This modification is essential for ghrelin activity, indicating the importance of the peptide portion surrounding Ser[3] in the binding of ghrelin to the growth hormone secretagogue receptor (GHS-R) (1). As no octanoyl modifications required for bioactive peptides and proteins have been previously described in mammals, studies of this modification may uncover a novel processing mechanism to produce active peptide hormones. The identity of the portions of the ghrelin peptide necessary for activity, however, remains unknown. Moreover, it is unclear whether the octanoyl modification at the Ser[3] is critical for ghrelin activity or additional acyl acid modifications can mediate equivalent activity. To address these issues, we examined the active core of ghrelin. Several ghrelin fragments were prepared by either partial enzymatic digestion of ghrelin or chemical synthesis to determine the active core. In addition, we investigated the role of the octanoyl group, its bonding structure and the identity of the third residue to examine the relationship between side-chain properties and the activity of ghrelin.

MATERIALS AND METHODS

Partial digestion of rat ghrelin peptide. Rat ghrelin (20 μg) was digested for 3 h with 400 ng of trypsin (Sigma, St. Louis, MO) in 100 μl of 50 mM Tris–HCl (pH 8.0) at 37°C. Rat ghrelin fragments [1–11] (compound **6**), [1–15] (compound **5**) and [16–28] (compound **12**) were purified from the tryptic digests by reverse-phase high-performance liquid chromatography (RP-HPLC). Ghrelin fragments [1–10] (compound **7**), [1–9] (compound **8**), and [1–8] (compound **9**) were prepared by the successive digestion of rat ghrelin [1–11] with carboxypeptidase A (200 ng) (Sigma) in 450 μl of 50 mM Tris–HCl (pH 8.0) at 37°C. The N-terminal fragment of ghrelin [1–4] (compound **10**) was

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prepared from rat ghrelin (3 μg) by a three-hour digestion with 200 ng of chymotrypsin in 100 μl of 50 mM Tris–HCl (pH 8.0) at 37°C. The digested peptides, purified by RP-HPLC, were subjected to electrospray ionization–mass spectrometry (ESI–MS) to confirm the identity of the peptides by molecular mass.

Identification of ghrelin fragments by mass spectrometric analyses. ESI-MS was performed utilizing a quadrupole mass spectrometer SSQ7000 (Finnigan, San Jose, CA), equipped with a Finnigan ESI source. A needle capillary was heated to 150°C to evaporate samples. Digested ghrelin peptide samples, dissolved in 50% (v/v) methanol/1% acetic acid, were introduced into the +4.5 kV (positive ionization) ion source by direct infusion with a syringe pump at a flow rate of 5 μl/min. The molecular masses of the purified peptides were calculated using the ICIS software, Bioworks, provided by Finnigan.

Peptide synthesis. N-α-Protected amino acids were obtained from either Applied Biosystems (Foster City, CA) or Watanabe Chemical Industries Ltd. (Hiroshima, Japan). Amino acids were of the L-configuration, unless otherwise mentioned. Fmoc amino acids included: Ser(tBu), Ser(Trt), Ser(*n*-octyl), Cys(*n*-octyl), Glu(OtBu), His(Boc), Gln(Trt), Arg(2,2,5,7,8-pentamethylchroman-6-sulfonyl), Lys(Boc), Phe, Leu, Pro, Val, Ala, Trp, Ile, β-Nal, and Nle. Boc-Gly was used for N-terminal incorporation. Peptide synthesis was performed using an Applied Biosystems Model 433A Peptide synthesizer on a 0.25 mmol scale, according to the manufacturer's standard protocol for Fmoc chemistry (HBTU/HOBt/DIPEA) (5, 6). Acylation at the target seryl hydroxyl group was achieved by exploiting the differential acid-sensitivity of *O*-Trt and *O*-tBu groups. The peptide resins were first treated with the solution of 1% (v/v) TFA, 5% (v/v) Tips in CH_2Cl_2 for 30 min to remove Trt group. Next, the liberated hydroxyl groups were reacted with a variety of carboxylic acids (acetic acid, *n*-butyric acid, hexanoic acid, octanoic acid, 3-octenoic acid, decanoic acid, lauric acid, palmitic acid, 4-methylpentanoic acid or 3-phenylpropionic acid) using EDC · HCl and DMAP. The O-acylated peptides were cleaved from the resins and all protecting groups were removed by the treatment with a cocktail of 88% (v/v) TFA, 2% (v/v) Tips, 5% (v/v) phenol, and 5% (v/v) H_2O. After 2 h, the peptides were isolated and purified by RP-HPLC using preparative C18 (YMC-Pack, ODS-A, 250 × 20 mm) and C4 (YMC-Pack, PROTEIN-RP, 250 × 20 mm) columns. Identity of the peptides was assured by an amino acid composition analyzer (Hitachi L-8500), an amino acid sequence analyzer (Applied Biosystems 492) and ESI-MS on a quadrupole mass spectrometer (Finnigan MAT TSQ 700).

Calcium-mobilization assays. Construction of a growth hormone secretagogue receptor (GHS-R)-expressing cell line has been described previously (1, 7). CHO-GHSR62 cells were plated at 4×10^4 cells/well in flat-bottom, black-wall 96-well plates (Corning Corstar Corp., Cambridge, MA) for 12–15 h prior to the assay. Cells were loaded for 1 h with 4 μM Fluo-4-AM fluorescent indicator dye (Molecular Probes, Inc., Eugene, OR) in assay buffer [Hanks' balanced salt solution (HBSS), 10 mM Hepes, 2.5 mM probenecid, 1% fetal calf serum (FCS)]. Following four washes in assay buffer without FCS, the intracellular calcium concentration ($[Ca^{2+}]_i$) changes were measured using a fluorometric imaging plate reader (FLIPR) (Molecular Devices, Sunnyvale, CA) (8). We used the maximum fluorescence change over baseline to determine agonist responses.

Growth hormone (GH) secretion activity in rats. Sprague–Dawley male rats (7 weeks, 250–280 g) were purchased from Charles River (Yokohama, Japan). Rat was anesthetized with Nembutal and cannulated into femoral artery. Rat ghrelin or [Cys^3(octyl)]-rat ghrelin was dissolved in saline and was injected into tail vein. Blood was withdrawn through femoral artery cannulae and was added with 50 mg EDTA, 5000U Trasylol/ml (1/50 volume of blood). Plasma was obtained after centrifugation. The GH plasma concentration was determined with Biotrak Rat GH RIA kits (Amersham, Buckinghamshire, UK).

RESULTS AND DISCUSSION

Minimum Structural Requirement for Ghrelin's Activity

The amino acid sequence surrounding the modified Ser^3 of ghrelin may encode a motif directing the octanoyl modification, as the 10 N-terminal amino acids are identical among the different mammalian species. The C-terminal portions of ghrelin, however, are varied, suggesting that the N-terminal, octanoyl-modified portion contains the ghrelin active core. To confirm the N-terminal portion of ghrelin as the active core, we examined the GHS-R stimulating activity of both enzymatically and chemically prepared ghrelin fragments. Rat ghrelin was partially digested with either trypsin, chymotrypsin or Arg-carboxypeptidase. Combinations of these enzymes generated fragments of rat ghrelin[1–15], [1–11], and ghrelin[1–10], [1–9], [1–8], [1–4] and [16–28] (compounds **5–10, 12**). After peptide purification by RP-HPLC, we confirmed the structures of these fragments by sequencing and mass spectrometric analyses. Human ghrelin, rat ghrelin, des-Gln^{14}-rat ghrelin (compound **3**), des-acyl-rat ghrelin (compound **4**) and ghrelin [1–3]-NH_2 (compound **11**) were chemically. Peptides were evaluated for the potency elevating intracellular calcium concentrations (Table 1). Compound **5** was less active than the complete peptide, possessing an EC_{50} of 8.6 nM compared to 1.5 nM for the intact ghrelin. The C-terminal fragment of ghrelin, ghrelin[16–28] (compound **12**), was completely inactive, indicating that the active core of ghrelin is located at N-terminal region. Beginning with the sequence of compound **5,** successive deletion of the C-terminal amino acid residue clarified the minimum structural requirements for ghrelin activity [1–4]. We reconfirmed the importance of an octanoyl group; des-acyl-rat ghrelin was 2300 times less active than the native peptide. When the peptide length was shortened to a tripeptide, no activity was observed, even in the presence of the octanoyl modification. These results demonstrated that the minimum core of ghrelin mediating GHS-R stimulating activity resides in the N-terminal tetrapeptide, absolutely requiring the octanoyl group at the third residue (9).

Contribution of the Third Residue and the Adjoining Side Chain of Ghrelin to Activity

Effect of fatty acid side chain length. We next evaluated the effect of both fatty acid length at Ser^3 and differing hydrophobic side chains on the GHS-R stimulating activity of ghrelin. A series of fatty acids possessing varying chain lengths were esterified onto the hydroxyl group of Ser^3. Beginning with the des-acyl-rat ghrelin (EC_{50} = 3500 nM), the adjacent fatty acid chain was systematically elongated by two carbon units. The activity was substantially enhanced by the introduc-

TABLE 1

Minimum Structural Requirement for Activity

No.	Peptide	Structure	EC_{50} (nM)
1	Human ghrelin	GSS(*O*-CO-C_7H_{15})FLSPEHQRVQQRKESKKPPAKLQPR	1.3
2	Rat ghrelin	GSS(*O*-CO-C_7H_{15})FLSPEHQKAQQRKESKKPPAKLQPR	1.5
3	[des-Gln^{14}]-Rat ghrelin	GSS(*O*-CO-C_7H_{15})FLSPEHQKAQRKESKKPPAKLQPR	1.5
4	[des-acyl]-Rat ghrelin	GSSFLSPEHQKAQQRKESKKPPAKLQPR	3,500
5	Rat ghrelin [1–15]	GSS(*O*-CO-C_7H_{15})FLSPEHQKAQQR	8.6
6	Rat ghrelin [1–11]	GSS(*O*-CO-C_7H_{15})FLSPEHQK	15
7	Ghrelin [1–10]	GSS(*O*-CO-C_7H_{15})FLSPEHQ	19
8	Ghrelin [1–9]	GSS(*O*-CO-C_7H_{15})FLSPEH	38
9	Ghrelin [1–8]	GSS(*O*-CO-C_7H_{15})FLSPE	100
10	Ghrelin [1–4]	GSS(*O*-CO-C_7H_{15})F	480
11	Ghrelin [1–3]-NH_2	GSS(*O*-CO-C_7H_{15})-NH_2	>10,000
12	Ghrelin [16–28]	KESKKPPAKLQPR	>10,000

Note. EC_{50} is the concentration of peptides or peptide derivatives at $[Ca^{2+}]_i$ increase on GHS-R-expressing cells.

tion of an acetyl (C2:0) group (EC_{50} = 780 nM), increasing as the function of the length of the fatty acid chain (Table 2). The maximum response was observed in the presence of an octanoyl (C8:0) group, supporting the structural observations with natural ghrelin. The activity was maintained with increasing side chain length up to a palmitoyl (C16:0) group (compound **18,** EC_{50} = 6.5 nM). The highest observed activity for the octanoyl (C8:0) modification suggests that the C8:0 unit was natural selected during molecular evolution. We are currently investigating the natural existence of endogenous C12:0 or C16:0-modified ghrelin in rats. The incorporation of an unsaturated or a branched fatty acid [3-octenoyl (C8:1): compound **19,** 4-methylpentanoyl: compound **20**] did not diminish ghrelin activity, displaying EC_{50}s of 1.7 and 4.4 nM, respectively. In addition, the aliphatic moiety was amenable to the substitution of aromatic groups, generating such active molecules as Ser^3(phenylpropionyl)- (compound **21,** EC_{50} = 1.4 nM) or [β-Nal^3]-human ghrelin (compound **25,** EC_{50} = 8.2 nM); these results indicate that hydrophobicity of the side chain at Ser^3, regardless of aliphaticity or aromaticity, is essential for the GHS-R stimulating activity of ghrelin.

Effect of the bonding structure of octanoic acid with the ghrelin peptide. To compare the effect of bonding structures on ghrelin activity, we altered the ester bond between octanoic acid and the Ser^3 side chain to a more chemically stable thioether and ether bonds. [Ser^3(octyl)]-human ghrelin (compound **22**) as well as [Cys^3(octyl)]-rat ghrelin (compound **23**) both retained GHS-R stimulating activity, possessing EC_{50} of 1.2 and 5.4 nM, respectively (Table 2). Compound **23** was also evaluated in rats for the ability to stimulate the secretion of growth hormone (GH) *in vivo.* The level of GH in plasma, stimulated by the intravenous injection of compound **23** (5 μg/head, iv), is equivalent to that produced by treatment with native rat ghrelin (5 μg/head, iv) (Fig. 1). The thioether derivative (compound

TABLE 2

Property of Ser^3 Side Chain of Ghrelin and Activity

No.	Peptide	Structure	EC_{50} (nM)
2	Rat ghrelin	GSS(*O*-CO-C_7H_{15})FLSPEHQKAQQRKESKKPPAKLQPR	1.5
4	[des-acyl]-Rat ghrelin	GSSFLSPEHQKAQQRKESKKPPAKLQPR	3500
13	[Ser^3(acetyl)]-Rat ghrelin	GSS(*O*-CO-CH_3)FLSPEHQKAQQRKESKKPPAKLQPR	780
14	[Ser^3(butyryl)]-Rat ghrelin	GSS(*O*-CO-C_3H_7)FLSPEHQKAQQRKESKKPPAKLQPR	280
15	[Ser^3(hexanoyl)]-Rat ghrelin	GSS(*O*-CO-C_5H_{11})FLSPEHQKAQQRKESKKPPAKLQPR	16
16	[Ser^3(decanoyl)]-Rat ghrelin	GSS(*O*-CO-C_9H_{19})FLSPEHQKAQQRKESKKPPAKLQPR	1.7
17	[Ser^3(lauroyl)]-Rat ghrelin	GSS(*O*-CO-$C_{11}H_{23}$)FLSPEHQKAQQRKESKKPPAKLQPR	2.4
18	[Ser^3(palmitoyl)]-Rat ghrelin	GSS(*O*-CO-$C_{15}H_{31}$)FLSPEHQKAQRKESKKPPAKLQPR	6.5
19	[Ser^3(3-octenoyl)]-Human ghrelin	GSS(*O*-$COCH_2CH{=}CH(CH_2)_3CH_3$)FLSPEHQRVQQRKESKKPPAKLQPR	1.7
20	[Ser^3(4-methylpentanoyl)]-Human ghrelin	GSS(*O*-CO-$CH_2CH_2CH(CH_3)_2$)FLSPEHQRVQQRKESKKPPAKLQPR	4.4
21	[Ser^3(3-phenylpropionyl)]-Human ghrelin	GSS(*O*-CO-CH_2CH_2Ph)FLSPEHQKAQQRKESKKPPAKLQPR	1.4
22	[Ser^3(octyl)]-Human ghrelin	GSS(*O*-C_8H_{17})FLSPEHQRVQQRKESKKPPAKLQPR	1.2
23	[Cys^3(octyl)]-Rat ghrelin	GSS(*S*-C_8H_{17})FLSPEHQKAQQRKESKKPPAKLQPR	5.4

Note. EC_{50} is the concentration of peptides or peptide derivatives at $[Ca^{2+}]_i$ increase on GHS-R-expressing cells.

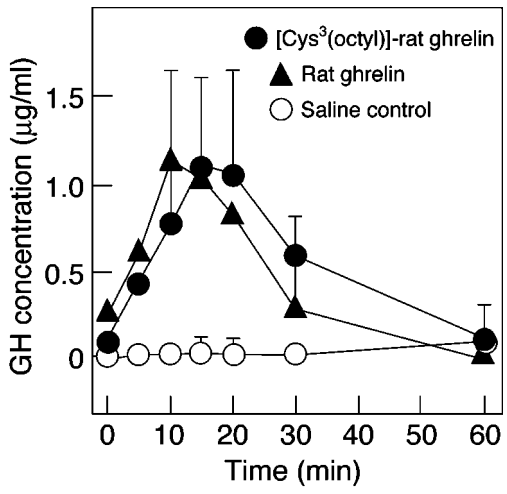

FIG. 1. Time course of plasma growth hormone concentrations following intravenous injection of [Cys^3(octyl)]-rat ghrelin and rat ghrelin into male rats. Blood samples were collected from cannulae in the femoral artery. GH concentrations were measured by radioimmunoassay. Data represent means (±SE) of five rats.

23), however, induced the prolonged onset of GH-stimulation, compared to rat ghrelin: We observed a 5- to 10 min delay in the T_{max}. The increased stability of the thioether bond at the third residue over the ester bond may contribute to the different time-course of GH stimulation, induced by [Cys^3(octyl)]-rat ghrelin.

Effect of amino acid replacement at Ser^3. We then investigated the effects of amino acid substitutions at the third position, replacing the octanoylated serine (Table 3). The replacement of this serine with an aromatic amino acid, such as Trp (compound **24**) or β-Nal (compound **25**), preserved ghrelin activity, with an EC_{50} of 31 nM or 8.2 nM, respectively. Incorporation of aliphatic amino acid, such as Val, Leu, Ile, and Nle (compound **27–30**), lead to a complete loss of potency. Thus, GHS-R prefers aromatic side chains over the shorter aliphatic chains.

To examine the influence of the configuration of third amino acid residue on ghrelin activity, we substituted D-conformation amino acids for the third residue serine. Incorporation of [D-β-Nal^3] into human ghrelin (compound **26**) resulted in severely reduced activity (EC_{50} > 10,000 nM). Unlike peptidyl hormone secretagogues (GHSs) (10), L-configuration of the third residue in ghrelin is critical for activity.

Charges of ghrelin and activity. We examined the effect of altering the charge of the N-terminal and C-terminal residues within ghrelin (Table 4). Amidation of the C-terminal carboxylic acid enhanced activity; ghrelin[1–8]-NH_2 (compound **32**) was approximately eight times more potent (EC_{50} = 13 nM) than native ghrelin[1–8] (compound **9,** EC_{50} = 100 nM). The insertion of a basic amino acid in the C terminus of ghrelin[1–7] (compound **33, 34**) also increased the potency of GHS-R stimulation. The EC_{50}s for ghrelin[1–7]-Arg-NH_2 (compound **33**) and ghrelin[1–7]-Lys-NH_2 (compound **34**) were elevated to 1.1 nM, comparable to that of native ghrelin possessing all 28 amino acid residues. [Lys^3]-human ghrelin (compound **35**) displayed a similar elevation of activity. The potency of both [Nle^3]-human ghrelin (compound **30,** EC_{50} = 2800 nM) and [Lys^3]-human ghrelin (compound **35**) was increased to an EC_{50} of 120 nM simply by adding an amino group to the side chain of Nle, substituting *n*-butyl for Nle, *n*-butyl amine for Lys. The elimination of positive charges at the N-terminus of ghrelin, however, lowered ghrelin activity, as observed for [N^{α}-acetyl]-ghrelin[1–10] (compound **31**). The incorporation of Tyr at the N-terminus of rat ghrelin (compound **36**) also lowered the activity by 80-fold. These results indicate the role of charge in ghrelin activity. Positive charges are essential in maintaining a high level of activity; negative charges inhibit activity.

In this study, we identified the minimum active core of ghrelin as the N-terminal tetrapeptide possessing the octanoyl modification at the third serine. We also discovered that the octanoyl ester bond can be effectively replaced by a more stable ether or thioether bond, an advantageous substitution creating long-term stability in pharmaceuticals. We demonstrated that

TABLE 3

Amino Acid Replacements at Ser^3 of Ghrelin and Activity

No.	Peptide	Structure	EC_{50} (nM)
1	Human ghrelin	GSS(*O*-CO-C_7H_{15})FLSPEHQRVQQRKESKKPPAKLQPR	1.3
24	[Trp^3]-Human ghrelin	GSWFLSPEHQRVQQRKESKKPPAKLQPR	31
25	[Nal^3]-Human ghrelin	GS-Nal-FLSPEHQRVQQRKESKKPPAKLQPR	8.2
26	[D-Nal^3]-Human ghrelin	GS-^{D}Nal-FLSPEHQRVQQRKESKKPPAKLQPR	>10,000
27	[Val^3]-Human ghrelin	GSVFLSPEHQRVQQRKESKKPPAKLQPR	1,600
28	[Leu^3]-Human ghrelin	GSLFLSPEHQRVQQRKESKKPPAKLQPR	4,400
29	[Ile^3]-Human ghrelin	GSIFLSPEHQRVQQRKESKKPPAKLQPR	>10,000
30	[Nle^3]-Human ghrelin	GS-Nle-FLSPEHQRVQQRKESKKPPAKLQPR	2,800

Note. EC_{50} is the concentration of peptides or peptide derivatives at $[Ca^{2+}]_i$ increase on GHS-R-expressing cells.

TABLE 4

Charges of Ghrelin Derivative Peptides and Activity

No.	Peptide	Structure	EC_{50} (nM)
2	Rat ghrelin	GSS(*O*-CO-C_7H_{15})FLSPEHQKAQQRKESKKPPAKLQPR	1.5
4	[des-acyl]-Rat ghrelin	GSSFLSPEHQKAQQRKESKKPPAKLQPR	3,500
7	Ghrelin [1–10]	GSS(*O*-CO-C_7H_{15})FLSPEHQ	19
9	Ghrelin [1–8]	GSS(*O*-CO-C_7H_{15})FLSPE	100
31	[N^{α}-acetyl]-Ghrelin [1–10]	Ac-GSS(*O*-CO-C_7H_{15})FLSPEHQ	>10,000
32	Ghrelin [1–8]-NH_2	GSS(*O*-CO-C_7H_{15})FLSPE-NH_2	13
33	Ghrelin[1–7]-Arg-NH_2	GSS(*O*-CO-C_7H_{15})FLSPR-NH_2	1.1
34	Ghrelin[1–7]-Lys-NH_2	GSS(*O*-CO-C_7H_{15})FLSPK-NH_2	1.1
35	[Lys^3]-Human ghrelin	GSKFLSPEHQRVQQRKESKKPPAKLQPR	120
36	[N^{α}-Tyr]-Rat ghrelin	YGSS(*O*-CO-C_7H_{15})FLSPEHQKAQQRKESKKPPAKLQPR	120

Note. EC_{50} is the concentration of peptides or peptide derivatives at $[Ca^{2+}]_i$ increase on GHS-R-expressing cells.

hydrophobicity surrounding the third amino acid residue, regardless of the aromaticity or aliphaticity, is essential in GHS activity, providing multiple approaches for designing novel GHSs.

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REFERENCES

1. Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402,** 656–660.
2. Arvat, E., Di Vito, L., Broglio, F., Papotti, M., Muccioli, G., Dieguez, C., Casanueva, F. F., Deghenghi, R., Camanni, F., and Ghigo, E. (2000) Preliminary evidence that ghrelin, the natural GH secretagogue (GHS)-receptor ligand, strongly stimulates GH secretion in humans. *J. Endocrinol. Invest.* **23,** 493–495.
3. Peino, R., Baldelli, R., Rodriguez-Garcia, J., Rodriguez-Segade, S., Kojima, M., Kangawa, K., Arvat, E., Ghigo, E., Dieguez, C., and Casanueva, F. F. (2000) Ghrelin-induced growth hormone secretion in humans. *Eur. J. Endocrinol.* **143,** R011–R014.
4. Takaya, K., Ariyasu, H., Kanamoto, N., Iwakura, H., Yoshimoto, A., Harada, M., Mori, K., Komatsu, Y., Usui, T., Shimatsu, A., Ogawa, Y., Hosoda, K., Akamizu, T., Kojima, M., Kangawa, K., and Nakao, K. (2000) Ghrelin strongly stimulates growth hormone release in humans. *J. Clin. Endocrinol. Metab.* **85,** 4908–4911.
5. Carpino, L. A., and Han, G. Y. (1972) The 9-fluorenylmethoxycarbonyl amino-protecting group. *J. Org. Chem.* **37,** 3404–3409.
6. Chang, C. D., Felix, A. M., Jimenez, M. H., and Meienhofer, J. (1980) Solid-phase synthesis of somatostatin using mild base cleavage of *N* alpha-9-fluorenylmethoxycarbonyl amino acids. *Int. J. Pept. Protein Res.* **15,** 485–494.
7. Hosoda, H., Kojima, M., Matsuo, H., and Kangawa, K. (2000) Purification and characterization of rat des-Gln14-Ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor. *J. Biol. Chem.* **275,** 21995–22000.
8. Schroeder, K. S., and Neagle, B. D. (1996) FLIPR: A new instrument for accurate, high throughput optical screening. *J. Biomol. Screening* **1,** 75–80.
9. Bednarek, M. A., Feighner, S. D., Pong, S. S., McKee, K. K., Hreniuk, D. L., Silva, M. V., Warren, V. A., Howard, A. D., Van Der Ploeg, L. H., and Heck, J. V. (2000) Structure–function studies on the new growth hormone-releasing peptide, ghrelin: Minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. *J. Med. Chem.* **43,** 4370–4376.
10. Nargund, R. P., Patchett, A. A., Bach, M. A., Murphy, M. G., and Smith, R. G. (1998) Peptidomimetic growth hormone secretagogues. Design considerations and therapeutic potential. *J. Med. Chem.* **41,** 3103–3127.