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Tryptic Hydrolysis of hGH-RH(1-29)-NH_2 Analogues Containing Lys or Orn in Positions 12 and 21

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Abstract: Two analogues of the 29 amino acid sequence of human growth hormone-releasing hormone, namely [Nle^{27}]hGH-RH(1-29)-NH_2 and [$Orn^{12,21}$,Nle^{27}]hGH-RH(1-29)-NH_2, have been synthesized and subjected to digestion by trypsin. The course of degradation was followed using RP-HPLC and ESI-MS. Several intermediates and final products of degradation were identified and conclusions regarding the rate of cleavages at different positions occupied by Lys and Arg residues were drawn. The analogue containing ornithine was found to be less susceptible to hydrolysis by trypsin: the 12-13 and 21-22 peptide bonds were completely resistant to the cleavage. The results show that by replacing Lys with Orn, a possibility exists to design new peptides, which could be more stable in biological fluids. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: GH-RH; ornithine analogues; solid-phase peptide synthesis; tryptic digestion

INTRODUCTION

Although peptides are involved in many important biological processes and dozens of peptides are used as pharmaceutical agents, it is often suggested that peptides are not effective as drugs because the peptide bond is enzymatically fragile. Rapid degradation by various proteases greatly reduces the biological effect, and consequently, it is necessary to increase the doses of peptide drugs. In order to transform a peptide lead into a useful drug, some modifications can be made. Properly selected modifications can result in more stable, and, in some cases, even more potent analogues.

It has been demonstrated that hGH-RH(1-44), hGH-RH(1-40), and its shortened synthetic fragment, hGH-RH(1-29)-NH_2, stimulate the release of growth hormone from the pituitary gland, accelerate growth velocity in GH-deficient children [1–6] and reverse age-related decreases in GH-level in men over 60 years old [7]. As medical applications of the releasing hormone have some medical advantages over the use of the more expensive GH, there is great demand for more active and more stable analogues of GH-RH, preferably GH-RH(1-29), which would make it possible to reduce doses and frequency of administration. Many analogues have been synthesized for studies of GH-releasing activity *in vitro* and receptor binding. Summaries of the cumulative efforts of various laboratories in this quest have been published [8,9].

It has also been reported that hGH-RH(1-29)-NH_2 is rapidly methabolized in the plasma, as a result of Ala^2-Asp^3 cleavage by dipeptidylpeptidase IV, to the inactive GH-RH(3-29)-NH_2 [10]. The other metabolites observed were characteristic of trypsin-like degradation between Arg^{11}-Lys^{12} and Lys^{12}-Val^{13} [11]. The cleavages observed are in agreement with the common knowledge that paired basic amino acids are the primary sites for the proteolytic processing of bioactive peptide precursors. The accumulation of two such pairs (Arg-Lys) makes the GH-RH molecule susceptible to trypsin-like proteases. A satisfactory solution for protecting the

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peptide bond between residues 2 and 3 has been found. The introduction of D-Ala at position 2 and desNH$_2$-Tyr at position 1 was found to protect the molecules from degradation by dipeptidylpeptidase IV [9]. Protection from trypsin-like degradation remains to be discovered. Basic amino acids in positions 11, 12, 20 and 21 are required in order for the molecule to exhibit biological activity and, therefore, modification possibilities are limited. The replacement of Lys residues by Arg residues resulted in a more active analogue [12], but the sequence Arg-Arg is expected to be less stable than Arg-Lys [13]. The substitution of Arg residues by Lys greatly decreases biological activity. The only successful modification at the 10-11 and 20-21 positions was the introduction of Orn residues in positions 12 and 21. Several GH-RH analogues containing Orn, with an additional modification in positions 1 and 29, express unusually high biological activity [14–16] after subcutaneous administration. One of these analogues [14] proved to be about 200 times more active than hGH-RH(1-29)-NH$_2$. Although the intention of that work was to obtain analogues more resistant to trypsin-like proteases, increased stability has not been demonstrated.

It is an accepted view that, apart from substitution at the *N*-terminus to prevent cleavage by DPP-IV, long-acting analogues of GH-RH would require some modifications in positions occupied by basic amino acid residues to avoid trypsin-like cleavage [9]. As our goal was to develop potent analogues resistant to trypsin-like degradation, we decided to study the degradation of two hGH-RH analogues, which are suitable models for comparing the course of degradation of peptides containing native -Arg-Lys- sequences, and those containing Orn residue instead of Lys:

Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-**Arg**^{11}-**Lys**^{12}-Val-Leu-Gly-Gln-Leu-Ser-Ala-**Arg**^{20}-**Lys**^{21}-Leu-Leu-Gln-Asp-Ile-Nle-Ser-Arg^{29}-NH$_2$ (**1**)

Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-**Arg**^{11}-**Orn**^{12}-Val-Leu-Gly-Gln-Leu-Ser-Ala-**Arg**^{20}-**Orn**^{21}-Leu-Leu-Gln-Asp-Ile-Nle-Ser-Arg^{29}-NH$_2$ (**4**)

To avoid chemical oxidation during digestion, Nle was introduced in position 27 instead of Met in both of these sequences. Because enzymatic degradation of peptides in biological fluids and homogenates gives a rather complex picture as a result of independent and successive cleavages by several types of proteases (dipeptidylpeptidases, chymotrypsin-like peptidases), we decided to use an isolated enzyme – trypsin as a representative of the trypsin-like enzymes. Trypsin itself was selected, as it has been demonstrated that trypsin-like degradation of GH-RH(1-29)-NH$_2$ in human plasma is prevented by a trypsin inhibitor, trasylol [11].

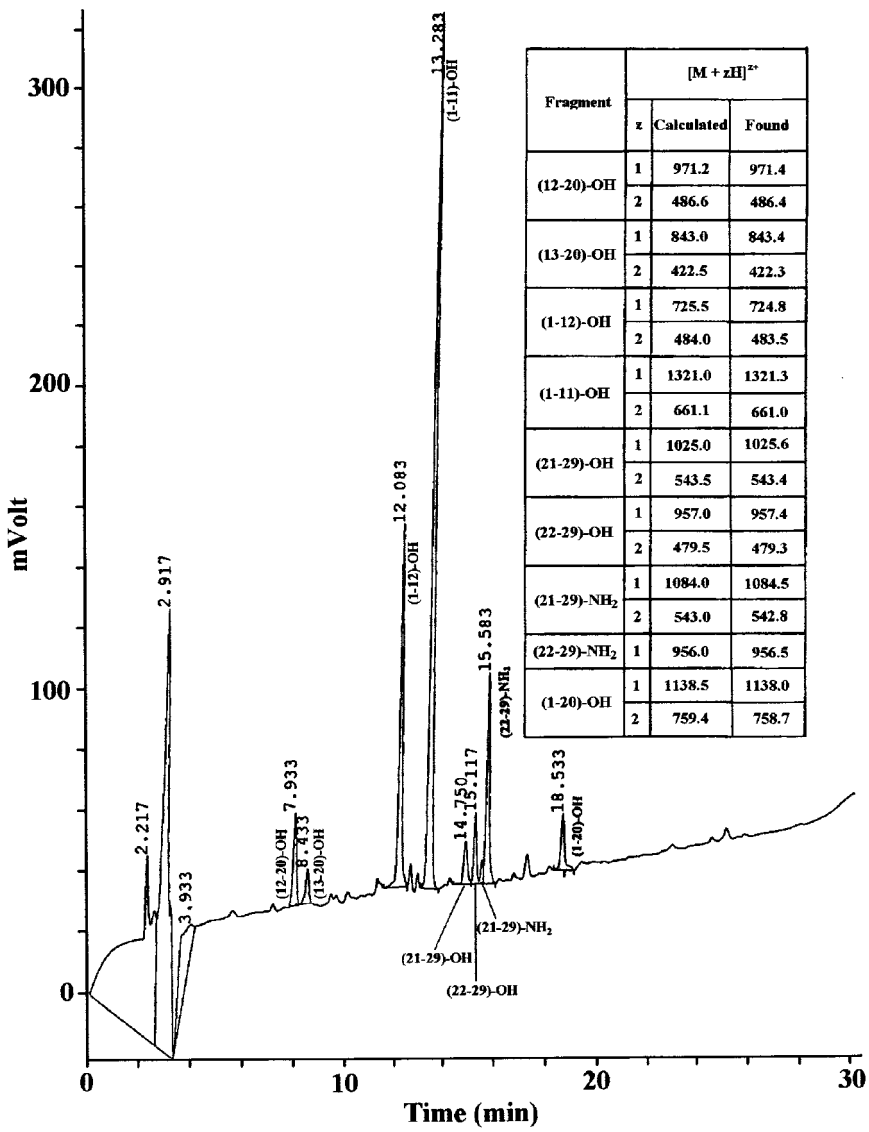
MATERIALS AND METHODS

Peptide Synthesis

Peptides were synthesized by a solid-phase method using the Boc strategy and carbodiimide (DcPC [17] or CIC [18]) as the coupling reagent. The side-chain functions of the Boc-amino acids were protected as follows: Arg(Tos), Asp(OcHx), Lys[Z(2Br)], Orn[Z(2Cl)], Ser(Bzl), Thr(Bzl), Tyr[Z(2Br)]. Peptides **1**–**3** were synthesized on a MBHA resin (0.51 mmol/g Bachem California). Peptide **8** was synthesized on a Merrifield resin (0.7 mmol/g, Fluka). The Boc-Arg(Tos)-resin was prepared using the cesium salt method [19]. The synthesis of peptides **4**–**7** was carried out on a MBHA resin (0.85 mmol/g Fluka); 55% TFA/DCM was used in all deprotection steps. Neutralizations were carried out with 5% DIEA/DCM. Boc amino acids (except Boc-Asn and Boc-Gln) were coupled by the symmetrical anhydride procedure using CIC. Boc-Asn and Boc-Gln were introduced by the carbodiimide (CIC)/HOBt method. Every coupling step was monitored with the Kaiser colour test. When needed, additional acylation was carried out. After the removal of the Boc group, samples of peptide resins were treated with HF (0°C, 1 h) in the presence of anisol. Crude peptides **2**–**8** were purified using a Waters System consisting of two Waters 501 pumps, an Automated Gradient Controller, a Waters 486 Tunable Absorbance Detector, and a Vydac C_{18} column 10×250 mm (5μ, 300Å). The column was eluted with a solvent system consisting of eluent A (0.1% aqueous TFA) and eluent B (80% MeCN in A). Fractions were eluted from the column with a linear gradient: peptide **2** and **7**, 20–50% B in 60 min; peptide **3** and **8**, 10–40% B in 60 min; peptide **4**, 35–40% B in 45 min; peptide **5**, 30–45% B in 50 min; peptide **6**, 25–35% B in 65 min; flow rate 2 mL/min; detection at 220 nm. Fractions were analysed with the same system, equipped with a Nucleosil 10 C_{18} 4×250 mm (Macherey–Nagel) column with gradient: peptide **2**, **3** and **7**, 20–50% B in 30 min; peptide **8**, 25–40% B in 15 min; peptide **4** and **5**, 30–70% B in 40 min; peptide **6**, 40–60% B in 20 min; flow rate 1 mL/min; detection at 220 nm.

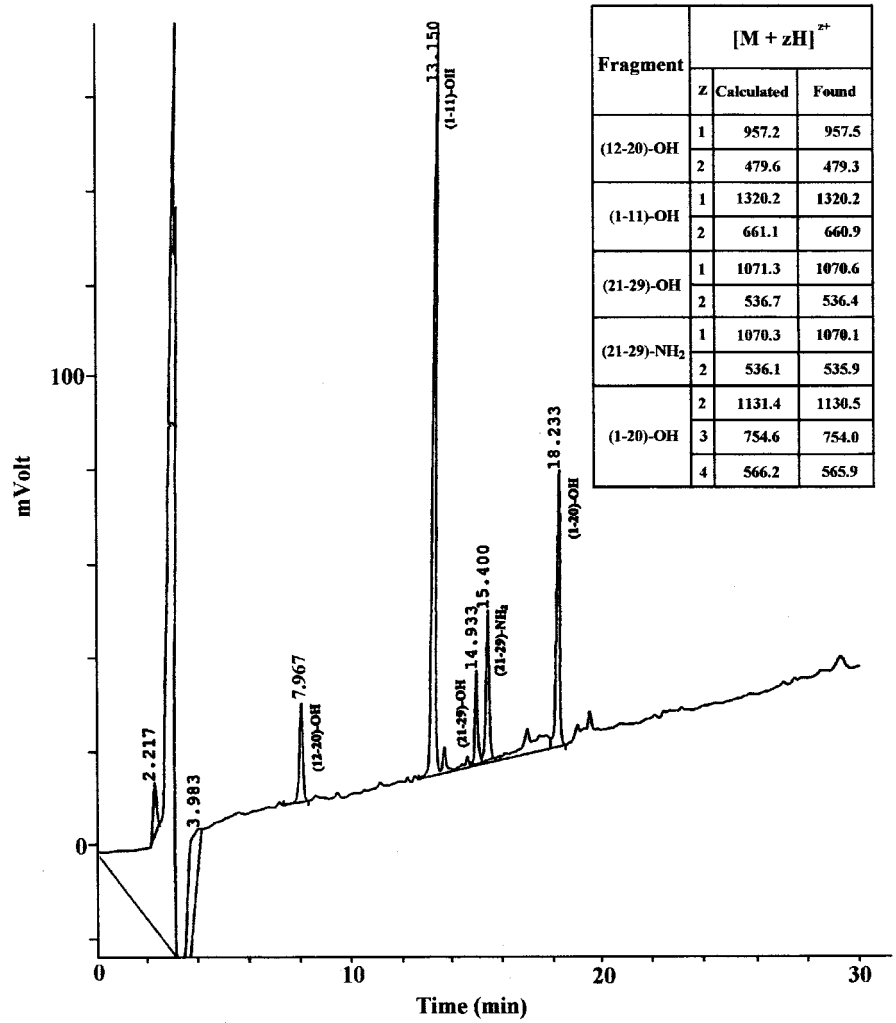
Tryptic Hydrolysis of Peptide 1 and 4

A sample of peptide (1.2 mg) was dissolved in 2.9 mL of 0.05 M ammonium acetate buffer (pH 8.5) and incubated at 37°C for 20 min. Then a solution (100 μL, 0.02 mg/mL) of trypsin (Serva, 36 U/mg) was added to obtain an enzyme: substrate ratio of 1:500 (w/w). The resulting solution was incubated at 37°C for 60 min and samples (500 μL) were collected after 5, 15, 30 and 60 min. All samples were diluted with 1 mL of 0.5 M AcOH and lyophilized. An analysis of products was performed by HPLC using a Knauer system with a Eurospher 100 C_{18} (4 × 250 mm, 5 μm) column; solvent system: (A) 0.1% TFA in water, (B) 80% MeCN in A; linear gradient 25–70% B in 30 min; flow rate 1 mL/min; detection at 220 nm. Fractions obtained from the sample digested for 15 min (Figures 1 and 2) were collected and peptides identified with electrospray ionization mass spectrometry (ESI-MS) using a Finnigan MAT 95S (Bremen, Germany). Mass spectrometry of the mixture obtained after the hydrolysis of **1** for 5 min revealed the presence of additional masses : (12-29)-NH_2 ($[M + 2H]^{2+}$, 1020.3 calculations 1020.0; $[M + 3H]^{3+}$, 680.3 calculations 680.0) and (13-29)-NH_2 ($[M + 2H]^{2+}$, 956.2 calculations 956.5; $[M + 3H]^{3+}$, 637.8 calculations 637.3), while in the case of **4**, it



Fragment	[M + zH]$^{z+}$		
	z	Calculated	Found
(12-20)-OH	1	971.2	971.4
	2	486.6	486.4
(13-20)-OH	1	843.0	843.4
	2	422.5	422.3
(1-12)-OH	1	725.5	724.8
	2	484.0	483.5
(1-11)-OH	1	1321.0	1321.3
	2	661.1	661.0
(21-29)-OH	1	1025.0	1025.6
	2	543.5	543.4
(22-29)-OH	1	957.0	957.4
	2	479.5	479.3
(21-29)-NH_2	1	1084.0	1084.5
	2	543.0	542.8
(22-29)-NH_2	1	956.0	956.5
(1-20)-OH	1	1138.5	1138.0
	2	759.4	758.7

Figure 1 HPLC profile of digestion products of [Nle^{27}]-hGH-RH(1-29)-NH_2 (trypsin:peptide ratio 1:500, 37°C for 15 min). Table shows ESI-MS data of separated products.



Fragment	$[M + zH]^{z+}$		
	z	Calculated	Found
(12-20)-OH	1	957.2	957.5
	2	479.6	479.3
(1-11)-OH	1	1320.2	1320.2
	2	661.1	660.9
(21-29)-OH	1	1071.3	1070.6
	2	536.7	536.4
(21-29)-NH_2	1	1070.3	1070.1
	2	536.1	535.9
(1-20)-OH	2	1131.4	1130.5
	3	754.6	754.0
	4	566.2	565.9

Figure 2 HPLC profile of digestion products of [$Orn^{12,21}$, Nle^{27}]-hGH-RH(1-29)-NH_2 (trypsin:peptide ratio 1:500, 37°C for 15 min). Table shows ESI-MS data of separated products.

revealed the presence of (12-29)-NH_2 ($[M + 2H]^{2+}$, 1013.2, calculations 1013.4). The course of this hydrolysis is illustrated in Figure 3. The structure of some peptides (**2**, **3** and **8** obtained from **1**, **5**, **7** and **8** obtained from **4**) was also confirmed by co-elution with synthetic peptides.

Tryptic Hydrolysis of Peptide 3, 4, 5, 6 and 7

A sample of peptide (0.2 mg) was dissolved in 1 mL of 0.05 M ammonium acetate buffer (pH 8.4) and incubated at 37°C for 20 min. Then 40 μL of trypsin (Serva, 36 U/mg) solution (0.05 mg/mL) in water was added to obtain an enzyme : peptide ratio of 1:100 (w/w). The mixture was incubated for 15 min at 37°C, then 2 mL AcOH was added, and the mixture was lyophilized.

The products of hydrolysis were analysed with a Knauer HPLC system using a reversed phase Nucleosil 100 C_{18} 4 × 200 mm column; a solvent system comprising (A) 0.1% TFA in water, (B) 80% MeCN in A; a linear gradient of 20–60% B in 40 min and 20%–40% B in 20 min; flow of 1 mL/min; detection at 220 nm.

RESULTS AND DISCUSSION

Peptides **1–8** (Table 1) were synthesized according to solid-phase protocols on the Merrifield resin (peptide **8**) or the benzhydrylamine resin (peptide **1–7**) using a Boc strategy and *N*,*N*′-dicyclopentylcarbodiimide or *N*-cyclohexyl-*N*′-isopropylcarbodiimide as coupling reagents. Boc-Asn and Boc-Gln were

Digestion products of [Nle^{27}]-GH-RH-(1-29)-NH$_2$

Time, min	(1-29)-NH$_2$	(1-11)-OH	(12-29)-NH$_2$	(1-12)-OH	(13-29)-NH$_2$	(1-20)-OH	(21-29)-NH$_2$	(22-29)-NH$_2$	(12-20)-OH	(13-20)-OH	(21-29)-OH	(22-29)-OH
5	+	+	+	+	+	+	+	-	+	+	-	-
15	-	+	-	+	-	+	+	+	+	+	+	+
60	-	+	-	+	-	-	+	+	+	+	+	+

Digestion products of [Orn^{12,21},Nle^{27}]-GH-RH-(1-29)-NH$_2$

5	+	+	+	-	-	+	+	-	+	-	-	-
15	-	+	-	-	-	-	+	-	+	-	+	-
60	-	+	-	-	-	-	+	-	+	-	+	-

Figure 3 Identified products of tryptic digestion.

introduced by the carbodiimide (CIC)/HOBt method. The peptides were cleaved from the resin by treatment with HF in the presence of anisole and purified by RP-HPLC.

Peptides **1** and **4** were incubated with trypsin in ammonium acetate buffer at pH 8.5. Samples of this solution were collected after 5, 15, 30 and 60 min. The course of the digestion was monitored by RP-HPLC. The results indicate that the peptides were rapidly cleaved to give several intermediate and final products. The samples obtained after digestion at 15 min (Figures 1 and 2) were separated and products corresponding to the major peaks were identified using electrospray-ionization mass spectrometry. The structures of peptides **2**, **3** and **8** obtained from **1**, and **5**, **7** and **8** obtained from **4** were confirmed by co-elution with synthetic peptides. During the digestion of peptide **1**, fragments resulting both from cleavage at the Arg residue and at the Lys residue were observed. Hydrolysis at Arg^{11} resulted in the formation of the fragments (1-11)-OH and (12-29)-NH$_2$; at Arg^{20} of the fragments (1-20)-OH and (21-29)-NH$_2$; at both Arg^{11} and Arg^{20} of the fragments (12-20)-OH. Fragment (1-20)-OH was observed only during the initial period of hydrolysis owing to further degradation to smaller fragments. Hydrolysis at Lys^{12} and Lys^{21} was also observed. The fragments (1-12)-OH and (22-29)-NH$_2$ were found and isolated. Excepting the *C*-terminal fragments mentioned above, deamidated peptides were also recognized: (21-29)-OH and (22-29)-OH. These peptides result from cleavage at Arg^{20} and Lys^{21}, respectively. A deamidation reaction could occur before or after these cleavages.

A comparison of peak intensities corresponding to peptides (1-11)-OH and (1-12)-OH indicates that

Table 1 hGH-RH(1-29)-NH$_2$ Analogues and Fragments

Number	Peptide	LSIMS		
		Calculated	Found	Yield (%)
1	[Nle^{27}]-GH-RH(1-29)-NH$_2$*	3340.9	3340.9	20
2	[Nle^{27}]-GH-RH(21-29)-NH$_2$	1083.3	1084.0	75
3	[Nle^{27}]-GH-RH(22-29)-NH$_2$	954.2	956.0	51
4	[Orn^{12,21},Nle^{27}]-GH-RH(1-29)-NH$_2$	3312.0	3315.0	14
5	[Orn^{12,21},Nle^{27}]-GH-RH(12-29)-NH$_2$	2011.0	2011.3	22
6	[Orn^{21},Nle^{27}]-GH-RH(13-29)-NH$_2$	1898.0	1898.6	15
7	[Orn^{21},Nle^{27}]-GH-RH(21-29)-NH$_2$	1069.0	1070.8	75
8	GH-RH(1-11)-OH	1320.4	1320.9	54

* Described previously [17].

cleavage at the Arg residue is more rapid than that at Lys. While the amount of peptide (1-11)-OH (calculated from HPLC profile) increases from 30% (at 5 min) to 65% (at 60 min), the proportion between (1-12)-OH and (1-11)-OH changes merely from 1:2.3 to 1:2.6. This is in agreement with our earlier finding [13] that both the Arg and Lys bonds in the sequence peptide-Arg-Lys-peptide are cleaved in the original sequence, while the following cleavage at Arg in the sequence peptide-Arg-Lys, to form peptide-Arg, is very slow.

In order to check whether all fragments were recognized during the separation of hydrolizates by HPLC, we also examined ES-MS spectra of the mixtures obtained after 5 min and 15 min digestion. A comparison of these spectra revealed that unstable peptides (12-29)-NH_2 and (13-29)-NH_2 were also present at an early stage of digestion.

As for the Orn analogue **4**, similar cleavages were observed at Arg^{11}, Arg^{20} and Arg^{29}. However, the ornithine-peptide bond seems to be completely resistant to trypsin. Peptides which could have resulted from cleavage at Orn residues were not observed, either among the separated fractions or in crude mixtures.

It is interesting to compare the course of release of *N*-terminal fragments during the digestion of **1** and its Orn analogue **4**. A hydrolysis of **1** for 15 min results in the formation of (1-11)-OH and (1-12)-OH. The contents estimated by integration of the peaks were 61 and 28%, respectively. The sum of these values (89%), compared with 59% of (1-11)-OH, the only product formed from the *N*-terminal portion of **4**, clearly indicate that the overall degradation of **1** in this part of the molecule is substantially higher than in the case of **4**. In addition, the increased stability of fragment 1-20, derived from peptide **4**, clearly indicates the beneficial effect of replacing Lys with Orn (see Figure 3).

The hydrolysis of synthetic peptides (21-29)-NH_2 (**7**) and (22-29)-NH_2 (**3**) at a higher concentration of trypsin (1:100 ratio) confirmed that deamidation is a slow reaction. About 10% of unchanged peptide was still present after digestion for 15 min. Orn in position 21 was not cleaved from peptide **7**.

CONCLUSIONS

Hydrolysis of peptide **1** by trypsin consists in the cleavage of peptide bonds at Arg^{11}, Arg^{20}, Lys^{12}, Lys^{21} and the amide bond at Arg^{29}. Cleavages at Arg^{11} and Arg^{20} are more rapid but cleavages at Lys residues have a noticeable impact on transforming the biologically active peptide to inactive fragments. Hydrolysis of the amide bond at Arg^{29} is slow but should also be taken into account. The replacement of Lys residues by Orn residues has resulted in the formation of a molecule (peptide **4**), which contains bonds (12-13) and (21-22) which are resistant to tryptic hydrolysis. As a result, overall hydrolysis is decreased.

It is obvious from these results that the replacement of Lys by Orn is a reasonable way to obtain long-acting analogues. The analogues of hGH-RH(1-29)-NH_2 containing Orn in positions 12 and 21 are indeed very potent after subcutaneous injection, up to 217 times more than the parent peptide. It should be noted, however, that these analogues contain desNH_2Tyr instead of Tyr in order to protect from DPP-IV degradation and agmatine instead of Arg-NH_2.

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