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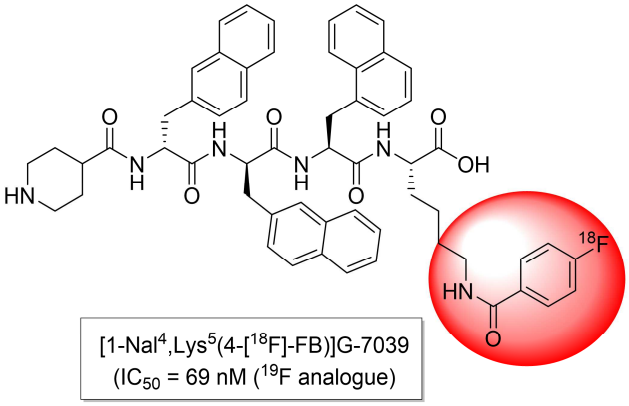


[1-Nal^4,Lys^5(4-[^{18}F]-FB)]G-7039
(IC_{50} = 69 nM (^{19}F analogue)

PET imaging of the ghrelin receptor

Peptidomimetic growth hormone secretagogue derivatives for positron emission tomography imaging of the ghrelin receptor

Milan M. Fowkes,[a,b†] Tyler Lalonde,[a,b] Lihai Yu,[a,b] Savita Dhanvantari,[c] Michael S. Kovacs[c] and Leonard G. Luyt[a,b,c*]

[a]Department of Chemistry, Western University, 1151 Richmond Street, London, Ontario N6A 5B7, Canada,

[b]London Regional Cancer Program, Lawson Health Research Institute, 790 Commissioners Road East, London, Ontario N6A 4L6, Canada,

[c]Imaging Program, Lawson Health Research Institute, 268 Grosvenor Street, London, Ontario N6A 4V2, Canada.

[†]Present address: Kennedy Institute of Rheumatology, Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Roosevelt drive, Oxford, OX3 7FY, United Kingdom.

[*]Corresponding author. London Regional Cancer Program, Lawson Health Research Institute, 790 Commissioners Road East, London, Ontario N6A 4L6, Canada.

E-mail address: lluyt@uwo.ca (L.G. Luyt).

Abbreviations: [^{18}F]FB, [^{18}F]fluorobenzoyl; [^{18}F]SFB, *N*-succinimidyl-4-[^{18}F]fluorobenzoate; [^{19}F]FBA, [^{19}F]fluorobenzoic acid; 7-TM, 7-transmembrane; AEEA, 2-(2-(2-aminoethoxy)ethoxy)acetic acid; Aib, 2-aminoisobutyric acid; cpm, counts per minute; D-1-Nal, D-1-naphthylalanine, D-2-Nal, D-2-naphthylalanine; D-2-Thi, D-2-thienylalanine; D-Trp(2-Me); D-2-methyltryptophan, Inp, isonipecotic acid; d.c., decay-corrected; DIPEA, *N*,*N*-diisopropylethylamine; Dpr, 2,3-diaminopropionic acid; EDC, *N*-(3-dimethylaminopropyl)-*N´*-ethylcarbodiimide; E_{max}, maximal response; Fmoc-SPPS Fmoc-solid phase peptide synthesis; GH, growth hormone; GHRH, growth hormone releasing hormone; GHSs, growth hormone secretagogues; HBSS, Hank's balanced salt solution; HCTU, 1-[Bis(dimethylamino)methylen]-5-chlorobenzotriazolium 3-oxide hexafluorophosphate; HEK293, human embryonic kidney 293; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Kryptofix K 2.2.2, 4,7,13,16,21,24-Hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane; MBHA, 4-Methylbenzhydrylamine hydrochloride salt resin; MeOTf, methyl trifluoromethanesulfonate; NHS, *N*-hydroxysuccinimide; RP reverse phase; TBMe, *tert*-butyl methyl ether; TFA, trifluoroacetic acid.

Abstract

The ghrelin receptor is a seven-transmembrane (7-TM) receptor known to have an increased level of expression in human carcinoma and heart failure. Recent work has focused on the synthesis of positron emission tomography (PET) probes designed to target and image this receptor for disease diagnosis and staging. However, these probes have been restricted to small-molecule quinalizonones and peptide derivatives of the endogenous ligand ghrelin. We describe the design, synthesis and biological evaluation of a series of 4-fluorobenzoylated growth hormone secretagogues (GHSs) derived from peptidic (GHRP-1, GHPR-2 and GHRP-6) and peptidomimetic (G-7039, [1-Nal^4]G-7039 and ipamorelin) families in order to test locations for the insertion of fluorine-18 for PET imaging. The peptidomimetic G-7039 was found to be the most suitable for ^{18}F-radiolabelling as its non-radioactive 4-fluorobenzoylated analogue ([1-Nal^4,Lys^5(4-FB)]G-7039), had both a high binding affinity (IC_{50} = 69 nM) and promising *in vitro* efficacy (EC_{50} = 1.1 nM). Prosthetic group radiolabelling of the precursor compound [1-Nal^4]G-7039 using *N*-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB) delivered the PET probe [1-Nal^4,Lys^5(4-[^{18}F]-FB)]G-7039 in an average decay-corrected radiochemical yield of 48%, a radio-purity ≥ 99% and an average molar activity of > 34 GBq/μmol. This compound could be investigated as a PET probe for the detection of diseases that are characterised by overexpression of the ghrelin receptor.

Keywords

Growth hormone secretagogues, peptidomimetics, positron emission tomography, ghrelin receptor, ^{18}F-radiochemistry.

1. Introduction

Growth hormone secretagogues (GHSs) are a class of compounds that stimulate the secretion of growth hormone (GH) by acting on the ghrelin receptor, also known as the growth hormone secretagogue receptor type-1a (GHS-R1a)[1]. This receptor is predominantly found in the hypothalamus and pituitary gland[1] and its secretion occurs through a route disparate from that of growth hormone-releasing hormone (GHRH)[2]. The first GHSs to show GH release *in vitro* were a series of Met-enkephalin analogues described by Bowers *et al* in 1980[3]. Further development of these analogues led to a hexamer that released GH *in vivo* in a number of animals[4, 5], including humans[6]. This peptide was later termed growth hormone-releasing peptide-6 (GHRP-6), and a

number of GHSs were subsequently synthesised in order to find alternatives to recombinant human GH therapy. These encompassed peptides (e.g. GHRP-1[7], hexarelin[8], KP-102 (later designated GHRP-2)[9]), peptidomimetics (e.g. G-7039[10], [1-Nal]^{4}G-7039[11] and ipamorelin[12]) and small-molecules (e.g. L-692,429[13] and MK-0677[14]). The amino acid sequences of the peptidic GHSs and the structures of the peptidomimetic and small-molecule GHSs are shown in Figure 1. The endogenous ligand for GHS-R1a is ghrelin, a 28-mer peptide with an *n*-octanoyl group on the Ser^3 side-chain, which was discovered in 1999[15] and exhibits a multitude of biological activities, such as the regulation of food intake and glucose metabolism[16, 17].

H-His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2

GHRP-6

H-Ala-His-D-2-Nal-Ala-Trp-D-Phe-Lys-NH_2

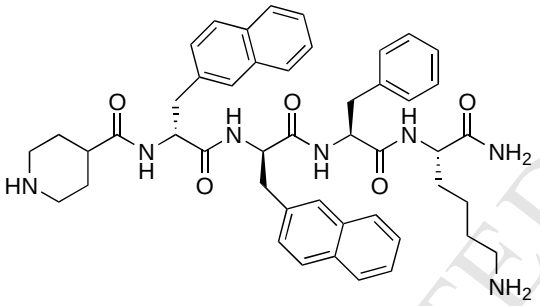
GHRP-1

H-His-D-Trp(2-Me)-Ala-Trp-D-Phe-Lys-NH_2

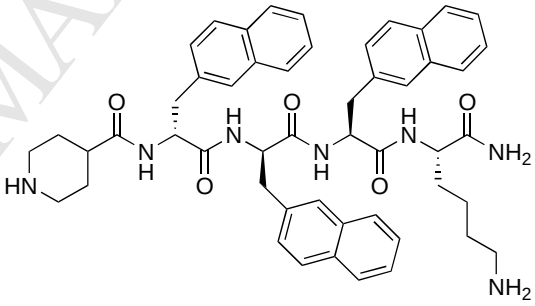
Hexarelin

H-D-Ala-D-2-Nal-Ala-Trp-D-Phe-Lys-NH_2

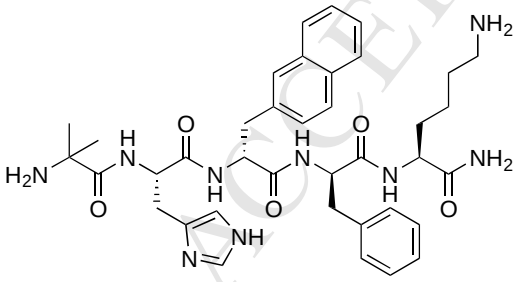
GHRP-2



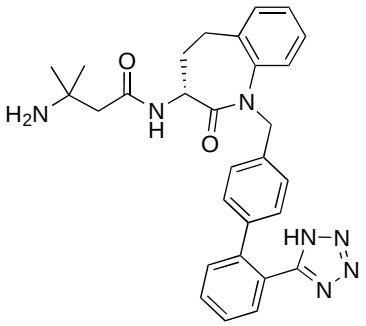
G-7039



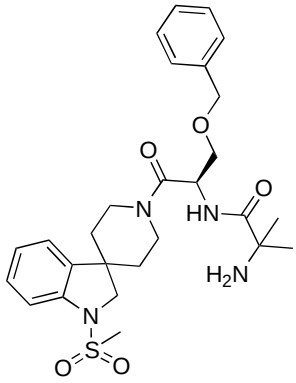
[1-Nal]^{4}G-7039



Ipamorelin



L 692,429



MK-0677

Figure 1. Peptidic, peptidomimetic and small-molecule growth hormone secretagogues. All amino acids are designated by the standard three-letter code. D-2-Nal, D-2-naphthylalanine; Inp, isonipecotic acid; D-Trp(2-Me); D-2-methyltryptophan.

The ghrelin receptor is expressed in a number of human malignancies including prostatic carcinoma cell lines[18, 19] and tissues[20], breast carcinoma tumours and cell lines[21], testicular tumours[22], and malignant ovarian cysts and tumours[23]. In addition, it has an elevated level of expression in tissues from patients with atherosclerosis[24] and in biopsies from those with chronic heart failure[25]. The ghrelin receptor thus represents a potential target for molecular imaging of carcinoma and cardiovascular disease. Among the imaging modalities most commonly utilised in the clinic, positron emission tomography (PET) combines a high spatial resolution with high sensitivity[26]. A number of recent publications have thus described the development of PET radiotracers for visualising the GHS-R1a receptor in disease[27-30]. For example, Hou and co-workers synthesized the first picomolar quinazolinone binder of the ghrelin receptor and successfully radiolabelled two nanomolar compounds in high radiochemical purity[30]. In another study on small-molecule derivatives, *in vivo* imaging in mice with a [^{11}C]radiotracer revealed a higher specific uptake in the pancreas compared to other organs[28]. However, these studies[27-30] and others[31-33] have primarily focused on either small-molecule quinazolinones[27, 28, 30, 31] or ghrelin-derived compounds[29, 32, 33]. To the best of our knowledge, a growth hormone secretagogue-based PET probe has not been reported to date. We reasoned that modification of peptidic and peptidomimetic GHSs should result in a clinically translatable PET agent with high target specificity, *in vivo* stability and favourable pharmacokinetic properties. Fluorine-18 was our preferred choice of radioisotope as it is a small innocuous unit that can be easily installed in the absence of a chelator using the [^{18}F]fluorobenzoyl ([^{18}F]FB) prosthetic group. This modification can be initially trialled with 4-[^{19}F]fluorobenzoic acid ([^{19}F]FBA), in order to determine the optimal location for the ^{18}F-radioisotope without significantly affecting peptide/peptidomimetic binding to the ghrelin receptor.

We describe herein the design, synthesis and biological evaluation of 4-fluorobenzoylated derivatives of GHSs with peptidic (GHRP-1, GHRP-2 and GHRP-6) and peptidomimetic (G-7039, [1-Nal^4]G-7039 and ipamorelin) structures; the determination of their IC_{50} values for the ghrelin receptor; the identification of the lead peptide [1-Nal^4,Lys^5(4-FB)]G-7039 with nanomolar IC_{50} and EC_{50} values; and the ^{18}F-radiolabelling of its precursor [1-Nal^4]G-7039 to furnish the peptidomimetic growth hormone secretagogue [1-Nal^4,Lys^5(4-[^{18}F]-FB)]G-7039, which could be applied to PET imaging of diseases *via* targeting of the ghrelin receptor.

2. Results and Discussion

2.1 Design strategy for peptidic and peptidomimetic growth hormone secretagogues

Initially, we sought to determine the location for ^{18}F insertion into the peptidic and peptidomimetic GHSs without significantly affecting receptor binding affinity. A study of the literature revealed that Huang and co-workers had designed a 3D pharmacophore for GHS activity using peptidic and non-peptidic compounds without inclusion of the lysine side-chain[34]. This led to the synthesis of a benzothiazepin compound with low nanomolar *in vitro* efficacy for the ghrelin receptor[34]. In addition, we recently reported a ghrelin receptor homology model based on neurotensin and opioid receptors with docking of the peptidomimetic G-7039[35]. The lysine residue was found to be unimportant for G-7039-GHS-R1a binding owing to an unfavourable interaction energy between its side-chain amino group and polar Glu^{197}/Arg^{199} residues[35]. This led us to select the lysine side-chain for ^{18}F-radioisotope insertion in the peptidic (GHRP-1, GHRP-2 and GHRP-6) and peptidomimetic (G-7039, [1-Nal^{4}]G-7039 and ipamorelin) GHSs using the [^{18}F]FB prosthetic group *via* the pre-activated *N*-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB)[36]. This modification was first tested by coupling a non-radioactive [^{18}F]FB mimic ([^{19}F]FBA) to the lysine side-chain of each individual GHS class using the orthogonal allyloxycarbonyl (Alloc) protecting group by standard Fmoc-solid phase peptide synthesis (SPPS). Any change in binding affinity for the ghrelin receptor (expressed in terms of the IC_{50}) was determined experimentally through a competitive receptor-ligand binding assay. Each parent growth hormone secretagogue (Figure 1) would be modified in turn until an IC_{50} < 100 nM was achieved.

2.2 Synthesis and characterisation of peptidic and peptidomimetic growth hormone secretagogues

The peptidic and peptidomimetic GHSs were synthesised through manual or automated Fmoc-SPPS and purified by reverse-phase (RP)-HPLC. Amino acids sequences for these GHSs are shown in the supporting information (Table S1), with characterisation data listed in Table 1.

Peptide/Peptidomimetic	HRMS (calculated) $[M + H]^+$	HRMS (found) $[M + H]^+$	% Purity	% Yield
Ipamorelin	712.3935	712.3959	> 97	19
[Lys^5(4-FB)]ipamorelin	834.4103	834.4133	> 99	15
[Lys^5(AEEA-4-FB)]ipamorelin	979.4842	979.4868	> 98	8
[D-2-Thi^4,Lys^5(4-FB)]ipamorelin	840.3667	840.3693	> 98	4
[Inp^1,Lys^5(4-FB)]ipamorelin	860.4259	860.4284	> 96	6
[Inp^1,D-2-Nal^4,Lys^5(4-FB)]ipamorelin	910.4416	910.4400	> 98	10
[Inp^1,D-2-Thi^4,Lys^5(4-FB)]ipamorelin	866.3824	866.3850	> 99	13
GHRP-1	955.4943	955.4964	> 99	19
GHRP-2	840.4204 $[M + Na]^+$	840.4173 $[M + Na]^+$	> 99	17
GHRP-6	873.4524	873.4531	> 97	14
[Lys^6(4-FB)]GHRP-6	1017.4511 $[M + Na]^+$	1017.4522 $[M + Na]^+$	> 96	7
[Dpr^6]GHRP-6	831.4055	831.4070	> 99	3
[Dpr^6(4-FB)]GHRP-6	953.4223	953.4237	> 98	14
G-7039*	798.4343*	798.4339*	> 99*	7*
[Lys^5(4-FB)]G-7039	920.4511	920.4529	> 97	6
[1-Nal^4]G-7039	848.4499	848.4501	> 99	23
[1-Nal^4,Lys^5(4-FB)]G-7039	970.4667	970.4693	> 96	9

Table 1. HRMS data, purities and yields for synthesised peptides and peptidomimetics. All amino acids are designated by the standard three-letter code. 4-FB, 4-fluorobenzoyl; AEEA, 2-(2-(2-aminoethoxy)ethoxy)acetic acid; D-2-Nal, D-2-naphthylalanine; D-2-Thi, D-2-thienylalanine; Dpr, 2,3-diaminopropionic acid; Inp, isonipecotic acid. *Characterisation data reported previously[35].

2.3 Structure-activity relationships of peptidic and peptidomimetic growth hormone secretagogues

The IC_{50} values of the peptidic and peptidomimetic GHSs were determined through receptor-ligand binding assays utilising human embryonic kidney 293 (HEK293)/ghrelin receptor

cells and [^{125}I]ghrelin as the competitive radioligand. In order to ascertain whether overexpression of the receptor in HEK293 cells had been achieved, the endogenous ligand ghrelin was assayed first. An IC_{50} value of 7.63 nM was obtained (Figure S1), indicating a satisfactory level of receptor expression and maintaining consistency with previously reported values[37].

The initial peptidomimetic that was chosen for ^{19}F-fluorobenzoylation was the pentapeptide ipamorelin (H-Aib-His-D-2-Nal-D-Phe-Lys-NH_2, compound **1**, Table 2). This is because it displayed a nanomolar binding affinity in previous work using HEK293/ghrelin receptor cells (K_i = 240 nM)[38].

Compound	Name	IC_{50}, nM
1	Ipamorelin	483
2	[Lys^5(4-FB)]ipamorelin	170
3	[Lys^5(AEEA-4-FB)]ipamorelin	474
4	[D-2-Thi^4,Lys^5(4-FB)]ipamorelin	161
5	[Inp^1,Lys^5(4-FB)]ipamorelin	688
6	[Inp^1,D-2-Nal^4,Lys^5(4-FB)]ipamorelin	1920
7	[Inp^1,D-2-Thi^4,Lys^5(4-FB)]ipamorelin	1170

Table 2. IC_{50} values of ipamorelin and a series of derivatives thereof. The listed IC_{50} values were determined in triplicate using HEK293/ghrelin receptor cells. For corresponding displacement curves, the reader is referred to the supporting information.

Introduction of a fluorobenzoyl moiety into the lysine side-chain caused the IC_{50} value of ipamorelin to decrease from 483 nM to 170 nM (compound **2**, [Lys^5(4-FB)]ipamorelin). This could be a consequence of the fluorobenzoyl group strengthening hydrophobic interactions with aromatic residues (e.g. Phe222 and Phe226) in the non-polar sub-pocket described in the open GHS-R1a homology model of Pedretti and co-workers[39]. Ipamorelin also possesses the same three C-terminal residues as G-7039 (the only difference being D-Phe^4 as opposed to Phe^4, respectively) and thus the additional fluorobenzene ring could also be interacting with residues in one of the hydrophobic sub-pockets (e.g. Phe286) detailed by Hou *et al*[35]. Extension of the lysine side-chain through a short mini-PEG linker (2-(2-(2-aminoethoxy)ethoxy)acetic acid, AEEA) prior to 4-fluorobenzoyl group coupling furnished [Lys^5(AEEA-4-FB)]ipamorelin, compound **3**. This had a

higher IC_{50} value compared to compound **2** (*cf*. 474 nM for [Lys^5(AEEA-4-FB)]ipamorelin to 170 nM for [Lys^5(4-FB)]ipamorelin). This may result from the additional flexibility afforded by the mini-PEG linker, which could cause a reduction in important hydrophobic interactions of the 4-fluorobenzoyl moiety when compared to the less flexible compound **2**. Replacement of the D-Phe residue with D-2-thienylalanine (D-2-Thi) was expected to improve binding affinity. This strategy was based on a study by Hansen and co-workers on dipeptide ipamorelin derivatives, where this alteration caused the EC_{50} value to improve by an order of magnitude[40]. Unfortunately, this modification furnished a compound with comparable affinity to the [Lys^5(4-FB)]ipamorelin analogue **2** with an IC_{50} = 161 nM ([D-2-Thi^4,Lys^5(4-FB)]ipamorelin, compound **4**).

The primary amine at the N-terminus of ipamorelin was then exchanged for the secondary amine isonipecotic acid (Inp) so as to make the subsequent compound ([Inp^1,Lys^5(4-FB)]ipamorelin, compound **5**) similar to the peptidomimetic G-7039, known to have an EC_{50} = 0.18 nM[10]. This led to a rise in the IC_{50} value (*cf*. [Lys^5(4-FB)]ipamorelin, IC_{50} = 170 nM, compound **2** to [Inp^1,Lys^5(4-FB)]ipamorelin, IC_{50} = 688 nM, compound **5**).

In order to probe the significance of the D-Phe residue in sub-pocket binding, this residue was replaced with D-2-Nal in [Inp^1,Lys^5(4-FB)]ipamorelin (**5**) to give [Inp^1,D-2-Nal^4,Lys^5(4-FB)]ipamorelin (IC_{50} = 1920 nM, compound **6**). This caused an approximately three-fold reduction in binding affinity for the ghrelin receptor compared to the parent compound **5**. This suggests that D-Phe may be taking part in π-π or hydrophobic interactions with Phe119 in TM3[39, 41], playing the same role as it does in the active tetra-peptide core of ghrelin[42]. The synthesis of compound **7** ([Inp^1,D-2-Thi^4,Lys^5(4-FB)]ipamorelin) and its corresponding IC_{50} value (1170 nM) could be seen as an approximately two-fold reduction in binding affinity for the ghrelin receptor from switching D-2-Thi for D-Phe in compound **5** ([Inp^1,Lys^5(4-FB)]ipamorelin); or an approximately seven-fold decrease in binding resulting from insertion of an unfavourable secondary amine (Inp) into compound **4** ([D-2-Thi^4,Lys^5(4-FB)]ipamorelin). In summary, the various synthesised derivatives of ipamorelin did not yield the desired IC_{50} of < 100 nM, despite some improvements to its initial IC_{50} value.

Next, our attention turned to the peptidic GHSs (GHRP-1, GHRP-2 and GHRP-6) due to the low nanomolar K_i values that were obtained for GHRP-2 and GHRP-6 (1.4 nM and 3.3 nM, respectively) using HEK293/ghrelin receptor cells[38]. The IC_{50} values of GHRP-1, GHRP-2 and GHRP-6 are shown in Table 3 (compounds **8-10**, respectively).

Compound	Name	IC_{50}, nM
8	GHRP-1	181
9	GHRP-2	449
10	GHRP-6	73
11	[Lys^6(4-FB)]GHRP-6	384
12	[Dpr^6]GHRP-6	397
13	[Dpr^6(4-FB)]GHRP-6	1060

Table 3. IC_{50} values of peptidic GHSs and their derivatives. The listed IC_{50} values were determined in triplicate using HEK293/ghrelin receptor cells. For corresponding displacement curves, the reader is referred to the supporting information.

It was found that GHRP-6, (H-His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2) had the strongest binding to the ghrelin receptor (IC_{50} = 73 nM, compound **10**) compared to GHRP-1 (IC_{50} = 181 nM, compound **8**) and GHRP-2 (IC_{50} = 449 nM, compound **9**). This peptide was therefore transformed into the fluorobenzoylated analogue [Lys^6(4-FB)]GHRP-6 (IC_{50} = 384 nM, compound **11**). Unfortunately, this alteration was not tolerated by the ghrelin receptor in spite of prior success with ipamorelin (*cf.* compound **11**, IC_{50} = 384 nM to compound **2**, IC_{50} = 170 nM). One possible explanation for this is that the lysine residue of GHRP-6 is involved in binding to polar sub-pocket residues (e.g. Arg199)[39].

Endeavouring to increase affinity for the ghrelin receptor, the lysine of GHRP-6 was replaced with 2,3-diaminopropionic acid (Dpr) to furnish [Dpr^6]GHRP-6 (IC_{50} = 397 nM, compound **12**). This increase in IC_{50} could be a product of the shorter side-chain of Dpr which could be decreasing lysine's charge-transfer interactions with Arg199 in the polar sub-pocket. This would also explain the greater loss in binding affinity after the attachment of the 4-fluorobenzoyl group ([Dpr^6(4-FB)]GHRP-6, IC_{50} = 1060 nM, compound **13**). Not only does the lysine side-chain appear to play an important role in receptor binding for GHRP-6 (a result contrary to that obtained for ipamorelin), but the length of the alkyl chain also appears to be essential. Further derivatisation of this class of peptides was not pursued owing to the high initial IC_{50} values of GHRP-1 (181 nM, compound **8**) and GHRP-2 (449 nM, compound **9**) and the assumption that the lysine residue in these peptides was also important for receptor interaction.

Finally, two Genentech 5-mers G-7039 (H-Inp-D-2-Nal-D-2-Nal-Phe-Lys-NH_2) and [1-Nal^4]G-7039 (H-Inp-D-2-Nal-D-2-Nal-1-Nal-Lys-NH_2) were investigated as a consequence of their reported low nanomolar *in vitro* efficacies (EC_{50} = 0.18 nM for G-7039[10] and 0.10 nM for [1-Nal^4]G-7039[11], respectively) and our recent computational study on the docking of G-7039 to GHS-R1a, which suggested that the lysine residue was not critical for receptor binding[35]. The parent compounds G-7039 (compound **14**) and [1-Nal^4]G-7039 (compound **16**) and their fluorobenzoyl congeners (compounds **15** and **17**, respectively) can be viewed in Table 4.

Compound	Name	IC_{50}, nM
14	G-7039*	5.2*
15	[Lys^5(4-FB)]G-7039	242
16	[1-Nal^4]G-7039	28
17	[1-Nal^4,Lys^5(4-FB)]G-7039	69

Table 4. IC_{50} values of Genentech peptidomimetics and their derivatives. The listed IC_{50} values were determined in triplicate using HEK293/ghrelin receptor cells. For corresponding displacement curves, the reader is referred to the supporting information. *Literature data[35].

Attaching G-7039 to *para*-fluorobenzoic acid had the effect of increasing the half-maximal inhibitory concentration (5.2 nM to 242 nM for [Lys^5(4-FB)]G-7039, compound **15**), whilst a smaller increase in the IC_{50} value was observed for [1-Nal^4]G-7039 (28 nM to 69 nM for [1-Nal^4,Lys^5(4-FB)]G-7039, compound **17**). The smaller increase observed for [1-Nal^4]G-7039 compared to G-7039 indicates that the introduction of an additional aromatic functionality (fluorobenzene) along with the already present 1-Nal^4 residue is simply strengthening hydrophobic sub-pocket binding, whereas for G-7039 this modification is less favourable; perhaps due to the reduction of salt-bridge interactions with Glu197 in the polar sub-pocket[35]. In spite of the increase in IC_{50} value for [1-Nal^4,Lys^5(4-FB)]G-7039, this peptidomimetic represents the first lead compound to be synthesised with the required nanomolar IC_{50} value (< 100 nM) making it the most suitable candidate for further study of physical and biochemical characteristics prior to [^{18}F]-radiolabelling with [^{18}F]SFB.

2.4 Determination of lipophilicity of peptidic and peptidomimetic growth hormone secretagogues

The log*P* value of [1-Nal^4,Lys^5(4-FB)]G-7039 as well as the other peptidic and peptidomimetic GHSs were computed using ACD/Log*P* software (Table S2). Inserting the 4-fluorobenzoyl moiety into any peptidomimetic parent compound resulted in an increase in hydrophobicity for all derivatives (+ 2.25). Most of the parent compounds exhibited a favourable log*P* value in the 1-3 unit range, except for G-7039 (5.28 ± 0.82) and [1-Nal^4]G-7039 (6.51 ± 0.82). This is virtue of the smaller size of these peptidomimetics compared to GHRP-1, GHRP-2 and GHRP-6, and the prevalence of more hydrophobic aromatic residues compared to ipamorelin (D-2-Nal and 1-Nal). Regrettably, the lead compound [1-Nal^4,Lys^5(4-FB)]G-7039 had a log*P* value of 8.76 ± 0.88, suggesting a lack of suitability for *in vivo* PET imaging due to potential solubility issues. Nevertheless, [1-Nal^4,Lys^5(4-FB)]G-7039 possesses a considerably lower IC_{50} value compared to the next best compound [D-2-Thi^4, Lys^5(4-FB)]ipamorelin (*cf.* 69 nM to 161 nM) and was thus investigated in further *in vitro* assays.

2.5 Determination of efficacy of lead peptidomimetic [1-Nal^4,Lys^5(4-FB)]G-7039

The EC_{50} value of [1-Nal^4,Lys^5(4-FB)]G-7039 was calculated in terms of the release of intracellular calcium ions (Figure 2). As expected, the control ligand ghrelin had a low *in vitro* potency of 1.6 nM, whilst the EC_{50} value of [1-Nal^4,Lys^5(4-FB)]G-7039 was determined as 1.1 nM. This low nanomolar efficacy signifies that the final compound remains a potent ghrelin receptor agonist despite blocking of the lysine side-chain with the 4-fluorobenzoyl group.

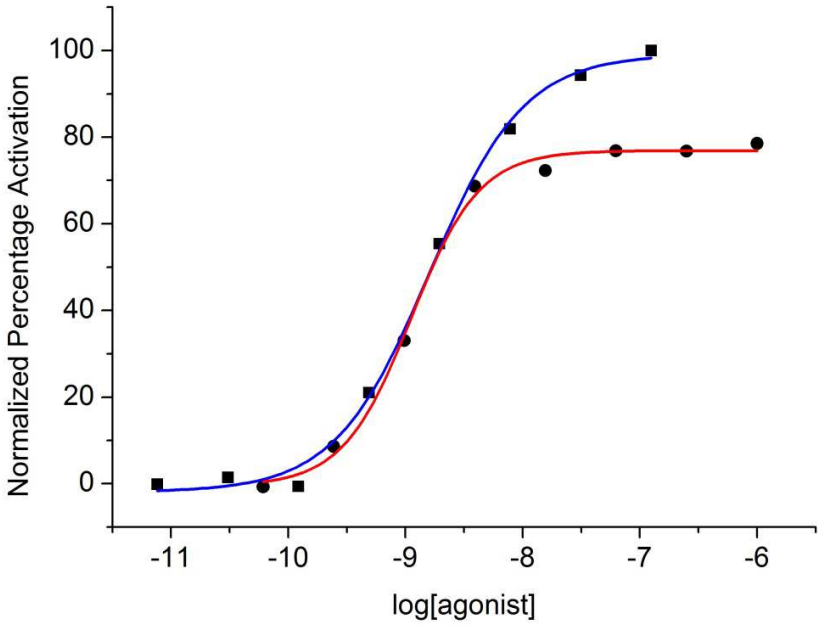


Figure 2. Dose-response curves for the lead peptidomimetic [1-Nal^4,Lys^5(4-FB)]G-7039 (red) and the control ligand ghrelin (blue). The EC_{50} value for these compounds was determined in terms of intracellular Ca^{2+} release. The percentage activation was normalised to the maximal response (E_{max}) of the control agonist ghrelin. Each assay concentration was performed in duplicate.

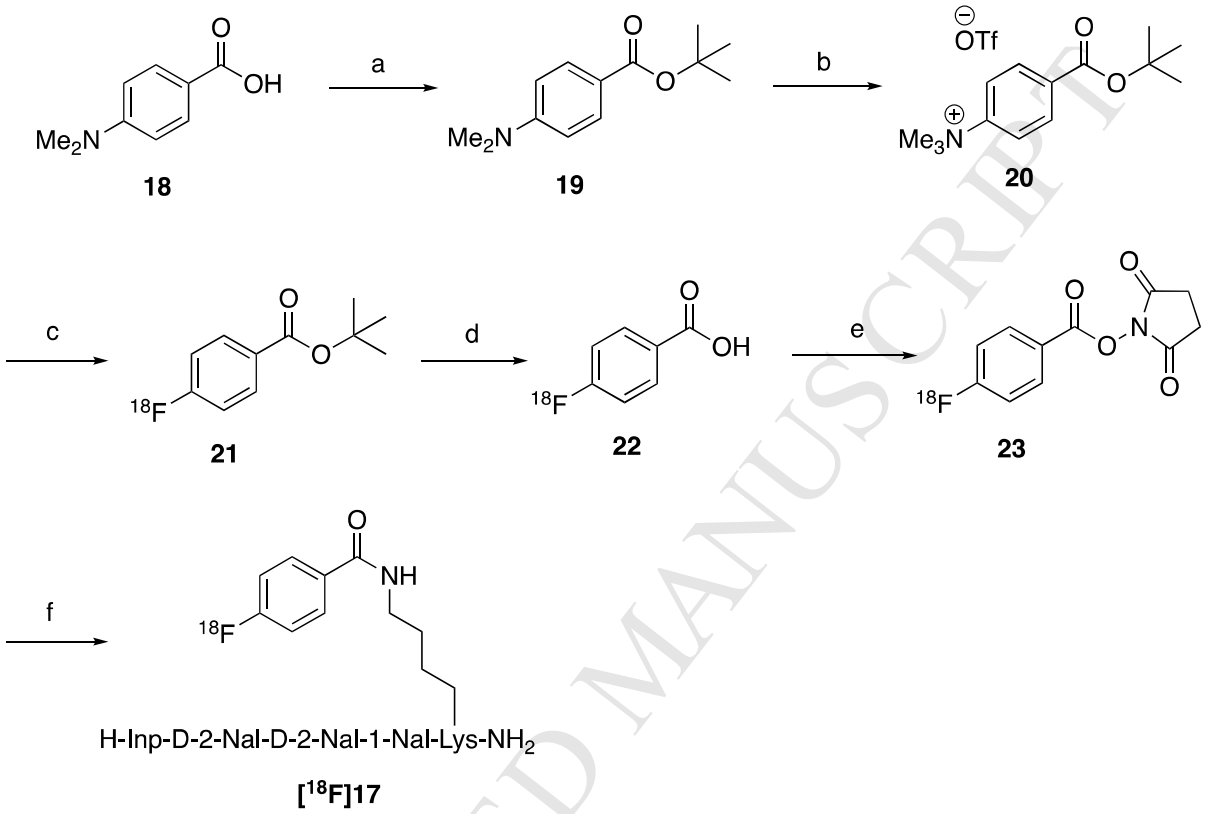
2.6 Stability of lead peptidomimetic [1-Nal^4,Lys^5(4-FB)]G-7039 in human serum

The lead peptidomimetic agonist [1-Nal^4,Lys^5(4-FB)]G-7039 was tested for stability in human serum in order to assess its biological half-life. This serum half-life was found to be 718 minutes, indicating a good potential time-frame for *in vivo* PET imaging in small animal models of carcinoma or cardiovascular disease. The [1-Nal^4]G-7039 lead precursor was thus carried forward for radiolabelling with [^{18}F]SFB.

2.7 Synthesis of [^{18}F]SFB and ^{18}F-radiolabelling of the lead precursor [1-Nal^4]G-7039

The synthesis of the [^{18}F]SFB prosthetic group is shown in scheme 1. Acid catalysed esterification of the 4-dimethylaminobenzoic acid starting material **18** furnished the intermediate t-butyl ester **19**. Methylation with trifluoromethanesulfonate (MeOTf) gave the triflate salt **20** in a yield of 38% and ≥ 95% purity by UHPLC. Having synthesized the precursor salt **20**, nucleophilic

aromatic substitution with the [$^{18}F^-$] anion (acquired from the PET cyclotron by irradiation of [^{18}O]H_2O) led to the formation of the radioactive compound **21**.



Scheme 1. Synthesis of the [^{18}F]SFB prosthetic group and subsequent ^{18}F-radiolabelling of [1-Nal^4]G-7039. *Reagents and Conditions*: a) i) trifluoroacetic anhydride, THF, 0°C ii) ^{t}BuOH, room temperature, 2 h; b) MeOTf, N_2, 0 °C, 1 h; c) $^{18}F^-$, K_2CO_3, Kryptofix 2.2.2, DMSO, 120 °C, 10 minutes; d) 6M HCl, 120 °C, 10 minutes; e) NHS, EDC, MeCN, room temperature, 15-20 minutes; f) [1-Nal^4]G-7039, *N*,*N*-diisopropylethylamine (DIPEA), MeCN/H_2O (1:1 ratio), 65 °C, 15-20 minutes.

Acid-catalysed de-esterification furnished [^{18}F]FBA (**22**) with an average d.c. (decay-corrected) radiochemical yield of 82%. The product was confirmed by co-injection with [^{19}F]FBA (see Figure S3 for representative radiochromatogram). Compound **22** was then coupled to *N*-hydroxysuccinimide (NHS) using the coupling reagent *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC) which led to the formation of the [^{18}F]SFB prosthetic group **23**. The crude

compound was purified by reverse-phase HPLC to give an average decay-corrected radiochemical yield of 71% and radiochemical purity ≥ 99% (see Figure S4 for representative radiochromatogram). The pure [^{18}F]SFB was added to the peptide precursor [1-Nal^4]G-7039 in a mixture of MeCN/H_2O using a small quantity of the hindered base DIPEA. After 15-20 minutes, the crude [1-Nal^4,Lys^5(4-[^{18}F]-FB)]G-7039 lead compound was obtained and purified by semi-preparative HPLC. The final radiolabelled peptide [1-Nal^4,Lys^5(4-[^{18}F]-FB)]G-7039 (**[^{18}F]17**) was obtained in an average decay-corrected radiochemical yield of 81%. For the entire synthesis, an overall average d.c. radiochemical yield of 48% was achieved (n = 3), an average molar activity of > 34 GBq/μmol and a radiochemical purity of ≥ 99%. Figure 3 displays a series of stacked chromatograms including the peptidomimetic precursor [1-Nal^4]G-7039 (**16**), the cold standard [1-Nal^4,Lys^5(4-FB)]G-7039 (**17**) as well as the radiolabelled [1-Nal^4,Lys^5(4-[^{18}F]-FB)]G-7039 peptidomimetic (**[^{18}F]17**).

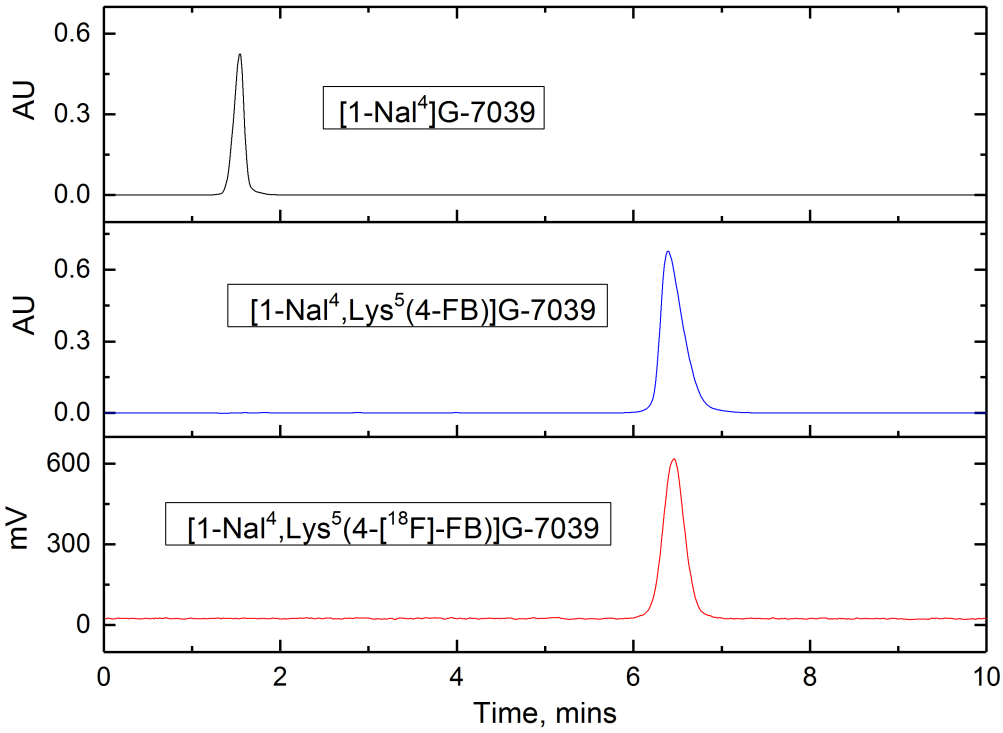


Figure 3. Stacked HPLC Chromatograms for [1-Nal^4]G-7039, [1-Nal^4,Lys^5(4-FB)]G-7039 (both λ = 254 nm) and [1-Nal^4,Lys^5(4-[^{18}F]-FB)]G-7039.

The retention times of the cold standard and "hot" peptide are almost identical ([1-Nal^4,Lys^5(4-FB)]G-7039, ^{t}R = 6.70 min compared to ^{t}R = 6.65 min for the "hot" peptide),

indicating that the precursor [1-Nal^4]G-7039 has been selectively radiolabelled at the amino group of the lysine side-chain. Further evidence for successful coupling of [^{18}F]SFB to [1-Nal^4]G-7039 *via* the lysine side-chain was provided by a co-injection of a pre-mixed solution of the cold standard and "hot" peptidomimetic (Figure 4).

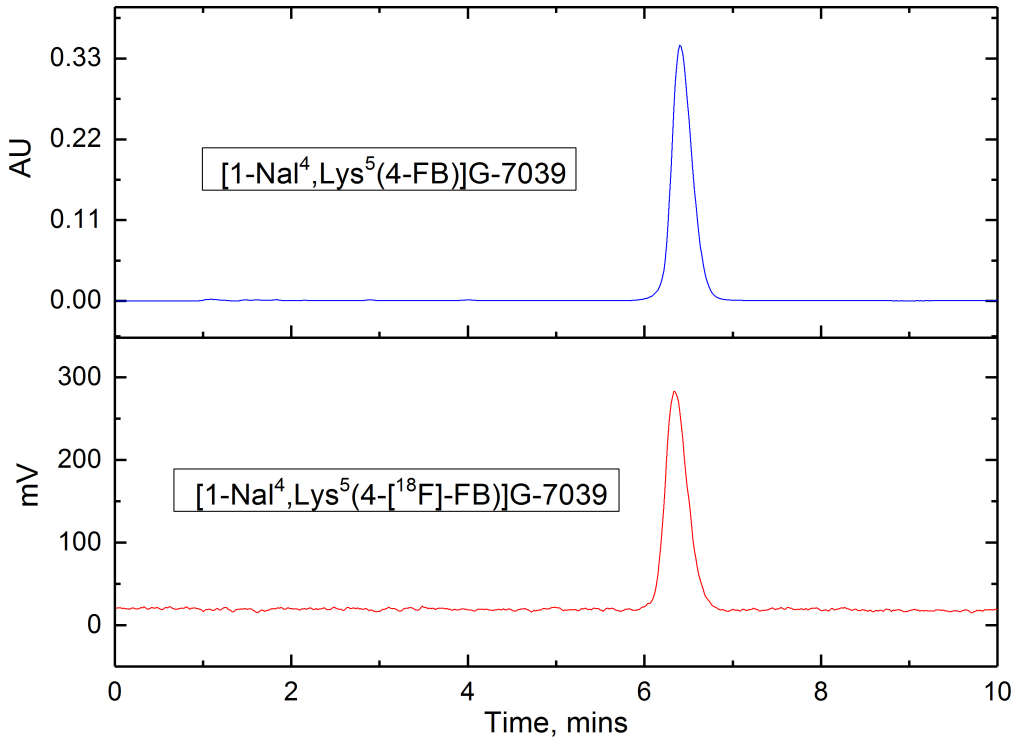


Figure 4. Stacked HPLC chromatograms resulting from the co-injection of cold standard [1-Nal^4,Lys^5(4-FB)]G-7039 (λ = 254 nm) and [1-Nal^4,Lys^5(4-[^{18}F]-FB)]G-7039.

Once again, this showed very similar retention times between the two peptidomimetic species ([1-Nal^4, Lys^5(4-FB)]G-7039 ^{t}R = 6.26 min and [1-Nal^4,Lys^5(4-[^{18}F]-FB)]G-7039 ^{t}R = 6.17 min) lending further credence to regioselective ^{18}F-fluorobenzoylation at the lysine side-chain.

3. Conclusions

In this study, several families of peptidic (GHRP-1, GHRP-2 and GHRP-6) and peptidomimetic (ipamorelin, G-7039, [1-Nal^4]G-7039) ghrelin receptor agonists were derivatised through 4-fluorobenzoylation of the lysine side-chain. This led to a range of half-maximal inhibitory concentrations ranging from 69 nM ([1-Nal^4,Lys^5(4-FB)]G-7039) to 1920 nM ([Inp^1,D-2-Nal^4, Lys^5(4-FB)]ipamorelin). The peptidomimetic [1-Nal^4]G-7039 was identified as the most

suitable candidate for ^{18}F-radiolabelling, as its ^{19}F-congener ([1-Nal^4,Lys^5(4-FB)]G-7039, compound **17**) had a nanomolar binding affinity (IC_{50} = 69 nM), high *in vitro* potency (EC_{50} = 1.1 nM) and good serum stability ($t_{1/2}$ = 718 min). This is most likely due to the presence of a core framework of aromatic amino acids that are involved in strong hydrophobic interactions with hydrophobic pockets of the ghrelin receptor. Prosthetic group radiolabelling of [1-Nal^4]G-7039 with [^{18}F]SFB delivered the radiolabelled peptidomimetic in an overall average decay-corrected radiochemical yield of 48%, a radio-purity ≥ 99% and an average molar activity of > 34 GBq/μmol. Further study of this compound would be required to assess its potential for *in vivo* PET imaging of diseases exhibiting heightened expression of the ghrelin receptor.

4. Materials and Methods

4.1 General information

All reagents were obtained from commercial suppliers and used without further purification. Peptides were either synthesised manually or through the use of a Biotage SyroWave automated peptide synthesizer. Peptide vessels were shaken using an IKA Vibrax VXR basic shaker with centrifugation performed on a Beckman Coulter Allegra X-30R or Fisher GS-6R centrifuge. In order to aid peptide dissolution, sonication of solutions was accomplished *via* a Bransonic 2510R-MTH or Fisher F5-14 ultrasonic cleaner. A Fisher 2052 Isotemp machine was used to heat test tubes in the Kaiser Test. Peptides were lyophilised using a Labconco FreeZone Freeze Dry System. Accurate weighing was carried out on a Mettler-Toledo XP6 microbalance. UV traces were obtained with a Waters 2487 UV/Vis Dual λ Absorbance Detector (170-900 nm) and low-resolution mass spectra with a Micromass Quattro *micro* API mass spectrometer (ESI-LC-MS). Peptide purification was achieved through HPLC (MeCN + 0.1% TFA, H_2O + 0.1% TFA solvent system). All peptides and small molecules obtained had a purity ≥ 95% as determined by HPLC or UHPLC analysis. A RP preparative C-18 column (SunFire OBD, 19 x 150 mm or Agilent Zorbax 21.2 x 150 mm) was used for preparative HPLC, whilst a C-18 RP column (SunFire, 4.6 x 150 mm or Agilent Zorbax, 4.6 x 150 mm) was used for analytical HPLC. Accurate mass spectrometry (HRMS) was carried out on a Finnigan MAT 8400 mass spectrometer (EI) for small molecules and on a Micromass LCT mass spectrometer (ESI-TOF) for peptides. ^{1}H NMR and ^{13}C NMR spectroscopy were performed on a Mercury VX 400 machine at 400 and 100 MHz respectively. Chemical shifts

are referenced to residual solvent, reported in ppm on a δ scale and all coupling constants quoted in hertz (Hz).

4.2 Manual Fmoc-SPPS

Rink amide MBHA resin (192 mg, 0.1 mmol, 1.0 equiv., 0.52 mmol g^{-1} loading) was vortexed in DCM (2.0 ml, 1 min.), allowed to swell (15 min) and solvent removed. This was followed by addition of DMF (2.0 ml), vortexing (1 min.) and removal of solvent. Deprotection of the Fmoc group was then performed. A solution of 20% piperidine/DMF (1.5 ml, v/v) was added to the resin and the subsequent mixture vortexed (2 min) and solvent removed. This was then repeated a second time with vortexing for 15 minutes. After solvent had been removed, the resin was washed of any unreacted by-products with DMF six times (2.0 ml, vortex 30 s). The desired amino acid or small molecule (0.3 mmol, 3.0 equiv.) and coupling reagent (HCTU, 0.12 g, 0.3 mmol, 3.0 equiv.) were then dissolved in DMF (1.5 ml) and added to the deprotected resin. After vortexing (30 s), DIPEA (111 μl, 0.6 mmol, 6.0 equiv.) was added and the final mixture vortexed (1-2 h). The resin was then washed with DMF (2.0 ml, 30 s vortex) a final four times. The deprotection/coupling cycle was then repeated unless the final amino acid in the sequence had been added, in which case the peptide was washed with DCM five times (2.0 ml, 30 s vortex) after washing with DMF and stored in a refrigerator. Removal of the N-terminal Fmoc-group was carried out in the same fashion as the deprotection cycle described previously, with resin washing occurring six times with DMF (2.0 ml, 30 s vortex) and four times with DCM (2.0 ml, 30 s vortex). Successful synthesis of the desired peptide was then ascertained *via* a microcleave prior to full cleavage of the peptide from the solid-support. This was carried out as follows: a solution of 95% TFA: 2.5% $(^{i}Pr)_3SiH$: 2.5% H_2O (300 μl) was added to a small number of resin beads (< 5 mg) and the subsequent mixture vortexed (3 h). The clear liquid was then evaporated under a stream of N_2. Analytical HPLC was then performed to determine whether the correct peptide had been synthesised. If the correct peptide had been obtained, a full cleavage was performed using a mixture of 95% TFA: 2.5% $(^{i}Pr)_3SiH$: 2.5% H_2O (2.0 ml) for 5-7 h. The subsequent solution was cooled in an ice-bath alongside *tert*-butyl methyl ether (TBMe, 40 ml). After 10 minutes, TBMe (20 ml) was added to the peptide solution, leading to the formation of a white precipitate. The precipitate was cooled further (10 min) and then centrifuged (7 min). Decanting of the supernatant was followed by addition of a second aliquot of TBMe (20 ml), vortexing (30 s) and final centrifugation (7 min). After decanting, a white solid was

obtained. This was then freeze-dried (20 min) to furnish crude peptide. Preparative HPLC was then used to purify the product peptide.

4.3 Deprotection of the Alloc protecting group

The resin-bound peptide was vortexed in DCM (4.5 ml, 30 s) and allowed to swell (10 min). Deprotection was carried out under a blanket of N_2. The swollen resin-bound peptide was stirred (5 min) before addition of $PhSiH_3$ (296 µl, 2.4 mmol, 24.0 equiv.). Further stirring (5 min) ensued prior to treating with $Pd(PPh_3)_4$ (12 mg, 0.01 mmol, 0.1 equiv.). After 5 minutes, the solution was vortexed (5 min), solvent removed and the brown-coloured resin washed four times with DCM (2.0 ml, 30 s vortex). The procedure was then repeated *ab initio*, with final resin washing occurring in the following order: DCM, DMF, MeOH, DMF and DCM (all 2.0 ml, 30 s vortex).

4.4 Kaiser Test

A small number of resin beads (< 5 mg) were taken and treated with phenol: EtOH (200 µl, 8:2 v/v), 0.001 M $KCN_{(aq.)}$: pyridine (200 µl, 2:98 v/v, 0.001 M aqueous KCN) and ninhydrin in EtOH (200 µl, 5% w/v), respectively. Tentagel resin (< 5 mg) was used as a control. Both test tubes were heated to 70 °C. The presence of free amine was indicated by blue resin beads whilst yellow or clear resin beads indicated the presence of protected amino groups.

4.5 Synthesis of peptidic- and peptidomimetic growth hormone secretagogues

All peptidomimetics were synthesised by the same general procedure described previously unless otherwise noted.

*4.5.1 H-Aib-His-D-2-Nal-D-Phe-Lys-NH_2: Ipamorelin (**1**)*

The product was purified by preparative HPLC (5-80% MeCN + 0.1% TFA). This furnished a white powder (20.5 mg, 19%): ^{1}H-NMR (400 MHz, CD_3OD); **D-2-Nal, D-Phe, His:** δ 7.99 (s, 1H, ArH), 7.80-7.76 (m, 1H, ArH), 7.71 (d, *J* = 8.6 Hz, 2H, ArH), 7.56 (s, 1H, ArH), 7.44-7.37 (m, 2H, ArH), 7.32-7.17 (m, 6H, ArH), 6.92 (s, 1H, His H_δ), 4.58 (m, 3H, H_α), 3.30-3.21 (m, 1H, D-2-Nal H_β), 3.15-3.00 (m, 2H, H_β), 2.96-2.72 (m, 3H, H_β), **Lys**: 4.11 (dd, *J* = 9.7, 4.2 Hz, 1H, H_α), 2.96-2.72 (m, 2H, H_ε), 1.75-1.64 (m, 1H, H_β), 1.54-1.40 (m, 3H, H_β, $2H_\delta$), 1.05-0.93

(m, 2H, H_γ), **Aib**: 1.48 (s, 3H, C$\underline{H}_3$) 1.44 (s, 3H, C$\underline{H}_3$) ppm. ESI-LC-MS *m/z* 356.9 $[M + 2H]^{2+}$; HRMS (ESI-MS) calcd. for $C_{38}H_{50}N_9O_5$ $[M + H]^+$ 712.3935, found 712.3959.

*4.5.2 H-Aib-His-D-2-Nal-D-Phe-Lys(4-FB)-NH_2: [Lys^5(4-FB)]ipamorelin (**2**)*

Purification by preparative HPLC (20-70% MeCN + 0.1% TFA) yielded a white powder (15.4 mg, 15%): ^{1}H-NMR (400 MHz, CD_3OD); **D-2-Nal, D-Phe, His, 4-FB:** δ 7.91 (s, 1H, ArH), 7.85-7.77 (m, 3H, ArH, 2F-ArH), 7.70 (d, *J* = 7.9 Hz, 2H, ArH), 7.54 (s, 1H, ArH), 7.45-7.40 (m, 2H, ArH), 7.29-7.19 (m, 6H, ArH), 7.14-7.07 (m, 2H, 2F-ArH), 6.93 (s, 1H, His H_δ), 4.65-4.52 (m, 3H, H_α), 3.30-3.27 (m, 1H, H_β), 3.23 (d, *J* = 4.0 Hz, 1H, H_β), 3.10 (dd, *J* = 13.5, 7.8 Hz, 1H, H_β), 3.04-2.74 (m, 3H, H_β), **Lys:** 4.12 (dd, *J* = 9.8, 4.2 Hz, 1H, H_α), 3.04-2.74 (m, 2H, H_ε), 1.77-1.67 (m, 1H, H_β), 1.58-1.42 (m, 3H, H_β, $2H_\delta$), 1.14-1.03 (m, 2H, H_γ), **Aib:** 1.50 (s, 3H, C$\underline{H}_3$), 1.46 (s, 3H, C$\underline{H}_3$) ppm. ESI-LC-MS *m/z* 418.0 $[M + 2H]^{2+}$; HRMS (ESI-MS) calcd. for $C_{45}H_{53}FN_9O_6$ $[M + H]^+$ 834.4103, found 834.4133.

*4.5.3 H-Aib-His-D-2-Nal-D-Phe-Lys(AEEA-4-FB)-NH_2:[Lys^5(AEEA-4-FB)]ipamorelin (**3**)*

Peptide purification by preparative HPLC (20-60% MeCN + 0.1% TFA) delivered an off-white powder (9.2 mg, 8%): ^{1}H-NMR (400 MHz, CD_3OD); **His, D-2-Nal, D-Phe, 4-FB:** δ 7.93 (s, 1H, ArH), 7.86-7.77 (m, 3H, ArH, 2F-ArH), 7.71 (d, *J* = 8.0 Hz, 2H, ArH), 7.55 (s, 1H, ArH), 7.46-7.41 (m, 2H, ArH), 7.31-7.18 (m, 6H, ArH), 7.17-7.10 (m, 2H, F-ArH), 6.94 (s, 1H, His H_δ), 4.65-4.58 (m, 2H, H_α), 4.55 (t, *J* = 7.5 Hz, 1H, H_α), 3.16-2.76 (m, 6H, H_β), **Lys:** 4.10 (dd, *J* = 9.7, 4.2 Hz, 1H, H_α), 3.16-2.76 (m, 2H, H_ε), 1.72-1.62 (m, 1H, H_β), 1.52-1.43 (m, 1H, H_β), 1.41-1.30 (m, 2H, H_δ), 1.07-0.97 (m, 2H, H_γ), **AEEA linker:** 3.93 (s, 2H, NHCOC$\underline{H}_2$O), 3.65-3.61 (m, 6H, C$\underline{H}_2$), 3.53 (t, *J* = 5.6 Hz, 2H, C$\underline{H}_2$), **Aib:** 1.50 (s, 3H, C$\underline{H}_3$), 1.47 (s, 3H, C$\underline{H}_3$) ppm. ESI-LC-MS *m/z* 490.4 $[M + 2H]^{2+}$; HRMS (ESI-MS) calcd. for $C_{51}H_{64}FN_{10}O_9$ $[M + H]^+$ 979.4842, found 979.4868.

*4.5.4 H-Aib-His-D-2-Nal-D-2-Thi-Lys(4-FB)-NH_2: [D-2-Thi^4,Lys^5(4-FB)]ipamorelin (**4**)*

The title peptide was synthesized by automated peptide synthesis and purified by preparative HPLC (20-80% MeCN + 0.1% TFA). This furnished a white solid (4.20 mg, 4%): ^{1}H-NMR (400 MHz, $(CD_3)_2SO$); δ 8.83 (s, 1H, ArH), 8.59 (d, *J* = 8.1 Hz, 1H, NH), 8.42 (t, *J* = 5.6

Hz, 1H, NH), 8.29 (d, *J* = 9.3 Hz, 1H, NH), 8.20 (d, *J* = 8.2 Hz, 1H, NH), 8.09 (d, *J* = 8.9 Hz, 1H, NH), 7.99 (s, 2H, NH), 7.86-7.81 (m, 2H, F-ArH), 7.79-7.75 (m, 1H, ArH), 7.72 (dd, *J* = 8.7, 3.7 Hz, 2H, ArH), 7.65 (s, 1H, ArH), 7.41-7.37 (m, 2H, ArH), 7.35-7.31 (m, 2H, ArH, NH), 7.26 (dd, *J* = 4.8, 1.5 Hz, 1H, Thi-H), 7.22-7.16 (m, 2H, F-ArH), 7.05 (s, 2H, NH, ArH), 6.88-6.84 (m, 2H, Thi-H), 4.73-4.66 (m, 1H, H_α), 4.62-4.51 (m, 2H, H_α), 4.19-4.12 (m, 1H, Lys-H_α), 3.21-3.12 (m, 4H, $2H_\varepsilon$, $2H_\beta$), 3.06-2.96 (m, 1H, H_β), 2.87-2.77 (m, 2H, H_β), 2.62-2.49 (m, 1H, H_β), 1.66-1.56 (m, 1H, Lys-H_β), 1.50-1.36 (m, 3H, Lys-H_β, $2H_\delta$), 1.26 (s, 3H, C$\underline{H}_3$), 1.20-1.10 (m, 2H, H_γ), 1.16 (s, 3H, C$\underline{H}_3$), ppm. ESI-LC-MS *m/z* 420.8 $[M + 2H]^{2+}$; HRMS (ESI-MS) calcd. for $C_{43}H_{51}FN_9O_6S$ $[M + H]^+$ 840.3667 found 840.3693.

*4.5.5 H-Inp-His-D-2-Nal-D-Phe-Lys(4-FB)-NH$_2$: [Inp^1,Lys^5(4-FB)]ipamorelin (**5**)*

The title peptide was synthesized *via* automated peptide synthesis and purified by preparative HPLC (20-70% MeCN + 0.1% TFA). The title compound was acquired as a white solid (6.30 mg, 6%): ^{1}H-NMR (400 MHz, $(CD_3)_2SO$); δ 8.79 (s, 1H, His H_ε), 8.72 (s, 1H, NH), 8.44 (dd, *J* = 10.8, 6.4 Hz, 2H, NH), 8.19 (d, *J* = 7.8 Hz, 1H, NH), 8.17-8.10 (m, 2H, NH), 7.87-7.81 (m, 2H, F-ArH), 7.80-7.76 (m, 1H, ArH), 7.71 (d, *J* = 7.9 Hz, 2H, ArH), 7.63 (s, 1H, ArH), 7.42-7.35 (m, 2H, ArH), 7.33-7.29 (m, 2H, NH), 7.22-7.18 (m, 6H, ArH), 7.17-7.11 (m, 2H, F-ArH), 7.06 (s, 1H, NH), 6.96 (s, 1H, His H_δ), 4.64-4.56 (m, 1H, H_α), 4.55-4.44 (m, 2H, H_α), 4.15-4.07 (m, 1H, Lys-H_α), 3.19-3.07 (m, 5H, C$\underline{H}_2$), 2.94 (dd, *J* = 13.6, 5.9 Hz, 1H, C$\underline{H}_2$), 2.87-2.67 (m, 5H, C$\underline{H}_2$), 2.58-2.48 (m, 1H, C$\underline{H}_2$), 2.38-2.29 (m, 1H, H_α), 1.67-1.34 (m, 8H, C$\underline{H}_2$), 1.15-1.00 (m, 2H, C$\underline{H}_2$), ppm. ESI-LC-MS *m/z* 430.9 $[M + 2H]^{2+}$; HRMS (ESI-MS) calcd. for $C_{47}H_{55}FN_9O_6$ $[M + H]^+$ 860.4259 found 860.4284.

*4.5.6 H-Inp-His-D-2-Nal-D-2-Nal-Lys(4-FB)-NH$_2$: [Inp^1,D-2-Nal^4,Lys^5(4-FB)]ipamorelin (**6**)*

The title peptide was made by automated peptide synthesis and purified by preparative HPLC (20-80% MeCN + 0.1% TFA). This delivered a white solid (11.3 mg, 10%): ^{1}H-NMR (400 MHz, $(CD_3)_2SO$); δ 8.77 (s, 1H, His H_ε), 8.51 (d, *J* = 7.5 Hz, 2H, NH), 8.36 (t, *J* = 5.5 Hz, 1H, NH), 8.19-8.10 (m, 2H, NH), 8.05 (d, *J* = 8.7 Hz, 1H, NH), 7.85-7.79 (m, 3H, 2F-ArH, ArH), 7.79-7.74 (m, 3H, ArH), 7.73 (s, 1H, ArH), 7.70 (s, 1H, ArH), 7.68 (d, *J* = 2.4 Hz, 2H, ArH), 7.59 (s, 1H, ArH), 7.43-7.35 (m, 5H, ArH), 7.31-7.27 (m, 2H, NH), 7.20-7.13 (m, 2H, F-ArH), 7.05 (s, 1H, NH), 6.93 (s, 1H, His H_δ), 4.67-4.60 (m, 2H, H_α), 4.52-4.44 (m, 1H, H_α), 4.16-4.08

(m, 1H, Lys-H_α), 3.17-2.93 (m, 7H, C$\underline{H}_2$), 2.88-2.66 (m, 4H, C$\underline{H}_2$), 2.53-2.47 (m, 1H, C$\underline{H}_2$), 2.34-2.25 (m, 1H, H_α), 1.63-1.34 (m, 6H, C$\underline{H}_2$), 1.32-1.23 (m, 2H, C$\underline{H}_2$), 1.07-0.95 (m, 2H, C$\underline{H}_2$), ppm. ESI-LC-MS *m/z* 455.9 $[M + 2H]^{2+}$; HRMS (ESI-MS) calcd. for $C_{51}H_{57}FN_9O_6$ $[M + H]^+$ 910.4416 found 910.4400.

*4.5.7 H-Inp-His-D-2-Nal-D-2-Thi-Lys(4-FB)-NH_2: [Inp[1],D-2-Thi[4],Lys[5](4-FB)]ipamorelin (**7**)*

The title peptide was prepared by automated peptide synthesis and purified by preparative HPLC (20-80% MeCN + 0.1% TFA). This furnished a white powder (14.0 mg, 13%): ^{1}H-NMR (400 MHz, CD_3OD); **His, D-2-Nal, D-2-Thi, 4-FB:** δ 8.23 (s, 1H, His H_ε), 7.86-7.81 (m, 2H, F-ArH), 7.80-7.77 (m, 1H, ArH), 7.73 (dd, *J* = 8.8, 3.3 Hz, 2H, ArH), 7.59 (s, 1H, ArH), 7.45-7.39 (m, 2H, ArH), 7.27 (dd, *J* = 8.5, 1.6 Hz, 1H, ArH), 7.22 (dd, *J* = 5.0, 1.2 Hz, 1H, Thi-ArH), 7.15-7.08 (m, 2H, F-ArH), 6.92 (s, 1H, His H_δ), 6.91-6.85 (m, 2H, Thi-ArH), 4.61 (dd, *J* = 10.3, 4.4 Hz, 1H, H_α), 4.53-4.47 (m, 2H, H_α), 4.22 (dd, *J* = 9.8, 4.2 Hz, 1H, Lys-H_α), 3.39-3.29 (m, 6H, C$\underline{H}_2$), 3.26-3.22 (m, 1H, C$\underline{H}_2$), 3.01-2.80 (m, 5H, C$\underline{H}_2$), 2.53-2.44 (m, 1H, Inp-H_α), 1.87-1.50 (m, 8H, C$\underline{H}_2$), 1.35-1.22 (m, 2H, C$\underline{H}_2$), ppm. ESI-LC-MS *m/z* 433.8 $[M + 2H]^{2+}$; HRMS (ESI-MS) calcd. for $C_{45}H_{53}FN_9O_6S$ $[M + H]^+$ 866.3824 found 866.3850.

*4.5.8 H-Ala-His-D-2-Nal-Ala-Trp-D-Phe-Lys-NH_2: GHRP-1 (**8**)*

The product was purified by preparative HPLC (15-80% MeCN + 0.1% TFA). This yielded a white powder (26.4 mg, 19%): ^{1}H-NMR (400 MHz, CD_3OD); **His, D-2-Nal, Trp, D-Phe:** δ 8.41 (s, 1H, His H_ε), 7.81-7.71 (m, 3H, ArH), 7.65 (s, 1H, ArH), 7.49 (d, *J* = 7.8 Hz, 1H, ArH), 7.46-7.40 (m, 2H, ArH), 7.35 (dd, *J* = 8.4, 1.7 Hz, 1H, ArH), 7.31 (d, *J* = 8.1 Hz, 1H, ArH), 7.24-7.13 (m, 3H, ArH), 7.10-7.04 (m, 4H, ArH), 7.00 (t, *J* = 7.5 Hz, 1H, ArH), 6.89 (d, *J* = 0.8 Hz, 1H, His H_δ), 4.67-4.60 (m, 2H, H_α), 4.38 (t, *J* = 7.4 Hz, 1H, H_α), 4.32 (t, *J* = 7.7 Hz, 1H, H_α), 3.25-2.68 (m, 8H, H_β), **Ala, Ala:** 4.17-4.10 (m, 1H, H_α), 3.94 (q, *J* = 7.0 Hz, 1H, H_α), 1.35 (d, *J* = 7.1 Hz, 3H, C$\underline{H}_3$), 1.10-1.01 (m, 3H, C$\underline{H}_3$), **Lys:** 4.17-4.10 (m, 1H, H_α), 3.25-2.68 (m, 2H, H_ε), 1.81-1.70 (m, 1H, H_β), 1.58-1.45 (3H, H_β, 2H_δ), 1.10-1.01 (m, 2H, H_γ), ppm. ESI-LC-MS *m/z* 478.5 $[M + 2H]^{2+}$; HRMS (ESI-MS) calcd. for $C_{51}H_{63}N_{12}O_7$ $[M + H]^+$ 955.4943 found 955.4964.

*4.5.9 H-D-Ala-D-2-Nal-Ala-Trp-D-Phe-Lys-NH_2: GHRP-2 (**9**)*

Purification by preparative HPLC (25-70% MeCN + 0.1% TFA) delivered the title peptide as a white powder (19.7 mg, 17%): ^{1}H-NMR (400 MHz, CD_3OD); **D-2-Nal**, **Trp, D-Phe**: δ 7.76 (d, *J* = 9.2 Hz, 2H, ArH), 7.73 (s, 1H, ArH), 7.65 (s, 1H, ArH), 7.51-7.47 (m, 1H, ArH), 7.44-7.37 (m, 2H, ArH), 7.35 (dd, *J* = 8.4, 1.7 Hz, 1H, ArH), 7.25-7.14 (m, 4H, ArH), 7.09-7.01 (m, 4H, ArH), 7.00 (s, 1H, ArH), 4.77 (dd, *J* = 10.9, 4.6 Hz, 1H, H_α), 4.49 (t, *J* = 6.8 Hz, 1H, H_α), 4.36 (t, *J* = 7.6 Hz, 1H, H_α), 3.11 (t, *J* = 6.7 Hz, 2H, H_β), 3.07 (d, *J* = 4.5 Hz, 1H, H_β), 2.88-2.70 (m, 3H, H_β), **D-Ala, Ala:** 4.23 (q, *J* = 7.1 Hz, 1H, H_α), 3.79 (q, *J* = 7.0 Hz, 1H, H_α), 1.27 (d, *J* = 7.2 Hz, 3H, C$\underline{H}_3$), 1.00 (d, *J* = 7.1 Hz, 3H, C$\underline{H}_3$), **Lys:** 4.16 (dd, *J* = 10.3, 4.1 Hz, 1H, H_α), 2.88-2.70 (m, 2H, H_ε), 1.82-1.72 (m, 1H, H_β), 1.56-1.43 (m, 3H, H_β, $2H_\delta$), 1.07-0.96 (m, 2H, H_γ), ppm. ESI-LC-MS *m/z* 409.9 $[M + 2H]^{2+}$; HRMS (ESI-MS) calcd. for $C_{45}H_{55}N_9O_6Na$ $[M + Na]^+$ 840.4204 found 840.4173.

*4.5.10 H-His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2: GHRP-6 (**10**)*

Purification by preparative HPLC (15-80% MeCN + 0.1% TFA) gave a white powder (19.7 mg, 14%): ^{1}H-NMR (400 MHz, CD_3OD); **His, D-Trp, Trp, D-Phe:** δ 8.46 (s, 1H, His H_ε), 7.54 (d, *J* = 7.8 Hz, 1H, ArH), 7.46 (d, *J* = 7.8 Hz, 1H, ArH), 7.28 (t, *J* = 7.7 Hz, 2H, ArH), 7.23-7.13 (m, 4H, ArH), 7.12-6.92 (m, 8H, 7ArH, His H_δ), 4.47 (t, *J* = 7.4 Hz, 2H, Trp H_α), 4.35 (t, *J* = 6.7 Hz, 1H, H_α), 4.28 (t, *J* = 7.9 Hz, 1H, H_α), 3.22-3.14 (m, 3H, H_β), 3.14-3.02 (m, 3H, H_β), 2.83 (d, *J* = 8.6 Hz, 2H, H_β), **Lys:** 4.03 (dd, *J* = 10.3, 3.9 Hz, 1H, H_α), 2.75 (t, *J* = 6.8 Hz, 2H, H_ε), 1.76-1.63 (m, 1H, H_β), 1.51-1.39 (m, 3H, H_β, $2H_\delta$), 0.97-0.88 (m, 2H, H_γ), **Ala:** 3.91 (q, *J* = 7.2 Hz, 1H, H_α), 0.86 (d, *J* = 7.3 Hz, 3H, C$\underline{H}_3$) ppm. ESI-LC-MS *m/z* 437.4 $[M + 2H]^{2+}$; HRMS (ESI-MS) calcd. for $C_{46}H_{57}N_{12}O_6$ $[M + H]^+$ 873.4524 found 873.4531.

*4.5.11 H-His-D-Trp-Ala-Trp-D-Phe-Lys(4-FB)-NH_2: [Lys^6(4-FB)]GHRP-6 (**11**)*

The product was purified by preparative HPLC (15-80% MeCN + 0.1% TFA). This yielded a white powder (9.60 mg, 7%): ^{1}H-NMR (400 MHz, CD_3OD); **His, D-Trp, Trp, D-Phe, 4-FB:** δ 8.50 (s, 1H, His H_ε), 7.88-7.80 (m, 2H, F-ArH), 7.57 (d, *J* = 7.9 Hz, 1H, ArH), 7.49 (d, *J* = 7.8 Hz, 1H, ArH), 7.30 (t, *J* = 8.2 Hz, 2H, ArH), 7.26-6.95 (m, 14H, 13ArH, His H_δ), 4.53-4.46 (m, 2H, H_α), 4.37-4.30 (m, 2H, H_α), 3.26-3.04 (m, 6H, H_β), 2.87 (d, *J* = 8.0 Hz, 2H, H_β), **Lys:** 4.04 (dd, *J* = 10.2, 4.0 Hz, 1H, H_α), 3.26-3.04 (m, 2H, H_ε), 1.81-1.70 (m, 1H, H_β), 1.58-1.43 (m, 3H, $2H_\delta$, H_β), 1.06-0.97 (m, 2H, H_γ), **Ala:** 3.94 (q, *J* = 7.3 Hz, 1H, H_α), 0.87 (d, *J* = 7.3 Hz, 3H,

C<u>H</u>$_3$) ppm. ESI-LC-MS *m/z* 498.4 $[M + 2H]^{2+}$; HRMS (ESI-MS) calcd. for $C_{53}H_{59}FN_{12}O_7Na$ $[M + Na]^+$ 1017.4511 found 1017.4522.

*4.5.12 H-His-D-Trp-Ala-Trp-D-Phe-Dpr-NH$_2$: [Dpr^6]GHRP-6 (**12**)*

Purification by preparative HPLC (15-80% MeCN + 0.1% TFA) furnished the title peptide as a white powder (4.7 mg, 3 %): ^{1}H-NMR (400 MHz, CD_3OD); 8.49 (s, 1H His H_ε), 7.55 (d, *J* = 7.9 Hz, 1H, ArH), 7.46 (d, *J* = 7.9 Hz, 1H, ArH), 7.30 (t, *J* = 8.6 Hz, 2H, ArH), 7.21-7.12 (m, 3H, ArH), 7.11-7.04 (m, 3H, ArH), 7.02-6.92 (m, 5H, ArH), 6.89 (d, *J* = 1.0 Hz, 1H, His H_δ), 4.62 (t, *J* = 6.8 Hz, 1H, H_α), 4.52 (t, *J* = 7.9 Hz, 1H, H_α), 4.42-4.33 (m, 2H, H_α), 4.28 (t, *J* = 6.7 Hz, 1H, Dpr-H_α), 4.00 (q, *J* = 7.3 Hz, 1H, Ala-H_α), 3.24-3.11 (m, 3H, C<u>H</u>$_2$), 3.12-2.92 (m, 5H, C<u>H</u>$_2$), 2.89-2.76 (m, 2H, C<u>H</u>$_2$), 0.94 (d, *J* = 7.3 Hz, 3H, Ala-C<u>H</u>$_3$), ppm. ESI-LC-MS *m/z* 831.4 $[M + H]^+$; HRMS (ESI-MS) calcd. for $C_{43}H_{51}N_{12}O_6$ $[M + H]^+$ 831.4055 found 831.4070.

*4.5.13 H-His-D-Trp-Ala-Trp-D-Phe-Dpr(4-FB)-NH$_2$: [Dpr^6(4-FB)]GHRP-6 (**13**)*

Preparative HPLC (25-70% MeCN + 0.1% TFA) gave the title compound as a white solid (19.8 mg, 14%): ^{1}H-NMR (400 MHz, CD_3OD); **His, D-Trp, Trp, D-Phe, 4-FB:** δ 8.58 (d, *J* = 1.3 Hz, 1H, His H_ε), 7.82-7.75 (m, 2H, F-ArH), 7.52 (d, *J* = 7.9 Hz, 1H, ArH), 7.40 (d, *J* = 7.9 Hz, 1H, ArH), 7.27 (dd, *J* = 8.6, 1.5 Hz, 2H, ArH), 7.15-7.07 (m, 5H, ArH), 7.06-6.98 (m, 6H, ArH), 6.97-6.89 (m, 3H, 2ArH, His H_δ), 4.54-4.45 (m, 1H, H_α), 4.44-4.30 (m, 3H, H_α), 3.22-2.98 (m, 6H, H_β), 2.93-2.74 (m, 2H, H_β), **Dpr:** 4.54-4.45 (m, 1H, H_α), 3.67 (dd, *J* = 13.8, 5.6 Hz, 1H, H_β), 3.54 (dd, *J* = 13.8, 7.8 Hz, 1H, H_β), **Ala:** 3.93 (q, *J* = 7.3 Hz, 1H, H_α), 0.86 (d, *J* = 7.3 Hz, 3H, C<u>H</u>$_3$) ppm. ESI-LC-MS *m/z* 477.4 $[M + 2H]^{2+}$; HRMS (ESI-MS) calcd. for $C_{50}H_{54}FN_{12}O_7$ $[M + H]^+$ 953.4223 found 953.4237.

*4.5.14 H-Inp-D-2-Nal-D-2-Nal-Phe-Lys-NH$_2$: G-7039* (**14**)*

Purification of the peptide proceeded through preparative HPLC (25-80% MeCN + 0.1% TFA). The title compound was obtained as a white powder (6.70 mg, 7%): ^{1}H-NMR (400 MHz, CD_3OD); 7.80-7.76 (m, 1H, ArH), 7.74 (dd, *J* = 6.1, 2.2 Hz, 2H, ArH), 7.70 (dd, *J* = 8.5, 3.5 Hz, 3H, ArH), 7.56 (s, 1H, ArH), 7.50 (s, 1H, ArH), 7.46-7.35 (m, 4H, ArH), 7.26 (dd, *J* = 8.5, 1.5 Hz, 1H, ArH), 7.20-7.10 (m, 6H, ArH), 4.66-4.56 (m, 2H, H_α), 4.50 (dd, *J* = 9.3, 5.4 Hz, 1H, H_α), 4.30 (dd, *J* = 9.5, 4.7 Hz, 1H, Lys-H_α), 3.20-2.80 (m, 10H, C<u>H</u>$_2$), 2.78-2.64 (m, 2H, C<u>H</u>$_2$), 2.33-2.24 (m,

1H, Inp-H_α), 1.92-1.82 (m, 1H, C$\underline{H}_2$), 1.74-1.65 (m, 1H, C$\underline{H}_2$), 1.64-1.44 (m, 5H, C$\underline{H}_2$), 1.44-1.24 (m, 3H, C$\underline{H}_2$), ppm. ESI-LC-MS *m/z* 399.8 $[M + 2H]^{2+}$; HRMS (ESI-MS) calcd. for $C_{47}H_{56}N_7O_5$ $[M + H]^+$ 798.4343 found 798.4339. HRMS data and yield has been reported previously[35], whereas the ^{1}H-NMR data has not.

*4.5.15 H-Inp-D-2-Nal-D-2-Nal-Phe-Lys(4-FB)-NH_2: [Lys^5(4-FB)]G-7039 (**15**)*

Preparative HPLC (35-80% MeCN + 0.1% TFA) furnished the title compound as a white solid (5.90 mg, 6%): ^{1}H-NMR (400 MHz, $(CD_3)_2SO$); δ 8.50 (d, *J* = 8.2 Hz, 1H, NH), 8.45 (t, *J* = 5.5 Hz, 1H, NH), 8.39 (s, 1H, NH), 8.15-8.09 (m, 2H, NH), 8.04 (d, *J* = 8.4 Hz, 1H, NH), 7.86-7.82 (m, 2H, F-ArH), 7.82-7.76 (m, 2H, ArH), 7.75-7.66 (m, 4H, ArH), 7.58 (s, 1H, ArH), 7.48 (s, 1H, ArH), 7.45-7.38 (m, 4H, ArH), 7.31 (dd, *J* = 8.5, 1.5 Hz, 1H, ArH), 7.28 (s, 1H, ArH), 7.26 (s, 1H, NH), 7.24-7.17 (m, 5H, ArH), 7.15-7.08 (m, 2H, ArH), 7.04 (s, 1H, NH), 4.66-4.60 (m, 1H, H_α), 4.60-4.53 (m, 2H, H_α), 4.20-4.14 (m, 1H, Lys-H_α), 3.25-3.14 (m, 2H, C$\underline{H}_2$), 3.13-3.02 (m, 3H, C$\underline{H}_2$), 2.98-2.91 (m, 1H, C$\underline{H}_2$), 2.90-2.83 (m, 1H, C$\underline{H}_2$), 2.81-2.56 (m, 5H, C$\underline{H}_2$), 2.30-2.22 (m, 1H, Inp-H_α), 1.75-1.65 (m, 1H, C$\underline{H}_2$), 1.62-1.55 (m, 1H, C$\underline{H}_2$), 1.54-1.43 (m, 6H, C$\underline{H}_2$), 1.38-1.19 (m, 2H, C$\underline{H}_2$), ppm. ESI-LC-MS *m/z* 452.5 $[M + 4H - F]^{2+}$; HRMS (ESI-MS) calcd. for $C_{54}H_{59}FN_7O_6$ $[M + H]^+$ 920.4511 found 920.4529.

*4.5.16 H-Inp-D-2-Nal-D-2-Nal-1-Nal-Lys-NH_2: [1-Nal^4]G-7039 (**16**)*

Peptide purification by preparative HPLC (25-80% MeCN + 0.1% TFA) delivered a white powder (24.9 mg, 23%): ^{1}H-NMR (400 MHz, CD_3OD); δ 8.12 (d, *J* = 8.4 Hz, 1H, ArH), 7.84 (d, *J* = 7.8 Hz, 1H, ArH), 7.78-7.72 (m, 2H, ArH), 7.68 (d, *J* = 8.5 Hz, 3H, ArH), 7.62-7.46 (m, 5H, ArH), 7.43-7.35 (m, 5H, ArH), 7.30 (d, *J* = 6.4 Hz, 1H, ArH), 7.27-7.21 (m, 2H, ArH), 7.02 (dd, *J* = 8.4, 1.5 Hz, 1H, ArH), 4.69 (dd, *J* = 9.4, 5.4 Hz, 1H, H_α), 4.65-4.55 (m, 2H, H_α), 4.32 (dd, *J* = 9.4, 4.8 Hz, 1H, Lys-H_α), 3.65 (dd, *J* = 14.4, 5.4 Hz, 1H, C$\underline{H}_2$), 3.20-3.10 (m, 2H, C$\underline{H}_2$), 3.05-2.94 (m, 3H, C$\underline{H}_2$), 2.92-2.80 (m, 5H, C$\underline{H}_2$), 2.76-2.62 (m, 2H, C$\underline{H}_2$), 2.30-2.23 (m, 1H, Inp-H_α), 1.92-1.81 (m, 1H, C$\underline{H}_2$), 1.75-1.29 (m, 9H, C$\underline{H}_2$), ppm. ESI-LC-MS *m/z* 424.8 $[M + 2H]^{2+}$; HRMS (ESI-MS) calcd. for $C_{51}H_{58}N_7O_5$ $[M + H]^+$ 848.4499, found 848.4501.

*4.5.17 H-Inp-D-2-Nal-D-2-Nal-1-Nal-Lys(4-FB)-NH_2: [1-Nal^4,Lys^5(4-FB)]G-7039 (**17**)*

The product was purified by preparative HPLC (25-90% MeCN + 0.1% TFA) which yielded a white powder (9.80 mg, 9%): ^{1}H-NMR (400 MHz, $(CD_3)_2SO$); δ 8.64 (d, *J* = 8.3 Hz, 1H, NH), 8.46 (t, *J* = 5.5 Hz, 1H, NH), 8.33 (s, 1H, NH), 8.25 (d, *J* = 8.4 Hz, 1H, ArH), 8.13 (d, *J* = 8.0 Hz, 1H, NH), 8.09 (d, *J* = 7.5 Hz, 1H, NH), 8.03 (d, *J* = 8.5 Hz, 1H, NH), 8.01-7.97 (m, 1H, NH), 7.89 (d, *J* = 8.2 Hz, 1H, ArH), 7.86-7.80 (m, 2H, F-ArH), 7.79-7.72 (m, 3H, ArH), 7.71-7.66 (m, 2H, ArH), 7.58-7.50 (m, 4H, ArH), 7.44 (d, *J* = 6.9 Hz, 1H, ArH), 7.42-7.34 (m, 5H, ArH), 7.31-7.25 (m, 2H, ArH), 7.21-7.15 (m, 3H, 2F-ArH, ArH), 7.07 (s, 1H, NH), 7.02 (d, *J* = 8.4 Hz, 1H, ArH), 4.72-4.65 (m, 1H, H_α), 4.62-4.50 (m, 2H, H_α), 4.19 (dd, *J* = 13.3, 8.4 Hz, 1H, Lys-H_α), 3.61 (dd, *J* = 14.3, 3.9, 1H, C$\underline{H}_2$), 3.24-3.00 (m, 5H, C$\underline{H}_2$), 2.97-2.89 (m, 1H, C$\underline{H}_2$), 2.82-2.49 (m, 5H, C$\underline{H}_2$), 2.29-2.19 (m, 1H, Inp-H_α), 1.78-1.66 (m, 1H, C$\underline{H}_2$), 1.66-1.54 (m, 1H, C$\underline{H}_2$), 1.53-1.40 (m, 5H, C$\underline{H}_2$), 1.40-1.16 (m, 2H, C$\underline{H}_2$), ppm. ESI-LC-MS *m/z* 477.4 $[M + 4H - F]^{2+}$; HRMS (ESI-MS) calcd. for $C_{58}H_{61}FN_7O_6$ $[M + H]^+$ 970.4667 found 970.4693.

4.6 Synthesis of 4-(tert-butoxycarbonyl)-N,N,N-trimethylbenzenammonium triflate (**20**)

4-dimethylaminobenzoic acid (1.00 g, 6.05 mmol, 1.0 equiv.) was added to THF (50 ml) and the resultant mixture stirred and cooled to 0°C. After 15 minutes, trifluoroacetic anhydride (1.85 ml, 13.3 mmol, 2.2 equiv.) was added dropwise and the consequent blue solution stirred (35 min). Addition of ^{t}BuOH (11.4 ml, 119 mmol, 19.7 equiv.) was followed by further stirring at room temperature (2 h). The solution was then poured into saturated $NaHCO_3$ (250 ml) and extracted with DCM (3x 100 ml, 3x 50 ml). The combined organic layers were dried ($MgSO_4$), filtered by gravity and residual solvent removed by rotary evaporation. This delivered a black oil which was eluted through a silica pad (DCM, 60 ml) and solvent removed by concentration *in vacuo*. The final yellow oil (1.12 g, crude) was then re-dissolved in DCM (dry, 30 ml) and cooled to 0°C under a blanket of N_2. This was followed by the addition of MeOTf (0.86 ml, 7.61 mmol, 1.5 equiv.) and stirring at 0°C (1 h). The reaction mixture was then poured into an ice-cold solution of Et_2O (200 ml) which caused instant precipitation of the product salt as a white crystalline solid (740 mg, 38%): ^{1}H-NMR (400 MHz, CD_3COCD_3); δ 8.24 (d, *J* = 9.3 Hz, 2H), 8.18 (d, *J* = 9.3 Hz, 2H), 3.91 (s, 9H, NMe_3), 1.60 (s, 9H, OC(C$\underline{H}_3$)$_3$); ^{13}C-NMR (100 MHz, CD_3COCD_3); δ 205.5 (CO) 163.6 (ArC), 133.7 (ArC), 131.0 (ArC), 120.9 (ArC), 81.7, 56.9, 27.3 (C$\underline{H}_3$CO) ppm. ESI-MS

m/*z* 165.1 $[C_{10}H_{13}O_2]^+$; HRMS (ESI-MS) calcd for $C_{14}H_{22}NO_2$ $[M\text{-}CF_3O_3S]^+$ 236.1651, found 236.1658.

4.7 Receptor-ligand binding assay

Competitive binding assays were carried out using HEK293/ghrelin receptor cells with [^{125}I]ghrelin as the competitive radioligand. A solution of binding buffer (50 ml, pH 7.4) was made up by adding 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, 0.3 g, 25 mM), $MgCl_2$ (0.051 g, 5 mM), $CaCl_2$ (7.4 x 10^{-3} g, 1 mM), EDTA (0.015 g, 2.5 mM) and BSA (0.2 g, 0.4%) to distilled water. The resultant solution was filtered through a 0.22 μm syringe filter and kept on ice.

An aliquot of frozen cells was thawed to room temperature (1.5 x 10^6 cells/pellet), centrifuged (3000 rpm, 10 min, room temperature) and the subsequent cell pellet re-suspended in binding buffer (2 ml) and placed on ice. A stock solution of peptide/peptidomimetic was diluted in binding buffer to acquire final concentrations between 10^{-5} to 10^{-11} M and added (30 µl) to these cells (50 µl) in triplicate.

[^{125}I]-ghrelin (10 µl) was added to binding buffer (3 ml) and vortexed. An aliquot of this [^{125}I]-ghrelin solution (20 µl) was counted on a Cobra II Auto-Gamma gamma counter and initial volume adjusted to obtain 14000-17000 counts per minute (cpm) per 20 µl aliquot. [^{125}I]-ghrelin (20 µl) was added to each peptide/peptidomimetic-cell mixture, to give a final volume of 300 µl in binding buffer. Each solution concentration was vortexed, followed by agitation (550 rpm, 20 min, 37 °C). After 20 minutes, samples were spun (13 000 rpm, 5 min, 4 °C) and placed on ice. The supernatant was removed and the cell pellet rinsed with ice-cold Tris-HCl (200 µl, 50 mM, pH 7.4). The samples were spun again (13,000 rpm, 5 min, 4 °C), cooled on ice, supernatant removed and cell pellet counted using a Cobra II Auto-Gamma gamma counter.

All binding assays were performed in triplicate for each concentration of peptide/peptidomimetic. Binding buffer (300 µl) and [^{125}I]-ghrelin (20 µl) alone were used as background controls. [^{125}I]-ghrelin (20 µl) and cells (50 µl) in binding buffer (230 µl) were used to calculate the percentage of bound [^{125}I]-ghrelin displaced by each concentration of peptide/peptidomimetic.

4.8 Computation of partition coefficients (LogP) for peptides and peptidomimetics

The *n*-octanol/water partition coefficients for all peptides and peptidomimetics were calculated using ACD/Log*P* prediction software from ACD/ChemSketch (Freeware) 2017.2.1.

4.9 Calcium flux dose-response assay

This assay was used to determine the EC_{50} value for [1-Nal[4],Lys[5](4-FB)]G-7039 in terms of intracellular Ca^{2+} release, with ghrelin used as the control ligand. The peptidomimetic compound was dissolved in DMSO (1 ml) at a concentration of 250 μM and solubility tested in Hank's balanced salt solution (HBSS): 1.26 mM $CaCl_2$, 0.493 mM $MgCl_2 \cdot 6H_2O$, 0.407 mM $MgSO_4 \cdot 7H_2O$, 5.33 mM KCl, 0.441 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 137.9 mM NaCl, 0.338 mM Na_2HPO_4, 5.56 mM D-Glucose, 20 mM HEPES, pH 7.4 with a final concentration of 1.2 % DMSO. Following successful solubility, human recombinant ghrelin receptor calcium-optimised stable cell lines (chem-1 cells) were loaded with a fluorescent calcium dye (Fluo-8 NW from ABD Bioquest 21080) and calcium flux detected in response to the agonist by a Molecular Devices FLIPR^{TETRA} instrument in a modified HBSS buffer (20 mM HEPES, 2.5 mM Probenecid, pH 7.4). Fluorescence values underwent baseline correction and the percentage activation was normalised to the maximum response (E_{max}) of the control ligand ghrelin. The agonist assay was performed for a total of 180 seconds and each assay concentration was performed in duplicate.

4.10 Serum stability study

A Waters Oasis HLB plate was used for both collection and extraction. The peptidomimetic [1-Nal[4],Lys[5](4-FB)]G-7039 was incubated in a solution of 25% human serum in PBS for 24 h. Aliquots were removed at 0, 20, 60, 240, 420, and 1440 minutes, with each time-point performed in triplicate. These aliquots were then mixed with 4% (v/v) ammonium hydroxide (NH_4OH) in water to denature peptidomimetic-serum (albumin) interactions. [1-Nal[4],Lys[5](4-FB)]G-7039 was then extracted from human serum using 20% MeOH/2% HCO_2H in water and quantified by UHPLC-MS. The amount was then expressed as the percentage of the area under the curve (AUC) in the UV chromatogram relative to time 0, plotted against time (minutes) and data fitted to a curve of exponential decay to determine the serum half-life ($t_{1/2}$).

4.11 Radiochemistry

4.11.1 General information

All reagents and solvents used for radiosynthesis were purchased from Sigma-Aldrich unless otherwise specified. ^{18}F-fluoride was produced *via* the ^{18}O(p,n) ^{18}F reaction in a GE

PETtrace 880 cyclotron (Lawson Health Research Institute, London, Ontario, Canada). A Biotage V-10 evaporator was used to remove solvent. A Waters HPLC with a 1525 binary pump system (solvent A = MeCN + 0.1% TFA, solvent B = H_2O + 0.1% TFA) and two detectors (Waters 2487 Dual λ absorbance detector set at 254 nm and a radioactive flow count detector) were utilised for product analysis and purification. An analytical column (Agilent Eclipse XDB-C18, 4.6 × 150 mm, 5 μm) was used for determining radiochemical purity and molar activity of [1-Nal^4,Lys^5(4-[^{18}F]-FB)]G-7039. A semi-preparative column (Agilent Zorbax SB-C18, 9.4 × 150 mm, 5 μm) was used for the purification of [^{18}F]SFB and [1-Nal^4,Lys^5(4-[^{18}F]-FB)]G-7039.

4.11.2 Synthesis of [^{18}F]FBA

Scheme 1 shows the synthetic pathway to [^{18}F]FBA. Aqueous [^{18}F]fluoride was trapped on a Waters Sep-Pak Accell Plus Light (46 mg) QMA Carbonate cartridge pre-activated with MilliQ water (10 ml). The initial radioactivity of the cartridge was measured using a dose calibrator (980-1300 MBq) A solution of acetonitrile/H_2O (1 ml, 25:75, v/v) containing potassium carbonate (2.1 mg, 0.015 mmol, 2.6 eq.) and 4,7,13,16,21,24-Hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane (6.0 mg, 0.016 mmol, 2.8 eq.) was used to elute [^{18}F]fluoride into the reaction vial. The solvent was removed azeotropically under vacuum at 50 °C. [^{18}F]fluoride was then dried a further two times under the aforementioned conditions by adding anhydrous acetonitrile (1 ml). 4-*Tert*-butoxycarbonyl)-*N*,*N*,*N*-trimethylbenzenammonium triflate (2.0 mg, 0.0057 mmol, 1.0 eq.) in anhydrous DMSO (400 μl) was added to dried [^{18}F]F$^-$ and the subsequent solution heated for 10 min at 120 °C. Aqueous HCl (1 ml, 6 M) was added and the resultant mixture heated further (10 min, 120 °C). Water (2 ml) was added to dilute the reaction mixture containing crude [^{18}F]FBA. This solution was then trapped on two inter-connected Waters Sep-Pak C-18 light cartridges sequentially pre-treated with ethanol (15 ml) and water (15 ml). Elution of product then occurred using anhydrous acetonitrile (800 μl) to deliver the title compound [^{18}F]FBA in an average d.c. radiochemical yield of 82% (n = 3). This compound was used in the next step without further purification. To confirm that the correct product had been acquired, a co-injection of 4-[^{19}F]-FBA and [^{18}F]-FBA in water was performed (20 μl, 30-70% MeCN + 0.1% TFA gradient system, see Figure S3 supporting information).

4.11.3 Synthesis of [^{18}F]SFB

[^{18}F]FBA was trapped onto two inter-connected Waters Sep-Pak C-18 light cartridges sequentially pre-treated with ethanol (15 ml) and water (15 ml) and eluted with acetonitrile (800 µl) into a vial charged with NHS (29 mg, 0.25 mmol.) and EDC (71 mg, 0.46 mmol.). The resultant solution was allowed to stand at room temperature for 15-20 minutes before being diluted with water + 0.1% TFA (1.2 ml) and purified by semi-preparative HPLC (30-70% MeCN + 0.1% TFA gradient system). After solvent removal (V-10 evaporator, 36 °C) dry [^{18}F]SFB was obtained in an average d.c. radiochemical yield of 71% (n = 3) and ≥ 99% radiochemical purity.

4.11.4 Synthesis of [1-Nal^4, Lys^5(4-[^{18}F]-FB)]G-7039

A solution of [1-Nal^4]G-7039 (2.0 mg, 1.86 x 10^{-3} mmol.) in acetonitrile (100 µl) and H_2O (100 µl) was added to the [^{18}F]SFB, followed by DIPEA (10 µl) and the resultant solution heated to 65 °C for 15-20 minutes. Crude [1-Nal^4, Lys^5(4-[^{18}F]-FB)]G-7039 began to precipitate out of solution as a white solid. The solvent was subsequently removed and the solid re-dissolved stepwise with DMF (300 µl), H_2O (500 µl) and MeCN (200 µl). This solution was purified by preparative HPLC (30-70% MeCN + 0.1% TFA gradient system) and delivered the title [^{18}F]radiolabelled peptidomimetic [1-Nal^4,Lys^5(4-[^{18}F]-FB)]G-7039 in an average d.c. radiochemical yield of 81% (n = 3), an overall average d.c. radiochemical yield of 48% (n = 3), a radiochemical purity ≥ 99% and an average molar activity of 34 GBq/µmol (n = 3).

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Appendix A. Supplementary data

Amino acid sequences, lipophilicity data, HPLC chromatograms, ^{1}H-NMR spectra and displacement curves for compounds **1-17**, the radio-chromatogram of [^{18}F]SFB, the displacement

curve for the endogenous ligand ghrelin and the UHPLC chromatogram, ^{1}H-NMR- and ^{13}C-NMR spectra of 4-(*tert*-butoxycarbonyl)-*N*,*N*,*N*-trimethylbenzenammonium triflate (**20**).

Conflicts of interest

Some of the peptidomimetic compounds in this work are the subject of the patent application "Peptidomimetics for Imaging the Ghrelin Receptor", U.S. Patent WO2016/191865 A1, December 8th 2016.

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Highlights:

- Fluorine-containing growth hormone secretagogues were synthesised
- A fluorinated G-7039 analogue was identified as a lead with an IC_{50} of 69 nM
- Prosthetic group radiolabelling yielded [1-Nal^4, Lys^5(4-[^{18}F]-fluorobenzoyl)]G-7039