Articles

Fluorine and Rhenium Substituted Ghrelin Analogues as Potential Imaging Probes for the Growth Hormone Secretagogue Receptor

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In our effort to create imaging probes targeting the growth hormone secretagogue receptor (GHSR), we now report on the design and synthesis of fluorine and rhenium containing ghrelin analogues through modification of the *n*-octanoyl Ser-3 side chain. Fluorine analogues were designed whereby the fluorine atom is situated at the terminus of an aliphatic chain using diaminopropionic acid (Dpr) as residue-3. Truncated ghrelin(1−5) and ghrelin(1−14) fluorine-bearing analogues were prepared, the best of which had a 28 nM IC_{50} for GHSR. Ghrelin(1−14) analogues were also prepared containing rhenium, as a surrogate metal for technetium-99m, with a cyclopentadienylrhenium tricarbonyl being situated at the terminus of the residue-3 side chain, yielding compounds the best of which had a 35 nM IC_{50}. This represents a rare case of incorporating rhenium into a peptide structure where the metal complex is required for biological activity. These fluorine and rhenium derivatives demonstrate the ability to modify the Ser-3 side chain of ghrelin in order to create imaging probes for the GHSR.

Introduction

The role of radiolabeled peptides in clinical diagnosis and therapy, especially in oncology, has been increasing in the past few decades. Peptides as targeting agents have several advantages over other types of targeting agents such as small molecules and antibodies.[1−3] The use of peptides as tumor imaging probes is based on targeting by the peptide ligand to specific cell surface receptors that are highly expressed in tumor cells but not in the surrounding normal cells. The binding of the peptide ligand to its receptor is often followed by internalization, leading to a high tumor-to-background ratio, which is further augmented by rapid tissue penetration and fast blood clearance. Peptides can be synthesized using an automated synthesizer from readily available starting materials which leads to simple and inexpensive large scale production.[1,4] However, despite the large number of peptides and their receptors that are currently under investigation, only a few are approved as imaging probes and marketed for clinical purposes, and their use in different types of tumors is limited.[1] Here we report on a preliminary investigation of the potential use of radiolabeled ghrelin analogues as cancer imaging probes targeting the growth hormone secretagogue receptor (GHSR[a]).

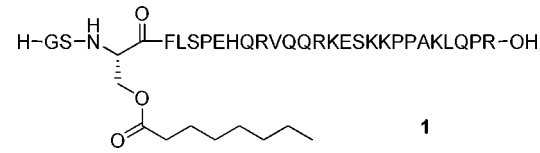

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Figure 1. Structure of human ghrelin.

GHSR, a member of the G protein-coupled receptor (GPCR) family, was first identified in 1996 as a seven-transmembrane domain 366 amino acid protein responsible for the regulation of growth hormone (GH) secretion.[5] This receptor is mainly expressed in the hypothalamus, pituitary cells, and a number of peripheral tissues.[5,6] Expression of the GHSR has been reported in various types of tumors, including breast carcinomas, prostate cancer cell lines, ovarian tumors, testicular tumors, pancreatic endocrine tumors, and intestinal carcinoids.[7−12] The presence of high affinity and specific binding sites in the neoplastic cells, but absence in the corresponding normal tissues, has been demonstrated in at least three different human breast carcinoma cell lines and pancreatic endocrine tumors.[7,11] This suggests that GHSR may be a suitable target for tumor imaging. According to our knowledge, the potential use of GHSR as a target in cancer imaging has not previously been reported.

Ghrelin, a 28 amino acid peptide hormone with a unique lipophilic *n*-octanoyl post-translational modification in its serine-3 residue (Figure 1), was discovered in 1999 by Kojima et al. and determined to be the endogenous ligand for the GHSR.[13] This hormone plays an important role in the stimula-

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[a] Abbreviations: Boc, *tert*-butoxycarbonyl; Cp, cyclopentadienyl; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Dpr, diaminopropionic acid; EI, electron ionization; ESI, electrospray ionization; Fmoc, 9-fluorenylmethoxycarbonyl; GH, growth hormone; GHSR, growth hormone secretagogue receptor; GPCR, G protein-coupled receptor; HBTU, 3-[bis-(dimethylamino)methyliumyl]-3*H*-benzotriazol-1-oxide hexafluorophosphate; HRMS, high resolution mass spectrometry; IC_{50}, half-maximal inhibitory concentration; MBHA, 4-methylbenzhydrylamine; Ms, mesyl; Mtt, methyltrityl; PET, positron emission tomography; Pyr, pyridine; RP HPLC, reverse phase high performance liquid chromatography; SPPS, solid phase peptide synthesis; SPECT, single photon emission computed tomography; TBAF, tetra-*n*-butylammonium fluoride; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TIS, triisopropylsilane; Trt, trityl; Ts, tosyl. The abbreviations for the common amino acids are in accordance with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature.

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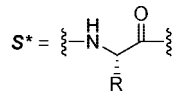
Table 1. Various Modifications to Human Ghrelin and Their Binding Affinities, As Reported by Bednarek et al[15]

2-8 H-GS**S***FLSPEHQRVQQRKESKKPPAKLQPR-OH
9 H-GS**S***FLSPEHQRVQQ-OH
10 H-GS**S***FL-NH_2
11 H-GS**S***F-NH_2

S* = 

compd	peptide sequence	R	IC_{50} (nM)[a]
1	human ghrelin	-CH_2O-CO-$(CH_2)_6CH_3$	0.25
	ghrelin(1−28)		
2	ghrelin(1−28)	-CH_2OH	>10000
3	ghrelin(1−28)	-CH_2O-CO-CH_3	>2000
4	ghrelin(1−28)	-CH_2O-CO-$(CH_2)_{14}CH_3$	0.87
5	ghrelin(1−28)	-CH_2O-CO-CH_2-adamantyl	0.12
6	ghrelin(1−28)	-CH_2O-CO-$(CH_2)_7Br$	0.08
7	ghrelin(1−28)	-CH_2O-CO-$(CH_2)_6NH_2$	>2000
8	ghrelin(1−28)	-CH_2NH-CO-$(CH_2)_6CH_3$	0.42
9	ghrelin(1−14)	-CH_2O-CO-$(CH_2)_6CH_3$	9.6
10	ghrelin(1−5)-NH_2	-CH_2O-CO-$(CH_2)_6CH_3$	55
11	ghrelin(1−4)-NH_2	-CH_2O-CO-$(CH_2)_6CH_3$	889

[a] IC_{50} values determined by [^{35}S]MK-0677 competitive binding assay.[15]

tion of GH secretion through binding with GHSR along with other functions including appetite regulation.[14] Ghrelin binds to the GHSR with high affinity and specificity, resulting in a complex formation of ghrelin−GHSR followed by internalization of the complex.[14] We intend to utilize the binding and internalization of the ghrelin−GHSR complex by developing a class of ghrelin based imaging agents targeting GHSR that are suitable for cancer imaging.

Design of Ghrelin Analogues as Imaging Probes

The relationship between ghrelin (**1**) structure and its binding activity to the GHSR has been previously studied by several groups, and the *n*-octanoyl side chain at Ser-3 was found to be crucial for binding.[15−17] These studies demonstrated that desacyl ghrelin **2** (Table 1), ghrelin without the *n*-octanoyl side chain, does not bind to the GHSR. Various modifications to ghrelin's *n*-octanoyl group were studied, and it was concluded that this side chain could be replaced by other groups as long as its lipophilicity and approximate size are maintained as in **4** and **5**.[15] When a short side chain was used as in **3**, poor binding was observed. In addition, a neutral halogen modification such as a bromo in **6** is well tolerated, while a charged group modification such as an amine in **7** leads to poor binding.[15]

There are two radionuclides that we are interested in incorporating as part of ghrelin based imaging agents: fluorine-18 (^{18}F) for application in positron emission tomography (PET) and technetium-99m (^{99m}Tc) for application in single photon emission computed tomography (SPECT). Since modification to the Ser-3 *n*-octanoyl is permitted as long as the requisite hydrophobic interaction to GHSR is maintained, we decided to integrate the radionuclide directly in the side chain of Ser-3. Careful design is needed to minimize negative interference caused by the addition of the radionuclides to this binding sensitive region of the molecule. The approach to integrate a radionuclide in a site that is crucial for the binding is not common for radiolabeled peptides, as the more common approach is to place the radionuclide far from the active binding site, in a pendant manner, thus reducing interference from the radionuclide complex with receptor binding. However, by placement of the radionuclide directly in the active binding site, the molecular weight of the agent will be minimized and further development into a peptidomimetic agent may be realized.

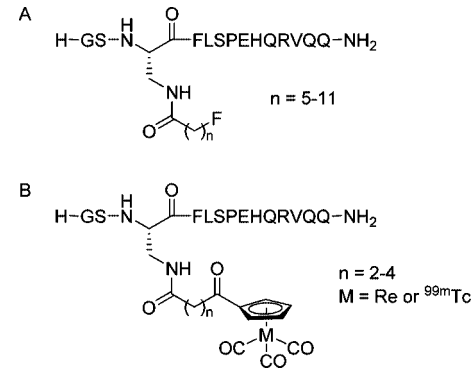


Figure 2. Design of ghrelin analogues for radioimaging: (A) fluorine-bearing ghrelin analogues for PET imaging; (B) rhenium or technetium-bearing ghrelin analogues for SPECT imaging.

In our design, the radionuclide ^{18}F will be incorporated directly into the end of the Ser-3 side chain (Figure 2A). The rationale behind this was that the similar bromine modification **6** is known to be well tolerated.[15] For the γ-emitting radionuclide ^{99m}Tc, we decided that a cyclopentadienyltechnetium tricarbonyl ($Cp^{99m}Tc(CO)_3$) complex would be a good choice for this integrated design, since not only is it small, nonchiral, and chemically and metabolically stable but it is also lipophilic,[18] a key requirement for the ghrelin side chain. For this reason, the $Cp^{99m}Tc(CO)_3$ complex was designed to be incorporated into the end of the side chain (Figure 2B). For chemical and biological analysis purposes, nonradioactive ^{19}F was used in the place of ^{18}F and $^{185/187}$Re was used in the place of ^{99m}Tc. Technetium does not have a stable isotope but is known to have similar chemical and biological properties to rhenium.[19] For binding optimization, the ghrelin analogues were designed with several different lengths of side chain in order to determine the optimal length.

The in vivo suitability of the peptides, including stability, tissue penetration, and fast clearance, is an important aspect of an imaging probe. In order to keep the size compact for better tissue penetration, as well as to reduce complication in synthesis, the minimum number of amino acid residues required for binding was used in the probe design. Previous structure−activity relationship studies have shown that the first four or five residues from the N-terminus of ghrelin are necessary for binding, although the binding affinity is greater when the chain is extended to include additional residues of the endogenous ghrelin, as shown in the cases of **9**, **10**, and **11**.[15−17] For that purpose, two different truncation lengths were chosen in this study: 5-mer and 14-mer peptides from the N-terminus (Figure 2). In order to increase the stability toward exopeptidases in vivo, the carboxylic acid of the C-terminus is replaced by an amide (Figure 2). In human ghrelin, the hydrophobic side chain is attached to the Ser-3 via an ester bond; however, this ester bond can undergo rapid hydrolysis.[15,16] According to a previous study, replacing the ester with an amide as in **8** has no detrimental effect on the binding capability;[15] therefore, in our design this ester bond was replaced with an amide bond. The non-natural amino acid diaminopropionic acid (Dpr), structurally similar to serine with an amine in the side chain instead of alcohol, was used in the place of Ser-3 to allow amide formation.

Results and Discussion

Fluorine Bearing Ghrelin Analogues. The first class of molecules that we developed were fluorine bearing ghrelin analogues for a preliminary study to investigate their potential

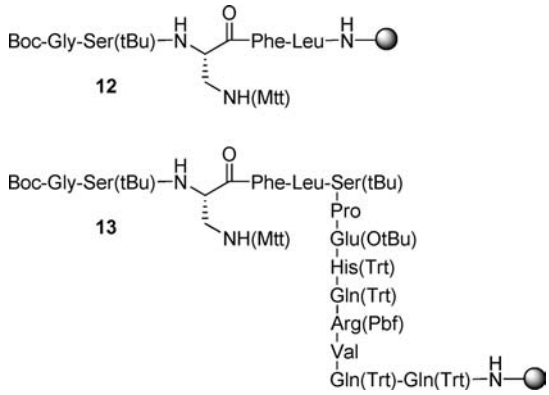
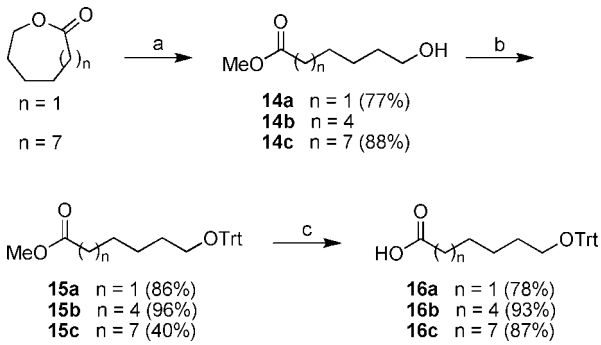


Figure 3. Truncated ghrelin analogues prior to modification.

Scheme 1. Synthesis of Side Chain Precursors[a]



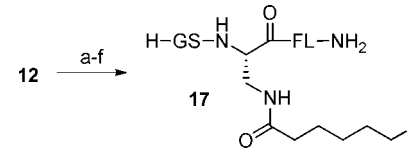
[a] Reagents and conditions: (a) H_2SO_4, MeOH, room temp; (b) TrtCl, pyridine, room temp; (c) $NaOH_{aq}$, THF, room temp.

use as PET imaging agents for cancer. The peptides were assembled following standard 9-fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis (SPPS) methods[20] on a polystyrene-based insoluble support, Rink amide 4-methylbenzhydrylamine (MBHA) resin, which provided an amide C-terminus upon cleavage. The Ser-3 of human ghrelin was replaced with Dpr, protected by the weak acid labile methyltrityl (Mtt), to provide an amide linkage in this position upon further modification. The fully protected resin-bound ghrelin(1−5) **12** and ghrelin(1−14) **13** prior to modification are shown in Figure 3.

At this point, the Dpr-3 of the peptide was ready for functionalization. Fluorine bearing analogues were designed with the fluorine attached to the end of the aliphatic side chain with variable length. Nonradioactive analogues using ^{19}F in the place of ^{18}F were used to study their chemical and biological properties, and side chain precursors were prepared in solution phase prior to attachment to the peptide, with fluorination of the side chain being conducted on-resin. Prior to attachment, side chain precursors had three important features: an aliphatic chain with length adjusted for binding affinity optimization, a carboxylic acid on one end of the chain as the attachment site to the peptide, and a protected alcohol group on the other end as the fluorination site.

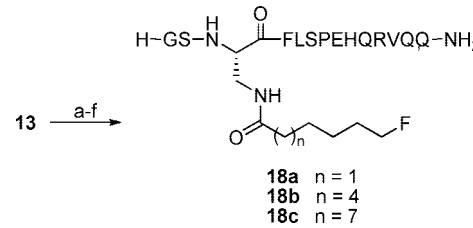
Three different side chain lengths were prepared: 6, 9, and 12 carbon length, indicated as 6C, 9C, and 12C, respectively. The 6C and 12C precursors were prepared from their cyclic lactones, with the first synthetic step being the opening of the lactone rings in methanol using an acid catalyst (Scheme 1). Methanol was chosen as media to provide methyl protection to the carboxylate group, as that was necessary during the next reaction step. The formed alcohols **14a** and **14c**, as well as commercially available methyl 9-hydroxynonanoate, the starting

Scheme 2. Synthesis of Fluorine Bearing Ghrelin(1−5)[a]



[a] Reagents and conditions: (a) 2% TFA, 5% TIS, CH_2Cl_2, room temp; (b) **16a**, HBTU, DIPEA, DMF, room temp; (c) 2% TFA, 5% TIS, CH_2Cl_2, room temp; (d) TsCl, pyridine, CH_2Cl_2, room temp; (e) TBAF, THF, room temp; (f) 88% TFA, 5% H_2O, 5% phenol, 2% TIS, room temp.

Scheme 3. Synthesis of Fluorine Bearing Ghrelin(1−14)[a]



[a] Reagents and conditions: (a) 2% TFA, 5% TIS, CH_2Cl_2, room temp; (b) **16a**−**c**, HBTU, DIPEA, DMF, room temp; (c) 2% TFA, 5% TIS, CH_2Cl_2, room temp; (d) MsCl, NEt_3, CH_2Cl_2, room temp; (e) TBAF, THF, room temp; (f) 88% TFA, 5% H_2O, 5% phenol, 2% TIS, room temp.

material for 9C, were then tritylated to obtain weak acid labile protection for the alcohol group, which was necessary during the side chain coupling to the peptide. Purification of the tritylated products **15a**, **15b**, and **15c** was problematic, and attempts to perform chromatography were made. The existence of excess trityl chloride and pyridinium salt made it difficult to dissolve the crude material, and in our early attempts methanol was added to help the solvation. Unfortunately methanol reacted with unused trityl chloride, caused acid formation, and resulted in trityl (Trt) deprotection from the product that reformed the starting materials. Instead, the byproducts were removed by filtration with organic solvents at 0 °C to obtain **15a** and **15b** or by flash chromatography without methanol to obtain **15c**.

Hydrolysis of the methyl ester of **15a**, **15b**, and **15c** was carried out under basic conditions, followed by an acidic workup, during which special attention was given to maintain the pH above 4 to prevent trityl deprotection. After recrystallization for solid product or flash chromatography for oil products, 6-trityloxyhexanoic acid **16a**, 9-trityloxynonanoic acid **16b**, and 12-trityloxydodecanoic acid **16c** were isolated, providing aliphatic side chains that were ready to be coupled to **12** and **13**.

The on-resin reaction series started with a selective deprotection of the acid labile amine-Mtt of Dpr-3, which was done with a series of treatments with a dilute trifluoroacetic acid (2% TFA) solution in CH_2Cl_2. After the free amine was obtained, the carboxylic acid group of the side chain precursor was coupled to this amine via *O*-benzotriazole (OBt) ester formation, following standard coupling procedures in peptide synthesis.[20] For the 5-mer **12**, only **16a** was coupled to the peptide (Scheme 2), while for the 14-mer **13**, all precursors previously made, **16a**, **16b**, and **16c**, were used (Scheme 3). Reaction completion was followed by the Kaiser test[21] and in some cases microcleavage to allow HPLC analysis and MS analysis to confirm the product mass.

After the side chain was attached to the peptide, the protected alcohol was transformed to a fluoro group via several steps. The first step was the selective deprotection of the alcohol-Trt with dilute acid (2% TFA) solution in CH_2Cl_2. The obtained

Table 2. Characterization and Binding Affinity Data of Fluorine Bearing Ghrelin Analogues

		m/z^b			
compd	purity (%)[a]		calcd	obsd	IC_{50} (nM)[c]
17	97	$[M + Na]^+$	646.3	646.3	>2000
18a	98	$[M + 2H]^{2+}$	857.4	857.5	147
18b	99	$[M + H]^+$	1755.9	1756.0	39.6
18c	99	$[M + 2H]^{2+}$	899.5	899.5	27.9

[a] Percent purity determined by RP HPLC with detection at 220 nm. [b] The *m*/*z* calculated and observed values are based on the prominent observed signals as determined by MS (ESI). [c] IC_{50} values (nM) determined by competitive radioligand binding assay to the GHSR (mean of two experiments) derived from human recombinant CHO-K1 cells according to published procedures.[23,24]

alcohol was activated prior to fluorination. For the ghrelin(1−5)-6C-OH, activation was done via tosylation in basic conditions. HPLC monitoring and MS analysis of the microcleaved product showed conversion of the starting material to the desired product ghrelin(1−5)-OTs (55%) and various byproducts.

The tosylation was also attempted on the ghrelin(1−14)-6C-OH; however, after several attempts HPLC and MS analyses clearly showed that tosylation did not work for the 14-mer peptide. This was likely caused by aggregation of the material on-resin, which could lead to incomplete solvation, matrix shrinkage, and poor reagent penetration. Since the nature of the peptide and the side chain could not be changed at this point, more reactive reagents were sought. Mesylation was chosen for the reason that mesyl chloride (MsCl) forms a highly reactive intermediate sulfene (SO_2CH_2), in which the sulfur is highly electrophilic and will react with any alcohol, even with tertiary alcohols that react very slowly with TsCl.[22] HPLC and MS analyses of the microcleaved products showed that mesylation was successfully applied to the 14-mer peptides with the 6C, 9C, and 12C side chains.

Fluorination for both ghrelin(1−5) and ghrelin(1−14) derivatives was performed using tetra-*n*-butylamonium fluoride (TBAF) in tetrahydrofuran (THF), monitored by HPLC and MS analyses of microcleaved products. Even though a competing reaction of elimination by F^- as a base during the treatment with TBAF was anticipated, this was not observed. The fluorination of the 14-mer analogues went more slowly than for the 5-mer analogue, likely because of some degree of aggregation that occurred in the 14-mer peptides as previously discussed. Fluorination was the last step that needed to be done on-resin, and at this point the peptides were ready to be cleaved off the resin and fully deprotected. The desired fluorine bearing ghrelin(1−5)-6C-F **17**, ghrelin(1−14)-6C-F **18a**, ghrelin(1−14)-9C-F **18b**, and ghrelin(1−14)-12C-F **18c** were isolated with yields of 9%, 7%, 2%, and 12%, respectively (Table 2).

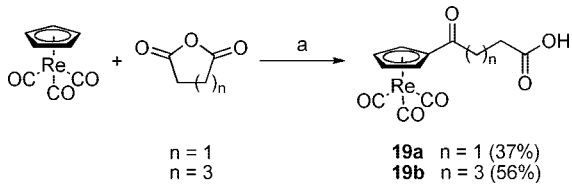
The binding affinities of these fluorine bearing ghrelin(1−5) and ghrelin(1−14) derivatives to the GHSR were evaluated according to the half-maximal inhibitory concentration (IC_{50}) values (Table 2) as determined by a radioligand binding assay.[23,24] The effect of the sequence length of the peptide was determined from the comparison of the 5-mer **17** and the 14-mer **18a** that had an identical side chain in their Dpr-3 residue. The IC_{50} value of **17** was determined to be greater than 2 μM, based on only a 20% inhibition at that concentration. This result showed that a 5-mer peptide with the fluorine modification lacks adequate affinity to the GHSR and therefore is not a suitable design for an imaging probe. In contrast, the IC_{50} of **18a** was found to be satisfactory with a value of 147 nM, showing its potential to be used as an imaging tracer. On the basis of these results, all optimizations throughout the study were conducted on 14-mer ghrelin analogues. Side chain length optimization of the fluorine bearing ghrelin(1−14) derivatives was evaluated on the basis of the binding data of three peptides **18a**, **18b**, and **18c** that had different side chain lengths: 6C, 9C, and 12C, respectively. All of these derivatives were found to have satisfactory nanomolar binding affinities that make them possible candidates for use as imaging agents. These analogues' IC_{50} values were found to decrease as the side chain length increases, indicating that the longer aliphatic chain is preferred for binding. This finding was in accordance with the previous study by Matsumoto et al. that to a certain length, binding affinity of ghrelin analogues improved as the side chain got longer.[16] On the basis of our findings in this preliminary study, we conclude that fluorine bearing 14-mer ghrelin analogues have potential to be developed as PET cancer imaging agents, with the 12C side chain analogue **18c** as the best candidate.

Rhenium Bearing Ghrelin Analogues. The second class of molecules designed were rhenium bearing ghrelin analogues for a preliminary study to investigate the suitability of ^{99m}Tc-radiolabeled ghrelin analogues as SPECT imaging agents for the GHSR. Re(I) and Tc(I) tricarbonyl complexes have been reported to have great stability in vivo and have been used in a variety of peptide and non-peptide imaging agents.[25] Among these systems, cyclopentadienyl tricarbonyl organometallic ($CpM(CO)_3$) species appear to be the best candidate for our imaging purpose because of the neutral, lipophilic, and stable properties of this complex. The ability to use the cyclopentadienylmetal complex in an integrated fashion has been demonstrated by Katzenellenbogen and co-workers through their investigation of rhenium containing estrogens, where the $CpRe(CO)_3$ unit is directly associated with the estrogen receptor binding site.[26] The neutral and lipophilic properties of the metal complex are important in our design because we plan to integrate this organometallic system as part of the residue-3 side chain that participates in the binding to the GHSR.

Metal complex incorporation into a site that is crucial for ligand−receptor interaction is rare for peptide based technetium radiopharmaceuticals. Typically, a metal complex is located far from the active binding site in a pendant fashion, in order to minimize the interference it might cause to receptor binding. Many such examples are reported in the literature for Tc/Re, including ^{99m}Tc-EDDA/HYNIC octreotide, Cp-TR-octreotide, and [^{99m}Tc$(CO)_3$-*N*α-histidinyl acetate]bombesin(7−14).[27−29] In contrast to this pendant (also referred to as conjugate) design, only a few examples are reported of an integrated design whereby the metal complex is part of the receptor binding region of the ligand. Bigott-Hennkens et al. reported on a Re(V) oxo inorganic complex that was successfully coordinated directly to the octreotide peptide backbone, and this metal coordination, replacing the function of a disulfide bridge, cyclized the peptide.[30] Although not located within the receptor binding pocket, the metal complex is a critical element for binding because of the resultant formation of a cyclic constraint to the peptide. Prior work reported on a similar cyclic strategy for rhenium and technetium α-melanocyte stimulating hormone analogues.[31] Thus, the success of our design would contribute to the rare cases of metal ligand incorporation into a region crucial for receptor binding of a peptide based pharmaceutical.

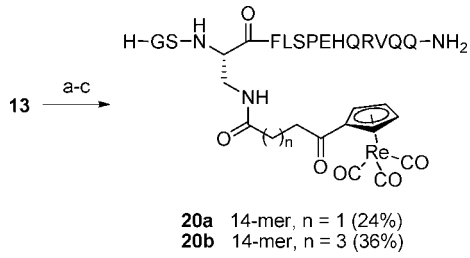
The side chains for the rhenium bearing ghrelin were prepared using solution-phase chemistry prior to attachment to the peptide. For optimization, two different lengths were prepared: four and six carbons long, counted from the amide carbon to the carbon in which Cp ring is attached and will be addressed as 4C and 6C. Both precursors were prepared from their cyclic anhydride, succinic anhydride, and adipic anhydride for 4C and

Scheme 4. Synthesis of Rhenium Side Chain Precursors[a]



[a] Reagents and conditions: (a) $AlCl_3$, CH_2Cl_2, room temp for **19a** or reflux for **19b**.

Scheme 5. Synthesis of Rhenium Bearing Ghrelin(1−14)[a]



[a] Reagents and conditions: (a) 2% TFA, 5% TIS, CH_2Cl_2, room temp; (b) **19a,b**, HBTU, DIPEA, DMF, room temp; (c) 88% TFA, 5% H_2O, 5% phenol, 2% TIS, room temp.

6C, respectively, and rhenium tricarbonyl cyclopentadienyl ($CpRe(CO)_3$) through Friedel−Crafts acylation using aluminum chloride ($AlCl_3$) (Scheme 4). The reaction for the 4C precursor went slowly and did not go to completion because of the low reactivity of the Cp ring when it is coordinated to Re(I). The starting material $CpRe(CO)_3$ was recovered during the extraction, and the product 4-(cyclopentadienylrhenium tricarbonyl)-4-oxobutanoic acid **19a** was isolated upon chromatography.

In the synthesis of 6C precursor, adipic anhydride was prepared from adipic acid through dehydration according to a literature procedure.[32] Various attempts to optimize the Friedel−Craft step were performed, including the use of different solvents (chloroform and dichloroethane), increased reaction temperature, portionwise addition of reagents, different Lewis acids, and increased reagent equivalencies. The best result was achieved when 2 equiv of adipic anhydride and 4 equiv of $AlCl_3$ were used, CH_2Cl_2 was used as the solvent, and the mixture was refluxed. This caused all starting material to be consumed within 2 days, and upon purification, 6-(cyclopentadienylrhenium tricarbonyl)-6-oxohexanoic acid **19b** and the dimer 1,6-di($CpRe(CO)_3$)-1,6-dioxohexane as a byproduct were isolated.

The side chain precursors **19a** and **19b**, already containing rhenium, were ready to be incorporated into the peptide at this point. Since the 14-mer binding data for fluorine bearing analogues were found to be superior to its 5-mer equivalent, rhenium bearing ghrelin analogues were made only with the 14-mer sequence. Fully protected on-resin **13** was prepared, and the Mtt group was removed from the amine of Dpr-3 using 2% TFA solution (Scheme 5). The side chains **19a** and **19b** were then coupled to this amine followed by cleaving from resin and complete deprotection. The rhenium bearing ghrelin(1−14)-4C-$CpRe(CO)_3$ **20a** and ghrelin(1−14)-6C-$CpRe(CO)_3$ **20b** were obtained with purified yields of 24% and 36%, respectively (Table 3).

The evaluation of **20a** and **20b** showed that both molecules have good binding affinity to the GHSR, marked by their nanomolar IC_{50} values: 35 and 174 nM, respectively (Table 3). The aliphatic chain between the Dpr-3 and the rhenium complex

Table 3. Characterization and Binding Affinity Data of Rhenium Bearing Ghrelin Analogues

		m/z[b]			
compd	purity (%)[a]		calcd	obsd	IC_{50} (nM)[c]
20a	98	$[M + 2H]^{2+}$	1008.4	1008.4	35
20b	99	$[M + H]^{+}$	2043.8	2043.8	174

[a] Percent purity determined by RP HPLC with detection at 220 nm. [b] Both ^{185}Re and ^{187}Re peaks were observed; however, values reported are based on the more abundant ^{187}Re. The m/z calculated and observed values are based on the prominent observed signals as determined by MS (ESI or MALDI-TOF). [c] IC_{50} values (nM) determined by competitive radioligand binding assay to the GHSR (mean of two experiments) derived from human recombinant CHO-K1 cells according to published procedures.[23,24]

adds to the bulkiness of the overall side chain, and by comparison of their binding affinity, it was found that the shorter **20a** has the optimal binding compared to the longer **20b**. This finding is in contrast with the general trend of fluorine bearing ghrelin(1−14) analogues **18a**, **18b**, and **18c**, for which the longer side chain leads to better binding. A possible cause is that the $CpRe(CO)_3$ has already contributed to a certain volume, and the extra length of the side chain of **20b** causes the overall size to be larger than what is required for optimal binding. Indeed, the previous study done by Matsumoto et al. showed that the binding affinity of ghrelin analogues improved as the side chain was increased, until at some point it started to decrease.[16]

Here we demonstrated that the incorporation of a metal complex in the crucial binding region of a peptide ligand was a success, with the key component being the neutral and lipophilic $CpRe(CO)_3$ mimicking the lipophilic property of the original ghrelin side chain. On the basis of these findings, we conclude that technetium-99m bearing 14-mer ghrelins have potential to be developed as SPECT cancer imaging agents, with the ghrelin−rhenium surrogate **20a** as our best candidate.

Conclusions

The aim of this preliminary study was to investigate the appropriateness of fluorine and rhenium functionalized ghrelin analogues as ligands for the growth hormone secretagogue receptor (GHSR). Several 5- and 14-mer ghrelin analogues were designed and synthesized with fluorine, or rhenium as $CpRe(CO)_3$ complex, that was attached to the end of the aliphatic side chain of residue-3, a region that is crucial for the binding activity. From the GHSR binding evaluation, it is revealed that 14-mer ghrelin analogues have high binding affinity, while a 5-mer analogue showed poor binding affinity. The fluorine derivative **18c**, containing a 12 carbon aliphatic chain at position-3 of the 14-mer ghrelin, has the best receptor affinity and is our lead candidate for PET imaging. The rhenium derivative **20a** has excellent receptor affinity and is, to our knowledge, the first example of incorporating rhenium into a peptide structure where the metal complex itself is critical for receptor binding. This sets the stage for future investigation by PET and SPECT of the ability to use the analogous ^{18}F and ^{99m}Tc ghrelin derivatives for the noninvasive imaging of GHSR expression.

Experimental Section

Materials and Equipment. Reagents and solvents were purchased from Sigma-Aldrich, Fluka, NovaBiochem, Peptides International, Strem Chemicals, Toronto Research Chemicals, Chem-Impex, Fisher Scientific, or VWR and were used without further purification unless noted. Dry CH_2Cl_2 was prepared by distillation from CaH under argon. Oven-dried or flame-dried apparatus and argon flow were used in all water sensitive reactions. Analytical HPLC was performed using a Grace Vydac protein/peptide RP-

C18 column 4.6 mm × 250 mm, 5 μm, and preparative HPLC was performed using a Grace Vydac protein/peptide RP-C18 column 22.0 mm × 250 mm, 10 μm. The absorbance was detected at wavelengths of 220 and 254 nm. A gradient system was used: H_2O + 0.1% of TFA (solvent A) and CH_3CN + 0.1% of TFA (solvent B). Flash column chromatography was performed using Merck silica gel 60 (230−400 mesh). Analytical TLC was carried out on EMD silica gel 60 F_{254} plates. ^1H and ^{13}C NMR data were obtained using a Varian Mercury 400, and the chemical shifts were referenced to solvent signals ($CDCl_3$, ^1H 7.25 ppm, ^{13}C 77.23 ppm) relative to TMS. Mass spectra were obtained using Finnigan MAT 8200 (HRMS-EI), Micromass LCT (MS-ESI), and Micromass MALDI-LR (MALDI-TOF) mass spectrometers. For compounds containing rhenium, both ^{185}Re and ^{187}Re peaks are observed, and the more abundant ^{187}Re mass is reported in this section. Melting points were determined in open capillary tubes on Mel-Temp apparatus without correction.

Peptide Assembly. Fully protected resin-bound peptides were synthesized according to the general procedures in Fmoc solid phase peptide synthesis[20] either manually or automatically using an APEX 396 peptide synthesizer. Fmoc protected rink amide MBHA resin (loading of 0.27 or 0.47 mequiv/g) was used as the solid support. *N*-Fmoc amino acids, with strong acid labile protecting groups for side chain functional groups, were used in general, and *N*-Boc amino acid was used for the N terminus. Fmoc-diaminopropanoic acid (Dpr), with the β-amine protected with methyltrityl (Mtt), was used for residue-3. Fmoc removal was achieved with treatments of 20% piperidine in *N*,*N*-dimethylformamide (DMF) for 10 and 20 min and successive washes using DMF and CH_2Cl_2 after each treatment. For each amino acid coupling, resin was treated once or twice with 3 equiv of Fmoc or Boc amino acids, 3 equiv of 3-[bis(dimethylamino)methyliumyl]-3*H*-benzotriazol-1-oxide hexafluorophosphate (HBTU), and 6 equiv of *N*,*N*-diisopropylethylamine (DIPEA) in 2 mL of DMF for 30 min to 4 h. Successive washes with DMF, CH_2Cl_2, and THF were done following the coupling. Using this general procedure, **12** and **13** were prepared.

Peptide Deprotection and Resin Cleavage. Selective deprotections of amine-Mtt and alcohol-Trt were achieved by shaking the resin with 2% TFA and 5% triisopropylsilane (TIS) in CH_2Cl_2 for 2 min, followed by successive washes with CH_2Cl_2. This treatment was repeated five times. During the solid phase reaction steps, the presence or absence of a free amine group was monitored by the Kaiser test.[21] When necessary, cleaving a small sample of resin beads (microcleave) was performed to obtain a small quantity of representative peptide, for which HPLC and MS analyses were conducted. After all modifications were done, the peptide was deprotected and cleaved from the resin by TFA containing the scavengers water (5% v/v), phenol (5% m/v), TIS (2% v/v) for 2−4 h. Resin was filtered and rinsed with a small amount of TFA. Peptide was precipitated from the TFA solution using *tert*-butyl methyl ether (TBME) and collected after centrifugation and decantating. Peptide was then rinsed using TBME and collected again. The resulting solid was redissolved in water with additional CH_3CN when necessary, frozen, and lyophilized to obtain crude peptide as a fine powder. Purification of the peptide was conducted through preparative HPLC runs, and the purity of the isolated material was determined by analytical HPLC.

Methyl 6-Hydroxyhexanoate (14a). This compound was prepared from ε-caprolactone (10.01 g, 87.7 mmol) according to a literature procedure.[33] The colorless oil **14a** was obtained with a yield of 77%. ^1H NMR (400 MHz, $CDCl_3$, δ_H ppm): 3.63 (3H, s, CO_2CH_3), 3.61 (2H, t, $^3J_{H-H}$ = 6.5 Hz, HO-CH_2), 2.30 (2H, t, $^3J_{H-H}$ = 7.4 Hz, CH_2CO_2), 1.50−1.68 (4H, m, 2CH_2), 1.31−1.44 (2H, m, CH_2).

Methyl 12-Hydroxydodecanoate (14c). Concentrated H_2SO_4 (0.2 mL) was added to a solution of oxacyclotridecan-2-one (850 mg, 4.3 mmol) in 20 mL of methanol and stirred for 1 day. Methanol was removed under reduced pressure, and the aqueous residue was extracted with diethyl ether 3 times. The combined organic layers were washed with saturated $NaHCO_3$, saturated NaCl and then dried over $MgSO_4$. The diethyl ether was removed under reduced pressure to yield 874 mg (88%) of **14c** as a white solid. ^1H NMR (400 MHz, $CDCl_3$, δ_H ppm): 3.65 (3H, s, CO_2CH_3), 3.63 (2H, t, $^3J_{H-H}$ = 6.6 Hz, HO-CH_2), 2.29 (2H, t, $^3J_{H-H}$ = 7.6 Hz, CH_2CO_2), 1.50−1.66 (4H, m, 2CH_2), 1.22−1.38 (14H, m, 7CH_2). HRMS (EI): *m*/*z* calcd 231.1955 ($[M + H]^+$, $C_{13}H_{27}O_3$), found 231.1954 $[M + H]^+$.

Methyl 6-Trityloxyhexanoate (15a). Trityl chloride (18.8 g, 67.4 mmol) was added to an ice cold (0 °C) stirring solution of **14a** (9.9 g, 67.4 mmol) in 80 mL of pyridine. The reaction mixture was warmed to room temperature and stirred under argon for 2 days, during which time a white byproduct formed. The solvent was removed under reduced pressure, and the resulting material was redissolved in ice cold THF. The insoluble byproduct was removed by filtration, and the filtrate was dried under reduced pressure to obtain 22.6 g (86%) of an orange oil, **15a**. ^1H NMR (400 MHz, $CDCl_3$, δ_H ppm): 7.43 (6H, m, *p*-Ar), 7.29 (6H, m, *m*-Ar), 7.22 (3H, m, *o*-Ar), 3.65 (3H, s, CO_2CH_3), 3.05 (2H, t, $^3J_{H-H}$ = 6.6 Hz, HO-CH_2), 2.29 (2H, t, $^3J_{H-H}$ = 7.5 Hz, CH_2CO_2), 1.54−1.71 (4H, m, 2CH_2), 1.33−1.45 (m, 2H, CH_2). HRMS (EI): *m*/*z* calcd 388.2038 ($C_{26}H_{28}O_3$), found 388.2039 $[M]^+$.

Methyl 9-Trityloxynonanoate (15b). The synthesis procedure of **15a** was followed, with 1.37 g (4.9 mmol) of trityl chloride, 0.46 g (2.5 mmol) of methyl 9-hydroxy-nonanoate, and 10 mL of pyridine used in the reaction. An insoluble byproduct was removed by filtration in CH_2Cl_2 and 10% ethyl acetate in hexanes. Upon solvent removal, 1.02 g (96%) of **15b** was obtained as a pale-yellow oil. ^1H NMR (400 MHz, $CDCl_3$, δ_H ppm): 7.44 (6H, m, *p*-Ar), 7.28 (6H, m, *m*-Ar), 7.22 (3H, m, *o*-Ar), 3.65 (3H, s, CO_2CH_3), 3.03 (2H, t, $^3J_{H-H}$ = 6.6 Hz, HO-CH_2), 2.29 (2H, t, $^3J_{H-H}$ = 7.5 Hz, CH_2CO_2), 1.55−1.65 (4H, m, 2CH_2), 1.18−1.40 (8H, m, 4CH_2). HRMS (EI): *m*/*z* calcd 430.2508 ($C_{29}H_{34}O_3$), found 430.2514 $[M]^+$.

Methyl 12-Trityloxydodecanoate (15c). The synthesis procedure of **15a** was followed. Trityl chloride (1.41 g, 5.1 mmol), **14c** (0.58 g, 2.5 mmol), and pyridine (10 mL) were used, and the reaction was prolonged to 3 days. The filtration step was omitted; instead, the crude material was purified by flash column chromatography (10% EtOAc in hexanes) yielding 477 mg (40%) of a pale-yellow oil, **15c**. ^1H NMR (400 MHz, $CDCl_3$, δ_H ppm): 7.44 (6H, m, *p*-Ar), 7.28 (6H, m, *m*-Ar), 7.21 (3H, m, *o*-Ar), 3.66 (3H, s, CO_2CH_3), 3.03 (2H, t, $^3J_{H-H}$ = 6.6 Hz, HO-CH_2), 2.29 (2H, t, $^3J_{H-H}$ = 7.5 Hz, CH_2CO_2), 1.55−1.65 (4H, m, 2CH_2), 1.10−1.39 (14H, m, 7CH_2). HRMS (EI): *m*/*z* calcd 472.2977 ($C_{32}H_{40}O_3$), found 472.2968 $[M]^+$.

6-Trityloxyhexanoic Acid (16a). An aqueous solution of 5 M NaOH (17.5 mL, 87.5 mmol) was added to a stirring solution of **15a** (20.1 g, 51.6 mmol) in 130 mL of THF and 52.5 mL of water at room temperature. After the mixture was stirred for 2 days, 3.1 mL of 5 M NaOH (15.5 mmol) was added, and stirring was continued for another 2 days. The THF was removed under reduced pressure. Then the aqueous residue was acidified with 1 M HCl to pH 5 and extracted with diethyl ether. The combined organic layers were washed with brine and dried over $MgSO_4$. The diethyl ether was removed under reduced pressure, and the resulting crude powder was recrystallized in hexanes to yield 15.1 g (78%) of white powder, **16a**. Mp 114−116 °C. ^1H NMR (400 MHz, $CDCl_3$, δ_H ppm): 7.43 (6H, m, *p*-Ar), 7.28 (6H, m, *m*-Ar), 7.22 (3H, m, *o*-Ar), 3.05 (2H, t, $^3J_{H-H}$ = 6.4 Hz, HO-CH_2), 2.33 (2H, t, $^3J_{H-H}$ = 7.5 Hz, CH_2CO_2), 1.56−1.68 (4H, m, 2CH_2), 1.36−1.47 (2H, m, CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, δ_H ppm): 180.26, 144.36, 128.62, 127.66, 126.79, 86.30, 63.24, 33.98, 29.63, 25.74, 24.48. HRMS (EI): *m*/*z* calcd 374.1882 ($C_{25}H_{26}O_3$), found 374.1883 $[M]^+$.

9-Trityloxynonanoic Acid (16b). Aqueous 0.5 M NaOH (13.2 mL, 6.6 mmol) was added to a stirring solution of **15b** (1.41 g, 3.3 mol) in 25 mL of THF. The reaction mixture was stirred for 3 days at room temperature, and then the solvent was removed by rotary evaporation. The purification was carried out by flash column chromatography (gradient 10% EtOAc in hexanes to 100% EtOAc) to obtain 1.28 g (93%) of a pale-yellow oil, **16b**. ^1H NMR (400 MHz, $CDCl_3$, δ_H ppm): 7.43 (6H, m, *p*-Ar), 7.28 (6H, m, *m*-Ar), 7.21 (3H, m, *o*-Ar), 3.03 (2H, t, $^3J_{H-H}$ = 6.6 Hz, HO-CH_2), 2.33 (2H, t, $^3J_{H-H}$ = 7.5 Hz, CH_2CO_2), 1.56−1.66 (4H, m, 2CH_2),

1.20−1.40(8H, m, 4CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, δ_H ppm): 180.26, 144.46, 128.63, 127.64, 126.74, 86.22, 63.55, 34.04, 29.95, 29.24, 29.13, 28.96, 26.15, 24.60. HRMS (EI): *m*/*z* calcd 416.2351 ($C_{28}H_{32}O_3$), found 416.2360 $[M]^+$.

12-Trityloxydodecanoic Acid (16c). The synthesis and purification procedures of **16b** were followed. Aqueous 0.5 M NaOH (4.6 mL, 2.3 mmol), **15c** (548 mg, 1.2 mmol), and THF (9.3 mL) were used, and the reaction duration was shortened to 2 days. After purification, 463 mg (87%) of **16c** was obtained as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$, δ_H ppm): 7.43 (6H, m, *p*-Ar), 7.28 (6H, m, *m*-Ar), 7.21 (3H, m, *o*-Ar), 3.02 (2H, t, $^3J_{H-H}$ = 6.7 Hz, HO-CH_2), 2.34 (2H, t, $^3J_{H-H}$ = 7.5 Hz, CH_2CO$_2$), 1.55−1.67 (4H, m, 2CH_2), 1.19−1.39 (14H, m, 7CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, δ_H ppm): 180.02, 144.53, 128.69, 127.64, 126.75, 86.25, 63.66, 34.02, 30.04, 29.52, 29.50, 29.48, 29.39, 29.22, 29.05, 26.25, 24.66. HRMS (EI): *m*/*z* calcd 458.2821 ($C_{31}H_{38}O_3$), found 458.2814 $[M]^+$.

Ghrelin(1−5)-6C-F (17). Selective Mtt amine deprotection was performed to residue-3 of **12** according to the general procedure, and the side chain precursor **16a** was coupled following the general procedure for coupling. Deprotection of the trityl containing side chain was conducted according to the general procedure to obtain free alcohol. This alcohol was then activated by an overnight treatment with tosyl chloride (47.66 mg, 0.25 mmol) in 2 mL of 50% CH_2Cl_2/50% pyridine mixture under argon to provide tosylated alcohol. Following this reaction, the resin was washed successively with CH_2Cl_2 and THF. Fluorination was then conducted by shaking the resin in 4 equiv of 0.1 M anhydrous TBAF in THF under argon for 2.5 h, twice. Successive THF and CH_2Cl_2 washes were performed after each fluorination step. Following this, final cleavage and deprotection were conducted according to the general procedure to obtain the crude peptide. Purification was performed by HPLC (gradient 20−40% solvent B in A) to obtain **17** as a white powder with an overall yield of 9% (3.4 mg). MS (ESI): *m*/*z* calcd 623.3, found 646.3 $[M + Na]^+$.

General Procedure for Fluorine Bearing Ghrelin(1−14) Analogues (18a−c). These peptides were made from on-resin peptide **13** following a procedure similar to the synthesis of **17**. Side chains **16a**, **16b**, and **16c** were coupled to the peptide to obtain **18a**, **18b**, and **18c**, respectively, after alcohol deprotection, activation, and fluorination steps. Mesylation was performed instead of tosylation as follows: resin was shaken with 5 equiv of MsCl and 15 equiv of NEt_3 in anhydrous CH_2Cl_2 under argon for 4 h, and then after washing the process was repeated once more overnight. Fluorination was conducted in a similar manner using up to 9 equiv of 0.1 M anhydrous TBAF/THF, and the process was repeated up to 6 times, as necessary for maximal fluorine incorporation.

Ghrelin(1−14)-6C-F (18a). Purification was performed by HPLC (gradient 12−30% solvent B in A) to obtain **18a** as a white powder with an overall yield of 7% (7.4 mg). MS (ESI): *m*/*z* calcd 1712.9, found 857.5 $[M + 2H]^{2+}$, 572.0 $[M + 3H]^{3+}$ (100%).

Ghrelin(1−14)-9C-F (18b). Purification was performed by HPLC (gradient 25−50% solvent B in A) to obtain **18b** as a white powder with an overall yield of 2% (2.2 mg). MS (ESI): *m*/*z* calcd 1754.9, found 1756.0 $[M + H]^+$, 889.5 $[M + Na + H]^{2+}$ (100%).

Ghrelin(1−14)-12C-F (18c). Purification was performed by HPLC (gradient 25−40% solvent B in A) to obtain **18c** as a white powder with an overall yield of 12% (12.1 mg). MS (ESI): *m*/*z* calcd 1797.0, found 899.5 $[M + 2H]^{2+}$ (100%).

4-(Cyclopentadienylrhenium Tricarbonyl)-4-oxobutanoic Acid (19a). This compound was prepared from $CpRe(CO)_3$ (1.00 g, 3.0 mmol) according to a literature procedure.[34] The yellow solid **19a** was obtained with a yield of 37% (0.49 g). ^{1}H NMR (400 MHz, $CDCl_3$, δ_H ppm): 6.00−6.04 (2H, m, Cp), 5.38−5.42 (2H, m, Cp), 2.87−2.93 (2H, m, CH_2), 2.72−2.78 (2H, m, CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, δ_H ppm): 192.97 (*C*O), 191.62 (*C*O), 177.12 (*C*O$_2$H), 95.15 (*Cp*-CO), 87.94 (Cp), 85.22 (Cp), 33.15 (*C*H$_2$), 27.44 (*C*H$_2$). HRMS (EI): *m*/*z* calcd 437.0029 ($[M + H]^+$, $C_{12}H_{10}O_6{}^{187}$Re), found 437.0022 $[M + H]^+$.

6-(Cyclopentadienylrhenium Tricarbonyl)-6-oxohexanoic Acid (19b). Anhydrous $AlCl_3$ (2.67 g, 20.0 mmol) and succinic anhydride (1.28 g, 10.0 mmol) were dissolved in 100 mL of anhydrous CH_2Cl_2 under argon flow. $CpRe(CO)_3$ (1.68 g, 5.0 mmol) was added, and mixture was refluxed for 1 day. Ice cold 5 M HCl_{aq} was added to the reaction mixture, and the organic layer was removed. The aqueous layer was extracted by CH_2Cl_2 three times, the combined organic layers were dried over $MgSO_4$, and the solvent was removed under reduced pressure. After addition of EtOAc, the insoluble byproducts were removed by filtration and purification was performed by column chromatography (20% EtOAc in hexanes, 50%, EtOAc in hexanes, then 1% HOAc in EtOAc) to obtain 1.30 g (56%) of yellow solid, **19b**. ^{1}H NMR (400 MHz, $CDCl_3$, δ_H ppm): 5.96−6.00 (2H, m, Cp), 5.34−5.41 (2H, m, Cp), 2.59−2.66 (2H, t, $^3J_{H-H}$ = 7.0 Hz, CH_2CO), 2.36−2.43 (2H, t, $^3J_{H-H}$ = 6.9 Hz, CH_2CO), 1.63−1.79 (4H, m, 2CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, δ_H ppm): 194.66 (*C*O), 191.72 (*C*O), 178.90 (*C*O$_2$H), 95.86 (*Cp*-CO), 87.85 (Cp), 85.22 (Cp), 38.28(*C*H$_2$CO), 33.58 (*C*H$_2$CO), 23.99 (*C*H$_2$), 23.52 (*C*H$_2$). HRMS (EI): *m*/*z* calcd 485.0139 ($[M + Na]^+$, $C_{14}H_{13}O_6{}^{185}$ReNa); found 485.0144 $[M + Na]^+$.

General Procedure for Rhenium Bearing Ghrelin(1−14) Analogues (20a and 20b). Selective amine-Mtt deprotection was conducted for residue-3 of **13** according to the general procedure, and then the side chain precursors **19a** and **19b** were coupled using the general coupling procedure. The peptides were cleaved off the resin and deprotected according to the general procedure to obtain crude peptides **20a** and **20b**.

Ghrelin(1−14)-4C-CpRe(CO)$_3$ (20a). Purification was performed by HPLC (gradient 25−50% of solvent B in A) to obtain white powder **20a** with an overall yield of 24% (55.6 mg). MS (ESI): *m*/*z* calcd 2014.8, found 1008.4 $[M + 2H]^{2+}$.

Ghrelin(1−14)-6C-CpRe(CO)$_3$ (20b). Purification was performed by HPLC (gradient 20−40% of solvent B in A) to obtain white powder **20b** with an overall yield of 36% (43.3 mg). MS (MALDI-TOF): *m*/*z* calcd 2042.8, found 2043.8 $[M + H]^+$.

Radioligand Binding Assay. Determination of IC_{50} values of ghrelin analogues **17**, **18a**−**c**, and **20a**,**b** for GHSR was conducted by radioligand binding assays according to published literature procedures.[23,24] Assays were performed using human recombinant CHO-K1 cells as receptor source and ^{125}I-ghrelin (human) as radioligand. Reference standards using ghrelin (human) were run to ensure the validity of the results. IC_{50} values were determined by a nonlinear, least-squares regression analysis using MathIQ (ID Business Solutions Ltd., U.K.). Exact IC_{50} value of ghrelin(1−5)-6C-F **17** was not determined, as at a concentration of 2000 nM only 20% inhibition was achieved; thus, these data are reported as >2000 nM. For ghrelin(1−14) analogues **18a**−**c** and **20a**,**b**, IC_{50} values were determined semiquantitatively according to the % inhibition at 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M concentration (all data duplicated). IC_{50} values are reported in Tables 2 and 3.

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Supporting Information Available: ^{1}H NMR spectra for compounds **16a**−**c** and **19a**,**b**; MS spectra and HPLC chromatograms for compounds **17**, **18a**−**c**, and **20a**,**b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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