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Report

High-Performance Liquid Chromatographic (HPLC) and HPLC–Mass Spectrometric (MS) Analysis of the Degradation of the Luteinizing Hormone-Releasing Hormone (LH-RH) Antagonist RS-26306 in Aqueous Solution

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The kinetics of the degradation of an LH-RH antagonist, RS-26306,1, in aqueous solution from pH 1 to pH 11 were studied by reverse-phase HPLC. The pH–rate profiles at 50, 60, and 80°C were U-shaped with the rate law of $k_{obs} = k_H a_H + k_w + k_{OH} a_{OH}$. The predicted 25°C shelf life at the pH of maximum stability, pH ~5, is greater than 10 years. The products from the degradation were analyzed by HPLC-MS using thermospray ionization. Below pH 3, the primary product, 2, forms from the acid-catalyzed deamidation of the C-terminal amide. Above pH 7, epimerization of the individual amino acids is the principal reaction. Between pH 4 and pH 6, intramolecular serine-catalyzed peptide hydrolysis becomes important, yielding a tripeptide, 3, and a heptapeptide, 4. At the pH of maximum stability all three pathways for degradation are observed.

KEY WORDS: luteinizing hormone-releasing hormone (LH-RH) antagonist; peptide degradation; deamidation; hydrolysis; epimerization; high-performance liquid chromatography (HPLC)–mass spectroscopy (MS); RS-26306.

INTRODUCTION

RS-26306, 1 (Scheme I), is an LH-RH antagonist being developed for potential treatment of endometriosis, breast cancer, prostate cancer, and fibroid tumors (1). It is a decapeptide containing five D amino acids and is acetylated at the N terminal and amidated at the C terminal. Although several reports on the stability of LH-RH and LH-RH analogues in aqueous solutions have appeared in the literature (2–6), product studies on these analogues are not complete, thus precluding the accurate assignment of the degradation pathways. In this study we have investigated the kinetics of the degradation of 1 in aqueous solution by HPLC and determined the degradation products by HPLC-MS.

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[4] In a mass spectrometric experiment the molecular ion and related ions which bear one or more heavy isotopes are resolved. The molecular weights listed in Table II are those of the principal isotopes which are the sum of the atomic weights of the constituent atoms (C = 12.000, H = 1.078, N = 14.003, O = 15.995, Cl = 34.969). They are different from the average molecular weights which are calculated from the sum of the weighted average atomic weights of all the isotopes (C = 12.011, etc.). As an example, the molecular weight of 1 is 1568.9, while the average molecular weight is 1570.4.

EXPERIMENTAL

Materials

RS-26306 was obtained as a mixture of its di- and triacetate salts from the Institute of Organic Chemistry, Syntex Research. The HPLC-grade solvents, ammonium acetate, potassium acetate, and potassium phosphate were purchased from J. T. Baker. Water was purified using the Barnstead Nanopure System. Benzyl alcohol was pharmaceutical grade.

Instrumentation

The pH measurements were made using a Radiometer Model PHM64 Research pH meter equipped with a Sensorex Model SG900C combination electrode. Ultraviolet-visible measurements were made on a Hewlett-Packard 8450A UV/VIS spectrophotometer. The HPLC system consisted of an HP 1090 pump and an autosampler connected to a UV-visible detector from Applied Biosystems (Model 783). The detector was interfaced to a Spectra-Physics 4000 integrator.

HPLC-MS analyses were performed on a TSQ-70 mass spectrometer equipped with a thermospray (TSP) ionization (7) source from Finnigan-MAT Corporation. The mass spectrometer was connected in series with a UV-visible detector from Applied Biosystems. Eluant was pumped using a Rainin Rabbit-HP solvent delivery system.

Determination of the pK_a values for 1

The dissociation constants of 1 were determined spec-

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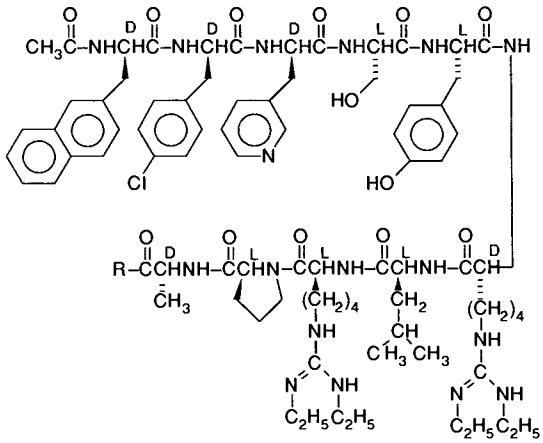


1, R=NH_2
2, R=OH
Scheme I

trophotometrically at a drug concentration of 40 μg/ml. The pH of the drug solution (3 ml) was adjusted with 0.30 *N* HCl and 0.10 *N* KOH for the determination of the pK_a of the 3-pyridinylalanine residue and the tyrosine residue, respectively. After the addition of each aliquot of base or acid (5–25 μl) the UV absorbance spectrum and pH of the drug solution were recorded.

Preparation of Samples for Kinetic and Product Studies

Buffer solutions of pH 4–5 and pH 6–8 were prepared with 0.01 *M* potassium acetate and potassium phosphate, respectively. Very acidic and basic solutions were prepared with HCl and KOH solutions, respectively. The ionic strength of each solution was adjusted to 0.15 *M* with potassium chloride and the pH was measured at the reaction temperatures. Benzyl alcohol (1%) was used as a preservative in samples with pH values of 3–8 at 50 and 60°C.

For the kinetic studies, drug solutions were prepared at 50 μg/ml and 0.5 ml aliquots were transferred to 1-ml clear colorless glass ampoules. The ampoules were flame-sealed and placed in 50, 60, and 80°C ovens. At fixed times, samples were removed from the ovens and stored at −4°C until they were assayed. For the HPLC-MS studies drug solutions were prepared at 1.0 mg/ml.

HPLC Methods

Two reverse-phase HPLC methods were developed and both methods used a Zorbax Rx octyl column (5 μ, 4.6 × 250 mm) from MacMod Analytical. Chromatography was performed at room temperature.

Method A was used to analyze the kinetic samples. The mobile phase consisted of 0.05 *M* ammonium phosphate buffer (pH 2)–acetonitrile (72.5:27.5). The flow rate was controlled at 1 ml/min, and the absorbance of the eluant monitored at 225 nm.

Method B was developed for use with the HPLC-MS experiments. The mobile phase consisted of 0.1 *M* ammonium acetate buffer (pH 6)–acetonitrile (58:42). The flow rate was controlled at 1.2 ml/min, and the eluant monitored at 230 nm. This method could not be used for the analysis of samples containing benzyl alcohol since benzyl alcohol interfered with the analysis of the degradation products. In the HPLC-MS experiments 100 μl (~100 μg) of sample was injected.

HPLC-MS Analysis

HPLC eluant was passed through a heated vaporizer (0.15-mm-i.d. tubing) into the high vacuum of the mass spectrometer. The vaporizer temperature was 65°C for the sample degraded in acid and 94°C for other samples. For all samples, the jet temperature was 240°C and the collector voltage was between 75 and 80 V. Centroid full-scan mass spectra were obtained by scanning the spectrometer between *m/z* 200 and *m/z* 1800 in 2 sec. Isotopic profiles were obtained by scanning a 20-mass unit window centered on the *m/z* values of the ion of interest over a 0.2-sec scan time.

RESULTS AND DISCUSSION

Dissociation Constants

The pK_a values for the two ionizable residues of **1**, the 3-pyridinylalanine and the tyrosine residues, were determined since these residues may act as general acid or base catalysts for the hydrolysis of peptide bonds or the epimerization of amino acids (8,9). Furthermore, ionization of these residues alters their electron withdrawing or electron donating ability, which could potentially affect the epimerization rate at these residues (10). Ionization may also change the secondary structure of the peptide which could influence reactivity (11).

The changes in UV absorbance that occur on ionization of the residues of **1** are shown in Fig. 1. Conversion of the pyridinium salt and ionization of the tyrosine residue to the free base were monitored by the absorbance changes at 265 and 242 nm, respectively. The pK_a values were determined by the best-fitting pH–absorbance data to the following equation:

$$pH = pK_a + \log (A_B - A_{obs})/(A_{obs} - A_{BH}) \quad (1)$$

where A_B is the absorbance of the basic form, A_{BH} is the absorbance of the acid form, and A_{obs} is the measured absorbance at a given pH. The pK_a value for the conjugate acid of the 3-pyridinylalanine residue in **1** thus obtained is 4.2 ± 0.1. This value is lower than that for methyl *N*-acetyl-3-pyridinylalaninate (12) and 3-pyridinylalanine (13), which both have a pK_a value of 4.65. The pK_a value of the tyrosine residue in **1** is 9.8 ± 0.1, which is also lower than that of tyrosine, which has a pK_a value of 10.2 (13). However, it is identical to that of the tyrosine residue in a related LH-RH analogue, nafarelin (2).

The pK_a values for the two diethylhomoarginines were not measured; however, it is expected that these residues would have pK_a values similar to that of arginine. The pK_a of arginine is 12.5 (13).

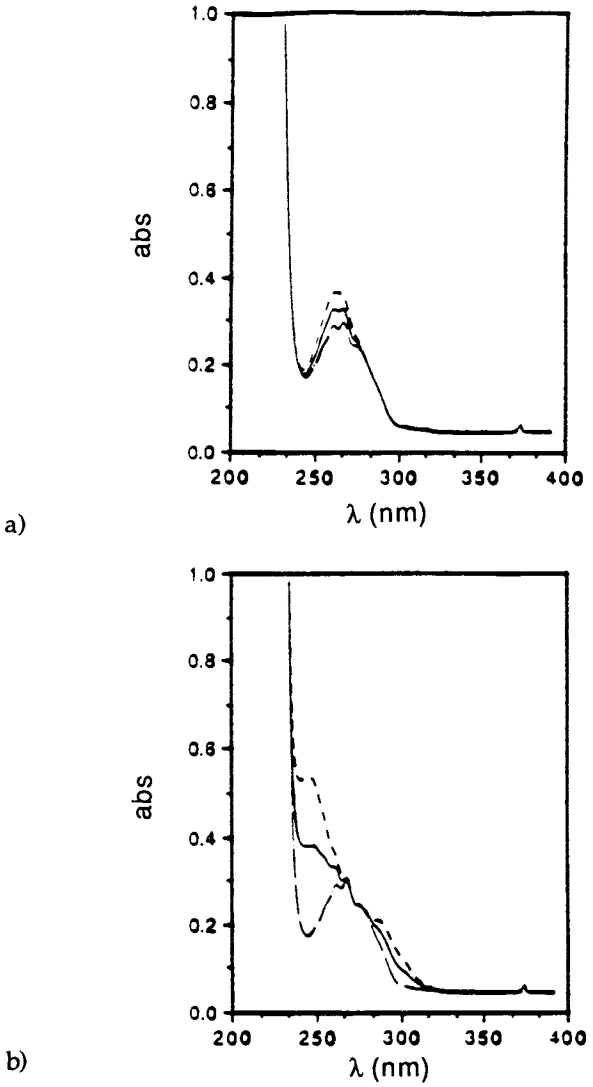


Fig. 1. UV-visible absorbance spectra of 1 as a function of pH showing (a) the protonation of the 3-pyridinylalanine residue (pK_a = 4.2) and (b) the ionization of the tyrosine residue (pK_a = 9.8). pH 2.0 (--), pH 4.1 (——), and pH 6.9 (— ·) are shown in a. pH 12.2 (--), pH 9.8 (——), and pH 6.9 (— ·) are shown in b.

Effect of pH on Degradation

The aqueous stability of 1 was studied in buffer solutions from pH 1 to pH 11 at 50, 60, and 80°C. The degradation kinetics were analyzed by HPLC (Method A) and were found to be pseudo first-order. Figure 2 shows typical first-order plots at 80°C and at pH 2, 5, and 10.5. The pH dependence of the degradation rate at 50, 60, and 80°C are shown in Fig. 3. There is no obvious curvature at the pK_a of the conjugate acid of 3-pyridinylalanine (pK_a = 4.2) or tyrosine (pK_a = 9.8), suggesting that these residues do not significantly affect the rate of decomposition of 1. The U-shaped pH–rate profiles can be fitted by a reaction mechanism that has specific-acid (k_H), water (k_w), and specific-base catalyzed (k_{OH}) terms.

$$k_{obs} = k_H a_H + k_w + k_{OH} a_{OH} \quad (2)$$

In Eq. (2), a_H and a_{OH} are, respectively, the hydrogen ion and hydroxide ion activities at the reaction temperatures. Values for various apparent rate constants at different temperatures were determined according to Eq. (2) using a nonlinear regression analysis method (14). The solid curves drawn in Fig. 3 were constructed from these apparent rate constants which are summarized in Table I. When the data are extrapolated to 25°C using the Arrhenius activation energies (Table I), a shelf life (t_{90}) of over 10 years is predicted at the pH of maximum stability, pH ~5. The extrapolated

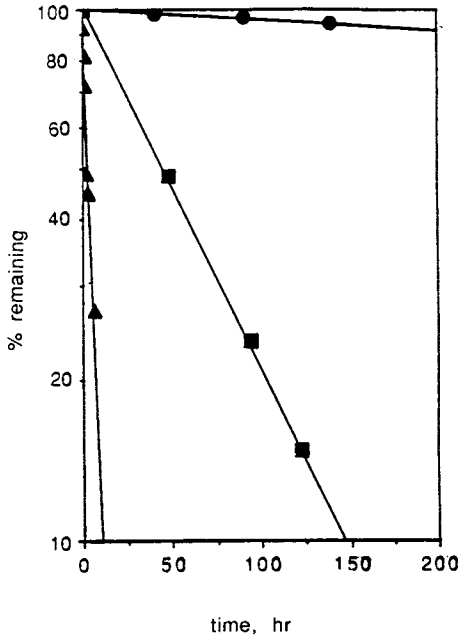


Fig. 2. First-order plot for the degradation of 1 at 80°C and pH2 (■), pH 5 (●), and pH 10.5 (▲).

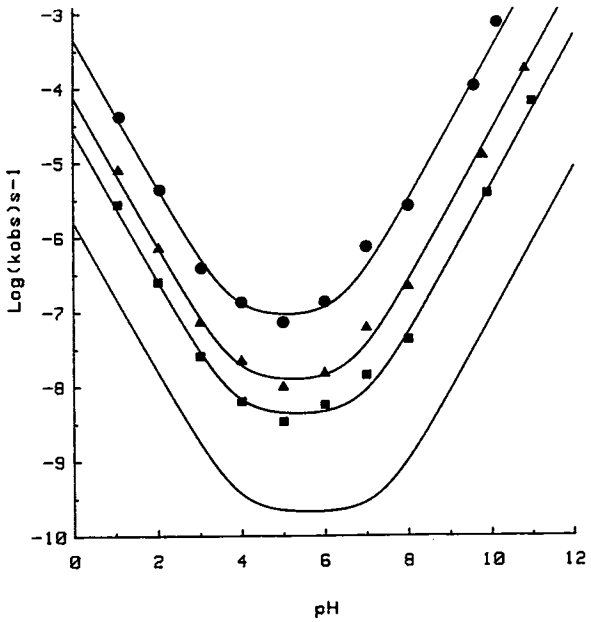


Fig. 3. pH–rate profiles for the degradation of 1 at 80°C (●), 60°C (▲), and 50°C (■). The calculated profile at 25°C is also shown with no data points.

Table I. Summary of the Activation Parameters and Observed Rate Constants for the Degradation of 1 in Aqueous Solution

k	50°C	60°C	80°C	E_a (kcal mol^{-1})	log A
k_H	2.6×10^{-5}	7.5×10^{-5}	4.6×10^{-4}	21.6 ± 0.4	10.0 ± 0.2
k_w	4.1×10^{-9}	1.2×10^{-8}	8.5×10^{-8}	22.9 ± 0.2	7.1 ± 0.2
k_{OH}	9.9×10^{-3}	2.5×10^{-2}	1.2×10^{-1}	18.9 ± 0.5	10.8 ± 0.3

data also predict that 1 would have a shelf life of over 2 years at pH values of 3–8.

Product Studies

The products from the degradation of 1 were studied by HPLC-MS using Method B (see Experimental). Typical HPLC chromatograms of the degradation solutions of 1 are shown in Fig. 4. The material balance of the reaction determined by area normalization of compounds 1*, 2, 2*, and 3 at 230 nm was over 98% under all conditions studied.

HPLC analysis of a pH 5.0 degradation sample detected the presence of the four components shown in Fig. 4a (1–3, 1*). Thermospray HPLC-MS also detected an additional component, 4, which eluted as a shoulder on the solvent front. At pH 2, one major product, 2, was observed by HPLC (Fig. 4b). Ten peaks were identified by HPLC from the sample degraded at pH 10.5 (Fig. 4c). The molecular weights of compounds 1–4 and 1* determined by HPLC-MS are listed in Table II (15).[4]

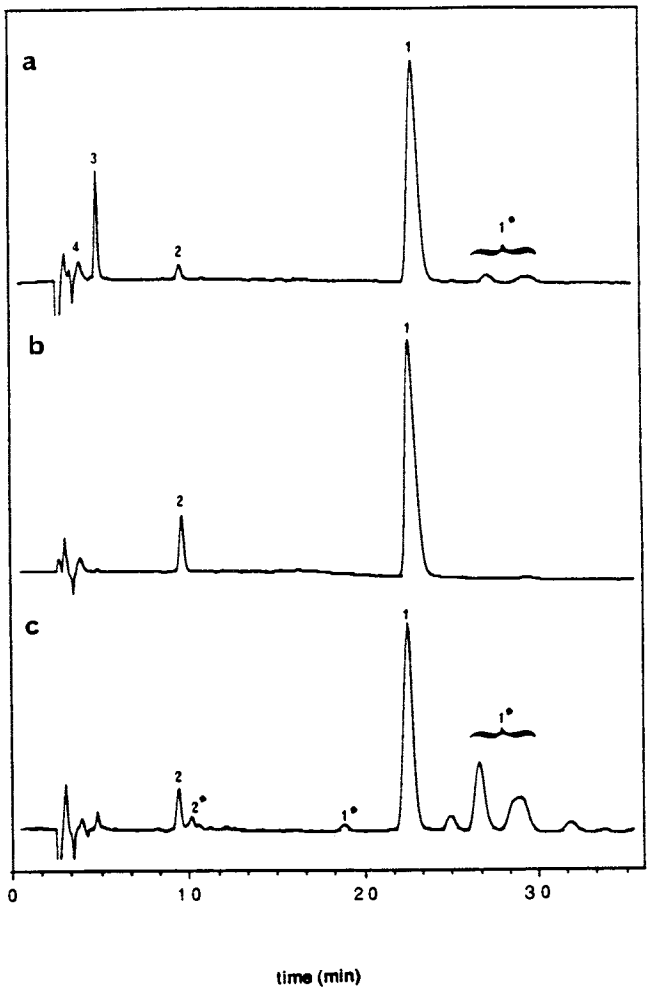


Fig. 4. HPLC chromatograms (Method B) of samples of 1 degraded in aqueous solution at 80°C and (a) pH 5.0 for 60 days, (b) pH 2 for 12 hr, and (c) pH 10.5 for 0.5 hr.

The thermospray mass spectrum of 1 yielded a singly charged $(M + H)^+$ ion at m/z of 1570 and a more intense doubly charged $(M + 2H)^{2+}$ ion at m/z of 785.5 [one-half of (1569 + 2)] (Fig. 5a). The doubly charged ion may result from the protonation of the strongly basic homoarginine residues in the mass spectrometer. To assure the m/z assignment of the protonated molecular ion, profile data of the isotopic cluster of the ion at m/z 1570 was obtained (Fig. 6a). The m/z value of the ion due to the principle isotopes (P) is 1569.8, which is consistent with the value calculated for $(M + H)^+$ of 1 (Table II). The species 1* have the same molecular weight as 1 and are assigned as its diasteriomers.

The full-scan mass spectrum of 2 is shown in Fig. 5b. The singly and doubly charged protonated molecular ions are observed at m/z of 1571 and 786, respectively. From the profile spectrum of the $(M + H)^+$ (Fig. 6b), it is determined that m/z of the principal isotopes is 1570.8, or one m/z unit higher than 1. The molecular weight measurements therefore suggest that 2 is the free acid of 1, which forms from the deamidation of the C-terminal amide. The species which elute immediately after 2, 2* (Fig. 4c) have molecular weights of 1570. These components are assigned to be the diasteriomers of 2.

The protonated molecular ion of 3 is observed at m/z 587 in the full-scan mass spectrum. The ratio of the relative intensities of the principal isotopes, P to $P + 2$ is approximately 3:1, suggesting the presence of a chlorine atom (the natural abundance of Cl^{35}:Cl^{37} is 3:1) in 3. This and the m/z value of 587 (Table II) suggest that 3 is a tripeptide consisting of the first three amino acids of 1.

The full-scan mass spectrum of 4 shows a protonated molecular ion, $(M + H)^+$, at m/z 1001 and a $(M + 2H)^{2+}$ ion at m/z 501. These data indicate the presence of a component with a molecular weight of 1000, consistent with the heptapeptide fragment derived from residues 4–10 of 1. This assignment also explains why 4 is not easily detected by HPLC. The UV absorbance of 4 at 230 nm is less than 7% of the absorbance of 1 or the degradation product 1*, 2, or 3(16) due primarily to the absence of the naphthalene chromophore.

Table II. Molecular Weights[a] of the Products Detected by HPLC-MS from the Degradation of RS-26306

Peak	MW
1	1568.8
1*	1568.8
2	1569.8
2*	1569.8
3	586.2
4	1000.4

[a] Of the principal isotopes.

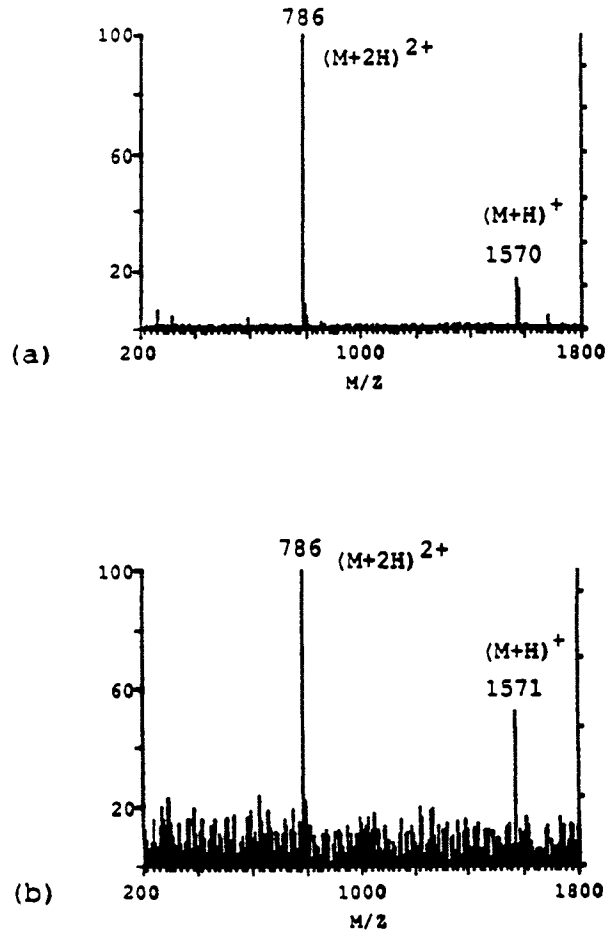


Fig. 5. Full-scan mass spectra of (a) 1 and (b) 2. Vertical axes are percentage relative intensity.

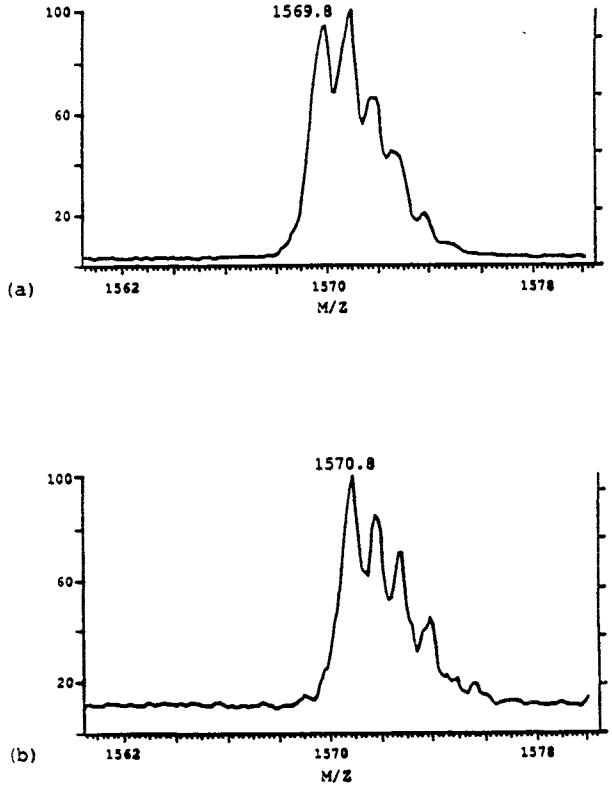
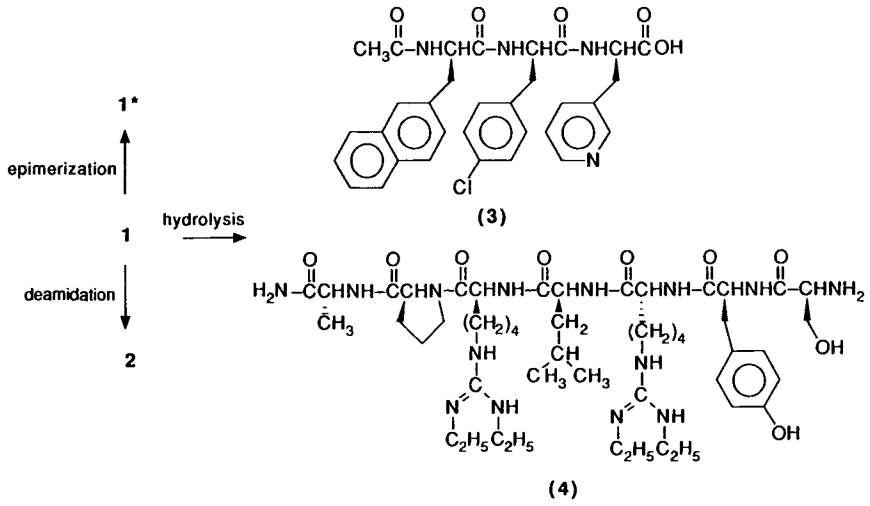


Fig. 6. Isotopic profile of the protonated molecular ion for (a) 1 and (b) 2. The vertical axes are relative intensity.

Mechanisms of Degradation

Based on the above product studies the principal degradation pathways of 1 are deamidation of the C-terminal amide to form the free acid 2, epimerization of the individual amino acids to form diasteriomers 1*, and hydrolysis of the peptide backbone to yield the tripeptide 3 and the heptapeptide 4 (Scheme II). Figure 7 shows the dependence of the three degradation pathways as a function of pH at 80°C. The data were constructed from the ratio of degradation products versus pH for samples which were less than 75% degraded to minimize secondary reactions. When these data are combined with the reaction kinetics (Fig. 3), pH–rate profiles can be constructed for each pathway in the regions where the pathway is significant. The results of these calculations are shown in Fig. 8.

The deamidation of the C-terminal amide dominates the degradation of 1 below pH 3. The second-order rate constant



Scheme II

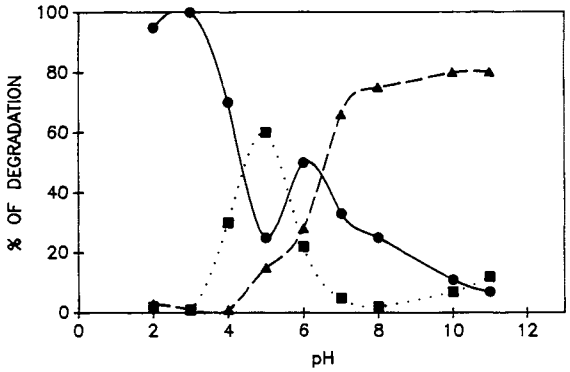


Fig. 7. The distribution of the degradation products 1* (▲), 2 (●), and 3 (■) as a function of pH at 80°C. The samples were analyzed by HPLC with greater than 75% remaining.

for the acid-catalyzed deamidation was calculated to be $\approx 5 \times 10^{-4}\ M^{-1}\ \text{sec}^{-1}$ at 80°C. This value is similar to the second-order rate constant of $1.0 \times 10^{-2}\ M^{-1}\ \text{sec}^{-1}$ for the acid catalyzed deamidation of the C-terminal amide of nafarelin (2). A base-catalyzed process (17,18) is observed above pH 6; however, at higher pH values deamidation becomes a less significant degradation pathway.

The epimerization of the amino acids of 1 is the major degradation pathway above pH 6. Base-catalyzed epimerization of amino acids is known to occur under these conditions (3,19,20). the reaction proceeds through a carbanion intermediate which is stabilized by the α-carbonyl bond of the peptide backbone. The rate constant for the base-catalyzed epimerization of 1 was calculated to be $\approx 5 \times 10^{-2}\ M^{-1}\ \text{sec}^{-1}$ at 80°C. This value can be compared to a base-catalyzed rate constant of $\approx 1\ M^{-1}\ \text{sec}^{-1}$ for the epimerization of polyserine at 100°C (20). Since the rate of epimerization of amino acids is determined by the electron withdrawing ability of the ß-substituent (10), it is probable that epimerization is occurring at the serine, 3-chlorophenylalanine, 3-pyridinylalanine, and tyrosine residues. Epimerization at any three of these residues would account for the five products observed.

The hydrolysis of the peptide bond joining the 3-pyridinylalanine and serine residues probably involves nucleophilic addition of the serine hydroxyl group to the neighboring amide bond, forming a cyclic tetrahedral intermediate (Scheme III) (21,22) which decomposes to an acylated serine intermediate in the rate-determining step (9). The rate of the reaction is pH independent from pH 3 to pH 8 with a rate constant of $\approx 4 \times 10^{-8}\ \text{sec}^{-1}$ at 80°C and the reaction becomes the major degradation pathway at pH 5.

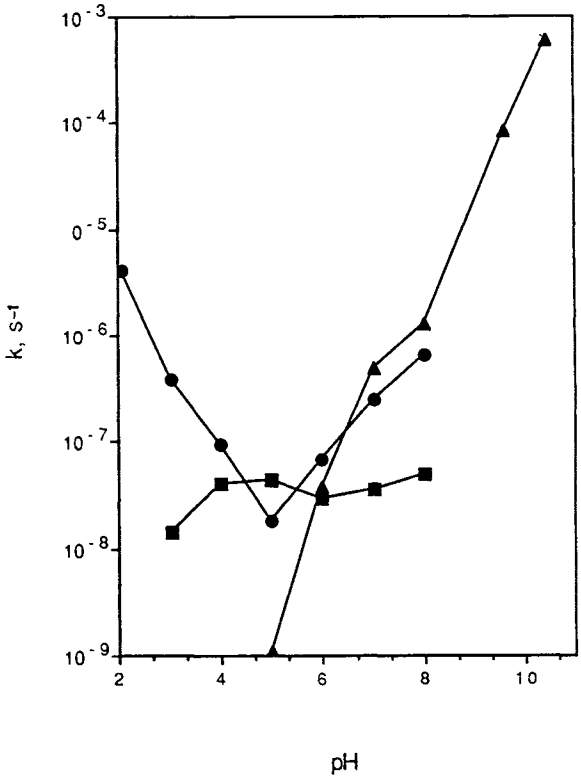
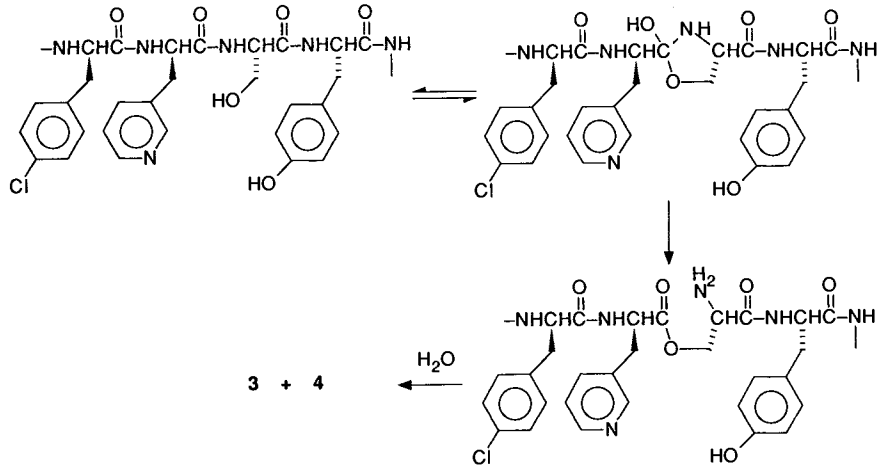


Fig. 8. pH–rate profiles for deamidation to 2 (●), epimerization to 1* (▲), and hydrolysis to 3 and 4 (■) as a function of pH at 80°C.

CONCLUSIONS

The kinetics, products, and mechanisms of the degradation of 1 in aqueous solution were identified. The methodology described, thermospray HPLC-MS, represents an



Scheme III

efficient way of characterizing the degradation products of small peptides without the isolation of individual products. This approach also made it possible to detect a degradation product **4** which was not readily observable by UV detection. The monitoring of the isotope profiles can further enhance the information obtained by the thermospray MS analyses by providing an unambiguous assignment of the molecular ion of each product.

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