ELSEVIER

Journal of Chromatography A, 729 (1996) 137–142

JOURNAL OF CHROMATOGRAPHY A

Liquid chromatography studies on the enzymatic degradation of luteinizing hormone-releasing hormone analogues with off-line identification by mass spectrometry

Antal Péter[a,*], Serge Devadder[b], Georges Laus[b], Dirk Tourwé[b]

[a]*Department of Inorganic and Analytical Chemistry, Attila József University, Dóm tér 7, H-6720 Szeged, Hungary*
[b]*Eenheid Organische Chemie, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium*

Abstract

Several agonists of luteinizing hormone-releasing hormone (LHRH) are currently used for different therapeutic purposes, but relatively little is known about their metabolic fate after administration. This paper describes the application of high-performance liquid chromatography combined with off-line fast atom bombardment mass spectrometry to identify the degradation products resulting from the incubation of LHRH analogues with proteolytic enzymes. Three analogues, containing a ψ(E,CH=CH) pseudo-peptide bond were synthesized and afforded to the assay to determine the resistance against α-chymotrypsin and subtilisin: [Tyr^5ψ(E,CH=CH)Gly^6]LHRH, [Gly^6ψ(E,CH=CH)D,L-Leu^7]LHRH and [Pro^9ψ(E,CH=CH)Gly^{10}]LHRH. The pattern of peptide metabolites identified by this method indicates that α-chymotrypsin degrades LHRH analogues at the Trp^3-Ser^4 and Tyr^5-Gly^6 bond, while subtilisin hydrolyzes only the Tyr^5-Gly^6 linkage. The results also indicate a possible stabilization of native amide bonds against enzymatic degradation by neighbouring ψ(E,CH=CH) modifications.

Keywords: Liquid chromatography–mass spectrometry; Enzymes; Luteinizing hormone-releasing hormone; Peptides; α-Chymotrypsin; Subtilisin

1. Introduction

Several agonists of luteinizing hormone-releasing hormone, Pyr-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (LHRH), are currently used for the treatment of prostate cancer, endometriosis and other sex-hormone dependent diseases [1–5], but only a few data are available concerning their metabolic fate after administration.

To increase the stability against proteolytic degradation, a number of analogues have been synthesized containing D-amino acids, or with a modified N- or C-terminus [6,8]. Although such modifications provide local stabilization against enzymatic cleavage, the peptide analogues are extensively degraded and thereby deactivated after administration.

In the present work, three LHRH analogues were synthesized by applying the pseudopeptide modification ψ(E,CH=CH) [9] (Fig. 1) and their resistance toward enzymatic degradation was assayed. The enzymes investigated were α-chymotrypsin and subtilisin, to which LHRH analogues are known to be highly susceptible [6,7].

The objectives of this study were (i) to obtain more information on the patterns of the degradation products of the three peptides, (ii) to investigate the

*Corresponding author.

SSDI 0021-9673(95)00948-5

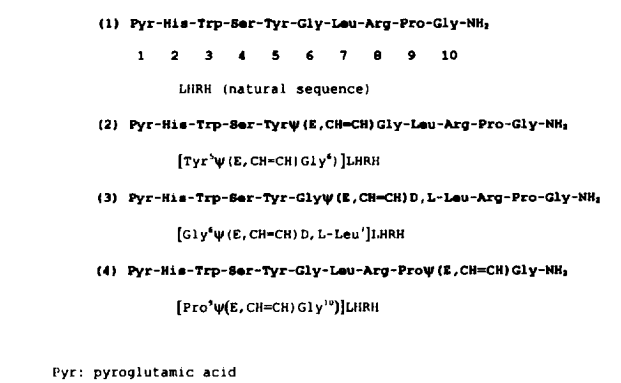

Fig. 1. Native LHRH and analogues studied.

influence of modifications on enzymatic resistance, and (iii) in addition to the direct effects to the modified peptide bond, to evaluate the stability of the neighbouring sites, since the literature data indicate that isosteric replacements by $\psi(CH_2-NH)$ and $\psi(CH_2-CH_2)$ bond may protect neighbouring amide bonds against proteolysis [10]. This effect would be very interesting since this may avoid difficult syntheses of isosteric dipeptides. Thus the Trp^3-Ser^4 peptide bond which is known to be a primary cleavage site by α-chymotrypsin was not modified.

2. Experimental

2.1. Chemicals and reagents

LHRH analogues (Fig. 1, compounds 2–4) were prepared [9] by using a solid-phase synthesis technique on a 4-methylbenzhydrylamine resin, with Boc main chain protection. The pseudo-dipeptides were obtained either by the method involving a Wittig reaction on Boc–Pro-aldehyde or Boc–Tyr(Bu)-aldehyde, or by alkylation of Boc–Glyψ(E,CH=CH)Gly-OEt [11,12]. Details will be published in a separate paper.

The native LHRH was obtained from Novabiochem (Switzerland). α-Chymotrypsin (EC 3.421.1) type I was purchased from Sigma (St. Louis, MO, USA) and subtilisin, Carlsberg type was obtained from UCB Bioproducts (Brussels, Belgium). Sodium phosphate, sodium chloride and trifluoroacetic acid (TFA) of analytical reagent grade and acetonitrile and methanol of HPLC grade were purchased from Merck (Darmstadt, Germany). Buffers were prepared with MilliQ water and were further purified by filtration on a 0.45-μm filter type HV (Millipore, Molsheim, France). Sep-Pak C_{18} cartridges were purchased from Millipore.

2.2. Chromatography

Analytical and preparative HPLC were performed on an SP-4000 low-pressure gradient pump with an SP-2000 variable-wavelength UV detector and an SP-4600 integrator (Spectra-Physics Analytical, Fremont, CA, USA). The injector was a Model 7125 (Rheodyne, Cotati, CA, USA) with 20-μl or 200-μl loops. Purification of synthetic peptides was performed on a Gilson Auto-Prep System equipped with type 302 and 303 pumps, a type 115 detector and a type 712 system controller (Gilson Medical Electronics, Villiers le Bel, France).

The columns used were Vydac 218TP54 C_{18} (250×4.6 mm I.D.), 5-μm particle size and Vydac 218TP1010 C_{18} (250×10 mm I.D.), 10-μm particle size (The Separations Group, Hesperia, CA, USA).

Gradient elutions were run with (A) a 0.1% aqueous solution of TFA and (B) a 0.1% aqueous solution of TFA–acetonitrile (50:50, v/v), ranging from 20% to 50% B within 30 min.

2.3. Enzymatic cleavage assay

Solutions of peptides (1–4) were prepared by dissolving 1.0 mg of each in 0.1 ml of methanol. A 50-μl aliquot of each solution was placed in an incubator at 37°C. The stock solutions of buffered α-chymotrypsin and subtilisin were prepared as follows: 0.03 *M* sodium phosphate–0.1 *M* sodium chloride solution was prepared and adjusted to pH 6.9. In 10.0 ml of sodium phosphate–sodium chloride solution, 0.2 mg of enzyme was dissolved and incubated for 15 min at 37°C. To each peptide, a 2.0-ml aliquot of buffered enzyme solution was added and the mixtures were incubated at 37°C. Then, 0.1-ml aliquots were removed from each sample at designated intervals [generally 0.1, 1, 5 or 10 min, but at 1, 2, 5, 10, 24, 48 h for the digestion of peptide (2) with subtilisin] and quenched into 0.5 ml of a solution of acetonitrile–water (7:3, v/v).

The peptide composition of each sample was

determined by analytical HPLC in two ways: (i) injection directly onto the HPLC column and (ii) prepurification on C_{18} cartridges. For this latter purpose, the sample was lyophilized, dissolved in 1 ml of a 0.1% aqueous solution of TFA and adsorbed on Sep-Pak C_{18} cartridges. The cartridge was washed with 5 ml of 0.1% TFA and the peptides were eluted with 5 ml of 0.1% TFA–acetonitrile (20:80, v/v). The solvent was removed by lyophilization and the residue was dissolved in the HPLC eluent. Since method (ii) resulted in better peak shapes and a better baseline, this method was used in the further study. The half-lives of the peptides were calculated by comparing the peak intensities of the intact peptide at the different incubation times to those at time zero.

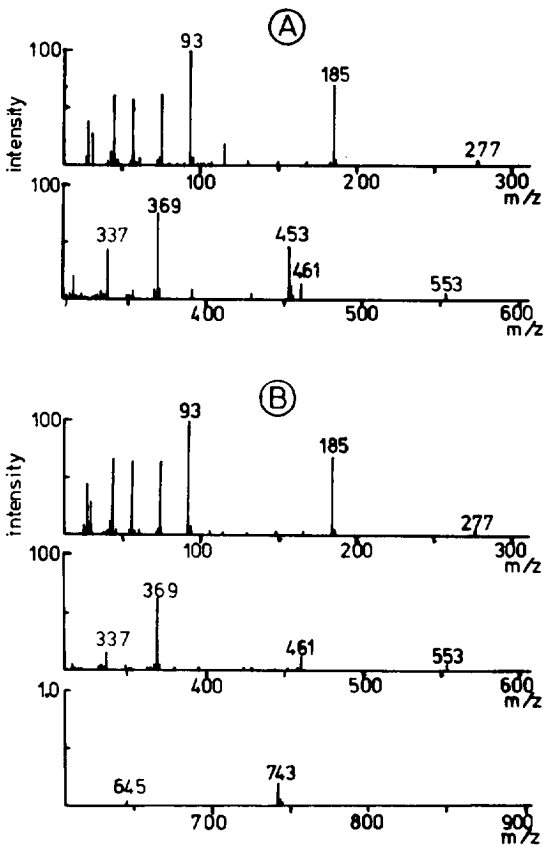

Fig. 2. FAB mass spectra of fragment 1–3 (A) and fragment 1–6 (B) of LHRH analogue (2), showing molecular ions at m/z=453 and 743, respectively. The ions at m/z=(n×92)+1 are due to the glycerol matrix.

For identification of the fragments, 1-mg samples of the peptides were incubated with enzyme, treated by method (ii) described above, and separated on a Vydac 218TP1010 column. Each peak was collected, lyophilized and identified by mass spectrometry.

2.4. Mass spectrometry

Fast atom bombardment (FAB) mass spectra were obtained on a double focussing MS 902 S spectrometer (A.E.I. Scientific Apparatus Division, Manchester, UK) at 8 kV, with xenon as bombarding gas. The off-line mass spectra were measured in positive-ion mode in a glycerol matrix. Peptides were identified via their molecular ion. An example of the identification of the 1–3 and 1–6 fragments of analogue (2) is shown in Fig. 2. No identification of the fragments was possible by direct application of mass spectrometry to the digestion mixture.

3. Results and discussion

Native LHRH and its modified analogues were incubated with the enzymes α-chymotrypsin and subtilisin. α-Chymotrypsin is known to be a very specific proteolytic enzyme to cleave the peptide bond after the aromatic amino acid residue. Subtilisin is a serine protease from *Bacillus subtilis*; it is a non-specific endopeptidase which acts on internal peptide bonds and which is generally used for the total hydrolysis of peptides or proteins.

3.1. Enzymatic digestion with α-chymotrypsin

The results of digestion are summarized in Table 1 and Fig. 3. Incubation of LHRH analogues with α-chymotrypsin results in degradation patterns which reflect the stabilizing effect of the peptide bonding modification. Fig. 3A reveals that for all analogues two major UV adsorption peaks appear after digestion for 1 min. Increase of the incubation time to 2 min results in the total disappearance of the peak of the original peptide and increases in the intensities of the fragment peaks. The observed fragments indicate that α-chymotrypsin digestion of

Table 1
Degradation products of LHRH analogues obtained by digestion with α-chymotrypsin

Analogue	t_R^a	m/z^b	$M_{r(calc.)}^c$	Sequence assignment	Sequence no.	$t_{(1/2)}^d$ (min)
(1) LHRH	4.5	–	–	Unidentified	–	
	8.1	453	452.2	Pyr-His-Trp	1–3	
	13.1	703	702.2	Pyr-His-Trp-Ser-Tyr	1–5	<0.1
	13.1	498	497.3	Gly-Leu-Arg-Pro-Gly-NH_2	6–10	
	17.4	–	1181.5	Intact peptide	1–10	
(2) [Tyr$^5\psi$Gly^6]LHRH	8.1	453	452.2	Pyr-His-Trp	1–3	
	9.1	731	730.4	Ser-TyrψGly-Leu-Arg-Pro-Gly-NH_2	4–10	<0.1
	20.3	–	1164.5	Intact peptide	1–10	
(3) [Gly$^6\psi$D,L-Leu^7]LHRH	8.1	453	452.2	Pyr-His-Trp	1–3	
	10.2	731	730.4	Ser-Tyr-GlyψD,L-Leu-Arg-Pro-Gly-NH_2	4–10	<0.1
	22.1	–	1164.5	Intact peptide	1–10	
(4) [Pro$^9\psi$Gly^{10}]LHRH	8.1	453	452.2	Pyr-His-Trp	1–3	
	13.1	731	730.4	Ser-Tyr-Gly-Leu-Arg-ProψGly-NH_2	4–10	
	13.1	703	702.2	Pyr-His-Trp-Ser-Tyr	1–5	<0.1
	13.1	481	480.3	Gly-Leu-Arg-ProψGly-NH_2	6–10	
	20.3	–	1164.5	Intact peptide	1–10	

[a] HPLC retention time in minutes.
[b] m/z values for the ions $[M+H]^+$ in the FAB mass spectra.
[c] Monoisotopic masses.
[d] Half-lives of peptides in minutes in the presence of α-chymotrypsin; incubation time, 1 min.

these LHRH analogues leads to rapid cleavage of the Trp^3-Ser^4 bond, as observed previously [6], with parallel cleavage of the Tyr^5-Gly^6 linkage. Fragment 4–10 resulting from hydrolysis of the Trp^3-Ser^4 bond in native LHRH (1) was not detected by the present experiment. This is probably due to rapid further degradation at residue 5.

As expected, the modified Tyr$^5\psi$Gly^6 bond in the pseudopeptide [Tyr$^5\psi$(E,CH=CH)Gly^6]LHRH (2) is fully resistant to enzymatic hydrolysis and the Trp^3-Ser^4 bond is the only degradation site. The native Tyr^5-Gly^6 linkage in [Gly$^6\psi$(E,CH=CH)D,L-Leu^7] LHRH (3) was also resistant, since no 1–5 or 6–10 fragmentation is observed. This indicates a stabilizing influence of the adjacent Gly$^6\psi$D,L-Leu^7 bond modification. This modification changes the degradation pathway, without increasing the overall stability. None of the three modified analogues (2), (3) and (4) possesses improved stability to α-chymotrypsin in comparison with native LHRH (1): the half-lives ($t_{1/2}$) of the peptides in the presence of enzyme are less than 0.1 min. All modified sites are at least two residues distant from the extremely labile Trp^3-Ser^4 bond, and consequently the substrate recognition by the enzyme may not be impaired.

3.2. Enzymatic digestion with subtilisin

Subtilisin degrades LHRH (1) and its analogues primarily at the Tyr^5-Gly^6 bond. We observed a half-life of less than 0.1 min for (1) and only the fragment 6–10 appeared in the HPLC chromatogram after digestion for 1 min. As expected, the analogue [Tyr$^5\psi$(E,CH=CH)Gly^6]LHRH (2) provided considerable resistance, with a half-life of 21 h (Fig. 4). Some degradation at the Trp^3-Ser^4 and Gly^6-Leu^7 linkages is detected in this analogue after digestion for 10 h. [Gly$^6\psi$(E,CH=CH)D,L-Leu^7]LHRH (3) has a half-life of 9 min. The weak stabilization of (3) may be due to a protecting effect of the neighbouring alkene isostere on the Tyr^5-Gly^6 bond. Degradation by subtilisin still occurs at the Tyr^5-Gly^6 bond, thereby producing fragment 6–10. In contrast with what was observed for α-chymotrypsin, the adjacent peptide bond modification did not change the cleavage site.

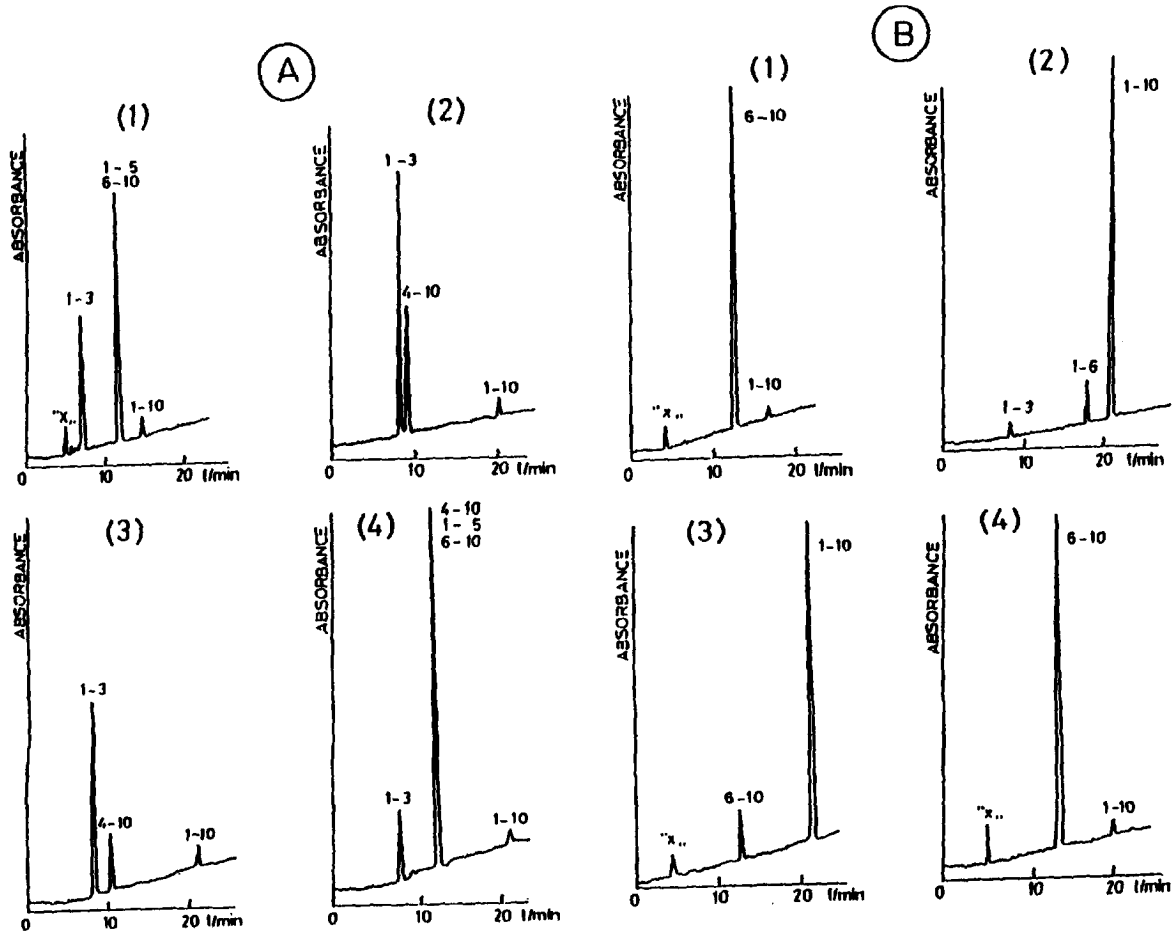

Fig. 3. HPLC separation of mixtures of degraded LHRH analogues after digestion with α-chymotrypsin (A) or subtilisin (B). Incubation time, 1 min (for the digestion of LHRH (2) with subtilisin, 10 h); column, Vydac 218TP54; detection, 210 nm; gradient elution, see Experimental,; (1) native LHRH, (2) [Tyr^{5}ψGly^6]LHRH; (3) [Gly^{6}ψD,L-Leu^7]LHRH, (4) [Pro^{9}ψGly^{10}]LHRH. Peak x is an unidentified sequence.

The C-terminal modified analogue (4) is readily degraded: the modified site is probably too far away to exert a protecting effect on the neighbouring amide bonds. In the cases of peptides (1), (3) and (4), the N-terminal sequence 1–5 peptide could not be identified in the incubation products. This fragment elutes at 13.1 min [see Table 1 and Fig. 3A (1)]. In the fractions collected at a retention time of 13.1 min (Table 2), the mass signal of the N-terminal fragment 1–5, $[M+H]^+=703$, was not observed. For these LHRH analogues a peak with a retention time of 4.5 min was found in the HPLC chromatogram, but its MS identification was unsuccessful.

4. Conclusions

The digestion of LHRH analogues containing a pseudopeptide modification with α-chymotrypsin led to hydrolysis of the Trp^3-Ser^4 and the Tyr^5-Gly^6 bonds, whereas subtilisin proved selective towards

Table 2
Degradation products of LHRH analogues obtained by enzymatic digestion with subtilisin

Analogue	t_R^a	m/z^b	$M^c_{r(calc.)}$	Sequence assignment	Sequence no.	$t^d_{(1/2)}$ min
(1) LHRH	4.5	–	–	Unidentified	–	
	13.1	498	497.3	Gly-Leu-Arg-Pro-Gly-NH_2	6–10	<0.1
	17.4	–	1181.5	Intact peptide	1–10	
(2) [Tyr$^5\psi$Gly^6]LHRH	8.3	453	452.2	Pyr-His-Trp	1–3	
	17.6	743	742.2	Pyr-His-Trp-Ser-TyrψGly	1–6	21 h
	20.3	–	1164.5	Intact peptide	1–10	
(3) [Gly$^6\psi$D,L-Leu^7]LHRH	4.5	–	–	Unidentified	–	
	13.3	481	480.3	GlyψD,L-Leu-Arg-Pro-Gly-NH_2	6–10	9
	20.3	–	1164.5	Intact peptide	1–10	
(4) [Pro$^9\psi$Gly^{10}]LHRH	4.5	–	–	Unidentified	–	
	13.1	481	480.3	Gly-Leu-Arg-ProψGly-NH_2	6–10	<0.1
	20.3	–	1164.5	Intact peptide	1–10	

[a] HPLC retention time in minutes.
[b] m/z values for the ions $[M+H]^+$ in the FAB mass spectra.
[c] Monoisotopic masses.
[d] Half-lives of peptides in minutes in the presence of subtilisin; incubation time, 1 min (in the case of (2) 10 h).

the Tyr^5-Gly^6 linkage. The incorporated modifications are insufficient for the design of α-chymotrypsin-resistant analogues. The E-alkene isosteric replacement of the Tyr^5-Gly^6 bond, however, leads to a spectacular stabilization against subtilisin digestion.

Further, these results indicate the stabilization of native amide bonds by neighbouring ψ(E,CH=CH) modifications toward proteolytic enzyme digestion. In vitro biological activity measurements are in progress to find a correlation between the stabilization effect and activity.

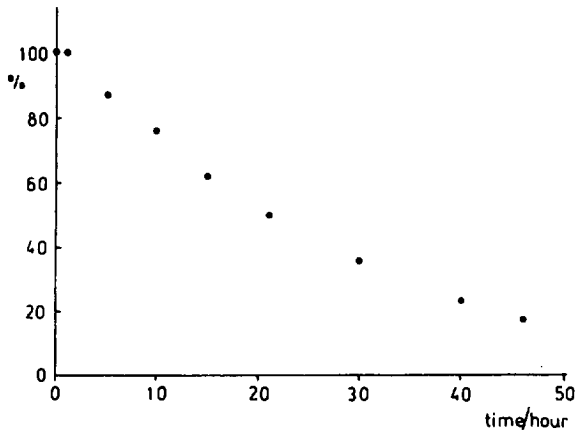

Fig. 4. Time course of the (1–10) peak intensity of LHRH analogue (2) on incubation with subtilisin.

References

[1] I. Sandow, W. König, R. Geiger, R. Uhman and W. Rechenberg, in D.B. Crightron, G.R. Foxcroft, N.B. Haynes and G.E. Lamming (Editors), Control of Ovulation, Butterworth, London, Boston, 1978, p. 49.
[2] M.J. Karten and J.E. Rivier, Endocr. Rev., 7 (1986) 44.
[3] A.S. Dutta, Drugs Future, 13 (1988) 43.
[4] M. Filicori and D. Flamigni, Drugs, 35 (1988) 63.
[5] A.J. Friedman, in R.L. Barbieri and A.J. Friedman (Editors), Gonadotropin Releasing Hormone Analogs, Applications in Gynecology, Elsevier, New York, Amsterdam, London, 1990, p. 1.
[6] F. Haviv, T.D. Fitzpatrick, C.J. Nichols, R.E. Swenson, E.N. Bush, G. Diaz, A. Nguyen, H.N. Nellans, D.J. Hoffman, H. Ghanbari, E.S. Johnson, S. Love, Van Cybulski and J. Greer, J. Med. Chem., 35 (1992) 3890.
[7] J. Rivier, J. Porter, C. Hoeger, P. Theobald, A.G. Craig, J. Dykert, A. Corrigan, M. Perrin, W.A. Hook, R.P. Siraganian, W. Vale and C. Rivier, J. Med. Chem., 35 (1992) 4270.
[8] M. Brudel, V. Kertscher, H. Berger and B. Mehlis, J. Chromatogr. A, 661 (1994) 55.
[9] S. Devadder, Ph.D Thesis, Vrije Universiteit, Brussels, 1994.
[10] D.E. Benovitz and A.F. Spatola, Peptides, 6 (1985) 257.
[11] A. Scanso, J. Degelaen, R. Viville, E. De Cock, M. Van Marsenille, L. Van Der Auwera, D. Tourwé and G. Van Binst, Bull. Soc. Chim. Belges, 100 (1991) 381.
[12] S. Devadder, J. C[illegible], H. Jaspers, M. Ceusters, D. Tourwé and G. Van Binst, Bull. Soc. Chim. Belges, 100 (1991) 407.