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A New Series of Highly Potent Growth Hormone-Releasing Peptides Derived from Ipamorelin

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A new series of GH secretagogues derived from ipamorelin is described. In an attempt to obtain oral bioavailability, by reducing the size and the number of potential hydrogen-bonding sites of the compounds, a strategy using the peptidomimetic fragment 3-(aminomethyl)benzoic acid and sequential backbone N-methylations was applied. Several compounds from this series release GH with high in vitro potency and efficacy in a rat pituitary cell assay and high in vivo potency and efficacy in anesthetized rats. The tetrapeptide NNC 26-0235 (3-(aminomethyl)-benzoyl-D-2Nal-N-Me-D-Phe-Lys-NH_2) shows, following iv administration, comparable in vivo potency to ipamorelin, GHRP-2, and GHRP-6 with an ED_{50} in swine at 2 nmol/kg. NNC 26-0235 demonstrated a 10% oral bioavailability in dogs, and NNC 26-0235 and ipamorelin were able to increase basal GH level by more than 10-fold after oral administration of a dose of 1.8 and 2.7 mg/kg, respectively. The tripeptide NNC 26-0323 (3-(aminomethyl)benzoic acid-N-Me-D-2Nal-N-Me-D-Phe-ol) which showed moderate in vitro potency but lacked in vivo potency demonstrated a 20% oral bioavailability in rats.

Introduction

Human growth hormone (hGH), a 191-amino acid hormone, is a pleiothrophic hormone interacting with most tissues in the human body. The most prominent effects of hGH are the growth-promoting effect, the protein anabolic effect and the lipolytic effect. Synthesis and release of GH from somatotrophs in the anterior pituitary are under tight control by two hypothalamic hormones: GH-releasing hormone (GHRH, 44 amino acids) which stimulates synthesis and release and somatostatin (SRIF, 14 amino acids) which inhibits GH release.[1]

In addition to the effect of GHRH, Bowers discovered in 1977 that release of GH can be induced by GH-releasing peptides (GHRPs).[2] It is widely believed that these compounds elicit their effect on both the hypothalamic and pituitary level and work synergistically with GHRH.[3] GH, GHRH, and SRIF are all polypeptides and incapable of being efficacious via the oral route.

The development of orally active drugs, with the capability of inducing GH secretion, has been speculated for some time. Such drugs could mediate their effect through the GHRP, GHRH, or SRIF pathway. An advantage of drugs mediating their effect through such pathways, compared to recombinant hGH, could be maintenance of a physiological pulsatile pattern of GH secretion.

The most extensively studied GHRPs are the hexapeptides GHRP-2 and GHRP-6, which demonstrated GH-releasing properties in vivo[4] and were efficacious for GH release in humans.[5] The Merck group has recently disclosed a number of nonpeptidyl mimics of GHRP-6, among those the benzolactam L-692,429 and the derivatized tripeptide MK677, which was the first potent GH secretagogue in vivo with good oral bioavailability in dogs.[6]

The medicinal importance of peptides as oral drugs is limited because peptides in general have two principle disadvantages compared with most other drugs: namely, high susceptibility to hydrolysis of the peptide bonds and poor intestinal absorption. To improve the oral bioavailability of peptides, a number of strategies have been applied, such as coating of peptides, use of penetration enhancers, or administration of peptidase inhibitors.[7] An alternative approach may be to chemically alter the peptidic nature by reducing the size of the molecule and by reducing the number of potential hydrogen-bonding sites, defined as heteroatoms with the ability to form hydrogen bonds.[8] We have previously reported the pentapeptide ipamorelin as a highly potent and selective GH releaser.[9]

Herein we report a structure−activity study of analogues of ipamorelin aiming at increased oral bioavailability and retained potency. Our strategy was to gradually reduce the size and polarity of the peptide by methylation of the amides, remove superfluous polar groups, and yet still retain the high potency by fixing the two aromatic group and the N-terminal amino group, previously identified as essential pharmacophores for GH-releasing activity.[10]

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Table 1. NMR Assignments of Protons of Ipamorelin

	NH	H_α	H_β, $H_{\beta'}$	other
Aib				1.5 (Me_β), 1.5 ($Me_{\beta'}$)
His	ND[a]	4.6	2.9, 2.8	6.8 (H_δ), 7.7 (H_ϵ)
D-2Nal	8.3	4.8	3.3, 3.0	7.7 (H_1), 7.4 (H_3), 7.9 (H_4), 8.0 (H_5), 7.6 (H_6, H_7), 7.9 (H_8)
D-Phe	8.4	4.6	3.1, 3.1	7.3 (H_2, H_6), 7.4 (H_3, H_5), 7.4 (H_4)
Lys-NH_2	8.2	4.1	1.7, 1.5	1.0 (H_γ, H_γ), 1.5 (H_δ, H_δ), 2.9 (H_ϵ, H_ϵ), 7.0, 7.4 (NH_2

[a] ND, not detected.

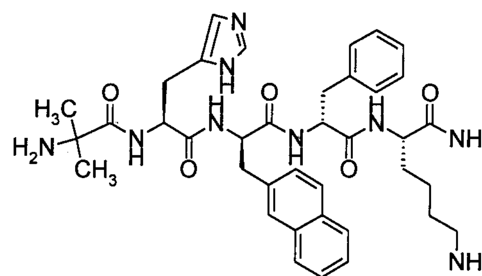
Table 2. NMR Assignments of Protons of the Major and Minor Isomer of NNC 26-0235

	NH	H_α	H_β, $H_{\beta'}$	other
			Major Isomer	
AMB				4.2 (CH_2), 7.7 (H_2), 7.3−7.6 (H_4, H_5, H_6)
D-2Nal	8.6	5.0	3.1, 3.1	6.6−7.6 (H_1−H_8)
N-Me-D-Phe		4.3	3.2, 2.7	2.6 (Me), 6.6−7.6 (H_2−H_6)
Lys-NH_2	7.6	4.2	1.7, 1.5	1.3 (H_γ, H_γ), 1.6 (H_δ, H_δ), 2.9 (H_ϵ, H_ϵ), 7.5 (NH_2)
			Minor Isomer	
AMB				4.1 (CH_2), 7.6 (H_2), 7.3−7.6 (H_4, H_5, H_6)
D-2Nal	8.7	4.8	2.9, 2.3	6.6−7.6 (H_1−H_8)
N-Me-D-Phe		5.0	2.9, 2.3	2.8 (Me), 6.6−7.6 (H_2−H_6)
Lys-NH_2	8.0	4.2	1.6, 1.5	1.2 (H_γ, H_γ), 1.5 (H_δ, H_δ), 2.8 (H_ϵ, H_ϵ), 7.5 (NH_2)

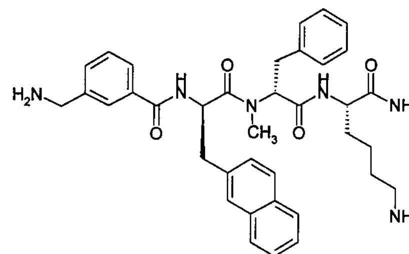
Chemistry

The compounds **1**−**3** and **5**−**12** were all prepared stepwise on solid support (Rink resin) using Boc- or Fmoc-peptide synthesis strategy based on commercially available Boc- or Fmoc-protected amino acids. Compounds **4** and **13** were prepared in a similar manner on a *N*-methyl-PAL resin.[11] Compound **14** was prepared in solution using traditional amide coupling (1-hydroxy-7-azabenzotriazole, 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide hydrochloride) conditions, starting from (*R*)-(methylamino)-3-phenylpropan-1-ol.[12] N-Methylation of the involved amino acids was obtained using sodium hydride and methyl iodide in THF.[13]

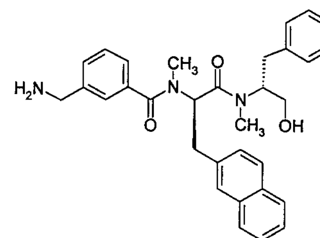
NMR Analysis of Ipamorelin and NNC 26-0235. The proton resonances of ipamorelin and NNC 26-0235 observed in water solution at 310 K have been assigned from a series of proton 2D-NMR spectra (DQF-COSY, TOCSY, and ROESY spectra). Probably due to fast exchange of amide protons with the water protons, only weak amide resonances were observable at this temperature. Only one conformer of ipamorelin was observed, while two distinct conformers of NNC 26-0235 were observed in solution as a result of restricted rotation around the *N*-methylated D-2Nal−D-Phe amide bond. The ratio between the two conformers was approximately 60:40. The presence of a strong NOE between methyl protons of N-Me-D-Phe and H_α of D-2Nal in the major conformer suggests that the predominant conformation of the peptide bond is trans. This is further substantiated by the observed (weak) NOE between H_α of D-2Nal and H_α of N-Me-D-Phe in the minor conformer (cis conformer), which is absent in the most populated conformation. The assignments of protons of ipamorelin and for the major and minor isomer of NNC 26-0235 are provided in Tables 1 and 2.



ipamorelin (**1**)



NNC 26-0235 (**9**)



NNC 26-0323 (**14**)

Results and Discussion

Structure−Activity Relationship. The GH-releasing properties of the compounds **1**−**14** are represented in Table 3 by EC_{50} as the potency in vitro in a rat pituitary cell assay and by ED_{50} representing the in vivo potency in rats. The efficacy E_{max} (maximal GH released) is given as percent of GHRP-6 (the maximal GH release induced by GHRP-6). In the rat model either male or female rats were used. No significant difference was observed of the potency in the two genders. When comparing the efficacy, a significantly lower E_{max} was observed for the female rat. Since all the E_{max} results are listed in percent of GHRP-6 E_{max}, these as well can be compared. The lead compound, ipamorelin, stimulates GH release from rat primary pituitary cells in a dose-dependent manner with an EC_{50} of 1.4 nM and an

Table 3. In Vitro[a] Potency and Efficacy in Primary Rat Pituitary Cells, in Vivo[b] Potency and Efficacy after iv Administration in Rats, and Chemical Characterization of the Prepared Growth Hormone Secretagogues

		rat pit cell		in vivo rats		HPLC t_R (min)		PDMS
no.	peptide sequence[a]	EC_{50}[b] (nmol)	E_{max} (%)	ED_{50}[c]	E_{max}	system A1	system B1	(m/z) (M + H$^+$)
GRHP-6	H-His-D-Trp-Ala-Trp-D-Phe-Lys-NH$_2$	2.2	100	115^M	100			
				60^F	100			
GHRP-2	H-D-Ala-D-2Nal-Ala-Trp-D-Phe-Lys-NH$_2$	1.8	85	30^M	100			
				30^F	90			
1	H-Aib-His-D-2Nal-D-Phe-Lys-NH$_2$ (NNC 26-0161, ipamorelin)	1.3	85	85^M	120	17.42	20.13	636.5
2	H-Aib-His-D-2Nal-N-Me-D-Phe-Lys-NH$_2$	2.0	100	1.8^M	90	21.78	23.53	726.0
3	H-Aib-His-N-Me-D-2Nal-N-Me-D-Phe-Lys-NH$_2$	1.5	85	14^F	85	21.08	22.10	740.1
4	H-Aib-His-N-Me-D-2Nal-N-Me-D-Phe-NH-Me	3.8	110			26.37	29.08	625.3
5	H-(2-AMB)-D-2Nal-D-Phe-Lys-NH$_2$	85	60			23.22	25.05	622.4
6	H-(3-AMB)-D-2Nal-D-Phe-Lys-NH$_2$	7.0	85	>4800^M		22.42	24.13	622.0
7	H-(N-Me-3-AMB)-D-2Nal-D-Phe-Lys-NH$_2$	10	85	65^M	30	26.72	27.82	635.5
8	H-(4-AMB)-D-2Nal-D-Phe-Lys-NH$_2$	110	50			22.22	23.50	621.9
9	H-(3-AMB)-D-2Nal-N-Me-D-Phe-Lys-NH$_2$ (NNC 26-0235)	0.5	80	80^M	90	26.78	27.88	636.5
10	H-(3-AMB)-N-Me-D-2Nal-N-Me-D-Phe-Lys-NH$_2$	5.0	80	146^F	100	25.25	26.25	650.8
11	H-(3-AMB)-D-2Nal-N-Me-D-Phe-NH$_2$	170	85	>1000^F		30.73	32.47	510.2
12	H-(3-AMB)-N-Me-D-2Nal-N-Me-D-Phe-NH$_2$	115	85	>266^F		28.68	30.05	521.6
13	H-(3-AMB)-N-Me-D-2Nal-N-Me-D-Phe-NH-Me	40	110			31.20	36.35	535.7
14	H-(3-AMB)-N-Me-D-2Nal-N-Me-D-Phe-ol (NNC 26-0323)	265	75	>1000^F		29.90	31.52	508.5

[a] Aib, aminoisobutyric acid; AMB, (aminomethyl)benzoic acid. [b] Rat pituitary cell assay (mean ± SEM of 1−5 separate experiments). E_{max} of GHRP-6 is 230 ± 50 ng/mL. [c] Rats (^{M}male rat; ^{F}female rat), mean ED_{50} values (±SEM) (n = 5−35). E_{max} of GHRP-6 in male and female rats are 1167 ± 120 and 732 ± 52 ng/mL, respectively.

E_{max} at 80% of GHRP-6 and in rats with an ED_{50} of 85 nmol/kg and an E_{max} at 120% after iv administration.

N-Methylation of D-Phe in ipamorelin afforded the compound **2**, which showed equipotency in vitro to ipamorelin but surprisingly showed a 50-fold increase in in vivo potency compared to ipamorelin. Further N-methylation of D-2Nal gave the dimethylated compound **3**, which also showed equipotency in vitro and increased in vivo potency, although to a lesser extent.

In an attempt to evaluate the bioavailability of the compounds following oral administration, the compounds were injected intravenously (iv) or administered orally via gavage to rats. In this model less than 1% of GHRP-2 and ipamorelin, respectively, was absorbed orally. The oral bioavailability of compounds **2** and **3** in this model was determined to ~1%, and we hypothesized at this stage that the low bioavailability of these compounds was caused not only by the number of secondary amide bonds but also to a much higher extent by the ionic nature of both the lysine in the C-terminal and the histidine in the N-terminal.

By replacing the N-terminal dipeptide fragment H-Aib-His-OH with the peptidomimetic fragments 2-(aminomethyl)benzoic acid (2-AMB), 3-(aminomethyl)benzoic acid (3-AMB),[14] or 4-(aminomethyl)benzoic acid (4-AMB), we discovered that H-Aib-His-OH could be replaced by 3-AMB (**6**) and still retain high in vitro potency. This compound **6** did not release GH in rats even at doses higher than 4800 nmol/kg, but driven by the information that N-methylation of D-Phe in ipamorelin increased the in vivo potency dramatically, we introduced the corresponding *N*-methyl group into **6**, affording NNC 26-0235 (compound **9**); indeed the same in vivo potency as that of ipamorelin was obtained. The increased in vivo potency caused by the N-methylation of ipamorelin (leading to **2**) and **6** (leading to NNC 26-0235), respectively, may be due to an increased pharmacokinetic stability, or it may be a result of an increased population of the cis conformer around the D-2Nal and D-Phe amide bond, as suggested by proton resonances of NNC 26-0235 (Table 2). The cis conformer may in particular be important for receptor recognition in compounds with the semirigid 3-AMB as the N-terminal amino acid, as exemplified by more than 10-fold increase of in vitro potency going from **6** to NNC 26-0235.

A further N-methylation of D-2Nal leading to the dimethylated compound **10** caused a slight decrease of in vitro and in vivo potency in rats. An investigation of the influence of the C-terminal lysine on the potency was carried out, and by removing the lysine from **9**, resulting in **11**, a 350-fold loss of in vitro potency and a total loss of in vivo activity were observed.

Assuming the C-terminal lysine amide was unfavorable for oral bioavailability and the fact that we did not totally lose the in vitro activity encouraged us to elaborate on **11** as the lead compound. An N-methylation at D-2Nal (compound **12**) slightly increased the activity, and a further N-methylation in the C-terminal (compound **13**) increased the in vitro potency 4−5-fold.

The hypothesis that reduced size and reduced number of hydrogen-bonding sites eventually would lead to oral bioavailability encouraged us to further reduce the polarity by replacing the C-terminal amide with an alcohol. The alcohol NNC 26-0323 (**14**), which has the "optimal" structure in this series, with only four hydrogen-bonding sites, released GH in vitro but with a lower potency. Although NNC 26-0323 was inactive in vivo, we were encouraged to test this compound for oral bioavailability in rats to prove our hypothesis. In rats the oral bioavailability (f_{po}) of NNC 26-0323 was determined to be 20%, indicating the importance of reduced size (MW 509.7) and reduced number of hydrogen-bonding sites.

In Vivo Characterization of NNC 26-0235 in Swine. The fact that NNC 26-0235 (**9**) showed good in vivo potency in rats and contained a reduced number of hydrogen-bonding sites encouraged us to determine the potency of NNC 26-0235 in species other than rats. Swine seems to be an interesting animal model because of their close similarity to humans regarding several physiological aspects, including GH endocrinology.[9]

Table 4. Effect of NNC 26-0235, Ipamorelin, GHRP-2, and GHRP-6 in 40-kg Female Danish Slaughter Swine[a]

compd	ED_{50} (nmol/kg)	E_{max} (% of GHRP-6)
NNC 26-0235	2.0 ± 0.3	70 ± 3*
ipamorelin	2.3 ± 0.03	88 ± 0.3
GHRP-2	0.6 ± 0.2*	75 ± 7
GHRP-6	3.9 ± 1.4	100 ± 9

[a] The values were calculated from dose−response curves based on mean plasma GH concentrations obtained 10 min after administration of test compounds. Each value is given as mean ± SEM ($n = 6-10$). *Significantly different from GHRP-6 value ($p < 0.05$). E_{max} of GHRP-6 is 74 ± 7 ng/mL.

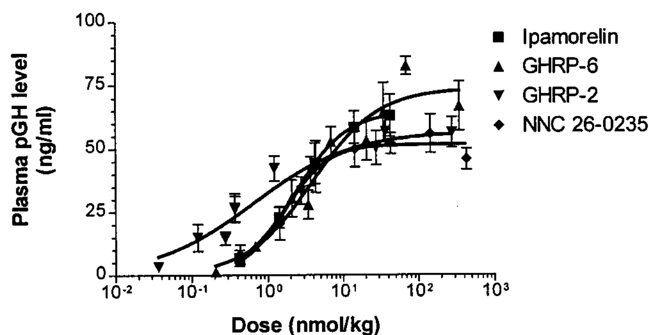


Figure 1. Dose−response curves for ipamorelin, GHRP-6, GHRP-2, and NNC 26-0235 in swine. Plasma pGH concentrations obtained 10 min after iv administration of test compound. The data are means ± SEM from six swine.

Consequently, NNC 26-0235, ipamorelin, GHRP-2, and GHRP-6 were tested intravenously in swine in doses ranging from 0.03 to 500 nmol/kg, and the results are presented in Table 4.

As shown in Table 4, NNC 26-0235 releases GH in swine in a dose-dependent manner (see Figure 1) with the same ED_{50} as ipamorelin and GHRP-6 although a slight but significant decrease in E_{max} compared to GHRP-6 was observed. The high potency of NNC 26-0235 together with its chemical structure, i.e., reduced number of hydrogen-bonding sites, tempted us to determine the oral bioavailability of this compound in dogs.

Oral Bioavailability of NNC 26-0235 and Ipamorelin in Dogs. To get a crude impression of the oral bioavailability of ipamorelin and NNC 26-0235, two dogs were dosed po and iv with ipamorelin and one dog was dosed po and iv with NNC 26-0235. Blood samples were drawn at intervals up to 6 h after dosing and assayed for growth hormone, ipamorelin, and NNC 26-0235, respectively. Table 5 shows doses, C_{max}, AUC(0−6 h), and oral bioavailability (f_{po}) after iv and oral administration of ipamorelin and NNC 26-0235 to beagle dogs. Recognizing the sparse data (only two and one dog used, respectively) and despite the fact that ipamorelin and NNC 26-0235 were able to significantly increase basal GH level by more than 10-fold after po administration, the low oral bioavailability discouraged us to further determine the GH release after po administration, although a significant increase of basal GH level may be expected with even lower doses.

Conclusion

A new series of GH secretagogues derived from ipamorelin has been described. The peptidomimetic fragment 3-(aminomethyl)benzoic acid has been shown to be isosteric to the much more hydrophilic dipeptide fragment Aib-His leading to NNC 26-0235. NNC 26-0235 demonstrated in vivo potencies in rats and swine comparable to those of ipamorelin and GHRP-6. NNC 26-0235 resulted in an increased basal GH level by more than 10-fold after oral administration in dogs in a dose of 1.8 mg/kg. Despite the reduced number of hydrogen-bonding sites of NNC 26-0235 compared to ipamorelin, only a slightly higher oral bioavailability was obtained.

With an oral bioavailability of 20% in rats, NNC 26-0323 proved the hypothesis that reduced size and reduced number of hydrogen-bonding sites would result in improved oral bioavailability. Although NNC 26-0323 lacked the in vivo potency in rats, it may be considered as a lead compound for future discovery.

Our main objective of this study was to design and prepare new GH secretagogues with high potency and high oral bioavailability. We obtained orally active compounds, and although the oral bioavailability was dissatisfactory, we created the basis for a new series of compounds with high oral bioavailability derived from ipamorelin. These compounds are described in the following paper.[15]

Experimental Section

Chemistry. General Methods. The compounds **1**−**13** were synthesized stepwise on a solid support using the Boc peptide synthesis strategy on an Applied Biosystems model 430A peptide synthesizer or using the Fmoc strategy on an Applied Biosystems model 431A peptide synthesizer. The standard protocols supplied from the manufacturer were used.

In all cases the crude peptides were purified using semipreparative RP-HPLC to a purity of >95% (based on HPLC with detection at 214 nm). The compounds were all characterized by analytical HPLC and plasma desorption mass spectrometry (see Table 3). The molecular mass found was in all cases in agreement with the expected structure.

The RP-HPLC analysis was performed using UV detection at 214, 254, 276, and 301 nm on a Vydac 218TP54 4.6-mm × 250-mm 5-μm C-18 silica column, which was eluted at 1 mL/min at 42 °C. Two different elution conditions were used. Method A1: The column was equilibrated with 5% acetonitrile in a buffer consisting of 0.1 M ammonium sulfate, which was adjusted to pH 2.5 with 4 M sulfuric acid. After injection the sample was eluted by a gradient of 5−60% acetonitrile in the same buffer during 50 min. Method B1: The column was equilibrated with 5% acetonitrile/0.1% TFA/water and eluted by a gradient of 5% acetonitrile/0.1% TFA/water to 60% acetonitrile/0.1% TFA/water during 50 min.

PDMS analysis was performed on a Bio-ion (Applied Biosystems) system using a Californium 252 (Cf252) source on a nitrocellulose matrix. NMR analysis was carried out in a 90% mixture of water and deuterium oxide. Proton spectra were recorded at 310 K on a BRUKER AMK-2 400 spectrometer. Mixing times of 68 ms (TOCSY) and 200 ms (ROESY) were applied. All chemical shifts are reported using the ppm scale and with the water resonance as chemical shift reference (δ = 4.7 ppm).

H-Aib-His-D-2Nal-D-Phe-Lys-NH$_2$ (1, Ipamorelin). A total of 4.53 g of the peptide resin Boc-Aib-His(Trt)-D-2Nal-D-Phe-Lys(Boc)-NH resin was prepared according to the Fmoc strategy on an Applied Biosystems 431A peptide synthesizer in two identical runs (to obtain sufficient material) in 1-mmol scale using the manufacturer-supplied FastMoc UV protocols which employ HBTU-mediated couplings in NMP and UV monitoring of the deprotection of the Fmoc protection group.

The starting resin used for the synthesis was 2 × 1.75 g of 4-((2′,4′-dimethoxyphenyl)(Fmoc-amino)methyl)phenoxy resin (Rink resin) (Novabiochem, Bad Soden, Germany, cat. #01−64−0013) with a substitution capacity of 0.55 mmol/g. The

Table 5. Doses, C_{max}, AUC(0−6 h), and Oral Bioavailability for Ipamorelin and NNC 26-0235 after iv and po Administration to Beagle Dogs

compd	dose (mg/kg)		basal GH (ng/mL)	C_{max} (ng/mL)		AUC (h·ng/mL)		f_{po} (%)
	iv	po		iv	po	iv	po	
ipamorelin (**1**)[a]	0.5	2.7	1.6/5.4	33/79	12/64	42/153	17/94	4/6
NNC 26-0235 (**9**)[b]	0.5	1.8	1.2	39	18	137	48	∼10

[a] Ipamorelin was tested in two dogs; both values are listed. [b] NNC 26-0235 was only tested in one dog.

protected amino acid derivatives used were Fmoc-Lys(Boc)-OH, Fmoc-D-Phe-OH, Boc-D-2Nal-OH, Boc-His(Trt)-OH, and Boc-Aib-OH. The peptide was cleaved from the 4.53-g peptide resin by treatment with 54 mL of TFA/phenol/ethanedithiol/thioanisole/water (40:3:1:2:2) for 3 h at room temperature.

Purification was done by semipreparative RP-HPLC, and the final product was characterized by amino acid analysis, analytical RP-HPLC, and plasma desorption mass spectroscopy. Amino acid analysis and mass spectrometry were in agreement with the expected structure within the experimental error of the method (mass spectrometry ± 0.9 amu, amino acid analysis ± 10%). In addition ^{1}H NMR analysis was performed, and the spectra were assigned (see Table 1). The NMR spectra fully supported the proposed structure.

Compounds 2, 3, and 5−12. Compounds **2**, **3**, and **5**−**12** were prepared in analogy to ipamorelin (**1**).

H-(3-AMB)-D-2Nal-N-Me-D-Phe-Lys-NH_2 (9, NNC 26-0235). ^{1}H NMR analysis was performed, and the spectra were assigned (see Table 2). The NMR spectra fully supported the proposed structure.

Compounds 4 and 13. Compounds **4** and **13** were prepared in analogy to ipamorelin (**1**) but using *N*-methyl-PAL-resin[11] instead of Rink resin.

H-(3-AMB)-N-Me-D-2Nal-N-Me-D-Phe-ol (14, NNC 26-0323). To a solution of Boc-N-Me-D-2Nal-OH (165.7 mg, 0.5 mmol), (2*R*)-(methylamino)-3-phenylpropan-1-ol[12] (165.2 mg, 1.0 mmol), and 1-hydroxy-7-azabenzotriazole (68.1 mg, 0.5 mmol) in a mixture of dimethylformamide (2 mL) and methylene chloride (4 mL) at 0 °C was added 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (115 mg, 0.6 mmol), and the mixture was stirred for 1 h at 0 °C and for 18 h at room temperature.

The methylene chloride was removed from the mixture by a stream of nitrogen, ethyl acetate (50 mL) was added, and the resulting mixture was extracted sequentially with 5% aqueous $NaHCO_3$ (100 mL), water (100 mL), 5% aqueous $KHSO_4$ (100 mL), and water (100 mL). The resulting organic phase was dried (Na_2SO_4) and concentrated in vacuo to an oil. The oil was dissolved in methylene chloride/trifluoroacetic acid (1:1) (4 mL) and stirred. After 10 min the mixture was concentrated by a stream of nitrogen, the resulting oil was redissolved in 70% acetonitrile/0.03 M hydrogen chloride (20 mL), water (480 mL) was added, and the mixture was lyophilized.

3-Boc-(aminomethyl)benzoic acid (502.6 mg, 0.5 mmol) was dissolved in methylene chloride (10 mL) by addition of 2 drops of dimethylformamide and then converted to the symmetrical anhydride by stirring with 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (191.6 mg, 1.0 mmol) for 10 min. A solution of the above lyophilized 2(*R*)-(H-N-Me-D-2Nal-N-Me)-3-phenylpropanol and diisopropylethylamine (342 mL) in methylene chloride (5 mL) was added to this mixture and reacted for 20 h at room temperature. The reaction mixture was concentrated to an oil and redissolved in ethyl acetate (50 mL). The solution was extracted sequentially with 5% aqueous $NaHCO_3$ (100 mL), water (100 mL), 5% aqueous $KHSO_4$ (100 mL), and H_2O (100 mL). The resulting organic phase was dried with Na_2SO_4 and concentrated in vacuo to an oil. The oil was then dissolved in methylene chloride/trifluoroacetic acid (1:1) (4 mL) and stirred. After 10 min the mixture was concentrated by a stream of nitrogen, and the resulting oil was redissolved in water (480 mL) and lyophilized.

The crude product was then purified by semipreparative HPLC in seven runs on a 25-mm × 250-mm column packed with 7-μm C-18 silica which was preequilibrated with 28% acetonitrile in 0.05 M $(NH_4)_2SO_4$, which was adjusted to pH 2.5 with 4 M H_2SO_4. The column was eluted with a gradient of 28−38% acetonitrile in 0.05 M $(NH_4)_2SO_4$, pH 2.5, at 10 mL/min during 47 min at 40 °C, and the compound-containing fractions were collected, diluted with 3 volumes of water, and applied to a Sep-Pak C18 cartridge (Waters part #51910) which was equilibrated with 0.1% trifluoroacetic acid. The compound was eluted from the Sep-Pak cartridge with 70% acetonitrile/0.1% trifluoroacetic acid and isolated from the eluate by lyophilization after dilution with water: crude yield, 31.5%; purified yield, 20.8%. HPLC: t_R = 29.90 min (A1); t_R = 31.52 min (B1). PDMS: m/z = 508.5 $(M + H)^+$

In Vitro Characterization in Rat Pituitary Cell Assay. The Sprague−Dawley male albino rats (250 ± 25 g) were purchased from Møllegaard, Lille Skensved, Denmark. The rats were housed in group cages (4−8 animals/cage) and placed in rooms with 12-h light cycle. The room temperature varied from 19 to 24 °C and the humidity from 30% to 60%.

The effect of different GH secretagogues on the GH release from primary rat pituitary cells was measured. The method is a modification of that of Heiman[16] and is described in Raun et al.[9] Briefly, the cells were isolated from rat pituitaries, and dispersed into single cells using trypsin, and cultured for 3 days. The cells were then washed and stimulated for 15 min with different GH secretagogues. The supernatant was decanted and assayed for GH content in a rat GH SPA assay.

In Vivo Characterization in Anesthetized Rats. The animals (male or female) were purchased and housed under the same conditions as described above.The method is described in Raun et al.[9] Briefly, pentobarbital dissolved in 0.9% NaCl (final concentration 50 mg/mL) was administered ip (dose volume 1.2 mL/kg) to male or female rats (250 ± 25 g). After 15 min, a blood sample of 0.4 mL was obtained from the orbital vein (sample "0"). Immediately after blood sampling, 2 mL/kg of a test compound solution was administered iv as a bolus injection through the tail vein. After 10 min, a new sample of 0.4 mL was obtained from the orbital vein (sample "10"). The samples were collected in heparin-coated vials and assayed for rat GH. At least five different doses of each compound were tested using six rats per dose level.

Calculations. Dose−response relations were constructed using the Hill equation (Prism, GraphPad). The efficacy (maximal GH released, E_{max}) was expressed in percent of maximal GH released by GHRP-6. The potency (EC_{50}/ED_{50}) was determined as the concentration inducing half-maximal stimulation of the GH release as measured from the basal level. For statistical comparisons Student's *t*-test was used. All parameters were tested for normality using the Gaussian distribution. If the variances (*F*-test) were significantly different, then Student's *t*-test with Welch's correction was used (Prism 2.0, GraphPad, Intuitive Software for Science).

Pharmacokinetics, Oral Bioavailability. Rats: The oral bioavailability of NNC 26-0323 was determined in 40 fasted male rats. For po administration the rats received 40 mg/kg via gavage and for iv administration 1.0 mg/kg via a tail vein. Blood was drawn by heart puncture in two animals per time point at intervals up to 6 h after dosing.

Dogs: Oral bioavailability studies were conducted in male and female Beagle dogs. The dogs were fasted overnight prior to dosing. Diet was withheld for at least 3 h postdosing. A 1-week washout period separated po and iv dosing. The compounds were administered in a vehicle of citrate/phosphate buffer, pH 5.0. For po administration the dogs received a dose of 2.7 mg/kg of body weight of ipamorelin and 1.8 mg/kg of body weight of NNC 26-0235 via gavage. For iv administration

the dogs received a dose of 0.5 mg/kg of body weight as a bolus in a hind leg vein. EDTA blood samples were drawn from a front leg vein at intervals up to 6 h after dosing.

Blood samples were placed on an ice−water bath immediately after sampling. Plasma was separated by centrifugation and stored frozen until analysis. Plasma was analyzed for GH using a porcine GH ELISA. An HPLC assay with UV detection and solid-phase extraction was developed for each compound. Analytical C8 columns and disposable C3 extraction columns were used. The oral bioavailability was calculated as the total area under the plasma concentration versus time curve following po administration divided by the area following iv administration, appropriately corrected for dose (eq 1):

$$f = \frac{\mathrm{AUC_{po}} \cdot \mathrm{dose_{iv}}}{\mathrm{AUC_{iv}} \cdot \mathrm{dose_{po}}} \quad (1)$$

In Vivo Characterization in Conscious Swine. For in vivo test of the GH secretagogues, six female 30−40-kg Danish slaughter swine of the breed Landrace Yorkshire cross (Lars Holmenlund, DK) were used. The model is described in Raun et al.[9] Briefly, jugular catheters were inserted and fixed under general halothane anesthesia at day 0. The test compound was dissolved in phosphate/citrate buffer diluted in saline containing 0.5% porcine serum albumin. Blood samples were drawn from the jugularis catheter at frequent intervals from 1 h prior to stimulation until 3 h poststimulation. The compounds were administered as single iv injections in increasing doses with 24-h intervals between the doses, five doses per experiment. Plasma was analyzed for porcine GH (pGH) by ELISA.

Calculations. The basal GH level, for the individual swine, was calculated as the average of the three GH values obtained prior to stimulation. Peak hormone levels (C_{max}) adjusted for basal level, obtained following administration of test compound, were used to characterize the hormone response of individual swine. Dose−response curve was constructed using C_{max} GH plasma concentrations. Fitting to the Hill equation or hyperbolic Michaelis−Menten equation was performed by nonlinear regression using Prism software (GraphPad). Using the obtained E_{max} values of the compound, the potency (ED_{50} values) was calculated as the dose inducing half-maximal stimulation, i.e., the increase in plasma GH from basal. All parameters were tested for normality using the Gaussian distribution. If the variances (*F*-test) were significantly different, then Student's *t*-test with Welch's correction was used (Prism 2.0, GraphPad, Intuitive Software for Science).

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